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Role of PIM oncogenes in the biology and chemoresistance of aggressive lymphomas

Role onkogenů PIM v biologii a chemoresistenci agresivních lymfomů

Diploma thesis

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Prohlášení:

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Abstract:

Proviral integration site for Moloney murine leukemia virus (PIM) kinases are serine/threonine kinases and oncoproteins involved in tumorigenesis of many solid and hematopoietic malignancies including mantle cell lymphoma (MCL) and diffuse-large B-cell lymphoma (DLBCL). They were shown to promote growth and survival of cancer cells by phosphorylation of proteins involved in cell cycle regulation, transcription, translation and apoptosis. Their potential as therapeutic target is, however, complicated by existence of overlapping signaling pathways. Here we show that inhibition of PIM kinases with AZD1208, highly selective pan-PIM inhibitor, reduces growth of cell lines derived from MCL and DLBCL patients. Inhibition of PIM kinases results in decreased phosphorylation of proteins involved in apoptosis and cap-dependent translation. We further showed that concurrent inhibition of PIM-kinases with AZD1208 and signaling from B-cell receptor with ibrutinib (a Bruton's tyrosine kinase (BTK) inhibitor) or idelalisib (a phosphatidylinositol 3-kinase (PI3K) inhibitor) synergistically reduces growth of MCL cell lines. Combination of AZD1208 and ibrutinib was accompanied by decreased phosphorylation of eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) and decreased expression of antiapoptotic MCL1. Despite significant improvement in the outcome of lymphoma patients achieved in recent 20 years new drugs and novel treatment approaches are needed for relapsed / refractory diseases. Our results thus show that PIM kinases present promising target for treatment of MCL and DLBCL patient especially in combination with ibrutinib or other inhibitors of BCR-mediated signaling.

Key words: PIM kinases, mantle cell lymphoma, diffuse-large B-cell lymphoma, cancer, AZD1208, ibrutinib, Bruton's tyrosine kinase

Abstrakt:

Proviral integration site for Moloney murine leukemia virus (PIM) kinázy jsou serin/threoninové kinázy a onkoproteiny jež se uplatňují při vzniku pevných a hematologických malignit včetně lymfomu z buněk pláště (MCL) a difuzního velkobuněčného lymfomu (DLBCL). PIM kinázy podporují růst a přežívání rakovinných buněk fosforylací proteinů regulujících buněčný cyklus, transkripci, translaci a apoptózu. Potenciál jejich využití jako léčebných cílů je však komplikován existencí signálních drah s částečně překryvnou funkcí. Naše výsledky ukazují, že inhibice PIM kináz za použití vysoce selektivního pan-PIM inhibitoru AZD1208 snižuje růst buněčných linií odvozených od pacientů MCL a DLBCL. Inhibice PIM kináz má za následek pokles proteinů regulujících apoptózu a cap-dependentní translaci. Dále jsme ukázali, že současná inhibice PIM kináz pomocí AZD1208 a signalizace z B-buněčného receptoru pomocí ibrutinibu (inhibitoru Brutonovy tyrozinkinázy) nebo idelalisibu (inhibitoru fosfatidylinositol 3-kinázy) společně snižuje růst MCL linií. Kombinace AZD1208 a ibrutinibu vedla ke snížení fosforylace eukaryotického translačního iniciačního faktoru 4E-vazebného proteinu 1 (4E-BP1) a k poklesu exprese antiapoptotického MCL1 proteinu. Přes to, že za posledních 20 let došlo k výraznému zlepšení léčby těchto onemocnění, řada pacientů prodělá návrat (relaps) choroby či je na léčbu primárně refrakterní. Proto je nutné hledat nové léčebné přístupy. Naše výsledky ukazují, je inhibice PIM kináz, obzvláště v kombinaci s dalšími léčivy jako je ibrutinib, představuje zajímavou možnost pro léčbu pacientů s MCL a DLBCL.

Klíčová slova: PIM kinázy, lymfom z buněk pláště, difuzní velkobuněčný lymfom, rakovina, AZD1208, ibrutinib, Brutnova tyozinkináza

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Abbreviations

4EBP1	eukaryotic translation initiation factor 4E binding protein 1
AA	amino acid
ABC	activated B-cell like
ABC	ATP-binding cassette
ABCG2	ATP binding Cassette Subfamily G Member 2
ACP/C	anaphase-promoting complex/cyclosome
AML	acute myeloid leukemia
AMPK	AMP-activated protein kinase
ARF1	adenosine diphosphate ribosylation factor-1
ASK1	apoptosis signal-regulating kinase 1
BAD	Bcl-2 associated death promoter
BCL2	B-cell lymphoma 2
BCL-XL	Bcl-extra large
BCR	B-cell receptor
BCRP	breast cancer resistance protein
BH3	Bcl-2 homology 3
BIM	Bcl2-interacting mediator of cell death
BTK	Bruton's tyrosine kinase
CDC25A	cell division cycle 25A
CDC25B	cell division cycle 25B
CDC25C	cell division cycle 25C
CDK2	cyclin-dependent kinase 2
CDK4	cyclin-dependent kinase 4
c-IAP	cellular inhibitor of apoptosis protein-1
CLL	chronic lymphocytic leukemia
DLBCL	diffuse large B-cell lymphoma
DLBCL, NOS	DLBCL not otherwise specified
DOX	doxycycline
EC	endothelial cells
eIF-4E	eukaryotic translation initiation factor
ES	embryonic stem cells
ETK	epithelial and endothelial tyrosine kinas
FL	follicular lymphoma
FLT3	fms-like tyrosine kinase 3
GAP	GTPase activating protein
GC	germinal center
GCB	germinal center B-cell
H3S10	histone 3 serine 10
hERG2	human ether-a-go-go-related protein

HSP90	heat shock protein 90
IC50	half maximal inhibitory concentration
IC	interassay control
IRES	internal ribosomal entry site
IκB	inhibitor of κB
JAK	Janus kinase
kDa	kilo Dalton
KID-1	kinase induced by depolarization 1
KO	knock-out
MAPK	mitogen activated protein kinase
MAX	Myc associated factor X
MCL	mantle cell lymphoma
MDM2	mouse double minute 2 homolog
MDR1	multidrug resistance protein 1
miRNA	micro RNA
MLV	murine leukemia virus
MM	multiple myeloma
MMVL	Moloney murine leukemia virus
mTORC1	mammalian target of rapamycin complex 1
MZL	marginal zone lymphoma
NF-κB	nuclear factor κB
NHL	non-Hodgkin lymphoma
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PIM	proviral integration site of MuLV
PP2A	protein phosphatase 2A
PRAS40	proline-rich Akt substrate of 40 kDa
RELA	Rel-like domain-containing protein
rpS6	ribosomal protein S6
RUNX	Runt-related transcription factor
S6K	ribosomal protein S6 kinase 1
SB	sleeping beauty
Ser	serine
STAT	signal transducer and activator of transcription
SUMO	small ubiquitin-like modifier
Thr	threonine
TKO	triple knock-out
TNFAIP3	tumor necrosis factor alpha-induced protein 3
TSC2	tuberous sclerosis complex 2
UTR	untranslated region
XIAP	X-linked inhibitor of apoptosis protein

1 Introduction

Non-Hodgkin lymphomas (NHL) represent the most common type of hematologic malignancies and account for approximately 3% of all newly diagnosed cancers. In 2018, more than half a million new cases were diagnosed worldwide (https://www.wcrf.org/dietandcancer/cancer-trends/worldwide-cancer-data 21.3.20119). NHLs are a heterogeneous group of lymphoproliferative malignancies, each subtype having different pathophysiology, therapeutic options and prognosis. Majority of NHLs arise as a result of malignant transformation of mature B and T lymphocytes at various stages of differentiation. According to their clinical behavior NHLs can be divided into indolent (slowly-growing), aggressive and highly aggressive lymphomas. While mean doubling time of indolent lymphomas can be counted in months, mean doubling time of highly-aggressive lymphomas may be less than 24 hours (Erlanson et al., 1995). Aggressive lymphomas comprise diffuse large B-cell lymphoma (DLBCL), the most common lymphoma subtype in the Western Hemisphere, mantle cell lymphoma (MCL) and systemic T-cell lymphomas. DLBCL and MCL both belong to B cell lymphomas and constitute approximately 30% and 3-6% of all NHL respectively (Morton et al., 2006; Zhou et al., 2008). Despite significant improvement in therapy of these during the past decades, a considerable portion of patients is either primarily resistant to currently available therapy or encounter relapse or progression of the disease after initial complete or partial response. Current therapy of B-NHL is still largely based on classical chemotherapy in combination with anti-CD20 monoclonal antibody rituximab. New targeted agents including tyrosine-kinase inhibitors or immunomodulatory agents recently approved for some NHL subtypes including MCL, significantly improved outcome of chemotherapy refractory patients (Dreyling et al., 2016).

Cancer cells depend on aberrant activation of various signaling pathways allowing them to proliferate and help to evade mechanisms that normally regulate their survival and migration. Identification of specific pathways essential for pathogenesis of particular cancer type or for development of drug resistance will allow use of targeted compounds to specifically interfere with components of the aberrantly activated oncogenic signaling. Use of targeted compounds could both improve response and reduce toxicity compared to less specific chemotherapeutical approaches. Better understanding of biology of particular cancer disease is therefore critical for the development, preclinical and clinical testing of novel targeted agents and for improved outcome of relapsed or refractory patients.

The PIM family consists of three serine/threonine protein kinases PIM1, PIM2 and PIM3. PIM kinases regulate hematopoiesis and B-cell development (Mikkers et al., 2004; Woodland et al., 2008) and are

expressed in many hematopoietic and solid cancers including DLBCL and MCL (Chen et al., 2005a, 2009b; Cibull et al., 2006; Cohen et al., 2004; Fujii et al., 2005; Gómez-Abad et al., 2011; Li et al., 2006; Zheng et al., 2008). They are connected to cancer progression, metastasis and poor prognosis in some of these malignancies (Alizadeh et al., 2000; Buchholz et al., 2005; Chen et al., 2005c; Cohen et al., 2004; Dai et al., 2005a; Hsi et al., 2008; Hüttmann et al., 2006; Schatz et al., 2011; De Vos et al., 2003; Wright et al., 2003).

PIM kinases regulate multiple pathways involved in cancerogenesis to promote proliferation and survival of malignant cell and thus present an interesting target for cancer therapy. They possess unique tertiary structure allowing for design of highly selective inhibitors that recently were shown to exert antitumor activity in various solid and hematopoietic malignancies (Chen et al., 2011; Garcia et al., 2014; Gómez-Abad et al., 2011; Horiuchi et al., 2016; Kirschner et al., 2015; Lin et al., 2010). Moreover, PIM kinases are dispensable for normal development so their inhibition could have limited toxicity to healthy tissues (Mikkers et al., 2004). This was confirmed in clinical trials of the first-in-class pan PIM kinase inhibitor AZD1208 conducted in solid and hematopoietic malignancies. AZD1208 was generally well tolerated and although the trials were terminated due to the lack of single-agent AZD1208 clinical response, they suggested PIM inhibition might be effective in particular cancer subtypes especially when rationally combined with other anti-cancer agents (Cortes et al., 2018).

2 Literature review

2.1 Diffuse large B-cell lymphoma

Diffuse large B-cell lymphoma is the most common type of non-Hodgkin lymphoma accounting for approximately one third of all NHL in western world. Median age of manifestation is 64 years with 55% male predominance (van Leeuwen et al., 2014; Morton et al., 2006; Shenoy et al., 2011). DLBCL cells can arise *de novo* (from mature B-cells) or can transform from low grade B-cell malignancies like follicular lymphoma or chronic lymphocytic leukemia. DLBCL is a heterogeneous disease that has been categorized into several subtypes including DLBCL not otherwise specified (DLBCL, NOS) (Swerdlow et al., 2016).

Based on gene expression profiling data, three subtypes of DLBCL, NOS were established connecting each subtype to a different stage of B-cell differentiation: activated B-cell like DLBCL (ABC-DLBCL) with expression profile similar to that of activated peripheral blood B-cells, germinal center B-cell like DLBCL (GCB-DLBCL) expressing genes that are typically expressed by germinal center B-cells and heterogeneous "third type" DLBCL with expression profile different from both, germinal center and activated B-cells. These subtypes differ significantly not only in gene expression but also in clinical behavior. Patients with ABC-DLBCL have statistically lower overall survival than patients with GCB-DLBCL but a great heterogeneity exists even within each subtype (Alizadeh et al., 2000; Rosenwald et al., 2002).

Transcription factor c-MYC and antiapoptotic B-cell lymphoma 2 (BCL-2) protein are often overexpressed in DLBCL due to increased gene copy number, chromosomal translocation, transcriptional or post-transcriptional mechanisms deregulation (Huang et al., 2002; Kawasaki et al., 2001; Sáez et al., 2003; Stasik et al., 2010; Wright et al., 2003). c-MYC rearrangement is present in 5-15% of DLBCL but c-MYC protein is overexpressed in 30-50% of DLBCL cases and its expression is connected with inferior survival of patients. Coexpression of c-MYC and BCL-2 is present in approximately 20-35% of DLBCL cases and leads to inferior clinical outcome compared with other DLBCL, NOS patients. As coexpression of both proteins is more common in ABC subtype, it could explain worse outcome of patients with this subtype (reviewed in Nguyen et al., 2017).

In accordance with gene expression profiling data and with the fact, that each subtype arises from B-cells in different stage of differentiation, DLBCL subtypes differ in oncogenic pathways that drive their pathogenesis. Aberrant activation of B-cell receptor (BCR), nuclear factor kappa B (NF-κB) and Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway is more often present in ABC subtype (Compagno et al., 2009; Davis et al., 2001, 2010; Lam et al., 2008) while defective

function of proteins involved in epigenetic regulation and mutation of genes of the phosphatidylinositol 3-kinase (PI3K/AKT) pathway is present in GCB subtype rather than ABC (Karube et al., 2018; Pasqualucci et al., 2011).

DLBCL is curable in 60-70% of cases, however, remaining 30-40% of patient encounter relapse of a disease or are primarily resistant to therapy (Coiffier et al., 2010). Prognosis of chemotherapy-refractory patients is dismal, and these patients represent unmet medical need for novel treatment options. Participation in clinical trials testing new agents is highly recommended for these patients.

2.2 Mantle cell lymphoma

Mantle cell lymphoma is an aggressive lymphoma that constitutes 3-8% of all non-Hodgkin's lymphoma with 74% male predominance and median age around 60 (1997; Zhou et al., 2008). MCL tumors are supposed to rise from naïve, mature B-cells but cases with low number of somatic mutations exist and are believed to be derived from cells intermediate between naïve and GC-B cells. Moreover, 15-45% of cases is derived from cells with hypermutated immunoglobulin genes suggesting role of antigen-selection in a development of subset of MCL (reviewd in Jares et al., 2012). Thus MCL is a heterogeneous group of diseases and despite some patients may present with an indolent disease, most MCL cases behave aggressively and required therapy. Patients with blastoid variant MCL suffer from especially aggressive disease that is often refractory to currently used treatments and have overall shorter survival than patient with more indolent variant (Bernard et al., 2001; Fisher et al., 1995; Gerson et al., 2018).

The genetic hallmark of MCL is the (11;14)(q13;q32) translocation, leading to aberrant expression of cyclin D1 driving cells through G1/S transition (de Boer et al., 1993; Li et al., 1999; Rosenwald et al., 2003). Rearrangements of c-MYC protein are rare in MCL (Chisholm et al., 2015) but are associated with high mortality and high disease stage (Hu et al., 2017; Setoodeh et al., 2013). Regardless of c-MYC rearrangement, c-MYC expression is present in majority of MCL cases and correlates with blastoid variant MCL (Chisholm et al., 2015; Dai et al., 2017). c-MYC was shown to drive proliferation of MCL cells and to be crucial mediator of survival in MCL. Thus, c-MYC appears as promising target for treatment of MCL (Dai et al., 2017; Lee et al., 2018).

Proliferation and survival is mediated via several mechanisms in MCL. BCR signaling activates NF-κB pathway leading to overexpression of several NF-κB target proteins including c-MYC, BCL2, BCLXL, and X-linked inhibitor of apoptosis protein (XIAP) (Dai et al., 2017; Pham et al., 2003; Roué et al., 2007).

Furthermore, phosphatidylinositol 3-kinase (PI3K)/AKT, JAK-STAT and WNT pathways are often activated in MCL leading to chemotherapy resistance and proliferation in MCL (reviewed v Jares et al., 2012).

With few exceptions, MCL is generally a chemosensitive disease. The current treatment approach comprises intensified immunochemotherapy regimen followed by consolidation with high-dose therapy and autologous hematopoietic cell transplantation, and maintenance therapy with anti-CD20 antibody rituximab. Despite considerable improvement of progression-free and overall survival in recent 20 years, prognosis of relapsed / refractory MCL remains poor and participation in clinical trials is thus often the best option for a patient (https://www.uptodate.com/contents/treatment-of-relapsed-or-refractory-mantle-cell-lymphoma 3.12.18).

2.3 PIM isoforms

PIM family of kinases constitutes three different serine/threonine kinases PIM1, PIM2 and PIM3. *Pim1* gene was originally described as gene frequently activated by insertion of murine leukemia virus (MLV) and to be associated with early T-lymphomagenesis. It was named Proviral integration site of MLV - PIM (Cuypers et al., 1984). Later on, *Pim2* gene was found to be another provirus integration site involved in tumorigenesis (Breuer et al., 1989). Ten years later Pim3 was described in rat neuroendocrine tumor cells as kinase related to Pim1 and Pim2 (Feldman et al., 1998).

2.3.1 PIM1

Pim1 gene was originally identified as a common proviral insertion site of Moloney murine leukemia (MMLV) virus in murine T-cell lymphoma. It was shown that *Pim1* is a weak oncogene which can, however, potently cooperate with other genes in tumorigenesis. Overexpression of *Pim1* in transgenic mice alone was not able to cause massive proliferation of hematopoietic cells (van Lohuizen et al., 1989) but sensitized mice to tumor development after infection by MMLV. Almost all of the tumors carried virus inserted near *c-Myc* gene, accompanied by high *c-Myc* expression, suggesting cooperation between these two genes (van Lohuizen et al., 1989; Scheijen et al., 1997). Similarly, infection of Eμ-*Myc* mice, overexpressing *c-Myc* under control of the immunoglobulin heavy chain enhancer, with MMLV induced B-cell tumorigenesis. In this case, one third of the tumors beard virus insertion in *Pim1* gene accompanied by *Pim1* mRNA expression (van Lohuizen et al., 1991). The extent of *Pim1* and *c-Myc* cooperation was demonstrated by concurrent overexpression of both genes. In mice, this led to embryonic lethality due to lymphoma development in utero (Verbeek et al., 1991) while coexpression of

both genes in prostate cells induced formation of larger tumors than did expression of either gene alone (Wang et al., 2010a).

To further understand its function, *Pim1* deficient mice were generated. They did not display any marked phenotype as their behavior, fertility, body weight and histology of main organs were not altered and only mild changes in hematopoietic cells were reported. The only phenotype observed was reduced mean cell volume of erythrocytes and impaired response of B-cells and bone derived mast cells to interleukins (Domen et al., 1993; Laird et al., 1993). This suggested existence of some overlapping regulatory pathway which can compensate for Pim1 function.

Human *PIM1* gene is located at human chromosome 6 and encodes three isoforms of serine/threonine kinases each translated from different start codons of one messenger RNA. Translation of PIM1 isoform-1 starts at classical AUG start codon giving rise to 313 amino acid (AA) long 34 kDa protein (Jinesh et al., 2015; Xie et al., 2005)**.** Translation of PIM1 isoform-2 starts at CUG codon upstream from classical AUG gaining 91 AA longer, 44 kDa protein with N-terminal extension (Saris et al., 1991). PIM1 isoform-3 is 40 kDa protein whose translation is proposed to starts from UUG codon situated between start codon of PIM1 isoform-1 and isoform-2 yielding 371 AA protein (Fig.1) (Jinesh et al., 2015; Xie et al., 2005). Isoform 1 and 2 both localize to the nucleus, mitochondria depleted cytoplasm and to the outer surface of mitochondria while isoform 3 localizes specifically inside mitochondria in cancer cell lines (Jinesh et al., 2015; Xie et al., 2005).

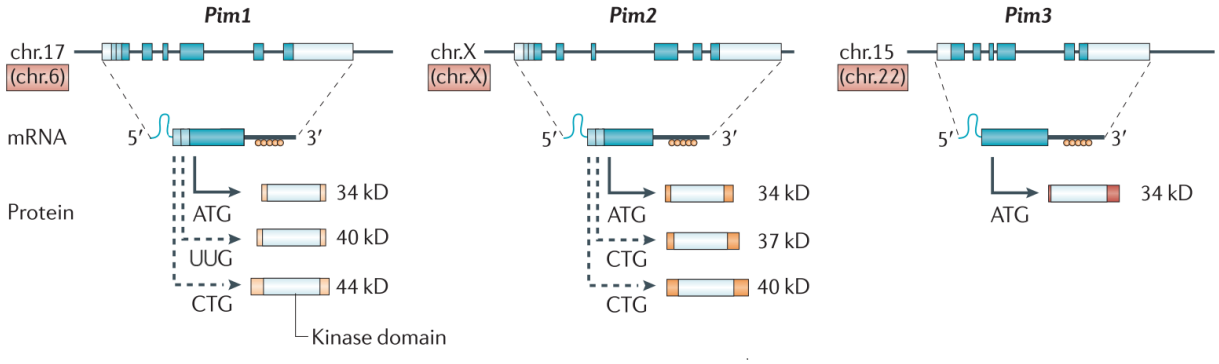


Figure 1. Genetic structure of PIMs
Localization of *PIM1*, *PIM2* and *PIM3 genes* on human (black characters in red boxes) and murine (black characters) chromosomes. Each PIM gene consists of 6 exons (blue boxes) and two untranslated regions (UTRs) (white boxes). 5´UTR is GC rich (blue loop) while 3´UTR contains several AU-rich destabilizing motifs (orange circles) which regulate PIM mRNA half-life ((Feldman et al., 1998; Fujii et al., 2005; van der Lugt et al., 1995)). Three PIM1 and three PIM2 isoforms are generated by translation from different start codons (arrows). *PIM3* gene codes for a single isoform. The isoforms have different molecular weights but all possess complete kinase domain (light blue) and kinase activity. PIM kinases lack regulatory domain and the kinase domain constitutes the majority of PIM protein. Adapted from Nawijn et al. (2011).

The abundance and localization of PIM1 isoforms differs between benign and cancer tissue and through the course of differentiation (Wang et al., 2002; Xie et al., 2005). Moreover nuclear localization of PIM1 was shown to be essential for Burkitt lymphoma tumorigenesis (Ionov et al.) as cited in (Kim et al., 2011) and to correlate with disease stage and aggressiveness of DLBCL (Brault et al., 2012).

2.3.2 PIM2

The mild phenotype of *Pim1* deficient mice suggested there must be another pathway or gene that can substitute for *Pim1* function. When trying to identify such a gene by proviral tagging in *Pim1* deficient mice, *Pim2* locus was found to be another common provirus integration site able to compensate for the loss of *Pim1*. In the presence of Eμ-*Myc* transgene, *Pim2* was activated in 90% of tumors from *Pim1*$^{-/-}$ mice. No proviral integration near *Pim2* was on the other hand detected in tumors from Eμ-*Pim1* (van der Lugt et al., 1995). Later on, *Pim2* was shown to induce lymphomagenesis in similar manner to *Pim1.* Both gene exerted weak oncogenic potential alone but strongly collaborated with *c-Myc*. In fact, both gene were activated with the same frequency in *c-Myc* overexpressing tumors (Allen et al., 1997; Scheijen et al., 1997). Finally, experiment with *Pim2* deficient mice showed that, as for *Pim1*, *Pim2* deficient mice are viable, healthy and fertile and do not exert any gross abnormalities (Mikkers et al., 2004). All these findings suggested functional redundancy of the two Pim family members.

PIM2 gene was mapped to X chromosome and codes for three protein isoforms translated from two CUG and one classical AUG start codons. The AUG codon codes for the shortest 311 AA long (34 kDa) PIM2-isoform 3. The two CUG codons lie upstream from the AUG codon and code for PIM2-isoform 2 (38 kDa) and isoform 1 (44 kDa) (Fig.1) (van der Lugt et al., 1995). All the isoforms localize to both, nucleus and cytoplasm in hematopoietic and solid tumors (Adam et al., 2015; Brault et al., 2012; Levy et al., 2012; Zhang et al., 2015) and increased nuclear localization is associated with prostate cancer (Dai et al., 2005a).

2.3.3 PIM3

PIM3 was first identified in brain as Kinase induced by depolarization 1 (KID-1) and a gene related to mammalian *PIM-1* (Feldman et al., 1998). Later on, when trying to identify genes that could substitute for *Pim1* and *Pim2* in lymphomagenesis it was shown that *Pim3* is selectively activated in tumors deficient for *Pim1* and *Pim2*. Findings in this study suggested redundancy of all the Pim family members in tumorigenesis and development. Because other genes that could substitute for *Pim1* or *Pim2* were of varying functions it was postulated that Pim kinases do not play a central role in signaling network but rather convey a crosstalk between synergistic proliferative pathways (Mikkers et al., 2002).

Further work showed that as for *Pim1* and *Pim2*, mice deficient for *Pim3* do not exert any marked phenotype (Mikkers et al., 2004). Moreover, similarly to *Pim1* and *Pim2*, overexpression of *Pim3* alone is not sufficient to induce tumorigenesis. It can, however, stimulate proliferation and sensitize liver cells to carcinogen treatment (Wu et al., 2010).

PIM3 gene is located on chromosome 22 and codes for a single 326 AA (34 kDa) isoform translated from a classical AUG start codon (Fig.1). PIM3 protein shares a sequence similarity with other PIM family members being 70% and 64% identical to PIM1 and PIM2 respectively (Fig.2). PIM3 was shown to localize to the lamelipodia of endothelial cells (EC) where it regulates EC spreading (Zhang et al., 2009b) and its localization in cancer tissue was also shown to be mostly cytoplasmic (Fujii et al., 2005; Li et al., 2006; Popivanova et al., 2007; Zheng et al., 2008).

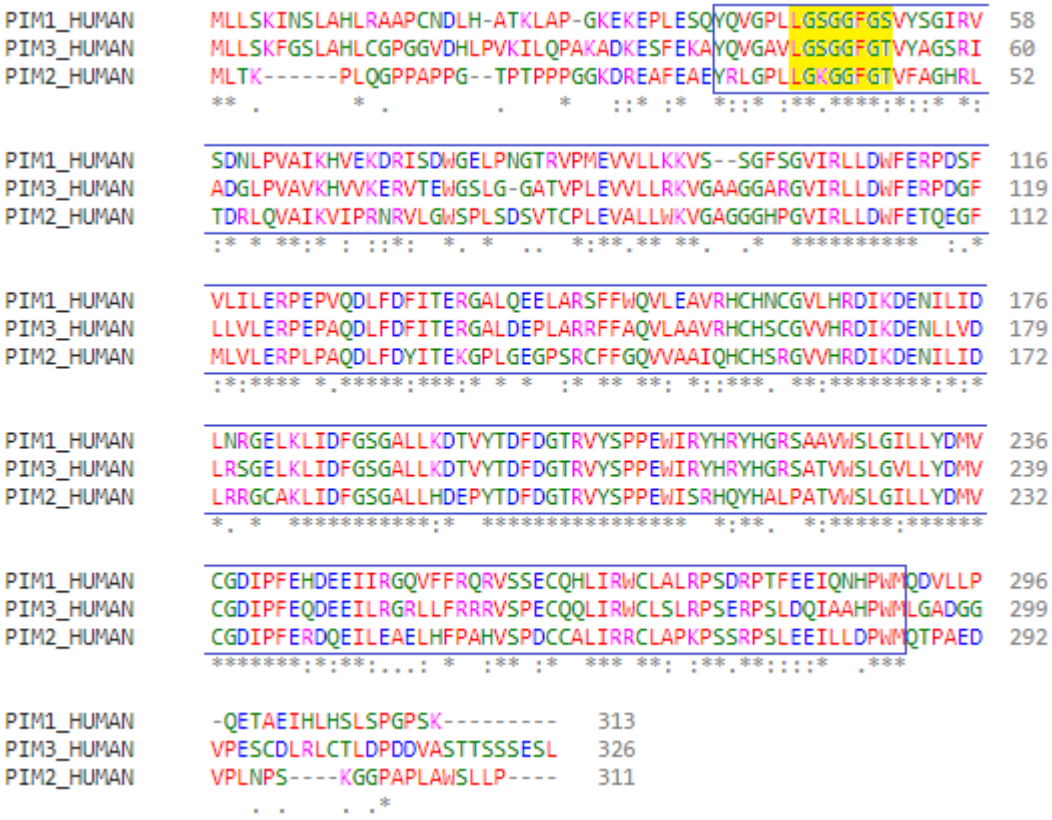


Figure 2. Amino acid alignment of human PIM family proteins
Alignment of PIM protein sequences from UniProt (accession numbers: P11309 (PIM1), Q9P1W9 (PIM2) and Q86V86 (PIM3)). The shortest PIM protein isoforms translated from classical AUG codon are shown. Human PIM proteins show high sequence identity. PIM1 and PIM3 are 70% identical, PIM1 and PIM2 are 56% identical and PIM2 and PIM3 are 64% identical at the AA level. The highest sequence identity is within the kinase domain (blue box). The ATP binding domain is shown in yellow. * indicates fully conserved residue; : indicates conservation of strongly similar properties; . indicates conservation of weakly similar properties

2.3.4 Functional redundancy of PIM kinases

The mutually exclusive activation of *Pim1* and *Pim2* genes in MLV-induced lymphomas (van der Lugt et al., 1995) and compensatory activation of other *Pim* genes in single or double *Pim* deficient mice (van der Lugt et al., 1995; Mikkers et al., 2002; Muraski et al., 2007), together with a finding that all three PIM kinases share very similar phosphorylation profile and consensus peptide substrate all suggested PIM kinases were functionally redundant (Bullock et al., 2005; Hutti et al., 2004).

More detailed studies however showed that in lymphomas, *PIM1* and *PIM2* genes share only few coregulated signaling pathways and that *PIM3* gene is associated with completely different signaling pathways than *PIM1* and *PIM2* (Gómez-Abad et al., 2011) which is surprising given the ability of *Pim3* to substitute for *Pim1* and *Pim2* in lymphomagenesis. Experiments with triple *Pim* knock-out (KO) mice also showed that in a context of hematopoiesis PIM kinases cannot fully substitute for each other and further experiment confirmed that each PIM kinase differentially affects cellular growth and survival (Hammerman et al., 2005; Mikkers et al., 2004; Muraski et al., 2007; Woodland et al., 2008).

Moreover, PIM kinases differ in their function even with respect to particular isoform. All PIM1 and PIM2 isoforms were reported to differ in their ability to suppress apoptosis, to phosphorylate Bcl-2 associated death promoter (BAD) and in their dependency on other apoptotic molecules to suppress apoptosis (Jinesh et al., 2015; Lilly et al., 1999; Xie et al., 2005; Yan et al., 2003). As all PIM1 and PIM2 isoforms only differ in the length of their N-termini it is likely that it is the very N-terminus that determines the details in function of each particular isoform – as indeed was shown on PIM1. The longest, 44 kDa isoform of PIM1 contains a proline-rich motive within its N-terminus that is not present in the shortest, 30 kDa isoform. This allows the 44 kDa isoform to interact with proteins that the shorter isoform cannot (Xie et al., 2005). The ability to interact with distinct molecules allows each isoform to localize to different compartments in cells and to modulate different signaling pathways. This mechanism can, at least in part, explain the different properties of particular isoforms (Jinesh et al., 2015; Levy et al., 2012; Saris et al., 1991; Xie et al., 2005).

Thus it appears that while PIM kinases share some overlapping substrates and are redundant in the ability to induce lymphomas, each of them fulfills somewhat distinct function within the cell through which it participates in both normal development and tumorigenesis.

2.4 Regulation of PIM expression

It was shown that PIM kinases in contrast to most of other kinases adopt constitutively active conformation and don´t rely on other kinases and signaling cascades to become activated (Bullock et al., 2009; Kumar et al., 2005; Qian et al., 2005). Given that PIM kinases are proto-oncogenes whose activity must be tightly regulated this regulation takes place on the level of their expression.

2.4.1 Transcription

PIM1, PIM2 and PIM3 mRNAs are expressed in generally low levels in many tissues with various expression patterns for each kinase. *PIM3* is normally expressed endothelial cells, heart, skeletal muscle, spleen, kidney, placenta and peripheral blood leukocytes (Fujii et al., 2005; Zhang et al., 2009b). PIM1 and PIM2 mRNAs are abundant in thymus and hematopoietic tissue and are strongly induced by variety of cytokines, hormones and mitogens (Allen et al., 1997; Buckley et al., 1995; Matikainen et al., 1999; Mikkers et al., 2004; Miura et al., 1994; Zippo et al., 2004) through JAK/STAT and NF-κB pathway (Li et al., 2001; Matikainen et al., 1999; Shirogane et al., 1999; Yip-Schneider et al., 1995; Zemskova et al., 2008; Zhu et al., 2002a). PIM1 mRNA expression is also induced by chemotherapeutical agents, irradiation and hypoxia to protect cells from apoptosis (Chen et al., 2009a; Kim et al., 2011; Kirschner et al., 2015; Weirauch et al., 2013).

2.4.2 Stability of PIM mRNA

The mRNA of all three PIM kinases contains several copies of AU-rich destabilizing motif within their 3´UTR (Feldman et al., 1998; Fujii et al., 2005; van der Lugt et al., 1995) which renders it very unstable (Buckley et al., 1995; Meeker et al., 1990; Yang et al., 2011). In tumors induced by integration of provirus in *Pim1* gene, the provirus was usually integrated in 3´UTR of PIM1 mRNA disrupting the destabilizing motive. This led to increase in both mRNA stability and abundance (Selten et al., 1985). PIM kinases were also reported to be regulated by micro-RNAs (miRNAs) (Huang et al., 2009; Liang et al., 2015; Nasser et al., 2008; Weirauch et al., 2013; Zhang, 2017) suggesting that proviral integration-induced tumorigenesis can act, besides disruption of the destabilizing motive, through disruption of miRNA binding site. Increase in PIM1 mRNA stability was reported in many transformed cell lines (Meeker et al., 1987 as cited in Losman et al., 2003) and can be induced by growth factors and cytokines (Yang et al., 2011; Yip-Schneider et al., 1995).

2.4.3 Translation

Expression of PIM proteins is regulated by cap-dependent and cap-independent mechanism. PIM mRNA contains long, GC rich 5´UTR which forms extensive stable secondary structures and was shown to inhibit translation of the mRNA in vitro (Fujii et al., 2005; Hoover et al., 1997). As a result, overexpression of eIF-4E or deletion of 5´UTR leads to strong increase in PIM1 protein level (Hoover et al., 1997) whereas inhibition of cap-dependent translation leads to decreased expression of PIM1 and PIM2 (Schatz et al., 2011).

With respect to particular PIM isoforms the AUG codon, form which the shortest PIM2 protein is translated, is surrounded by poor Kozak consensus sequence. Moreover, additional CUG codons upstream from the AUG codon contain more favorable sequence. As a consequence, only a fraction of translation initiation occurs on the AUG codon and the shortest PIM2 isoform is thus the less expressed compared to the other two isoforms (van der Lugt et al., 1995).

Furthermore PIM1 mRNA contains internal ribosomal entry site (IRES) which allows, similarly to some other genes connected to proliferation and survival, translation of PIM1 mRNA in situation when cap dependent translation is reduced or inhibited (Chiluiza et al., 2011; Johannes and Sarnow, 1998; Johannes et al., 1999). Whether all three PIM1 isoforms or other PIM kinases are target of IRES translation, however, is not unknown.

2.4.4 Activity

In contrast to most of the kinases PIM1 and PIM2 adopt a constitutively active conformation. They possess no regulatory domain and no upstream kinase has been reported except for one study where epithelial and endothelial tyrosine kinas (ETK) expression induced PIM1 phosphorylation and kinase activity (Kim et al., 2004). PIM kinases were shown to autophosphorylate in vitro (Hoover et al., 1991; Losman et al., 2003; Palaty et al., 1997) but conflicting findings on whether and how does the auto/phosphorylation influence PIM kinase activity were reported (Bullock et al., 2005; Jacobs et al., 2005; Ma et al., 2007; Palaty et al., 1997). PIM kinases thus don´t probably rely on other kinases or signaling cascades to be activated and are constitutively active following translation (Bullock et al., 2009; Kumar et al., 2005; Qian et al., 2005). Concordantly, there exists a tight correlation between PIM protein expression and kinase activity (Fox et al., 2003; Zhu et al., 2002a). No crystallographic study on PIM3 has been published up to date but given high sequence similarity between PIM kinases it is likely that PIM3 shares main structural and regulatory features with the other two members.

2.4.5 Protein stability

PIM proteins are rather unstable with half-life ranging from 5 min to 1 h (Adam et al., 2015; Liang et al., 1996; Losman et al., 2003). This, together with the fact that PIM kinases seem to be constitutively active, suggests that protein degradation is involved in inhibition of PIM activity. Concordantly, increased protein stability is one of the mechanisms responsible for tumor induction. It was shown that while PIM1 half-life is shorter than 5 min in primary blood and splenic cells (Kuo et al., 2016; Liang et al., 1996; Losman et al., 2003) it is significantly prolonged in transformed cells (Liang et al., 1996; Ma et al., 2007; Shay et al., 2005).

Two chaperons were described to bind and regulate PIM1 protein stability. Heat shock protein 90 (HSP90) was shown to protect PIM1 from degradation and also to increase PIM1 kinase activity. The latter, however, was not confirmed by another group (Mizuno et al., 2001; Shay et al., 2005). Heat shock protein 70 (HSP70) is another chaperon that binds PIM1 and was proposed to mediate its degradation (Shay et al., 2005).

Another molecule that regulates PIM protein stability is protein phosphatase 2A (PP2A) which was shown to interact with and dephosphorylate PIM kinases in vitro. Increasing expression of PP2A leads to decrease in expression of all three PIM kinases (Losman et al., 2003; Ma et al., 2007) but it is not clear wheatear PP2A directly dephosphorylates PIM kinases to induce their degradation or whether its function is indirect in vivo. If the former is true, autophosphorylation or phosphorylation by other cellular kinases could regulate the stability of PIM kinase. No other kinase than ETK, however, was reported to phosphorylate PIM kinases and, at least in the case of PIM3, autophosphorylation does not have any effect on protein stability (Losman et al., 2003). The role of either autophosphorylation or phosphorylation by other cellular kinase in regulation of PIM activity and stability thus remains to be clarified.

Degradation of PIM kinases is mediated via the ubiquitin-proteasome pathway but details of the process remain elusive (Losman et al., 2003; Ma et al., 2007; Shay et al., 2005). Recently, modification by small ubiquitin-like modifier (SUMO) was suggested to regulate PIM1 ubiquitylation and degradation via SUMO-targeted E3 ubiquitin ligase RNF-4 (Iyer et al., 2017).

2.5 Biological functions of PIM kinases

PIM kinases are expressed in hematopoietic tissues during development of birds and mice (Eichmann et al., 2000; Gapter et al., 2006). To address their function in development, tipple *Pim* KO (TKO) mice were

generated. As for the single *Pim* KO mice, TKO mice were viable and fertile. Their body size however was reduced compared to WT mice due to overall decreased in cell numbers and partial reduction of cell size. The whole blood analysis did not show alterations in the numbers of any type of white blood cells and there was a slight increase in the number of erythrocytes. The bone marrow cells however showed impaired cytokine induce myeloid development and B-cell proliferation. PIM kinases are thus important for normal B-cell development and homeostasis (Mikkers et al., 2004; Woodland et al., 2008).

Besides their role in development and hematopoiesis, PIM1 and PIM3 play an important role in self-renewal capacity of embryonic stem cells (ES) and their depletion leads to decreased expression of pluripotent genes and increased differentiation (Aksoy et al., 2007). Both are also involved in angiogenesis as they mediate proliferation and migration of endothelial cells (Zhang et al., 2009b; Zippo et al., 2004). PIM1 plays further role in vascular smooth muscle cell proliferation after injury (Katakami et al., 2004) and is expressed in failing human heart and in murine cardiomyocytes after pathological injury to protect heart cell from apoptosis (Muraski et al., 2007). Given the mild phenotype of TKO mice, the importance of PIM kinases in these processes is probably limited, suggesting for redundancy in the PIM network.

2.6 PIM kinases in cancer: the molecular basis of PIM and c-MYC cooperation

Activation of oncogene often results in apoptosis or senescence of a cell as part of a mechanism by which multicellular organism defend itself from cancer development. *c-MYC* proto-oncogene is commonly overexpressed in cancer and codes for a transcription factor that regulates expression of many genes involved in cell cycle progression, cellular metabolism and cell death. Because its overexpression triggers programmed cell death of a cell (Eischen et al., 1999; Evan et al., 1992) a logical suggestion for the mechanism of PIM and c-MYC cooperation was that PIM kinases are able to reverse the lethal consequence of c-MYC activation and to permit proliferation of the damaged cell. And indeed, this turned out to be true as *Pim1* was shown to induce survival of cells overexpressing *c-Myc* through *Bcl-2* induction (Shirogane et al., 1999). Later on, several other mechanisms responsible for the cooperation of c-MYC and PIM kinases in tumorigenesis were described including direct regulation of c-MYC transcriptional activity, cell cycle progression and regulation of protein synthesis.

2.6.1 c-MYC activity

One of the possible explanations for PIM-MYC cooperation was that PIM kinases regulate c-MYC activity. And it really was shown that PIM1 and PIM2 directly interact with c-MYC after growth factor stimulation

(Zhang et al., 2008; Zippo et al., 2007) and that PIM1 regulates c-MYC transcriptional activity (Kim et al., 2010). PIM1 associates with c-MYC/MYC associated factor X (MAX) complex and localizes with this complex to c-MYC-target gene enhancers where it is responsible for phosphorylation of histone 3 serine 10 (H3S10) and activation of transcription of some c-MYC target genes. By doing so PIM1 regulates transcription of approximately 20% of c-MYC-regulated genes, particularly of those connected to nucleic acid metabolism, transcriptional regulation, RNA processing and oncogenesis (Zippo et al., 2007). The phosphorylation of H3S10 by PIM1 induces recruitment of positive elongation factor and release of promoter-proximal paused RNA polymerase. Thus, by phosphorylation of H3S10, PIM1 stimulates transcription elongation of c-MYC target genes (Zippo et al., 2009) and cooperates with c-MYC in cell transformation (Zippo et al., 2007).

Another possible mechanism to regulate c-MYC activity is to control c-MYC expression. While knock down of PIM1 leads to decrease in c-MYC protein (Zhang et al., 2008), increased expression of PIM3 can stimulate c-MYC protein translation by inducing association of c-MYC mRNA with heavy polysomes (Beharry et al., 2011). Because decrease of c-MYC protein after PIM1 knock down was not accompanied by decrease in mRNA levels (Zhang et al., 2008), it is likely that regulation of c-MYC by PIM1 does not involve transcription of the gene or mRNA stability but rather functions through regulation of mRNA translation or protein stability. One study reported, however, increase in c-MYC mRNA level in PIM1 overexpressing prostate cancer cells (Chen et al., 2005c) so the role of PIM kinases in c-MYC mRNA expression remains unclear perhaps depending on cellular context.

PIM kinases directly and indirectly regulate c-MYC stability. It was shown that PIM1 and more efficiently PIM2 phosphorylate c-MYC on serine (Ser) 329 to stabilize the protein (Zhang et al., 2008). Both kinases also indirectly regulate phosphorylation of c-MYC on Ser62, which is known to stabilize the protein, and on threonine (Thr) 58 which mediates normal c-MYC degradation (Sears et al., 2000). Overexpression of PIM1 and to a lesser extent of PIM2 leads to increase in Ser62 phosphorylation and decreased phosphorylation on Thr58. Despite differential efficiency of Ser329, Ser62 and Thr58 phosphorylation, both PIM kinases stabilize c-MYC protein and consequently increase c-MYC transcriptional activity (Zhang et al., 2008). The molecular mechanism by which PIM1 and PIM2 regulate Ser62 phosphorylation is not known but could involve PP2A phosphatase which was shown dephosphorylates Ser62 of c-MYC to promote its degradation (Yeh et al., 2004) since PIM1 and PIM2 negatively regulate activity of PP2A (Chen et al., 2005c). Furthermore, c-MYC can directly regulate expression of PIM kinases. In some human and mouse lymphomas PIM3 is direct target of c-MYC and is expressed in all lymphomas with

overexpressed c-MYC (Plym Forshell et al., 2011) suggesting possibility of existence of positive feedback loop between these proteins in some context.

PIM1 was also shown to phosphorylate Runt-related transcription factor 1 (RUNX1) and to potentiate its transcriptional activity (Aho et al., 2006). RUNX1 is transcription factor that is expressed at G1-S transition in hematopoietic cells and that regulates hematopoiesis and lymphocyte development. Its amplification occurs in child B-lineage acute leukemia and it cooperates with c-MYC in lymphomagenesis (reviewed in Ito et al., 2015).

2.6.2 Protein synthesis

Finding that mice deficient in all three PIM kinases have reduce body and cell size (Mikkers et al., 2004) suggested PIM kinases play an important role in metabolism and growth. It was shown that PIM kinases can function in parallel to but independently of protein kinase B (PKB)/AKT signaling – that both kinases share multiple targets to activate translation, promote growth, survival and lymphomagenesis (Hammerman et al., 2005). AKT and PIM kinases regulate translation partly by controlling mammalian target of rapamycin complex 1 (mTORC1) pathway that is involved in cancer pathogenesis and whose components are mutated in many cancers and familiar cancer syndromes (reviewed in Laplante and Sabatini, 2012).

Upstream from mTORC1 is Tuberous sclerosis complex 2 (TSC2) which functions as negative regulator of mTORC1 signaling (Tee et al., 2003). PIM2 and AKT can modulate mTORC1 pathway by phosphorylating TSC2 to prevent its inhibition of mTORC1 (Lu et al., 2013; Manning et al., 2002). Moreover, both PIM1 and ATK can regulate mTORC1 activity by mediating dissociation of its inhibitory subunit proline-rich AKT substrate of 40 kDa (PRAS40) (Kovacina et al., 2003; Zhang et al., 2009a). PIM thus function in parallel to AKT with both kinases preferentially regulating different levels of mTORC1 signaling. In MM, PIM kinases are more important regulators of TSC2, while AKT preferentially regulates PRAS40 (Lu et al., 2013).

Downstream targets of mTORC1 are ribosomal protein S6 kinase 1 (S6K1) and eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1). S6K1 regulates translation initiation and elongation and mRNA biogenesis while 4E-BP1 acts as negative regulator of eIF4E, a key component of translation initiation complex for cap-dependent translation (reviewed in Laplante et al., 2012). Unphosphorylated 4E-BP1 binds eIF4E but dissociates after phosphorylation and enables eIF4E to engage in translation activation (Brunn et al., 1997). The importance of regulation of 4E-BP1 in tumorigenesis was demonstrated on mice with constitutively active AKT expressed in T-cells. The expression of AKT led

to increased phosphorylation of 4E-BP1, protein synthesis and increased tumor formation. Conversely inhibition of 4E-BP1 activity by expression of non-phosphorylatable 4E-BP1 led to decreased protein synthesis and decreased tumor size in these mice (Hsieh et al., 2010).

It is thus important that PIM2 can regulate both: 4E-PB1 and eIF4E. By phosphorylating 4E-BP1 on Ser65 PIM2 activates translation and promotes lymphomagenesis. PIM2 can further, in contrast to AKT, maintain phosphorylation of 4E-BP1 and eIF4E even in the presence of rapamycin, an mTORC1 inhibitor, and thus protect lymphoma cells from inhibition of translation by this drug. PIM2 however cannot protect cells from inhibition of translation by non-phosphorylatable 4E-BP1 (Fox et al., 2003; Schatz et al., 2011; Tamburini et al., 2009) suggesting 4E-BP1 is an important PIM translation target. Furthermore, PIM2 can induce eIF4E protein expression and phosphorylation (Hammerman et al., 2005) and together with AKT phosphorylates eIF4B to induce translation initiation complex formation (Cen et al., 2014). Thus, by regulation of molecules downstream of mTORC1, PIM kinases can substitute for some of its functions and confer resistance to mTORC1 inhibition.

All Pim kinases further regulate mTORC1 pathway via AMPK (Beharry et al., 2011). AMPK functions as an sensor of cellular AMP:ATP ratio and when the ATP level in cell is low, AMPK inhibits mTORC1 complex and reduces protein synthesis (reviewed in Shaw, 2009). PIM3 was shown to induce mTORC1 activity and subsequently overall protein synthesis by regulation AMP:ATP ratio. Expression of PIM3 kinase in *Pim*-TKO MEFs is sufficient to activate protein synthesis and drive growth of these cells to a similar level as in wild type MEFs (Beharry et al., 2011).

Moreover, PIM kinases, but not AKT, can regulate cap-independent translation of proteins involved in tumorigenesis including BCL-2 and several RTKs (An et al., 2015; Cen et al., 2013) and thus it is possible that PIM kinases could drive IRES-mediated c-MYC translation during apoptosis (Stoneley et al., 2000).

Despite PIM kinases and AKT share many substrates and regulate similar pathways in cells their function is at least partially independent of each other: a) components of Akt signaling pathway were not activated by proviral integration in Pim deficient mice (Mikkers et al., 2002), b) antiapoptotic function of PIM2 and AKT are additive and c) antiapoptotic function of PIM2 is independent of upstream components of PI3K/AKT cascade d) PIM kinases do not substantially influence AKT expression, phosphorylation or activity (Fox et al., 2003; Le et al., 2016; Song et al., 2016). On the other hand, PIM kinases and components of PI3K/AKT pathway are mutually exclusively activated breast cancer (Le et al., 2016) suggesting partial redundancy of these two pathways that could be of great importance in clinics.

2.6.3 Apoptosis and drug resistance

PIM kinases regulate survival on multiple levels and were shown to protect cells from many death-inducing stimuli including cytokine withdrawal, oxidative stress, hypoxia, irradiation and anticancer drugs treatment (Aho et al., 2004; Chen et al., 2009a; Gu et al., 2009; Kim et al., 2011; Schatz et al., 2011; Song et al., 2015; Weirauch et al., 2013; Xie et al., 2008, 2010; Yan et al., 2003). PIM kinases not only directly modulate function of apoptotic mediators, but also increase expression of prosurvival proteins through regulation of transcription and translation factors like c-MYC, RUNX, STAT, NF-κB and eIF4E. Moreover, besides their ability to regulate apoptosis-related proteins, PIM kinases can further protect cancer cells by stimulating drug efflux though ATP-binding cassette (ABC) transporters. Thus PIM kinases not only interfere with the process of apoptosis itself but can prevent triggering of the process by eliminating death inducers to further enhance the drug resistance phenotype.

One of the proteins directly regulated by PIM kinases is BAD, a pro-apoptotic BCL-2 family member protein which induces apoptosis via binding to anti-apoptotic family members BCL-2, B-cell lymphoma extra-large (BCL-XL) and BCL-W (Chen et al., 2005b; Kuwana et al., 2005; Letai et al., 2002). Murine BAD contains evolutionary conserved serine residues Ser112, Ser136 and Ser155 [1] which can be phosphorylated and whose phosphorylation status regulates BAD function. Prosurvival signaling leads to phosphorylation of these residues and sequestration of Bad by 14-3-3 protein in cytosol (Datta et al., 2000; Zha et al., 1996; Zhou et al., 2000). AKT and all three PIM kinases phosphorylate BAD on these sites. PIM1 and PIM2 phosphorylate Ser112 much more efficiently than the other two residues whereas PIM3 prefers Ser136 and Ser155 over Ser112. Nevertheless phosphorylation of BAD by all three PIM kinases leads to dissociation of BAD from BCL-XL and binding to 14-3-3 (Macdonald et al., 2006) to induce apoptotic resistance (Aho et al., 2004; Yan et al., 2003). BAD also plays an important role in cellular metabolism as part of a larger complex that regulates glycolysis (Danial et al., 2003). PIM2 was shown to regulate this BAD function and to increase aerobic glycolysis in colorectal carcinoma (Zhang et al., 2015). The function of PIM kinases in cellular metabolism is, however, out of the focus of this study.

Moreover, PIM1 regulates BCL-2 gene expression in cooperation with c-MYC (Shirogane et al., 1999) and together with AKT can further inhibit apoptosis by downregulation of apoptosis signal-regulating kinase 1 (ASK1) (Gu et al., 2009; Kim et al., 2001).

[1] Nomenclature in this work will be based upon the murine sequence; corresponding human residues are Ser75, Ser99 a Ser118 respectively

The 44 kDa isoform of PIM1 can also inhibit p53 mediated apoptosis induced by chemotherapeutical agents in prostate cancer cells via binding of its N-terminal proline-rich motive to SRC homology 3 (SH3) domain of ETK kinase. This interaction disrupts interaction of ETK with p53 leading to ETK activation and apoptotic resistance (Jiang et al., 2004; Xie et al., 2005). This presents an example of kinase-independent function of PIM1, where PIM1 functions as p53 binding competitor. Aberrant expression of PIM1 may thus disrupt the balance between these two molecules and contribute to cancerogenesis by antagonizing p53 tumor suppressing functions regardless of its kinase activity.

Besides direct regulation of apoptosis PIM kinases can indirectly affect apoptotic proteins by regulating their expression. PIM3 but not PIM1 or PIM2 can induce phosphorylation and activation of STAT3 lading to expression of pro-survival protein Survivin in prostate cancer (Chang et al., 2010). Moreover, PIM1 and PIM2 were shown to activate NF-κB pathway leading to increased cellular inhibitor of apoptosisprotein-1 (c-IAP) and BCL-XL expression and increased IL-3 independent survival. In fact, the ability of PIM2 to synergize with c-MYC in tumorigenesis is dependent on sustained NF-κB activation (Hammerman et al., 2004a; Zemskova et al., 2008). There are several mechanisms through which PIM kinases regulate NF-κB pathway. PIM1 can regulate NF-κB signaling by direct stabilization of Rel-like domain-containing protein (RELA)/p65 and stimulation of its transcriptional activity (Nihira et al., 2010) or by downregulation of negative regulator of NF-κB signaling tumor necrosis factor alpha-induced protein 3 (TNFAIP3) (Kim et al., 2010) while PIM2 induces phosphorylation and degradation of inhibitor of κB (IκB) (Hammerman et al., 2004b).

Another indirect function PIM kinases exert with respect to survival is control of cap-dependent translation as discussed in previous section. eIF4E, which is regulated by PIM2, mediates survival via increased translation of a specific subset of mRNA connected to survival, growth and proliferation, e.g. PIM1, cyclin-dependent kinase 2 (CDK2), cyclin-dependent kinase (CDK4) or cell division cycle 25B (CDC25B) (Hoover et al., 1997; Larsson et al., 2007; Mamane et al., 2007). Inhibition of cap-dependent translation, and of PIM kinases, on the other hand leads to decreased expression of proto-oncogenes like Cyclin-D, c-MYC, MCL1 (Peters et al., 2016; Schatz et al., 2011; Tamburini et al., 2009; Yang et al., 2012).

Human ABC superfamily of transporters codes for 48 transmembrane proteins involved in transport of various substrates. Their physiological role is to protect body from xenobiotic but, when aberrantly expressed, they can promote cancer resistance by excluding drugs from cells (reviewed in Szakács et al., 2006). PIM1 induces membrane expression and efflux activity of ATP Binding Cassette Subfamily G Member 2/ breast cancer resistance protein (ABCG2/BCRP) leading to resistance to chemotherapeutical

drugs in prostate cancer (Natarajan et al., 2013; Xie et al., 2008) while inhibition of PIM1 leads to increased accumulation of anticancer drug mitoxanton in breast cancer cells (Darby et al., 2015). PIM1 was also shown to induce cell surface expression of multidrug resistance protein 1 (MDR1) and inhibition of PIM1 sensitized cells to doxorubicin in acute myeloid leukemia (AML) (Natarajan et al., 2013; Xie et al., 2010). However, whether the doxorubicin resistance was consequence of enhanced membrane expression of MDR1 mediated by PIM1 or of another function of PIM1 was not tested and a later study on breast cancer showed that inhibition of PIM1 had no effect on sensitivity to doxorubicin mediated by MDR1 (Darby et al., 2015).

2.6.4 Cell cycle progression

A proper regulation of cell cycle is critical for maintaining tissue homeostasis and deregulation of this process may cause uncontrolled proliferation and genetic instability. Abnormalities in gene dosage can lead to aberrant expression of oncogenes and tumor-suppressors and subsequent death of affected cell or development of cancer. PIM kinases regulate cell cycle on multiple levels.

First, they regulate S-phase entry. PIM kinases, and AKT, phosphorylate p27 to induce its nuclear export and proteasomal degradation while PIM1 can further reduce p27 transcription. Downregulation of PIM1 thus leads to accumulation of p27 and impaired cell proliferation (Cen et al., 2010; Fujita et al., 2002; Morishita et al., 2008). Moreover PIM1 and AKT phosphorylate p21 on Thr145 to induce its nuclear exclusion leading to increased growth of transforming cells (Wang et al., 2002; Zhang et al., 2007; Zhou et al., 2001). PIM2 was also shown to phosphorylate p21 at Thr145 and to stabilize p21 protein. However, p21 stabilized by PIM2 localizes to the nucleus and induces cell cycle arrest in HCT116. Such a different outcome of expression of these two kinases which in general have similar function can be explained by a different genetic background on which both kinases were tested as in HCT116 cells neither PIM1 was able to induce cytoplasmic translocation of p21 (Wang et al., 2010b). Nevertheless, inhibition of PIM kinases in general induces cell cycle arrest (Beharry et al., 2009; Gómez-Abad et al., 2011; Keeton et al., 2014; Lin et al., 2010).

PIM1 can further promote S phase entry by negative regulation of anaphase-promoting complex/cyclosome (APC/C^{Cdh1}). Pim1 can also phosphorylate cell division cycle 25A (CDC25A) to induce its biological activities and to promote cellular transformation (Mochizuki et al., 1999) while c-Myc regulates transcription of CDC25A (Galaktionov et al., 1996) and their synergistic action was shown to promote cell cycle progression and prevent apoptosis (Shirogane et al., 1999).

Next, PIM kinases regulate M-phase entry and mitosis. PIM1 can inhibit C-TAK1 kinase which inactivates cell division cycle 25C (CDC25C) phosphatase or directly phosphorylate CDC25C. Both events lead to increased CDC25C activity and drive cells to mitosis through G2/M checkpoint (Bachmann et al., 2004, 2006). During mitosis, PIM1 localizes to spindle poles, where it phosphorylates several proteins and promotes mitosis progression (Bhattacharya et al., 2002). Overexpression of PIM1 can mediate mitotic spindle defects and spindle checkpoint dysfunction leading to genetic instability as a consequence of abnormal chromosome segregating (Roh et al., 2003).

Thus PIM kinases directly regulate function of many proteins to protect cells from apoptosis and to drive cell cycle progression. Moreover, they regulate activity of several transcription factors involved in tumorigenesis to mediate expression of proto-oncogenes and finally they mediate cap-dependent and cap-independent translation of proteins involved in survival and proliferation. By all these means PIM kinases may cooperate with c-MYC to promote cancer development and tumor growth.

2.7 PIM kinases in cancer therapy

2.7.1 Expression in cancer

Since their discovery, PIM kinases were shown to be aberrantly expressed in many cancers: PIM1 and PIM2 are overexpressed in many hematological malignancies including chronic lymphocytic leukemia (CLL), DLBCL, MCL, follicular lymphoma (FL), marginal zone lymphoma (MZL) but also in solid cancers like prostate adenocarcinoma (Chen et al., 2009b; Cibull et al., 2006; Cohen et al., 2004; Gómez-Abad et al., 2011), while PIM3 is overexpressed predominantly in solid cancers including hepatocellular carcinoma, pancreatic and gastric cancer (Chen et al., 2005a; Fujii et al., 2005; Li et al., 2006; Zheng et al., 2008).

PIM1 was shown to be a target of aberrant somatic hypermutation in DLBCL (Pasqualucci et al., 2001), suggesting for its role in malignant transformation in DLBCL. As these mutations increased but also decreased PIM1 in vitro kinase activity it is likely that PIM1 has also some kinase-independent function in cancerogenesis (Kumar et al., 2005). Moreover PIM2 is important for cell transformation in AML (Mizuki et al., 2003). This, together with findings that expression of PIM1 and PIM3 is higher in precancerous lesions than in cancerous tissue in some solid cancers (Fujii et al., 2005; Li et al., 2006; Popivanova et al., 2007; Valdman et al., 2004; Yang et al., 2005; Zheng et al., 2008) suggests PIM kinases are involved in development of both, hematopoietic and solid cancers. The fact that PIM3 and PIM1 are more expressed in precancerous than cancerous tissue implies both kinases play more important role in early stages of tumor development that in later events, at least in some cancers.

When expressed, PIM kinases are connected to disease progression, metastasis and poor prognosis in many hematological malignancies including MCL, CLL, FL and DLBCL (Alizadeh et al., 2000; Cohen et al., 2004; Hsi et al., 2008; Hüttmann et al., 2006; Schatz et al., 2011; De Vos et al., 2003; Wright et al., 2003) as well as in solid cancer (Buchholz et al., 2005; Chen et al., 2005a; Dai et al., 2005b). Moreover, mutations in PIM1 gene are connected with transformation of CLL and FL to more aggressive DLBCL (Rossi et al., 2006).

In lymphomas, expression of PIM2 gene was shown to be associated with some of the pathways most important for B-cell tumorigenesis including NF-κB, mitogen activated protein kinase (MAPK) or JAK-STAT pathway. PIM3 on contrary was shown to negatively correlate with pathways involved in proliferation or tumorigenesis (Gómez-Abad et al., 2011). In a clinical setting, this may mean that isoform-specific inhibitors would be beneficial in some types of cancer.

2.7.2 PIM inhibition

Given the importance of PIM kinases in many types of cancer a broad range of inhibitors was developed some of which have entered clinical trials. Various PIM inhibitors reduce growth of cell lines and tumors derived from both solid and hematological malignancies including T-cell and B-cell leukemia and lymphomas, MM, prostate and breast cancer (Chen et al., 2011; Garcia et al., 2014; Gómez-Abad et al., 2011; Horiuchi et al., 2016; Kirschner et al., 2015; Lin et al., 2010).

Recent study in breast cancer showed that knockdown of PIM1 or PIM2 induced cell death and reduced proliferation of breast cancer cells and that both effects significantly correlated with c-MYC and PIM1 protein expression. The connection between PIM and c-MYC was confirmed in mouse xenograft as inhibition of PIM kinases with pan-PIM kinase inhibitor SGI-1776 significantly reduced only the growth of tumors with high c-MYC expression. Furthermore, PIM inhibition decreased expression, phosphorylation and transcriptional activity of c-MYC both in vitro and in PDX models (Horiuchi et al., 2016).

SGI-1776 further induced cell cycle arrest and apoptosis in CLL, AML and prostate cancer and even sensitized prostate cancer cells to taxane treatment (Chen et al., 2009b, 2011; Mumenthaler et al., 2009; Natarajan et al., 2013). However, to what extent these effects are the consequence of inhibition of PIM kinases is not certain as SGI-1776 was shown to inhibit not only PIM kinases but also fms-like tyrosine kinase 3 (FLT3) kinase (Chen et al., 2009b) and drug efflux transporters MDR1 and ABCG2 (Natarajan et al., 2013). Nevertheless, clinical trials on SGI-1776 in prostate cancer and NHL were terminated in phase 1 due to cardiac toxicity (NCT00848601). The reason for this toxicity could be a cardio protective

function of PIM1 (Muraski et al., 2007) but more likely the ability of SGI-1776 to inhibit human ether-a-go-go-related protein (hERG) potassium channel (Foulks et al., 2014) as two other PIM inhibitors are well tolerated (Cortes et al., 2018; Raab et al., 2014).

Another PIM inhibitor, AZD1208 reduced growth of prostate cancer xenografts comparably to docetaxel, a commonly used anticancer drug. Immunohistochemistry of the grafts showed increased apoptosis with reduced BAD phosphorylation together with decreased proliferation, c-MYC phosphorylation and concomitant downregulation of c-MYC target genes. AZD1208 treatment led also to the suppression of p53 signaling and sanitized prostate cancer cells to irradiation (Kirschner et al., 2015).

In a preclinical study in AML, inhibition of PIM by AZD1208 led to inhibition of cell growth in sensitive cell lines while the sensitivity of the cell lines correlated with high PIM expression and STAT5 phosphorylation. PIM inhibition was able to induce cell cycle arrest in G0/G1, p27 accumulation and decreased BAD phosphorylation in some of the sensitive cell lines but was able to induce apoptosis only in one of five sensitive cell lines. AZD1208 reduced colony formation of primary AML cells and reduced growth of mouse xenografts to the same extent as standardly used drug cytarabine in monotherapy and even more in combination with cytarabine.

On a molecular level decrease of BAD phosphorylation did not correlate with the sensitivity of cell lines which may reflect the genetic heterogeneity of AML cell lines and thus their differential dependence on BAD apoptotic function. Decreased phosphorylation of S6K, ribosomal protein S6 (rpS6) and 4E-BP1 was however connected with the sensitivity of cell lines. Decrease in 4E-BP1, S6K and rpS6 phosphorylation in sensitive lines led to decrease of translation initiation complex and polysome formation, decreased translation and reduced cell size. It is thus likely that in AML PIM kinases exert their function though regulation of translation rather than apoptosis (Keeton et al., 2014).

AZD1208 entered Phase I clinical study in AML and solid cancers but it was terminated with AZD1208 having no clinical response. Pharmacodynamical study however confirmed the activity of AZD1208 in a subset of patients (NCT01588548) (Cortes et al., 2018).

2.7.3 Cancer resistance

2.7.3.1 Resistance to BCL-2 mimetics

Deregulated expression of BCL-2 family apoptotic regulators, especially BCL-2 protein, is a common feature of many cancers. The family consists of both, pro-apoptotic and anti-apoptotic proteins that

interact with each other through their BCL-2 homology (BH) domains. Their interactions constitute complex network whose fine regulation ensures tissue homeostasis. Aberrant expression of antiapoptotic proteins leads to increased survival of cells and cancerogenesis. Moreover, resistance of cancer cells to apoptosis poses a great problem for treatment of cancer as it limits the effects of cytotoxic drugs (Yip and Reed, 2008). Thus compounds that would mimic the Bcl-2 homology 3 (BH3) domain of pro-apoptotic BCL-2 family protein and disrupt pathological interactions between pro-apoptotic and anti-apoptotic BCL-2 family members were proposed to exert efficacy in cancer treatment.

One such molecule, ABT-737, was developed to induce apoptosis and consequently to inhibit growth of hematological and solid cancer cell lines and xenografts. However, as ABT-737 binds and inhibits function of antiapoptotic proteins BCL-2, BCL-XL and BCL-W but not MCL-1 (Oltersdorf et al., 2005), some cancers develop resistance to ABT-737 which is mediated by increased expression of MCL-1 (Konopleva et al., 2006; Tahir et al., 2001; Yecies et al., 2010). It was thus necessary to find a way to target MCL-1 to overcome this resistance. In prostate cancer PIM inhibition turned out to be an option. Three different PIM inhibitors, SMI-4a, Pimi-14j and K00135, were shown to mediate degradation of MCL-1 protein, to reduce global and MCL-1 protein synthesis, and to upregulate NOXA, a pro-apoptotic BCL-2 family member that antagonize MCL-1. Combination of ABT-737 with these inhibitors was thus able to synergistically kill prostate cancer cell lines and to reduce prostate tumor growth (Song and Kraft, 2012).

Later it was shown however, that by inhibiting BCL-XL, ABT-737 induces apoptosis in platelets, leading to sever thrombocytopenia in mice and thus limiting the use of ABT-737 in human cancer therapy (Kuo et al., 2016). In order to reduce this side effect a BCL-2-selective inhibitor, ABT-199 (Venetoclax), was designed also showing antitumor activity and sparing platelets (Souers et al., 2013). Venetoclax thus became the first-in-class BH3 mimetic approved for the treatment of cancer, specifically CLL.

As for ABT-737, resistance to ABT-199 can occur through upregulation of other BCL2 family proteins, namely BCL-XL and MCL-1. And as for ABT-737, inhibition of MCL-1 helps overcome this resistance (Kuo et al., 2016). It is thus possible, that combination of ABT-199 and inhibitors of PIM kinases could be effective in some types of cancer. Moreover PIM inhibition could be more effective than direct targeting of MCL-1 given that PIM kinases regulate apoptosis on several additional levels and increase expression of NOXA that can inhibit the remaining MCL-1.

In CLL patient samples however, simultaneous inhibition of PIM1 and BCL2 protein with three inhibitors of PIM kinases and two of BCL2 exerted in general only mild synergism in the ability to induce cell death.

None of the three PIM inhibitors was able to change expression of PIM targets such as p4E-BP1 and pS6K and PIM inhibition did not change expression of antiapoptotic proteins MCL1, BCL-XL and BCL2 (Cervantes-Gomez et al., 2016) suggesting that the synergism between BCL-2 and PIM inhibitors could be cell-type specific.

2.7.3.2 Resistance to PI3K/AKT pathway inhibition

The PI3K/AKT pathway is a signaling cascade which controls cell cycle, survival and metabolism and which is commonly mutated in several human cancers. Many compounds targeting different levels of this pathway have been developed and tested in clinical trials. Targeting PI3K/AKT pathway through mTORC1 complex with agents like rapamycin, temsirolimus or everolimus was, however, associated with resistance mediated by multiple molecules including AKT and PIM kinases (Carracedo et al., 2008; Fox et al., 2005; O'Reilly et al., 2006; Schatz et al., 2011; Shah et al., 2004). Moreover, resistance to pharmacological inhibition of AKT is mediated via PIM1 and inhibition of both kinases synergistically blocks tumor growth in prostate cancer (Cen et al., 2013, 2014). Most recently, PIM kinases were shown to mediate resistance to PI3K inhibition in breast cancer (Le et al., 2016). Taken together, these findings show that co-targeting components of PI3K/AKT pathway and PIM kinases could present rational therapeutic approach in tumors dependent on PI3K signaling.

2.7.3.3 BCR

Ibrutinib, a Bruton's tyrosine kinase (BTK) inhibitor, is commonly used for treatment of relapsed/refractory MCL patients. BTK is involved in BCR signaling to mediate proliferation and survival of B-cells. It is highly expressed in MCL and ibrutinib treatment reduces BTK-mediated growth and survival in MCL cell lines (Cinar et al., 2013; Dai et al., 2017). Even though ibrutinib significantly improves outcome of MCL patients, a great portion of patients is primarily resistant to ibrutinib or acquire resistance it the course of treatment (reviewed in Hershkovitz-Rokah et al., 2018). Understanding and overcoming this resistance is thus essential for improving outcome in MCL patient. In DLBCL, PIM1 was shown to mediate resistance to ibrutinib via NF-κB pathway and combination of ibrutinib with PIM inhibition synergistically reduced growth of tumors in mice (Kuo et al., 2016). Suggesting PIM kinases may play an important role in this resistance.

2.7.4 PIM kinases in MCL

Gene expression profiling showed that *PIM1* and *PIM2* but not *PIM3* are overexpressed in MCL (Gómez-Abad et al., 2011) and that *PIM1* and *PIM2* together with c-MYC, *BCL2* and *HSP90* are enriched in

blastoid variant MCL (De Vos et al., 2003; Zhu et al., 2002b), an aggressive disease variant characterized by frequent chemoresistance, high proliferative rate and dismal prognosis (Bernard et al., 2001; Fisher et al., 1995; Tiemann et al., 2005). Furthermore, expression of PIM1 protein was shown to be a negative predictive factor associated with shorter survival in MCL (Hsi et al., 2008). All these findings indicate that PIM kinases play an important role in MCL pathogenesis.

Inhibition of PIM kinases by pan-PIM inhibitor SGI-1776 induced apoptosis of MCL cell lines and reduced phosphorylation of PIM target c-MYC and 4E-BP1 – proteins that regulate transcription and control translation in cell. Decreased c-MYC phosphorylation correlated with overall decrease in RNA synthesis and with increased cell death. Decreased 4E-BP1 phosphorylation correlated with decreased expression of MCL-1. This suggest that transcription and translation are important targets of PIM kinases in MCL (Yang et al., 2012).

MCL cells depend on c-MYC expression that is partially mediated by NF-κB pathway. Because NF-κB pathway is activated by constitutive BCR signaling in subset of MCL, ibrutinib was shown to counteract this signal and to downregulate c-MYC protein leading to reduce viability of some MCL-cell lines (Dai et al., 2017). Moreover, direct downregulation of c-MYC was shown to be universally toxic to MCL cell lines independently of their ibrutinib sensitivity (Lee et al., 2018). PIM kinases on the other hand regulate c-MYC stability, transcriptional activity and NF-κB pathway (Hammerman et al., 2004a; Zemskova et al., 2008). Indeed, the ability of PIM kinases to cooperate with c-MYC is NF-κB dependent (Kim et al., 2010; Sears et al., 2000; Zhang et al., 2008). It is thus possible that inhibition of PIM kinases together with BTK-mediated BCR signaling would be efficient in reducing c-MYC protein and viability in MCL cells as it is in DLBCL (Kuo et al., 2016).

2.7.5 PIM kinases in DLBCL

Gene expression profiling of NHL patients showed that *PIM1* is aberrantly expressed in 32% and *PIM2* in 45% of DLBCL cases while no differences in expression between healthy lymph nodes and lymphomas were observed for *PIM3* gene. On a protein level 23% of DLBCL patients had strong expression of PIM2 (Gómez-Abad et al., 2011). *PIM1* is target of aberrant somatic hypermutation in DLBCL (Pasqualucci et al., 2001) and is one of the most frequently mutated genes in DLBCL patients (Zhang et al., 2013). Moreover, expression of PIM1 is connected to transformation of indolent follicular lymphoma to DLBCL (Rossi et al., 2006) suggesting a role of PIM1 in DLBCL tumorigenesis.

Furthermore, *PIM1* and *PIM2* are amongst top 20 genes associated with ABC-DLBCL subtype which exerts more aggressive behavior compared to GCB-DLBCL (Alizadeh et al., 2000; Care et al., 2013; Wright et al., 2003). Mutations of *PIM1* gene are more often in ABC-DLBCL than GCB-DLBCL and can increase PIM1 stability and confer resistance to chemotherapeutic drug ibrutinib (Karube et al., 2018; Kuo et al., 2016). These findings led to hypothesis that PIM kinases are more important in ABC rather than GCB-DLBCL and that PIM inhibition would be specifically potent in the first subtype.

While DLBCL cell lines more or less recapitulate result from gene expression profiling: ABC-DLBCL cell lines express higher levels of both PIM1 and PIM2 mRNA and protein than GBC-DLBCL cell lines, while PIM3 is expressed almost equally between both subtypes (Brault et al., 2012; Peters et al., 2016), immunohistochemistry of lymphoma samples showed that PIM2 was expressed in 27% of ABC and 18% of GCB patients (Gómez-Abad et al., 2011). This finding was confirmed by another study, where patients with ABC a GCB subtypes did not differ in expression of PIM kinases. PIM3 was expressed almost equally in both subtypes and the difference of PIM1 and PIM2 expression between GC and non-GC subtype did not reach statistical significance (Brault et al., 2012). This raises a question what is the cause of the difference between PIM gene and protein expression in both subtypes, especially when there exists tight correlation between PIM2 mRNA and protein expression in DLBCL cell lines (Gómez-Abad et al., 2011) and how it will influence sensitivity of patients of both subtypes to PIM inhibition.

Inhibition of PIM kinases with pan-Pim inhibitor ETP-39010 induced apoptosis in ABC-DLBCL cell lines, G1 accumulation and decreased 4E-BP1 phosphorylation while genetic inhibition of solely PIM2 was not able to induce any significant apoptosis (Gómez-Abad et al., 2011). Later it was shown, that sensitivity of ABC-DLBCL cell lines to PIM inhibition is connected with decreased S6K, 4E-BP1, TSC2 and EIF4B phosphorylation and decreased expression of c-MYC, likely result of reduced protein translation (Peters et al., 2016).

Expression of PIM2 together with BCL-2 in IL-3 dependent cell leads to transformation to IL-3 independent cells, while expression of PIM2 alone or coexpression of PIM1 and BCL-2 confers strong selective advantage during IL-3 withdrawal but does not permit transformation to IL-3 independent cells (Peters et al., 2016). Furthermore, expression of PIM1 is connected with Ibrutinib sensitivity in ABC-DLBCL (Kuo et al., 2016) suggesting that both kinases play an important role in DLBCL.

Thus PIM kinases are together with c-MYC expressed in many cancers of various origins and both are often connected with poor prognosis. Targeting c-MYC constitutes a key challenge in cancer treatment

yet no clinical-grade inhibitors of c-MYC protein are available up to date. In hematological malignancies PIM kinases potently cooperate with c-MYC protein but are not essential for normal function of the organism. Thus, PIM inhibition presents a possible way to inhibit c-MYC function and to specifically eradicate cancer cells while sparing normal, healthy tissues. PIM kinases possess a unique hinge region with unique ATP-binding pocket that permits design of highly selective inhibitors some of which entered clinical trials (Bullock et al., 2005, 2009; Qian et al., 2005). Moreover, the synergism between PIM kinases and c-MYC is not restricted to hematological malignances but was also described in solid cancers (Wang et al., 2010a; Zhang et al., 2008) suggesting targeting c-MYC through PIM inhibition could be of more general use.

PIM kinases also regulate survival pathways and drug efflux transport, two features that contribute to significant clinical problem of cancer drug resistance. Therefore, combination of PIM inhibitors with other chemotherapeutical or targeted compounds can overcome resistance to conventional treatment (Cen et al., 2013; Kuo et al., 2016; Song and Kraft, 2012).

3 Aims:

1. The first aim was to test effects of pharmacological inhibition of PIM kinases by pan-PIM kinase inhibitor AZD1208 in aggressive B-cell lymphomas (MCL and DLBCL):

- to determine pro-apoptotic (LD50) and anti-proliferative (IC50) effect of PIM inhibition by AZD1208
- to correlate sensitivity/resistance with expression of PIM1/2/3, expression of MYC, and expression/phosphorylation of AKT

2. The second aim was to test in vitro rational anti-lymphoma combination of AZD1208 and selected targeted anti-lymphoma drugs**:**

- ibrutinib (BTK inhibitor)
- ABT-199/venetoclax (BCL2 inhibitor)
- idelalisib (PI3K inhibitor)

3. Last aim was to analyze effect of PIM2 overexpression on sensitivity of lymphoma cell lines to AZD1208, ibrutinib and idelalisib

4 Materials and methods:

4.1 Reagents:

ABT-199, ibrutinib, idelalisib, AZD1208 (MedChemExpress); dissolved in DMSO

RIPA lysis buffer:

- 1% Triton X-100 (Sigma-Aldrich)
- 0,1% SDS (Promega)
- 50 mM Tris-HCL pH7,4
- 150 mM NaCl (Lach-Ner)
- 1 mM EDTA (Sigma)
- 0,5% Sodium Deoxycholate (Sigma)

Blotting buffer:

- 25 mM Tris
- 192 mM glycine (Sigma-Aldrich)
- 20% (v/v) Methanol (PENTA)

Culture medium:

- IMDM (LM-I1090, Biosera)
- 15% FBS (Biosera)
- 100 U/mL Penicillin, 100 U/µg Streptomycin (Sigma-Aldrich)

LB medium and LB agar:

- 25 g/L of LB Broth (Miller) powder
- + 15 g/L agar when intended for agar plates
- supplemented with ampicillin, final concentration 100 µg/mL (Sigma-Aldrich)

PBST:

- PBS (Armesco)
- 0,1% (v/v) Tween 20 (Sigma-Aldrich)

Ponceau S:

- 0,1% Ponceau S (Sigma-Aldrich)
- 5% Acetis acid (PENTA)

Running buffer:

- 25 mM Tris
- 192 mM glycine (Sigma-Aldrich)
- 1% (w/v) SDS (Promega)

Separating buffer:

- 1,5M Tris/HCl pH 8,3

Stacking buffer:

- 0,5M Tris/HCL pH 6,8

TAE buffer:

- 40 mM Tris
- 1 mM EDTA pH=8,0
- 20 mM Acetic acid

pH adjusted to 8,5

4.2 Plasmids:

PIM2 (NM_006875) Human Tagged ORF Clone (OriGene, cat. number: RG201933)

pCMV(CAT)T7-SB100 (Addgene, cat. number: 34879)

pSBtet-Pur (Addgene, cat. number: 60507)

4.3 Cell culture:

Cells were purchased form ACCT (MINO, Z138) or DSMZ (GRANTA-519, JEKO, MAVER1, HBL2, NU-DUL1, RIVA, OCI-LY18, OCI-LY19 and SU-DHL4) or were obtained as a kind gift from prof. Georg Lenz (REC1, U2932, HBL1, OCI-LY3, TMD8, BJAB, HT, K422, WSU-DLCL2). UPF4D cell line was derived in our laboratory from patient with GCB-DLBCL.

Cell lines were authenticated using Multiplex Cell Authentication by Multiplexion (Heidelberg, Germany) as described previously (Castro et al., 2013).The SNP profiles matched known profiles or were unique.

The purity of cell lines was validated using the Multiplex cell Contamination Test by Multiplexion (Heidelberg, Germany) as described previously (Schmitt and Pawlita, 2009). No Mycoplasma, SMRV or interspecies contamination was detected.

4.3.1 Cultivation:

All cells were cultured in IMDM culture media supplemented with 15% fetal bovine serum (FBS) or 15% human plasma (OCI-LY3, TMD8, HBL1) and penicillin-streptomycin antibiotic. Cells were cultivated at 37°C in a humidified atmosphere with 5% CO2. Cells were checked with microscope and passaged every 2 or 3 days.

4.3.2 Seeding for experiment:

Cells were mixed in the cultivation flask with pipet and counted in Bürker counting chamber. Aliquot containing desired number of cells was moved to new sterile tube, centrifuged for 5 min at 4°C and 300 g and resuspended in final volume of fresh medium.

4.3.3 Harvesting:

Cells were mixed with pipet in cultivating flask, counted in Bürker chamber and aliquot containing desired number of cells was centrifuged for 5 min at 4°C and 300 g. The medium was removed and pellet was washed twice with ice-cold PBS. After last centrifugation all the PBS was removed and pellets were stored in -80°C.

4.4 Protein manipulation:

4.4.1 Preparation of protein samples

RIPA lysis buffer supplemented with inhibitors of phosphatases (PhosSTOP, ROCHE) and proteases (P8340 Protease Inhibitor Coctaile, Sigma) was used to lyse the pellets. Lysates were incubated for 30 min on ice, then centrifuged for 15 min at 4°C and 13 000 g. The supernatant was transferred to new clean microtube and sonicated. Concentration of protein samples was measured with Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Prior to SDS-PAGE samples were diluted to the same concentration, mixed with 4x Laemmli Sample Buffer (Bio-Rad) supplemented with 10% 2-mercapthoethanol (Sigma) and boiled for 5 min at 95°C.

4.4.2 SDS-PAGE:

Separating gel of desired concentration was prepared (Table 1). The mixture was immediately poured between glasses of gel apparatus and covered with isopropanol to restrain oxygen from inhibiting polymerization. After 30 min, isopropanol was removed and stacking gel prepared according to Table 1 was added to fill the rest of the space between glasses. A 10 or 15 well comb was inserted and gel was left to polymerize for another 30 min.

The glass with polymerized gel was transferred to running tank, the comb was removed and the tank was filled with fresh running buffer. Precision Plus Protein™ Dual Color Standards (Bio-Rad) was loaded to first well to estimate protein molecular weight. 10 to 50 μg of total protein per well was loaded. Electrophoresis was performed with Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Bio-Rad) and PowerPacTM Basic Power Supply (Bio-Rad) at constant 130V for 1.5 h.

Table 1. Composition of polyacrylamide gels.

Separating gels	**10%**	**12.5%**	**Stacking gel**	
dH2O (mL)	1.29	1.02	dH2O (mL)	2.5
Separating buffer (mL)	0.85	0.85	Stacking buffer (mL)	0.45
30% Acrylamide-BIS (mL)	1.11	1.38	30% Acrylamide-BIS (mL)	0.6
10% SDS (μL)	33	33	10% SDS (μL)	36
10% APS (μL)	33	33	10% APS (μL)	36
TEMED (μL)	3	3	TEMED (μL)	3.6

4.4.3 Western blot:

Following SDS-PAGE, the gel was removed from the glass and washed with transfer buffer. Polyvinylidene difluoride (PVDF) membrane was activated for 10 s in methanol and then incubated for 1 min in transfer buffer. Meanwhile, a blotting cassette was assembled as follows: fiber pad, 3 pieces of filter paper, PVDF membrane, gel, 3 pieces of filter paper, fiber pad. The whole cassette was then transferred to the blotting tank and the tank was filled with ice-cold blotting buffer. Transfer ran for 1 h at constant 100V in Mini Trans-Blot® cell (Bio-Rad). Ponceau S staining was used to confirm a proper protein transfer. The membrane was then washed in PBST and incubated on a mini-rocker shaker for 20 min in 10% (w/v) non-fat dry milk in PBST to block nonspecific binding of antibody.

4.4.4 Protein immunodetection

Membrane was incubated in 4°C overnight with primary antibody (Table 2) diluted in 2% (w/v) non-fat dry milk in PBST. Next day the membrane was washed three times for 5 min in PBST then incubated at room temperature for 1 h with secondary antibody (Table 2) diluted in 2% (w/v) non-fat dry milk in PBST. The membrane was next washed three times for 5 min in PBST. All steps were performed while slowly shaking on mini-rocker shaker.

Proteins were visualized by incubating the membrane with Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific) for 1 min and documented by exposing the membrane to CL-XPosure™ Film

(Thermo Fisher Scientific). The films were developed with Foma LP-T and fixed with Fomafix solution (Foma Bohemia).

Table 2. List of antibodies used for protein immunodetection.

Primary antibody	Source, clonality	Purchased from	Cat. No.	Dilution
4EBP1	rabbit, mono	Cell signaling	9644	1:1000
AKT	rabbit, poly	Cell signaling	9272	1:1000
β-ACTIN	mouse, mono	Abcam	ab6276	1:10000
BAD	mouse, mono	Santa Cruz	Sc-8044	1:500
MCL1	rabbit, poly	Santa Cruz	Sc-819	1:1000
c-MYC	rabbit, mono	Abcam	ab32072	1:10000
PIM1	rabbit, mono	Abcam	ab75776	1:10000
PIM2	rabbit, mono	Cell signaling	4730	1:1000
PIM3	rabbit, mono	Cell signaling	4165	1:1000
phospho-4EBP1 (Ser65)	rabbit, poly	Cell signaling	9451	1:1000
phospho-AKT (Ser473)	rabbit, mono	Cell signaling	4060	1:500
phospho-BAD (Ser112)*	rabbit, mono	Cell signaling	5284	1:500

Secondary antibody	Source, label	Purchased from	Cat. No.	Dilution
Anti-mouse IgG	donkey, HRP	Jackson ImmunoResearch	715-036-150	1:10000
Anti-rabbit IgG	donkey, HRP	Jackson ImmunoResearch	711-036-152	1:10000

* The Ser112 nomenclature is based upon the mouse sequence. The analogous phosphorylation site is Ser75 in human

4.4.5 Western blot densitometry analysis

The western blot analysis was done using GS-800 Calibrated Densitometer (Bio-Rad) with integrated Quantity One software version 4.6.5 (Bio-Rad) according to the software documentation instructions. The X-ray films were scanned at 400 dpi as 8-bit grayscale and saved in TIFF format. All bands were normalized to the β-actin loading control from the same membrane. No image adjustment was done prior to densitometry analysis.

4.5 DNA cloning

4.5.1 DNA cloning logic

The goal was to create a cell line expressing the 34 kDa isoform of PIM2 protein using Sleeping Beauty (SB) system. The Sleeping beauty system consists of vectors. First contains transposon-derived insertion cassette containing tetracycline inducible expression system and two SfiI restriction sites allowing for

one-directional insertion of gene of interest and its tetracycline-regulated expression (pSBtet-Pur vector, Addgene plasmid #60507). It further contains genes for puromycin and ampicillin resistance, allowing selection in mammalian and bacterial cells, respectively. Second plasmid is mammalian expression plasmid coding for transposase (pCMV(CAT)T7-SB100 vector, Addgene plasmid #34879), which mediates transposition of the whole insertion cassette from SB plasmid randomly into host genome (Kowarz et al., 2015).

The source of PIM2 cDNA was PIM2 (NM_006875) Human Tagged ORF Clone expression plasmid (OriGene) from which the cDNA sequence was amplified by PCR reaction. Primers for PCR reaction were designed to contain tails with restriction sites for SfiI restriction enzyme which enables the PCR product to be inserted to the SB plasmid.

After the PCR reaction, the PCR mixture was digested by DpnI restriction enzyme to eliminate methylated plasmid DNA and then purified to remove PCR and digestion reactions components. Because of a low PCR reaction yield, the PCR product was first captured into pSC Amp/Kan vector (Agilent) followed by bacteria transformation. Amplified plasmid was then isolated from selected colonies by miniprep protocol, digested with SfiI enzyme and separated by agarose electrophoresis to check for presence of SfiI restriction sites and proper length of the insert. Plasmids with insert of predicted length, therefore with intact restriction sites, were sequenced to exclude eventual PCR introduced mutations and one was selected for further use.

The selected plasmid was digested with SfiI enzyme, separated by agarose electrophoresis and PIM2 cDNA corresponding band was isolated from the gel. Isolated fragment was ligated into pre-opened SB plasmid, introduced into bacteria and amplified. PIM2 cDNA containing pSBtet-Pur plasmid was than transfected together with transposase coding plasmid pCMV(CAT)T7-SB100 into JEKO1 and MINO cells generating inducible PIM2 expressing cells. Empty SB plasmid expressing cells were generated as a control and fluorescent mCardinal protein expressing cells were generated for optimization of gene induction.

4.5.2 Amplification of Pim2 gene by PCR

Desired DNA fragment was amplified from PIM2 (NM_006875) Human Tagged ORF Clone expression plasmid (RG201933; OriGene) by PCR reaction using Advantage® HD Polymerase mix (TAKARA). The OriGene clone ORF sequence corresponds to Ensemble human PIM2 gene long transcript PIM2-201 (ENST00000376509.4) having 933 nt coding for 311 AA and is followed by linker bound GPF tag

sequence. Primers were designed to contain tail with SfiI restriction site for subsequent insertion into the SB plasmid and to exclude the GFP-tag sequence. Stop codon was introduced between PIM2 sequence and SfiI restriction site. Composition of PCR mixture and setting of PCR program are listed in Table 3 and Table 4. Five separate samples were made, each amplified with different program setting (gradient of annealing temperature).

The OriGene expression vector ORF sequence:

Red = cloning site, green = tags, blue = PIM2 ORF

TTTTGTAATACGACTCACTATAGGGCGGCCGGGAATTCGTCGACTGGATCCGGTACCGAGGAGATCTGCCGCCGC
GATCGCCATGTTGACCAAGCCTCTACAGGGGCCTCCCGCGCCCCCCGGGACCCCCACGCCGCCGCCAGGAGGCAA
GGATCGGGAAGCGTTCGAGGCCGAGTATCGACTCGGCCCCCTCCTGGGTAAGGGGGGCTTTGGCACCGTCTTCGC
AGGACACCGCCTCACAGATCGACTCCAGGTGGCCATCAAAGTGATTCCCCGGAATCGTGTGCTGGGCTGGTCCCCC
TTGTCAGACTCAGTCACATGCCCACTCGAAGTCGCACTGCTATGGAAAGTGGGTGCAGGTGGTGGGCACCCTGGC
GTGATCCGCCTGCTTGACTGGTTTGAGACACAGGAGGGCTTCATGCTGGTCCTCGAGCGGCCTTTGCCCGCCCAGG
ATCTCTTTGACTATATCACAGAGAAGGGCCCACTGGGTGAAGGCCCAAGCCGCTGCTTCTTTGGCCAAGTAGTGGC
AGCCATCCAGCACTGCCATTCCCGTGGAGTTGTCCATCGTGACATCAAGGATGAGAACATCCTGATAGACCTACGC
CGTGGCTGTGCCAAACTCATTGATTTTGGTTCTGGTGCCCTGCTTCATGATGAACCCTACACTGACTTTGATGGGAC
AAGGGTGTACAGCCCCCCAGAGTGGATCTCTCGACACCAGTACCATGCACTCCCGGCCACTGTCTGGTCACTGGGC
ATCCTCCTCTATGACATGGTGTGTGGGGACATTCCCTTTGAGAGGGACCAGGAGATTCTGGAAGCTGAGCTCCACT
TCCCAGCCCATGTCTCCCCAGACTGCTGTGCCCTAATCCGCCGGTGCCTGGCCCCCAAACCTTCTTCCCGACCCTCAC
TGGAAGAGATCCTGCTGGACCCCTGGATGCAAACACCAGCCGAGGATGTACCCCTCAACCCCTCCAAAGGAGGCC
CTGCCCCTTTGGCCTGGTCCTTGCTACCCACGCGTACGCGGCCGCTCGAG - GFP Tag – GTTTAA

Primers used for PCR reaction:

Red = stop codon, blue = sequence complementary to plasmid DNA, green = SfiI restriction site

PIM2_Forward (initial Tm 64.2)

5´-AAAGGCCTCTGAGGCCACCATGTTGACCAAGCCTCTACAGG -3´

PIM2_Reverse (initial Tm 64.2, before reverse complementing)

5´-GCCTGGTCCTTGCTACCCTAAGGCCTGTCAGGCCAAG-3´

Table 3. Composition of PCR reaction.

Component	Stock concentration	Final	1 sample (µL)
5x Advantage Buffer (Mg2+)	5x	1x	5
Primer 1	10 pmol/µL	0,25 pmol/µL	0,6
Primer 2	10 pmol/µL	0,25 pmol/µL	0,6
dNTP	4x10 mM	200 µM	0,6
Advantage HD	2.5 U/µL	1x	0,2
plasmid DNA	1 ng/µL	1 ng	1
ddH2O		up to 25µL	17

Table 4. PCR program setting.

Cycles	Temperature (°C)	Time (s)
1	95	0:30
	98	0:15
30	56-64	0:15
	72	1:10
1	72	10:00
1	10	forever

4.5.3 DNA electrophoresis

To check a proper PCR amplification, DNA electrophoresis was performed using 1% agarose gel, 1x TEA buffer, Enduro™ Horizontal Gel Boxes (Labnet International) and 1x SYBR™ Safe (Invitrogen) dye to visualize the DNA. Samples were mixed with DNA Gel Loading Dye (ThermoFisher) prior to loading into wells in 5:1 ratio. To determine DNA fragment length, 1 kb DNA Ladder (Biosystem) was loaded into the first well. Electrophoresis ran in TAE buffer at constant 80 V and the DNA was visualized using UV-transluminator. In all but one well, a band of approximately 1 kb was clearly visible suggesting successful PCR reaction.

4.5.4 PCR product digestion

The PCR product was digested with DpnI enzyme (NEB) at 37°C for 1 h directly in PCR reaction mixture to digest and, therefore, eliminate the template methylated plasmid DNA.

4.5.5 PCR product purification

The PRC product was purified using PureLink™ PCR Purification Kit (Invitrogen) according to the manufacturer´s instructions.

4.5.6 PCR product ligation

The PCR product was cloned into pSC-B-Amp/Kan cloning vector using StrataClone Ultra Blunt PCR Cloning Kit (Agilent) according to the manufacturer´s instructions with vector to insert ratio being approximately 1:3.

4.5.7 Transforming competent bacterial cells

The vector DNA was introduced into StrataClone SoloPack competent cells (Agilent) by heat shock transformation. Competent cells were thawed on ice and the cloning reaction mixture was added. This transformation mixture was mixed gently and incubated on ice for 20 min. The bacteria were heat-shocked at 42°C for 45 seconds and then incubated on ice for 2 min. After 2 min, 700 μL of pre-warmed LB medium was added to the transformation mixture and the bacteria were left for 1 h at 37°C in shaking incubator to recover and to express the Ampicillin resistance. Finally, 100 μL of the bacterial suspension was transferred on agar selection plates with ampicillin and spread around. To allow blue-white screening of successful PCR insertion into the pSC Amp/Kan plasmid, 40 μL of gal solution (20 mg/ml) was spread on the plate before bacterial culture plating. The plates were incubated at 37°C overnight.

4.5.8 Plasmid DNA isolation – Miniprep protocol

Selected white bacterial colonies that grew on the agar plate were transferred into 5 ml of LB medium with ampicillin and incubated overnight at 37°C with agitation. The following day the plasmid DNA was isolated using QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer´s instructions. The plasmid DNA was eluted into H2O and the DNA concentration was measured by NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific).

4.5.9 Plasmid digestion

Plasmid DNA was digested with SfII enzyme (ThermoFisher) at 50°C for 1 h. The composition of reaction mix is listed in Table 5. Digested plasmid DNA was separated by agarose electrophoresis to check for the length of the insert. Two bands, one corresponding to plasmid backbone and one approximately 1 kb

long corresponding to PIM2 sequence, were seen in 5 out of 6 samples suggesting that the proper insert with correct restriction sites was present in these samples.

Table 5. Composition of SfII restriction mix.

Nuclease free water	15 µL
10x FastDigest Buffer	2 µL
Plasmid DNA	2 µL (1 µg)
FastDigest enzyme	1 µL
Total volume:	20 µL

4.5.10 DNA sequencing

Plasmid DNA was sequenced by CF OMICS facility in Biocev, using Sanger sequencing method and one plasmid was selected for further use.

4.5.11 DNA isolation from agarose gel

The selected plasmid was digested with SfII enzyme and separated by agarose electrophoresis according to the same protocol as previously. The 1 kb DNA fragment was cut out of the gel and isolated with GeneJET Gel Extraction Kit (ThermoFisher) according to the manufacturer´s instructions.

4.5.12 Ligation of PIM2 sequence into SB plasmid

The isolated DNA fragment was then ligated into pre-opened SB plasmid using T4 DNA Ligase (NEB). Ligation products were introduced into DH5a competent cell (TAKARA) according to the same heat-shock protocol as previously and bacteria were seeded on agar plates containing ampicillin.

4.5.13 Plasmid DNA isolation – Midiprep protocol

Selected colonies that grew on agar plate were transferred into ampicillin containing LB medium and grown overnight. Plasmid DNA was isolated using QIAGEN Plasmid Midi Kit (Qiagen).

4.6 Electroporation

Cells in optimal confluence (approximately 70%) in log phase of growth and in fresh medium were used for electroporation. On the day of electroporation, desired amount of plasmid DNA, listed in the Table 6 was transferred into a sterile 1.5 ml microtube. For each cell line, three reactions were made. For each reaction, the transposase coding pCMV(CAT)T7-SB100 vector was mixed with either the empty pSBtet-Pur vector, the PIM2 coding pSBtet-Pur vector or the mCardinal coding pSBtet-Pur vector.

Cells were counted and an aliquot containing 1.2 million of cells per one electroporation was transferred into a 15 ml tube. The cells were centrifuged at 300 g for 5 min, the supernatant was discarded and the

pellet was washed in 10 ml of PBS. The suspension was centrifuged again, the supernatant was discarded and the pellet was resuspended in 120 μL of resuspension buffer per electroporation reaction. Resuspended cells were transferred into the 1.5 ml tube containing the plasmid DNA mix.

Table 6. Plasmids used for electroporation reactions.

Transposase coding vector	Amount (μg)	Gene of interest	Amount (μg)
pCMV(CAT)T7-SB100	4	pSBtet Pur empty	6
pCMV(CAT)T7-SB100	4	pSBtet Pur mCardinal	6
pCMV(CAT)T7-SB100	4	pSBtet Pur PIM2	6

Cells were electroporated using Neon™ Transfection System (Invitrogen) according to the manufacturer´s instructions. Specific electroporation conditions for each cell line are listed in Table 7.

Table 7: **Electroporation conditions for JEKO1 and MINO cell line.**

Cell line	Pulse voltage (V)	Pulse length (ms)	Number of pulses	Buffer
MINO	1300	30	1	R*
JEKO1	1500	20	1	R

*R buffer was used as it is recommended for electroporation of established suspension cell lines by manufacturer

Four days after electroporation, puromycin was added at final concentration of 2 μg/mL and cells were selected for 8 to 14 days. In parallel, control WT cells were also treated with puromycin at final concentration 2 μg/mL and no cell growth was detectable after two weeks.

4.7 Proliferation assay and IC50 calculation

Proliferation was assessed using Quick Cell Proliferation colorimetric Assay Kit Plus (BioVision) as follows: 10 μL of WST solution was added to 90 μL of cell suspension and incubated for 3 h in 37°C. Absorbance was measured at 450 nm using Sunrise™ 96 well microplate reader (Tecan). DMSO was used as control in all experiment.

For the IC50 determination, $1*10^4$ to $3*10^4$ cells per well were seeded in final 300 μL of fresh medium. AZD1208 was dissolved in DMSO and applied to the cells in concentration ranging from 1 nM to 100 μM. Proliferation was assessed in 72 or 96 h. Relative viability was counted as the ratio of absorbance of AZD1208 treated versus control cells. Each concentration was tested in duplicate and at least three independent experiments were performed to construct a dose-response curve for each cell line. The half maximal inhibitory concentration (IC50) values were calculated using four-parameter dose-response curve fitting in GraphPad Prism Version 8 software (GraphPad software, San Diego, CA).

In experiment using combination of AZD1208 and Ibrutinib, $1*10^5$ cells per mL was seeded in cultivation flask. AZD1208, ibrutinib or DMSO was applied to the cells. After 48 h an aliquot for western blot analysis was taken and viability was measured after 72 h.

In experiments with doxycycline (DOX)-induced PIM2 expression doxycycline in final 100 ng/mL concentration was used. Cells were cultivated with or without doxycycline for 24 h, then $3*10^4$ cells per well were seeded, with or without doxycycline, on 96 well plate in final 300 µL of fresh medium. AZD1208, ibrutinib, idelalisib or DMSO was applied to the cells and absorbance was measured after 72 h. Relative viability was counted as the ratio of absorbance of treated versus control cells.

4.8 Apoptosis assay

Different stages of cell death are characterized by distinct biological changes within the cell. In early stages of apoptosis cell membrane is intact but loses its asymmetry. Lipid phosphatidylserine is translocated from the inner to the outer layer of plasma membrane. Annexin V is a protein that has high affinity for negatively charged phosphatidylserine in the cell membrane. Annexin V conjugated to fluorescent protein can thus mark apoptotic cells and be detected by flow cytometry.

While early apoptotic cells still possess intact plasma membrane, late apoptotic and necrotic cells lose integrity of the membrane allowing extracellular molecules to enter the cell. Propidium iodide (PI) is fluorescent DNA intercalating agent which is excluded from living cells but can enter cells whose membrane integrity is lost. Thus, apoptotic ($AnnexinV^+PI^+$) cells can be distinguished from viable ($AnnexinV^-PI^-$) and necrotic ($AnnexinV^-PI^+$) cells.

For apoptotic assay, 100 000 cells/well was seeded on 96 well plate in a final 300 µL of fresh medium. Medium was supplemented with AZD1208, ATB199 or with combination of both. Vehicle (DMSO) was used as a control. Cells were incubated for 24 h, then the plate was centrifuged and medium was removed. Cells were washed with 300 µL of annexin binding buffer (Apronex), centrifuged and the buffer was discarded. 100 µL of Annexin binding buffer with 0.5 µL AnnexinV-FITC (Apronex) per well was added. Following 15 min of incubation in dark on ice, 200 µL of Annexin binding buffer with PI in final concentration 2 µg/ml was added. The fluorescence was measured by BD FACSCanto™ II using 488 nm blue laser for excitation and BA1 780/60 nm or BE4 530/30 nm filter for PI or AnnexinV-FITC detection respectively. The data was analyzed in FlowJo® X 10.0.7r2 analysis software.

4.9 Statistical analysis

Statistical analysis was performed in GraphPad Prism software (version 8). Unpaired t-test was used to determine p values. $P < 0.05$ was considered to indicate statistical significance. Because of small number of repetitions (3 or 4 in all experiments) data normality could not be determined but was assumed in accordance with common practice (Horiuchi et al., 2016; Le et al., 2016; Zemskova et al., 2008)

5 Results

5.1 MCL, ABC and GCB-DLBCL cell lines are differentially sensitive to pan-PIM kinase inhibition with AZD1208

We analyzed the effect of pan-PIM kinase inhibitor AZD1208 on a panel of 7 MCL, 6 ABC-DLBCL and 8 GCB-DLBCL cell lines (Figure 3). In contrast to GCB-DLBCL, ABC-DLBCL and MCL cells express high levels of PIM1 and PIM2 mRNA (Alizadeh et al., 2000; Care et al., 2013; Cohen et al., 2004; Gómez-Abad et al., 2011; Peters et al., 2016; Wright et al., 2003). As activity of PIM kinases should depend almost exclusively on the level of their protein expression (Bullock et al., 2009; Fox et al., 2003; Kumar et al., 2005; Qian et al., 2005; Zhu et al., 2002a) we assumed that the differences in mRNA and protein expression should correlate with sensitivity to PIM kinase inhibition.

Lymphoma cells were treated with DMSO or increasing concentrations of AZD1208 in vitro and cell viability was measured after 72 or 96 hours using a standard WST-8 based cell proliferation assay. IC50 for each cell line was calculated from a dose-response curve. In all but one (GRANTA519) tested cell lines AZD1208 reduced cell viability in a dose dependent manner. In concordance with previously published data (Peters et al., 2016), ABC-DLBCL cell lines were more sensitive to PIM kinase inhibitor (mean IC50 = 4.2) compared to GCB-DLBCL (mean IC50 = 13.0). Interestingly, MCL cell lines were even more sensitive than ABC-DLBCL (mean IC50 = 1.6) (Figure 3). IC50 for GRANTA519 was not determined.

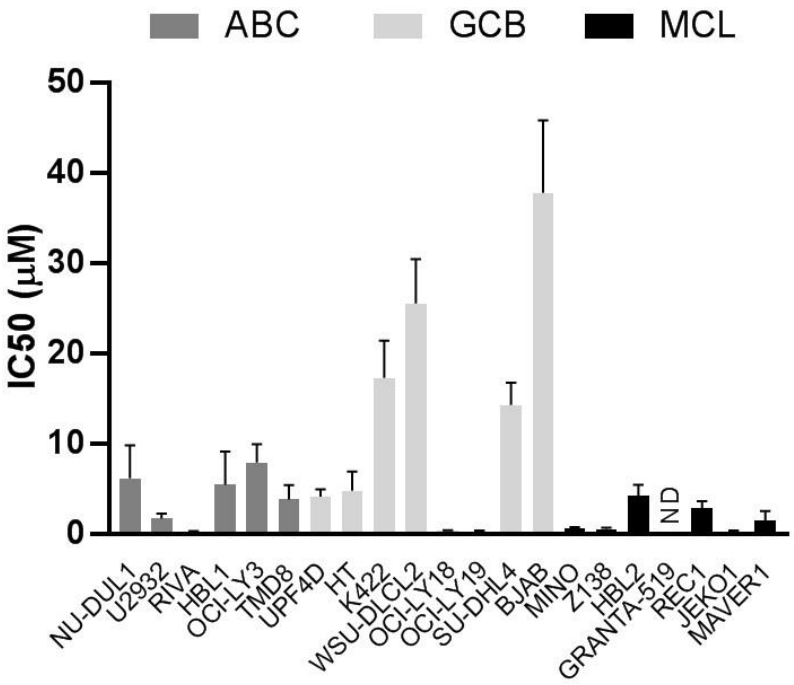


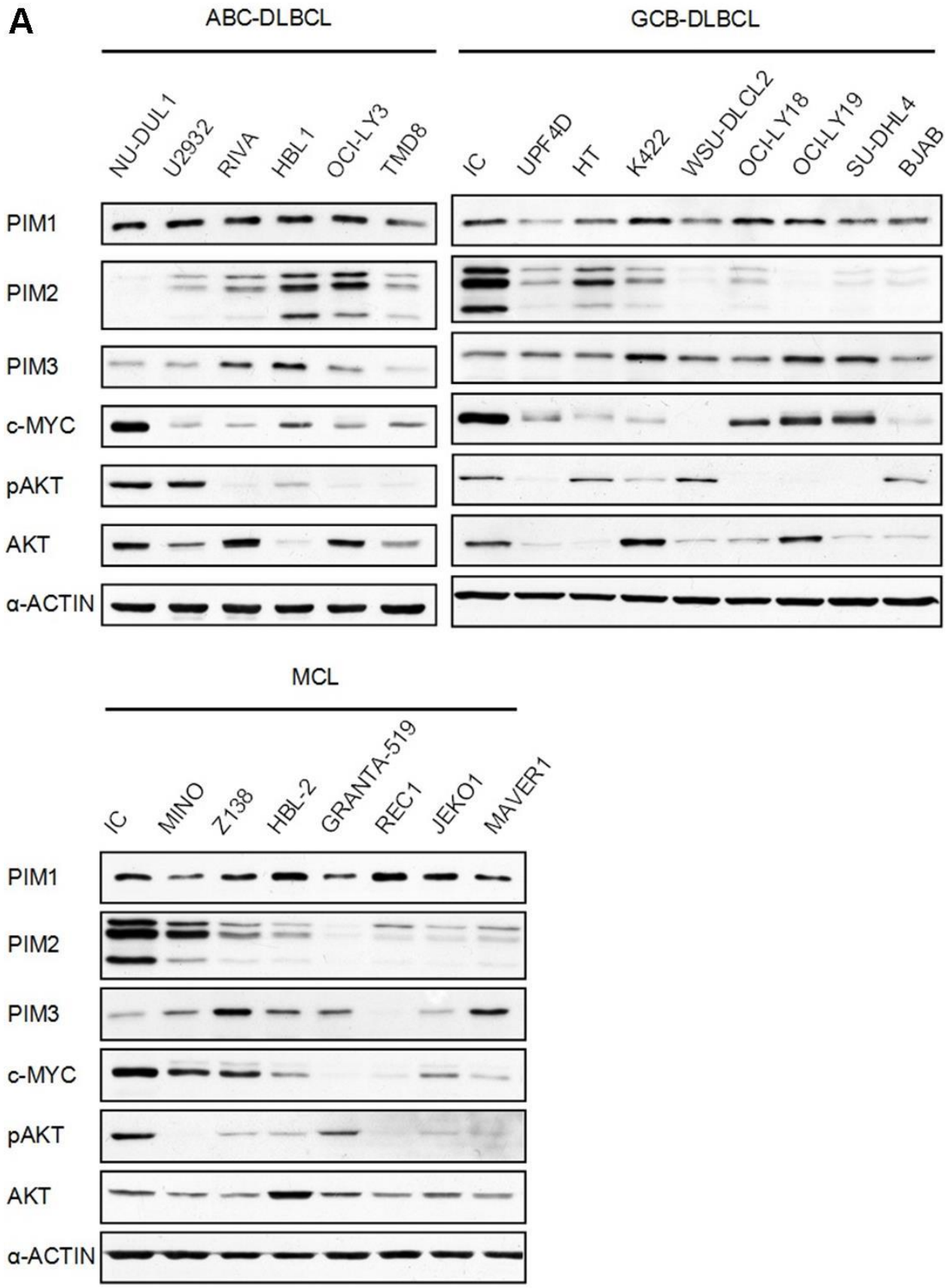
Figure 3. PIM inhibition with AZD1208 in lymphoma cell lines
IC50 values for ABC, GCB-DLBCL and MCL cell lines (y axis). All experiments were done in duplicates; IC50 for each cell line was calculated from at least three independent experiments using non-linear curve fit regression in GraphPad Prism software. Shown is the mean IC50 for each lymphoma cell line ±SD. ND = not determined.

5.2 Sensitivity correlation with PIM, c-MYC or AKT protein expression

Though the difference in *PIM1* and *PIM2* gene expression between MCL, ABC-DLBCL and GCB-DLBCL was confirmed by several groups, immunohistochemistry of patient tumors samples showed that the difference in mRNA levels might not be conserved on protein level (Brault et al., 2012; Gómez-Abad et al., 2011). We analyzed the panel of lymphoma cell lines for expression of PIM kinases by western blotting (Figure 4A). We wanted to see whether PIM protein expression would correlate with the reported differential gene expression between ABC and GCB-DLBCL. Importantly, because PIM kinases are constitutively active and are not regulated by phosphorylation, we hypothesized that sensitivity of lymphoma cell lines to PIM inhibition would correlate with the level of PIM protein expression. Lysates from untreated cells were probed for PIM1, PIM2 and PIM3 protein and band intensities were measured by densitometry. PIM2 protein was expressed at higher levels in ABC compared to GCB-DLBCL cells while no difference in expression of PIM3 was observed. PIM1 was differentially expressed between both DLBCL subtypes but the diffidence was rather in variability of PIM1 expression level. PIM kinase expression pattern of MCL cell lines was similar to that of GCB-DLBCL (Figure 4A-4B).

Sensitivity of MCL and DLBCL cell lines to AZD1208 was correlated with expression of the three PIM kinases using Pearson correlation. Sensitivity of DLBCL cell lines did not correlate with expression of any of PIM kinases. In contrast, sensitivity of MCL cell lines was negatively correlated with PIM1 protein expression (Figure 4C).

It was shown that *PIM* kinases and *c-MYC* cooperate in lymphomagenesis (van Lohuizen et al., 1989; Scheijen et al., 1997; Shirogane et al., 1999; Wang et al., 2010a) and that in breast cancer high c-MYC expression correlates with sensitivity of cell lines to PIM inhibition (Horiuchi et al., 2016; Keeton et al., 2014). Moreover, PI3K-AKT pathway that integrates prosurvival signaling from B-cell receptor and other key cell surface receptors, functions in parallel with PIM kinases (Hammerman et al., 2005). We thus hypothesized that expression of c-MYC and/or AKT would correlate with AZD1208 sensitivity. In our study we analyzed expression and activity of c-MYC and AKT in the panel of the tested lymphoma cell lines. Activity of AKT was measured by Ser473 phosphorylation (rewieved in Hemmings and Restuccia, 2012). Band intensity was measured by densitometry and Pearson correlation was used to evaluate relationship with sensitivity to AZD1208. In contrast to breast cancer, level of expression of c-MYC did not correlate with sensitivity of lymphoma cells to AZD1208. The same results were obtained for AKT or pAKT expression.


A
ABC-DLBCL
GCB-DLBCL
NU-DUL1
U2932
RIVA
HBL1
OCI-LY3
TMD8
IC
UPF4D
HT
K422
WSU-DLCL2
OCI-LY18
OCI-LY19
SU-DHL4
BJAB
PIM1
PIM2
PIM3
c-MYC
pAKT
AKT
α-ACTIN
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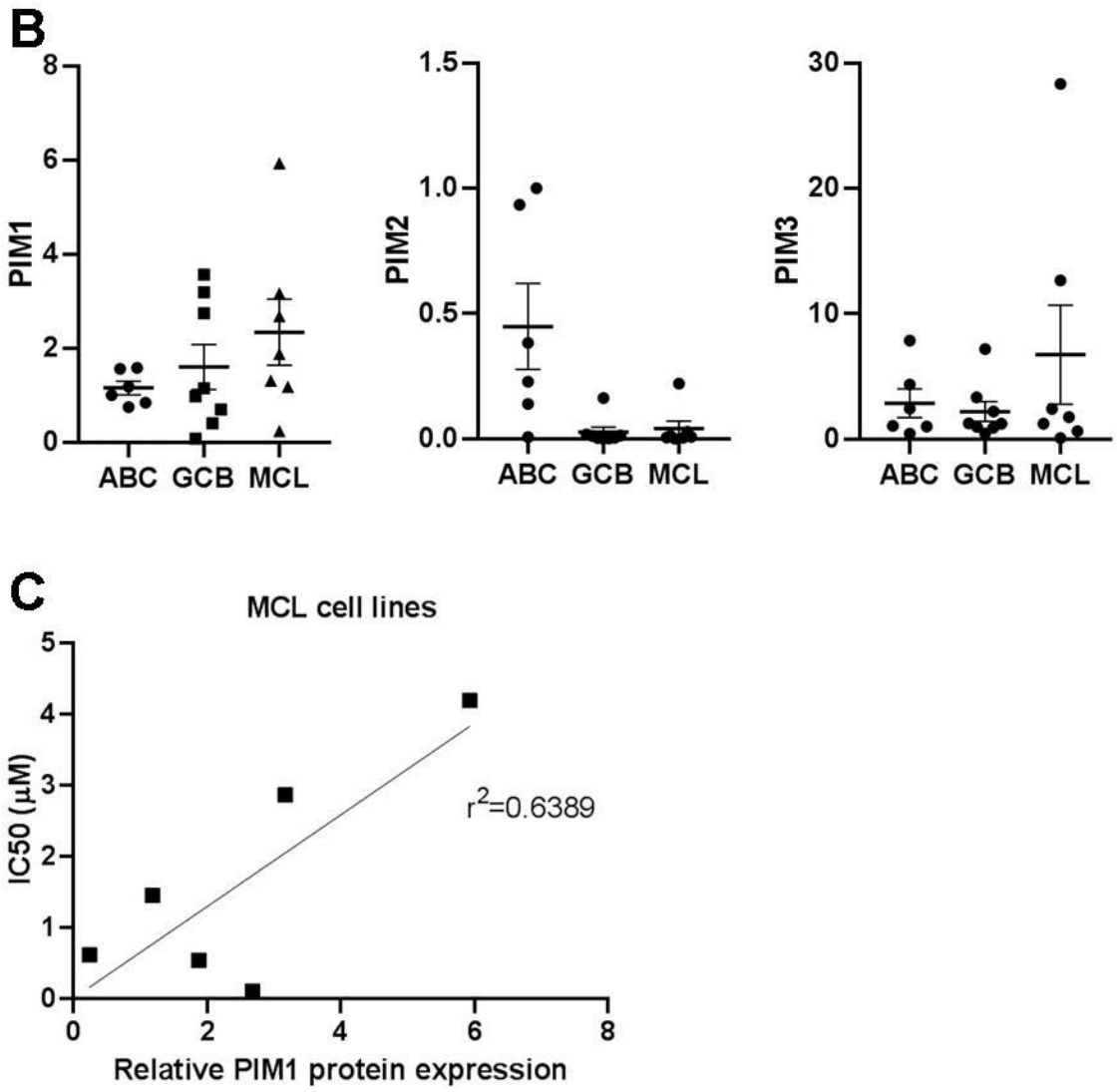


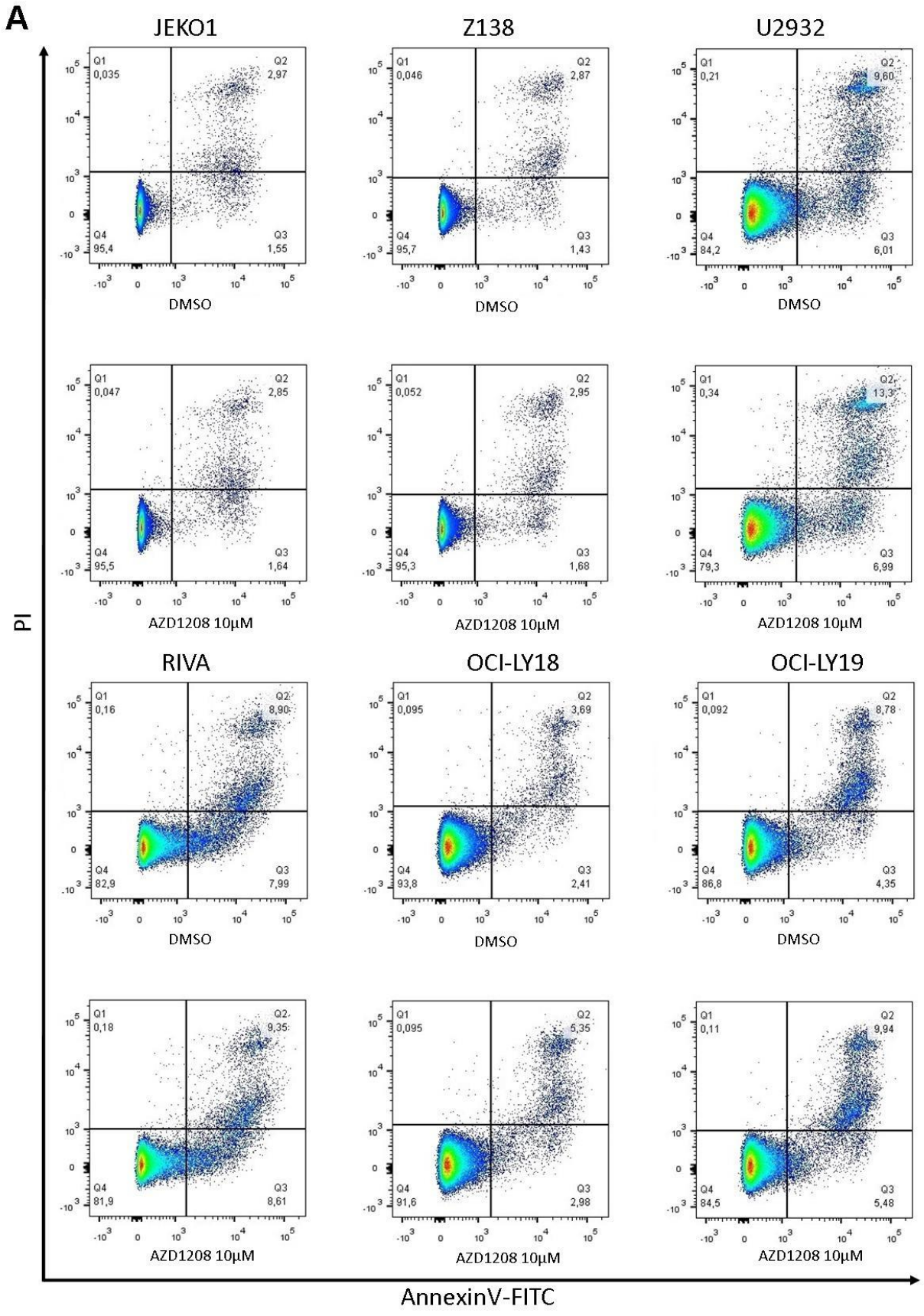
Figure 4. Screen for protein expression and sensitivity of lymphoma cell lines

(A) Analysis of protein expression in ABC-DLBCL, GCB-DLBCL and MCL cell lines. A representative example of two independent experiments is shown. IC (interassay control) = HBL1 for PIM2 and NU-DUL1 for the rest of proteins). pAKT = AKT phosphorylated on Ser473. (B) Band densities were analyzed in Quantity One software and normalized to actin loading control from the same gel. Normalized densities were than compared with expression of interassay control, to express relative protein expression. Each dot indicates single cell line; y axis shows protein expression relative to IC. Shown is mean relative expression of each lymphoma subtype ±SD. No image adjustment was made prior to densitometry analysis. Relative expression for each cell line was calculated as mean of two independent experiments. (C) PIM1 protein level is linearly correlated with AZD1208 sensitivity in MCL cell lines. Spearman correlation was applied to analyze the relationship between the relative PIM1 protein expression and AZD1208 sensitivity expressed as IC50.

5.3 AZD1208 has limited proapoptotic activity in lymphoma cell lines and does not augment death-inducing activity of BCL2 inhibitor ABT-199/venetoclax

PIM kinases regulate apoptosis on multiple levels and their inhibition was shown to induce cell death in both, solid and hematopoietic cancers (Chen et al., 2009b; Horiuchi et al., 2016; Keeton et al., 2014; Kirschner et al., 2015; Szydłowski et al., 2017). We measured cellular apoptosis following AZD1208 treatment by Annexin V-FITC and PI staining in six most sensitive lymphoma cell lines including JEKO1 and Z138 (MCL), RIVA and U2932 (ABC-DLBCL), and OCI-LY18 and OCI-LY19 (GCB-DLBCL) (Figure 5). AZD1208 was not able to induce any significant apoptosis in any cell line tested (Figure 5A).

The inability of AZD1208 to induce apoptosis in lymphoma cell lines, not even at the concentration high above their calculated IC50, was surprising but similar results were reported for multiple myeloma (MM). Inhibition of PIM kinases reduced proliferation of MM cell lines, but did not trigger significant cell death, so it was hypothesized that in MM PIM are not essential for survival of unstimulated MM cells, but that they exert antiapoptotic function in the presence of death promoting stimuli (Lu et al., 2013). This is supported by the finding that PIM inhibition can sensitize cancer cell to chemotherapeutical agents (Kirschner et al., 2015; Natarajan et al., 2013; Song and Kraft, 2012; Xie et al., 2010). Targeted inhibition of BCL2 with ABT-199/venetoclax represents a novel, highly effective approach to selectively eliminate leukemia and lymphoma cells that (over)express BCL2 protein. ABT-199/venetoclax is currently used to treat patients with chronic lymphocytic leukemia and mantle cell lymphoma, and is clinically tested in relapsed/refractory NHL including DLBCL (Davids et al., 2017). Recently, PIM kinases were shown to mediate resistance to BCL-2 inhibition via MCL-1 (Song and Kraft, 2012). In our study, we treated lymphoma cell lines with AZD1208 in combination with BCL-2 inhibitor ABT-199/venetoclax (Figure 5B). Three ABT-199/venetoclax sensitive and three resistant cell lines were treated with AZD1208 and ABT-199/venetoclax, single-agent or in combination, to evaluate their potential cytotoxic synergy. In one MCL and both GCB-DLBCL cell lines neither AZD1208 nor ABT-199/venetoclax did induce any significant response and combination of both was not able to overcome this resistance. In OCI-LY3, TMD8 and MINO cell line ABT-199 induced cell death to 43, 29 and 49% respectively, but inhibition of PIM kinases did not increase this effect.


A
JEKO1
Z138
U2932
Q1 0,035
Q2 2,97
Q4 95,4
Q3 1,55
DMSO
Q1 0,046
Q2 2,87
Q4 95,7
Q3 1,43
DMSO
Q1 0,21
Q2 9,60
Q4 84,2
Q3 6,01
DMSO
Q1 0,047
Q2 2,85
Q4 95,5
Q3 1,64
AZD1208 10µM
Q1 0,052
Q2 2,95
Q4 95,3
Q3 1,68
AZD1208 10µM
Q1 0,34
Q2 13,3
Q4 79,3
Q3 6,99
AZD1208 10µM
RIVA
OCI-LY18
OCI-LY19
Q1 0,16
Q2 8,90
Q4 82,9
Q3 7,99
DMSO
Q1 0,095
Q2 3,69
Q4 93,8
Q3 2,41
DMSO
Q1 0,092
Q2 8,78
Q4 86,8
Q3 4,35
DMSO
Q1 0,18
Q2 9,35
Q4 81,9
Q3 8,61
AZD1208 10µM
Q1 0,095
Q2 5,35
Q4 91,6
Q3 2,98
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Q3 5,48
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PI
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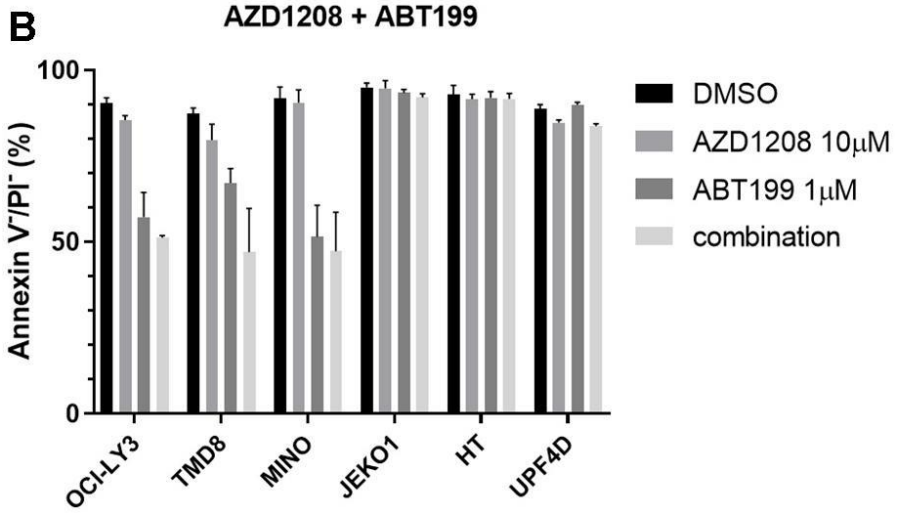

Figure 5. Inhibition of PIM kinases alone or in combination with BCL-2 inhibitor ABT-199 does not induce cell death in MCL and DLBCL cell lines
(A) Six lymphoma cell lines were treated with 10 μM AZD1208 or vehicle (DMSO) for 24 hours. Cells were stained with Annexin V-FITC and PI and analyzed by flow cytometry. Each experiment was performed in duplicates. Shown is representative of three independent experiments. (B) Six lymphoma cell lines were treated with 10 μM AZD1208, 1 μM ABT-199, with combination of both or with vehicle (DMSO) for 24 hours. Cells were stained with Annexin V-FITC and PI and analyzed by flow cytometry. Each experiment was performed in duplicates. Shown is mean ±SD of three independent experiments.

5.4 Combination of AZD and Ibrutinib

Prosurvival signaling from B-cell receptor (BCR) belongs to key signals indispensable for survival of malignant B-cells including MCL and ABC-DLBCL (reviewed in Raedler, 2015). Inhibition of BTK, a key mediator of BCR signaling, with ibrutinib, belongs to milestones of targeted therapy in hematooncology. Based on its excellent anti-tumor activity ibrutinib was approved for the therapy of patients with relapsed chronic lymphocytic leukemia and mantle cell lymphoma. Increased PIM kinase activity was reported in ibrutinib-resistant malignant B-cells (Kuo et al., 2016) suggesting that increased prosurvival signaling from PIM kinases might compensate for the inhibited prosurvival signaling from BCR. We proposed that concurrent blockage of BCR signaling with ibrutinib and PIM kinases with AZD1208 might induce cytotoxic synergy in MCL.

Cells were treated with AZD1208 (1 μM), ibrutinib (1 μM) or the combination of both agents for 72 hours and cell viability was measured with WST8-based cell proliferation assay. Combination of AZD1208 and ibrutinib reducer cell viability significantly more than either compound alone (Figure 6A). In all cell lines except for JEKO1, the combination of AZD1208 with ibrutinib reduced viability to greater extent than was

a simple sum of effects of each compound alone (Figure 6B). In JEKO1 cells, both compounds at the tested concentrations reduced viability more than 50% so the synergistic effect could not be determined.

To gain insight into the molecular mechanisms behind these observations, we analyzed the effects of both compounds on expression of their target proteins by western blotting (Figure 6C). First, we examined the expression of PIM proteins. Exposure to AZD1208 increased PIM3 and PIM2 protein in both cell lines. Exposure to ibrutinib increased PIM2 protein expression in REC1 cells, but reduced PIM2 in JEKO1 cells. Exposure to both AZD1208 and ibrutinib resulted in significant increase in PIM2 expression.

Next, we assessed expression and phosphorylation status of PIM target protein BAD. BAD is phosphorylated on Ser112 by all three PIM kinases (Macdonald et al., 2006) and AZD1208 inhibited BAD phosphorylation in both cell lines thereby confirming an effective inhibition of PIM kinase activity despite their increased expression after exposure to AZD1208.

AKT belongs to established downstream targets of BTK (Craxton et al., 1999; Kitaura et al., 2000). Inhibition of BTK with ibrutinib reduced AKT phosphorylation in JEKO1 cells. Unexpectedly, in REC1 cells, exposure to ibrutinib significantly increased AKT phosphorylation.

Finally, we analyzed the impact of AZD1208 and ibrutinib on cap-dependent translation. Protein translation was shown to be a key target of PIM inhibitors and it mediates survival of lymphoma cells via expression of several pro-survival proteins including c-MYC and MCL1 (Schatz et al., 2011; Yang et al., 2012). Western blots were probed for total and phosphorylated 4E-BP1, a known regulator of cap-dependent translation regulated by AKT and PIM2 (Fox et al., 2003; Schatz et al., 2011; Tamburini et al., 2009). This protein was hyperphosphorylated in both cell lines and while ibrutinib had only minimal effect on 4E-BP1 phosphorylation, treatment with AZD1208 reduced p4E-BP1 in both cell lines. Importantly, combination of AZD1208 and ibrutinib reduced phosphorylation of 4E-BP1 to greater extent than did AZD1208 alone in both cell lines but this effect was much more prominent in JEKO1 cells. The decrease of 4E-BP1 phosphorylation accompanied by decrease of MCL1 protein in JEKO1 cells, but there was only minimal decrease of MCL1 level in REC1 cells only after treatment with both compounds together. c-MYC expression, however, was changed in neither cell line.

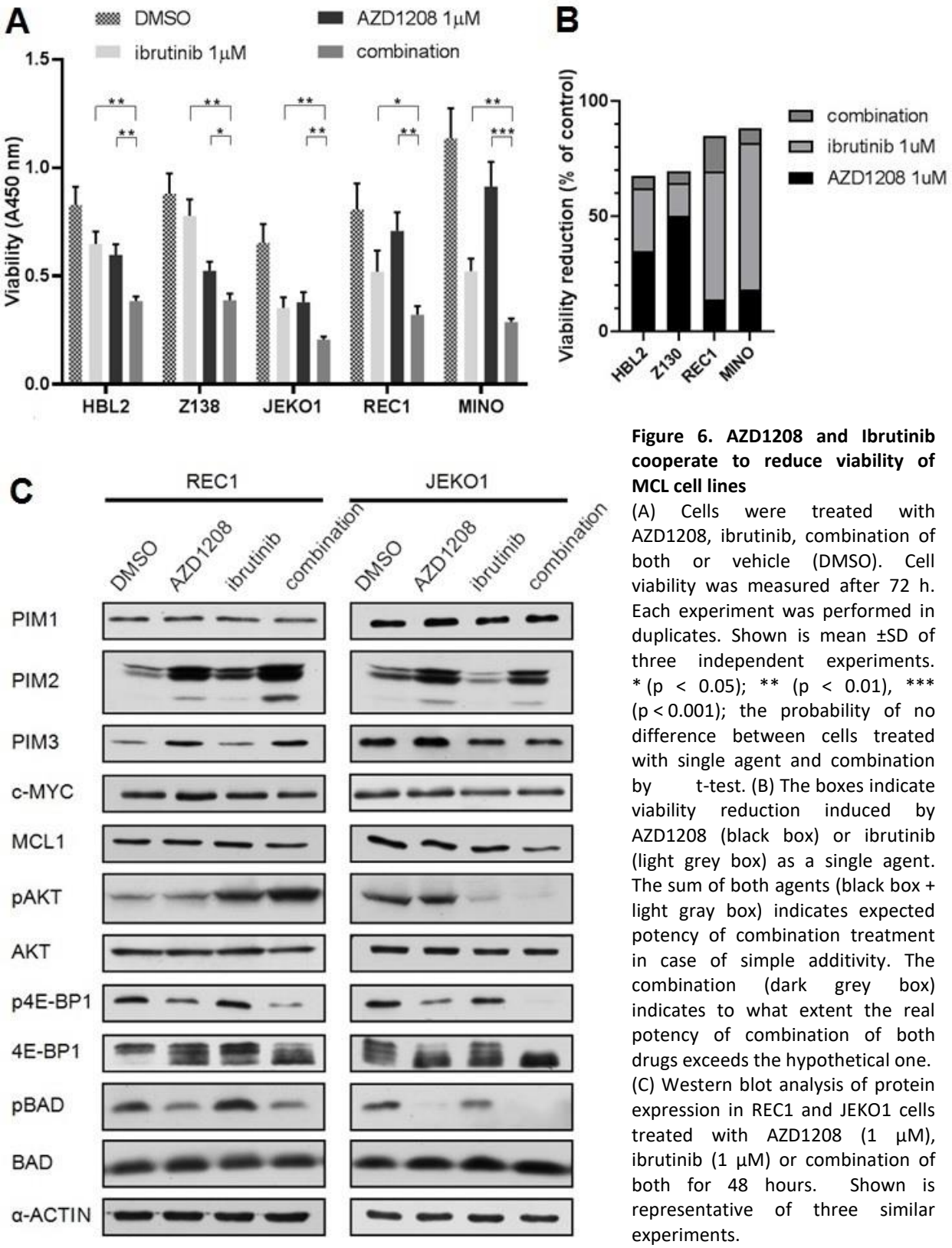

Figure 6. AZD1208 and Ibrutinib cooperate to reduce viability of MCL cell lines

(A) Cells were treated with AZD1208, ibrutinib, combination of both or vehicle (DMSO). Cell viability was measured after 72 h. Each experiment was performed in duplicates. Shown is mean ±SD of three independent experiments. * ($p < 0.05$); ** ($p < 0.01$), *** ($p < 0.001$); the probability of no difference between cells treated with single agent and combination by t-test. (B) The boxes indicate viability reduction induced by AZD1208 (black box) or ibrutinib (light grey box) as a single agent. The sum of both agents (black box + light gray box) indicates expected potency of combination treatment in case of simple additivity. The combination (dark grey box) indicates to what extent the real potency of combination of both drugs exceeds the hypothetical one. (C) Western blot analysis of protein expression in REC1 and JEKO1 cells treated with AZD1208 (1 μM), ibrutinib (1 μM) or combination of both for 48 hours. Shown is representative of three similar experiments.

5.5 Overexpression of PIM2 34 kDa isoform

PIM2 is critical for B-cell homeostasis (Woodland et al., 2008) and is more strongly associated with pathways important for B-cell lymphomagenesis than PIM1 or PIM3 (Gómez-Abad et al., 2011). Moreover, cap-dependent translation is regulated by PIM2 and PIM2 is an important mediator of survival in hematological malignancies (Hammerman et al., 2005; Schatz et al., 2011; Yang et al., 2012). Moreover, the cytotoxic synergy between AZD1208 and ibrutinib was accompanied by marked overexpression of PIM2 isoform. This led us to investigate the role of PIM2 overexpression in sensitivity of MCL cell lines to anticancer drugs. We generated JEKO1/PIM2 and MINO/PIM2 cells that express the shortest (34 kDa) PIM2 isoform in a doxycycline-inducible manner. We also generated JEKO1/empty and MINO/empty cells expressing empty vector as a control.

First, we analyzed lysates from these cells to confirm PIM2 protein expression and kinase activity (Figure 7A). There was a marked overexpression of the 34 kDa isoform in both cell lines accompanied by significant increase in phosphorylation of BAD od Ser112, a known pharmacodynamic target of this PIM2 isoform (Yan et al., 2003). There was, however, no change in phosphorylation of 4E-BP1 another PIM target (Fox et al., 2003; Schatz et al., 2011; Tamburini et al., 2009) which was affected by PIM inhibition.

Next, we tested effect of doxycycline and doxycycline-induced PIM2 expression on viability of the two cell lines (Figure 7B). Neither doxycycline nor doxycycline-induced PIM2 expression had any effect on viability of JEKO1/PIM2 and JEKO1/empty compared to WT or doxycycline untreated cells. In MINO cells however, doxycycline reduced viability of MINO/empty compared untreated and WT cells, but expression of PIM2 reduced this effect.

Finally, we tested the effect of PIM2 overexpression on sensitivity of the cells to three anticancer drugs – AZD1208, ibrutinib and idelalisib (Figure 7C). Idelalisib is a PI3Kδ inhibitor that is approved for treatment of patients with relapsed CLL, follicular lymphoma and small lymphocytic lymphoma (Raedler, 2015). Cells were treated with ibrutinib (1 μM), AZD1208 (10 and 1 μM), idelalisib (10 and 1 μM) or combination. Combination of AZD1208 with idelalisib exerted similar cooperation as combination with ibrutinib. There was however, no difference in sensitivity to any of these compounds between empty vector or PIM2 expressing cells.

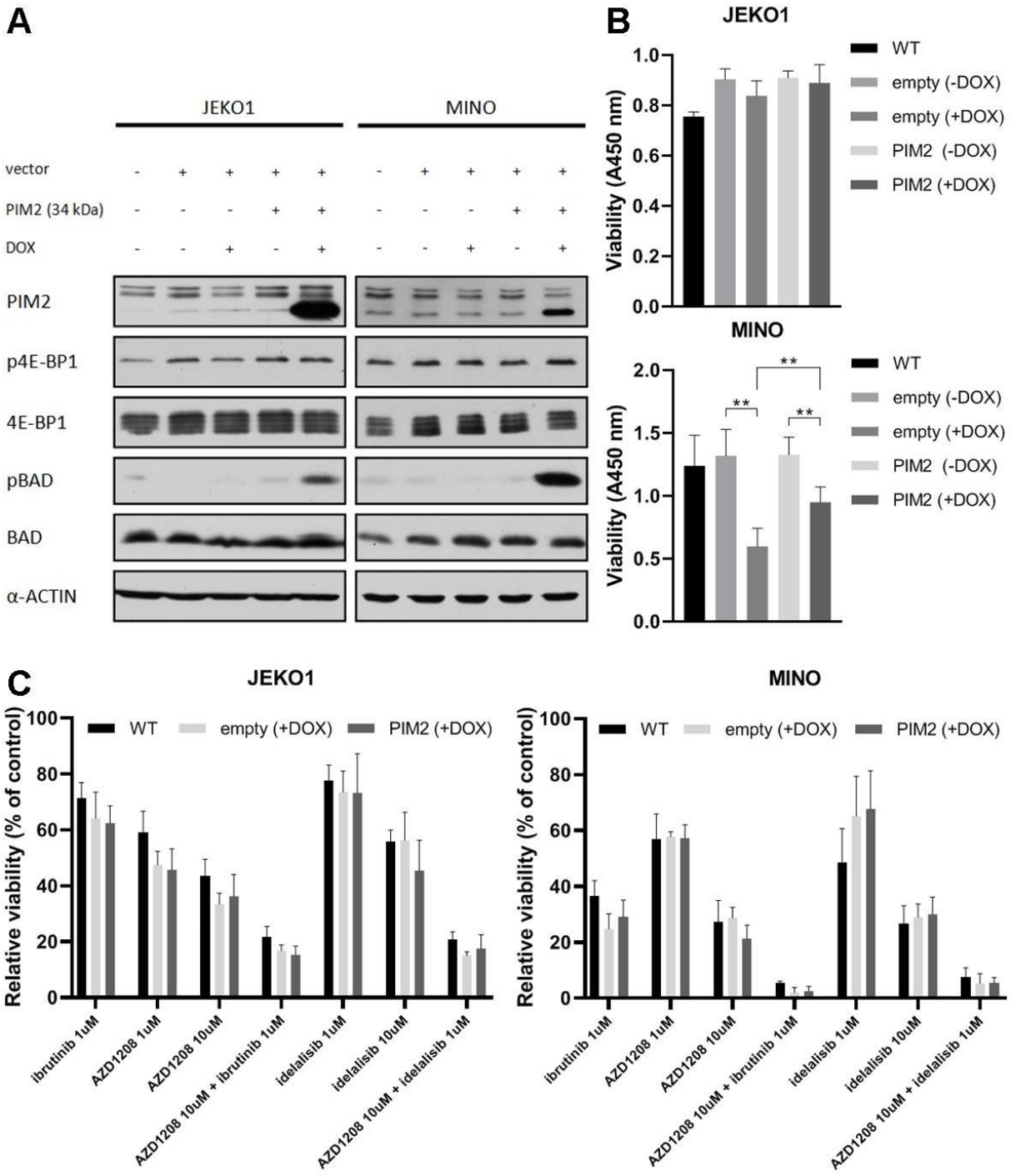

Figure 7. Overexpression of 34 kDa PIM2 isoform does not alter sensitivity of MCL cells to anticancer drugs
(A) JEKO1 and MINO cells containing empty or PIM2 expressing vector were incubated with or without DOX for 72h and protein expression was analyzed compared to WT cells (firs line). Shown is representative of three similar experiments. (B) JEKO1 and MINO cells containing empty or PIM2 vector were incubated with or without DOX for 24h to induce PIM2 expression, and then seeded on 96 well plates with or without DOX. Viability was measured after 72h with WST-8 assay. Shown is mean ±SD of four independent experiments. ** ($p<0.01$), the probability of no difference between viability of cells by t-test. (C) JEKO1 and MINO cells containing empty or PIM2 vector were incubated with DOX for 24 hours, than seeded for experiment in medium containing DOX. All cells were treated

with AZD1208, ibrutinib, idelalisib, combination of drugs or vehicle (DMSO) for 72h. Cell viability was measured with WST-8 assay. Relative viability was calculated as ratio of treated versus control cells. Shown is mean ±SD of three independent experiments.

6 Discussion

6.1 MCL, ABC and GCB-DLBCL cell lines are differentially sensitive to pan-PIM kinase inhibition with AZD1208

The association of PIM kinases with an aggressive behavior in many cancers including MCL (blastoid variant) and DLBCL (ABC subtype) (Alizadeh et al., 2000; Hsi et al., 2008; De Vos et al., 2003; Wright et al., 2003) and the fact that their inhibition induced apoptosis in ABC-DLBCL (Gómez-Abad et al., 2011) and MCL cells (Yang et al., 2012) imply that PIM kinases play an important role in both lymphomas. We therefore expected MCL and ABC-DLBCL cell lines to be sensitive to PIM inhibitor. Our results show that while ABC-DLBCL and MCL cell are sensitive to AZD1208 with mean IC50 < 10 µM, a group of GCB cell lines exists that is similarly sensitive to ABC-DLBCL cell lines but that there are also cell lines which are much less sensitive. Similar results were obtained by another group using different pan-PIM inhibitor PIM447 (Peters et al., 2016). These results suggest that while ABC-DLBCL cell lines respond homogenously to AZD1208, a great variance exists among GCB-DLBCL that does not seem to correspond to the uniformly low expression of *PIM1* and *PIM2* gene in this subtype (Alizadeh et al., 2000; Care et al., 2013; Cohen et al., 2004; Gómez-Abad et al., 2011; Peters et al., 2016; Wright et al., 2003). *PIM1* is mutated more frequently but not exclusively in ABC-DLBCL compared to GCB-DLBCL and some of these mutations were shown to increase PIM1 stability and to confer resistance to chemotherapeutical drug ibrutinib (Karube et al., 2018; Kuo et al., 2016). It was thus possible that the heterogeneity among GCB-DLBCL cell lines was caused by mutations in *PIM1* or *PIM2* gene that lead to stabilization of PIM protein with mRNA levels unchanged. Thus, GCB-DLBCL cell lines sensitive to AZD1208 would be those whose pathogenesis is driven, at least partly, by expression of mutated, aberrantly stable PIM1 or PIM2 protein. This was however disproved by our finding, that there was no correlation between the level of PIM1 or PIM2 protein expression and sensitivity to PIM inhibition.

MCL cell lines were even more sensitive than ABC-DLBCL cells with all the cell lines tested having IC50 < 5 µM. IC50 for GRANTA-519 was not determined. These cells show only limited dose-dependent response to AZD1208. Moreover, this cell line is positive for Epstein-Barr virus. EBV positivity was show to affect tumorigenic potential and behavior of the cells and was proposed to perplex interpretation of results acquired with the use of EBV-positive MCL cell lines (reviewed in Salaverria et al., 2006).

6.2 Sensitivity correlation with PIM, c-MYC or AKT protein expression

The panel of lymphoma cell lines was analyzed for PIM1, PIM2 and PIM3 protein expression. PIM3 was expressed almost equally between all three lymphoma subtypes. This was not surprising as *PIM3* gene was not shown to be overexpressed in MCL or DLBCL patients and as both the protein and the gene are equally expressed in all DLBCL cell lines and patients (Brault et al., 2012; Gómez-Abad et al., 2011; Peters et al., 2016). It is further consistent with PIM3 being more connected to solid cancers than hematopoietic malignancies and to be associated with completely different pathways than PIM1 and PIM2 (Fujii et al., 2005; Li et al., 2006; Popivanova et al., 2007; Yang et al., 2005; Zheng et al., 2008).

Higher levels of PIM2 on the other hand were detected in ABC-DLBCL compared to GCB-DLBCL cells. This was also not surprising, given the differential *PIM2* gene expression in both subtypes (Alizadeh et al., 2000; Care et al., 2013; Cohen et al., 2004; Gómez-Abad et al., 2011; Peters et al., 2016; Wright et al., 2003) and that tight correlation between PIM2 mRNA and protein level exists in DLBCL cell lines (Gómez-Abad et al., 2011).

With respect to PIM1 protein, both DLBCL subtypes seemed to rather differ in the variability than in overall level of PIM1 expression. Our results suggest, however, slightly higher expression of PIM1 in the GCB subtype. Our finding thus supports the theory that the clear difference between ABC and GCB-DLBCL in *PIM1* and *PIM2* gene expression is not so well conserved on a protein level, at least in the case of PIM1. The difference in sensitivity to PIM inhibition between both subtypes that was shown by another group and by our data, thus most likely isn't a simple consequence of differential expression of both *PIM* genes. First, there is no significant difference in PIM1 protein expression between both DLBCL subtypes in patients (Brault et al., 2012; Gómez-Abad et al., 2011) and cell lines (our data). Second, there is no correlation between expression of any of PIM kinases and AZD1208 sensitivity in DLBCL cell lines.

The absence of significant correlation between PIM protein level and AZD1208 sensitivity is rather surprising given that a) activity of PIM kinases is believed to be regulated exclusively by their protein expression level (Fox et al., 2003; Zhu et al., 2002a), b) their tumorigenic potential was shown to be dose-dependent (Allen and Berns, 1996; van der Lugt et al., 1995), c) in triple negative breast cancers that express, similarly to DLBCL, high levels of PIM1 and c-MYC such correlation exists (Horiuchi et al., 2016). This conundrum can be at least partially explained by the following findings. First, *PIM1* gene is a target of aberrant somatic hypermutation in DLBCL (Pasqualucci et al., 2001) and mutations of *PIM1* found in DLBCL patients were shown to both increase and decrease PIM1 kinase activity (Kumar et al., 2005). Thus, PIM1 protein level does not have to directly reflect PIM1 kinase activity. Next it was

suggested that PIM kinases possess some kinase-independent function in lymphoma (Kreuz et al., 2015; Kumar et al., 2005). Hence simple inhibition of their kinase activity would not be sufficient to prevent all their biological functions in the lymphoma cell. And last, it is possible that subcellular localization of PIM kinases plays more important role in their pathogenesis rather than the level of the protein expression. Localization of PIM kinases is different in malignant and benign tissue (Xie et al., 2005) and while PIM1 protein is expressed with almost the same frequency in both DLBCL subtypes, it predominantly localizes to the nucleus in ABC-DLBCL cells and its nuclear localization correlates with disease progression (Brault et al., 2012). Thus, the sensitivity of DLBCL to inhibition of PIM-signaling seems to depend on multiple factors that differentially affect PIM protein function and localization.

MCL is mostly derived from naive B-cells. These cells did not undergo process of somatic hypermutation and therefore possess unmutated or minimally mutated variable region of immunoglobulin genes. Concordantly, *PIM1* mutations that were shown to be mediated by aberrant somatic hypermutation in almost half of DLBCL cases are not present in MCL patients (Pasqualucci et al., 2001). It is thus likely that PIM1 protein expressed in MCL is not mutated and the level of PIM1 protein more closely reflects PIM1 kinase activity. There was indeed, correlation of PIM1 protein with AZD1208 sensitivity in MCL cell lines. There was no such correlation for PIM2, which could mean that PIM1 plays more important role in MCL than PIM2.

Furthermore, because PIM kinases were shown to cooperate with *c-MYC* in lymphomagenesis (Allen et al., 1997; van Lohuizen et al., 1989, 1991; van der Lugt et al., 1995; Scheijen et al., 1997; Verbeek et al., 1991) and because *PIM1* and *PIM2* expression correlates with that of *c-MYC* in aggressive variants of several diseases (Alizadeh et al., 2000; Care et al., 2013; De Vos et al., 2003; Wright et al., 2003; Zhu et al., 2002b), we hypothesized that sensitivity to PIM inhibition would correlate with high c-MYC expression. Our hypothesis was supported by previously reported findings that such a correlation exists in breast cancer, where sensitivity to PIM1 knock down correlates more closely with expression of c-MYC protein than with PIM1 protein (Horiuchi et al., 2016). Existence of such correlation would be of great importance as high c-MYC expression is connected with poor prognosis in patients with malignant lymphomas and specific inhibitors of c-MYC are still missing while there are plenty of PIM inhibitors being developed and tested. In either DLBCL or MCL cell lines, however, no such correlation was present. One possible explanation is that, given the proposed kinase-independent function of PIM kinases, presence of inhibited protein in the cell is sufficient to maintain some PIM function and elimination of the protein is required to see such dose-dependent effect as was seen after PIM1 knock-down. But at

least for MCL, this is not sufficient explanation. Another possibility is that PIM kinases play important role in early stages of tumor development to cooperate with c-MYC but that they are dispensable for c-MYC function in later stages in these lymphomas.

PI3K/AKT pathway is often aberrantly activated in MCL and DLBCL (Hasselblom et al., 2010; Rudelius et al., 2006). Specifically, GCB and ABC-DLBCL subtypes are sensitive to AKT and PI3K inhibition respectively (Erdmann et al., 2017) while in MCL inhibition of both kinases induces apoptosis and inhibits proliferation of MCL (Rudelius et al., 2006). PIM kinases and AKT share many common substrates. Moreover, PIM and PI3K/AKT pathway were shown to be at least partially redundant in breast cancer and expression of PIM kinases mediated resistance to inhibition of PI3K/AKT signaling (Le et al., 2016). We thus hypothesized, that high AKT activity could counteract effects of the PIM inhibitor and mediate resistance to PIM inhibition. We assessed expression of AKT protein and its activity (measured as phosphorylation on Ser473) in lymphoma cell lines. Despite the fact there was no significant correlation between the levels of AKT or pAKT and sensitivity to AZD1208 in no lymphoma subtype, 3 of 4 AZD1208 resistant (IC50>10 μM) GCB-DLBCL cell lines had high pAKT expression, while 3 of 4 AZD1208 sensitive (IC50<10 μM) GCB-DLBCL cell lines had no detectable pAKT. In ABC-DLBCL, there was no such association. Given that GCB-DLBCL cell, in contrast to ABC cells, depend of AKT activity (Erdmann et al., 2017) it is possible that high activity of AKT in GCB-DLBCL cell lines confers resistance to PIM inhibition.

6.3 AZD1208 has limited proapoptotic activity in lymphoma cell lines and does not augment death-inducing activity of BCL2 inhibitor ABT-199/venetoclax

AZD1208 did not induce any apoptosis in neither MCL nor DLBCL cell lines. This was surprising given that inhibition of PIM kinases by different pan-PIM inhibitors, SGI-1776 and ETP-39010, induced apoptosis in MCL and DLBCL cell lines respectively (Gómez-Abad et al., 2011; Yang et al., 2012). Both compounds, however, exert strong off-target activity towards FLT3 kinase (Saluste et al., 2012), which is absent in AZD1208 (Keeton et al., 2014) and which is probably responsible for at least part or the reported effect of these inhibitors. Concordantly, AZD1208 did not effectively reduce growth of two DLBCL cell lines in another study (Kreuz et al., 2015). Similarly, while SGI-1776 induced apoptosis in 3 out of 3 tested AML cell lines (Chen et al., 2011) AZD1208 only induced apoptosis in 1 out of 5 tested AML cell lines in more recent study (Keeton et al., 2014). It is thus question to what extent the reported effects of these inhibitors are consequence of their off-target activity.

We next tested the hypothesis that PIM kinases can protect cells from apoptosis induced by other chemotherapeutical agents. PIM kinases were shown to mediate resistance to multiple

chemotherapeutical and targeted agents including ABT-199 (An et al., 2015; Kirschner et al., 2015; Kuo et al., 2016; Natarajan et al., 2013; Schatz et al., 2011; Song and Kraft, 2012; Weirauch et al., 2013). Resistance to ABT-199 is mediated by overexpression of antiapoptotic BCL-XL and MCL1 proteins and inhibition of MCL1 can overcome this resistance (Kuo et al., 2016). PIM inhibitors were shown not only to downregulate MCL1 but also to induce expression of NOXA, an MCL1 antagonist (Song and Kraft, 2012; Yang et al., 2012). We thus hypothesized that combination of ABT-199 and AZD1208 would sensitize ABT-199 resistant cells to ABT-199 or augment ABT-199-induced cell death. AZD1208 however did not induce cell death in ABT-199 resistant cells, nor did it enhance cell death induced by ABT-199 in ABT-199 sensitive cells. Analysis of protein expression showed that AZD1208 only moderately reduced MCL-1 level in JEKO1 and even less in REC1 cells which is probably not sufficient to overcome the ABT-199 resistance. Moreover, it is possible that, in our system, AZD1208 did not induce NOXA to further inhibit MCL1.

The complete inability of AZD1208 to induce cell death even in such concentration that potently inhibited growth of all tested cell lines suggests that PIM kinases are not crucial mediators of survival in these lymphomas. Because Pim1 was shown to cooperate with c-Myc via induction of Bcl-2 (Shirogane et al., 1999), it is likely that PIM kinases play an important role in early stages of tumorigenesis to protect cell from c-MYC induced cell death, but that they are dispensable for later survival of lymphoma cells even in the presence of another death-inducer.

6.4 Combination of AZD and ibrutinib

Ibrutinib is commonly used for treatment of MCL patients and resistance to ibrutinib was shown to be driven partly by NF-κB-mediated c-MYC expression (Dai et al., 2017). PIM kinases were reported to mediate ibrutinib resistance in DLBCL via NF-κB pathway (Kuo et al., 2016) and to mediate expression and stability of c-MYC (Cen et al., 2013; Zhang et al., 2008). Thus, because MCL cells depend on c-MYC signaling (Dai et al., 2017; Lee et al., 2018), we hypothesized that combination of both inhibitors would synergistically inhibit c-MYC expression, reduces growth of ibrutinib sensitive MCL cells and that PIM inhibition would be effective even in ibrutinib resistant cells.

Indeed, combination of AZD1208 and ibrutinib potently reduced viability of all MCL cell lines tested. Synergistic index was not assessed but it is possible that, at least in REC1 cell line, such effect exists. It is noteworthy that AZD1208 enhanced effect of ibrutinib in Z138 cell line, which is derived from blastoid variant of MCL and that a possible synergism between these two compounds exists in REC1 cell, another

blastoid-MCL cell line. Thus our next step is to test how this combination affects growth of MCL-derived xenograft in mice.

AZD1208 induced marked increase in PIM2 and to a lesser extent in PIM3 and PIM1 protein levels. This phenomenon was reported previously in DLBCL and BL cell lines (Kreuz et al., 2015) and could limit use of PIM inhibitors in these lymphomas. REC1 and JEKO1 cell express similar basal levels of PIM2 protein but REC1 cell are less sensitive to AZD1208. Concordantly, AZD1208 increased PIM2 protein level in REC1 cells to more extent than in JEKO1 cells and reduced BAD a 4E-BP1 phosphorylation less efficiently in REC1 than in JEKO1 cells. It is thus possible that the extent of stabilization of PIM kinases after PIM inhibition affects sensitivity of particular cell line to AZD1208 more than basal PIM protein expression. Ibrutinib, on the other hand, increased PIM2 protein in REC1 cell but decreased it in JEKO1 cells. BTK activates NF-κB pathway (Petro et al., 2000) that regulates expression of PIM1 and PIM2 (Li et al., 2001; Zhu et al., 2002a). Thus it is possible that the decrease in PIM2 protein was due to inhibition of NF-κB pathway by ibrutinib. Given the differential effect of ibrutinib on expression of PIM kinases in both cell lines, this mechanism is probably dependent on cellular context.

Because AKT is downstream target of BTK (rewieved in Merolle et al., 2018) , we analyzed the impact of ibrutinib on AKT phosphorylation. Surprisingly, ibrutinib reduced AKT phosphorylation only in JEKO1 cell, while it increased pAKT in REC1 cells and the effect was even stronger when combined with AZD1208. The mechanism by which ibrutinib increased AKT phosphorylation is not known, but it was not due to increased AKT expression which remained unchanged in both cell lines. Given the significant changes in AKT activation in JEKO1 and REC1 induced by ibrutinib and the minimal impact of these changes on 4E-BP1 phosphorylation, it is likely that AKT is not a key regulator of 4E-BP1 phosphorylation in these cells. Furthermore, AZD1208 did not influence AKT phosphorylation in neither cell line, which is consistent with both kinases being independent regulators of growth and survival (Fox et al., 2003; Le et al., 2016; Song et al., 2016).

Cap-dependent translation is regulated independently by AKT and PIM kinases and is an important mediator of survival in hematological malignancies (Hammerman et al., 2005; Schatz et al., 2011; Yang et al., 2012). We assessed the impact of AZD1208 and ibrutinib on phosphorylation of translation regulator 4E-BP1 and on expression of its target protein MCL-1. Treatment with AZD1208 reduced 4E-BP1 phosphorylation on Ser65, a known PIM target (Fox et al., 2003; Tamburini et al., 2009), which further confirms inhibition of PIM kinase activity. Ibrutinib only minimally reduced Ser65 phosphorylation in both cells. Combination of both compounds, however, reduced total and Ser65 phosphorylation of 4E-

BP1 to much greater extent than did AZD1208 alone. Because ibrutinib alone had a limited impact on phosphorylation of 4E-BP1, but markedly increased the impact of AZD1208, it appears that ibrutinib might regulate 4E-BP1 phosphorylation in AKT-independent way. AKT-independent regulation of translating mediated by ibrutinib have been reported previously in DLBCL (Ezell et al., 2014), but molecular mechanisms responsible for these findings remained elusive.

Our findings suggest that PIM kinases are more important regulators of 4E-BP1 than AKT in this cellular context. Decrease of 4E-BP1 phosphorylation was accompanied by decrease in MCL1 protein in JEKO1 cell line suggesting that cap-dependent translation was reduced by both compounds in these cells. On the other hand in REC1 cells, cap-dependent translation was inhibited much less efficiently, as indicated by moderate decrease of 4E-BP1 phosphorylation and only slight decrease in MCL1 expression by combination of both drugs.

Ibrutinib was shown to downregulate c-MYC protein via proteasomal degradation in a subset of MCL cell lines (Dai et al., 2017) but we did not observe such effect in our experiments. The reason for this discrepancy is not known. PIM kinases were shown to stabilize c-MYC (Zhang et al., 2008). Even though AZD1208 inhibited PIM kinases, as indicated by reduced phosphorylation of their targets BAD and 4E-BP1, neither AZD1208 alone, nor combination with ibrutinib reduced c-MYC protein level in the tested cell line. In contrast, SGI-1776, another inhibitor of PIM kinases, reduced c-MYC protein level in JEKO cells (Yang et al., 2012). It is possible that AZD1208 was not able to inhibit PIM-mediated stabilization of c-MYC in our experiment setting, or that the effect reported previously was due to the off-target activity of SGI-1776. It is possible that SGI-1776 inhibited either c-MYC protein translation or stability by PIM-independent mechanism and that PIM kinases are not a key regulator of c-MYC expression in MCL. PIM1, however, was shown to directly regulate c-MYC transcriptional activity via H3S10 so it would be interesting to assess phosphorylation status of H3S10 to see whether AZD1208 has potential to reduce c-MYC transcriptional activity. If so, it could at least partially elucidate the role of PIM kinases in MCL. Moreover, because c-MYC specific inhibitors are not currently available, indirect targeting of c-MYC via HSP90 inhibition was shown to induce cell death and cell cycle arrest in both ibrutinib sensitive and resistant MCL cells (Lee et al., 2018). Because PIM1 was shown to be stabilized by HSP90 (Mizuno et al., 2001; Shay et al., 2005) it is possible, that degradation of PIM1 and perhaps other PIM proteins, contributes to effects of HSP90 inhibition. Inhibition of HSP90 thus presents another interesting option to co-target PIM kinases and c-MYC with a single inhibitor.

For the last, we wanted to address effect of both inhibitors on BAD protein. All three PIM kinases phosphorylate BAD (Macdonald et al., 2006) and AZD1208 reduced BAD phosphorylation in both cell lines. Ibrutinib treatment increased pBAD levels in REC1 cells and reduced it in JEKO1 cells, which is in concordance with elevated and decreased PIM2 level induced by ibrutinib in these cells respectively. The fact that 10 µM AZD1208 markedly reduced BAD phosphorylation and viability, but did not induce apoptosis in MCL cell lines suggests BAD is not a relevant regulator of PIM mediated survival in MCL.

Our results show, that PIM inhibition can lead to cytotoxic synergy with ibrutinib in both, ibrutinib resistant and sensitive cells. The molecular mechanism behind the observed synergy remains elusive. Decreased phosphorylation of 4E-BP1 correlated with reduced viability after AZD1208 or combination treatment while it did not correlate with reduced viability after ibrutinib treatment in both cell lines. Contrary to reduced p4E-BP1, no c-MYC and only moderate MCL1 downregulation was observed after combination treatment. We thus have to ask a question to what extent AZD1208 inhibits overall protein synthesis or c-MYC transcriptional activity and whether its role both as a single agent and in combined therapy is dependent on these two processes.

6.5 Overexpression of PIM2 34 kDa isoform

The fact that overexpression of the 34 kDa PIM2 isoform led to marked increase in BAD phosphorylation at Ser112, which is known target of this isoform (Yan et al., 2003) suggests, that the expressed protein was kinase-active and functional in these cells. PIM2 overexpression had no effect on proliferation of JEKO1 cells but reduced the negative effect of doxycycline on viability of MINO cells. Weather doxycycline induced cell death or reduced proliferation of MINO cells was not tested, but our findings suggest that the shortest PIM2 isoform exerts some cell-protective function in this context.

Furthermore, expression of the shortest PIM2 isoform in JEKO1 and MINO cells did not mediate any difference in sensitivity of these cells to AZD1208, ibrutinib or idelalisib. This suggests that this particular isoform, though able to efficiently phosphorylate its targets, is not sufficient to protect cells from negative effects of these drugs.

Because in the previous experiment 4E-BP1 was shown to be regulated by PIM kinases and PIM inhibition reduced viability of these cells, it is possible that the lack of any obvious changes in the sensitivity of PIM2 overexpressing cells the compounds tested was due to the inability of this particular isoform to regulate 4EBP1 and consequently cap-dependent translation. It is thus possible that the function of PIM kinases in MCL is partially dependent on their ability to regulate cap-dependent

translation which is known to be an important target in lymphoma (Hammerman et al., 2005; Schatz et al., 2011).

The ability of AZD1208 to cooperate in addition to ibrutinib also with idelalisib, another antagonist of BCR signaling, suggest more general mode of action of this cooperation. Concurrent inhibition of PIM kinases and BTK or PI3K-mediated BCR signaling thus present interesting opportunity in treatment of hematological malignancies which depend on BCR signaling.

Inhibition of PIM kinases with AZD1208 results in decreased phosphorylation of BAD, a known PIM target. The decrease in pBAD, however, is not accompanied by increased cell death in JEKO1 cell. Indeed, inhibition of PIM kinases with AZD1208 does not induce cell death in any of the cell lines tested. Moreover, AZD1208 has no effect on cell death in a context of BCL2 inhibition. PIM inhibition on the other hand reduced MCL1 expression in JEKO1 cells which was accompanied by reduced viability. These finding are in accordance with MCL1 being prominent BCL2 family member regulated by PIM kinases as part of overall protein synthesis in MCL (Yang et al., 2012). Our data thus support theory that apoptosis is not an important target in MCL, while the role of cap-dependent translation remains elusive.

7 Conclusions

Our results are in accordance with previous finding that inhibition of PIM kinases reduces growth of DLBCL cell lines and that ABC-DLBCL cells are more sensitive to PIM inhibition than GCB-DLBCL cells. We further show that MCL cell lines are even more sensitive to PIM inhibition than ABC-DLBCL.

Despite AZD1208 reduced growth of all tested cell lines, it did not induce apoptosis in either DLBCL or MCL cells suggesting apoptosis is not a key PIM target in these lymphomas. Combination of PIM inhibitors with other, apoptosis-inducing agent might nevertheless improve PIM inhibition efficacy.

We demonstrated that PIM1 and PIM2 are differentially expressed between two major DLBCL subtypes, i.e. GCB and ABC subtypes. Our data suggest that the observed differences in protein expression do not directly recapitulate differential gene expression. Importantly, levels of expression of PIM kinases do not directly correlate with sensitivity to PIM inhibition in DLBCL. Expression of PIM1, however, correlated with sensitivity in MCL. This might be due to the fact that PIM1 is commonly mutated in DLBCL but not in MCL and that in MCL PIM1 protein level more closely reflects PIM kinase activity.

We analyzed cytotoxic efficacy of the combination of AZD1208 and three clinically approved anticancer drugs including venetoclax, ibrutinib and idelalisib. Combination of AZD1208 and venetoclax was not associated with a measurable increase in cytotoxicity toward either DLBCL or MCL cells. Interestingly, combined inhibition of PIM kinases and B-cell receptor signaling with ibrutinib (a BTK inhibitor), or idelalisib (a PI3K inhibitor) significantly reduced growth of MCL cell lines compared to single-agent approaches. Combination of AZD1208 and ibrutinib was effective in all tested MCL cell lines including aggressive blastoid-variant and ibrutinib resistant MCL cell lines. On a protein level, the combined exposure of MCL cells to AZD1208 and ibrutinib led to markedly decreased phosphorylation of 4E-BP1, key regulator of cap-dependent translation, and decreased expression of MCL1 protein in two MCL cell lines suggesting for reduced cap-dependent translation.

Finally, we showed that overexpression of the shortest PIM2 isoform impacts phosphorylation of PIM target BAD, but does not affect sensitivity of MCL cells to AZD1208, ibrutinib or idelalisib, nor does it affect phosphorylation of 4E-BP1. The data suggest that PIM kinases exert their function, at least in part through regulation of cap-dependent transition.

In conclusion, our work confirmed PIM kinases as promising innovative therapies for mantle cell lymphoma and ABC-subtype diffuse large B-cell lymphoma patients. Importantly, our data provide sound

preclinical substantiation for experimental treatment strategy based on the combination of AZD1208 and ibrutinib in mantle cell lymphoma.

8 References

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