Multiple outcomes for PI3K/Akt/mTOR targeting in non-Hodgkin lymphoma

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Dipl. Biol. Anja Müller

Präsident der Humboldt-Universität zu Berlin

Prof. Dr. Jan-Hendrik Olbertz

Dekanin/Dekan der Lebenswissenschaftlichen Fakultät

Prof. Dr. Richard Lucius

Gutachter/innen:
1. Prof. Dr. Wolfgang Uckert
2. PD. Dr. Christian Scholz
3. Prof. Dr. Claus Scheidereit

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Abstract

The PI3K/Akt/mTOR pathway is a key element in cell orchestration and affects almost all cellular processes including the one important for proliferation, growth, survival and cell death. Constitutive active PI3K/Akt/mTOR signaling, a common event in lymphomas, promotes tumor progression, is associated with therapy resistance and, thereby, gives a poor prognosis. Thus, chemotherapeutical targeting of PI3K/Akt/mTOR signaling has gained high priority in the last years. However, the reported effects of PI3K/Akt/mTOR inhibitors on outcome and molecular regulation are contradictory. Here, we tested the molecular response of an *in vitro* lymphoma model to the newly developed pan class I PI3K inhibitor BKM120 and explored the therapeutic potential of combining PI3K/Akt/mTOR inhibitors with cytotoxic agents.

In that model, it has been shown for the first time that BKM120 induces G2/M arrest by activating the Chk2/Cdc25C axis of the G2/M checkpoint. This is accompanied by the upregulation of the BH3-onlys Puma and Hrk and subsequent cell death via the intrinsic apoptosis pathway. Apoptotic resistance, however, causes the BKM120 dependent formation of giant polyploid cells. In contrast to previous reports, this polyploidy requires cooperate loss of the apoptosis regulators Bax, Bak and p53. However, cell cycle controlling factors, presumably involved in polyploidy, are regulated by BKM120 equally in apoptotic and non-apoptotic cells. This includes the inactivation of Chk1, the downregulation of Cyclin A and the activation of MEK1/2. MEK1/2, thereby, acts downstream of ATM/ATR. Both, BKM120-mediated apoptosis and polyploidy, are controlled by ATM/ATR and MEK1/2. Inhibition of either ATM/ATR of MEK1/2 synergistically enhances apoptosis but prevents polyploidy. It remains to clarify, which of these BKM120 effects are particularly PI3K dependent or a result of off-target effects. Nevertheless, these data clearly show that BKM120 has high impact on cell cycle regulation and that its uniform signaling still can have different outcomes due to differences in the genetic profile. Therefore and as polyploidy often is a preliminary stage to aneuploidy, a condition associated with tumor progression and therapy resistance, the use of BKM120 in the clinic needs to be evaluated.

One strategy to prevent therapeutic resistance is the horizontal combination of chemotherapeutics. Here in that *in vitro* model, several inhibitors of the PI3K/Akt/mTOR pathway were combined with conventional and potential cytotoxic agents. Depending on the death stimulus, namely TRAIL, cytarabine, cisplatin and mitoxantrone, pretreatment with PI3K/Akt/mTOR inhibitors protected from apoptosis. Thereby, particularly low concentrations abrogated the initiation of cell death, while high concentrations restored or even sensitized apoptosis. Additional inhibition of NFκB neutralized this effect. The antiapoptotic NFκB target Pim-2 was identified as possible candidate. Here it is shown for the first time that PI3K/Akt/mTOR signaling controls Pim-2 expression and that this expression is

congruent to the observed oscillation in the apoptotic response. Yet, it remains to clarify whether NFκB is the connection. The use of the chemical pan Pim kinases inhibitor LGB321 could not reveal the role of Pim-2 for apoptotic resistance but allowed the discovery of a new feedback loop within the PI3K/Akt/mTOR pathway. Referring to this, inhibition of PI3K or mTOR induces the expression of Pim-2, which in turn, is reported to negatively regulate TSC2. Hence, inhibition of PI3K /mTOR promotes the reactivation of mTOR via Pim-2. This is of crucial importance as mTOR signaling is an important mechanism of involved in therapy resistance. Furthermore, these data show that in the combinatory therapy the use of PI3K/Akt/mTOR inhibitors needs to be evaluated as well.

Zusammenfassung

Der PI3K/Akt/mTOR Signalweg spielt eine zentrale Rolle in der Orchestrierung von Zellabläufen und beeinflusst fast alle wichtigen Prozesse, einschließlich derer die Vermehrung, Wachstum, Überleben und Zelltod steuern. Die konstitutive Aktivierung der PI3K/Akt/mTOR Signalkaskade, wird häufig in hämotologischen Tumoren beobachtet und steht im Zusammenhang mit Tumorprogression, Therapieresistenz, und einer generell schlechteren Prognose. Die Hemmung dieses Signalweges mit Chemotherapeutika scheint daher eine logische Konsequenz und vielversprechende therapeutische Strategie zu sein. Jedoch finden sich in der Literatur widersprüchliche Berichte über die Auswirkungen und die molekulare Regulation von PI3K/Akt/mTOR Inhibitoren auf z.B. Zellzyklus und Apoptose. Im Rahmen dieser Arbeit wurden daher zwei mögliche Strategien der PI3K/Akt/mTOR Inhibition in einem *in vitro* Lymphommodell erprobt. Zum einen wurde die Antitumorwirkung des neu entwickelten PI3K Inibitors BKM120 sowie die zugrunde liegenden molekularen Mechanismen untersucht und zum anderen das Potential von PI3K/Akt/mTOR Inhibitoren in horizontaler Kombination mit zelltoxischen Agenzien erforscht.

Im untersuchten Lymphommodell wird erstmalig gezeigt, das BKM120 die Chk2/Cdc25C Achse des G2/M Checkpoints aktiviert und damit reversiblen G2/M Arrest induziert. Dies geht mit einer Hochregulation der BH3-onlys Puma und Hrk sowie der Aktivierung des intrinsischen Apoptosesignalweg einher. Apoptoseresistenz dagegen führt zur Bildung gigantischer und polyploider Zellen. Im Gegensatz zu bisherigen Beobachtungen ist dabei für die Genese von Polyploidie ein kombinierter Verlust der apoptotischen Regulatoren Bax,Bak und p53 nötig. Zellzyklus regulierende Faktoren, die im Zusammenhang mit DNA Replikation und Polyploidie stehen, werden jedoch von BKM120 in apoptotischen und nicht apoptotischen Zellen gleichermaßen reguliert. Dazu gehören die Inaktivierung von Chk1, die Herunterregulation von Cyclin A und die Aktivierung von MEK1/2. Dabei steht MEK1/2 stromabwärts von ATM/ATR. Beide, Apoptose und Polyploidie, sind sowohl ATM/ATR als auch MEK1/2 abhängig. Die Inhibition von ATM/ATR oder MEK1/2 fördert synergistisch BKM120 induzierte Apoptose, wogegen Polyploidie gehemmt wird. Ob diese Ergebnisse eine direkte Folge von BKM120 vermittelter PI3K Inhibition sind oder eher auf unspezifische Nebeneffekte zurückzuführen, muss noch geklärt werden. Nichtdestrotrotz zeigen diese Daten, dass BKM120 entscheidenden Einfluss auf die Zellzyklusregulation hat. Außerdem ergeben sich weitere Hinweise darauf, der gleiche Signalweg aufgrund differierender genetische Prolife zu einem unterschiedlichen Ausgang führen kann. Aufgrundessen und der Tatsache das Polyploidie häufig eine Vorstufe zur Aneuploidie ist und somit Tumorporgression und Therapieresistenz begünstigt, gehört die Verwendung von BKM120 genauestens abgewägt.

Zur Vermeidung von Resistenzen wird die Strategie der horizontalen Kombination verfolgt. Um deren Wirkung im Lmphommodell zu untersuchen, wurden verschiedene PI3K/Akt/mTOR Inhibitoren mit gängigen und potentiellen Chemotherapeutika kombiniert. Abhängig vom Stimulus, in diesem Fall TRAIL, Cytarabin, Cisplatin und Mitoxantron, schützte die Kombination mit PI3K/Akt/mTOR Inhibitoren vor Apoptose. Dabei verhinderten besonders niedrige Konzentrationen die Initiation von Zelltod, während hohe Konzentrationen die Sensitivität wiederherstellten oder sogar verstärkten. Eine zusätzliche Hemmung von NFκB neutralisierte diesen Effekt. In der Folge wurde das antiapoptotische NFκB Target Pim-2 als mögliche Ursache für den Schutzmechanismus identifiziert. Hier konnte erstmalig gezeigt werden, das der PI3K/Akt/mTOR Signalweg die Expression von Pim-2 kontrolliert und dies kongruent zum beobachteten Schutzmechanismus verläuft. Ob diese Verbindung über NFκB hergestellt wird, konnte nicht eindeutig geklärt werden. Die genaue Aufgabe von Pim-2 im Schutzmechanismus konnte durch chemische Inhibition mit LGB321 zwar nicht weiter aufgedeckt werden, führte aber zur Entdeckung eines neuen Rückkopplungsmechanismus innerhalb des PI3K/Akt/mTOR Signalweges. Diesbezüglich führt die Inhibition von PI3K oder mTOR zur Hochregulation von Pim-2, welches bekanntermaßen TSC2 negativ reguliert. Somit führt die Hemmung des PI3K/Akt/mTOR Signalweges, über Pim-2, zur Reaktivierung von mTOR. Dies ist von Bedeutung, da der mTOR Signalweg in Zusammenhang mit Therapieresistenzen steht. Außerdem zeigen diese Ergebnisse, dass auch im Fall der kombinierten Therapie eine sorgfältige Abwägung vonnöten ist.

Table of Contents

1 INTRODUCTION

The PI3K/Akt/mTOR pathway plays a fundamental role in promoting metabolism, growth and survival of eukaryotic cells. Thus, activation of this pathway contributes to a variety of diseases including cancer. The constitutive signaling of the PI3K/Akt/mTOR pathway has prognostic relevance for non-Hodgkin lymphoma and is implicated in tumor progression and therapy resistance [1,2]. This is the virtue of two major mechanisms, the promotion of cell cycle progression and the suppression of apoptotic cell death. Massive cell expansion, accumulation of mutations and genome instability are the consequences. Targeting the PI3K/Akt/mTOR pathway with chemical agents is considered as a promising strategy to improve cancer therapy and patient survival. However, the complex network within and around the pathway is an obstacle in predicting the outcome of PI3K/Akt/mTOR inhibition in cancer cells. This introduction, therefore, outlines PI3K/Akt/mTOR signaling and the main intersection points to cell cycle regulation and cell death control.

1.1 THE PI3K/AKT/MTOR PATHWAY

Within the pathway, PKB/Akt is the major regulator of the important downstream processes, controlling growth, proliferation, cell cycle regulation, and survival [3]. Although many ways to regulate this kinase have been identified, the classical pathway via PI3K/PIP_3/PDK1 is still most important. Here, the major components of the pathway are introduced and the cascade of the classical pathway is explained.

1.1.1 The major components

1.1.1.1 Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)

The family of PI3 kinases comprises three classes, of which only class I participates in Akt regulation. Class I PI3 kinases become activated upon receptor signaling and, depending on the receptor, a further subdivision in class IA and class IB kinases is made. So far, however, only receptor tyrosine kinases (RTK) mediated activation of class IA kinases is implicated in cancer. Class IA PI3 kinases are complexes composed of a p85 regulatory and a p110 catalytic subunit. The regulatory unit corresponds with the receptor and is required for the activation of the protein complex. p85 contains two SRC homology 2 (SH2) domains that recognize phosphorylated tyrosine in context with a specific amino acid sequence. Thus, it interacts with auto-phosphorylated receptors directly or via adapters and co-localizes the catalytic unit to the membrane-bound substrate [4]. Three catalytic units have been identified so far, p110α, p110β, and p110δ. Among them, mutations or amplifications were found only for the gene encoding for p110α, PI3KCA [5]. However, attempting a correlation between PI3KCA mutations and

prognosis is difficult as shortened survival and therapeutic resistance, but also an increased response to PI3K/Akt/mTOR inhibitors is reported [6,7,8,9,10,11,12]. Class IA PI3 kinases have both protein kinase and lipid kinase function. However, the preferred substrate is the membrane-bound phospholipid phosphatidylinositol (PtdIns). Phosphoinositides, the phosphorylated forms of PtdIns, are important for downstream signaling and the activation of PKB/Akt.

1.1.1.2 Protein Kinase B (PKB/Akt)

PKB/Akt occurs in the three isoforms, AKT1, AKT2 and AKT3, which share 80% homology, although, they are encoded by three different genes [13]. Each isoform has distinct functions. Thus, mice lacking AKT1 show high prenatal mortality and reduced body weight, while AKT2 negative mice show dysfunction of the glucose metabolism and AKT3 deficient mice have smaller brains [14,15,16,17,18]. Within the cell, their mode of actions seems to be determined by their localization. AKT1 is found mainly in the cytoplasm, whereas AKT2 and AKT3 are localized at the mitochondrial and nuclear membrane, respectively [19]. Alterations in all three isoforms were found in cancer [20,21,22], although the overall incidence of Akt mutations in tumors is low [23]. The Akt protein comprises an n-terminal pleckstrin homology (PH) domain, a catalytic domain and a c-terminal regulatory domain. With the PH domain, Akt is recruited to the cellular membrane, a necessary step to become activated by phosphorylation. Two phosphorylations are associated with activity. Phosphorylation of T308 within the catalytic domain is mediated by Phosphoinositide-dependent kinase-1 (PDK1) at the cellular membrane. Phosphorylation of S473 within the regulatory domain is now broadly accepted to be performed by mammalian target of rapamycin complex 2 (mTORC2) [24]. Thereby, T308 phosphorylation is reported to depend on S473 phosphorylation [25]. However, whether both phosphorylations are required for full activity or determine distinct functions of Akt, is still discussed [25,26,27,28,29].

1.1.1.3 Mammalian Target of Rapamycin (mTOR)

It took almost twenty years from the isolation of the fungicide rapamycin out of *Streptomyces hygroscopicus* to the discovery of its target FRAP/mTOR [30,31]. FRAP/mTOR was found to bind a complex of rapamycin and the intracellular receptor FKBP12. This abrogates mTOR's activity and affects downstream signaling. The main targets of mTOR are p70S6K and 4EBP1. The ribosomal protein S6 kinase (p70S6K) phosphorylates S6, a component of the 40S ribosomal subunit. S6 is required for the specific translation of mRNAs encoding proteins of the translational machinery. The activation of S6K requires hierarchical phosphorylation by several factors including mTOR [32]. In contrast, eIF4E binding protein 1 (4EBP1) controls a more general process of translation. The eukaryotic translation initiation factor 4E (eIF4E) that positions mRNAs within the translational complex, is bound and inhibited by 4EBP1. mTOR dependent phosphorylation of 4EBP1 causes its dissociation and releases

eIF4E [33]. As these two processes are crucial for cap-dependent translation, mTOR is one of the most important supervisors of protein synthesis.

These effects, described above, are conducted by mTOR when it is bound to Raptor, Deptor, mLST8 and PRAS40, a complex termed as mTORC1. A second, rapamycin insensitive, mTOR complex was discovered in 2004 [34]. In this complex, mTORC2, mTOR is bound to Rictor, Deptor, mLST8, Sin-1 and Protor. The duties of this complex are poorly understood, but according to current knowledge very different to those of mTORC1. Besides its established function in Akt activation (chapter 1.1.1.2), only the regulation of PKCs and SGK1 is reported; proteins associated with ion transport and cytoskeleton organization [24]. In addition, the events leading to mTORC2 activation are still under investigation. Recently, the mTORC2 complex component Sin-1 was reported to be a direct target of Akt [35]. However, for the activation of mTORC2, a PI3K/PIP_3 dependent, but TSC1/TSC2 independent mechanism was suggested [36,37]. Interestingly, although mTORC2 is initially reported to be rapamycin insensitive, prolonged rapamycin treatment also affects mTORC2 activity [38]. This circumstance most likely attributes to the fact that the assembly of both complexes is balanced by the shared use of mTOR. Thus, treatment with PI3K or mTOR inhibitors not only affects mTORC1 signaling but also has impact on mTORC2.

1.1.2 From PI3K to Akt to mTOR

In the classical pathway, class I PI3 kinases trigger the activation of Akt. However, several intermediate steps are necessary (Figure 1). To become activated itself, class I PI3 kinases require the receptor mediated activation of their regulatory subunit (chapter 1.1.1.1). Once activated, the lipid kinase function of PI3K then recognizes its substrate phosphatidylinositol (PI) within the cellular membrane and performs its conversion into phosphatidylinositol 3-phosphate (PIP), phosphatidylinositol (3,4)-bisphosphate (PIP_2) and phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). For Akt activation only the phosphorylation step from PIP_2 to PIP_3 is relevant, which is also the preferred step *in vivo* [39]. Akt equally binds PIP_2 and PIP_3 through its pleckstrin homology (PH) domain [40,41]. This recruits Akt to the cellular membrane and triggers a conformational change but does not activate it [42]. The key event that triggers Akt activation is the additional recruitment of Phosphoinositide-dependent kinase-1 (PDK1). PDK1 possesses a PH domain as well, but contrary to Akt, it binds PIP_3 with much higher affinity than PIP_2. The binding of PDK1 to PIP_3 does not affect its kinase activity, but the co-localization to PKB/Akt is suggested to enable the direct phosphorylation and activation of Akt by PDK1 [43].

One of the major downstream targets of Akt is the translational regulator mTORC1 (Figure 1). The Akt mediated activation of mTORC1 occurs by direct and indirect mechanisms. Phosphorylation of the complex protein FRAP/mTOR at S2448 is directly mediated by Akt [44] and promotes its binding to

raptor [45]. Besides this, Akt promotes mTORC1 activity by disabling several negative regulatory mechanisms. PRAS40 is a component of mTORC1 and suggested to control mTORC1 activity negatively by covering the binding sites for downstream substrates [46]. Akt phosphorylates PRAS40 at T246, which prevents this inhibitory effect on mTORC1 [47]. The most prominent mechanism to control mTORC1, yet, occurs via the TSC1/TSC2 complex. TSC1/TSC2 negatively regulates mTORC1 activity by preventing Rheb dependent inactivation of the mTORC1 inhibitor FKB38 [48,49]. Phosphorylation of TSC2 by Akt inactivates the TSC1/TSC2 complex and promotes mTORC1 activation.

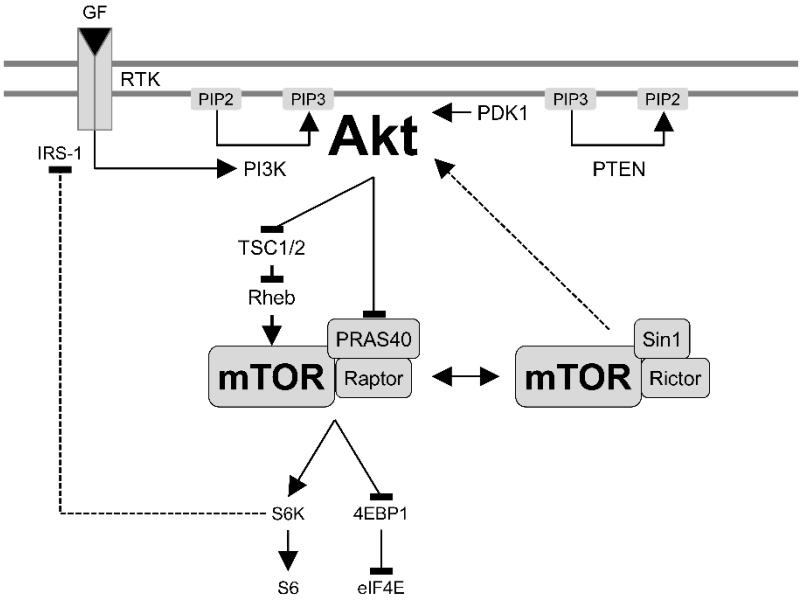

Figure 1: The PI3K signaling pathway. PI3K signaling is initiated when growth factors bind and activate their counterpart receptors, mostly receptor tyrosine kinases (RTK). Class I PI3Ks are recruited to the receptor and become phosphorylated and activated by direct and indirect mechanism (e.g. IRS-1). PI3K, then, catalyzes the conversion of PIP2 into PIP3. PIP3 acts as an anchor for Akt and PDK1, bringing them in close proximity and enabling the phosphorylation of Akt at T308 by PDK1. The major downstream target of Akt is mTORC1. Akt promotes mTORC1 activation in three ways. Firstly, it directly phosphorylates the central protein mTOR, which facilitates Raptor binding. Secondly, PRAS40, an mTORC1 component that occupies the substrate binding sites, is phosphorylated and inactivated by Akt. Thirdly, the TSC1/TSC2 complex that inhibits Rheb-GTP dependent mTORC1 activation, is inhibited by Akt as well. Once activated, mTORC1 mainly regulates translation by controlling the activity of the ribosomal protein S6 and the translational complex component eIF4E. S6 is positively regulated by p70S6K. S6K itself, is regulated by mTORC1 dependent phosphorylation at T389 and S371. eIF4E in turn, is negatively regulated by 4EBP1. Inactivation of 4EBP1 through mTORC1 mediated phosphorylation, releases and activates eIF4E. Regulatory feedback loops keep the pathway in balance. Firstly, S6K signals back to IRS-1 and prevents further PI3K activation. Secondly, PTEN reverses PIP3 into PIP2 and prevents further activation of Akt. Thirdly, the balance between mTORC1 and mTORC2 affects phosphorylation and activation of Akt. mTORC1 prevents Akt activation, while mTORC2 promotes Akt activation.

1.1.3 Restricting PI3K/Akt/mTOR signaling

PI3K/Akt/mTOR signaling is restricted by several mechanisms (Figure 1). One crucial step in the pathway is the PI3K dependent conversion of PIP_2 into PIP_3 that is required for Akt activation. This step

is reversed by phosphatase and tensin homolog (PTEN). PTEN is a protein tyrosine phosphatase that eliminates PIP_3 from the system by dephosphorylating it back to PIP_2. This step prevents Akt activation and excessive signaling. The importance of PTEN for cell homeostasis is demonstrated by the frequent loss of PTEN function in cancer.

In addition, feedback loops modulate the signaling of the PI3K/Akt/mTOR pathway [50]. The most prominent feedback is mTORC1 negatively signaling back to PI3K via IRS-1. IRS-1 is an endogenous substrate of the insulin receptor and an important adaptor for PI3K activation. Tyrosine phosphorylated IRS-1 interacts with the SH2 domains of PI3K's regulatory subunit and promotes its activation [51]. In contrast, phosphorylation of IRS-1 on serine/threonine sites determines its proteasomal degradation. These phosphorylations are rapamycin dependent and conducted by mTORC1 and its downstream target S6K. Additionally, mTORC1 and S6K are involved in the transcriptional repression of IRS-1. Thus, mTORC1 activation prevents a further activation of PI3K and restricts PI3K/Akt/mTOR signaling.

The balance between mTORC1 and mTORC2 also provides a mechanism of regulation. Akt signaling promotes the assembly of mTORC1, which reduces the amount of mTORC2. This feedback prohibits a further mTORC2 dependent activation of Akt. Vice versa, inhibition of mTORC1 diverts signaling towards mTORC2, which up regulates Akt phosphorylation and signaling [52,53].

The PI3K/Akt/mTOR pathway promotes growth and proliferation by facilitating cell cycle progression and positively regulating its superior regulation. The impact of PI3K/Akt/mTOR signaling on the cell cycle has been investigated extensively and intersection points to all phases of the cell cycle have been identified. Impairing PI3K/Akt/mTOR signaling with chemical inhibitors mostly induces G1 arrest and, thus, the influence of PI3K/Akt/mTOR signaling on G1/S transition is best characterized. In some cases, however, inhibition of PI3K/Akt/mTOR leads to arrest in G2/M [54,55]. The required conditions for these events and the underlying mechanisms are poorly understood.

This chapter provides and introduction into cell cycle and checkpoint regulation and furthermore summarizes the most important intersection points of the PI3K/Akt/mTOR pathway, discovered so far.

1.1.4 The cell cycle and its regulation

Proliferation of somatic cells occurs by cell growth, duplication of the genetic information and organelles followed by the subsequent division into two daughter cells. These processes are under the control of the mitotic cell cycle. Four phases are determined: growth phase I (G1), DNA synthesis phase (S), growth phase II (G2) and cell division by mitosis (M). For quiescent cells, an additional G0 phase is defined. The progression through this cell cycle is conducted by the family of Cyclin dependent kinases (CDKs) in conjunction with the periodically expressed Cyclins. A superior mechanism, the checkpoint

control, monitors the cell cycle performance. In case of external and internal disturbances, the checkpoint induces the “pause” of the cell cycle until the conditions for progression are re-established.

1.1.4.1 Cycling with CDKs

Cyclin dependent kinases (CDKs) are a huge family of serine/threonine kinases, of which only four are directly involved in passing through the cycle, CDK4, CDK6, CDK2 and CDK1. As implicated, CDKs depend on Cyclins to perform properly. Binding of a Cyclin to a CDK rearranges the CDK’s T-loop and permits the ATP binding, required for substrate phosphorylation [56]. Therefore, Cyclins are often designated as the regulatory subunits. Cyclins are, as the name suggests, cyclically expressed and, thus, restrict the action of the CDK/Cyclin complex to a distinct point in the cell cycle (Figure 2). Mainly six CDK/Cyclin complexes coordinate the progression through the cell cycle. Their functions will be outlined in the following (mostly reviewed from [57]). CDK4/Cyclin D and CDK6/Cyclin D coordinate the G0/G1 transition and early events of the G1 phase. Both complexes positively regulate the transcription factor E2F by inactivating the tumor suppressor retinoblastoma protein (pRb). E2F, then, induces the expression of Cyclin E and Cyclin A. These two Cyclins interact with CDK2. CDK2/Cyclin E is involved in the G1/S transition and suggested to initiate DNA replication by conducting the nuclear translocation and stabilization of the origin of replication licensing factor Cdc6 [58]. When Cyclin E is downregulated after S phase entry, Cyclin A steps into the complex. CDK2/Cyclin A, then, monitors the DNA synthesis and promotes the exit into G2 phase, a process that is suggested to be associated with the relocation of Cdc6 to the cytoplasm [59]. During S/G2 transition Cyclin A is also bound to CDK1. CDK2/Cyclin A and CDK1/Cyclin A share several substrates that participate in cell cycle progression. Upon entry into G2 phase, however, only the CDK1/Cyclin A persists. CDK1/Cyclin A is present during G2 phase and implicated in the activation and stabilization of the so-called M phase complex CDK1/Cyclin B [60,61]. Activation of CDK1/Cyclin B spares the need for CDK1/Cyclin A and initiates the degradation of Cyclin A before metaphase-anaphase transition [62,63]. Finally, CDK1/Cyclin B1 prepares and controls the separation of the chromosomes by coordinating the assembly of the mitotic spindle. Inactivation of CDK1/Cyclin B1 is initiated by degradation of Cyclin B1, a necessary step for sister chromatid separation and completion of mitosis [64].

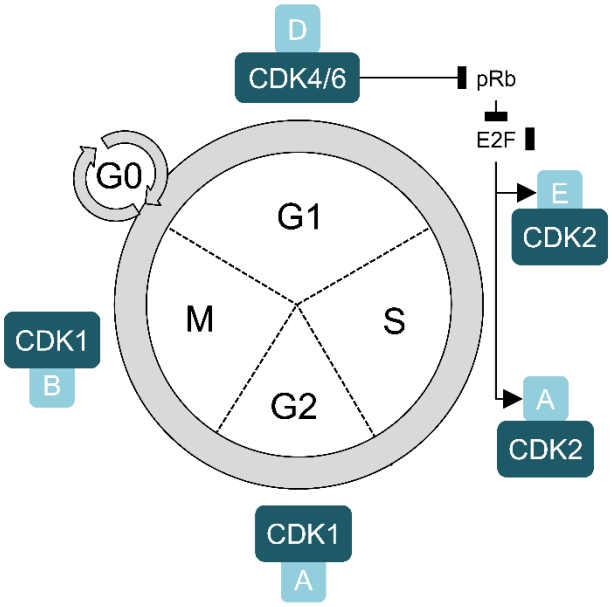

Figure 2: The eukaryotic cell cycle. The cell cycle consists of four phases coordinating cell growth (G1), DNA duplication (S), preparation for cell division (G2) and cell division (M). Non-proliferating cells are assumed to be situated in G0. The transition from one phase to the next is regulated by Cyclin dependent complexes (CDKs) in conjunction with the Cyclins D, E, A and B. The complexes CDK4/Cyclin D and CDK6/Cyclin D promote G1 progression by inhibiting pRb. This releases and activates the transcription factor E2F and leads to the expression of Cyclin E and Cyclin A. Cyclin E in complex with CDK2 also inhibits pRB and promotes G1/S transition and initiation of DNA replication. In S phase, CDK2/Cyclin A monitors DNA replication and the exit into G2. In G2 phase, CDK1/Cyclin A coordinates G2 progression. CDK1/Cyclin A dependent stabilization of CDK1/Cyclin B promotes mitotic entry and progression until metaphase. The subsequent transition from metaphase into anaphase and exit of mitosis requires the degradation of CDK1/Cyclin B. With the mitotic exit, one cycle is completed.

1.1.4.2 Modulation of CDK/Cyclin complexes

Two main mechanisms additionally determine the activity of CDK/Cyclin complexes and prevent premature activation. Firstly, CDK inhibiting proteins (CKIs) bind and inhibit CDK/Cyclin complexes. Secondly, phosphorylation of the CDK subunits has both, activating and inhibiting function.

The most prominent members of the CKI family are p21WAF1/CIP1 and $p27^{Kip1}$. p21 is a direct target of the tumor suppressor p53 and, thus, expressed upon cellular stress or DNA damage. Once induced, p21 either promotes G1 arrest by inhibiting CDK2/Cyclin E and the subsequent degradation of pRB [65] or induces S phase arrest by abrogating CDK2/Cyclin A mediated Cdc6 degradation [66]. The other CKI member, p27, likewise binds and inhibits CDK2 containing complexes, particularly CDK2/Cyclin E, and prevents G1 progression. In contrast to p21, p27 is constantly expressed and its mode of action is rather controlled by cellular location and degradation.

The activation of CDK/Cyclin complexes further requires the phosphorylation of CDKs at a specific threonine residue within the T-loop, T161 in CDK1, T160 in CDK2 and T172 in CDK4 [67]. This

phosphorylation is mediated by the CDK-activating kinase (CAK). However, additional phosphorylations on T14 and Y15 negatively regulate CDKs and prevent premature activation [68,69]. These two phosphorylations are conducted by the kinases Wee-1 and Myt-1 [70,71]. Full activation of the CDK/Cyclin complexes occurs by two mechanisms. On the one hand, the phosphatases of the Cdc25 family directly dephosphorylate existing CDK/Cyclin complexes on T14 and Y15 [72]. On the other hand, inactivation of Wee-1 and Myt-1 prevents the phosphorylation of newly formed complexes.

1.1.4.3 Checkpoint control

Perturbing events like nutrient deprivation, cellular stress or DNA damage trigger the pause or arrest of the cell cycle by inducing the checkpoint control system (Figure 3). Primarily, this system provides the opportunity to sustain starvation and to repair damages. In case of irreversible damages, however, checkpoint signaling can also induce programmed cell death. The checkpoints have overlapping functions in monitoring nutrient availability, DNA damage, chromosome organization or cytoskeleton assembly, although DNA damage dependent signaling is best characterized. The central proteins of checkpoint regulation are the checkpoint kinases Chk1 and Chk2 that trigger cell cycle arrest by preventing the activation of the current CDK/Cyclin complex. In case of DNA damage, they become phosphorylated and activated by the DNA damage sensors ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and RAD3-related (ATR). ATM/ATR mediated phosphorylation of Chk2 at T68 causes its dimerization and is necessary for Chk2 auto-phosphorylation and the release of activated monomers [73]. Chk1 in turn, requires ATM/ATR dependent phosphorylation at S317 and S345, with S345 being essential for Chk1 activity [74,75,76]. Once activated, Chk1 and Chk2 abrogate CDK/Cyclin activities by maintaining them phosphorylated at T14 and Y15. This mainly occurs by triggering the nuclear export and proteasomal degradation of the phosphatases Cdc25C and Cdc25A [77,78]. In addition, Chk1 stabilizes T14/Y15 phosphorylation and prevents G2/M transition by phosphorylating the kinase Wee-1 [79]. The levels of Wee1 activity, thereby, determine the timing of entry into mitosis [80]. During G1 phase, the tumor suppressor p53 functions as checkpoint regulator as well. As previously described (chapter 1.1.4.2), p53 modulates the activity of CDK2/Cyclin complexes, and thereby G1/S progression, by controlling p21 expression. In response to DNA damage, p53 becomes activated via ATM/ATR signaling by multiple mechanisms. Firstly, ATM phosphorylates and inactivates MDM-2, the negative regulator of p53. Secondly, ATM, and probably ATR, directly phosphorylate p53 at S15, which prevents binding to MDM-2[81]. Thirdly, the ATM/ATR downstream target Chk2 phosphorylates p53 at S20, which importantly regulates p53's stability and function [82,83].

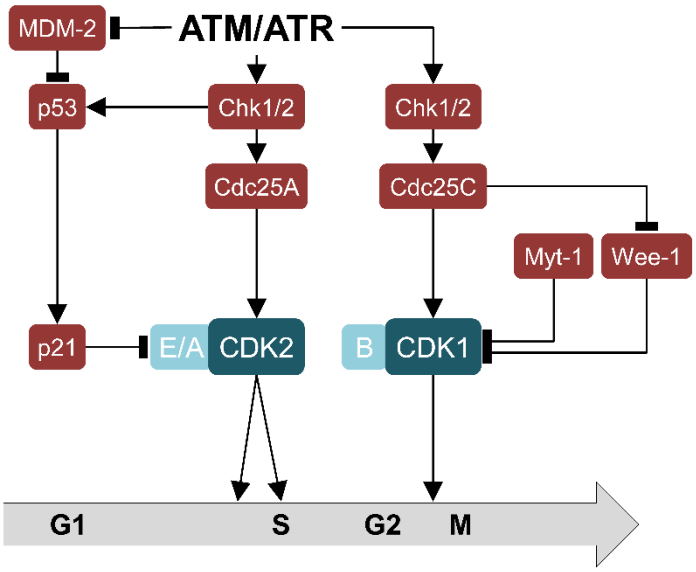

Figure 3: ATM/ATR DNA damage checkpoint regulation. ATM/ATR are activated at DNA breaks. During G1/S phase, this leads to the activation of p53. p53 is a transcription factor that induces the expression of the Cyclin-dependent kinase inhibitor (CKI) p21. p21 directly inhibits the activity of CDK2/Cyclin complexes and arrests cells in G1 and S phase. In a second path, ATM/ATR activates the checkpoint kinases Chk1 and Chk2. Both negatively regulate Cdc25. Inactivation of Cdc25 prevents activation of CDK2 and arrests cells at G2/S phase. During G2/M phase, ATM/ATR mediated activation of Chk1 and Chk2 causes inactivation of Cdc25, which prevents activation of the M phase CDK1/Cyclin B complex. Chk1 also inactivates Wee-1, which activates CDK1 by phosphorylation at T161.

1.1.4.4 The spindle assembly checkpoint and mitotic catastrophe

To prevent chromosome aberrations, the classical DNA damage checkpoints arrest the cell cycle before entering mitosis. However, in 1955 cell cycle arrest at the stage of metaphase was described in a plant model for the first time [84]. The cells did not enter anaphase until all chromosomes were aligned in the equatorial plate. Further investigations revealed the existence of a mitotic checkpoint, also called spindle assembly checkpoint (SAC). This checkpoint also targets the corresponding CDK/Cyclin complex, precisely CDK1/Cyclin B1. However, in contrast to DNA damage checkpoints, it does not prevent but promote its activation. As described before (chapter 1.1.4.1), CDK1/Cyclin B1 is required for the entry into mitosis and the assembly of the mitotic spindle. In order to promote metaphase-anaphase transition and mitotic exit, however, the CDK1/Cyclin B1 complex needs to be inactivated and degraded. Thus, mitotic arrest is mediated by keeping CDK1/Cyclin B1 active, which prevents metaphase-anaphase transition [85,86,87].

During the metaphase, the microtubules of the mitotic spindle attach to the centers of the chromosomes, the kinetochores, and align them along the equatorial plate. This is a crucial step that, during the following anaphase, ensures the equal distribution of the sister chromatids between the two daughter cells. The mitotic checkpoint complex (MCC) monitors the alignment of the

chromosomes. The kinetochores of non- or miss-attached chromosomes are bound by proteins of the MCC and prevent tension to the spindle poles. Upon correct alignment, the proteins of the complex degrade and, thus, allow chromosome separation. The actual separation, however, requires further steps. The separation of the sister chromatids is negatively controlled by Securin (Pds1). Securin inhibits the cysteine protease Separase (Esp1) and thereby prevents the cleavage of the cohesin protein complexes that hold together the sister chromatids [88]. Securin in turn, is under the control of the anaphase promoting complex/cyclosome (APC/C), a highly conserved complex of at least eight subunits, first identified in clam oocytes [89,90]. Its central enzyme is an E3 ubiquitin ligase that promotes the proteasomal degradation of mitosis inhibiting proteins. Induction of the APC/C triggers degradation of Securin, Separase release, cohesion cleavage and movement of the sister chromatids to the spindle poles [91]. Besides Securin, Cyclin B1 is one of the most important targets of the APC/C. APC/C mediated degradation of Cyclin B1 inactivates CDK1/Cyclin B1 and promotes mitotic progression [91,92]. To prevent miss-segregation by premature separation, the APC/C is under negative control by the MCC. Only the correct alignment of the chromosomes and the subsequent downregulation of the MCC give rise to the activation of the APC/C, chromatid separation and mitotic progression [93,94].

Defects in mitosis regulation and chromatin segregation activate a program called mitotic catastrophe that leads to mitotic cell death. As morphology features giant non-viable cells with two nuclei or multiple micronuclei have been described, features that derive from premature envelope formation around chromosome fragments and the omitting of cytokinesis [95]. The mechanisms of mitotic cell death induction are still a matter of controversy. Many apoptotic characteristics were observed in the process of mitotic catastrophe and initially suggested that mitotic catastrophe triggers apoptosis [96]. However, the unique morphology of mitotic catastrophe and the fact that inhibition of apoptosis by overexpression of Bcl-2 or deletion of Bax/Bak even promotes mitotic catastrophe, implicates another type of programmed cell death distinct from apoptosis [97,98].

Evading mitotic arrest and mitotic catastrophe is associated with premature mitotic exit and termed as mitotic slippage. The consequences are polyploidy and aneuploidy, followed by genome instability [99]. Thus in cancer, polyploidy is associated with aggression stage, malignancy and poor prognosis [100]. Many anti-mitotic drugs such as cisplatin, vincristine and nocodazole were found to induce polyploidy [101,102,103]. This is problematic as cisplatin and vincristine are approved for the treatment of NHL.

The regulatory mechanisms that supervise the spindle assembly checkpoint and mitotic catastrophe are largely unknown. However, the checkpoint kinase Chk2 seems to play a crucial role. Activation of Chk2 delays mitotic exit and protects from mitotic catastrophe [104,105]. In contrast, loss of Chk2 is associated with genome instability [106,107]. Another key event seems to be the loss of p53 and

homologs. Cells of $p53^{-/-}$ knockout mice show genome instability and polyploidy, which is even more pronounced when p73 is knocked out as well [108,109]. Conversely, polyploid cells respond better to chemotherapeutics when p53 is overexpressed [110].

Dipping into the field of endoreplication also might be helpful to understand mitotic catastrophe, mitotic slippage and polyploidy. During endoreplication, polyploid cells are formed by skipping mitosis at different stages, a normal phenomenon observed throughout fauna and flora [111,112]. In mammals, the most prominent examples are the hematopoietic megakaryocytes and differentiated liver cells. The underlying mechanisms are still under investigation. Yet, inhibition of the CDK1 activity seems to be a crucial event [113,114]. In addition, two signaling pathways are implicated in promoting endoreplication, the MAPK pathway and Notch signaling [115,116].

1.1.5 The involvement of PI3K/Akt/mTOR

The PI3K/Akt/mTOR pathway promotes cell cycle progression by interfering with the CDK/Cyclin machinery on multiple levels (Figure 4). Best characterized is the PI3K/Akt/mTOR dependent progression through G1 phase, a circumstance resulting from the fact that cells treated with inhibitors of the PI3K/Akt/mTOR pathway mostly arrest in G1phase. The first mechanism how PI3K/Akt/mTOR forwards G1 transition was discovered, when interleukin-2 (IL-2) was found to activate the transcription factor E2F in a PI3K dependent manner [117]. As described before (chapter 1.1.4.1), E2F mediated transcription of cell cycle progressing components is inhibited by pRB. Hyper-phosphorylation inactivates pRB and releases E2F. In line with this, inhibition of PI3K or mTORC1 down regulates hyper-phosphorylation and prevents inactivation of pRB. This is probably mediated by mTOR dependent inhibition of specific phosphatases [118]. A second mechanism was provided by the discovery of Cyclin D1 as target of the PI3K/Akt/mTOR pathway [119]. Cyclin D1 is required for the formation of CDK4/Cyclin D1 and CDK6/Cyclin D1 and, thus, G1 progression. The translation of Cyclin D1 is promoted by the Akt downstream target mTORC1 [120]. In addition, transcription and degradation of Cyclin D1 are under the control of another downstream target of Akt, Glycogen synthase kinase-3beta (GSK3β). GSK3β not only inhibits ß-catenin dependent Cyclin D1 transcription, it also marks Cyclin D1 for nuclear export and proteasomal degradation by direct phosphorylation at T286 [121,122]. The induction of PKB/Akt signaling inhibits the activity of GSK3β and thereby promotes expression and stabilization of Cyclin D1. In sum, PI3K/Akt/mTOR increases the protein levels of Cyclin D1, which supports the assembly of G1 phase CDK complexes and hyper-phosphorylation of pRB.

Hyper-phosphorylation of pRB promotes the E2F dependent transcription of Cyclin E and Cyclin A. Thus, PI3K/Akt/mTOR signaling is also involved in G1/S transition and S phase progression. Furthermore, PI3K/Akt/mTOR controls the activity of the G1/S phase CDK2. Via direct interaction, the

center pathway protein PKB/Akt phosphorylates CDK2 at T39, which enhances its binding to Cyclin A [123]. Furthermore, PKB/Akt enhances the activity of the S phase CDKs by negatively regulating the CKIs p21 and p27. The direct binding of p21 to CDK2 is prevented by PKB/Akt dependent phosphorylation of p21 at T145 [124]. PKB/Akt furthermore stabilizes MDM-2, which inhibits p53 dependent p21 gene expression [125,126]. Similarly, p27 is phosphorylated by Akt at T157, which promotes its dissociation from CDK2/Cyclin E and its nuclear export [127].

Thus, the usual observations with PI3K/Akt/mTOR inhibitors are G1 arrest accompanied by downregulation of the Cyclin D1 protein level and upregulation of p21 and p27 [128,129,130].

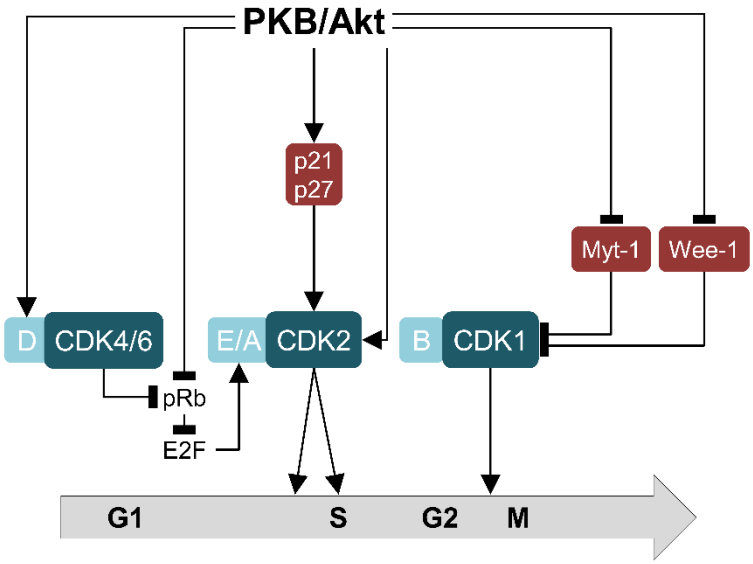

Figure 4: The involvement of PKB/Akt in cell cycle progression. Akt signaling primarily promotes G1/S progression. Early G1 phase progression is supported by inhibition of pRB and upregulation of Cyclin D1. During late G1 progression and G1/S transition, Akt supports the binding of CDK2 to Cyclins and inhibits the CKI family members p21 and p27. The impact on G2 phase and M phase progression is poorly understood. Yet, Akt dependent inhibition of Myt-1 and Wee-1 is suggested to activate CDK1/Cyclin B1, the M phase CDK complex.

The importance of the PI3K/Akt/mTOR pathway for G1/S progression is demonstrated by another phenomenon. PI3K/Akt/mTOR signaling was shown to participate in the tetraploidization of liver cells and the EZH2-mediated genomic instability and polyploidy of breast cancer cells [131,132]. Thinking this forward, initiation of DNA replication and possibly endoreplication are PI3K/Akt/mTOR dependent. However, endoreplication is accompanied by skipping or escaping mitosis. A role for PI3K/Akt/mTOR signaling in that might be possible. Recent reports demonstrated that activation of PKB/Akt overcomes the DNA damage induced G2/M checkpoint [54,55,133]. This event is associated with the inactivation of Wee-1 and Myt-1, the two kinases that are responsible for the inhibitory phosphorylation of CDKs at T14/Y15.

1.2 PI3K/AKT/MTOR AND PROGRAMMED CELL DEATH

PI3K/Akt/mTOR signaling promotes survival and protects cells from programmed cell death. Thereby, PI3K/Akt/mTOR attenuates the execution of a variety of cell death programs, such as apoptosis, autophagy and possibly mitotic catastrophe. In this introduction part, the basics of the cell death programs are outlined. Furthermore, the known implications of PI3K/Akt/mTOR signaling on these programs are illustrated.

1.2.1 Apoptosis

Apoptosis is a physiological process to eliminate unneeded, damaged or dangerous cells. Based on studies in the model system *Caenorhabditis elegans,* a highly conserved and molecular programmed mechanism with a distinct morphology has been uncovered, widespread among fauna and flora [134,135,136]. In animal cells, the early features are cell shrinking, chromatin condensation and translocation of phosphatidylserine to the outer membrane. Subsequently, cell blebbing is initiated and the caspase-activated DNase (CAD) cleaves the DNA into nucleosomal units of 180bp and multiples. Finally, the cell decomposes into apoptotic bodies. The early presentation of phosphatidylserine attracts phagocytes but, at the same time, inhibits their inflammatory response. So apoptotic cells are engulfed by the immune system without causing an inflammation.

On molecular level, these morphological processes of apoptosis are conducted by a proteolytic signaling cascade. The main players of this cascade are the cysteine-dependent aspartate-directed proteases (caspases). Caspases are synthesized as inactive zymogens containing an n-terminal prodomain, and a c-terminal protease domain. Death signaling triggers the auto-proteolytic cleavage of the prodomain and further cleavage within the protease domain, which produces a large and a small subunit. A hetero-tetramer of two small and two large subunits, then, forms an active caspase complex. Two groups of caspases, initiator caspases (caspase-2, -8, -9, -10) and effector caspases (caspase-3, -6, 7), establish a hierarchical signaling cascade that finally leads to the execution of apoptosis. Thereby, the initiator caspases perceive the death signal and pass it on to the downstream effector caspases. Activation of the initiator caspases occurs by two distinct mechanisms, extrinsic death receptor signaling or intrinsic stress signaling (Figure 5).

1.2.1.1 The Extrinsic pathway

Forcing cells into programmed cell death is an important regulatory mechanism to eliminate, for instance, auto-reactive immune cells, infected cells or cancer cells. The apoptotic program of such cells is induced by the superfamily of tumor necrosis factors (TNFs). FasL/CD95L and tumor necrosis factor alpha (TNFα) are the best-characterized members of this family. However, TNF-related apoptosis-inducing ligand (TRAIL) is of special interest due to its ability to induce apoptosis selectively in tumors

[137]. Four TRAIL receptors have been identified so far, of which only TRAIL-R1 and TRAIL-R2 have a death domain (DD) carrying cytosolic region. This domain is essential for the activation of initiator caspases and apoptotic signaling. Upon ligand-receptor binding, the adaptor proteins FADD and TRADD are recruited to the cytosolic region of the receptor by their DD. These adapter proteins contain multiple protein binding domains that can link protein partners together. In case of the receptors of the TNF superfamily, the two major adapter proteins are TNFR-associated death domain (TRADD) [138] and Fas-associated death domain (FADD) [139]. TRADD mainly interacts with growth and survival pathways like MAPK and NFκB, while FADD primarily coordinates apoptotic signaling, although, also a function in cell cycle progression has been reported [140]. FADD possess a death domain (DD) at the c-terminus [141] which, directly interacts with the trimerized intracellular DDs of Fas and TRAIL-R or, in case of TNFR, uses the DD of TRADD as an adapter. At the n-terminus, FADD carries a death effector domain (DED). By homotypic interaction, the DED of FADD recruits other DED carrying proteins, like procaspase-8 or -10, to the receptor to form the death inducing signaling complex (DISC). Multiple caspases are recruited to the receptor by DED protein-protein interaction and, thereby, are brought in close proximity to each other. Now, by autoproteolytic activity, the initiator caspases are cleaved and become activated. The FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (Flip) also possess two DEDs and, as well, is recruited to the DISC. Binding of Flip to FADD displaces the initiator procaspases and prevents their cleavage and activation of the caspase cascade. Flip, usually a short-lived protein regulated by proteasomal degradation, was found to be up regulated in many tumors and is correlated with poor prognosis [142]. Conversely, downregulation of Flip by siRNA, ER stress or translational suppression sensitizes for death receptor induced apoptosis [143,144,145,146]. Initiator caspase activation requires homodimerization followed by a stepwise cleavage of the prodomain and the small subunit [147]. Then, two large and two small subunits form an active initiator-caspase complex. Once the caspase cascade is activated, rapid transmission of the apoptotic signal by cleavage of the downstream effector caspases-3, -6, and -7 occurs. In type I cells DISC triggered initiator caspase activation is sufficient to activate downstream effector caspases directly. However, in type II cells effector caspases are not sufficiently activated, and the caspase cascade needs amplification by the intrinsic pathway. In this case, it is thought that the family of IAPs, especially XIAP, inhibits effector caspases like caspase-3. Even when caspase-3 is cleaved and activated by caspase-8, the presence of XIAP rapidly causes caspase-3 degradation and prevents forwarding of the caspase cascade. Downregulation of XIAP can turn type II cells into type I cells and overcomes intrinsic pathway resistance [148].

1.2.1.2 The Intrinsic pathway

Activation of the intrinsic initiator caspases is triggered by Apaf-1. Apaf-1 owns three functional units, a domain to bind caspases (Caspase activation and recruitment domain - CARD), a domain to bind ATP (nucleotide-binding and oligomerization domain - NOD) and a domain to bind cytochrome c (WD40 region). Under normal conditions, Apaf-1 is locked in an inactive state in the cytosol [149]. In the presence of cytochrome c and ATP, seven Apaf-1 monomers oligomerize and form a complex, the apoptosome. Thereby, the Apaf-1 CARDs are oriented in the center of the complex. A variety of procaspases, such as initiator caspase-9, has a corresponding CARD within their prodomain. By CARD-CARD interaction, the procaspases of caspase-9 are recruited to the center of the complex and colocalized to each other. The close proximity then enables autoproteolytic cleavage and activation of caspase-9. The exact mechanism is still not clearly resolved. A caspase-9 dimerization model and the apoptosome as holoenzyme are discussed, although, dimers have been shown to be much less active than the holoenzyme [150,151,152]. Once activated, caspase-9 triggers the cleavage and activation of effector caspases similar to capase-8 and caspase-10.

Usually, the binding partner of Apaf-1, cytochrome c, is a central protein within the respiratory chain and has O_2-oxidoreductase function. Accordingly, it is associated with the inner membrane of the mitochondria. This localization impairs spontaneous apoptosome formation and caspase-9 activation. Release of cytochrome c into the cytosol is thought to be caused by pore formation in the outer mitochondrial membrane. This enables the influx of molecules, swelling of the mitochondria, disruption of the proton gradient and subsequently the release of cytochrome c. Therefore, mitochondrial membrane permeabilization is an essential event for apoptosome formation and intrinsic signaling. The permeabilization of the mitochondria depends on apoptotic signals and requires the participation of the Bcl-2 family.

1.2.1.3 The Bcl-2 family

Intrinsic stress signaling and mitochondrial depends on the Bcl-2 family, termed by the first member discovered in b-cell follicular lymphomas [153]. The Bcl-2 family proteins share homology in one to four regions, named the Bcl-2 homology (BH) domains. Based on the present domains, the family is subdivided into three groups. The antiapoptotic Bcl-2 family, including Bcl-2, Bcl-xL and Mcl-1, possesses all four domains. The proapoptotic multi-domain or Bax family lacks the BH4 domain, and proapoptotic proteins that contain only the BH3 domain, such as Puma, Bim and Nbk, are designated as BH3-only family.

The Bcl-2 members interact in a complex hierarchical system to coordinate survival or apoptosis. In living cells, proapoptotic Bax and Bak, the executors of apoptosis, are held in check by the

antiapoptotic Bcl-2 members. Triggering apoptosis initiates the activation of BH3-onlys, which then compete with Bax and Bak for their binding sites within the antiapoptotic proteins. As the antiapoptotic Bcl-2 members have a higher affinity to BH3-only proteins, Bax and Bak are released. Free Bax and Bak, now, forward the apoptotic signal on to the mitochondria. This postulated processing is referred to as indirect activator model. A second model, called direct activator model, is discussed where BH3-only proteins are further divided into activators and sensitizers. Activators are proposed to directly bind and activate Bax and Bak, similar to antiapoptotic Bcl-2 members, whereas sensitizers only inhibit the antiapoptotic family [154,155]. Although direct interaction of Bax with Bid has been shown this model is critical as cells deficient for the activators still are able to induce apoptosis [156,157].

How Bax and Bax cause mitochondrial outer membrane permeabilization (MOMP) is still debated. The conformational change of Bax and Bak, however, seems to be crucial [158]. In the inactive state, the transmembrane domains, as well as the BH3 domain, of Bax are masked inside the hydrophobic core. Apoptosis induction causes the exposure of the BH3 domain, which allows oligomerization, and the exposure of the n-terminal residue, which allows integration into the mitochondrial membrane. One postulated mechanism is, that integration of Bax and Bak into the membrane and subsequent oligomerization leads to the formation of transmembrane pores that might, or might not, be big enough to permit the release of apoptotic factors [159,160]. The other mechanism is that Bcl-2 family members interact with the permeability transition pore complex (PTPC), consisting of the voltage-dependent anion channel (VDAC) in the outer membrane, the adenine nucleotide translocase (ANT) in the mitochondrial inner membrane and cyclophilin D (CYPD) in the mitochondrial matrix. Binding of Bcl-2 to the PTPC only permits the exchange of metabolites, while capturing of Bcl-2 by BH3-onlys enables Bax to bind the PTPC, which causes massive influx of water and ions, swelling of the mitochondria, rupturing of the outer membrane and, finally, release of apoptotic factors [161,162,163].

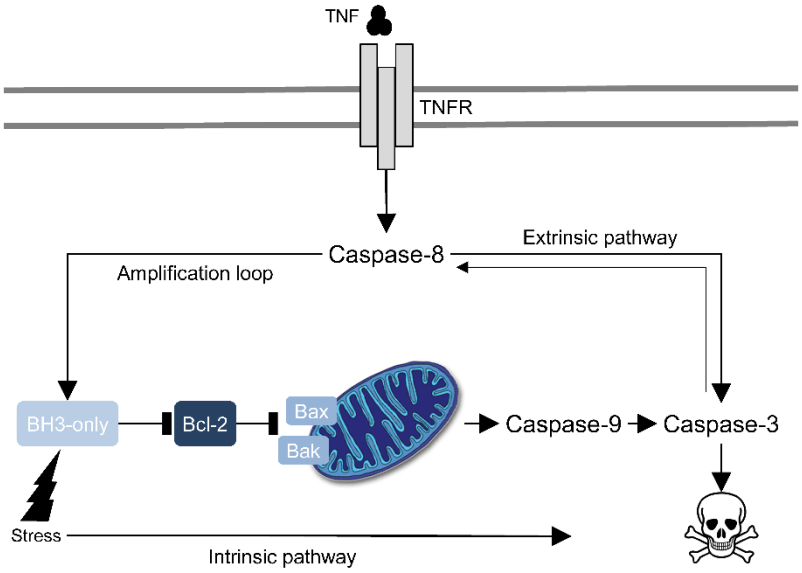

Figure 5: Apoptotic signaling. Two distinct pathways execute apoptosis. Extrinsic signaling is triggered by external death ligands and transition of the death signal into the cell. Initiator caspase-8 is recruited and activated at the receptor. Initiator caspases directly cleave and activate effector caspases, which executes apoptosis. Intrinsic signaling is a response to cellular stress signals, which causes the activation of BH3-onlys. BH3-onlys compete for binding to antiapoptotic Bcl-2 members by replacing Bax and Bak. Unbound Bax and Bak form pores in the mitochondria, which releases intra-mitochondrial factors into the cytoplasm and leads to the activation of intrinsic initiator caspases. Both pathways are linked, as caspase-8 can trigger the intrinsic pathway by directly activating the BH3-only Bid.

1.2.1.4 The role of PI3K/Akt/mTOR

The PI3K/Akt/mTOR pathway promotes survival by negatively regulating apoptotic signaling on multiple levels. Thereby, mainly intrinsic signaling is attenuated by PI3K, Akt and mTOR (Figure 6).

The intrinsic initiator caspase, caspase-9, is under direct and indirect control of PI3K/Akt. Direct PKB/Akt mediated phosphorylation at S196 inhibits the protease activity of caspase-9 and prevents cytochrome c dependent processing [164,165]. In this way, cells become resistant to stimuli that promote apoptosis via the mitochondrial pathway, like staurosporine and etoposide. In addition, the PI3K/PDK1/PKCζ axis promotes another inhibitory phosphorylation at S144 [166,167].

The sensoring of stress signals by the proapoptotic Bcl-2 family subgroup of BH3-onlys, which is required for mitochondrial apoptosis, is negatively regulated by PI3K/Akt/mTOR signaling as well. Best characterized is the Akt mediated modulation of the BH3-only Bad. Akt phosphorylates Bad at S136, which initiates its binding 14-3-3 and, thus, prevents its interaction with and inactivation of antiapoptotic counterparts [168,169,170,171,172]. In contrast, withdrawal of growth factors abrogates PKB/Akt signaling and revokes the inactivation of Bad. The strongest inducer of apoptosis, however, is Bim. Bim is also a target of Akt/mTOR signaling. Direct phosphorylation at S87 attenuates

Bim's proapoptotic function and is mediated by PKB/Akt [173]. Moreover, the transcription of Bim is under the negative control of the Akt/mTOR/FoxO axis [174].

Furthermore, PI3K/Akt/mTOR signaling abrogates the transition of proapoptotic stress signals down to the mitochondria by promoting expression and stabilization of antiapoptotic Bcl-2 members. Growth factors initiate the expression of antiapoptotic Bcl-2, Mcl-1 and Bcl-xL by several transcription factors such as CREB, STATs and NFκB [175,176,177]. All these transcription factors are reported targets of the PI3K/Akt/mTOR pathway [175,178,179,180]. The translational control of antiapoptotic Bcl-2 members contributes to mTOR. For instance, upregulation of Mcl-1 translation by mTOR is associated with apoptotic resistance [181]. In contrast, stabilization of antiapoptotic Bcl-2 family proteins, for instance Mcl-1, the PI3K/Akt/mTOR pathway conducts by inhibiting the function of GSK3β, an enzyme marking proteins for proteasomal degradation [182].

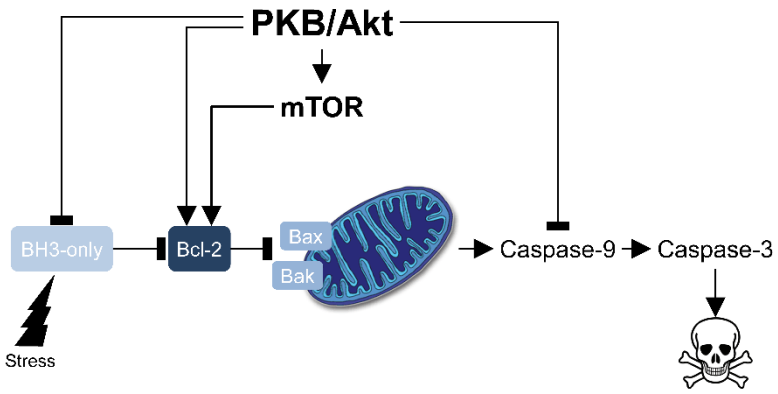

Figure 6: The prosurvival impact of the PI3K/Akt/mTOR pathway on apoptosis signaling. PKB/Akt negatively regulates the activity of proapoptotic BH3-only proteins (red), promotes the expression and activity of antiapoptotic Bcl-2 members (green) and abrogates the activation of caspases. mTOR is likewise involved in the modulation of antiapoptic Bcl-2 family members.

1.2.2 Autophagy

Autophagy is reported to be both, a mechanism to preserve times of nutrient deprivation and a form of programmed cell death. During autophagy, organelles and proteins are degraded in autophagosomes. This mechanism provides nutrients and promotes survival in terms of ER stress or starvation [183,184]. It also constitutes a mechanism to remove damaged organelles and missfolded proteins. Excessive digestion of organelles, however, is suggested to cause cell death. Autophagy and apoptosis are closely linked and it is still not clear whether autophagy leads to apoptosis or rather provides a distinct form of programmed cell death. The autophagosome formation is orchestrated by the large family of autophagy related genes (ATGs). ATG6, Beclin-1 in mammals, together with class III PI3 kinases is needed for the initiation of autophagy. Beclin-1, however, also is a BH3-only protein and can be inhibited by antiapoptotic Bcl-2 members. Inhibition of Beclin-1 by Bcl-2 abrogates autophagy

and prevents cell death [185]. In line with this, inhibition of autophagy with pharmaceuticals or by downregulating of Beclin-1, promotes apoptosis [186]. This would suggest that autophagy primarily is a survival mechanism and that in the absence of apoptosis autophagy is impaired as well. However, knockout of Bax and Bak induces autophagy in etoposide or irradiation treated cells, which suggests autophagy as an alternative form of cell death [187,188]. Moreover, caspase-3 can inactivate Beclin-1 by cleavage and, thus is a negative regulator of autophagy [189].

One of the key regulators of autophagy is mTOR. mTOR signaling negatively regulates Beclin-1 activity and thereby prevents phagosome formation. Nutrient deprivation inhibits mTOR activity and promotes autophagy. The same is observed when mTOR is directly inhibited with rapamycin or RAD001 [190,191]. Interestingly, autophagy that is induced by mTOR inhibition protects from apoptosis, which is consistent with the hypothesis of autophagy as survival mechanism [192,193].

1.2.3 Mitotic death

As described above (chapter 0), the question whether mitotic death is a distinct type of programmed cell death still needs to be answered. Features assigned to apoptosis were also observed during mitotic death, like DNA fragmentation, release of mitochondrial factors and activation of caspase-3 [96]. However, caspase dependent and caspase independent mitotic death is reported [194], which doubts apoptosis as the terminus of mitotic catastrophe. This is further underlined by the different morphology of cells undergoing mitotic death or apoptosis. Apoptotic cells are characterized by chromatin condensation and cytoplasm shrinking, while cells in mitotic death show cell expansion and uncondensed chromatin. Furthermore, the typical DNA ladder of apoptotic cells is usually not detected during mitotic death [95].

The involvement of the PI3K/Akt/mTOR pathway in mitotic cell death is poorly understood. A recent report demonstrated that the PI3K inhibitor Wortmannin induces both interphase and mitotic cell death [195]. Furthermore, PI3K inhibition promotes cell death induced by the anti-mitotic drug nocodazole. One possible mechanism provides the observation that PI3K/Akt signaling promotes microtubule stabilization and that PI3K inhibition causes missorientation of the mitotic/meiotic spindle [196,197].

1.3 PI3K/AKT/MTOR INHIBITION AS A THERAPEUTIC OPTION IN NON-HODGKIN LYMPHOMAS

Constitutive activation of the PI3K/Akt/mTOR pathway is a frequent event in non-Hodgkin lymphomas. For instance, S473 phosphorylation of Akt is detectable in 40% to 50% of diffuse large b-cell lymphoma (DLBCL), the most common form of NHL [2,198]. This activation of PI3K/Akt signaling highly correlates

a *PTEN* gene deletion-dependent loss of protein expression, but only in the germinal center b-cell like (GCB) subtype of DLBCL [199,200]. In contrast, frequent copy number variation within the genes encoding for PI3K and Akt were found in both, GCB and non-GCB [9]. Downstream of PI3K/Akt, a high expression rate of mTOR correlates with gender, age and shorter survival rate [201].

Likewise, activation of Akt is observed in 40% to 80% of mantle cell lymphoma (MCL) [1,202]. Thereby, all aggressive blastoid variants of MCL are positive for Akt phosphorylation, but only 30% of the classical MCL [1]. This Akt activation, again, correlates with the loss of PTEN function. Although, loss of protein expression is observed, the loss of function rather contributes to inactivation by phosphorylation [1,202]. In addition, MCL show high expression of the *PI3KCA* gene product p110α, the prevalent catalytic subunit of class IA PI3K, and relapse of MCL significantly correlates its increased expression [203,204]. This high expression contributes to an increase in the gene copy number of *PI3KCA* and the associated upregulation of the mRNA level. In consequence to Akt activation, the downstream target mTOR is activated as well [1,205].

In the NHL subtype of Burkitt lymphoma (BL), the mechanisms of constitutive PI3K/Akt/mTOR signaling are more complex [206]. Only a minority of 7% show mutation of *PTEN*. Another indirect mechanism affects PTEN expression. The *MYC* gene, constitutively expressed in BL, controls the mir-17-92 miRNA cluster, of which miR-19a/b attenuates PTEN expression. c-Myc itself is a downstream target of the Akt/GSK3β pathway. GSK3β phosphorylates c-Myc at T58 and promotes its proteasomal degradation [207]. PKB/Akt inhibits GSK3β and, thereby, stabilizes c-Myc. In this way, c-Myc and PI3K/Akt mutually maintain their signaling. Activation of the PI3K/Akt/mTOR pathway further depends on sustained B-cell-receptor (BCR) signaling. In BL, frequent expression of the transcription factor TCF-3 upregulates BCR expression and promotes its signaling.

Initial success to target the constitutive activate PI3K/Akt/mTOR pathway in non-Hodgkin lymphomas was achieved with the mTOR inhibitor rapamycin *in vitro*. Rapamycin induces G1 arrest and inhibits the proliferation of cell lines derived from DLBCL, MCL and BL [208,209,210]. However, rapamycin is poorly soluble in water and unstable in PBS or HEPES [211,212]. This and the fact that rapamycin is metabolized by the cytochrome P450 3A4, contribute to its low bioavailability [213,214]. For clinical use, second-generation analogs, so called rapalogs, were developed. For instance, in phase II clinical trials, the rapalog RAD001/everolimus has moderate overall response rates of about 20% to 30% in relapsed NHL [215,216]. Likewise, the overall response rate to the rapalog CCI-779/temsirolimus was about 28% in DLBCL [217]. However, in relapsed MCL, temsirolimus reached overall response rates of about 40% in phase II trials and 22% in a phase III trial [218,219,220]. Thereupon, temsirolimus was approved for the treatment of refractory and relapsed MCL.

Yet, all rapalogs share two disadvantages, the incapacity to kill tumor cells and the reactivation of PKB/Akt via mTORC2 or the S6K/IRS-1/PI3K axis. As already observed in renal cell carcinoma (RCC), prolonged exposure to temsirolimus promotes resistance, a mechanism most certainly depending on the mTORC2 mediated Akt activation [221]. Therefore, inhibiting PI3K/Akt/mTOR signaling focused on the upstream PI3 kinase. The first PI3K inhibitors were LY294002 and Wortmannin that still have their use in basic research. LY294002, for example, inhibits Akt signaling and induces apoptotic cell death in cell line models of DLBCL, MCL and BL [2,205,222,223]. However, both failed in clinical trials, due to dermal and liver toxicity, low bioavailability and instability. On the search for suitable PI3K inhibitors, the screening for structures binding the ATP site of the class I catalytic subunits lead to discovery of 6-hydroxyphenyl-2-morpholino pyrimidines. By further modifications, improving potency and solubility, the new inhibitor NVP-BKM120 (BKM120) was raised [224]. BKM120 is highly selective to all four class I catalytic subunits of PI3K and decreases the level of phosphorylated Akt in various cell line models [225,226,227,228]. Moreover, BKM120 was shown to induce cell death. However, the terms for BKM120 mediated cell death are not clear as features of apoptosis, autophagy and mitotic catastrophe were reported [227,228,229]. Further examination is strongly required before considering BKM120 for the clinical use. Nevertheless, the current recruiting for phase II clinical trials demonstrates the interest in BKM120 for the treatment of NHL (NCT01693614, NCT01719250).

Dysregulation of the PI3K/Akt/mTOR pathway commonly accounts for the general and acquired resistance against several therapeutic strategies. Therefore, another approach is the combination of PI3K or mTOR inhibitors with established therapeutics. Most promising for NHL seems to be the combination with the CD20 antibody rituximab. In DLBCL cell line models, rituximab and rapamycin synergistically downregulate PI3K/AKT/mTOR signaling and enhance cytotoxicity [209,230]. Moreover, in a phase II study an overall response of 38% was achieved [231]. In a phase II trial in MCL, combination of rapamycin with rituximab even gained overall response rates of 59% [232]. Also in BL combination of rituximab with mTOR inhibitors is a suggested strategy as acquired resistance to rituximab is associated with a deregulated PI3K/Akt/mTOR pathway [233]. In cell line models of other hematological malignancies, PI3K/mTOR inhibitors were shown to synergize with radiation, proteasome inhibitors and cytostatics such as cytarabine, vincristine and doxorubicin [234,235,236]. Those cytostatics are also part of the treatment protocols for DLBCL, MCL and BL. Moreover, the proteasome inhibitor bortezomib has approval for the treatment of NHL in the USA and shows high sensitivity especially in MCL [237]. Therefore, their combination with PI3K/mTOR inhibitors might be effective in NHL as well. Indeed, in MCL combination of RAD001 with doxorubicin, vincristine and bortezomib were synergistically cytotoxic [238].

Recently, the combination of PI3K/mTOR inhibitors with agents that target apoptotic signaling has been reported. So called BH3-mimetics, inhibitors of the antiapoptotic Bcl-2 family, kill NHL in cooperation with rapamycin or LY294002 [239,240]. In general, targeting apoptotic signaling is a popular strategy to kill tumors of any entity. Besides BH3-mimetics, also the ligand TRAIL is in this focus due to its preference to kill tumor but not normal cells [137]. Synergistic combination of TRAIL and PI3K/Akt/mTOR inhibitors is reported for several cancer types and, therefore, might be an interesting strategy for the treatment of NHL [241,242,243].

2 STUDY AIMS

The prosurvival PI3K/Akt/mTOR pathway is constitutively active in many cancers, including NHL. Therefore, targeting this pathway is a promising therapeutic strategy for the treatment of NHL. So far, however, PI3K/Akt/mTOR inhibitors hardly reached clinical trials or even got approval. In part one of this study, the aim was to find out whether the newly developed pan-PI3K inhibitor NVP-BKM120 (BKM120) is suitable for the therapy of NHL. In detail, it was to be examined whether BKM120 inhibits PI3K/Akt/mTOR signaling and has antitumor activity in NHL cell lines. Moreover, the impact of BKM120 on cell cycle regulation, particularly G2/M transition and mitosis, was to be explored.

Compensatory activation of the PI3K/Akt/mTOR pathway is implicated in the resistance to chemical therapeutics. Therefore, the combination of PI3K/Akt/mTOR inhibitors with established cytotoxic agents is a promising strategy to prevent resistance and enhance the cytotoxic effect. In addition, the discovery of new combinations is suggested to expand the therapeutic field. In part two of this thesis, therefore, the combined administration of a panel of PI3K/Akt/mTOR inhibitors with several cytotoxic agents was investigated.

3 RESULTS

3.1 BIVALENT FUNCTION OF BKM120 IN NON-HODGKIN LYMPHOMAS

Based on structural comparison analysis, the pan-PI3K class I inhibitor NVP-BKM120 (BKM120) was developed (Novartis Pharma GmbH). BKM120 is a 2,6-dimorpholino pyrimidine derivate, which competitively inhibits the ATP binding site of PI3K. The specificity of BKM120 has previously been tested by protein kinase assay and revealed a highly selective inhibition of all class I PI3K isoforms at concentrations between 52nM and 262nM (Table 1) [225]. However, *in vitro* concentrations of 500nM to 1,000nM are needed to observe dephosphorylation and inactivation of downstream Akt in a variety of malignancies [225,228,244]. Nevertheless, in all these tumors BKM120 was shown to have antiproliferative effect. In the following part of this thesis, the impact of BKM120 on distinct non-Hodgkin lymphoma cell lines was analyzed. Precisely, the conditions for BKM120 to induce programmed cell death were examined. Beyond this, reasons and consequences of resistance were explored.

Table 1: BKM120 selectively inhibits the p110 subunit of class I PI3Ks. IC_{50} values against all PI3K classes and PI3K distinct kinases picturing 50% inhibition of kinase activity measured by four different methods [225].

	Enzym	IC_{50} [nmol/L]	Enzym	IC_{50} [nmol/L]	Enzym	IC_{50} [nmol/L]
Class I PI3Ks	p110α	52 ± 37 (n=7)	p110α-H1047R	58 ± 2 (n=2)	p110α-E545K	99 ± 6 (n=2)
	p110β	166 ± 29 (n=3)	p110δ	116 (n=1)	p110γ	262 ± 94 (n=7)
Class III PI3Ks	Vps34b	2410 ± 150 (n=19)				
Class IV PI3Ks	mTOR	2866 ± 1671	DNA-PK	> 10000	ATR	8091 ± 2038
PI4K	PI4Kβ	>25000 (n=22)				
Protein kinases	Axl	>10000	Fak	>10000	VEGFR2/Kdr	>10000
	Jak2	>10000	HER1/ErbB1	>10000	c-Abl	>10000
	IGF1-R	>10000	c-Src	>10000	EphB4	>10000
	PKA	>10000	Ret	>10000	Akt1/PKBα	>10000
	CSF1R	582	PDK1	>10000	c-Met	>10000
	B-Raf^{V599E}	9,2	K650E-FGFR	>10000	CDK2/cyclin A	>10000

3.1.1 BKM120 inactivates mTOR targets

First, the impact of BKM120 on PI3K signaling was investigated in the non-Hodgkin lymphoma (NHL) model system. Therefore, the two cell lines MINO and GRANTA-519 were incubated with increasing concentrations of BKM120 for 6h and the phosphorylation status of PI3K downstream kinases was captured using immunoblotting (Figure 7). In both cell lines, the phosphorylation of the downstream target PKB/Akt was reduced. However, to achieve this dephosphorylation a concentration of 2,000nM was required. In contrast, downstream of Akt, BKM120-dependent dephosphorylation of the mTORC1 targets S6K and 4EBP1 could be observed in both cell lines at lower concentrations. The target sites of mTORC1 on S6K are T389 and S371. To prevent phosphorylation at T389, 1,000nM or 1,500nM of BKM120 were needed in GRANTA-519 or MINO, respectively. Dephosphorylation at S371 was achieved with 1,000nM BKM120 in MINO and 2,000nM BKM120 in GRANTA-519. The second mTORC1 target, 4EBP1, is phosphorylated by mTORC1 at T37/46. In MINO, dephosphorylation at this site was detected with 1,000nM, while 1,500nM were required in GRANTA-519. According to a previous report [245], the multiple bands of 4EBP1 represent its phosphorylation status. Briefly, the upper bands correspond to hyperphosphorylated 4EBP1, whereas the lowest band displays hypophosphorylated 4EBP1. This assumes a BKM120 dependent dephosphorylation and possibly activation of 4EBP1. Altogether, concentrations of about 1,500nM of BKM120 were capable to downregulate but not to fully block PI3K and downstream signaling.

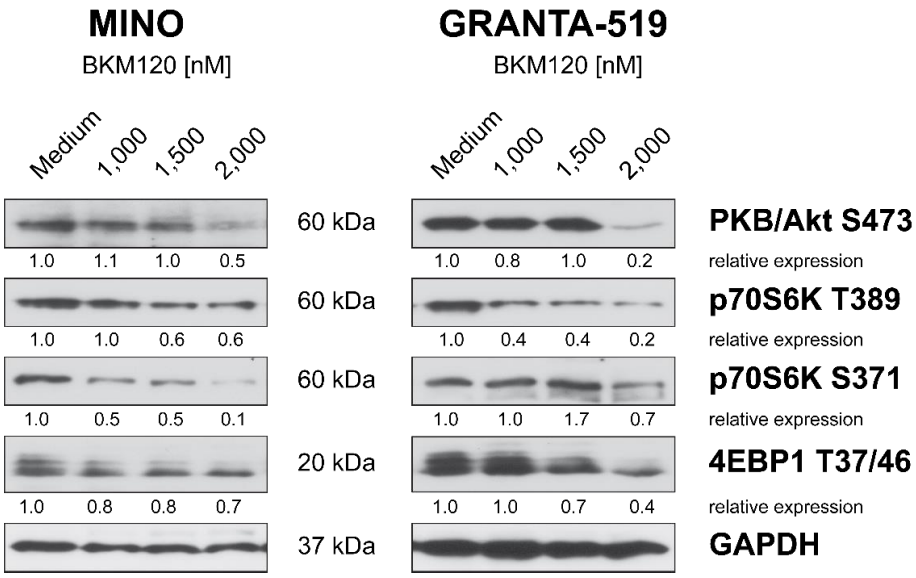

Figure 7: BKM120 inhibits PI3Kdownstream signaling. The two non-Hodgkin lymphoma cell lines MINO and GRANTA-519 were incubated with the indicated concentrations of BKM120 for 6h. The BKM120 dependent inactivation of PI3K downstream targets was probed by imaging their phosphorylation status with western blot analysis. The relative protein expression was determined in relation to the housekeeping protein GAPDH using Image J. Untreated cells were set to 1,0.

3.1.2 BKM120 inhibits proliferation and induces cell death

Next, the antitumor potential of BKM120 was examined. Therefore, eight NHL cell lines were incubated with increasing concentrations of the inhibitor for 72h. The impact on proliferation was determined by measuring the metabolic activity of the mitochondria using the XTT-assay (Figure 8A). BKM120 dose dependently inhibited the proliferation of the tested cell lines with an average IC_{50} value of about 1,000nM. Proliferation was completely abrogated at concentrations of 1,500nM and more.

Furthermore, the capacity of BKM120 to kill NHL cell lines was explored (Figure 8B). Therefore, the loss of the cell membrane integrity was detected by measuring the uptake of the membrane impermeable DNA intercalating dye propidium iodide (PI). PI uptake was observed in the cell lines MINO, REC-1, JEKO-1, GRANTA-519 and MAVER-1 in a dose dependent manner. In the cell lines CA-46, DG-75 and SU-DHL-10, however, PI uptake was barely detectable. Thus, a BKM120 death sensitive and a BKM120 death resistant subgroup were identified. In the sensitive subgroup, the average LC_{50} was about 1,400nM. This correlates with the observed concentration for maximal proliferation inhibition. In sum, BKM120 has the capacity to kill NHL cell lines.

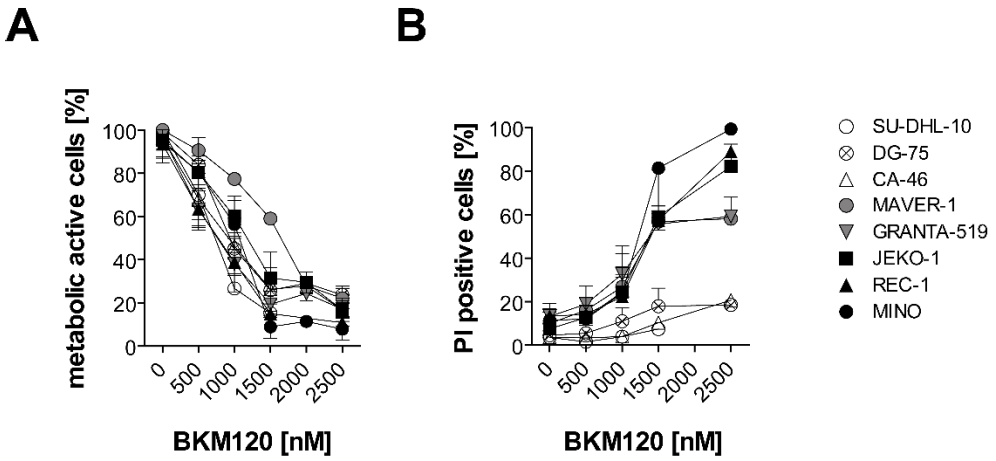

Figure 8: BKM120 inhibits the proliferation of NHL cell lines and has killing potential. Eight NHL cell lines were incubated with increasing concentration of BKM120 for 72h. (A) The proliferation rate was measured by detecting the metabolic activity with XTT assay (B) Cell death was determined by detecting the loss of membrane integrity through PI uptake.

3.1.3 BKM120 induces apoptotic cell death via the intrinsic pathway

Cell death can proceed in different ways, whereby apoptosis is the most common and best investigated. Typical characteristics of apoptosis are DNA condensation and externalization of phosphatidylserine at early stages, followed by cleavage of the DNA into 180bp fragments and blebbing of the cells into apoptotic bodies at late stages. To analyze, whether BKM120 induces cell death by apoptosis, the occurrence of fragmented DNA was measured. Therefore, the eight NHL cell

lines were incubated with increasing concentrations of BKM120 for 72h. Subsequently, the DNA was labeled with PI and the percentage of sub-G1 cells, representing cells with fragmented DNA, was detected by flow cytometry (Figure 9A). In the BKM120 death sensitive cell lines MINO, REC-1, JEKO-1 and GRANTA-519 a dose dependent increase of DNA fragmented cells was observed. The death sensitive cell line MAVER-1, however, showed very slight DNA fragmentation only at 2,000nM. As expected, in the death resistant cell line CA-46 no DNA fragmentation was detected. In the two BKM120 death resistant cell lines DG-75 and SU-DHL-10, surprisingly, the amount of cells with a sub G1 content increased, together with the concentration of BKM120.

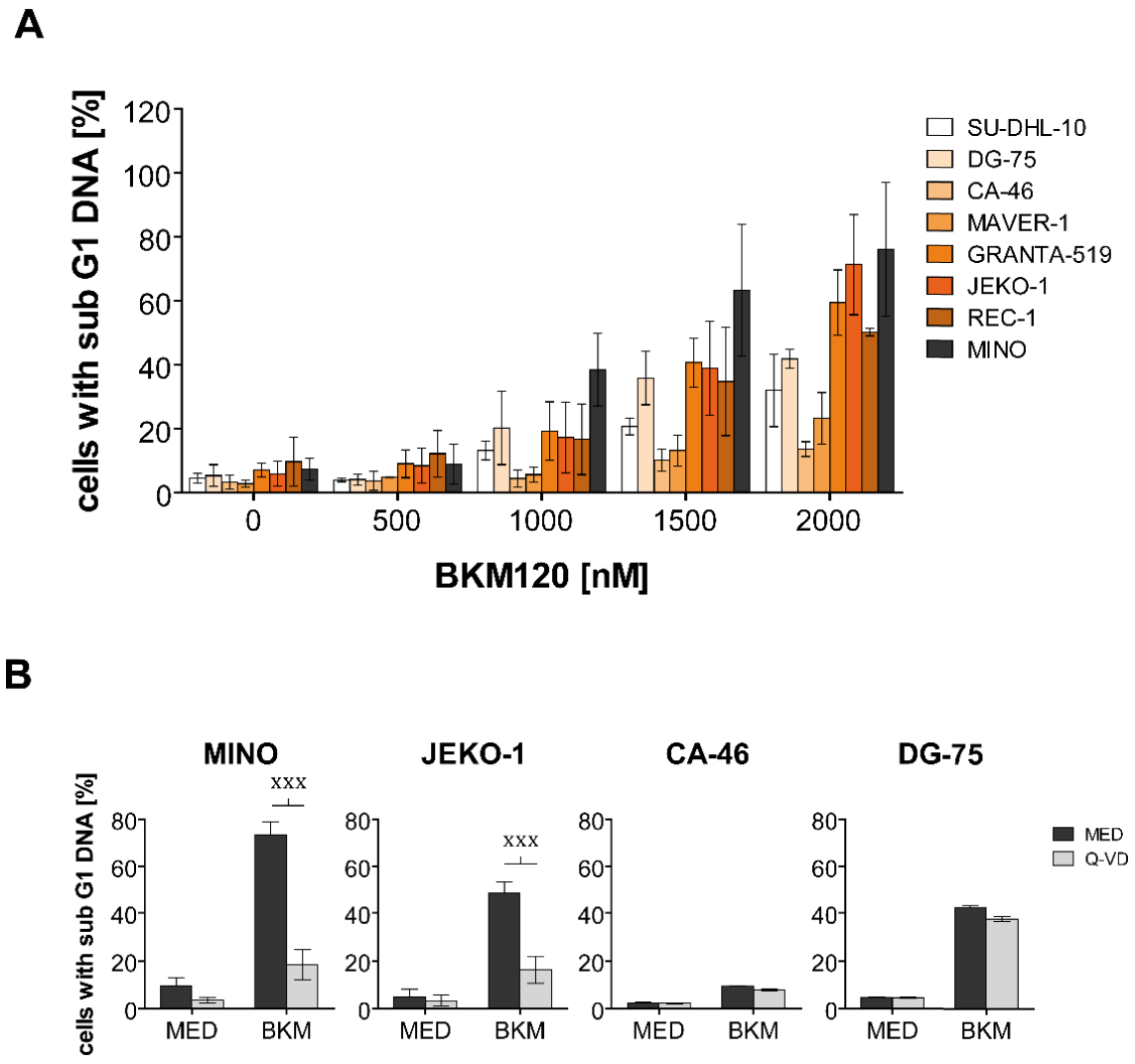

Figure 9: BKM120 death sensitive cells undergo caspase-dependent apoptosis. Diminished levels of DNA in response to BKM120 in the presence or absence of caspases. (A) Eight NHL cell lines were incubated with increasing concentration of BKM120 for 72h.The apoptotic rate was measured by detecting cells with a sub G1 content, representing DNA fragmentation. (B) Four representitive cell lines were pretreated with 10µM of the pan-caspase inhibitor Q-VD-OPh for 4h and then incubated with 1,500nM of BKM120 for another 72h. The apoptotic rate was detected by measuring DNA fragmentation.

This discrepancy might contribute to the fact that a diminished amount of DNA is not a unique feature of apoptotic cell death. By contrast, the participation of caspases is specific for apoptosis. To verify caspase dependency and apoptosis, caspases were blocked with the pan-caspase inhibitor Q-VD-OPh (Q-VD) in two BKM120 death sensitive and two BKM120 death resistant cell lines (Figure 9B). Therefore, the cell lines were pretreated with Q-VD for 4h and then incubated with 1,500nM BKM120 for another 72h. Again, the impact on the DNA level was examined. In the two BKM120 death sensitive cell lines MINO and JEKO-1, caspase inhibition attenuated the appearance of cells with sub-G1 DNA. In this case, the diminished amount of DNA correlates with DNA fragmentation. Thus, it was concluded that the BKM120 death sensitive subgroup undergoes caspase-dependent apoptotic cell death. In the death resistant cell lines CA-46 and DG-75, however, the amount of cells with sub-G1 DNA was not affected by caspases inhibition. Especially the 40% of sub-G1 cells in DG-75 were not reduced by Q-VD. This suggests that, here, the decreased levels of DNA do not account for caspase dependent apoptosis.

Apoptosis occurs by either extrinsic or intrinsic signaling. The PI3K/Akt/mTOR pathway mostly is involved in preventing intrinsic apoptotic signaling: Therefore, the ability of BKM120 to induce apoptosis via the intrinsic pathway was investigated. Accepted methods to verify intrinsic apoptosis are measuring the activation of Bax and Bak, the mitochondrial breakdown and the specific activation of the intrinsic initiator caspase-9. At first, the activation of Bax and Bak was explored. Upon activation, Bax and Bak proteins undergo a conformational change that reveals a certain epitope within the n-terminus. This epitope is detectable by specific antibodies. To examine the activation of Bax and Bak, the BKM apoptotic sensitive cell lines MINO and JEKO-1 were incubated with increasing concentrations of BKM120 for 48h. The binding of the specific antibodies to the exposed epitopes was visualized with flow cytometry using fluorescence labeled secondary antibodies. In the two cell lines MINO and JEKO-1, upon BKM120 treatment, Bax and Bak were dose dependently activated, whereby Bax activation was more pronounced (Figure 10A). Next, the breakdown of the mitochondrial membrane potential ($\Delta\psi m$) was examined. The potentiometric dye JC-1 potential-dependent accumulates in the mitochondria and thereby changes its emitting color from green to red. A reduced mitochondrial membrane potential is associated with a reduced red fluorescence. Again, the two sensitive cell lines MINO and JEKO-1 were treated with increasing concentrations of BKM120 for 48h. The changes in $\Delta\psi m$ were detected by administration of JC-1 and measurement of the red fluorescence by flow cytometry. In both cell lines, treatment with BKM120 resulted in a dose dependent reduction of the mitochondrial membrane potential, which indicated breakdown of the mitochondria (Figure 10B). Finally, it was tested whether BKM120 activates the intrinsic pathway specific caspase-9. Caspase-9 activation is associated with cleavage of the 47kDA proform into a large subunit (35/37kDa) and a small subunit (10kDa). To verify caspase-9 activation, the sensitive cell line MINO was incubated with

increasing concentration of BKM120 for 24h and caspase-9 cleavage was examined by western blot. Indeed, upon BKM120 treatment a 37kDa fragment appeared, which demonstrated cleavage and activation of caspase-9 (Figure 10C). Taken together, BKM120 induced apoptotic signaling occurs via the intrinsic pathway accompanied by Bax/Bak activation, breakdown of $\Delta\psi m$ and caspase-9 cleavage.

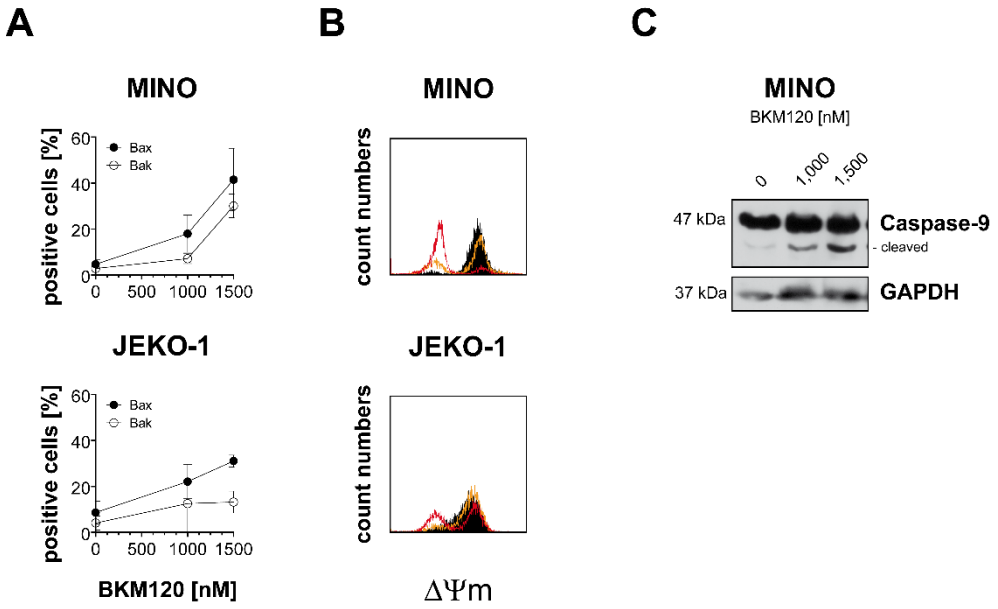

Figure 10: BKM120 induces apoptosis via the intrinsic pathway. (A) Activation of Bax and Bak in response to BKM120. JEKO-1 and MINO were incubated with 1,000nM or 1,500nM of BKM120 for 48h and conformational change of Bax or Bak was detected with specific antibodies. (B) Loss of mitochondrial membrane potential ($\Delta\Psi m$) in response to BKM120. JEKO-1 and MINO were left untreated (black) or incubated with 1,000nM (orange) or 1,500nM (red) BKM120 for 48h and $\Delta\Psi m$ was detected using JC-1 assay. (C) Caspase activation in response to BKM120. MINO cells were treated with BKM120 for 24h and protein expression was assessed using western blot analysis.

3.1.4 BKM120 dependent apoptosis is associated with upregulation of Puma and Hrk

Apoptosis via the intrinsic/mitochondrial pathway, as observed with BKM120 (chapter 3.1.3), usually requires the coordinated and hierarchical interplay of the Bcl-2 family. A BKM120 dependent activation of Bax and Bak already has been observed (chapter 3.1.3). Under normal conditions, the activation of the proapoptotic executers Bax and Bak is inhibited by binding to antiapoptotic Bcl-2 members. Apoptotic stimuli activate the proapoptotic Bcl-2 family subgroup of BH3-onlys, which also binds to antiapoptotic Bcl-2 members but with higher affinity. This releases Bax and Bak and promotes intrinsic apoptosis. Thus, it was next investigated whether BKM120 modulates the expression of proapoptotic BH3-onlys or antiapoptotic Bcl-2 members. Therefore, the BKM120 apoptotic sensitive cell line JEKO-1 was incubated with 1,500nM of BKM120 for different times. The mRNA was isolated, synthesized

into cDNA and quantified by realtime PCR. A threshold of at least 3-fold expression was set to eliminate unspecific fluctuations.

First, the expression of proapoptotic BH3-onlys was examined (Figure 11, upper row). Strong and early upregulation was observed for the BH3-onlys Puma and Hrk, followed by a late and moderate upregulation of Nbk and Bmf. A 5-fold increase in the mRNA expression of the BH3-only Puma was measured within 6h. This was followed by a nearly 15-fold increase after 12h/24h and a complete downregulation of Puma mRNA expression after 36h. Likewise, Hrk mRNA expression was upregulated nearly 6-fold within 6h/12h, which decreased back to nearly 3-fold after 24h/36h of BKM120 treatment. For Nbk and Bmf in turn, a clear mRNA upregulation was observed only after 24h and 36h respectively. Taken together, this implies that Puma and Hrk are the main regulators of BKM120-dependent apoptosis, while Nbk and Bmf signaling is of minor importance. Furthermore, the early Puma/Hrk mRNA expression presumes direct BKM120-mediated regulation, whereas the late expression of Nbk/Bmf might be a result of signal amplification by feedback looping. The most potent BH3-only, Bim, was not expressed in JEKO-1.

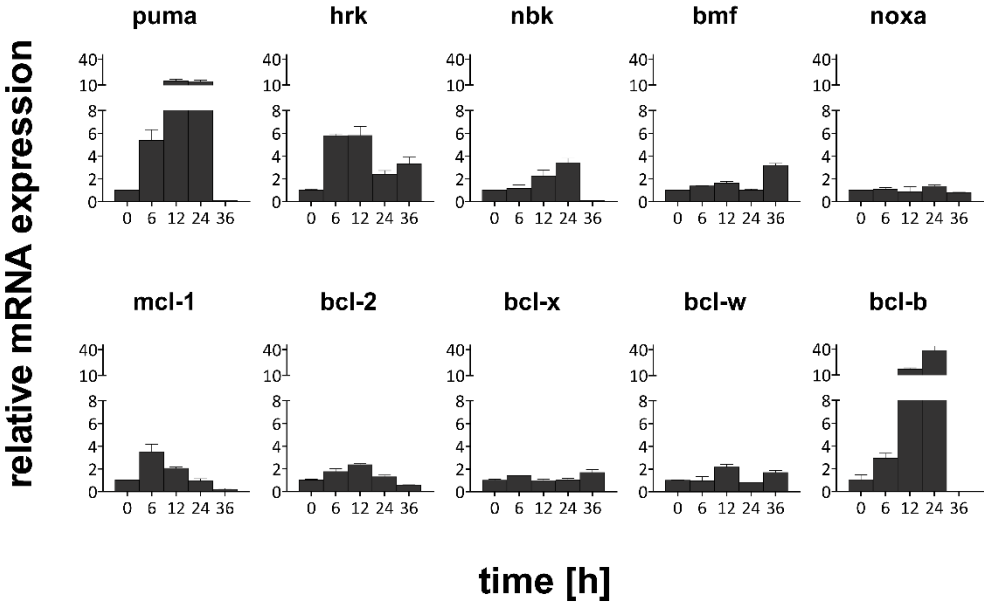

Figure 11: BKM120 treatment upregulates Puma and Hrk. Relative mRNA Expression level (x-fold) of Bcl-2 family members in JEKO-1 cells after treatment with 1,500nM BKM120 for the indicated time points [h].

Next, the expression of the antiapoptotic Bcl-2 members was explored (Figure 11, lower row). Treatment with BKM120 caused a strong upregulation of Bcl-b mRNA expression after 12h and 24h, followed by a complete degradation after 36h. For Mcl-1 and Bcl-2, a slight mRNA upregulation was detected after 6h and 12h respectively. Bcl-x_L and Bcl-w mRNA expression, however, was not affected by BKM120. Mcl-1 and Bcl-2 are binding partners of Puma, Hrk, Nbk and Bmf. Considering the strong

upregulation of Puma and Hrk, the expression levels of Mcl-1 and Bcl-2 most certainly did not prevent their apoptotic signaling. Nbk and Bmf expression in turn, might be compensated by Mcl-1 and Bcl-2, especially as Mcl-1 and Bcl-2 were upregulated before Nbk and Bmf. Furthermore, Nbk is sequestered by Bcl-b. The slight upregulation of Nbk, thereby, might be compensated by the strong Bcl-b upregulation. This leaves only Puma and Hrk as potential candidates for BKM120-mediated apoptosis.

3.1.5 Apoptotic resistance correlates with the loss of Bax and Bak

In the previous experiments, a BKM120 apoptotic death insensitive subgroup was identified (chapter 3.1.2). This strongly suggested aberrations in the apoptotic pathway. To analyze this, the base line expression levels of central apoptotic proteins were compared among the eight NHL cell lines (Figure 12).

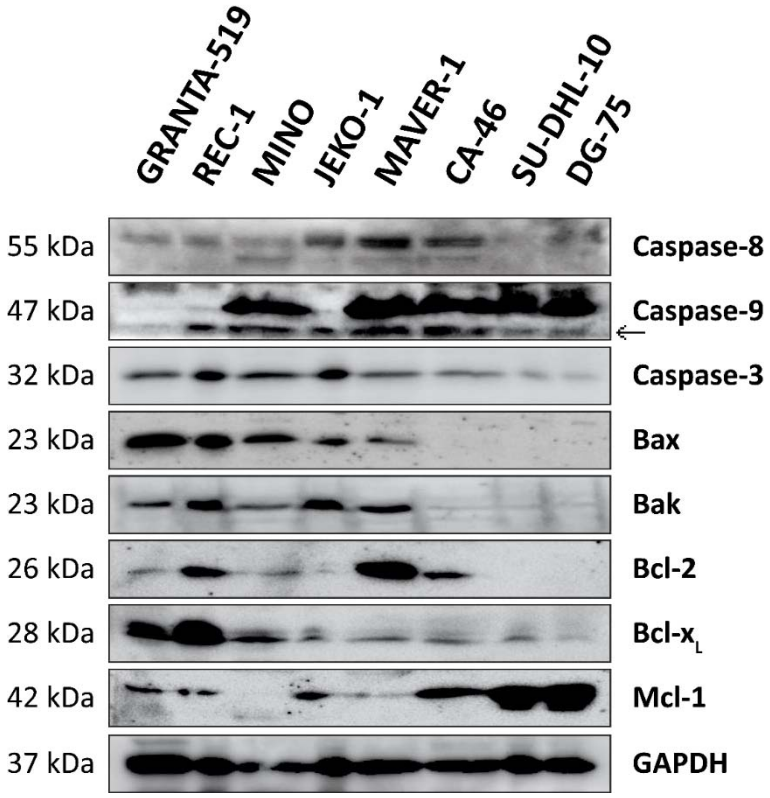

Figure 12: BKM120 apoptosis resistant cells show aberrations within the intrinsic apoptotic pathway. Basal expression of apoptosis related proteins. Protein expression was assessed using western blot analysis.

In the apoptotic sensitive cell lines, the major extrinsic initiator caspase-8 and the intrinsic initiator caspase-9 were expressed to a moderate or high level. In the apoptotic sensitive cell line GRANTA-519, however, caspase-9 expression was not detectable. The major executer caspase-3 likewise was expressed in all sensitive cell lines. With regard to the Bcl-2 family, a heterogeneous expression of the antiapoptotic Bcl-2 members was observed. Very high Bcl-2 expression was observed in MAVER-1, moderate expression in REC-1 and little or no expression in GRANTA-519, MINO and JEKO-1. Bcl-x_L was

highly expressed in GRANTA-519 and REC-1 but very little in MINO, JEKO-1 and MAVER-1. Mcl-1 in turn, was expressed weakly in all apoptotic cells lines. In contrast, the proapoptotic executers Bax and Bak were expressed throughout the apoptotic sensitive subgroup. Thus, all proteins required for the execution of apoptosis were present in the apoptotic sensitive subgroup. In the apoptotic resistant subgroup, consisting of CA-46, SU-DHL-10 and DG-75, caspase-8 was clearly detectable in CA-46 and very slightly in DG-75 but not in SU-DHL-10. In contrast, caspase-9 could be detected in all cell lines, although SU-DHL-10 and DG-75 showed much weaker expression. A similar situation was observed for caspase-3. When regarding the expression of the antiapoptotic Bcl-2 family members, expression of Bcl-2 was detected in CA-46 but not SU-DHL-10 or DG-75. Bcl-xL was expressed weakly in all three cell lines. Mcl-1, however, was highly expressed in the whole subgroup of apoptotic resistant cells. Among the proapoptotic counterparts, no expression of either Bax or Bak was detectable. In total, loss of Bax and Bax expression or overexpression of Mcl-1 might play an important role in the regulation of resistance mechanisms against BKM120-mediated apoptosis.

3.1.6 Loss of Bax and Bak protects from BKM120 induced apoptosis

The previous results strongly indicated that BKM120 induced apoptosis requires Bax and Bak. By abrogating Bax/Bak expression, this presumption was examined. In the tested NHL cell line system, the downregulation of protein expression by transfection of siRNA with the Amaxa system is established [222]. This downregulation, however, is incomplete. As complete loss of Bax/Bak expression was necessary to test the supposition, the HCT116 Bax/Bak double knockout model system was used. HCT116 wild type (WT) or Bax/Bak double knockout cells ($BB^{-/-}$) were incubated with increasing concentrations of BKM120 for 72h and the apoptotic rate was detected by measuring DNA fragmentation with flow cytometry (Figure 15B, left panel). In HCT116 WT cells, BKM120 dose dependently induced apoptotic cell death. With 1,500nM BKM120 30% of the cells were apoptotic, which increased to 70% with 2,000nM. Knockout of Bax and Bak clearly prevented apoptosis. Here, at 1,500nM, only 14% of cells were apoptotic, whereas 2,000nM induced apoptosis only in about 40% of the cells. Thus, Bax and Bak are required to conduct BKM120 mediated apoptosis.

3.1.7 BKM120 induces G2/M arrest and polyploidy

Besides its important role in apoptosis execution, PI3K/Akt/mTOR signaling also has high impact on cell cycle regulation. In consequence, inhibitors of PI3K/Akt/mTOR were found to affect cell cycling potentially. Above, BKM120 was observed to inhibit proliferation irrespective of cell death induction (chapter 3.1.2). This suggested that BKM120 attenuates cell cycling as well. To examine the effect of BKM120 on cell cycle progression, the eight NHL cell lines were incubated with increasing concentrations of BKM120 for 72h. Changes in the cell cycle were pictured by measuring the DNA

content with PI and quantifying it with the software ModFit LT (Figure 13). Somatic cells in G1 phase have two chromosome sets, defined as 2n cells. After DNA replication in S phase, cells in G2 or M phase carry four chromosome sets and are defined as 4n cells. All untreated cell lines were majorly situated in G1 or S phase. Depending on the cell line, only 4% to 16% of the cells were in G2 or M phase. Treatment of the death sensitive subgroup with BKM120 increased the amount of G2/M cells and, thus, caused G2/M arrest in a dose dependent manner. In MINO and JEKO-1, about 30% to 40% of the cells were arrested in G2/M when treated with 1,500nM or 2,000nM of BKM120. In GRANTA-519, REC-1 and MAVER-1 clear G2/M arrest was only detected with 2,000nM BKM120. Similar was observed for the death resistant subgroup. 1,500nM and 2,000nM BKM120 strongly induced G2/M arrest in all three cell lines. However, in SU-DHL-10 and DG-75, already 1,000nM of BKM120 were sufficient to induce G2/M arrest.

Despite G2/M arrest, a second phenomenon was observed. Especially, in the two death resistant cell lines DG-75 and SU-DHL10, BKM120 dose dependently caused the formation of cells with an 8n and more DNA content. These cells were assumed polyploid. For instance, at 1,500nM, 35% of SU-DHL-10 cells and 60% of DG-75 cells were polyploid. However, at a higher concentration of 2,000nM, small amounts of polyploid cells were also detected in CA-46, REC-1 and JEKO-1.

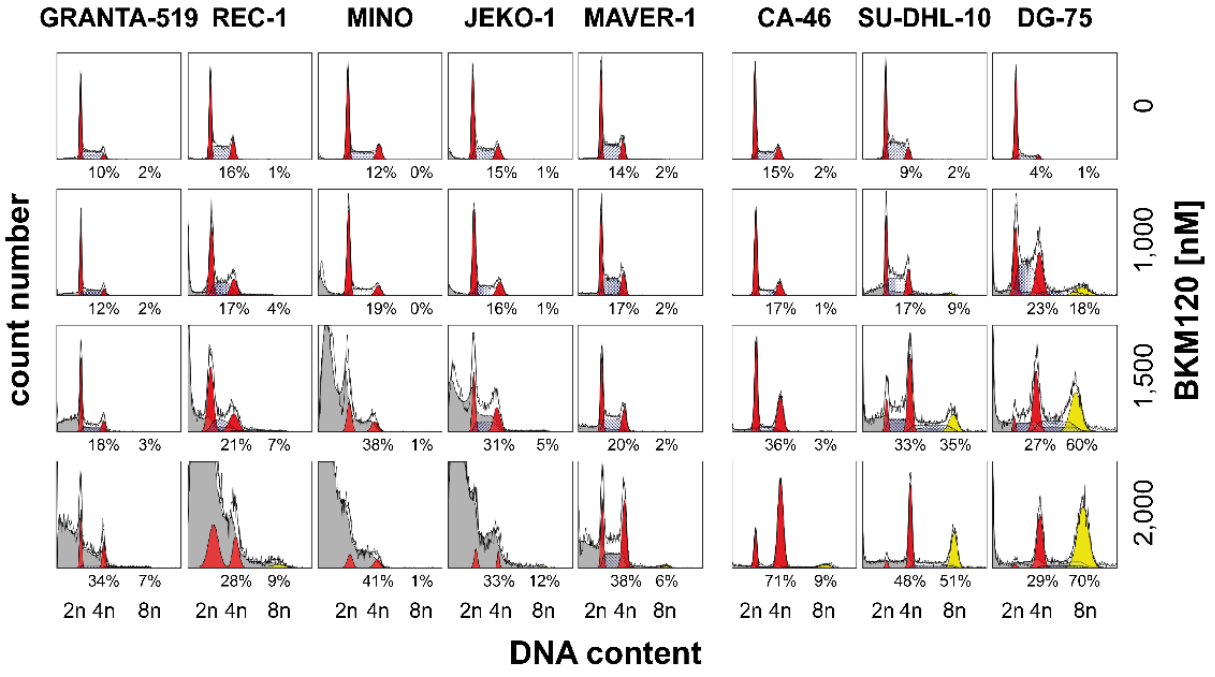

Figure 13: BKM120 induces G2/M arrest and polyploidy. (A) The eight NHL cell lines were treated with the indicated concentrations of BKM120 for 72h. The cell cycle phases were pictured by labeling the DNA with PI and measuring the DNA content with flow cytometry. Quantification of the cell cycle phases was performed using ModFit LT. 2n/diploid cells in G0/G1 phase (red, left), 4n/tetraploid cells in G2/M phase (red, middle), 8n/polyploid cells (yellow), S phase cells (blue grid), debris/apoptotic cells (gray).

To further characterize polyploidy, the two cell lines CA-46 and DG-75 were incubated with BKM120 for a period of 216h (Figure 14). Prolonged treatment with BKM120 strongly increases the amount of cell with a >4n content in DG-75 cells. After 72h cells with an 8n DNA content appeared. After 144h, even cells with a 16n DNA content were observed. In CA-46 cells the appearance of 8n cells occurred after 144h. Thus, polyploidy is always associated with the exact doubling of the genome. However, in parallel to polyploidy, the amount of cells with a <2n content increases.

Altogether, BKM120 promotes G2/M phase arrest in all cell lines, irrespective of death sensitivity. In death resistant cells, however, BKM120 additionally induces polyploidy.

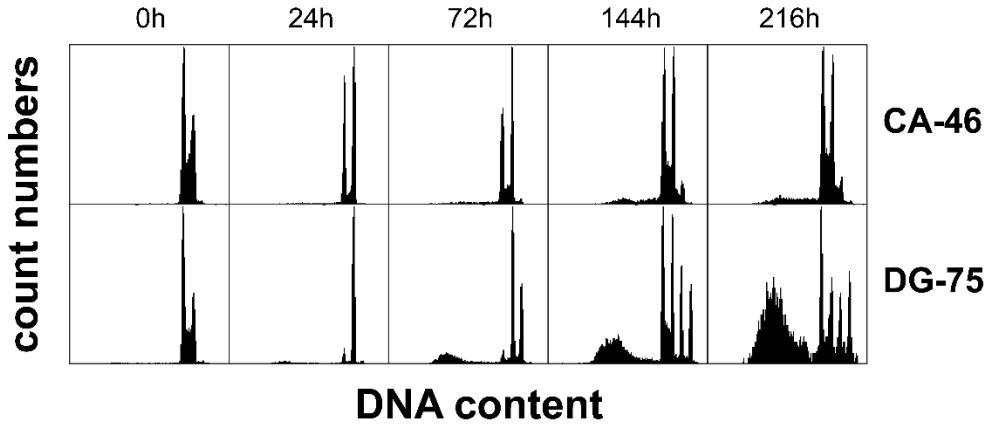

Figure 14: Longtime treatment of CA-46 and DG-75 with BKM120. Both cell lines were incubated with 1,500nM of BKM120 for the indicated time points. The DNA content was measured by flow cytometry using PI and plotted with Cell Quest Pro.

3.1.8 BKM120-induced polyploidy is Bax/Bak and p53 dependent

The present results revealed that resistance to BKM120 induced apoptosis correlated with the loss of Bax and Bak expression (chapter 3.1.5 and chapter 3.1.6). Furthermore, this inability to undergo apoptotic cell death was accompanied by the induction of BKM120 dependent polyploidy in the cell lines DG-75 and SU-DHL-10 (chapter 3.1.6). This suggested that Bax and Bak might play a prominent role in BKM120 mediated polyploidy. To further examine this, the HCT116 Bax/Bak double knockout model system was used. HCT116 WT and $BB^{-/-}$ cells were treated with increasing concentrations of BKM120 and the ploidy status was assessed by measuring the DNA content with flow cytometry (Figure 15). Treatment of HCT116 WT with BKM120 caused a slight formation of polyploid cells at 1,500nM and 2,500nM. Similar was observed in the in the HCT116 $BB^{-/-}$ cells. In contrast to the hypothesis, knockout of Bax and Bak did not further increase the amount of polyploid cells. Thus, BKM120 induced polyploidy, however, was Bax/Bak independent in this setting.

Besides Bax and Bak, loss of p53 is described as an important initiator of polyploidy. Of note, in the current NHL cell line model, all three BKM120 apoptotic resistant and polyploid cells forming cell lines

carry p53 mutations. Thus, to analyze the role of p53 for the BKM120 response, p53 was downregulated with siRNA in the HCT116 model system. In this system, downregulation of protein expression with p53 siRNA was complete (Figure 15A). Downregulation of p53 slightly sensitized for BKM120-mediated apoptosis at 1,000nM and 1,500nM (Figure 15B, middle panel). The amount of polyploid cells, however, was not increased by p53 downregulation (Figure 15C). Thus, BKM120 induced polyploidy, however, was p53 independent in this setting.

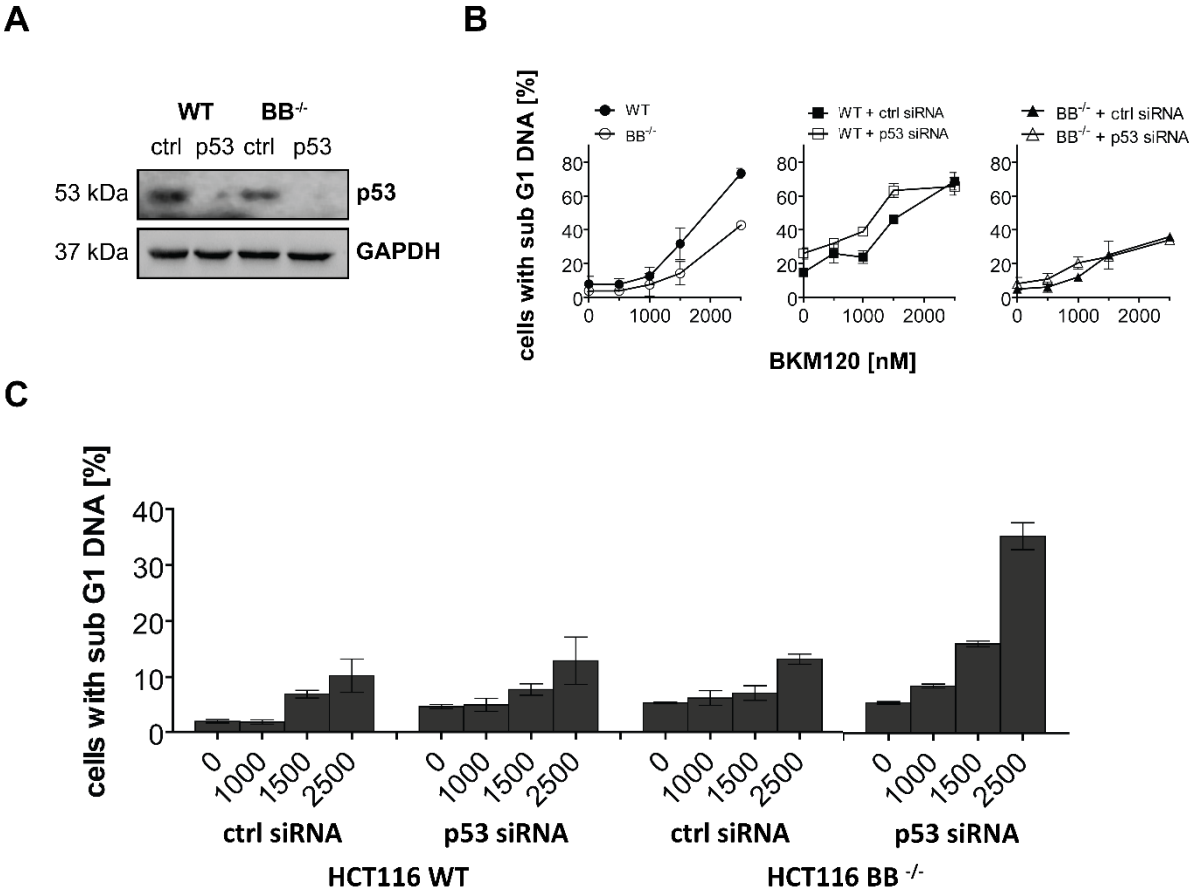

Figure 15: BKM120 mediated polyploidy is Bax, Bak and p53 dependent. HCT116 model system. Treatment of wild type (WT) or Bax/Bak double knockout (BB$^{-/-}$) HCT116 with increasing concentrations of BKM120 for 72h. Additional downregulation of p53 by transfecting p53 siRNA with the Amaxa system. (A) Downregulation of p53. Transfection of HCT116 WT and HCT116 BB$^{-/-}$ with either scrambled siRNA (ctrl) or p53 siRNA (p53) and subsequent incubation for 24h. Protein expression was assessed with western blot. (B) Apoptotic rate of untransfected HCT116 WT and BB$^{-/-}$ cells when treated with increasing concentrations of BKM120 for 72h (left panel). HCT116 WT transfected with either scrambled siRNA (ctrl siRNA) or p53 siRNA (p53 siRNA) (middle panel). HCT116 BB$^{-/-}$ transfected with either ctrl siRNA or p53 siRNA (right panel). (C) Polyploidy status was assessed by measuring the DNA content with flow cytometry.

In the NHL cell line system, loss of Bax/Bak and mutations within p53 occurred side by side in the polyploid cell lines. Thus, a context between Bax/Bak expression and p53 activity was considered. To test this, the possibility of Bax/Bak and p53 to cooperate in ploidy regulation was analyzed. Therefore, HCT116 WT and BB$^{-/-}$ cells were incubated with BKM120 in the absence and presence of p53 (Figure

15C). As described above, loss of either Bax/Bak or p53 did not affect ploidy. In conjunction, however, BKM120-induced formation of polyploid cells was strongly enhanced. This effect was additive at 1,500nM and synergistic at 2,500nM. Thus, threefold loss of Bax, Bak and p53 is crucial for BKM120-dependent polyploidy.

3.1.9 G2/M arrest is the initial event leading to either apoptosis or polyploidy

BKM120 mediated G2/M arrest was observed in all tested NHL cell lines irrespective of undergoing apoptosis or polyploidy. This suggested G2/M arrest to be the trigger for both events. To investigate this, the early BKM120 mediated effects on the cell cycle were assessed. Therefore, the apoptosis sensitive cell line MINO and the apoptosis resistant cell line DG-75 were treated with 1,500nM BKM120 for 3h, 6h, 12h, 24h, and 48h and cell cycle analysis was performed by flow cytometry and quantified with ModFit LT (Figure 16). In MINO cells, treatment BKM120 time dependently induced arrest in G2/M phase. The amount of G2/M cells increases from initial 14% to 24% within 6h. After 12h, 32% of the cells population was situated in G2/M phase. A maximum of 41% cells situated in G2/M phase was reached after 48h (Figure 16 A, upper histograms).

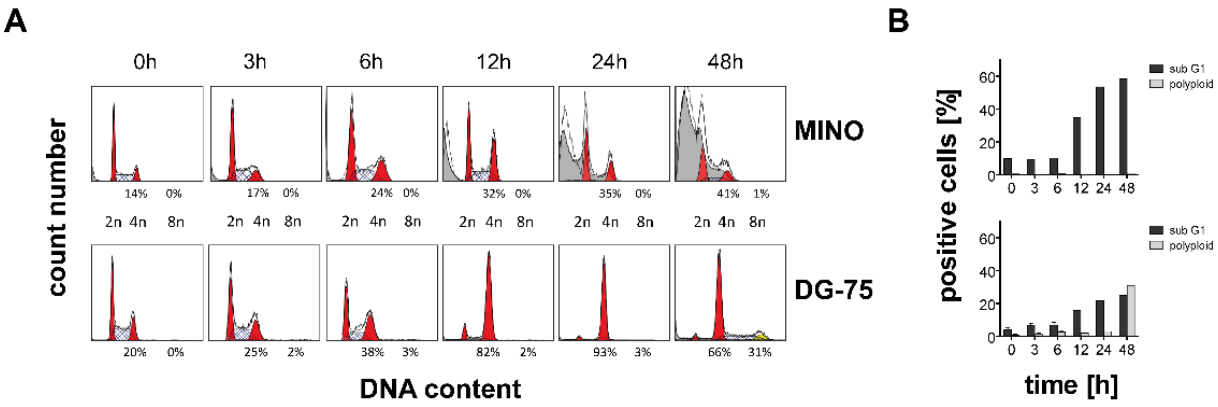

Figure 16: BKM120 induces G2/M arrest prior to apoptosis in sensitive cells. (A) MINO (upper panel) and DG-75 (lower panel) cells were incubated with 1,500nM of BKM120 and incubated for the indicated time. The cell cycle status was assessed by measuring the DNA content by flow cytometry. 2n cells are situated in G0 or G1 phase (red, left peak), 4n cells in G2 or M phase (red, right peak) and 8n cells are designated as polyploid (yellow). Cells in S phase (blue grid), debris (grey). Analysis was performed with ModFit LT. (B) Hypodiploidy (sub G1) and polyploidy status of MINO and DG-75 cells measured with flow cytometry.

In contrast, an increase for cells was observed after 12h with about 35% and reached nearly 60% after 48h (Figure 16 B, upper graph). In DG-75 cells, the amount of G2/M cells increased from 20% to 38% within 6h and reached a maximum of 93% after 24h. After 48h of incubation, a decrease of G2/M cells was observed that was accompanied by the appearance of 8n cells (Figure 16A, lower histograms). An increase in the amount of sub G1 cells from about 5% to nearly 20% was observed after 12h (Figure 16B, lower graph). Further incubation for 48h increased the level of sub G1 cells only to 25%. These

results showed that in both cell lines, G2/M arrest occurred prior to hypodiploidy and, thus, is suggested to be the initial event. Moreover, polyploid cells raised from cells arrested in G2/M phase as the amount of G2/M cells decreased in the same way the amount of polyploid cells increased.

3.1.10 BKM120 affects checkpoint signaling

It has been demonstrated above that PI3K inhibition by BKM120 causes G2/M cell cycle arrest in all tested cell lines and that this G2/M arrest seems to be the decision point directing cells into either apoptosis or polyploidy (chapter 3.1.7). As G2/M cell cycle arrest is under the control of the checkpoint machinery, next, it was analyzed whether BKM120 treatment affects the checkpoint signaling in the two cell lines MINO and DG-75 (Figure 17).

The central proteins of the checkpoint cascade are Chk1 and Chk2, phosphorylated and activated by ATM/ATR as response to DNA damage. The kinase activity of Chk1 depends on phosphorylation at S345, while that of Chk2 requires phosphorylation at T68 [73,74,75,76]. When treated with BKM120, in both cell lines dose dependent phosphorylation of Chk2 at T68 was observed. In contrast, Chk1 was dephosphorylated at Ser345 in both cell lines. This suggests BKM120 mediated activation of Chk2 but, however, inactivation of Chk1.

The checkpoint kinases have impact on cell cycle progression by triggering the dephosphorylation CDKs at T14/Y15. As G2/M transition is mediated by CDK1, its phosphorylation status in response to BM120 was analyzed. Treatment of MINO or DG-75 with BKM120 hardly affected the phosphorylation of CDK1 at T161, which is required for its activation. Surprisingly, the inhibitory phosphorylation at Y15 was dose dependently reduced by BKM120 in both cell lines. This suggested that BKM120 rather promoted G2/M transition and arrest in mitosis.

The CDK1 complexes further require Cyclin A for G2 progression and Cyclin B1 for mitosis. To verify BKM120 mediated mitotic arrest, the protein levels of these two Cyclins were investigated by western blot analysis. Treatment with BKM120 degreased the protein levels of Cyclin A in a dose dependent manner in both cell lines. In DG-75, however, this downregulation was much more pronounced and associated with abrogation of Cyclin A expression at 1,500nM. In contrast, the expression of Cyclin B1 was dose dependently upregulated upon BKM120 administration. In MINO, a nearly fourfold increase was observed with 1,500nM BKM120, whereas Cyclin B1 expression reached twofold expression in DG-75. Considering that Cyclin A is degraded before metaphase/anaphase transition and upregulation of Cyclin B1 prevents metaphase/anaphase progression, this suggests that BKM120 causes mitotic arrest at the metaphase/anaphase checkpoint [62,63,85,86,87].

In total, BKM120 manipulates checkpoint signaling and promotes G2/M transition leading into mitotic arrest between metaphase and anaphase.

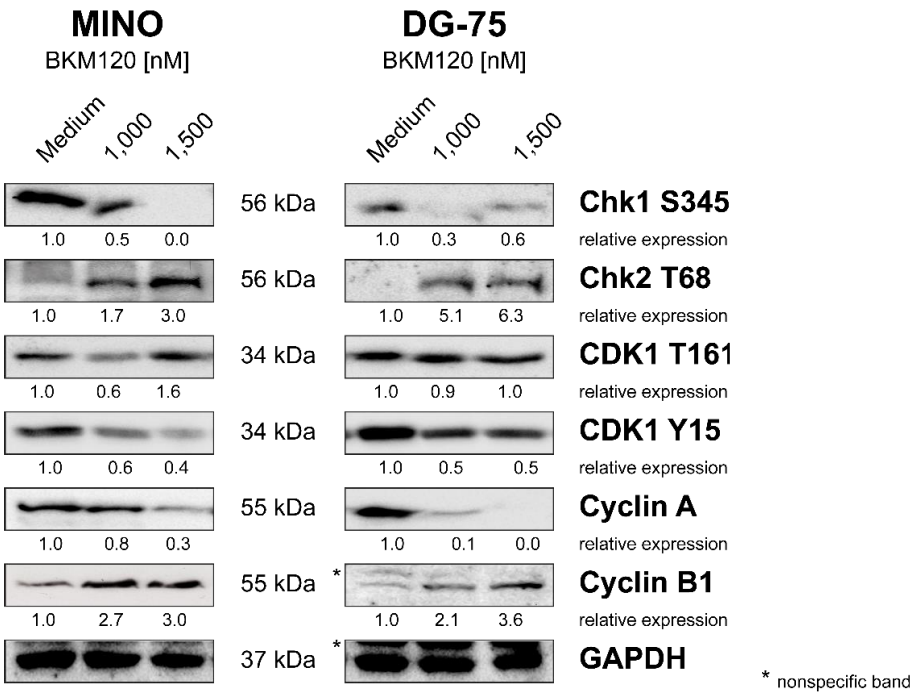

Figure 17: Expression of checkpoint proteins in response to BKM120. Treatment of MINO and DG-75 with 1,000nM or 1,500nM of BKM120 for 24h. Protein expression and phosphorylation status were assessed by western blot analysis.

3.1.11 BKM120 activates MEK1/2

Blocking PI3K/mTOR signaling can be associated with the activation of MAPK signaling by an Akt-Raf crosstalk or the S6K/IRS-1 feedback [246]. Activation of the MAPK pathway in turn is reported to induce G2/M arrest [247,248,249,250]. Thus, the observed BKM120 mediated events might be a result of inverse MAPK activation. Therefore, the effect of BKM120-dependent PI3K inhibition on MAPK signaling was investigated in the two cell lines MINO and DG-75 (Figure 18). BKM120 clearly induced the phosphorylation of MEK1/2 at S217/S221 in both cell lines. These phosphorylation sites are required for MEK's activation. Thus, BKM120 treatment activates the MAPK pathway. This not only provided a possible link for BKM120 mediated checkpoint regulation but also could offer an explanation for polyploidy in DG-75 cells.

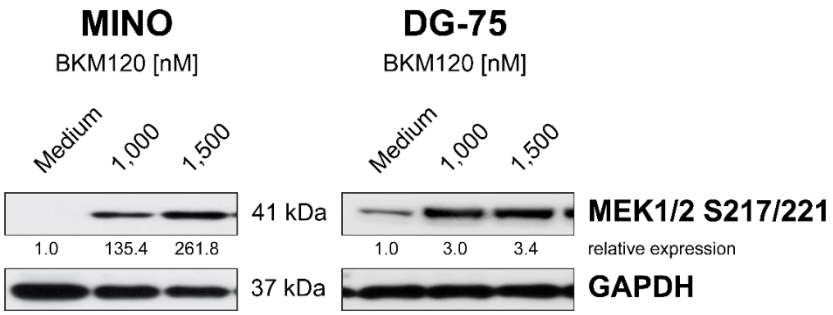

Figure 18: BKM120 dependent activation of MAPK signaling. MINO and DG-75 cells were incubated with BKM120 at the indicated concentrations for 3h and the activity status of MEK1/2 was measured by detecting its phosphorylation status by immunoblotting.

3.1.12 MEK1/2 signals downstream of ATM/ATR

Above BKM120 was shown to activate MEK1/2 (chapter 3.1.11). Although MAPK signaling is implicated in G2/M arrest, little is known about the underlying mechanisms. Previous findings suggest a connection to checkpoint regulation as ATM dependent ERK phosphorylation and, vice versa, ERK dependent ATM phosphorylation were reported [248,251]. To investigate the role for BKM120 dependent MEK1/2 activation on G2/M checkpoint regulation, MEK1/2 and ATM/ATR were blocked with the chemical compounds U0126 and Caffeine, respectively (Figure 19). Again, the two cell lines MINO and DG-75 were used. As expected, U0126 reduced MEK1/2 phosphorylation in DG-75 cells. In MINO, however, pretreatment with U0126 strongly increased the amount of phosphorylated MEK1/2. This compensating upregulation of phosphorylated MEK1/2 upon treatment with U0126 has been reported before [252]. Inhibition of ATM/ATR with Caffeine in turn, decreased MEK1/2 phosphorylation in both cell lines. This clearly showed that MEK1/2 phosphorylation requires ATM/ATR and, in consequence, proved that MEK1/2 acts downstream of ATM/ATR. Moreover, this suggested that BKM120 promotes MEK1/2 activation in an ATM/ATR dependent manner.

Further, BKM120 mediated checkpoint regulation was shown to be mediated via Chk2 (chapter 3.1.10). To test, whether this effect is MEK1/2 or ATM/ATR dependent, the two cell lines MINO and DG-75 were treated with U0126 or Caffeine for 4h and subsequently incubated with 1,500nM BKM120 for another 24h. Treatment with the ATM/ATR inhibitor Caffeine clearly decreased BKM120 mediated phosphorylation of Chk2 at T68 in both cell lines. Inhibition of MEK1/2, however, decreased the phosphorylation of Chk2 in DG-75 but not MINO cells. Here, Chk2 T68 was upregulated similarly to MEK1/2 S217/221. Yet, this showed that the phosphorylation of Chk2 depended on the activity of MEK1/2.

BKM120 further affected the phosphorylation of the G2/M phase CDK1 at Y15 (chapter 3.1.10). As the upstream occurring checkpoint kinase Chk2 was shown to be ATM/ATR and MEK1/2 dependent, the

same was suggested for CDK1. The activating phosphorylation of CDK1 at T161 was attenuated by inhibition of either ATM/ATR or MEK1/2 in both cell lines alone or in combination with BKM120. However, similar observations were made for the inhibitory phosphorylation at Y15. As T161 is essential for CDK1 activity and entry into mitosis, Caffeine and U0126 prevented CDK1 activation. This suggested that BKM120 mediated MEK1/2 activation promoted mitotic entry.

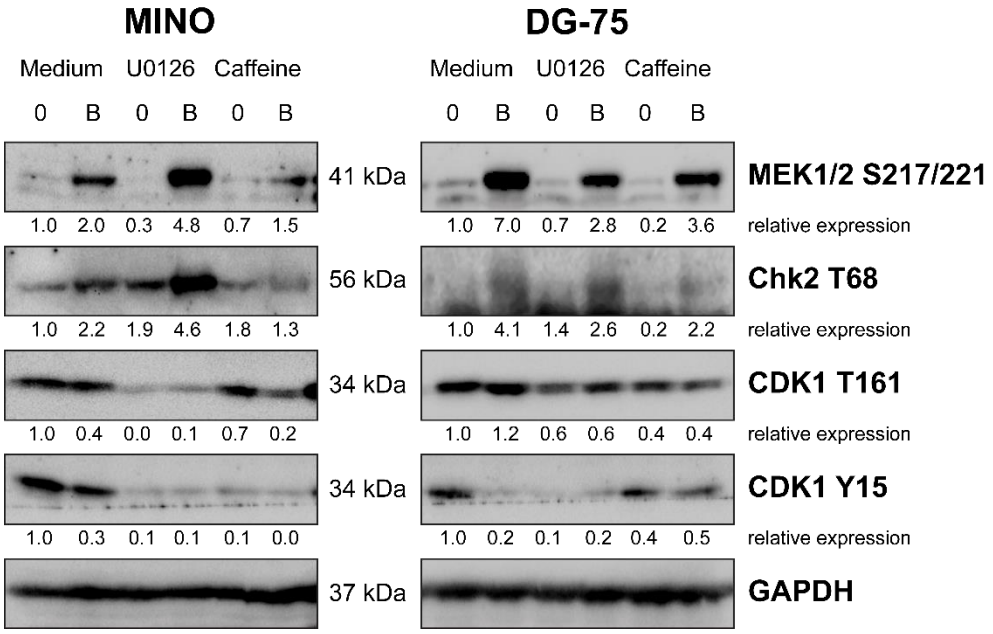

Figure 19: MEK signals downstream of ATM/ATR. MINO and DG-75 were pretreated either with 10,000nM of the MEK inhibitor U0126 or with 500,000nM of the ATM/ATR inhibitor Caffeine for 4h followed by incubation with 1,500nM of BKM120 for another 24h. Protein expression and phosphorylation status were assessed by western blot analysis.

3.1.13 BKM120-induced polyploidy is MEK and ATM/ATR dependent

Above, BKM120 was shown to activate the MAPK pathway (chapter 3.1.11). Furthermore, this activation was ATM/ATR dependent and affected BKM120 mediated checkpoint regulation (chapter 3.1.12). Interestingly, MAPK signaling is implicated in polyploidy, while PI3K/mTOR inhibition rather protects from polyploidy [115,247,253,254]. Therefore, BKM120 mediated polyploidy might be a result of MEK1/2 activation.

To examine this, MEK1/2 was inhibited using the chemical compound U0126 and the impact on BKM120 mediated mitotic arrest and polyploidy was explored in MINO and DG-75 cells. Further, to analyze the MEK/ERK pathway in the context of checkpoint regulation, ATM and ATR were blocked using the inhibitor Caffeine (Figure 20A). In MINO cells, pretreatment with 50,000nM U0126 promoted BKM120-mediated G2/M arrest and nearly doubled the amount of 4n cells. Pretreatment with Caffeine, however, did not alter cell cycle distribution. In DG-75 cells, inhibition of MEK1/2 with U0126 prevented the formation of 8n, polyploid, cells. This was accompanied by an increase of 4n cells. The

inhibition of ATM/ATR with Caffeine reduced polyploidy and increased G2/M phase arrest similar to U0126. Thus, BKM120 mediated polyploidy in DG-75 cells is MEK1/2 and ATM/ATR dependent. Considering the U0126 dependent attenuation of mitotic entry observed in both cell lines before (chapter 3.1.12), it is suggested that the increase of G2/M cells is associated with an arrest at the upstream G2/M checkpoint. Thus, BKM120 induces mitotic arrest, while U0126 promotes G2/M arrest. Furthermore, the observation of increased cells in G2/M phase is consistent with the previous result of G2/M arrest as initiator of either apoptosis or polyploidy.

In consequence, it was expected that pretreatment with U0126 likewise protected from BKM120 induced apoptosis in MINO cells by detaining cells in G2/M phase. To test this, the sub G1 levels of MINO and DG-75 cells in response to U0126, Caffeine and BKM120 were assessed (Figure 20B). In contrast to the presumption, U0126 synergistically sensitized MINO cells for BKM120-induced apoptosis. This effect was observed only with 1,000nM but not 1,500nM BKM120. The same could be detected when the cells were pretreated with the ATM/ATR inhibitor Caffeine, instead. In contrast, in DG-75 cells no such sensitization could be detected, neither with the MEK1/2 inhibitor nor with the ATM/ATR inhibitor.

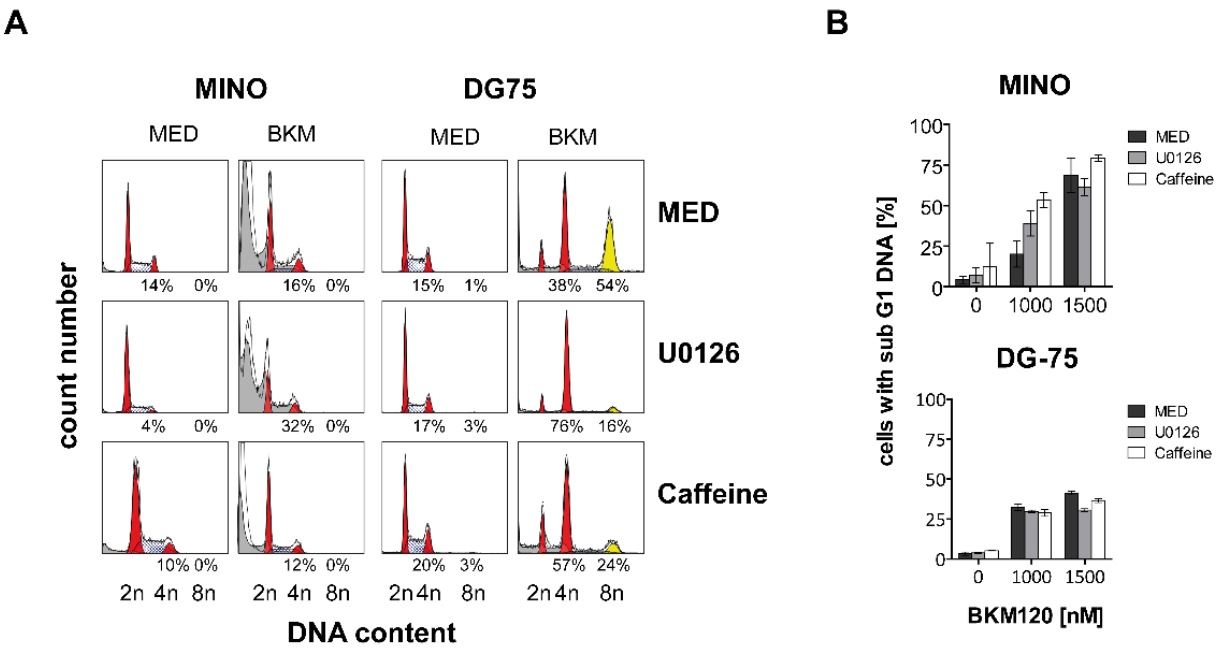

Figure 20: BKM120-induced polyploidy is ATM and MEK dependent. (A) MINO and DG-75 were pretreated with 50,000nM of the MEK inhibitor U0126 or with 500,000nM of the ATM/ATR inhibitor Caffeine for 4h and subsequently incubated with 1,500nM of BKM120 for another 72h. The cell cycle status was assessed by measuring the DNA content with flow cytometry and analysis was performed using ModFit LT. (B) MINO and DG-75 were pretreated with U0126 or Caffeine and subsequently incubated with the indicated concentrations of BKM120 for another 72h. The amount of hypodiploid cells was measured with flow cytometry.

Thus, inhibition of either ATM/ATR or MEK1/2 enhances apoptotic cell death in sensitive cells and protects from polyploidy in resistant cells. As both are involved in controlling Chk2 and CDK1 activity, this effect is suggested to be G2/M checkpoint dependent.

3.1.14 BKM120 induced events are reversible

In the resistant cell line DG-75, the level of hypodiploid cells increased time dependently (chapter 3.1.9) but was independent of caspases, ATM/ATR or MEK1/2 (chapter 3.1.3 and chapter 3.1.13). Furthermore, the observed G2/M arrest and polyploidy are features implicated in mitotic catastrophe and mitotic cell death. Cell death generally is suggested to be irreversible, once activated. Here, the same is assumed for mitotic cell death. Consequently, BKM120-mediated polyploidy should be irreversible in apoptotic resistant cell lines. To test this, the two apoptotic resistant and polyploid cell lines CA-46 and DG-75 were incubated with 1,500nM of BKM120 for an indicated time and transferred back to medium to incubate for an overall period of 9 days. As the first formation of polyploid cells was observed after 48h (chapter 3.1.9), BKM120 was removed after 24h (BKM24) or 72h (BKM72) respectively. As control, both cell lines where incubated with BKM120 throughout the entire time (BKM). Proliferation was assessed by viable cell count measurement and cell cycle status was detected using flow cytometry and ModFit LT.

First, the BKM120-dependent cell cycle distribution was analyzed. In CA-46, continuous incubation with BKM120 resulted in strong G2/M arrest with a highest value of 43% measured after 72h of incubation. Longtime incubation, however, reduced the amount of cells arrested in G2/M but slightly increased the amount of polyploid cells from 5% to 14% after 216h. When BKM120 was removed after 24h, the amount of G2/M cells strongly reduced from 41% to 24% after 72h and was almost back to basal level after 144h. Furthermore, the formation of polyploid 8n cells was abrogated. Removement of BKM120 after 72h led to nearly similar results. The level of G2/M cells was reduced from 47% to 19% after 144h. However, the amount of polyploid cells first increased from 5% to 10% after 144h and then decreased back to 4% after 216h (Figure 21A, upper panel). In DG-75, as observed before, almost all cells (85%) arrested in G2/M already after only 24h of BKM120 treatment. With the decrease of G2/M cells down to 51% after 72h, additional polyploidy was observed in 30% of the cells. Continuous incubation strongly increased the amount of polyploid cells up to 49% after 144h. However, after 216h, the percentage of polyploid cells decreased. Removing BKM120 after 24h, time dependently reduced the amount of G2/M cells down to basal level after 216h. Polyploid cells, however, appeared after 72h but were not detected after 144h or 216h. When BKM120 was removed after 72h, the number of G2/M cells was reduced from 58% to approximately 17% after 144h/216h. In contrast the amount of polyploid cells, increased from 37% to 56% after 144h and then dropped back to 29% after 216h (Figure 21A, lower panel).

Next, proliferation in response to BKM120 was investigated. In both cell lines medium treated cells proliferated and the cell number increased constantly. Continuous treatment with BKM120 abrogated proliferation in both cell lines. When BKM120 was replaced by medium after 24h, both cell lines restarted proliferation. Replacement with media after 72h, however, promoted CA46 but not DG-75 to proliferate (Figure 21B).

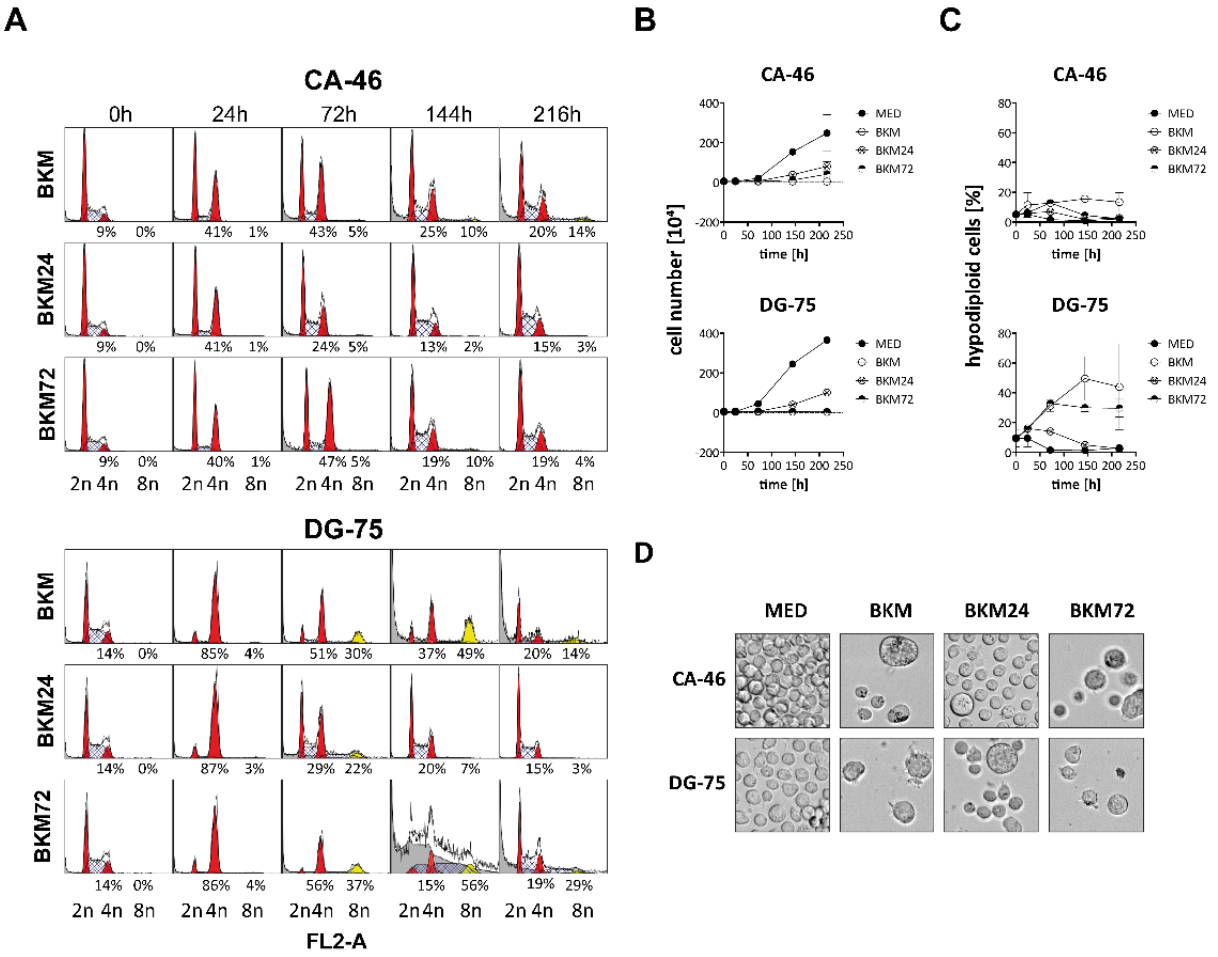

Figure 21: BKM120-induced events are reversible. (A) Cell cycle analysis of the Burkitt lymphoma cell lines CA-46 and DG-75 after to longtime treatment with 1,500nM BKM120 (BKM) and release from BKM120 treatment after 24h (BKM24). diploid cells in G1 or G2 phase (red), hyperdiploid cells (yellow), cells in s phase (blue grid). (B) Proliferation of CA-46 and DG-75 as measured by cell count after longtime treatment with medium (MED) BKM120 (BKM) and release from BKM120 after 24h (BKM24) or 72h (BKM72). Analysis was performed with ModFit. (C) Light microscopy of CA-46 and DG-75 cells after 216h of culture in medium (MED), BKM120 (BKM) or after release from BKM120 after 24h (BKM24) or 72h (BKM72) of treatment. (D) Light microscopy of CA-46 and DG-75 cells in response to BKM120 and after release fromBKM120 after 24h or 72h.

Next, the BKM120 dependent hypodiploid cell formation was assessed. In CA-46, continuous incubation with BKM120 slightly increased the amount of hypodiploid cells. Replacement of BKM120 after 24h or 72h decreased the level of hypodiploid cells back to untreated. In DG-75, a strong BKM120-

dependent increase of hypodiploid cells was observed. Here however, only removement of BKM120 after 24h reversed hypodiploidy whereas replacement after 72h did not (Figure 21C).

Finally, the morphology of BKM120 treated cells was examined. In both cell lines, longtime incubation with BKM120 forced the formation of giant cells, almost ten times bigger than normal cells. Removal of BKM120 after 24h but not 72h increased the amount of normally sized cells and decreased the amount of giant cells in both cell lines (Figure 21D).

These observations clearly show that BKM120-mediated events were reversible. Thereby, the possibility to rescind these events strictly was time-dependent. Release from BKM120 before polyploidy was induced (after 24h in DG-75 and 72h in CA-46), restored cell cycle distribution and proliferation. Moreover, the formation of polyploid and hypodiploid cells reversed. However, whether the decrease of giant cells is a result of proliferation-related cell shrinking, increased proliferation of normally sized cells or distinct dell death remains an open question. In contrast, release from BKM120 after polyploidy induction (after 72h in DG-75), restored cell cycle distribution. In addition, polyploidy was reduced. However, proliferation could not be reinitiated and cells with a <2n DNA content did not disappear.

3.2 TARGETING PI3K/AKT/MTOR AS A COMBINATORIAL STRATEGY IN THE NHL SUBTYPE MCL

Acquired resistance to chemotherapeutic drugs is a common event in the onset of MCL therapy and has always been a serious problem. Vertical and horizontal drug combination is an attempt to overcome resistance. The combination of established cytostatics with inhibitors of the PI3K/mTOR pathway has shown to be affective in many tumors *in vitro*, and might be a promising strategy for the treatment of MCL.

3.2.1 Therapeutic drugs induce apoptosis in MCL

Chemotherapeutics, approved or currently tested for the treatment of MCL, were tested for their capacity to inhibit proliferation and viability of MCL cell lines (Figure 22).

Vincristine is a vinca alkaloid that arrests proliferating cells in mitosis by inhibiting the assembly of microtubules. Vincristine is approved for the treatment of non-Hodgkin lymphomas. In the present MCL cell line system, vincristine likewise had antiproliferative activity. All tested cell lines, except REC-1 (IC_{50} = 100nM), showed very high sensitivity to vincristine with an average IC_{50} value of 0.7nM. Vincristine also induced apoptotic cell death. Thereby, the cell lines MINO and JEKO-1 (average LC_{50} = 0.6nM) were more sensitive than GRANTA-519 and MAVER-1 (average LC_{50} = 1.4nM). REC-1 (IC_{50} =

100nM) cells, however, were not killed by low concentration of vincristine but behaved similar to GRANTA-519 and MAVER-1 at 100nM and similar to MINO and JEKO-1 at 100,000nM. In all cell lines, the very same concentrations that inhibited proliferation also induce apoptosis. This suggests that the vincristine-mediated decrease in metabolic activity is directly associated with cell death.

Bendamustine is a synthetically designed agent that has alkylator and antimetabolic activity. It causes cell cycle arrest by inducing DNA damages. Bendamustine is rapidly withdrawn by cytochrome p450 metabolism and albumin binding. The culture medium supplement FCS contains a high amount of albumin, which only can be compensated by the administration of high doses of bendamustine. Previous studies established a working concentration between 25,000 and 75,000nM for *in vitro* experiments. In the tested MCL cell line model, even higher concentrations of 100,000nM were needed to inhibit proliferation, whereby JEKO-1 and MINO were the most sensitive cell lines. REC-1, MAVER-1 and GRANTA-519 were much less responsive. Proliferation inhibition directly correlated with apoptosis induction. Strong apoptosis was observed in MINO, followed by JEKO-1 and REC-1. MAVER-1 and GRANTA-519 were apoptosis resistant.

Bortezomib is approved in the USA for the treatment refractory and relapsed mantle cell lymphomas [255]. Proteasome inhibition by bortezomib affects the proper degradation of circulating cellular proteins. This shifts the balance between pro- and anti-survival proteins and can lead to cell death. In the MCL cell lines tested in the current model, bortezomib inhibited proliferation and induced apoptosis to a similar extent. All cell lines were highly sensitive with an average IC_{50}/LC_{50} value of about 0.5nM. The effect on metabolic activity, therefore, was directly associated with apoptosis induction. Resistance to bortezomib was not detected.

Cisplatin is an alkylating agent that causes cross-linking of the DNA. It already has approval for distinct malignancies of the reproductive system, lung and skin and is currently tested in phase II trials for the treatment of MCL. However, cisplatin requires metabolic activation within the kidney [256]. Therefore, much higher concentrations were needed to gain metabolic activity *in vitro*. In the presented cell line model system, cisplatin inhibited proliferation only at concentrations beyond 1,000nM and induced apoptosis at concentrations of 10,000nM and more. Again, JEKO-1 and MINO cells were most sensitive for apoptosis induction, while cisplatin had an average effect on REC-1 and GRANTA-519. Only a slight impact on apoptosis was detected in MAVER-1.

Mitoxantrone is a type II topoisomerase inhibitor. Topoisomerases cover the DNA breaks, which are required to uncoil the DNA for replication. Consequently, topoisomerase inhibition leaves unrepaired DNA breaks. Furthermore, mitoxantrone disrupts DNA synthesis and DNA repair, by intercalation between the DNA bases [296]. In the tested MCL cell lines, treatment with mitoxantrone dose

dependently inhibited proliferation in all tested cell lines. Thereby, the cell lines could be divided into a highly sensitive (MINO, JEKO-1, MAVER-1) and a less sensitive (GRANTA-519, REC-1) subgroup. With regard to apoptosis, MINO was the most sensitive cell line with a LC_{50} value of about 0.5nM. In the less sensitive cell lines JEKO-1, GRANTA-519, REC-1 and MAVER-1 the LC_{50} was reached at 7.5nM, 50nM, 50nM and 100nM, respectively.

Cytarabine is a chemotherapeutic agent that displaces and replaces the nucleotide cytidine within the DNA. This leads to DNA damage and blocks DNA repair. High dose cytarabine has turned out to be an effective adjuvant to CHOP treatment [257] in MCL. In the current system, single treatment of MCL cell lines with cytarabine inhibited proliferation and induced apoptotic cell death in a dose dependent manner. Proliferation inhibition was most pronounced in MINO, JEKO-1 and MAVER-1 with IC_{50} values of about 50nM. GRANTA-519 and REC-1 were less sensitive with an IC_{50} of 500nM and 1,000nM, respectively. With regard to apoptosis, MINO and JEKO-1 were most sensitive, followed by GRANTA-519 and REC-1. MAVER-1 was the least sensitive cell line.

The tumor necrosis factor-α related apoptosis inducing ligand (TRAIL) recently received much attention as a possible anti-cancer candidate. It preferentially kills tumor cells by targeting the receptors DR4 and DR5. However, many tumors are resistant to TRAIL treatment. In the present MCL model system, strongest proliferation inhibition was observed in JEKO-1 cells with an IC_{50} value of about 2ng/ml. Moderate inhibition was observed in MINO, GRANTA-519 and REC-1. Again, MAVER-1 was the least sensitive cell line. Surprisingly, when focusing on apoptosis induction, only two sensitive cell lines could be identified, MINO and JEKO-1. In both cell lines, TRAIL induced apoptosis with LC_{50} values of about 5ng/ml. Although TRAIL treatment inhibited proliferation of GRANTA-519, REC-1 and MAVER-1 cells, no apoptosis induction could be detected in GRANTA-519 and MAVER-1. In REC-1, only a slight increase of hypodiploid cells was noticed.

Overall, MINO and JEKO-1 was the most sensitive cell lines irrespective of the agent used. GRANTA-519 and REC-1 showed average response to cytostatics and were resistant to TRAIL. MAVER-1 was the most resistant cell line and adequate apoptosis could only be induced with vincristine, mitoxantrone and bortezomib. This leaves bortezomib as the most potent agent to kill the MCL cell lines of the presented model system.

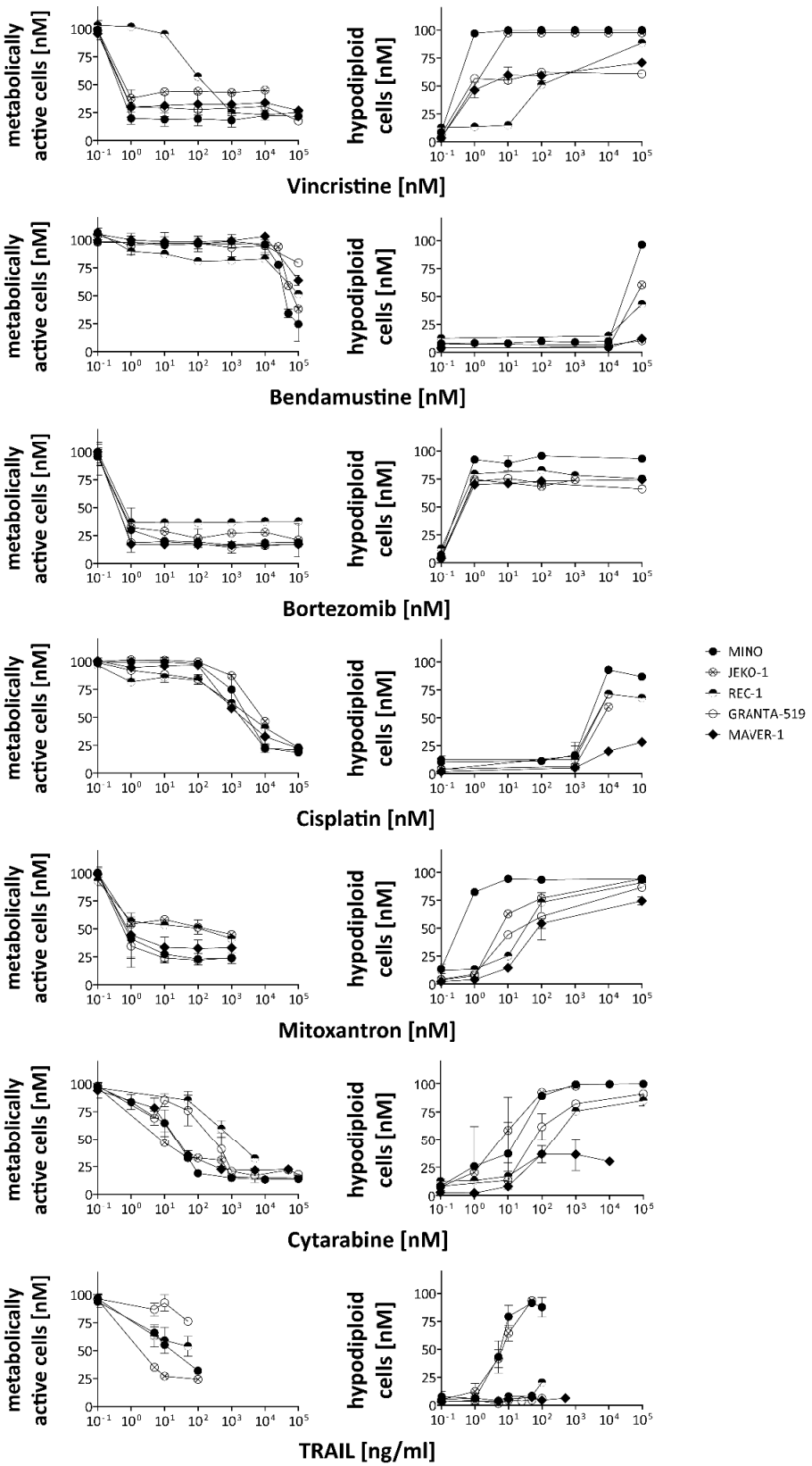

Figure 22: Response of MCL to biochemical therapeutics. Dose dependent response of the indicated anticancer agents on proliferation and viability of the mantle cell lymphoma cell lines MINO, JEKO-1, REC-1, GRANTA-519 and MAVER-1. The cell lines were incubated with increasing concentrations of BKM120 for 72h and proliferation was assessed by measuring the metabolic activity with XTT-assay while viability was assessed by measurement the amount of hypodiploid cells with flow cytometry.

3.2.2 TRAIL downregulates PI3K signaling

To investigate the apoptotic response of the used MCL cell lines to TRAIL, key apoptotic markers were evaluated in sensitive and resistant cell lines (Figure 23). As expected, the sensitive cell lines JEKO-1 and MINO showed downregulation of the BH3-only Bid, probably caused by truncation into tBid, and PARP cleavage. PARP cleavage is associated with its inactivation and conducted by effector caspase-3. Thereby, PARP cleavage marks the execution of apoptosis. Truncation of Bid causes amplification of the apoptotic signal by additional activation of intrinsic signaling. That suggests that JEKO-1 and MINO are type II cells. In contrast, no Bid activation or PARP cleavage was detectable in the resistant cell lines REC-1 and GRANTA-519.

One possible mechanism for TRAIL resistance is the activation of NFκB by TRAIL itself [258]. NFκB activity is negatively regulated by IκBα and downregulation of IκBα results in NFκB activation. Of the two resistant cell lines, only REC-1 showed downregulation of IκBα (Figure 23). Surprisingly, in the sensitive cell lines IκBα was downregulated as well. This eliminates NFκB as a possible key factor for TRAIL resistance in this model system.

Another possible resistance mechanism is constitutive PI3K signaling [259]. As already shown, Akt is constitutive active in these MCL cell lines [222]. By analyzing the phosphorylation status of downstream targets, the activity of Akt in response to TRAIL was assessed (Figure 23). Treatment with TRAIL caused downregulation of S6K phosphorylation at S371 in all four cell lines, irrespective of sensitivity. However, only in the sensitive cell lines JEKO-1 and MINO overall expression of S6K was abrogated, whereas S6K was still expressed in the resistant REC-1 and GRANTA-519. Furthermore, phosphorylation of 4EBP1 at S65 was clearly downregulated in JEKO-1 and slightly in MINO but not in the resistant REC-1 and GRANTA-519. This shows a different regulation of mTOR signaling in TRAIL sensitive and resistant cell lines and suggests a role for PI3K/Akt/mTOR signaling in TRAIL resistance.

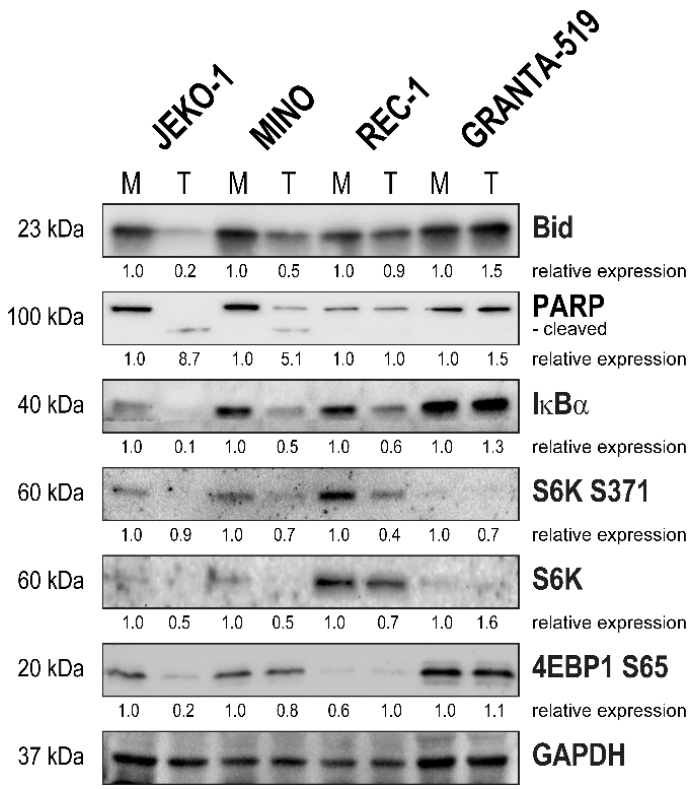

Figure 23: Response to TRAIL induced apoptosis. Downregulation of Mcl-1, Bcl-2, Bid, Iκbα, S6K in the sensitive cells MINO and JEKO-1 treated with TRAIL (T) for 24h or left untreated (M). Furthermore, dephosphorylation of 4EBP1 and S6K. Execution of apoptosis is proven by PARP cleavage.

3.2.3 mTOR inhibition protects from apoptosis

The lack of mTOR target regulation upon TRAIL treatment in resistant cell lines emerged the question whether inhibition of mTOR would overcome resistance and sensitize these cells for TRAIL. Therefore, mTOR signaling was blocked with the rapamycin analog RAD001 prior to TRAIL treatment. Surprisingly, pretreatment of GRANTA-519 or MAVER-1 with RAD001 did not sensitize for TRAIL (Figure 24B). This initially suggested that TRAIL mediated downregulation of mTOR signaling does not correlate with TRAIL's apoptotic potential. However, the ability of mTOR or PI3K inhibition to synergize with TRAIL in sensitive cells is documented [145,241,242,243]. To test the capacity of the rapamycin analog RAD001 to sensitize for TRAIL induces apoptosis, JEKO-1 and MINO cells were pretreated with 500nM of RAD001 for 4h and subsequently incubated with 5ng/ml TRAIL for another 72h. The apoptosis rate was evaluated by measuring the amount of hypodiploid cells with flow cytometry (Figure 24A, B). Surprisingly, and in contrast to the published data [145,241,242,243], combination of RAD001 with TRAIL did not sensitize but protected both cell lines from TRAIL induced apoptosis and reduced the amount of hypodiploid cells. In MINO cells, apoptosis was abrogated almost completely. To investigate whether this effect is restricted to TRAIL or might a general effect, the drug panel examined before (chapter 3.2.1) was combined with RAD001 as well (Figure 24A). In MINO cells, pretreatment with

RAD001 did not affect apoptosis induction by vincristine or bendamustine. For bortezomib and cisplatin, as slight, but not significant, decrease was observed. In contrast, significant protection from apoptosis occurred when RAD001 was combined with mitoxantrone or cytarabine. In the other cell lines, RAD001 mediated apoptosis protection was observed as well. In JEKO-1, pretreatment with RAD001 downregulated apoptosis mediated by cisplatin, mitoxantrone and cytarabine, however, only in cisplatin treated cells protection was significant (Figure 24B). In GRANTA-519, RAD001-mediated apoptosis protection was detected with cisplatin and mitoxantrone, whereas, again, only protection from cisplatin was significant (Figure 24B). MAVER-1 cells were non-significantly protected from mitoxantrone and cytarabine mediated apoptosis (Figure 24B). Thus, the protective capacity of RAD001 varies substantially between the used drugs and cell lines so that no clear picture of RAD001's behavior could be assembled.

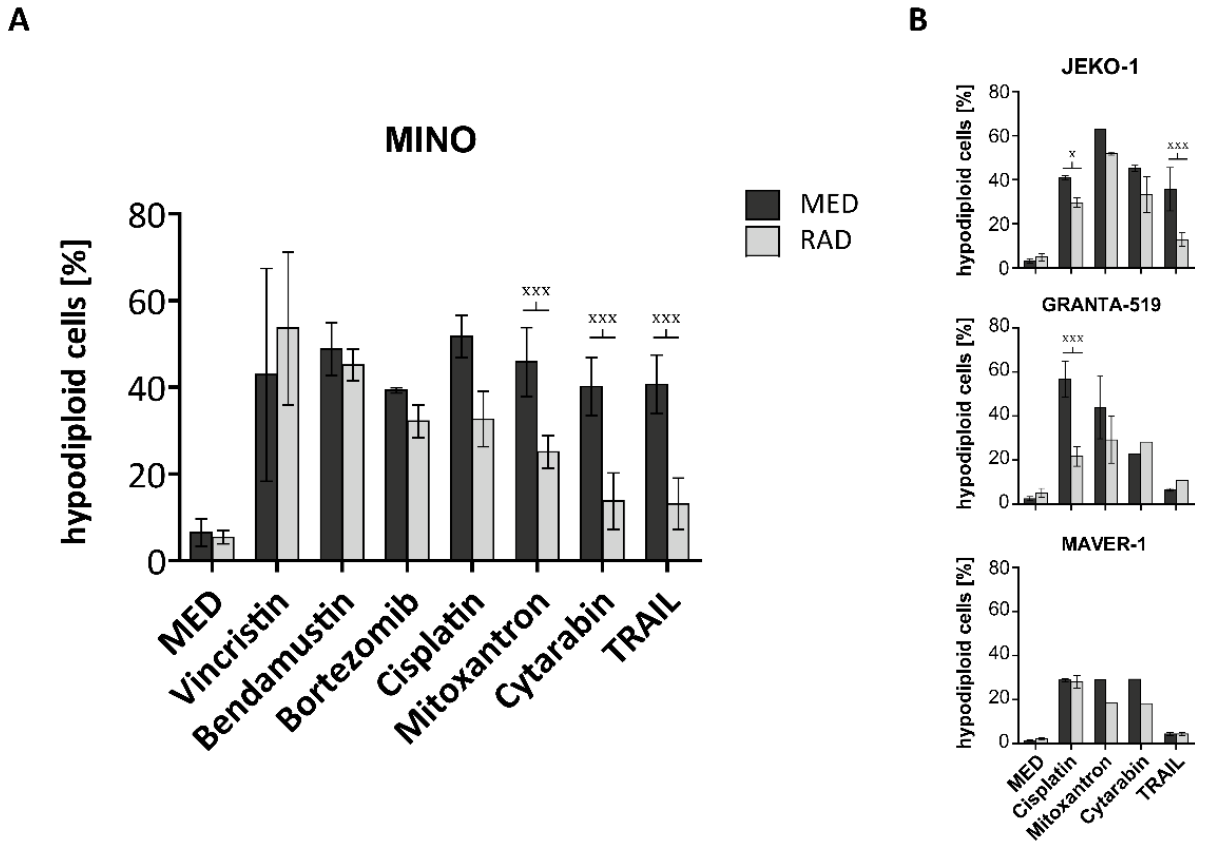

Figure 24: Pretreatment with RAD001 protects from apoptosis. (A) MINO cells were treated with 500nM of RAD001 for 4h and then incubated with medium (MED), 0,5nM vincristine, 50000nM bendamustine, 0,5nM bortezomib, 5000nM cisplatin, 0,5nM mitoxantrone, 30nM cytarabine or 5ng/ml hrTRAIL for another 72h. (B) JEKO-1 cells were treated with 500nM of RAD001 for 4h and then incubated with medium (MED), 5000nM Cisplatin, 100nM Mitoxantrone, 10nM cytarabine or 5ng/ml hrTRAIL. GRANTA-519 cells were treated with 500nM of RAD001 for 4h and then incubated with medium (MED), 5000nM Cisplatin, 100nM Mitoxantrone, 50nM cytarabine or 50ng/ml hrTRAIL. MAVER-1 cells were treated with 500nM of RAD001 for 4h and then incubated with medium (MED), 5000nM cisplatin, 100nM mitoxantrone, 100nM cytarabine or 50ng/ml hrTRAIL. Apoptosis was assessed by measuring the amount of hypodiploid cells.

3.2.4 Apoptosis protection by mTOR inhibition is dose dependent

The observation that mTOR inhibition protects from apoptosis induction, conflicts with published data. In previous reports, concentrations of 1nM to 100nM were used, which led to the assumption, that the RAD001 effect might be dose dependent. To examine this, a wide concentration range of RAD001 was combined with TRAIL (Figure 25, fifth panels). In MINO cells, strong apoptosis protection was detected between 5nM and 500nM RAD001. Surprisingly, very high concentrations of 5,000nM abrogated protection and restored sensitivity to TRAIL. In JEKO-1 cells, 5nM of RAD001 had a small, albeit but not significant, effect on TRAIL induced apoptosis. Significant cell death inhibition was observed with 500nM RAD001. Here, with high doses of RAD001 not only abrogation of apoptotic protection and restore of sensitivity was observed, but also synergistic sensitization for TRAIL could be detected. This clearly showed that RAD001 has dual function on apoptosis regulation and can induce opposite effects, depending on its concentration. For cancer therapy, this could be critical as some drug combinations might be contraindicated.

3.2.5 Inhibition of the PI3K pathway on different levels protects from TRAIL

mTOR generally acts downstream of PI3K and Akt signaling. However, due to several feedback loops, mTOR inhibition can affect PI3K/Akt activity itself. The complex signaling, impedes the determination of mTOR as key regulator of the observed effect. Systematic inhibition of upstream-located kinases could help to identify the central protein. Therefore, the TRAIL sensitive cell lines MINO and JEKO-1 were preincubated with increasing concentrations of either BKM120 (PI3K inhibitor), Wortmannin (PI3K inhibitor), LY294002 (PI3K inhibitor), Akt inhibitor VIII (Akt inhibitor) or BEZ235 (double PI3K and mTOR inhibitor) (Figure 25). As observed before with RAD001 (chapter 3.2.4), the inhibition of upstream signaling protected from TRAIL induced apoptosis. The Akt inhibitor Akt inhibitor VIII strongly downregulated TRAIL induced apoptosis in both cell lines at low concentrations from 1,000nM to 5,000nM in MINO and 100nM to 1,000nM in JEKO-1. However, only for 1,000nM in MINO significance was proven. In contrast, high concentrations of 5,000nM in JEKO-1 and 10,000 in MINO had synergistic effect to TRAIL and sensitized for apoptosis. Upstream of mTOR and Akt, the response to signal inhibition was heterogeneous. In MINO cells, all three PI3K inhibitors (BKM120, Wortmannin, LY294002) and even the PI3K/mTOR double inhibitor BEZ235 significantly protected from TRAIL induced apoptosis at 5-500nM (BKM120), 100nM (Wortmannin), 1,000-5,000nM (LY294002) or 5nM (BEZ235). Sensitization to TRAIL was observed only with high concentrations of Wortmannin. In JEKO-1 cells, only treatment with 1,000nM LY294002 significantly protected from apoptosis. In contrast, sensitization to TRAIL with high concentrations was observed for all inhibitors. This suggests that PI3K/Akt/mTOR signaling plays a crucial role in triggering TRAIL mediated apoptotic cell death. Furthermore, the consistant response to mTOR and Akt inhibition in distinct cell lines and the mixed

response to PI3K inhibition suggests that mTOR probably is the key molecule in controlling apoptosis initiation. Thus, mTOR signaling seems to be redunant among our MCL cell lines, while PI3K signaling is variable.

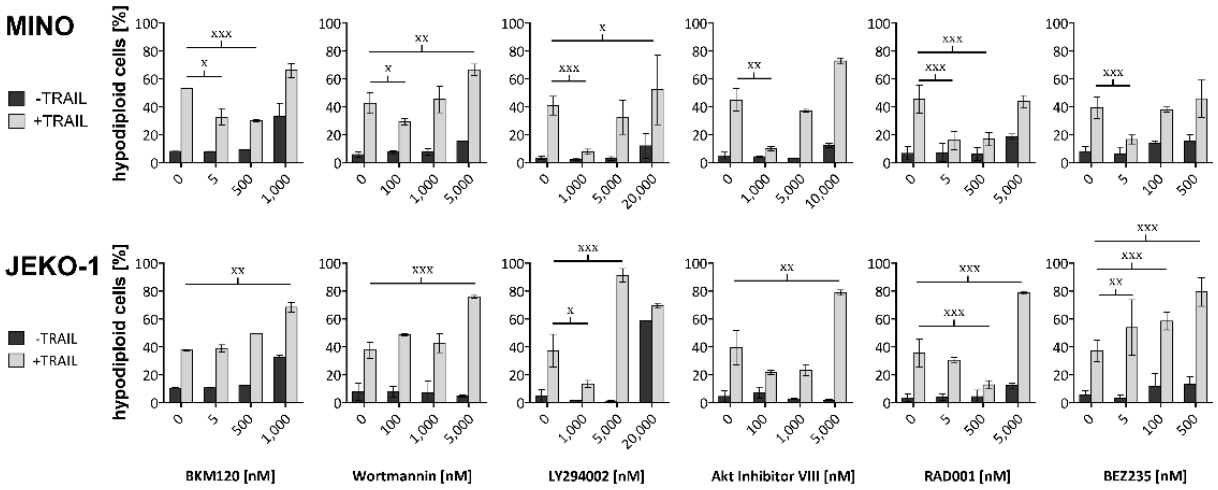

Figure 25: Inhibition of the PI3K pathway on different levels protects from TRAIL. The TRAIL sensitive cell lines MINO and JEKO-1 were incubated with increasing concentrations of either BKM120, Wortmannin, LY294002, Akt inhibitor VIII, RAD001 or BEZ235 for 4h, subsequently treated with 5ng/ml of hrTRAIL (+TRAIL) and incubated for another 72h. The apoptotic rate was assessed by measuring hypodiploid cell with flow cytometry.

3.2.6 Apoptosis protection is independent of autophagy

mTOR was suggested to be the key regulator of TRAIL protection (chapter 3.2.5). One of its main functions is the regulation of autophagy. Therefore, we presumed autophagy to be the mechanism that prevents apoptosis induction in TRAIL treated cells. This is supported by previous reports of autophagy protecting against death receptor induced apoptosis [260,261,262]. A hallmark of autophagy is the cleavage and lipidation of LC3-I into LC3-II, which is detectable by immunoblotting. To examine the ability of PI3K and mTOR inhibitors to induce autophagy in our model system, LC3 processing was analyzed (Figure 26). However, no LC3-II formation was detected with any of the inhibitors used. Although LC3-I was dose dependently downregulated with BEZ235, no LC3-II band appeared. This suggested that in our model system, PI3K/mTOR inhibition did not induce autophagy and thereby ruled out the possibility that autophagy might protects from apoptosis.

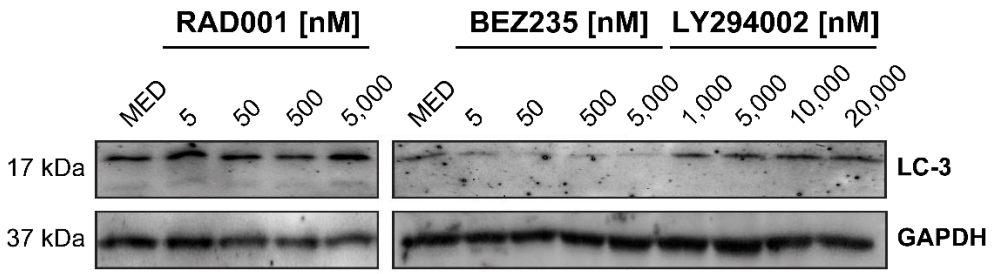

Figure 26: Apoptosis protection is independent of autophagy. Treatment of JEKO-1 cells with RAD001, BEZ235 or LY294002 at the indicated concentrations [nM] for 24h. The protein expression was assessed using western blot analysis. The predicted sizes for LC3-I and LC3-II are 17 and 15kDA, respectively.

3.2.7 The antiapoptotic PI3K inhibition can be reversed by blocking NFκB

Another important antiapoptotic factor is NFκB. TRAIL and Rapamycin, are reported to regulate NFκB [180,263]. Consequently, downregulation of NFκB sensitizes for TRAIL [258,264] and might do so for PI3K/mTOR inhibitors. In addition, NFκb activation is involved in cytarabine and cisplatin resistance [265,266]. Therefore, inhibition of NFκB was assumed to overcome RAD001 mediated apoptosis resistance and resensitizes cells for TRAIL and cytarabine. To analyze this, NFκB was blocked with the inhibitor BAY117082 at the effective but non-toxic concentration of 500nM (Figure 27A). BAY117082, RAD001 and TRAIL were administered at intervals of 4h and cells were incubated for 72h. The apoptosis rate was assessed by counting hypodiploid cells with flow cytometry. Combination with BAY117082, did not affect the response to RAD001 in JEKO-1 or MINO cells. Combinatorial treatment with BAY117082 and TRAIL seemed to protect JEKO-1 cells slightly from TRAIL induced apoptosis and sensitized MINO cells for TRAIL, but none of these effects was significant. Similar, MINO cells, incubated with BAY117082 and cytarabine, showed slightly less apoptosis that cell treated with cytarabine alone. This was also not significant. As observed before, combination of RAD001 with TRAIL or cytarabine significantly protected from apoptosis. Administration of all three drugs, BAY117082, RAD001 and TRAIL, overcame RAD001 mediated apoptosis protection and restored TRAIL sensitivity in both cell lines tested. In case of cytarabine, treatment with NFκb inhibitor, reversed RAD001 protection but to a much lesser extent than observed with TRAIL. Full sensitivity to cytarabine could not be reinstalled. Thus, NFκB signaling is suggested to play a prominent role in regulating mTOR-dependent sensitivity to apoptosis inducing agents like TRAIL or cytarabine.

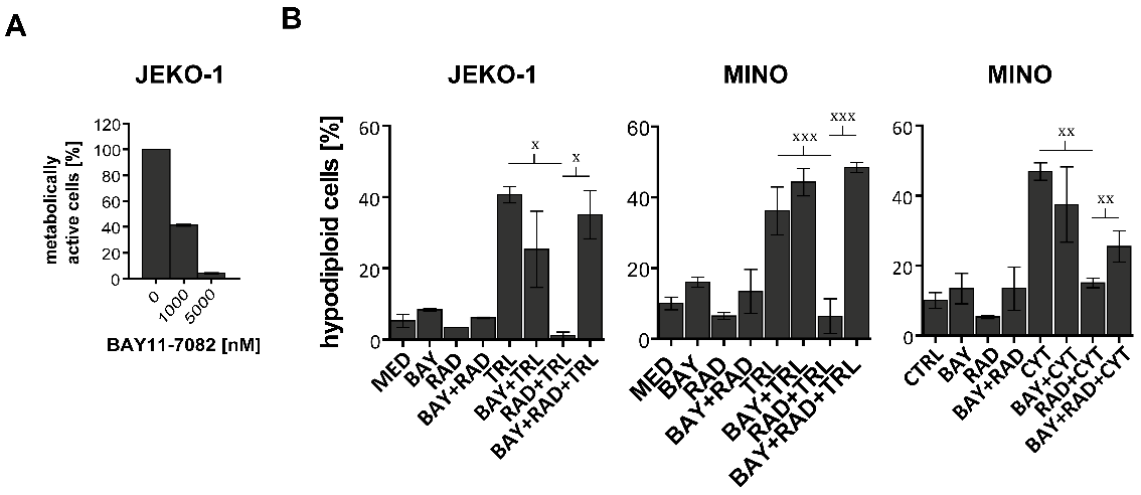

Figure 27: NFκB Inhibition with BAY11-7082 reverses RAD001 protective effect on TRAIL and Cytarabine induced apoptosis. (A) Dose dependent proliferation inhibition in JEKO-1 cells treated with the NFκB inhibitor BAY11-7082 for 72h. (B) Triple treatment of JEKO-1 and MINO cells with 500nM of BAY11-7082 (BAY), 500nM RAD001 (RAD) and 5ng/ml of hrTRAIL (TRL) or 5nM of cytarabine (CYT) in intervals of 4h and subsequent incubation for another 72h. The Apoptotic rate was assessed measuring hypodiploid cells with flow cytometry.

3.2.8 RAD001 prevents TRAIL induced caspase-8 cleavage and upregulates Flip and Pim-2

To examine the underlying mechanism of RAD001 mediated apoptosis protection and the possibility of NFκB inhibition to restore sensitivity, apoptotic signaling was evaluated on the protein level using the immunoblotting technique. Briefly, the sensitive cell line MINO was pretreated with 500nM of BAY11-7082 for 4h, RAD001 was administered at 500nM for another 4h and finally TRAIL was added at 5ng/ml and incubated for 24h. When examining the effects of single drug administration, apoptotic signaling was observed after treatment with TRAIL, but not BAY11-7082 or RAD001. Only TRAIL induced apoptosis associated cleavage of initiator caspase-8 and PARP. With regard to dual treatment, as expected, pretreatment with RAD001 abrogated TRAIL-dependent caspase-8 cleavage and reduced PARP cleavage. In contrast, BAY11-7082 did not affect TRAIL-induced caspase-8 or PARP cleavage. Upon triple treatment, however, BAY117082 counteracted RAD001's effects and reinduced caspase-8 as well as PARP cleavage in TRAIL treated cells (Figure 28). Thus, mTOR was suggested to have impact on apoptotic signaling and that NFκB interferes with that.

The previous results suggested that the TRAIL sensitive cells of the used model are type II cells and, thereby, require signal amplification via the intrinsic pathway (chapter 3.2.2). The upregulation of antiapoptotic Bcl-2 members abrogates intrinsic signaling and can cause TRAIL resistance. In fact, many antiapoptotic Bcl-2 members are reported targets of NFκB and mTOR signaling, including the major proteins Bcl-2 [175,176] and Mcl-1 [181,267]. Thus, NFκB and mTOR could control TRAIL sensitivity by

regulating antiapoptotic Bcl-2 members in the used MCL model. To test this, we evaluated the expression of Bcl-2 and Mcl-1 in response to single or combinatorial treatment with TRAIL, BAY117082 and RAD001 in MINO cells (Figure 28). As observed before (chapter 3.2.2), TRAIL treatment slightly downregulated Mcl-1 and Bcl-2 expression. In contrast, single mTOR inhibition with RAD001 did not affect Bcl-2 or Mcl-1 expression. Finally, BAY117082 downregulated Bcl-2 slightly, but not Mcl-1. However, both, double and triple inhibition downregulated Bcl-2 but not Mcl-1 (Figure 28). Thus, neither Bcl-2 nor Mcl-1 controls NFκB and/or mTOR dependent TRAIL sensitivity.

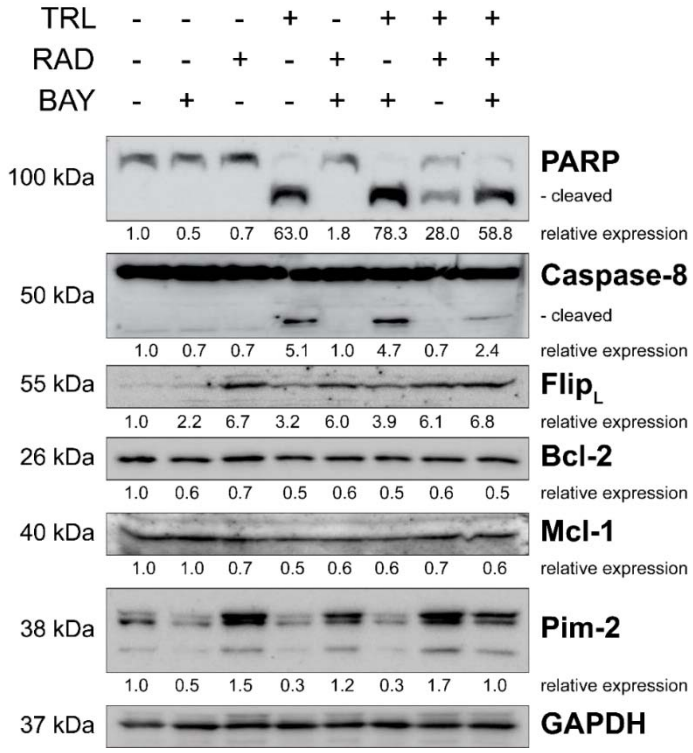

Figure 28 Expression of apoptosis related proteins in response to triple treatment. MINO cells were treated with 500nM of the NFκB inhibitor BAY11-7082 (BAY), 500nM of the mTOR inhibitor RAD001 (RAD) and 5ng/ml of the TRAIL-receptor ligand hrTRAIL (TRL) in intervals of 4h and incubated for another 24h. Protein expression was assessed using western blot analysis.

Caspase-8 cleavage and subsequent TRAIL sensitivity, among others, depends on Flip expression, which is known to be regulated by mTOR [145,241] and is also a target of NFκB [268]. Thus, we presumed that the interaction between mTOR, NFκb and TRAIL might be regulated by Flip. To examine this, Flip expression in response to single or combined treatment with TRAIL, BAY117082 and RAD001 was measured. TRAIL treatment alone did not regulate Flip expression considerably. Surprisingly and in contrast to previous reports [145,241], treatment with RAD001 clearly upregulated Flip expression. This was also observed in combination with TRAIL. Treatment with BAY117082, however, had no effect on the Flip level, neither alone nor in dual combination with TRAIL nor in triple combination with

RAD001 and TRAIL. Thus, Flip upregulation by RAD001 could be a reason for TRAIL resistance but did not correlate with restoration of sensitivity upon additional NFκB inhibition in this model.

Another suggested target is the Pim-2 kinases. The family of Pim kinases was identified as oncogenes frequently overexpressed in many malignancies [269]. Thereby, a high expression is associated with apoptosis resistance and tumorigenesis. On the one hand, Pim kinases are known targets of NFκB and, on the other hand, have overlapping functions with Akt/mTOR [269]. In lymphomas, especially Pim-2 expression seems to be relevant [270]. Thus, the regulation of Pim-2 might hold an explanation for these observations. To investigate this, Pim-2 expression in response to TRAIL, RAD001 and BAY117082 has been evaluated. Treatment with BAY117082 alone downregulated Pim-2 expression and thereby confirmed Pim-2 as a target of NFκB. Interestingly, single treatment with TRAIL downregulated Pim-2 expression as well. However, combination of BAY117082 and TRAIL did not further reduce Pim-2 expression. In contrast and to our surprise, RAD001 alone caused upregulation of Pim-2 expression. This was also observed in combination with TRAIL. Combination of RAD001 with BAY117082 decreased Pim-2 and did so in additional combination with TRAIL (Figure 28). In total, Pim-2 is regulated in accordance to the observed viability pattern of combinatorial TRAIL, RAD001 and BAY117082 treatment. Thus, Pim-2 could be the factor that determines TRAIL sensitivity.

3.2.9 The NFκB target Pim-2 is regulated dose dependently

Treatment of mantle cell lymphoma with RAD001 revealed a strong upregulation of the highly conserved NFκB targets Pim-2 and Flip (chapter 3.2.8). However, only Pim-2 expression seemed to correlate with BAY117082-mediated recovery from RAD001-induced TRAIL resistance. As protection from TRAIL induced apoptosis by PI3K pathway inhibition is dose dependent and occurs only at lower concentrations, we analyzed whether the expression of Pim-2 is expressed dose dependently as well. Therefore, MINO cells were treated with increasing concentrations of RAD001, LY294002 or BEZ235 (Figure 29). Treated with RAD001, dose dependently regulated Pim-2 expression in MINO. Low concentration of 5nM and 50nM clearly increased the Pim-2 proteins level. At 500nM, Pim-2 levels dropped slightly below base line. With the dual PI3K/mTOR inhibitor BEZ235, Pim-2 expression was strongly induced at 5nM and 50nM and slightly dropped at 500nM and 5,000nM (Figure 29). Thus, RAD001, LY294002 and BEZ235-mediated Pim-2 expression highly correlates with the inhibitor dependent TRAIL resistance. For instance, RAD001 protects from TRAIL induced apoptosis at 5nM and in consistence Pim-2 expression is upregulated, while concentrations of 5000nM restored sensitivity and even sensitized for TRAIL (chapter 3.2.5). Altogether, Pim-2 expression fluctuates in line with the protective behavior of all PI3K pathway inhibitors used in the MCL model.

Next, it was analyzed whether PI3K/Akt/mTOR controls NFκB, as Pim-2 is a reported NFκB target and NFκB inhibition neutralized the PI3K/Akt/mTOR mediated apoptosis protection (chapter 3.2.7). Inhibition of either mTOR (RAD001), PI3K (LY294002) or PI3K/mTOR (BEZ235) dose dependently downregulated the negative regulator of NFκB, IκBα (Figure 29). Likewise, a dose dependent upregulation of NFκB was observed for LY294002 and BEZ235 but not RAD001. However, the protein levels of neither IκBα nor NFκB were compatible to Pim-2. Thus, there were doubts whether PI3K/Akt/mTOR controls Pim-2 expression via NFκB.

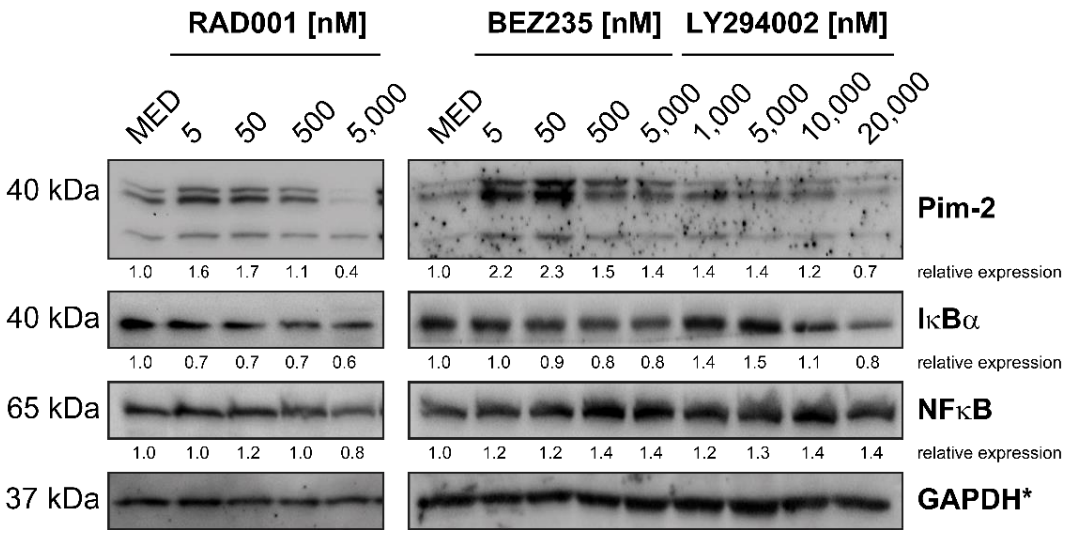

Figure 29: Dose dependent expression of Pim-2. MINO cells were incubated with increasing concentrations (nM) of either RAD001, BEZ235 or LY294002 for 24h and protein expression was assessed using western blot analysis. The same Blot was used for Figure 26 and Figure 29. Therefore, the housekeeping control GAPDH is the same (*).

3.2.10 Downregulation of Pim-2 protects from TRAIL induced apoptosis

As Pim-2 expression correlated with the protective effect of PI3K inhibitors, it was suspected that abrogating Pim-2 activity would sensitize cells for TRAIL induced apoptosis. To examine this, Pim-2 expression was blocked by the use of the pan-Pim inhibitor LGB321 from Novartis. The two cell lines MINO and JEKO-1 were treated with increasing concentration of LGB321 for 4h and incubated in TRAIL for another 72h. The apoptotic response was evaluated by flow cytometry and measurement of hypodiploid cells. Surprisingly, the pan-Pim inhibitor LGB321 protected both cell lines from TRAIL induced apoptosis (Figure 30A). This protective response was similar to the observations with PI3K inhibitors. At low concentrations of 5nM and 500nM, TRAIL induced apoptosis was reduced while the high concentration of 5,000nM sensitized for TRAIL. To analyze this effect in more detail, Pim-2 signaling was examined by western blot (Figure 30B). Interestingly, when MINO cells were treated with LGB321, Pim-2 expression was not down- but upregulated. In contrast, LGB321 inhibited S6K phosphorylation at S371 and 4EBP1 phosphorylation at S65 and T37/46. That, on the one hand,

showed that LGB321 affected mTOR signaling but, on the other hand, questioned whether LGB321 inhibited Pim-2 activity. Yet, assuming LGB321 to inhibit Pim kinases activity but not to cause their degradation, an increase in Pim-2 expression would be associated with the accumulation of inactive Pim kinases/LGB321 complexes. Thus, these results do not clarify whether LGB321 affects Pim-2 activity.

Overall, the inhibitor LGB321 might not be the right tool to investigate the role of Pim-2 in TRAIL resistance. Downregulation by siRNA, the expression of a dominant negative protein or genetic knockout should be considered. Nevertheless, as the activity of S6K and 4EBP1 was demonstrated to be Pim-2 dependent, RAD001 mediated upregulation of Pim-2 expression provided a dose dependent feedback loop that reactivated signaling downstream of mTOR.

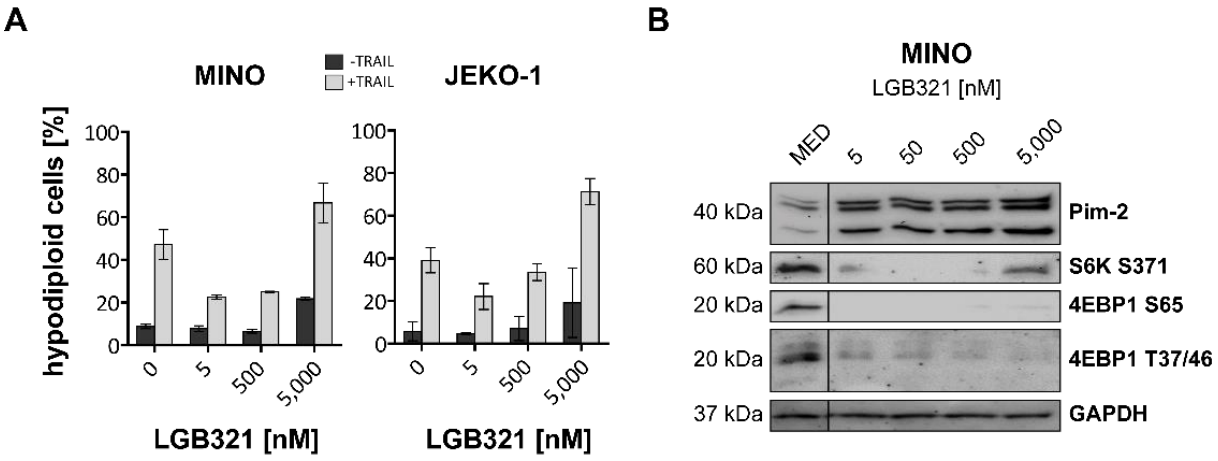

Figure 30: Pim inhibition protects from hrTRAIL induced apoptosis. (A) Protein expression of MINO cells in response to LGB321. MINO cells were incubated with the indicated concentrations of the pan-Pim inhibitor LGB321 for 24h and protein expression was assessed by western blot analysis. (B) Apoptotic response to combined treatment with LGB321 and hrTRAIL. MINO and JEKO-1 cells were incubated with increasing concentrations of LGB321 for 4h and treated with 5ng/ML hrTRAIL for another 72h. Apoptosis was assessed by measuring the amount of hypodiploid cells by flow cytometry.

4 DISCUSSION

4.1 BKM120 IN THE TREATMENT OF NHL

PI3K signaling balances cells towards survival and proliferation by influencing a wide range of cellular processes and interacting with many other pathways. Aberrations in PI3K signaling are associated with tumor development and tumor progression and, therefore, are found in many tumor entities [271]. To target PI3K signaling has become an important field in the development of new cancer strategies. Several agents, that block signaling at different stages of the PI3K pathway, were shown to have an antiproliferative impact in many tumors. Rapamycin (Sirolimus) and the rapalogs RAD001 (Everolimus) and CCI779 (Temsirolimus), inhibitors of the PI3K downstream signaling protein mTORC1, are first generation inhibitors that have proven its virtue in the treatment of many solid and hematological malignancies and improve the progression free survival of patients with, for instance, relapsed MCL with manageable side effects [220,272,273]. The mode of action of these mTOR inhibitors relies on their binding to the small cytosolic molecule FK-binding protein 12 (FKBP12). In complex with FKBP12, rapalogs bind and inhibit mTORC1 activity [274,275]. Their potential to kill cells, however, appears to be limited, which in part is due to reactivation of PI3K signaling by several negative feedback loops [209,276,277]. To improve the efficacy of pathway inhibition, the development of second-generation inhibitors focuses on targeting upstream PI3K to circumvent feedback signaling. Furthermore, newly designed inhibitors are competitive for the ATP binding side of their targets, which enhances their affinity.

BKM120, a pan-PI3K class I inhibitor developed by Novartis, is currently tested in phase I and II clinical trials against breast, lung, prostate cancer, leukemias and b-cell lymphomas. In parallel, the molecular effects of BKM120 on tumor cells are investigated in *in vitro* and *in vivo* studies. Here, the utility of BKM120 for the treatment of b-cell non-Hodgkin lymphomas was examined in an *in vitro* cell line model. Therefore, a panel of mantle cell lymphoma cell lines, two Burkitt lymphoma cell lines and a diffuse large b-cell lymphoma cell line were used.

4.1.1 BKM120's impact on PI3K/Akt/mTOR signaling and viability

In initial reports, BKM120's capacity to inhibit PI3K activity was proven with the downregulation of downstream Akt S473 phosphorylation at concentrations of up to 2,000nM [224,225,226,244,278]. In addition, the mTOR downstream targets p70S6K and 4EBP1 were dephosphorylated. This suggests that BKM120 not only inhibits PI3K but also, directly or indirectly, affects PI3K downstream signaling by inhibiting mTOR activity. Indeed, protein kinase assays predict that mTOR becomes a direct target of BKM120 at 2,866 ± 1,671 nM [225]. In the chosen NHL model system, BKM120 was demonstrated to

dephosphorylate Akt S473 at 2,000nM. Moreover, the inactivation of the downstream target mTOR was proven by detecting dephosphorylation of S6K and 4EBP1. This dephosphorylation, however, occurred already at concentrations of about 1,000 to 1,500nM. Thus, BKM120 inhibits PI3K activity and downstream signaling in NHL cell lines.

The PI3K/Akt/mTOR pathway promotes growth, proliferation and survival of cells. In consequence, blocking PI3K/Akt/mTOR signaling with established inhibitors such as LY294002, Wortmannin, or rapamycin affects the viability of tumor cells. The same was observed for the newly developed PI3K inhibitor BKM120. Here, BKM120 was shown to inhibit the metabolic activity and, thus, proliferation and viability of the used NHL cell lines in a dose dependent manner. Furthermore, treatment with BKM120 killed NHL cells. Thereby, apoptosis was identified as the preferred type of programmed cell death and was characterized by DNA fragmentation and caspase dependency. This is consistent with previous data from gliomas, breast carcinoma, lung cancer, myeloma and chronic lymphoid leukemia [226,228,279,280]. For apoptotic signaling two pathways are established, the extrinsic and the intrinsic pathway. Intrinsic or mitochondrial signaling is associated with pore formation in the outer membrane of the mitochondria, loss of the mitochondrial membrane potential and release of proapoptotic factors. These events are initiated by the proapoptotic Bax subgroup of the Bcl-2 family. Activation of Bax/Bak and breakdown of the mitochondrial membrane potential have already been shown for the PI3K inhibitors LY294002 and PI103 and the dual PI3K/mTOR inhibitor BEZ235 [222,281,282]. Here, for the first time, the activation of Bax and Bak and the subsequent loss of the mitochondrial membrane potential are demonstrated for BKM120. Furthermore, mitochondrial breakdown was associated with the cleavage of the intrinsic caspase-9. This clearly shows that BKM120 likewise uses the intrinsic pathway to induce apoptosis.

4.1.2 BKM120 mediated apoptosis and the involvement of the Bcl-2 family

The activation of Bax and Bak, in turn, depends on the neutralization of the antiapoptotic Bcl-2 members by the proapoptotic subgroup of BH3-onlys. Under healthy conditions, intrinsic signaling is prevented by negative regulation of the BH3-onlys on distinct levels, including transcription, translation, phosphorylation and proteasomal degradation. Many of these modifications are conducted by the PI3K/Akt/mTOR pathway. For instance, the expression of BH3-onlys is negatively regulated by Akt/mTOR dependent inhibition of transcription factors such as FoxO3a and p53 [283,284,285]. In consequence, established inhibitors of the PI3K/Akt/mTOR pathway promote apoptosis by upregulating the expression of BH3-onlys like Bim and Puma [174,222,286,287]. Here, the impact of BKM120 on the expression of BH3-onlys was investigated in the NHL model system. BKM120 caused a strong and initial upregulation of Puma and Hrk followed by a less pronounced upregulation of Nbk and Bmf. This suggested a two-step mechanism in the induction of intrinsic apoptosis by

BKM120, with Puma and Hrk being the initiators. The Puma peptide is capable to neutralize five out of six antiapoptotic Bcl-2 members and its exogenous expression strongly induces apoptosis [288,289,290]. Therefore, it is assigned to as potent inducer of apoptosis [291]. Puma's action is transcriptionally controlled mainly by p53 [292]. PI3K/Akt signaling stabilizes the negative regulator of p53, MDM2, and promotes its translocation into the nucleus [125,126]. In addition, GSK3β, a direct target of PI3K/Akt was shown to be required for p53 dependent Puma expression [293,294]. This would mean that PI3K/Akt inhibition promotes p53 dependent Puma expression due to MDM2 degradation and GSK3β activation. However, here in the tested lymphoma model system, the majority of cell lines carry mutations for p53 [295,296], which makes that way of regulation unlikely. Thus in this model system, a p53 independent Puma regulation via FoxO3a is more likely. FoxO3a is a direct transcription factor of Puma expression and negatively controlled by Akt and mTOR mediated phosphorylation [283,284,297,298]. It is therefore reasonable that BKM120 induces Puma expression by activating FoxO3a.

Similar to Puma, the Hrk peptide was reported to interact with five of six antiapoptotic Bcl-2 members, whereas binding to Bcl-w, Bcl-x_L and A1/Bfl is clearly preferred [289]. However, Hrk induced apoptosis can be attenuated likewise by overexpression of Bcl-x_L or Bcl-2 [299,300]. The regulatory mechanisms that control Hrk are largely unknown. Yet in hematopoietic progenitors, growth factor withdrawal was shown to induce Hrk expression and apoptosis [301,302]. As growth factors mainly signal via the PI3K/Akt/mTOR pathway, this strongly implies the participation of PI3K/Akt/mTOR in Hrk regulation. Indeed, especially in cells with low NFκB expression, a SYK/PI3K dependent Hrk regulation is implicated as SYK inhibition with R406 as well as PI3K inhibition with LY294002 upregulates Hrk on the transcriptional level [303]. In this study, BKM120 dependent induction of Hrk expression is reported for the first time and assumed to be involved in BKM120 mediated apoptosis.

In contrast to Puma and Hrk, late and weak upregulation was observed for Nbk and Bmf. Due to their restricted binding of antiapoptotic Bcl-2 members, both are assigned to as weak inducers of apoptosis [291]. According to published coimmunoprecipitation data, Nbk/Bik binds Bcl-x_L and Bcl-2 but not Mcl-1. This is associated with entirely Bax dependent apoptotic signaling, a circumstance that certainly affects the killing potential of Nbk [289,304,305]. Yet, overexpression of Nbk induces programmed cell death and sensitizes for apoptotic stimuli [305,306,307,308,309]. Endogenous Nbk seems to be less effective. In mature B-cells, the Nbk levels increase upon BCR stimulation and correlate with apoptosis [310,311]. However, deletion of Nbk does not affect their response to apoptotic stimuli. Therefore, Nbk appears to be dispensable for programmed cell death in B-cells. The expression of Nbk is, like Puma, usually p53 dependent [312,313,314]. In the NHL model system, used here, a different regulatory mechanism is suspected, due to the frequent p53 mutations in the tested cell lines. Nbk

expression is reported to be alternatively induced by the cell cycle transcription factor E2F, an indirect target of the PI3K/Akt/mTOR pathway [315,316]. Yet, PI3K/Akt signaling promotes the activity of E2F, while PI3K inhibition generally prevents E2F dependent transcription and would probably do so for Nbk expression. This is supported by reports demonstrating LY294002 mediated block of Nbk transcription [308,310] but conflicts with the presented findings for BKM120. Other possible candidates are the FoxO transcription factors. Recently, Nbk was shown to be upregulated upon FoxO activation [317]. The FoxO transcription factors are negatively regulated by Akt/mTOR mediated phosphorylation and become dephosphorylated and activated upon administration of PI3K/Akt/mTOR inhibitors including BKM120 [283,284,318]. Thus in the presented NHL model system, BKM120 more likely induces Nbk expression in a FoxO dependent manner.

Similar to Nbk, Bmf was weakly upregulated at later time points. Bmf preferentially antagonizes Bcl-2, Bcl-w and Bcl-x_L, although binding to Mcl-1 is reported under certain circumstances [289,319,320,321]. Bmf is clearly expressed at all stages of B-cell development with exception for pre-B-cells [322]. The relevance of Bmf in B-cells is further underlined by the fact that knockout of Bmf in mice promotes the development of b-cell hyperplasia. However, Bmf's participation in programmed cell death seems to be restricted to certain stimuli such as glucocorticoids, HDAC inhibitors or anoikis [322,323]. Bmf is under negative control of the PI3K/Akt/mTOR pathway. Constitutive expression of PI3K represses Bmf RNA expression, whereas inhibition of PI3K/Akt/mTOR signaling with LY294002, NVP-BEZ235, rapamycin or RAD001 upregulates the Bmf protein level [321,323,324]. Moreover, apoptosis induced by PI3K/mTOR inhibition requires Bmf as deletion of Bmf prevents LY294002 or rapamycin mediated DNA fragmentation. The observations made for BKM120 in the tested NHL model system are congruent. Transcriptional upregulation of Bmf was detected after 36h, which is consistent with the reported LY294002 dependent increase of the Bmf protein level after 48h. This clearly supports the presumption that the PI3K/Akt/mTOR pathway controls Bmf on the transcriptional level.

Nevertheless, in the tested NHL model system, the late and weak upregulation of Nbk and Bmf after 24 and 36h, respectively, rather suggests a minor role for Nbk or Bmf in BKM120 mediated apoptosis. This is supported by the fact, that in the NHL subtype of burkitt lymphomas, Nbk expression in repressed by EBV [325]. In contrast, Puma and Hrk were rapidly and strongly upregulated within 6h and, thus, more likely participate in BKM120 induced apoptosis.

Interestingly, the most potent BH3-only, Bim, was not detectable on RNA level. The tested cell line JEKO-1 is described to be negative for Bim previously, due to due to homozygous deletion [295,326]. Loss of Bim is a common event in certain subtypes of NHL, whereas in mantle cell lymphoma homozygous deletion are prevalent, and in Burkitt lymphoma, the Bim gene is mainly silenced by hypermethylation [295,327,328]. Although, Bim was shown to be important for BKM120 mediated

apoptosis in CLL [318], this does not seem to be the case for the NHL model system used here. Especially as in this model system, Bim positive cell lines such as SUDHL10, CA-46 or DG-75 [329,330,331] were resistant to BKM120 induced apoptosis.

According to the general opinion, BH3-only proteins induce apoptosis by neutralizing the antiapoptotic Bcl-2 family members. However, high expression of antiapoptotic members, such Bcl-x_L, can protect from programmed cell death [332,333]. In the tested NHL model, a BKM120 dependent upregulation of antiapoptotic Bcl-2 members was not expected, as PI3K/Akt/mTOR signaling is known to promote antiapoptotic Bcl-2, Bcl-x_L and Mcl-1 [175,334,335,336,337]. In line with this, inhibition of PI3K signaling downregulates Mcl-1 protein levels [222,227,280]. Thus, as expected, in the NHL model system, treatment of the cell line JEKO-1 with BKM120 for 36h downregulated the levels of Mcl-1, Bcl-2 and Bcl-b RNA. Yet, at earlier time points, strong upregulation of Bcl-b RNA and slight upregulation of Mcl-1 and Bcl-2 RNA was detected. Mcl-1 and Bcl-2 are targets of the BH3-only Puma [289]. The strong upregulation of Puma RNA implicates a strong expression on protein level, which most certainly neutralizes upregulated Mcl-1 and Bcl-2. Bcl-b in turn, is only targeted by Bim and Nbk [290]. Thus, its expression cannot be neutralized by upregulated Puma or Hrk. Nevertheless, as in the tested model system, BKM120 treated JEKO-1 cells undergo apoptotic cell death, its capacity to protect from apoptosis is of no significance. In line with this, previous reports demonstrated the incompetence of Bcl-b to protect from anticancer regimens induced cell death, probably due to instability and rapid, steady-state or drug-induced proteasomal degradation [338,339]. Thus, little is known about the function of Bcl-b. Like Bcl-2 and Bcl-x_L, Bcl-b was shown to bind Beclin-1 and to inhibit autophagy, while knockdown of Bcl-b triggers autophagic cell death [340]. However, this does not provide an explanation for BKM120 dependent upregulation of Bcl-b in the tested model system as recent reports demonstrate that BKM120 also mediates autophagy [227,341].

Altogether, BKM120 is shown to upregulate the BH3-only proteins Puma and Hrk, which is associated with intrinsic apoptotic signaling via Bax and Bak. The subsequent mitochondrial breakdown promotes the cleavage and activation of caspase-9 and finally leads to DNA fragmentation and apoptotic cell death. Moreover, PI3K/Akt/mTOR dependent Bcl-b regulation was shown for the first time. Yet, the relevance of this discovery still needs to be clarified.

4.1.3 BKM120 in cell cycle regulation and checkpoint signaling

The involvement of PI3K/Akt/mTOR signaling on proliferation by promoting cell cycle progression is well documented. Therefore, inhibitors of the pathway are shown to induce cell cycle arrest, namely at G1 phase. The hierarchical level is not relevant as G1 arrest is performed likewise by inhibitors of mTOR, Akt or PI3K [1,128,130,342]. Observation of G2/M arrest with these inhibitors is rather rare

[343,344]. For BKM120, however, both G1 arrest and G2/M arrest are reported, whereby publications of G2/M arrest are prevalent. In myeloma and lung cancer cells, BKM120 induce G1 arrest [279,345]. In contrast, G2/M arrest was observed for glioma cells, gastrointestinal cancer cells, T-ALL cells and melanoma cells [228,229,346,347]. Thereby, at least for melanoma cell lines, microscopy implicated an arrest at an early stage of mitosis. Here in this thesis, measuring the DNA content revealed that BKM120 likewise induces G2/M arrest in the chosen NHL cell line model. Moreover, the protein biochemical analyses also strongly indicate arrest at mitosis rather than G2 phase. The phosphorylation of mitotic CDK1 at T161 and its BKM120 mediated dephosphorylation at Y15 in the two cell lines MINO and DG-75 clearly demonstrated activation of CDK1 and, thus, highly implicate transition from G2 phase into mitosis. Further, mitosis is coordinated by active CDK1 bound to Cyclin A and Cyclin B. Cyclin A essentially conducts the entry into mitosis but delays the metaphase-anaphase events of chromosome alignment and chromatid separation [61,63]. Thus, Cyclin A is degraded after mitotic entry in an APC/c dependent but SAC independent manner [348]. Likewise, Cyclin B1 is required for the transition into mitosis but in contrast to Cyclin A further promotes kinetochore attachment and chromosome alignment [87,349]. Yet, right after the chromosomes are aligned Cyclin B1 is degraded as well, a step necessary for anaphase onset and mitotic exit [85,350]. Consequently, constitutive Cyclin B1 expression arrests cells at the metaphase-anaphase transition and attenuates mitotic exit [64,87]. Also delayed degradation of Cyclin B1 correlates with reduced mitotic exit after DNA damage [351,352]. Applying this knowledge to the results found in the chosen NHL model system, the downregulation of Cyclin A and accumulation of Cyclin B1 further promote the suggestion of BKM120 inducing mitotic arrest.

Managing cell cycle arrest upon cellular stress is the matter of checkpoint signaling. The checkpoint kinases Chk1 and Chk2 are the messengers of cellular stress such as DNA damage. Chk1 and Chk2 negatively regulate the activation of the CDKs by inhibiting the Cdc25 proteases. In the used NHL cell line model, BKM120 activated checkpoint signaling via Chk2 while dephosphorylating and inactivating Chk1. Despite Chk2 activation, BKM120 was still capable to dephosphorylate and activate CDK1. This weak competence of Chk2 mediated signaling underlines the suggestion of Chk2 being just an amplifier kinase [353]. Several reports conclude that rather Chk1 has the key responsibility [354,355,356]. First, knockout of Chk1 but not that of Chk2 is mostly lethal, proving an essential role for Chk1 in G2/M DNA damage response and coordinating DNA replication. Second, Chk1 can replace Chk2 functions, which is not operative for the reverse. Third, Chk2 has only a low tumor suppressor function in tumorigenesis. Fourth, Chk1 is essential for genome integrity, while Chk2 is optional. Moreover, loss or inhibition of Chk1 forces mitotic entry, associated with activation of CDK1/Cyclin B, in various scenarios [357,358,359]. Phosphorylation of Chk1 at S345 thereby has an essential function, as Chk1-S345 mutants promote premature mitosis [360]. Considering all this for the NHL cell line system, a model is

proposed, where BKM120 stimulates CDK1 activation and mitotic entry by dephosphorylating/inactivating Chk1 at S345.

In conclusion, BKM120 forces mitotic entry and mitotic arrest at the metaphase-anaphase transition by controlling checkpoint signaling and the activation of the M phase complex CDK1/Cyclin B1.

4.1.4 BKM120 in mitotic catastrophe and polyploidy

Aberrant mitosis, including prolonged arrest during the metaphase-anaphase transition, is a hallmark of mitotic catastrophe. When treated with BKM120, additional features of mitotic catastrophe were observed in the tested NHL cell lines CA-46, SU-DL-10 and DG-75, namely giant cell formation and polyploidy. These findings strongly support previous reports, were BKM120 already was assumed to induce mitotic catastrophe [228,229]. Other PI3K inhibitors such as LY294002, Wortmannin and 3-methyladenine are demonstrated to induce mitotic catastrophe as well [195,361]. However, a clear definition for the term of mitotic catastrophe is still missing. The morphologic typical multinucleated giant cells were initially reported to be non-viable, which implicated mitotic catastrophe to be a new type of cell death, distinct from apoptosis [95,362,363]. Yet, cells undergoing mitotic death also show clear features of apoptosis such as chromatin condensation, mitochondrial membrane permeabilization and caspase cleavage. According to this, mitotic catastrophe was assumed a special subtype of apoptosis [96,364]. Inhibition of apoptosis, however, fails to prevent the formation of giant cells with multiple nuclei, which rather suspects apoptosis to be secondary or unrelated to mitotic catastrophe [97,365,366]. Later on, also features of necrosis and senescence were reported in the context of mitotic catastrophe. Thus, mitotic catastrophe is now rather seen as a cell death pre-stage or oncosuppressive mechanism that prevents genome instability by causes either senescence or inducing various types of cell death [367,368]. This is underlined by the observation that the typical multinucleated/polyploid giant cells can be temporally viable and even have the capacity to undergo de-polyploidization. Presumably, mitotic catastrophe functions as a sensor of aberrant mitotic events, such as premature mitotic entry, prolonged mitotic arrest or malfunctioning of mitotic checkpoint regulation, and has tumor suppressor function.

According to the Nomenclature Committee on Cell Death, loss of plasma membrane integrity is a defined criterion of cell death [369]. In the NHL model system, this was measured by detecting PI positive cells with flow cytometry. In the cell lines CA-46, DG-75 and SU-DHL-10, only weak PI uptake was observed in response to BKM120, which assumes that these cells have an intact membrane and are viable. Therefore, the giant polyploid cells detected in these cell lines were estimated to be viable as well. This strongly supports the hypothesis of mitotic catastrophe not being a distinct type of cell death but a pre-stage. Furthermore, BKM120 treated DG-75 cells adhered in the 4n stage of mitosis

for a minimum of 24h. The amount of mitotic cells reduced, contemporaneously to the appearance of polyploid 8n cells after 48h. Prolonged incubation with BKM120 further increased the DNA content and led to the formation of cells with a 16n content. This assumes exit from mitosis without or with incomplete cytokinesis, reentry into the cell cycle and reinitiation of DNA replication with exact and continuous doubling of the genome, a process also known as endoreplication, endomitosis or endocycle. Similar has been observed in nocodazole treated fibroblasts and CML as well as irradiation treated BL cells [370,371,372]. In these settings, the initiation of DNA re-replication required the loss of p53 function. In line with this, all three polyploid cell lines of the NHL model carry p53 mutations. This strongly suggests a switch from mitotic catastrophe to endoreplication in the polyploid cell lines of the NHL model system, which again supports the pre-stage hypothesis. Moreover, this assumes that the described morphology of multinucleated polyploid giant cells rather contributes to the process of endomitosis or endoreplication than to mitotic catastrophe.

Endocycling also seems to provide a survival mechanism for tumor cells with the capacity to repair DNA damages [372,373]. That might be one reason why polyploidy in tumors contributes to malignancy and is associated with a poor prognosis [100,374,375,376]. In line with this, polyploidy is suggested as a drug resistance mechanism. Resistance to taxol induces polyploidy in the leukemia cell line K562 [377]. In another report, melanoma cells treated with methotrexate for one week form polyploid cells, but restore a regular ploidy level within two week after methotrexate removal [378]. The same was observed for irradiation and cisplatin induced polyploidy [101,379]. The data that were collected as part of the thesis in the NHL model take the same line. In the polyploid cell lines CA-46 and DG-75, removal of BKM120 from the culture media partially reverted BKM120 induced events. Release from BKM120 clearly reduced the amount of 4n/mitotic cells, overcame cell cycle arrest and re-induced proliferation. This demonstrates that BKM120 induced mitotic arrest and mitotic catastrophe is reversible and not an endpoint cell death mechanism. This further supports the hypothesis of a cell death pre-stage. Beyond this, although polyploidy increases shortly after BKM120 removal, prolonged culture causes the reduction of polyploid cells. This is associated with a decrease of giant cells as observed by microscopy. That reduction might be due to resumption of mitosis, cytokinesis and subsequent de-polyploidization. Alternatively, it is possible that the cells with a regular 2n or 4n DNA content restart proliferation and overgrow the subpopulation of polyploid giant cells, once BKM120 is removed. Yet, once polyploidy is induced, release from BKM120 does not allow reinitiation of proliferation in giant cells. Moreover, the percentage of cells with a DNA content of <2n increases concomitantly to the polyploidy, a correlation that becomes more obvious after prolonged treatment with BKM120. These sub-G1 cells could contribute to loss of chromosomes because of unequal chromosome separation. That would support the suspicion of reinduced cell division. Yet, the polyploid DNA content is always an exact multiple of 2n, which does not imply the loss of

chromosomes. DNA fragmentation due to programmed cell death is also likely, but conflicts with the assumption of viability that have been made by the observation of an intact plasma membrane. In addition, the formation of sub-G1 cells in the polyploid cell lines CA-46 and DG-75 is caspase independent, which eliminates apoptotic cell death as possible mechanism. Thus, it is not clear, whether BKM120 induced polyploidy is a temporal survival event or contributes to an endpoint and maybe death mechanism.

As pointed out before, premature mitotic entry and prolonged mitotic arrest are linked to the CDK1/Cyclin B complex. Consequently, aberrant activation of CDK1/Cyclin B1 induces mitotic catastrophe [364]. As the activity of CDK1 is inhibited by the DNA damage response, mitotic catastrophe should also be under the control of the checkpoint kinases Chk1 and Chk2. Indeed, both checkpoint kinases negatively regulate mitotic catastrophe by preventing anaphase onset and mitotic exit [104,351,357,359,380,381]. Moreover, downregulation of Chk1 overcomes irradiation induced mitotic arrest in HeLa cells and promotes mitotic exit associated with downregulation of Cyclin B1 [351]. Likewise, Chk1 deficiency in taxol treated DT40 cells induces downregulation of Cyclin B2 and correlates with mitotic exit, chromosome missegregation and chromosome instability [359]. Even cells with haploinsufficient Chk1 are capable to bypass mitosis [380]. This function of Chk1, again, strictly depends on its phosphorylation at S345. During mitosis, Chk1 becomes phosphorylated particularly at S345 and accumulates in the metaphase and anaphase of unperturbed cells [382]. In line with this, mutation of Chk1 at S345 is capable to induce mitotic catastrophe in embryonic stem cells [360]. Similarly, in yeast cells S345 mutants overcome bleomycin mediated checkpoint arrest, which leads into an unequally cell division [383]. For Chk2, a redundant function is reported. In yeast, the Chk2 homologue Rad53 prevents mitotic catastrophe by suppressing the APC/c factor Cdc20p [384]. In mammals, Chk2 colocalizes and interacts with SAC proteins such as Plk1 and Aurora B at the centrosomes, which strongly suggests an important function of Chk2 in spindle assembly and genome integrity [381,385,386,387]. Moreover, Chk2 was demonstrated to phosphorylate and to control a wide panel of other SAC proteins and cell cycle regulating transcription factors, such as p53 and E2F1 (reviewed in [364]). Inhibition of Chk2 overrides cell cycle arrest, induces mitotic catastrophe and leads into cell death with apoptotic features. Beyond this, inhibition or mutation of either Chk1 or Chk2 is also linked to genome instability and cancer. Both Chk1 and Chk2 are required for the DNA replication checkpoint. Thus, elimination of Chk1 or Chk2 abrogates this checkpoint and initiates DNA replication and endoreplication [380,381,388,389,390]. In combination with mitotic catastrophe, this causes multiple rounds of DNA replication and leads to the typical morphology of giant polyploid cells. In the tested NHL cell line model, BKM120 contrariwise inhibits Chk1 by dephosphorylation at S345 and activates Chk2 by phosphorylation at T68. Regarding the described effects of Chk1 inhibition, it is strongly implicated that BKM120 induced mitotic catastrophe and polyploidy involves, and possibly

requires, inactivation of Chk1. In contrast, activation of Chk2 is suspected to be negligible as the earlier discussed inability of Chk2 to prevent CDK1 activation also assumes the inability to affect mitotic catastrophe or polyploidy. To further elucidate the role of Chk1 and Chk2 in BKM120 mediated mitotic catastrophe and polyploidy, the use of biochemical inhibitors, such as UCN-01 (Chk1 inhibitor) or XL844 (Chk1/Chk2 inhibitor), might be useful.

In the NHL model, BKM120 activated Chk2. In line with this, inhibition of ATM/ATR with Caffeine prevented BKM120 induced Chk2 phosphorylation. That strongly implicates that BKM120 signals via ATM/ATR. Moreover, ATM/ATR is required to induce polyploidy in DG-75 cells, which implicates a prominent role of ATM/ATR signaling in the initiation of DNA replication. In line with this, a previous report demonstrated that activation of ATM/ATR promotes over-replication in response to bleomycin [391]. Moreover, the BK polyomavirus induces polyploidy in human renal proximal tubule epithelial cells wherefore it requires ATM/ATR to replicate its DNA [392]. In the NHL model, inhibition of ATM/ATR activation with Caffeine attenuates polyploidy by inactivating CDK1 and thereby preventing mitotic entry. This leads to the accumulation of cells arrested at the G2/M checkpoint. Yet, it is contradictory that activation of ATM/ATR promotes polyploidy, while the downstream targets Chk1/Chk2 need to be inactivated to reinitiate DNA replication (see previous section).

Polyploidy requires escape from mitosis and the reinitiation of DNA replication. This has been reported to be associated with the downregulation of Cyclin B1 [393,394]. Conversely, for the onset of DNA replication Cyclin A is required [395]. However, in *Drosophila* Schneider D2 cells silencing of Cyclin A but not Cyclin B forms giant polyploid cells and, moreover, is necessary for the exact doubling of the genome from 4n to 8n [396]. In the NHL model, BKM120 promoted the degradation of Cyclin A. This downregulation might be required for the re-initiation of DNA replication and formation of polyploid giant cells in the NHL cell line system.

In conclusion, BKM120 induces reversible mitotic catastrophe. Prolonged exposure to BKM120 facilitates escape from mitotic catastrophe and causes the formation of giant polyploid cells. This implicates the re-initiation of DNA replication in the absence of cytokinesis, a process possibly supported by BKM120 dependent inactivation of Chk1 and degradation of Cyclin A. Moreover, polyploidy is ATM/ATR dependent and can be prevented with Caffeine by abrogating the CDK1 dependent mitotic entry.

4.1.5 ATM in BKM120 induced apoptosis

The cell line MINO shows a similar molecular response to BKM120 treatment. The Chk2 is activated, Chk1 is inactivated and CDK1 becomes dephosphorylated at Y15. Therefore, MINO cells likewise undergo mitotic catastrophe. In this cell line, however, BKM120 induces apoptosis instead of mitotic

slippage and polyploidy. Inhibition of mitotic catastrophe by administration of Caffeine similarly prevents mitotic entry by attenuating CDK1 activation, which arrests cells at the G2/M transition and increases the amount of 4n cells. When mitotic catastrophe induces apoptosis, it is expected that inhibition of mitotic entry protect from apoptosis. However, treatment with Caffeine synergistically promotes apoptosis. Similar has been described for the combination of Caffeine with nocodazole, radiation or the MDM-2 antagonist Nutilin-3 [397,398,399]. That suggests that activation of ATM/ATR during mitosis might have a survival function. In fact, in Drosophila the ATM orthologous dATM is required for viability and fertility [400]. This protective mechanism might be associated with the prevention of DNA damage induced apoptosis.

In summary, BKM120 induces mitotic catastrophe, which leads to apoptosis. Concomitant inhibition of ATM/ATR synergistically promotes apoptosis. Thus, for tumor therapy the combination of BKM120 with ATM/ATR inhibitors might be a promising strategy. Moreover, as ATM/ATR inhibition attenuates polyploidy, this combination might prevent genome instability and cancer progression in resistant tumors.

4.1.6 Apoptosis versus Polyploidy

In the tested NHL cell line model, mitotic arrest in response to BKM120 was observed likewise in apoptotic and polyploid cells. In cells that undergo cell death, like MINO, the amount of 4n cells increases 6h before the execution of apoptosis. In contrast, viable cells, such as DG-75, adhere in M phase for at least 42h before polyploidy is detectable. This strongly suggests mitotic arrest and mitotic catastrophe as the initiator of both events and supports the hypothesis of mitotic catastrophe as an oncosuppressive pre-stage. Previous reports and the data presented as part of this thesis, together, implicate apoptosis as the preferred outcome of mitotic catastrophe [96,401,402,403,404]. In turn, the inability to undergo apoptotic cell death seems to be the trigger for polyploidy. Apoptosis can easily be inhibited by elimination of the apoptosis executing Bcl-2 members Bax and Bak [405,406]. In line and with this, protein expression analysis revealed that the three polyploid cell lines CA-46, SU-DHL-10 and DG-75 were deficient for Bax and Bak. That not only implicated the requirement of Bax and Bak for BKM120 mediated apoptosis, it also suggested that the absence of Bax and Bak promotes polyploidy. Previous reports underline this presumption. Transformed baby mouse kidney (BMK) cells deficient for Bax and Bak form carcinomas with a high mitotic rate and many tumor giant cells [407]. In addition, treatment of FDM cells with the microtubule inhibitor paclitaxel causes polyploidy only after knockout of Bax and Bak [98]. To confirm the necessity of Bax and Bak loss for BKM120 mediated polyploidy, the HCT116 model system, were Bax/Bak double knockouts were available, was used. Knockout of Bax and Bak did inhibit BKM120 mediated apoptosis, but did not induce polyploidy. That initially implicated that BKM120 induced polyploidy is Bax/Bak independent. Another important

regulator of apoptosis and polyploidy is p53. Expression of p53 augments polyploidy by inducing apoptosis [408]. In contrast, loss of p53 function not only protects from apoptosis, it is also relevant for mitotic catastrophe and giant cell formation [95,363,409,410,411,412]. However, p53 independent polyploidy is also reported [96,98,413]. According to the databases of IARC and canSAR (http://p53.iarc.fr/CellLines.aspx and https://cansar.icr.ac.uk/), most cell lines of the tested NHL model system carry p53 mutations. The two cell lines GRANTA-519 and REC-1 are wild type for p53, while p53 is defective in MINO, JEKO-1, MAVER-1, CA-46, DG-75 and SU-DHL-10. As BKM120 likewise induces cell death in the p53 wild type cell lines (GRANTA-519, REC-1) and p53 defective cell lines (MINO, JEKO-1, MAVER-1), BKM120 mediated apoptosis and polyploidy were suggested to be p53 independent. Experiments in the HCT116 model system promoted this suggestion. Here, downregulation of p53 expression by the use of siRNA did not protect from BKM120 mediated apoptosis and did not induce polyploidy. However, p53 is known to be a negative regulator of DNA replication [414,415,416] and thereby might prevent endoreplication and polyploidy in BKM120 treated Bax/Bak deficient HCT116 cells. To test this, p53 was also downregulated in Bax/Bak double knockout cells. Indeed, combination of p53 siRNA with knockout of Bax/Bak remarkably increased the amount of polyploid cells. These results clearly demonstrate for the first time that both factors are required for mitotic catastrophe dependent endoreplication and polyploidy, loss of Bax/Bak to protect from apoptosis upon mitotic arrest and loss of p53 to reinitiate DNA replication. As loss of Bax and Bak is observed in high grade NHL and chemotherapeutic resistant NHL, treatment with BKM120 appears to be of limited use [417,418,419].

4.1.7 The link between BKM120, ATM and MEK1/2

The cross-talk between PI3K/Akt/mTOR and MAPK-ERK signaling leads to inverse activation of both pathways. Inhibition of Akt causes the activation of MEK/ERK via Raf [420,421]. In addition, mTORC1 inhibition promotes MEK activation by via the S6K/IRS/PI3K axis [246]. In the NHL model, however, studies with Caffeine revealed that BKM120 mediated MEK1/2 activation is ATM/ATR dependent. Moreover, combination of BKM120 with either Caffeine or U0126 similarly prevented polyploidy in DG-75 cells and sensitized for apoptosis in MINO cells. This clearly demonstrated that ATM/ATR and MEK1/2 signal in the same pathway. The MAPK pathways are known targets of ATM/ATR signaling. For instance, in fibroblasts, the induction of double strand breaks or the treatment with etoposide leads to the phosphorylation of ERK in an ATM dependent manner [251,422]. However, MEK/ERK is also reported to act upstream of ATM. ERK is required for the phosphorylation of ATM at S1981. In line with this, the phosphorylation of the ATM downstream targets Chk1 and Chk2 depends on ERK [248,423]. In the NHL model, ATM/ATR inhibition (Caffeine) or MEK inhibition (U0126) prevented BKM120 mediated Chk2 T68 phosphorylation. This strongly suggests that MEK1/2 signals downstream of ATM

but upstream of Chk2 and, thus, between ATM and Chk2. Yet, as Caffeine mediated dephosphorylation of Chk2 is much more pronounced, ATM is assumed to additionally phosphorylate Chk2 in a MEK1/2 independent manner.

The observation of BKM120 inducing MEK1/2 phosphorylation in an ATM dependent manner strongly suggests that ATM signals downstream of PI3K. This, however, is contrary to the previous knowledge. Although PKB/Akt is described to inhibit DNA damage induced checkpoint signaling, it is always shown to do so downstream of ATM/ATR [424]. Therefore, a different regulation is suspected for BKM120. Recent data demonstrate that mTOR suppresses ATM gene expression via S6K signaling and the regulation of specific miRNAs [425]. mTOR inhibition with either AZD8055 or rapamycin, therefore, upregulates ATM mRNA levels. This mechanism also provides a possible link from BKM120 to ATM/ATR. BKM120 mediated PI3K inhibition prevents downstream mTOR signaling, which causes the upregulation of ATM. ATM then promotes the activation of MEK1/2.

This thesis demonstrates for the first time that PI3K inhibition by BKM120 activates MEK1/2 in an ATM/ATR dependent manner. More importantly, the presented data strongly imply that PI3K signaling is involved in the regulation of ATM/ATR.

4.1.8 The role of the MAPK pathway in BKM120 mediated events

The PI3K/Akt/mTOR pathway controls proliferation and survival, inter alia, by cross-talk with other signaling pathways. One of these is the MAPK-ERK pathway. MAPK-ERK signaling is positively and negatively regulated by the PI3K/Akt/mTOR pathway. In mouse embryo fibroblasts and human keratinocytes, an inverse relationship between Akt and ERK activity was demonstrated [426]. Both, etoposide and doxorubicin inhibited the phosphorylation of Akt and induced the phosphorylation of ERK. In line with this, the expression of constitutive active Akt prevented doxorubicin induced phosphorylation of ERK. This correlation is a result of multiple mechanisms. First, Akt directly phosphorylates and inhibits the activity of upstream Raf [420]. Second, Akt signaling activates Rheb, a member of the Ras family, which binds and inhibits Raf [421]. Third, experiments with the mTORC1 inhibitor RAD001 revealed that inverse ERK activation is mediated via S6K/IRS-1/PI3K feedback signaling [246]. That supports previous reports where PI3K/PDK1 signaling, in contrast to Akt/mTOR signaling, facilitated ERK activation (reviewed in [50,427]). Here, in the NHL cell line model system BKM120 was shown to induce MEK1/2 phosphorylation at S217/S221, which strongly indicated activation of the MAPK-ERK pathway. BKM120 induced ERK phosphorylation has been demonstrated before for a gastric carcinoma cell line, which clearly supports the presented results [244].

Like the PI3K/Akt/mTOR pathway, MAPK-ERK signaling is involved in proliferation and survival. Both pathways have overlapping functions. For instance, PI3K/Akt/mTOR and MEK/ERK together negatively

regulate apoptosis by phosphorylating the BH3-onlys Bad and Bim and the intrinsic caspase-9 [164,169,173,428,429,430]. In addition, the transcription factors FoxO3a and CREB are targeted by both pathways [178,431,432,433]. Therefore, inverse activation of MEK/ERK signaling upon PI3K/Akt/mTOR inhibition can be a compensatory mechanism that protects from apoptotic cell death and causes cell resistance. In the used NHL model system, BKM120 dependent activation of MEK1/2 was observed likewise in cells undergoing apoptotic cell death and viable polyploid cells. That strongly implied that MEK1/2 is involved in BKM120 mediated mitotic catastrophe and might regulate both, apoptosis and polyploidy. Indeed, inhibition of MEK with the chemical compound U0126 synergistically sensitized for BKM120 induced apoptosis and, on the other hand, protected from polyploidy. That clearly demonstrated that MEK1/2 activation is important for BKM120 mediated events.

Synergistic induction of apoptosis by concomitantly inhibiting PI3K/Akt/mTOR and MAPK/ERK signaling already has been proven with other PI3K/mTOR inhibitors such as rapamycin, LY294002, GDC-0941 and NVP-BEZ235 [434,435,436,437]. The synergy between the PI3K inhibitor BKM120 and the MEK inhibitor U0126, demonstrated here for the first time, is consistent with that and, once again, underlines that combined administration of PI3K/Akt/mTOR inhibitors with MEK/ERK inhibitors is a promising strategy in the therapy of cancer. Moreover, this verifies MEK1/2 as a negative regulator of apoptosis and implies that compensatory activation of MEK1/2 attenuates BKM120 induced apoptosis. Yet, U0126 was incapable to overcome resistance to BKM120 induced apoptosis in the cell line Bax/Bak deficient DG-75. This, again, demonstrates that both pathways primarily trigger cell death via intrinsic apoptotic signaling, which requires Bax and Bak.

Interestingly, U0126 prevented BKM120 dependent polyploidy in the cell line DG-75 and enhanced the formation of 4n cells. That clearly demonstrated that MEK1/2 inhibition reverses BKM120 mediated events, which strongly suggests that MEK1/2 is the key regulator of BKM120 induced mitotic entry, mitotic catastrophe and endoreplication. The importance for MAPK signaling in endoreplication and polyploidy was demonstrated before. MAPK signaling is known to be required for the formation of polyploid cells during normal mammalian development. For instance, the thrombopoietin driven differentiation of diploid hematopoietic stem cells into polyploid megakaryocytes is MEK/ERK dependent [115,438,439]. However, the data presented here highly implicate an important role for MAPK signaling in the polyploidy of cancer cells. This is supported by recent data demonstrating that prolonged nuclear localization of MEK1/2 forces polyploidy and neoplastic transformation of colon cancer cells [440]. In addition, Pectenotoxin-2 induced polyploidy in human leukemia cell lines is associated with phosphorylation of JNK, ERK and p38 [247]. Inhibition of JNK with SP600125 or MEK with PD98059 decreases the polyploidy of these cells. The mechanism how the MAPK pathway controls cancer transformation and ploidy status is poorly understood. Yet, a critical point seems to be the

MEK/ERK dependent checkpoint regulation. On the one hand, the MAPK pathway is essential for G1/S progression and DNA replication [441,442]. Moreover, activation of MEK/ERK signaling upon treatment with neurotoxic agents or viral infection forces cells to re-enter S phase and enhance DNA replication [443,444]. This provides the possibility that in the NHL model system BKM120 mediated MEK1/2 activation likewise cause reinitiation of DNA replication, endoreplication and polyploidy in the cell lines CA-46, DG-75 and SU-DHL-10. In fact, inhibition of MEK with U0126 attenuates the BKM120 dependent formation of cells with an 8n DNA content in the cell line DG-75. This strongly demonstrates that BKM120 requires MEK1/2 to initiate DNA replication and polyploidy. On the other hand, MEK/ERK signaling promotes G2/M progression and mitotic entry in many tumor models [445,446,447]. As forced mitotic entry is a common cause for mitotic catastrophe [368], BKM120 might induce mitotic catastrophe in the NHL model system by activating MEK1/2, which in conjunction with reinitiation of DNA replication supports mitotic slippage and polyploidy. This mechanism is prevented by inhibition of MEK1/2. Thus, U0126 is suggested to increase the amount of 4n cells by arresting cells at G2/M phase and preventing mitotic entry. Moreover, the U0126 mediated inhibition of DNA replication probably protects from mitotic slippage and polyploidy. This is underlined by the previous reported observation of MEK and PI3K inhibitors cooperatively inhibiting DNA synthesis and arresting cells in G2/M phase [434].

This hypothesis is further corroborated by the observation that U0126 downregulates the phosphorylation of CDK1 at T161. This most certainly abrogates activation of CDK1, although it is further dephosphorylated at Y15, and prevents transition from G2 into mitosis. MAPK signaling was demonstrated to be necessary for the phosphorylation of CDK2 at T160 [448], which strongly implicates the same mechanism for the T161 phosphorylation of CDK1. In addition, the cytokine Bone morphogenesis protein 4 (BMP4) induces the expression of CDK1 and Cyclin B1 in a MEK/ERK dependent manner [449]. Thus, in the NHL cell line model, inhibition of MEK1/2 with U0126 prevents BKM120 mediated mitotic entry by inactivating the CDK1/Cyclin B1 complex.

The data generated in the NHL model system demonstrated that the upstream checkpoint regulator Chk2 is modulated by MEK1/2 signaling as well. Treatment with the MEK inhibitor U0126 abrogated the BKM120 induced phosphorylation of Chk2 at T68, which clearly demonstrated that BKM120 controls the activity of Chk2 in a MEK1/2 dependent manner. This is consistent with a previous report of U0126 abrogating Gemcitabine induced Chk2 and Chk1 activation in hepatocellular and cholangiocellular carcinoma cells [423]. Likewise, downregulation of ERK1 or ERK2 affects etoposide-induced phosphorylation of Chk2 at T68 and reduces the inhibitory phosphorylation of Cdc25C at S216 in the breast cancer cell line MCF7 [248]. In line with this, in the tested NHL model, inactivation of Chk2

correlated with an increased U0126 dependent dephosphorylation of CDK1 at Y15. This implies that BKM120 mediated Chk2 activation does have a minor but insufficient negative impact on CDK1 activity.

In the apoptotic sensitive cell line MINO, treatment with U0126 increased the amount of 4n cell as observed in the polyploid cell line DG-75. Moreover, U0126 similarly downregulated the phosphorylation of CDK1 at Y15 and T161. Thus, like seen in the context of polyploidy in DG-75 cells, MEK inhibition prevents the entrance into mitosis and arrest BKM120 treated cells at the G2/M checkpoint. This again demonstrates that mitotic arrest and mitotic catastrophe by BKM120 is universal for all cells and strongly supports the hypothesis of mitotic catastrophe as an oncosuppressive mechanism. The further outcome is not restricted and rather depends on the genetic profile of the cell. However, the prevention of mitotic catastrophe by inhibition of MEK does not protect from BKM120 induced apoptosis. In contrast, in sensitizes for apoptotic cell death. Thus, mitotic arrest and probably mitotic catastrophe might have an additional anti-apoptotic function. This is supported by a report demonstrating that the intrinsic caspase-9 is periodically inhibited by CDK1/Cyclin B1 dependent phosphorylation at T125 during normal mitotic progression [403]. In addition, MEK/ERK itself has a protective impact on apoptosis by preventing DNA damage. In consequence, inhibition of MEK with PD98059 sensitizes Chk1 inhibitor treated human myeloma cell lines or hyperoxic rat alveolar epithelial cells for apoptosis [450,451].

Paradoxically, administration of MEK inhibitor U0126 causes the BKM120 dependent accumulation of phosphorylated MEK1/2 in the cell line MINO. A similar phenomenon has been described for U0126 before [252]. U0126 directly inhibits the catalytic activity of phosphorylated MEK1, which abrogates phosphorylation and signaling of downstream ERK1/2 [452]. This suggests that in MINO complexes of inactive phosphorylated MEK1/2 bound to U0126 accumulate. Why this regulation is different among the cell lines remains to be clarified. Nevertheless, as all other U0126 mediated effects, including enhanced G2/M arrest and CDK1 inhibition, were equally observed in DG-75 and MINO, MEK1/2 was considered to be inhibited in MINO as well.

In conclusion, MEK1/2 controls BKM120 mediated mitotic entry and mitotic catastrophe. Furthermore, MEK1/2 is required for polyploidy in apoptotic resistant cells. Consequently, inhibition of MEK1/2 arrests cells at the G2/M checkpoint, which impedes subsequent events, such as mitotic catastrophe and polyploidy. In apoptosis competent cells, G2/M arrest by inhibition of MEK1/2 sensitizes for BKM120 induced cell death. In both cases, G2/M arrest is mediated by preventing BKM120 induced activation of CDK1.

4.1.9 The specificity of BKM120

The involvement of PI3K/Akt/mTOR signaling in supporting cell cycle progression is well documented. Inhibition of the PI3K/Akt/mTOR pathway primarily induces G1/S arrest by affecting the activity of G1 phase and S phase CDK/Cyclin complexes. Rapamycin for instance, an inhibitor of mTORC1, downregulates the expression of Cyclin D, Cyclin E and Cyclin A in the early and late G1 phase [130,453,454]. In addition, rapamycin inhibits the activation of the Cyclin dependent kinases CDK1, CDK2 and CDK4 [453,455,456]. Similar has been described for the PI3K inhibitor LY294002 [129,457,458]. Yet, CDK1 and CDK2 also have a pivotal role in controlling G2/M transition and mitotic progression, which would implicate that PI3K/Akt/mTOR inhibition induces G2/M arrest as well. Indeed, LY294002 is reported to impair G2/M transition and even to affect mitosis, which leads cells into mitotic cell death [195,344,361,459]. In accordance to this, the PI3K product PIP_3 was shown to be required for spindle orientation in HeLa cells and mouse oocytes [196,197]. Likewise, inhibition of downstream Akt with the compound a-443654 prevents mitotic progression [460]. In contrast, studies in mice and rats revealed an important role of the PI3K/Akt pathway in the developmental tetraploidization process of liver cells [254]. The polyploidization of mammalian megakaryocytes likewise depends on mTOR, probably by controlling the nuclear location of Cyclin D [253]. Another possible explanation might be that PI3K/Akt/mTOR signaling promotes polyploidy by controlling the cell size [461,462]. The pan-PI3K inhibitor BKM120 consistently induces mitotic arrest but also promotes polyploidy in the tested NHL model. This behavior is more congruent to microtubule-active drugs such as nocodazole and taxol. The specificity of BKM120, therefore, seems to be a critical point. Although, the inhibition of kinases others than PI3K is described to mostly occur at concentrations above 2000nM [225], side effects cannot be precluded. Studies in A2058 melanoma cells revealed a deviant transcriptomic profile of BKM120 at high concentrations and postulate off-target effects above 2000nM [229]. Moreover, in these settings BKM120 is demonstrated to directly bind tubulin and to inhibit tubulin polymerization, which is suspected to be the off-target effect that leads to changes in mitotic genes expression and G2/M arrest. Yet, PI3K is known to constitutively bind α/β-tubulin, and γ-tubulin in response to insulin [463]. In line with this, inhibition of PI3K/Akt signaling with LY294002 or dominant negative Akt destabilizes the microtubules in migrating NIH3T3 cells [464]. This implies that BKM120 does not directly bind to tubulin but to PI3K that is located at tubulin, which causes a BKM120-tubulin colocalization. Thus, BKM120 mediated tubulin binding and inhibition of tubulin polymerization is not necessarily an off-target effect.

A concentration of 2,000nM seems to be the threshold for the specificity of BKM120. Transfection of a constitutive active Akt delays BKM120 induced proliferation inhibition, but only at concentration below 2,000nM [229]. Moreover, this concentration induces cell death in cells with mutated PTEN and

thus constitutive active Akt signaling. In the tested NHL model, 50% growth inhibition was observed at an average concentration of 1,000nM BKM120, whereas 1,400nM of BKM120 were needed for 50% death induction. For a clear dephosphorylation of the PI3K target Akt, however, 2,000nM BKM120 were needed. In contrast, dephosphorylation of mTOR targets was possible with 1,000nM to 1,500nM. BKM120 apparently does block mTOR more efficiently PKB/Akt.

The downstream target PKB/Akt directly phosphorylates Chk1 at S280 [465]. This prevents the activation of Chk1 in response to DNA damage and replication arrest. Moreover, this phosphorylation abrogates ATM/ATR mediated phosphorylation and activation at S345. That is contrary to the observed dephosphorylation of Chk1 in response to the PI3K inhibitor BKM120 in the NHL model system. Either BKM120 is incapable to inhibit Akt properly, or the BKM120 mediated Chk1 regulation is PI3K/Akt independent.

The BKM120 mediated phosphorylation of MEK1/2 was shown to be ATM/ATR dependent in the NHL model, which implies the participation of PI3K signaling in the regulation of ATM/ATR. This is novel and contrary to previous reports, where the PI3K target Akt is consistently demonstrated to be downstream of ATM/ATR [466,467,468]. Therefore, BKM120 mediated ATM/ATR regulation could be an indication for off-target effects. More recent data, however, show that inhibition of the PI3K target mTOR with either AZD8055 or rapamycin upregulates ATM mRNA levels, a mechanism involving S6K and the miRNAs microRNA-18a and microRNA-421 [425]. This suggests that BKM120 mediated ATM/ATR regulation is PI3K dependent and not a result of an off-target effect.

The ATM/ATR inhibitor Caffeine synergizes with nocodazole to induce apoptosis but protects from apoptosis induced by the PI3K inhibitor LY294002 [397,469]. This further promotes the suggestion of BKM120 behaving more like an anti-microtubule agent than a PI3K inhibitor.

In conclusion, BKM120 inhibits the phosphorylation of the PI3K downstream target Akt and the mTOR downstream targets 4EBP1 and S6K. Thus, BKM120 is approved to be an inhibitor of the PI3K/Akt/mTOR pathway. Furthermore, BKM120 mediates MEK1/2 activation via ATM/ATR, which most likely is PI3K dependent as ATM is a confirmed target of downstream mTORC1. Mitotic catastrophe has been demonstrated for the PI3K inhibitor LY294002 as well and consequently is a result of BKM120 mediated PI3K inhibition. However, PI3K/Akt activation rather than inhibition is suggested to promote polyploidization, which implicates that BKM120 might also have off-target effects.

4.1.10 Model for BKM120 mediated signaling

Based on the observations in the NHL cell line system, a model of BKM120 induced signaling is proposed (Figure 31). BKM120 activates the DNA damage sensors ATM and ATR. This induces Chk2

checkpoint signaling on the one hand and the MAPK cascade on the other hand. However, Chk2 signaling is insufficient to prevent the dephosphorylation of CDK1 at Y15 and therefore cannot impede its activation. The induction of the MAPK cascade further facilitates the activation of CDK1 by promoting its phosphorylation at T161. Moreover, BKM120 signaling upregulates the expression of CDK1 complex collaborate Cyclin B1. In consequence, BKM120 positively regulates the activation of the M phase CDK1/Cyclin B1 complex. Activation of CDK1/Cyclin B1 then promotes the transition from G2 phase into mitosis. However, the BKM120 dependent increase of cells with a 4n DNA content implicates the inability to complete mitosis and suggests mitotic arrest, probably between metaphase and anaphase. Mitotic arrest leads the cells into mitotic catastrophe followed by programmed cell death via apoptosis. This apoptotic signaling occurs via the intrinsic pathway and involves the activation of Bax/Bak, loss of the mitochondrial membrane potential and the subsequent cleavage of intrinsic caspase-9. The link between mitotic catastrophe and apoptotic signaling still needs to be clarified but might involve the BH3-onlys Puma and Hrk. Inhibition of ATM/ATR or MEK1/2 prevents mitotic entry and causes arrest at the G2/M checkpoint. This demonstrates that mitotic catastrophe requires ATM/ATR and MEK1/2 signaling. However, this does not protects from cell death, in contrast, it synergistically sensitizes for BKM120 induced apoptosis. Thus, mitotic catastrophe is a discrete mechanism distinct and upstream from apoptotic cell death, which supports the hypothesis of a pre-stage. Nevertheless, apoptosis is the preferred executing mechanism. However, as ATM/ATR and MEK1/2 signaling have an anti-apoptotic function, mitotic catastrophe also could have a pro-survival role. The combination of BKM120 with inhibitors of either ATM/ATR or MEK1/2 might be a promising therapeutic strategy for the treatment of NHL (Figure 31A).

Cells with a defective apoptotic signaling pathway still undergo mitotic arrest and mitotic catastrophe but remain viable. In p53 deficient cells, this promotes the formation of giant cells with a polyploid DNA content. Polyploidy implicates the re-initiation of DNA replication in the absence of cytokinesis, which leads to the typical morphology of giant multinucleated cells. Yet, polyploidy provides a possible mechanism for genomic instability and tumor progression. The single use of BKM120 as therapeutic agent therefore might have a disadvantage in tumors with disrupted apoptotic signaling. Inhibition of ATM/ATR or MEK1/2 protects from polyploidy, which demonstrates that mitotic catastrophe is the initial event to polyploidy. Moreover, this underlines the pro-survival function of ATM/ATR/MEK signaling. Thus, the combination of BKM120 with an ATM/ATR or MAPK inhibitor also might be a strategy in tumor with defective apoptosis. BKM120 inhibits tumor proliferation and additional inhibition of ATM/ATR or MAPK prevents genome instability and tumor progression.

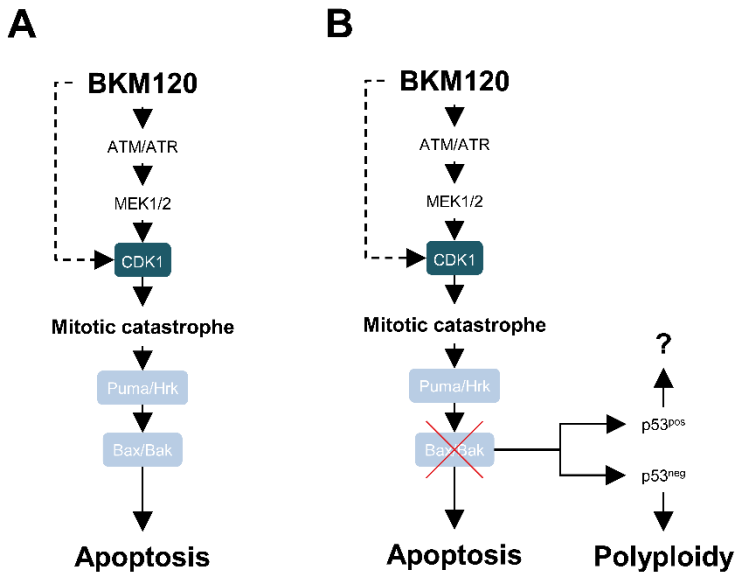

Figure 31: Proposed model for BKM120 mediated signaling.

4.2 Inhibition of the PI3K/Akt/mTOR pathway as combinatorial strategy

Chemotherapy is the most common treatment modality for the NHL subtype of mantle cell lymphoma (MCL). The administration of cytotoxic drugs such as cyclophosphamide, bendamustine, mitoxantrone, cisplatin or cytarabine causes DNA damages. This activates the DNA damage checkpoints and induces cell cycle arrest. Prolonged cell cycle arrest or massive DNA damage lead the tumor cells into death via the intrinsic apoptosis pathway. Other approved cytotoxic strategies that likewise induce cell cycle arrest and apoptotic cell death are the disruption of mitotic progresses with the vincristine (in the USA and EU) and the inhibition of proteasomal degradation with bortezomib (in the USA). The standard protocols for newly diagnosed MCL intend a combination of the CD20 antibody rituximab with cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP) in alteration with high dose cytarabine (R-CHOP). Although this protocol improved the overall survival, most of the patients relapse. Among others, this may contribute to the fact that resistance acquirement among these cytostatics is common. The activation of the PI3K/Akt/mTOR pathways seems to be a common source for this resistance acquirement [470,471,472,473,474].

Another strategy to kill tumors is targeting the extrinsic pathway of apoptosis. Thereby, targeting the receptor of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has become a promising strategy as its corresponding receptors induce apoptosis preferentially· in cancer [137]. Monoclonal antibodies against TRAIL-R1 (HGS-ETR1) and TRAIL-R2 (HGS-ETR2) have been tested in phase I clinical trials against solid tumors (reviewed in [475]). In lymphomas, preclinical trials proved growth inhibition and apoptosis in response to HGS-ETR1 and HGS-ETR2 [476]. In addition, the human recombinant

Apo2L/TRAIL is well tolerated in a phase I study of patients with low grade NHL [477]. However, resistance acquirement against hrTRAIL is also observed [259,478,479,480]. Again, activation of PI3K/Akt/mTOR signaling is involved [259,474,479,480,481,482].

Therefore, the combination of approved cytostatics or hrTRAIL with PI3K/Akt/mTOR inhibitors is suspected to be a promising therapeutic strategy to sensitize for cell death and overcome resistances.

4.2.1 The response of MCL to hrTRAIL

The antiproliferative and apoptotic activity of recombinant human Apo2L/TRAIL has been approved for a wide range of hematologic malignancies, including MCL [478,483,484,485]. Consistent results were obtained in the MCL cell line model system chosen for this thesis. TRAIL inhibited the proliferation of all tested cell lines. However, apoptosis was induced only in two out of five cell lines. As seen for many other cell lines before, TRAIL mediated apoptosis occurs via the intrinsic pathway, as shown by Bid activation, and results in PARP cleavage.

Activation of the PI3K/Akt/mTOR pathway is one suggested mechanism for TRAIL resistance. TRAIL can directly activate PKB/Akt and subsequent mTOR signaling, which contributes to apoptosis resistance in many cell lines [479]. However, in the MCL model dephosphorylation of the mTOR targets S6K and 4EBP1 was observed in TRAIL sensitive and resistant cell lines. This demonstrates that TRAIL inactivates PI3K/Akt/mTOR signaling in MCL, irrespective of apoptotic signaling. Thus, the PI3K/Akt/mTOR pathway is not involved in apoptotic resistance to TRAIL in this MCL model. A possible mechanism for TRAIL resistance in MCL is provided by the observation that resistant MCL cell lines have elevated levels of c-Flip and that downregulation of c-Flip with siRNA sensitizes for TRAIL induced apoptosis [485].

4.2.2 Protecting from apoptosis with PI3K/Akt/mTOR inhibitors

Resistance against chemotherapy is a severe problem in the treatment of MCL. Even with combinatorial strategies, appreciable relapse and cross-resistance are observed [486]. Activation of the PI3K/Akt/mTOR pathway has been identified as one of the main sources for therapy resistance. Therefore, joint administration of established cytostatics with inhibitors of the PI3K/Akt/mTOR signaling pathway is a new promising strategy. Indeed, various preclinical studies demonstrated that PI3K/mTOR inhibitors like LY294002, ZSTK474, PI103, rapamycin or RAD001 sensitize solid and soft tissue tumors for vincristine, cisplatin and bortezomib induced apoptotic cell death [487,488,489,490]. In hematologic malignancies, inhibition of PI3K/mTOR likewise has been approved to enhance the effects of chemotherapy [234,271,474,491,492]. Moreover, resistance to vincristine can be overcome by PI3K/Akt/mTOR inhibition [472,473].

Activation of PI3K/Akt/mTOR signaling also contributes to the resistance against the TRAIL-R ligand Apo2L/TRAIL. In line with this, the PI3K inhibitor PI103 and the mTOR inhibitor rapamycin have been demonstrated to sensitize for TRAIL induced apoptosis and even to break TRAIL resistance [145,241,242,243]. However, in the MCL cell line model tested in this thesis, TRAIL reduced the activity of Akt/mTOR signaling likewise in sensitive and resistant cell lines. That implied that additional PI3K/mTOR inhibition would not further sensitize cells for TRAIL induced apoptosis or break TRAIL resistance. In fact, pre-treatment with the mTOR inhibitor RAD001 did not break the TRAIL resistance of the MCL cell lines GRANTA-519 and MAVER-1. This demonstrated that TRAIL resistance in this MCL model is independent of mTOR.

In the TRAIL sensitive cell lines MINO and JEKO-1, however, administration of RAD001 protected from TRAIL induced apoptosis, which was associated with abrogated caspase-8 and PARP cleavage. Moreover, RAD001 also prevented apoptosis in cisplatin, mitoxantrone and cytarabine treated cells. Thereby, apoptosis protection by RAD001 varies among the cell lines and among the cytostatics. This phenomenon was not restricted to mTOR inhibition. The attenuation of upstream signaling at the level of PKB/Akt or PI3K gave similar results. Even concurrent inhibition of PI3K and mTOR with BEZ235 protected from apoptosis. Thus, in contrast to the previous observations, combination of PI3K/Akt/mTOR inhibitors with cytostatics or TRAIL does not seem to be a suitable strategy for the treatment of MCL.

It is of special interest that the characteristics of all PI3K/Akt/mTOR inhibitors were highly dose dependent. Only at low concentrations, administration of PI3K/Akt/mTOR inhibitors attenuated TRAIL or cytarabine induced apoptosis. In contrast, very high concentrations of each inhibitor additively or synergistically enhanced apoptotic cell death. In clinical trials, the mTOR inhibitors such as RAD001 are administrated at doses of up to 10mg per day. This corresponds to a rapamycin concentration of about 20ng/ml in the plasma and about 20nM in the tumor tissue [493]. Therefore, low concentrations of PI3K/Akt/mTOR inhibitors have relevance in clinical therapy, at least for rapamycin derivates.

4.2.3 Evaluating possible protective mechanisms

The protective mechanism of PI3K/Akt/mTOR inhibition is a rare but not novel observation [192,494,495,496,497,498]. To find an explanation for this phenomenon, several scenarios are worth considering. First, PI3K/mTOR signaling involves two major feedback loops causing re-activation of Akt when mTOR is inhibited and activation of PI3K/Akt/mTOR is a known reason for apoptosis resistance [499,500]. Inhibition of signaling upstream of mTOR by the use of Akt or PI3K inhibitors and dual inhibition of PI3K and mTOR by BEZ235 prevents Akt activation via mTORC2 feedback signaling and inhibits PI3K activation via the S6K/IRS-1 feedback. Thereby re-activation of the PI3K/Akt/mTOR

pathway via the major feedback loops is prohibited. In the chosen MCL model, however, dual inhibition of PI3K/mTOR with BEZ235 still protects from TRAIL induced apoptosis. Therefore, mTOR/S6K/IRS-1 feedback signaling and reactivation of PI3K/Akt is not involved in the protective mechanism against TRAIL induced apoptosis. Second, the induction of autophagy can be a protective event, by so far unknown mechanisms. The removement of damaged mitochondria or the provision of energy by producing amino acids out of endogenous proteins are discussed reasons to preserve time of starvation and stress [501,502]. In line with this, autophagy has been shown to protect against TRAIL induced apoptosis [260]. mTOR is a known negative regulator of autophagy and its inhibition activates the cascade for lysosome formation. In consequence, mTOR inhibition attenuates cell death by inducing autophagy [192,496,497]. In the tested MCL cell line model, however, blocking PI3K/mTOR signaling with RAD001, BEZ235 or LY294002 did not induce autophagy at any concentration. Therefore, autophagy as mechanism protecting mantle cell lymphomas from TRAIL or cytostatic induced apoptosis can be excluded. Third, PI3K and mTOR signaling regulate a variety of pro- and antiapoptotic family members and, thereby, can influence apoptosis. Usually, inhibition of PI3K/Akt/mTOR signaling sensitizes for apoptosis by downregulating antiapoptotic and upregulating pro-apoptotic Bcl-2 family members. However, PI3K inhibition with LY294002 can also attenuate okadaic acid-induced apoptosis by increasing Bcl-2 levels [494]. Likewise, rapamycin dependent upregulation of Bcl-2 protects from taxol induced cell death [495]. In the tested MCL cell line system, however, treatment with TRAIL downregulated the expression of Bcl-2 and Mcl-1, but this was not prevented by pre-treatment with RAD001. Thus, the protective mechanism does not contribute to antiapoptotic Bcl-2 family regulation. Previously, studies in MCL cell lines demonstrated dose dependent downregulation of the pro-apoptotic BH3-only member Bad in response to rapamycin and BEZ235 [222]. However, in some cases of this setting, the expression of Bad was re-established with higher concentrations. Thus, by so far unknown mechanism, Bad regulation might provide a possible resistance mechanism. Yet, this hypothesis requires further examination. Forth, the expression of antiapoptotic factors, such as c-FLIP is usually promoted by PI3K/Akt/mTOR signaling, whereas inhibition of mTOR with rapamycin downregulates FLIP expression [145,503]. Thereby, $FLIP_S$ but not $FLIP_L$ expression depends on mTOR and sensitizes for TRAIL induced apoptosis in glioblastoma multiforme cells. In the MCL model, however, mTOR inhibition with RAD001 upregulates the expression of $FLIP_L$. This implicates that the protective effect of PI3K/Akt/mTOR inhibitors might contribute to the regulation of $FLIP_L$. This is supported by previous reports that $FLIP_L$ equally protects from TRAIL induced apoptosis [143,144,485,504,505]. Yet, the mechanism of RAD001 dependent $FLIP_L$ regulation in this setting still needs to be examined.

4.2.4 The involvement of NFκB

One of the most important pro-survival proteins is the transcription factor NFκB. NFκB attenuates the impact of cell death stimuli by directly promoting the expression of antiapoptotic proteins such as Flip, XIAP or members of the antiapoptotic Bcl-2 family [506]. Interestingly, death receptor signaling via TRAIL-R or TNF-R also activates NFκB, which is a postulated source for resistance against death ligands [263,507]. This is supported by the demonstration that NFκB inhibition sensitizes for TRAIL induced apoptosis [258,264]. In addition, resistance against chemotherapeutics like cytarabine is associated with an upregulation of NFκB [265]. In the tested MCL model, TRAIL administration also caused the degradation of the NFκB inhibitor IκBα in the two TRAIL sensitive cell lines MINO and JEKO-1 but also in the resistant cell line REC-1. Only in the TRAIL resistant cell line GRANTA-519, IκBα expression was stable. This initially implicated that TRAIL-R mediated NFκB activation does not correlate to TRAIL sensitivity in the chosen MCL model. However, administration of non-toxic concentrations of the NFκB inhibitor BAY11-7082 slightly, albeit not significantly, prevented from TRAIL or cytarabine induced apoptosis. That, in contrast, implicated that NFκB has an important role in mediating TRAIL induced apoptosis and demonstrated that NFκB also has a proapoptotic function. In line with this, previous studies demonstrated that NFκB is required to execute UV radiation, H_2O_2 or doxorubicin induced apoptosis [508,509,510]. In support of this, inhibition of NFκB with BMS-345541 counteracts etoposide induced apoptosis in T-ALL, which is associated with the downregulation of FAS, Bim and Bcl-x_L mRNA levels [511]. Similar has been observed with bortezomib and vincristine in the same setting. In AML, BAY117082 mediated NFκB inhibition protects from TNF dependent apoptosis by inducing the expression of the catabolic enzyme heme oxygenase-1 (HO-1), which is considered to facilitate tumor progression [512].

The PI3K/Akt/mTOR pathway usually facilitates NFκB signaling via stimulation of IKK and induction of IκBα degradation, the negative regulator of NFκB [180,513]. In line with this, mTOR inhibition by rapamycin attenuates nuclear translocation of NFκb and sensitizes for TNFα or doxorubicin induced cell death [514,515]. LPS mediated NFκb activation, however, is prevented by the mTOR inhibitor rapamycin but enhanced by the PI3K inhibitors LY294002 and Wortmannin [516,517,518]. Thereby, rapamycin mediated inhibition of NFκb protects from cell death. This furthermore underlines that NFκb has anti- and proapoptotic function. In contrast, enhanced NFκb activity due to PI3K inhibition seems to be involved in inflammatory but not apoptotic responses. Enhanced LY294002/Wortmannin dependent activation of NFκB has also been observed in murine lymphoma cell lines that were resistant to vincristine or doxorubicin [472]. It is postulated, but not proven, that this might compensate cell death signaling. In the MCL model, inhibition of PI3K/mTOR signaling with RAD001, BEZ235 or LY294002 led to degradation of IκBα. Yet, only with BEZ235 and LY294002, this was

accompanied by upregulation of NFκB expression. Thus, in the MCL model PI3K inhibition rather induces NFκB signaling than preventing it. Moreover, inverse activation of NFκB signaling in response to PI3K/Akt inhibition might provide a possible protective mechanism against apoptosis induced by TRAIL and cytotoxic agents. To test this, NFκB signaling was additionally inhibited with BAY117082. Non-toxic NFκb inhibition alone did not affect RAD001 signaling but, somehow, counteracted RAD001 mediated protection and restored sensitivity against TRAIL induced apoptosis, which was accompanied by re-initiation of caspase-8 and PARP cleavage. This strongly implicates that the protective effect of PI3K/Akt/mTOR inhibition is NFκB dependent. Moreover, the proapoptotic function of NFκB signaling on TRAIL and cytarabine induced apoptosis seems to switch to an antiapoptotic function when these death stimuli are combined with PI3K/Akt/mTOR inhibitors.

That mTOR inhibition mediated resistance can be reverted by additional NFκB inhibition, suggests that the underlying mechanism must be inversely controlled by both pathways. One possible mechanism is the regulation of the antiapoptotic protein c-Flip. Flip supplants caspase-8 at the DISC and, thereby, prevents its cleavage and activation. Consequently, TRAIL triggered extrinsic signaling cannot take place. High Flip expression is shown for many tumors and compromises apoptosis [519,520,521,522]. In turn, downregulation of Flip sensitizes for apoptosis [504,505,523,524]. Both pathways positively control Flip protein levels, NFκB by transcription and mTOR by translation [145,268,485,503]. In the MCL model system, however, mTOR inhibition with RAD001 did not inhibit but induce c-$Flip_L$ expression. This alone was already suspected to be a possible resistance mechanism above (chapter 4.2.3). Yet, additional NFκb inhibition did not counteract RAD001 induced c-$Flip_L$ expression. Therefore, c- Flip regulation is not responsible for the BAY117082 mediated reversibility of RAD001 dependent apoptosis protection. Besides, c-Flip negatively regulates the extrinsic apoptotic signaling of TRAIL but not the intrinsic apoptotic signaling of cytotoxic agents like cytarabine. Thus, the protective mechanism in cytarabine induced apoptosis, anyway, would be c-Flip independent.

4.2.5 The role of Pim-2 in apoptosis protection

The dose dependent protection versus sensitization character of PI3K/mTOR inhibition suggests that this regulation depends on dose dependent expression or activation of proteins. In the MCL model, one protein was identified whose expression is congruent to this dose dependent pattern against TRAIL induced apoptosis: Pim-2. Low concentrations of PI3K, Akt, mTOR single or dual inhibitor strongly increased Pim-2 expression while increasing concentrations brought it back to basal levels. Pim-2 was identified as a proto-oncogene while inducing T cell lymphomas in mice by using Moloney murine leukemia virus (MuLV) [525]. In human two transcripts were identified of which the smaller is highly expressed in hematopoietic tissues and in leukemia and lymphomas and considerably expressed in testis, small intestine, colon and human colorectal adenocarcinoma cells [526]. A larger transcript is

expressed in spleen, thymus, small intestine and colon. Pim-2 acts as a serine threonine kinase, which is functionally redundant to and can compensate for Pim-1, which synergizes with c-Myc in the development of b-cell leukemias [527,528]. Transfection of Pim-2 also enhances tumor growth of prostate cancer cells [529]. Therefore, targeting Pim kinases has become a promising strategy in treatment of cancer and the development of new therapeutic drugs has high priority [530,531,532,533,534]. The pan-Pim inhibitor SGI-1776 inhibits translation and induces autophagy in multiple myeloma, induces apoptosis in mantle cell lymphoma by downregulating Mcl-1 and Cyclin D1, sensitizes for bendamustine and has proven to enhance sunitinib activity by reducing c-Myc levels [535,536,537,538]. Interestingly, Pim-2 kinase regulates many proteins that are also controlled by PI3K/Akt/mTOR signaling. First, the proapoptotic BH3-only protein Bad, usually phosphorylated and controlled by Akt, is also phosphorylated and inhibited by Pim-2 [539,540]. Second, the mTOR target 4EBP1, an inhibitor of translation, is activated by Pim-2 [540]. Third, negative modulation of TSC2 by direct phosphorylation is reported [541]. Therefore, Pim-2 steps in for Akt and mTOR signaling and can compensate for mTOR inhibition resulting in rapamycin resistance [542]. Vis versa, however, effects based on ectopic expression of Pim-2, cannot be overcome by rapamycin suggesting that both signaling pathways do not fully overlap and that Pim-2 activity does not depend on PI3K/Akt signaling [540]. This is supported by reports that Akt and Pim-2 phosphorylate the targets at distinct sites. For instance, Bad phosphorylation by Akt occurs at S136, by Pim-2 at S112 and TSC2 phosphorylation by Akt occurs at T1462, by Pim-2 at S1798 [169,539,541,543].

Unlike other kinases, Pim-2 does not need posttranslational modification in form of phosphorylation and is already active when expressed [540]. So far only few transcription factors regulating Pim expression have been identified, STAT3/STAT5, NFκB and the forkhead transcription factors FoxN3 and FoxP3 [544,545,546,547,548]. Of these transcription factors, NFκB is also a target of Pim kinases [549,550]. Therefore, increased expression of Pim-2 is correlated with high levels of NFκB in AML and ALL [551]. In the model system of mantle cell lymphoma, NFκB dependent Pim-2 regulation was confirmed as inhibition of NFκB with BAY11-7082 downregulated Pim-2 expression. Interestingly, TRAIL treatment also caused downregulation of Pim-2, whereas pre-incubation with RAD001 strongly induced Pim-2 expression in TRAIL treated cells. Therefore, Pim-2 upregulation highly correlates with TRAIL mediated resistance. As Pim-2 kinase signaling affects intrinsic signaling, its regulation is also a possible explanation for the resistance against cytostatics. This resistance can be overcome by additional NFκB inhibition which abrogates RAD001 mediated Pim-2 induction. As BAY1107982 alone does not sensitize for TRAIL, this recommends that in this setting other NFκB dependent pro- or antiapoptotic mechanisms are negligible and that NFκB's only mode of action is to inhibit Pim-2 expression. However, downregulation was incomplete assuming that, Pim-2 expression might be

regulated by more than one transcription factor. The family of STAT kinases, especially STAT3 and STAT5 [544], are known control Pim-2 expression. STATs, in turn, are under positive [552,553] and negative [554,555] control by mTOR, whereby negative regulation is probably mediated via the S6K/IRS-1 feedback loop [556]. Therefore, mTOR inhibition might induce Pim-2 expression via activation of STATs. The third group of transcription factors, that are known to negatively regulate Pim-2, are the forkhead transcription factors, which, again, act downstream of Akt/mTOR signaling [547,548]. Akt/mTOR signaling usually inhibits forkhead transcription factors by phosphorylation, which, in case of mTOR inhibition, would activate forkhead transcription factors and prevent Pim-2 expression. However, mTOR inhibition, somehow, also can inhibit forkhead transcription factors [557]. Moreover, in our models system RAD001 dependent inactivation of FoxO3a has been observed, which might provide a possible mechanism for Pim-2 regulation.

In the MCL model, the findings that Pim-2 expression is a possible candidate for RAD001 mediated resistance, suggests that preventing the upregulation of Pim-2 would restore TRAIL sensitivity. Therefore, Pim kinases were inhibited with the pan-Pim inhibitor LGB321. Surprisingly, similar to PI3K/mTOR inhibition, LGB321 did not sensitize but protect from TRAIL induced apoptosis in the MCL cell line system. Thereby, the same concentration based behavior was observed. This is contrary to an *in vivo* study, where LGB321 synergistically sensitizes for the death stimuli cytarabine [534]. When analyzing the response to LGB321 on protein level, no downregulation of Pim-2 expression could be observed. In contrast, Pim-2 expression dose dependently increased. Similar has been observed for LGB321 in myeloma cells [534]. On the one hand, inhibitor bound Pim-2 might accumulate in the cell, on the other hand, feedback signaling might increase Pim-2 expression and, thereby, protect cells from apoptosis. In that case, Pim-2 feedback expression should re-activate mTOR and thereby S6K and 4EBP1 phosphorylation, which is not the case in the MCL model. Only at high LGB321 concentrations, re-phosphorylation of S6K was detected. Although, Pim-2's dose dependent expression perfectly fits to the observed protective behavior of PI3K/mTOR inhibition, there is doubt on its relevance for apoptosis resistance. The observation that both, PI3K/mTOR and Pim-2 inhibition, promote apoptosis protection, rather suggests that the regulator acts downstream of mTOR signaling. Interestingly, inhibition of S6K protects from aging, extends the life span of *C. elegans* and *Drosophila* and protects cells from apoptosis by inhibiting cell growth [558,559,560,561,562]. Reinitiation of S6K also restores sensitivity to apoptosis. In line with this, at high concentrations of LGB321, S6K phosphorylation and TRAIL sensitivity are restored in the MCL model. Thus, S6K1 rather seems to play the prominent role [563,564]. S6K1-/- cells show constitutive downregulation of caspase-8 and Bid and fail to activate caspase-8 or caspase-3 upon TNFR or Fas stimulation. In contrast, knockout of S6K1 and S6K2 is lethal [565]. Furthermore, S6K activity increases upon G1 entry and, thereby, is important for cell cycle progression [566,567]. This matches with findings that cell cycle arrest in G1 protects from apoptosis

[568,569]. This leads to the suggestion that, in the MCL model system, PI3K/mTOR or Pim-2 inhibition causes inhibition of downstream S6K1, which may protect from apoptosis by abrogating caspase activation. However, this does not yet explain how additional NFκB inhibition might counteract this effect.

4.2.6 The discovery of a new feedback loop in the PI3K/Akt/mTOR pathway

In the MCL model, PI3K/Akt/mTOR inhibitors and Pim kinases inhibitors have the similar effect on the execution of apoptosis. Moreover, inhibition of either pathway downregulates the phosphorylation and activation of the mTOR targets S6K and 4EBP1. This supports previous observations [534,540]. For Pim-2 and PI3K/Akt signaling overlapping functions, yet separate pathways, are postulated as both share the same targets but modulate different phosphorylation sites. In contrast, mTOR and Pim-2 share the same targets and the same phosphorylation sites. This strongly suggests mTOR and Pim-2 signaling are connected. In fact, in the MCL model, mTOR inhibition upregulates Pim-2 expression, which demonstrates that mTOR controls Pim-2 expression. The same was observed for the PI3K inhibitor LY294002 and the dual PI3K/mTOR inhibitor BEZ235. Thus, Pim-2 also depends on upstream PI3K signaling. This demonstrates for the first time a direct connection between the Pim kinases pathway and the PI3K/Akt/mTOR pathway. Moreover, as Pim-2 was reported to regulate the TSC2 activity [541], the findings in this thesis revealed the existence of another negative feedback loop within the PI3K/Akt/mTOR pathway (Figure 32). Usually, activation or constitutive signaling of the PI3K/Akt/mTOR pathway negatively regulates Pim-2 expression. Inhibition of PI3K/mTOR signaling, however, upregulates the expression of Pim-2. Pim-2 then inactivates the TSC1/TSC2 complex by phosphorylating TSC2. This releases mTOR from its negative regulation via TSC1/TSC2 and promotes its reactivation. Thus, Pim-2 not only replaces PKB/Akt in the regulation of downstream mTOR, it also maintains mTOR signaling in case of PI3K/Akt inhibition. Therefore, Pim-2 acts upstream of mTOR and positively regulates mTOR activity.

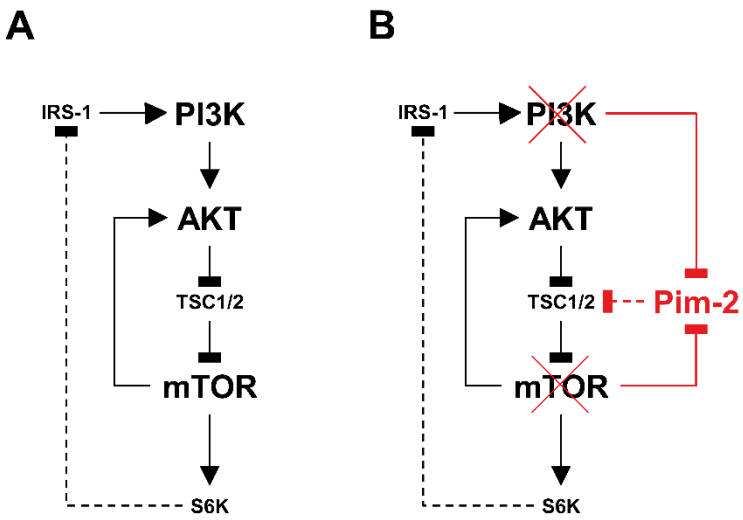

Figure 32: Suggested feedback loop that controls mTOR signaling via Pim-2. A new negative feedback loop has been identified. Inhibition of mTOR causes upregulation of Pim-2 which causes the phosphorylation and inactivation of TSC2 and, thereby, reactivation of mTOR signaling.

5 PERSPECTIVES

BKM120 was demonstrated to strongly upregulate the antiapoptotic Bcl-2 member Bcl-b. However, little is known about the role of Bcl-b in apoptotic signaling and its regulation by the PI3K/Akt/mTOR pathway. In addition, Bcl-b might possible be important for mitotic catastrophe and polyploidy. Downregulation of Bcl-b by siRNA in the presence of BKM120 might give insight into this.

In the three polyploid cell lines, augmented expression of Mcl-1 was observed. Moreover, the Mcl-1 expression correlated with the degree of polyploidy. Compared to CA-46, SU-DHL-10 and DG-75 showed higher levels of Mcl-1 and polyploidy. Previous reports demonstrated an increased rate of mitotic slippage and polyploidy when an ovarian cancer cell line with elevated levels of Mcl-1 was treated with vincristine [570]. This highly implicates an important role for Mcl-1 in BKM120 induced apoptosis and polyploidy, a role that will be investigated in further studies.

The increase of polyploid cells was coupled to an increase of cells with a sub G1 DNA content. This might be a result of DNA fragmentations via apoptotic or mitotic cell death but also can be caused by missegregation of chromosomes during mitosis. Further experiments will be required to clarify this.

The checkpoint kinases Chk1 and Chk2 seem to have importance in the execution of mitotic catastrophe and polyploidy. Specific Chk inhibitors like UCN-1 or XL844 might be helpful to investigate Chk1 and Chk2 signaling in this.

BKM120 controls the activity of CDK1 in a MEK1/2 dependent manner. CDK1 in turn seems to play a very important role in regulating mitotic catastrophe and polyploidy. Downregulation of CDK1 with siRNA could enlighten the role of CDK1 in this.

The combination of BKM120 with the MEK1/2 inhibitor U0126 or the ATM/ATR inhibitor Caffeine synergistically sensitized for apoptotic cell death. Therefore, these combinations might be a promising strategy in treating NHL and should be further investigated in primary cell models and *in vivo* studies.

The inhibition of PI3K/Akt/mTOR signaling can protect tumor cells from cell death by a panel of cytotoxic agents. This is of crucial importance for clinical administration. However, the presented results could not clarify the role of the prosurvival Pim-2 kinase in this. Moreover, a rather downstream regulation via S6K was suggested. This needs further investigation.

6 EXPERIMENTAL PROCEDURES

6.1 CELL BIOLOGY METHODS

6.1.1 Cell culture

6.1.1.1 Culture cultivation and passaging

A panel of human non-Hodgkin lymphoma cell lines was chosen for the performed studies as they guarantee constant results and reproducibility. The cell lines used are listed in Table 2.

Table 2: Specifications of the cell lines. MCL – mantle cell lymphoma, DLBCL – diffuse large b-cell lymphoma, BL – burkitt lymphoma, CC – colon carcinoma, wt – wild type, ko – knockout, neg – negative, pos - positive

Cell line	Cell type	Safety level	EBV status
JEKO-1	MCL	S1	Neg
MINO	MCL	S1	Neg
GRANTA-519	MCL	S2	Pos
REC-1	MCL	S1	Neg
MAVER-1	MCL	S1	Neg
SU-DHL-10	DLBCL	S1	Neg
DG-75	BL	S1	Neg
CA-46	BL	S1	Neg
HCT 116 wt	CC	S1	Neg
HCT 116 Bax/Bak ko	CC	S1	Neg

The cell lines JEKO-1, GRANTA-519, REC-1, MAVER-1, DG-75, CA-46, SU-DHL-10 and HCT 116 were purchased from DSMZ (Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). The cell line MINO was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). The HCT116 Bax ko/Bak ko cell line was kindly provided from Dr. Richard J. Youle [571] (Surgical Neurology Branch, NINDS, Porter Neuroscience Research Center, Bethesda, MD, USA). According to their EBV (Epstein-Barr virus) status, positive cells were cultured at safety level S2. The lymphoma cell lines were cultured in RPMI medium supplemented with 100 U/ml Penicillin, 100 µg/ml Streptomycin and either 10% (MINO, GRANTA-519, REC-1, SU-DHL-10, DG-75, CA-46) or 20% (JEKO-1, MAVER-1) fetal calf serum (FCS). The HCT 116 wt and Baxko/Bakko were cultured in DMEM medium supplemented with 100 U/ml Penicillin, 100 µg/ml Streptomycin and 10% FCS. All media components were purchased from Gibco, Invitrogen, Carlsbad, CA, USA. The cell lines were held at 37°C with 5% CO_2 and passaged every 2 to 3 days. After a culture time of maximal 3 months the cell line were replaced.

6.1.1.2 Storage of cells

For long time storage, $5x10^6$ cells per cell line were centrifuged at 300xg for 5min and resuspended in 1,5ml freezing medium containing 50% medium, 40% FCS and 10% DMSO (SERVA Electrophoresis GmbH, Heidelberg, Germany). The cell were put in cryovials (Simport Beloeil, QC, Canada) and transferred to -20°C for 2h. Then the vials were moved to -80°C for two days. For long time storage, the cells were transferred to liquid nitrogen. To thaw the cells the cryovials were gently warmed in a 37°C water bath and the cells were transferred into 10 ml of pre-warmed full media (supplemented with 100U/ml Penicillin, 100µg/ml Streptomycin and 20% FCS). To remove the DMSO cells were centrifuged at 300xg for 5min. The pelleted cells were resuspended in 10ml of pre-warmed full media and cells were passaged twice before experimental use.

6.1.2 Inhibitor treatment

To target PI3K signaling, a panel of chemical inhibitors was used. The pan-PI3K inhibitor LY294002 was purchased from BioVision Inc. (Milpitas, CA, USA). The mTOR inhibitor RAD001, the pan-PI3K inhibitor NVP-BKM120 (BKM120) and the dual inhibitor NVP-BEZ235 (BEZ235) were kindly provided from Novartis AG (Basel, Switzerland). The pan-PI3K inhibitor Wortmannin was purchased from Calbiochem (Merck KGaA, Darmstadt, Germany). For caspase inhibition, the pan-caspase inhibitor Q-VD-Oph from Calbiochem was used. The anti-cancer therapeutics vincristine, bendamustine, cisplatin, cytarabine, mitoxantrone and bortezomib were ordered at the pharmacy (Charité-Universitätsmedizin Berlin, Berlin, Germany). The human recombinant TRAIL, ligand of the TRAIL-receptor family, was purchased from R&D Systems Inc. (Minneapolis, MN, USA). To inhibit NFκB signaling, the IKK inhibitor BAY117082 from Calbiochem (Merck KGaA, Darmstadt, Germany) was used. Pim kinase inhibition was performed using LGB321 from Novartis (Basel, Switzerland). All inhibitors were solved in DMSO (SERVA Electrophoresis GmbH, Heidelberg, Germany).

For inhibitor treatment $5x10^4$ cells (MINO, DG-75, SU-DHL-10, CA-46, REC-1) or $10x10^4$ cells (JEKO-1, GRANTA-519, MAVER-1) were resuspended in 1ml of culture medium, seeded in 24 well plates and incubated over night. The inhibitors were added at the indicated concentrations and incubated for the assays specific times. In case of co- or pre-treatment, the inhibitors were administered in intervals of 4h.

6.1.3 RNA interference and transfection

To examine the role of a single protein or gene within a complex signaling pathway downregulation by small interfering RNAs had become the methods of choice. RNA interference is a natural mechanism in eukaryotes to regulated gene expression. So far, 3 forms of short double stranded RNAs were found *in vivo*: micro RNAs, small interfering RNAs and *Piwi* interacting RNAs. The primary transcripts are

processed into small about 20 nucleotides long pieces by the dicer protein family, which then interact with the AGO protein family to inhibits complement target structures. This technique was being exploited to establish the use of synthetic siRNAs in scientific research.

6.1.3.1 siRNAs

For downregulation of Bax and Bax, siRNAs from Dharmacon (Thermo Fisher Scientific Inc., Waltham, MA, USA) were used. The siRNA for p53 was kindly provided from Scheidereit AG (MDC, Berlin, Germany) and was purchased from Eurogentec S.A. Liège (Seraing, Belgium).

6.1.3.2 Transfection

Transfection of the siRNA into the target cell was performed by using the Amaxa system from Lonza Group AG (Basel, Switzerland). The principal bases on a special form of electroporation where the siRNA is inserted directly into the nucleus. Especially lymphomas benefit from this method as their nuclear-cytoplasmic ratio is relatively high and standard procedures fail to efficiently transfect those types of cells.

The transfection was performed according to the protocols of Lonza. In detail, for transfection, $3x10^6$ HCT116 wt or HCT116 Bax/Bak ko cells were centrifuged at 90xg for 10min and re-suspended in 100µl freshly prepared transfection solution V (72µl transfection solution plus 18µl supplement). 2µg of the suitable siRNA were added and the solution was transferred into the cuvette. For transfection, the program D-032 was used. Subsequently, 500µl of prewarmed medium were added and the cells were transferred to 6 wells containing 2,4ml of prewarmed medium. To verify the transfection efficiency the cells were co-transfected with 2µg of the kit included pmaxGFP plasmid. The transfection efficiency was tested by measuring the amount of GFP positive cells by flow cytometry after 24h. Viability was assessed by adding propidium iodide. The transfections efficiency was about 70% with a viability of 70%. The downregulation of the particular gene was verified by immunoblotting after 24h of incubation.

6.1.4 Proliferation

6.1.4.1 XTT assay

Growth and cell division are important markers for measuring the viability of cells. The XTT assay is a colorimetric assay to measure viable cells. The tetrazole XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) is metabolically reduced to a water-soluble formazan by the mitochondrial dehydrogenase of viable cells and, thereby, changes color from yellow to red, which emits at 450nm. The optical dense can be measured with photometric systems and is direct proportional to the amount of metabolic active cells.

To perform the XTT assay (Roche Holding AG, Basel, Switzerland), $5x10^4$ cells treated with the specific inhibitor for 72h were harvested, centrifuged at 300xg for 5min, resuspended in 100µl 1xPBS (Gibco, Invitrogen, Carlsbad, CA, USA), and transferred to 96 wells. The XTT solution were mixed at a ratio of 1:50 as described in the manufacture protocol (Roche Holding AG, Basel, Switzerland) and 50µl were added to each well. The culture plate was incubated at 37°C for 30min to 4h depending on the cell line. Absorption at 485nm minus reference at 690nm was measured using TriStar 2 from Berthold Technologies GmbH & Co.KG (Bad Wildbad, Germany). For normalization, untreated cells were set to 100%.

6.1.5 Cell death

6.1.5.1 Propidium iodide uptake

All types of cell death impair the integrity of the cell membrane *in vitro*. Propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO, USA), a membrane impermeable dye, enters the cells and integrates into the DNA when the cell membrane is damaged and is generally used as marker for cell death and viability.

Inhibitor treated cells were harvested after 72h of incubation and centrifuged at 300xg for 5min. The cells were resuspended in 1ml of 1xPBS (Gibco, Invitrogen, Carlsbad, CA, USA), centrifuged again and finally resuspended in 100µl propidium iodide (40µg/ml in 1x PBS) and incubated for 5min. Measurement of PI positive and, thereby dead cells, was performed by flow cytometry in channel FL2-H using FACSCalibur (Becton Dickinson AG, Franklin Lakes, NJ, USA).

6.1.5.2 Detection of DNA Fragmentation

Fragmentation of the DNA is a characteristic feature of apoptosis. During apoptosis, execution endonucleases cleave the condensed chromatin DNA between the histones creating fragments of 180bp and multiples of 180bp. Permeabilization of the cell membrane by detergents causes leaking of these fragments and leaves cells with reduced DNA content or hypodiploid cells. By labeling the DNA with the dye propidium iodide, apoptotic cells can be analyzed and quantified by flow cytometry. Viable cells have typically 2 peaks, cells in G0/G1 phase with 2n DNA content and cells in G2/M phase with 4n DNA content. Apoptotic cells with reduced DNA content are called hypodiploid and show up as peak left of the G0/G1 phase. However, necrotic cells also show DNA fragmentation, whereas the DNA is cleaved randomly and leaves fragments of different size. Unfortunately, these both types of cell death cannot be differentiated by this method why additional assays to verify apoptosis are needed.

Inhibitor treated cells were harvested after 72h of incubation and centrifuged at 300xg for 5min. The cells were resuspended in 200µl of ice cold 70% Ethanol and incubated at -20° for 2h. 1ml of 1xPBS (Gibco, Invitrogen, Carlsbad, CA, USA) was added and the cells were centrifuged at 300xg for 5min. The

cells were re-suspended in 100µl of RNAse solution (40µg/ml in H_2O) and incubated at 37°C for 2h. 200µl of propidium iodide (40µg/ml in H_2O) were added and, after incubation for 5min, apoptotic cells were quantified by flow cytometry in channel FL2-H using FACSCalibur (Becton Dickinson AG, Franklin Lakes, NJ, USA).

6.1.5.3 Conformational change of Bax/Bak

Cells undergoing type II apoptotic cell death require the activation of the proapoptotic Bcl-2 family members Bax and Bak. They oligomerize in the outer mitochondrial membrane and form pores causing the release of proapoptotic factor into the cytosol. Activation of Bax and Bak is associated with their conformational change exposing the n-terminus to the surface, which can be detected by specific antibodies and quantified by the use of a FITC labeled secondary antibodies by flow cytometry.

Inhibitor treated cells were harvested after 48h of incubation and centrifuged at 300xg for 5min. The pellet was resuspended in 200µl 2% formaldehyde and incubated at 20°C for 30min. 1ml of 1xPBS (Gibco, Invitrogen, Carlsbad, CA, USA) containing 2%FCS (Gibco, Invitrogen, Carlsbad, CA, USA) was added and the cells were centrifuged at 300xg for 5min. The pellets were resuspended in 100µl of PBS/2%FCS solution containing 0,1µg of either anti-Bax (804-224-c100, Alexis Biochemicals, Enzo Life Sciences GmbH, Lörrach, Germany) or anti-Bak (AM04, Calbiochem (Merck KGaA, Darmstadt, Germany) antibody and incubated at 20°C for 1h. 1ml of 1xPBS/2%FCS was added and the cells were centrifuged at 300xg for 5min. The pellets were resuspended in 100µl of PBS/2%FCS solution containing 0,1µg of FITC labeled F(ab)2 secondary antibody (goat anti-rabbit: 111-096-003, goat anti-mouse 115-096-003, Jackson Immuno Research, West Grove, PA, USA) and incubated at 20°C for 1h in the dark. 1ml of 1xPBS/2%FCS was added and the cells were centrifuged at 300xg for 5min. The cells were resuspended in 200µl 1xPBS and FITC fluorescence was measured by flow cytometry in channel FL1-H by using FACSCalibur (Becton Dickinson AG, Franklin Lakes, NJ, USA).

6.1.5.4 Mitochondrial membrane potential

Cells undergoing apoptosis via the mitochondrial pathway show loss of the mitochondrial membrane potential as pore formation in the outer membrane by Bax and Bak cause its permeabilization. The lipophilic membrane permeable dye JC-1 (Molecular Probes, Leiden, The Netherlands) is usually a monomer and emits green light at about 525nm. In an electric field of 80 to 100mV JC-1 aggregates to polymers emitting red light at about 595nm (Figure 33). The mitochondrial membrane potential of viable cells produces such an electric field leading to the accumulation of JC-1 polymers. Apoptotic cells with reduced mitochondrial membrane potential accumulate less JC-1 and the amount of red fluorescent cell decreases.

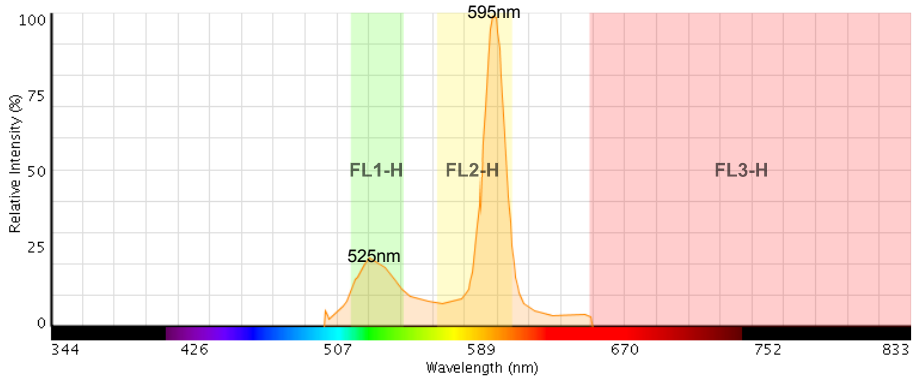

Figure 33: Emission spectrum of JC-1. Monomers emit green light at 525nM and are detected in FL1-H, polymers emit red light at 595nM and are detected in FL2-H when measured by flow cytometry.

JC-1 is hardly soluble. Therefore, dissolving of JC-1 was performed by shaking the culture medium in a 50ml falcon tube on a vortexer and adding JC-1 drop wise to the rotating medium. The freshly prepared JC-1 solution was added to the cell inhibitor treated for 48h at a final concentration of 2µg/ml and the cells were incubated at 37°C for 1h. The cells were harvested and centrifuged at 300xg for 5 min. The pellet was washed twice in 1xPBS and centrifuged at 300xg for 5min. Finally, the cells were re-suspended in 200µl 1xPBS and mitochondrial membrane potential was analyzed by flow cytometry measuring the red fluorescent JC-1 polymers at FL2-H with compensating green fluorescent monomers in FL1-H using FACSCalibur (Becton Dickinson AG, Franklin Lakes, NJ, USA).

6.2 BIOCHEMICAL METHODS

6.2.1 Immunoblotting

To gather information about changes in protein expression and protein modifications, immunoblotting is used. Therefore, the characteristic of antibodies to recognize specific epitopes within a protein is utilized. A primary antibody targets the proteins of interest. A secondary antibody is labeled with a signal-generating component and targets the primary antibody. By detecting the signal, alterations in the protein expression can be pictured. Prior to the actual immunodetection, the proteome of a cell needs to be separated by electrophoresis and transferred onto a blotting membrane. This creates space at the epitope and ensures binding of the specific antibody.

6.2.1.1 Protein extraction

To isolate whole cell protein, inhibitor treated cell were harvested after 6h or 24h of incubation and centrifuged at 300xg for 5min. The pellets were resuspended in 1xPBS, transferred to 1,5ml Eppendorf tubes and centrifuged at 300xg for 5min. The pellets were frozen over night at -80°C. After thawing on ice, the pellets were resuspended in about 50 to 100µl of RIPA lysis buffer and incubated on ice for

30min. For extraction of the proteins, the lysate was centrifuged at 10 000xg for 15min and supernatant was transferred to a fresh tube. The protein concentration was measured using BCA method. In detail, 2µl of protein were diluted 1:5 in ddH_2O and 200µl of BCA Protein Assay (Thermo Fisher Scientific Inc., Waltham, MA, USA) were added. After incubation at 37°C for 30min, optical density was measured at 560nm with TriStar 2 from Berthold Technologies GmbH & Co.KG (Bad Wildbad, Germany) and protein concentration was calculated using a BSA standard line. The protein samples were equalized by diluting with 2x Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA, USA) and denatured at 95°C for 5min.

RIPA lysis buffer: 150mM NaCl (Carl Roth GmbH + Co. KG, Karlsruhe, Germany)

50mM Tris pH8.0 (AppliChem GmbH, Darmstadt, Germany)

1% Triton X100 (Sigma-Aldrich Corporation, Saint Louis, MO, USA)

1% Sodium desoxychol acid (Carl Roth GmbH + Co. KG, Karlsruhe, Germany)

0,1% SDS (Carl Roth GmbH + Co. KG, Karlsruhe, Germany)

Protease inhibitor Cocktail (Complete Mini, Roche, Holding, Basel, Switzerland)

Phosphatase Inhibitor Cocktail (PhosStop, Roche Holding, Basel, Switzerland

6.2.1.2 SDS-PAGE

Each protein possesses an individual profile depending on its molecular mass, electric charge and three-dimensional conformation. When separating proteins in an electric field by **p**oly**a**crylamide **g**el **e**lectrophoresis (**PAGE**), these three factors regulate the migration through the gel, whereby charge acts direct and molecular mass as well as conformation act indirect proportional. Addition of the detergent **s**odium **d**odecyl **S**ulfate (**SDS**) disrupts the three dimensional conformation and denatures the proteins, thereby unfolding them into their primary linear structure. SDS also binds to the hydrophobic regions, overlapping the individual charge, and gives the protein a negative net charge. Therefore, SDS-PAGE separates proteins only by their molecular mass, which clearly simplifies the technique.

Depending on the molecular mass of the protein of interest, continuous gels were prepared for separation as described in Table 3. The gel electrophoreses was performed with the Mini-PROTEAN® System (Bio-Rad Laboratories, Hercules, CA, USA) using Tris-glycine buffer (25mM Tris pH8.3, 192mM glycine, 0,1% SDS) as running buffer. The proteins were stacked at 120V for 15min and separated at 180V for 1-2h.

Table 3: Preparation of continuous polyacrylamide gels for SDS-PAGE

Solution	**Stock concentration**	**Separating Gel**			**Stacking Gel**
		30-120kDa	15-80kDa	10-50kDa	
		10%	12%	14%	5%
H_2O		4800µl	4300µl	3800µl	2260µl
Tris-HCl pH 8.8	1,5M	2500µl	2500µl	2500µl	
Tris-HCl pH 6.8	0,5M				990µl
Acrylamide-bis 40% (29:1)	100%	2500µl	3000µl	3500µl	670µl
SDS	10%	100µl	100µl	100µl	40µl
TEMED	100%	4µl	4µl	4µl	4µl
Ammonium persulfate (APS)	10%	100µl	100µl	100µl	40µl

6.2.1.3 Western Blot

Electrophoretic separated proteins are embedded in the gel where specific antibodies cannot reach their epitope. Therefor prior to immunodetection the separated proteins need to be transferred to a nitrocellulose membrane which exposes the proteins and their epitopes at the surface. The transferring occurs by western blot where proteins migrate in an electric field and attach to the membrane by ionic and polar interactions.

The electrophoretic separated proteins were transferred to a 0,2µm nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) by the use of the Trans-Blot® System from Bio-Rad Laboratories (Hercules, CA, USA). Blotting was performed using ice cold Tris-glycine buffer [572] (25mM Tris pH8.3, 192mM glycine, 1% Methanol) as blotting buffer with additional cooling by an ice tank and cooling units. Transferring of the proteins was performed at 250mA for 2-4h.

6.2.1.4 Immunodetection

After protein transfer by wet blot, the nitrocellulose membranes were incubated in 1x western blocking reagent (Roche Diagnostics GmbH, Mannheim, Germany) at 20°C for 30min on a shaker. Binding of the primary antibody (Table 4) was performed in 50ml falcon tubes at 4°C over night on the GFL rotator 3025 (Gesellschaft für Labortechnik GmbH, Burgwedel, Germany). Primary antibodies were diluted in 1xTBS-T (0,02M Tris pH7.6, 0,14M NaCl, 0,1% Tween20) as described in Table 4. After primary antibody incubation, the membranes were washed twice with TBS-T for 15min and incubated in horseradish peroxidase labeled secondary antibody diluted in TBS-T at 20°C for 2h on a shaker. The membrane was washed twice with TBS-T for 15min and chemiluminescence was generated by using the SuperSignal® West Pico Chemiluminescent Substrate from Thermo Scientific (Rockford, IL, USA). Immunodetection was performed using the ChemiDoc™ MP System (Bio-Rad Laboratories, Hercules, CA, USA).

Table 4: Antibodies for immunodetection

Antibody	dilution	company	Host	clone	Catalog number
4EBP1 S65	1:500	Cell Signaling	Rabbit	Polyclonal	9451
4EBP1 T37/46	1:500	Cell Signaling	Rabbit	Polyclonal	9459
Akt S473	1:500	Cell Signaling	Rabbit	Polyclonal	9271
Bak	1:1000	Santa Cruz	Rabbit	Polyclonal	7873
Bax	1:1000	Cell Signaling	Rabbit	Polyclonal	2772
Bcl-2	1:500	Cell Signaling	Rabbit	Polyclonal	2876
Bcl-xL	1:500	Cell Signaling	Rabbit	Monoclonal	2764
Bid	1:500	Cell Signaling	Rabbit	Polyclonal	2002
Caspase-3	1:1000	R&D Systems	Goat	Polyclonal	AF-605-NA
Caspase-8	1:1000	Cell Signaling	Rabbit	Monoclonal	4790
Caspase-9	1:1000	R&D Systems	Goat	Polyclonal	AF8301
CDK1 T161	1:500	Cell Signaling	Rabbit	Polyclonal	9114
CDK1 Y15	1:500	Cell Signaling	Rabbit	Polyclonal	9111
Chk1 S345	1:500	Cell Signaling	Rabbit	Polyclonal	2341
Chk2 T68	1:500	Cell Signaling	Rabbit	Polyclonal	2661
Cyclin A	1:500	BD Pharmingen	Mouse	Monoclonal	611269
Cyclin B1	1:500	BD Pharmingen	Mouse	Monoclonal	554177
Flip	1:500	Exalpha	Rabbit	Polyclonal	X1129P
GAPDH	1:2000	Santa Cruz	Rabbit	Polyclonal	25778
IκBα	1:1000	Santa Cruz	Rabbit	Polyclonal	371
LC3	1:1000	Novus Biologicals	Rabbit	Polyclonal	NB100-2331
Mcl-1	1:500	Santa Cruz	Rabbit	Polyclonal	20679
MEK1/2 S217/221	1:1000	Cell Signaling	Rabbit	Polyclonal	9121
NFκb	1:1000	Santa Cruz	Rabbit	Polclonal	372
p53	1:1000	BD Pharmingen	Mouse	Monoclonal	554253
PARP	1:1000	Cell Signaling	Rabbit	Polyclonal	9542
Pim-2	1:1000	Cell Signaling	Rabbit	Monoclonal	4730
S6K	1:500	Cell Signaling	Rabbit	Polyclonal	9202
S6K S371	1:500	Cell Signaling	Rabbit	Polyclonal	9208
S6K T389	1:500	Cell Signaling	Rabbit	Polyclonal	9205

6.2.2 Quantitative realtime PCR

By realtime PCR, nucleic acids can be amplified. The additional use of fluorescence dyes, labeling DNA, or fluorescence labeled templates enables quantification. Thereby, the fluorescence signal increases directly proportional to the amount of amplification product. This method is used to detect changes in the gene expression by measuring the amount of cDNA which is synthesized out of the cells mRNA.

6.2.2.1 RNA isolation and cDNA synthesis

Inhibitor treated cell were harvested at the indicated time points and total RNA was isolated using the NucleoSpin® RNA II Kit from Machery Nagel GmbH & Co. KG (Düren, Germany) following protocol 5.1.

RNA concentrations were measured and equalized with DEPC treated water. The samples were stored at -80°C. For cDNA synthesis, first 3µl of random hexamer (Bioline, London, UK) were added to 1µg of total RNA diluted in 9µl DEPC treated water and incubated for 10min at 25°C. Then the samples were put on ice and 8µl of master mix were added. Synthesis of cDNA was performed at 40°C for 1h. Polymerase was inactivated by incubation at 70°C for 10min and samples were stored at -20°C.

Master mix: 2.7µl DEPC treated H_2O

4µl 5x reaction buffer (Bioline, London, UK)

0.8µl 50mM dNTP master mix (Invitek GmbH, Berlin, Germany)

0.25µl RNAse inhibitor (Bioline, London, UK)

0.25µl Bioscript polymerase (Bioline, London, UK)

6.2.2.2 Primer

Primer design was performed by the workgroup AG Daniel and primers were synthesized from TIB Molbiol Syntheselabor GmbH (Berlin, Germany). The used primers are listed in Table 5.

Table 5: Realtime PCR primer

Gene	Primer	Sequence
Abl	Template	6FAM-CCATTTTTGGTTTGGGCTTCACACCATT-TMR
	Forward	5'-TGGAGATAACACTCTAAGCATAACTAAAGGT
	Reverse	5'-GATGTAGTTGCTTGGGACCCA
Gus	Template	6FAM- CCAGCACTCTCGTCGGTGACTGTTCA-DB
	Forward	5'-GAAAATACGTGGTTGGAGAGCTCATT
	Reverse	5'-CCGAGTGAAGATCCCCTTTTTA
Puma	Template	6FAM-CTCATCATGGGACTCCTGCCCTTACCC-TMR
	Forward	5'-AGACAAGAGGAGCAGCAGCGG
	Reverse	5'-ACCTAATTGGGCTCCATCTCGG
hrk	Template	6FAM-CGGAGCCGAGACCCAGCCG-TMR
	Forward	5'- GCAGGCGGAACTTGTAGGAA
	Reverse	5'- TTTCTCCAAGGGACACAGGGTTT
nbk/bik	Template	FAM-CATCCCTGATGTCCTCAGTCTGGTCGTCT-PH
	Forward	5'-CACAGCCTGGGTCTGGCTT
	Reverse	5'-TTAAGTGTGGTGAAACCGTCCA
noxa	Template	6FAM-AAGTCGAGTGTGCTACTCAATCA-TMR
	Forward	5'-GCAAGAACGCTCAACCGAG
	Reverse	5'-GCAGAAGAGTTTGGATATCAG
bmf	Template	FAM-CAGATTGCCCGAAAGCTTCAGTG-TMR
	Forward	5'-TGGCAACATCAAGCAGAGGT
	Reverse	5'-CTGCTGGTGTTGCTGCACA
bcl-2	Template	6FAM-CCC CTG GTG GAC AAC ATC GCC CT-TMR

	Forward	5’-AGAGCGTCAACCGGGAGATGT
	Reverse	5’-TGCCGGTTCAGGTACTCAGTCA
bcl-x	Template	6FAM-TCGGATCGCAGCTTGGATGGCCXT-PH
	Forward	5’-CACTGTGCGTGGAAAGCGT
	Reverse	5’-TGTTCCCATAGAGTTCCACAAAAGTAT
bcl-w	Template	6FAM-TCAACAAGGAGATGGAACCACTGTXT-PH
	forward	5’-CTGCACTGTGTGCTGAGAG
	Reverse	5’-CTGTGAACTCCGCCCAGC
bcl-b	template	6FAM-CAGCTGGTCCAGGCTTTTCTGTCATGCT-TMR
	Forward	5’-CTTTCCACTGGCTTTTTGGAGAAA
	Reverse	5’-TCCAGAGATAAATGAAGGCTGTTGTTAAC
mcl-1	Template	6FAM-AGGACGAGTTGTACCGGCAGTCGCTG-TMR
	Forward	5’-GCCGCTGCTGGAGTTGGT
	Reverse	5’-GCT CCC GAA GGT ACC GAG AGA T

6.2.2.3 Quantitative realtime PCR

To perform quantitative realtime PCR, the cDNA was diluted 1:5 in TBE buffer. 6µl of a Master-mix, containing forward, reverse primer and template to target the RNA in question, were put in a 96well optical Reaction plate (Applied Biosystems, Foster City, CA, USA) and 5µl of the diluted cDNA were added. Reverse transcriptase PCR was performed using TaqMan from Eppendorf AG (Hamburg, Germany) pictured in Figure 34.

Master-mix: 5µl 2x TaqMan® Fast Universal PCR Master Mix

0,25 µl forward primer (10 µM)

0,25 µl reverse primer (10 µM)

0,3 µl template (5 µM)

0,2 µl DEPC H_2O

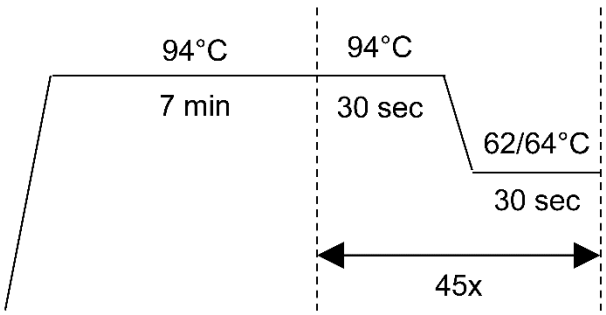

Figure 34: Amplification steps of quantitative realtime PCR.

6.2.2.4 Evaluation

For relative quantification of the realtime PCR results, the ΔΔCt method was used. The Ct value (Cycle threshold) marks that cycle at which the fluorescence of the PCR product significantly exceeds background fluorescence. The Ct values were normalized to untreated controls (ΔΔCt) and n-fold expression was assessed with the formula: n-fold expression = $2^{-\Delta\Delta Ct}$.

6.3 STATISTICS

Statistics concerning the analysis of average and standard deviation were performed using GraphPad Prism 5 for Windows Version 5.03 (GraphPad Software, Inc., La Jolla, CA, USA). Significance tests were performed with the student's t-test and evaluated with the calculator of GraphPad (http://www.graphpad.com/quickcalcs/ttest1/).

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