PIM serine/threonine kinases in the pathogenesis and therapy of hematologic malignancies and solid cancers

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ABSTRACT

The identification as cooperating targets of Proviral Integrations of Moloney virus in murine lymphomas suggested early on that PIM serine/threonine kinases play an important role in cancer biology. Whereas elevated levels of PIM1 and PIM2 were mostly found in hematologic malignancies and prostate cancer, increased PIM3 expression was observed in different solid tumors. PIM kinases are constitutively active and their activity supports *in vitro* and *in vivo* tumor cell growth and survival through modification of an increasing number of common as well as isoform-specific substrates including several cell cycle regulators and apoptosis mediators. PIM1 but not PIM2 seems also to mediate homing and migration of normal and malignant hematopoietic cells by regulating chemokine receptor surface expression. Knockdown experiments by RNA interference or dominant-negative acting mutants suggested that PIM kinases are important for maintenance of a transformed phenotype and therefore potential therapeutic targets. Determination of the protein structure facilitated identification of an increasing number of potent small molecule PIM kinase inhibitors with *in vitro* and *in vivo* anticancer activity. Ongoing efforts aim to identify isoform-specific PIM inhibitors that would not only help to dissect the kinase function but hopefully also provide targeted therapeutics. Here, we summarize the current knowledge about the role of PIM serine/threonine kinases for the pathogenesis and therapy of hematologic malignancies and solid cancers, and we highlight structural principles and recent progress on small molecule PIM kinase inhibitors that are on their way into first clinical trials.

Key words: PIM kinases, leukemia, solid cancer, structure, small molecule inhibitors

Citation: Brault L, Gasser C, Bracher F, Huber K, Knapp S, and Schwaller J.PIM serine/threonine kinases in the pathogenesis and therapy of hematologic malignancies and solid cancers. Haematologica 2010;95:1004-1015. doi:10.3324/haematol.2009.017079

Identification of the PIM family of constitutively active proto-oncogenic serine/threonine kinases

Over two decades ago, cloning of retroviral integration sites in murine Moloney leukemia virus induced lymphomas has led to the identification of the PIM (Proviral Integration Moloney virus) gene locus.[1] Over 50% of early T-cell lymphomas showed integrations near the PIM1 locus leading to deregulated expression of the PIM1 mRNA. The *PIM1* gene locus was mapped to mouse chromosome 17, and to short arm of chromosome 6 (6p21) in the human genome. Further analysis revealed that the open reading frame of PIM1 encoded for a protein of 313aa extending over 6 exons, with highest homology to serine/threonine kinases.[2] Predisposition to lymphomagenesis in PIM1 transgenic mice through cooperation with c-myc and N-myc demonstrated the proto-oncogenic activity of PIM1.[3] Subsequent studies have characterized PIM1 as synergizing oncogene with over-expressed BCL2, GFI1, loss of FAS-L, or in collaboration of a leukemogenic fusion gene (*E2A-PBX1*).[4] The *PIM1* gene encodes for two isoforms of 34 and 44kD through the use of alternative initiation sites. Both isoforms contain the kinase domain and exhibited comparable *in vitro* kinase activity.[5] PIM1 was found ubiquitously expressed and to function as a protein with a short half-life. Interestingly, the half-life of PIM1 (< 5 min) observed in normal peripheral leukocytes was significantly increased in K562, a Philadelphia chromosome-positive leukemia cell line derived from chronic myeloid leukemia

Funding: JS holds a research professorship from the Gertrude Von Meissner Foundation and is further supported by the Swiss Cancer League, the Swiss National Research Foundation, the Susy-Rueckert Foundation/Krebsliga beider Basel, Wilhelm-Sander Foundation (Munich) and the Novartis Foundation for Biomedical Research (Basel). SK is an investigator of the Structural Genomics Consortium (SGC), a registered charity (no. 1097737) funded by the Wellcome Trust, GlaxoSmithKline, Genome Canada, the Canadian Institutes of Health Research, the Ontario Innovation Trust, the Ontario Research and Development Challenge Fund, the Canadian Foundation for Innovation, VINNOVA, The Knut and Alice Wallenberg Foundation, The Swedish Foundation for Strategic Research, and Karolinska Institutet.
Manuscript received on October 28, 2009. Revised version arrived on December 1, 2009. Manuscript accepted on December 21, 2009.
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The online version of this article has a Supplementary Appendix.

in blast crisis.[6] Abundant levels of PIM1 were found in hematopoietic cells. Moreover, sustained PIM1 expression was induced by cytokines that signal through structurally related receptors such as IL-3, GM-CSF, G-CSF or IL-6.[7] Subsequently, several studies have documented that PIM1 is a major downstream target of the signal transducer and activator of transcription (STATs) induced by a large variety of additional receptors such as IL-2, IL-7, IL-9, IFNγ, EPO, FLT3 or TPO.[7] PIM1 expression is not only regulated at the transcriptional, but also at the posttranscriptional, translational and posttranslational levels (Figure 1). Other studies have shown that PIM1 kinase is significantly protected from proteasomal degradation by heat shock proteins (Hsp70, Hsp90).[8,9] Moreover, it has been proposed that micro-RNAs, miR-1 and miR-210, might be implicated in regulation of PIM1 expression.[10,11]

Germline inactivation of the *PIM1* gene was associated with a mild phenotype as PIM1 deficient mice are ostensibly normal, healthy and fertile. However, subtle functional defects of the hematopoietic system have been identified: PIM1$^{-/-}$ mice showed erythrocytic microcytosis and PIM1$^{-/-}$ B cells and bone marrow-derived mast cells were impaired in interleukin-7 (IL-7) or IL-3 induced proliferation.[12,13]

Retroviral insertion site cloning in secondary transplants of Moloney murine virus induced lymphomas revealed PIM2 as a frequent but late event in tumorigenesis.[14] Interestingly, proviral tagging in *c-myc* transgenic mice lacking PIM1 has led to compensatory activation of PIM2. The *PIM2* gene located on chromosome Xp11 comprises 6 exons and is 53% identical to PIM1 at the amino acid level and shares preference and usage of non-AUG alternative initiation codons leading to 3 different isoforms. PIM2 is ubiquitously expressed with highest levels in brain and lymphoid cells, and like PIM1, PIM2 also potently synergizes in c-MYC induced lymphomagenesis.[15]

Through high throughput retroviral tagging in tumors of *c-myc* transgenic mice lacking PIM1 and PIM2, Mikkers and colleagues found selective activation of PIM3 suggesting that PIM3 can substitute for PIM1 and PIM2 in MuLV-induced lymphomagenesis.[16] The *PIM3* gene is located on chromosome 22q and encodes for a serine/threonine kinase with over 60% homology to PIM1 and PIM2, that is ubiquitously expressed with highest levels in kidney, breast and brain.[17]

PIM1, PIM2 and PIM3 compound knockout mice that survived the perinatal period displayed a profound reduction in body size suggesting that PIMs are important for body growth. Colony forming assays with bone marrow from PIM1$^{-/-}$PIM2$^{-/-}$PIM3$^{-/-}$ mice demonstrated that PIMs act redundantly in clonogenic growth in response to IL-3, IL-5, SCF and TPO. However, PIM1 seems to be the most crucial isoform for these responses. Despite these defects, it was possible to establish PIM compound knockout mice that were viable and fertile suggesting that the PIM family of serine/threonine kinases is important but dispensable for growth factor signaling.[18]

The oncogenic activity of PIM serine/threonine kinases is mediated by multiple cellular substrates

Expression of recombinant PIM1 protein demonstrated its activity as serine/threonine kinase. Interestingly, in addition to phosphorylation of cellular substrates, *in vitro* experiments also suggested autophosphorylation activity of PIM1.[7,19] Examination of the substrate sequence specificity of PIM1 revealed strong preference for peptides containing (K/R)3-X-S/T-X (X=neither basic nor large hydrophobic residue)[20] and positional peptide library screens identified a consensus sequence (ARKRRRHPSGPPTA) that bound with low nM affinity to PIM kinases.[21,22] Mass-spectrometric analysis of *Xenopus laevis* PIM1 identified potential autophosphorylation sites including serine 190 and threonine 205 both conserved between species.[23] We have recently observed phosphorylation of PIM1 after heterologous expression in *E.coli* located on residue Ser261. However, this site does not seem to be phosphorylated *in vitro*, instead other putative phosphorylation sites were found of which one would map to Ser8 located in the unstructured N-terminus of PIM1 (S Knapp, unpublished observation, 2009). Negative regulation of the stability of PIM kinases by the protein phosphatase 2A (PP2A) suggested that autophosphorylation and/or phosphorylation by so far unknown regulatory kinases might be important determinants of PIM function.[24,25] Modification of PIM1 function through phosphorylation by an upstream kinase has also been proposed in interleukin-6 (IL-6)-treated prostate cancer cell lines. Phosphorylation of PIM1 on Tyr218 by the ETK tyrosine kinase was required for IL-6-induced activation

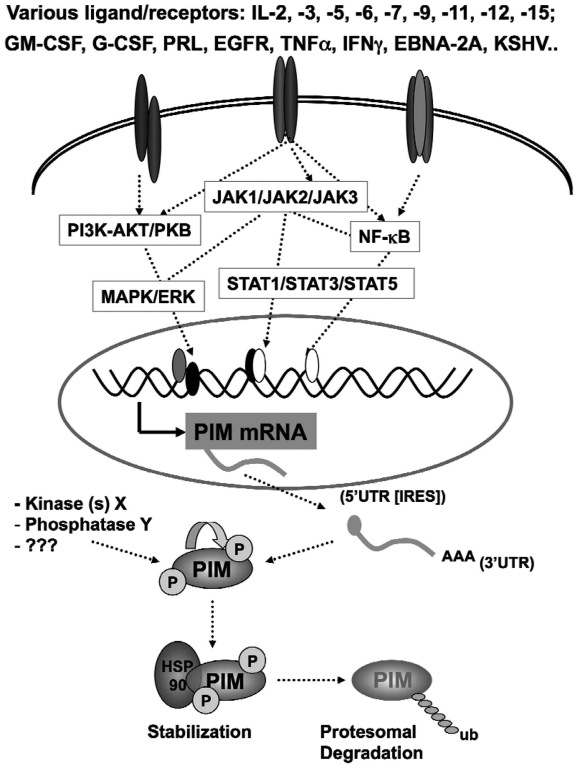

Figure 1. Regulation of PIM1 expression. Binding of several ligands leads to activation of a complex network of signaling pathways that results in upregulation of PIM1 mRNA. Binding of PIM1 to heat shock protein 90 (HSP90) protects from proteosomal degradation. Most experimental data has been generated using PIM1; very little is known about regulation of PIM2 and PIM3. There is increasing evidence for modification of PIM kinases through as yet unkown protein kinases and/or phosphatases.

of androgen receptor-mediated transcription.[26,27] As PIM proteins harbor over 35 potential recognition sites for a large variety of kinases, the impact of PIM autophosphorylation or heterologous phosphorylation by upstream regulators for the oncogenic function needs further clarification (Figure 2).

Gain of function studies through overexpression of human or mouse PIM cDNAs in different cell types showed that PIM kinases act as survival factors by blocking apoptotic cell death through regulation of pro- and anti-apoptotic members of the BCL2 protein family.[7,28] Several studies revealed a key role of PIM1 in cytokine-mediated survival signaling in murine mast cells and primary human basophils and eosinophils.[13,29] The pro-survival activity of all three PIM kinases in several cell types might be (at least in part) explained by inactivation of the pro-apoptotic protein BAD through phosphorylation of the Ser112 gatekeeper residue and presumably also Ser136 and Ser155.[30-33] Most recent work suggests that phosphorylation of the proline-rich Akt substrate 1 (PRAS1) might also have the potential to block apoptosis of murine factor-dependent hematopoietic progenitor cells (FDCP-1).[34] In addition, PIM1 seem to impair the activity of the apoptosis signaling kinase 1 (ASK1), by direct phosphorylation, resulting in protection from H_2O_2-induced cell death of H1299 lung cancer cells.[35]

Intriguingly, overexpression of PIM1 also stimulated cell death signaling in Rat1 fibroblasts elicited by c-MYC most probably through interaction and modification of the Cdc25A cell cycle phosphatase.[36] A series of *in vitro* experiments demonstrated that collaboration of PIM1 with *c-myc* is essential for STAT3-mediated cell cycle progression and survival of hematopoietic Ba/F3 cells.[37] Recent *in vitro* work using human vascular endothelial cells suggested that functional cooperation of PIM kinases with *c-myc* might be based on PIM-mediated phosphorylation of histone H3. PIM1 seems to be recruited to the E-box elements of MYC leading to a MYC-MAX-PIM1 complex. This complex phosphorylates H3-S10 stimulating then the binding of RNA polymerase II that contributes to transcriptional activation of a subset of MYC target genes.[38] However, it is currently not known which of the PIM1-co-regulated MYC target genes might be essential for transformation or whether H3-S10 can also be phosphorylated by PIM2 or PIM3. Interestingly, phosphorylation of H3 by PIM1 seems to provide essential docking sites for acetylation of Histone H4 at lysine 16 (H4K16ac) by the MOF histone acetyltransferase. The resulting nucleosomal mark then allows binding of the BRD4 bromodomain protein and the positive elongation factor b (p-TEFb) mediating transcriptional elongation.[39]

Murine hematopoietic cells (FDCP-1) were protected

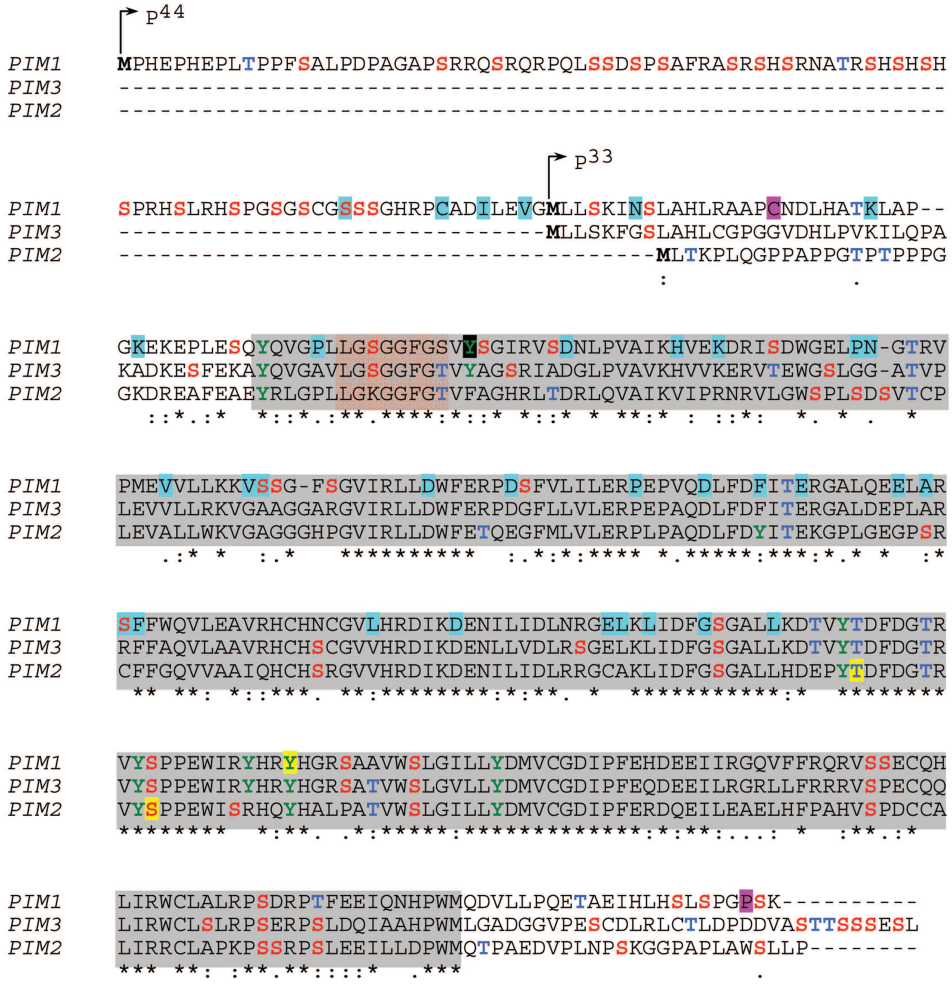

Figure 2. Human PIM kinases amino acid sequences alignment. ClustalW2 alignment with protein sequence from Swiss-Prot (accession numbers: P11309 (PIM1), Q9P1W9 (PIM2) and Q86V86 (PIM3)). The kinase domain is shown in gray; the ATP binding domain in orange. S (Serine, = red), T (Threonine, =blue), Y (Tyrosine, =green) are potential phosphorylation sites. Reported phosphorylation sites are shown in yellow. Reported mutation in a case of colon cancer is indicated in black (PIM1Y144H); reported point mutations in DBA cases are shown in pink (PIM1C17Y and PIM1P311T); residues that have been reported to be modified by somatic hypermutation in human B-cell malignancies are marked in blue.

from irradiation or adriamycin-induced apoptosis by overexpression of PIM1. Interestingly, cellular protection was associated with nuclear localization of a large fraction of the short (p33) but not the long (p44) PIM1 isoform suggesting the existence of functionally important isoform-specific cellular substrates.[40] A PIM1 consensus site (RKR-RQTSM) was found in the cell cycle regulator $p21^{Cip1/WAF1}$ (CDKN1A). PIM1 associated with and phosphorylated $p21^{Cip1/WAF1}$ on Thr145 resulting in stabilization and nuclear translocation. These observations made in various cell lines suggested that deregulated PIM1 activity might contribute to tumorigenesis at least in part by regulation of $p21^{Cip1/WAF1}$.[41,42] PIM kinases seem also to regulate the $p27^{KIP1}$ (CDKN1B) cyclin-dependent kinase inhibitor. All three PIM kinases bound and directly phosphorylated $p27^{KIP1}$ at residues Thr157 and Thr198 that allows binding of $p27^{KIP1}$ to 14-3-3 proteins resulting in its nuclear export and proteosome-dependent degradation. Through phosphorylation and inactivation of FoxO1a and FoxO3a, PIM kinases seem to directly repress $p27^{KIP1}$ transcription as shown in solid cancer and leukemia cell lines.[43] PIM kinases seem not only to interfere with G1-S (through modification of Cdc25A, p21 and p27) but also with the G2-S transition of the cell cycle by phosphorylating Cdc25C phosphatase and the Cdc25C-associated kinase (C-TAK1).[44,45]

Recognition motif-based searches as well as protein-protein interaction screens resulted in identification of several putative PIM substrates including SND1 (p100), PAP-1 (RP9), HP1γ (CBX3), SNX6, SOCS-1 and -3, RPS19, RUNX-1 and -3, ABCG2/BRC, API5, MYB, MYC, NFAT1, NUMA, PTPRO and p65/REL-A[46-61] (*Online Supplementary Table S1*). Although most of these proposed substrates have not been validated as being *in vivo* executors of the proto-oncogenic function of the PIM kinases, some of them are of special interest.

The SOCS proteins are well-characterized regulators of the JAK/STAT signal transduction pathway. Two groups have reported that the stability of SOCS1 is regulated through interaction and phosphorylation by PIM serine/threonine kinases.[50,51] Structure functional analysis showed that the N-terminal 79aa of SOCS1 are essential for interaction with PIM. However, the targeted S/T residues (>30 potential phospho-motifs are present but no apparent PIM recognition site can be found in this region) remain unknown. Malignant transformation by the *v-Abl* oncogene was associated with phosphorylation of SOCS1 on non-tyrosine residues and inhibition of SOCS1 mediated proteasomal targeting of activated JAK kinases. Interestingly, *v-Abl* was not able to efficiently transform primary bone marrow cells from $PIM1^{-/-}PIM2^{-/-}$ compound knockout mice but combined deficiency of PIM1/PIM2 and SOCS1 resulted in partial restoration of the *v-Abl* transformation efficiency. These observations suggested that modulation of SOCS1 by PIM kinases might play a key role in *v-Abl* mediated malignant transformation.[62] Taken together, PIM kinases might participate on several levels in a complex feedback mechanism regulating the JAK2/STAT5 signaling pathway that plays an essential role in induction and maintenance of hematologic malignancies.[63]

Mutations in the gene encoding the ribosomal protein RPS19 account for about 25% of patients with Diamond Blackfan anemia (DBA). This is a congenital bone marrow failure syndrome that typically results in macrocytic anemia within the first year of life, associated with birth defects and increased incidence of cancer.[64] Possible interaction and phosphorylation of RPS19 in the ribosome by PIM1 is interesting as *PIM1* gene dosage seems to directly influence the size of the erythrocytes: $PIM1^{-/-}$ mice have a decreased erythrocyte size, whereas *PIM1* transgenic mice exhibit an increase in the median cellular erythrocyte volume.[12,52] Although the PIM1 targeted residues in RPS19 have not been mapped, the most common missense mutations in RPS19 are located in a serine/threonine-rich region predicted to be targeted by kinases.[64] To address whether *PIM1* could be a candidate gene for DBA forms without RPS19 mutations, Chiochetti and colleagues performed a mutational screen involving 99 DBA patients and found 2 missense mutations Pro311Thr (C/A) and Cys17Tyr (G/A).[52] These mutations located at the very 5' and 3' end of PIM1 and their functional consequence remains to be studied.

Increasing evidence supports functional links between the integrity of ribosomal proteins and the p53 tumor suppressor. In mice, mutations in RPS19 and RPS20 caused dark skin and reduced erythrocyte count in mice through activation of p53.[65] Whether PIM1 modulates p53 through interaction of RPS19 is currently not known. However, elevated PIM1 levels seemed to induce the p53 pathway in different cancer cell lines and murine embryonic fibroblasts (MEFs) and also correlated with increased Mdm2 levels in mantle cell lymphoma patients' biopsies. PIM1 associated with and phosphorylated Mdm2 at Ser166 and Ser186 leading to stabilization of both proteins.[66] More work is needed to validate the impact of PIM-mediated p53 regulation for induction and/or maintenance of malignant transformation.

PIM serine/threonine kinases in hematologic malignancies and solid cancers

Hematologic malignancies

PIM1. Early studies demonstrated overexpression of PIM1 in a significant fraction (30%) of human myeloid and lymphoid leukemia (including 51 primary patients' samples and 19 leukemia cell lines) in absence of any apparent gene rearrangements or amplifications.[67] In cellular models of malignant myeloproliferative disorders, PIM1 and PIM2 were both found to be up-regulated and proposed to be a mediator of anti-apoptotic properties of oncogenic protein tyrosine kinases (PTKs) such as BCR/ABL, FLT3-ITD, or the JAK2V617F mutant, most probably mediated through aberrant JAK2/STAT5 activity.[68-73] We and others have observed that overexpression of PIM1 was sufficient to induce IL-3 independence in murine hematopoietic Ba/F3 cells.[74,75] Microarray experiments revealed upregulation of PIM1 expression in acute myeloid leukemia harboring alterations of the mixed-lineage leukemia (*MLL*) gene such as the *MLL/ENL* or *MLL/AF9* fusion genes[76] (J Schwaller, unpublished observation, 2009). Elevated PIM1 levels in acute myeloid leukemia are most likely the consequence of FLT3 activation (by overexpression or mutation) and/or of aberrant activation of HOXA9, a direct transcriptional regulator of PIM1 [69,70,72,77] (Figure 3).

To address the role of PIM kinases for induction of PTK-mediated leukemic disorders, we have performed bone marrow reconstitution experiments using PIM knockout cells. Transplantation of wild-type or $PIM2^{-/-}$ bone marrow retrovirally expressing the FLT3-ITD mutant led to induc-

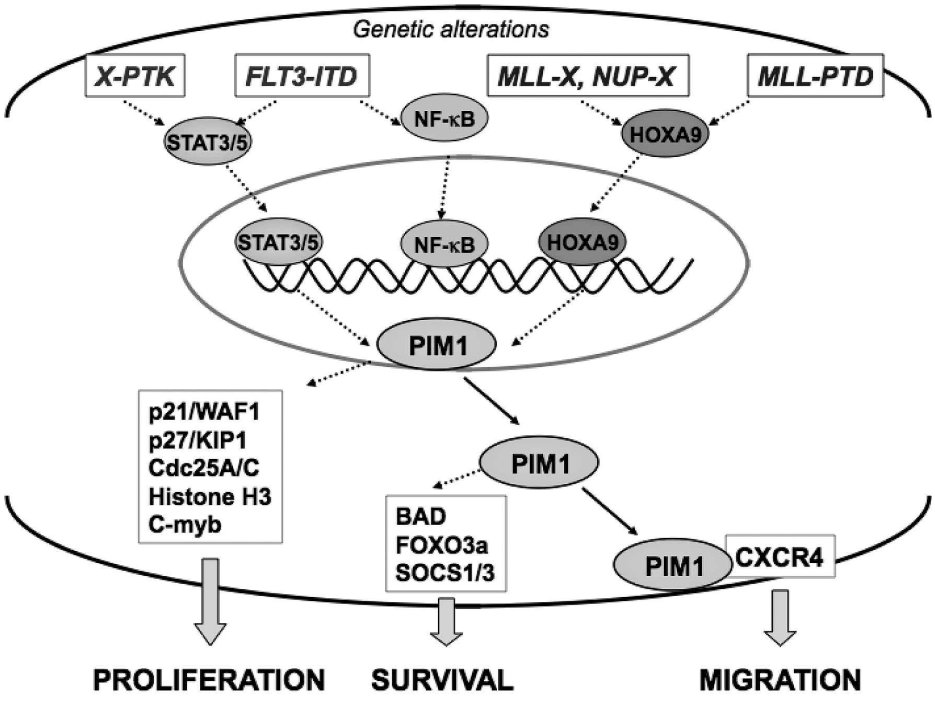

Figure 3. Potential downstream substrates of overexpressed PIM1 in hematologic malignancies. Functionally collaborating genetic alterations such as mutated protein tyrosine kinases and fusion genes involving transcriptional regulators such as mixed lineage leukemia (MLL) lead to constitutive activation of major signaling mediators, like STAT5 or HOXA9, shown to be transcriptional activators of PIM1. Elevated PIM1 levels support cellular proliferation through modification of cell cycle regulators, survival through modification of regulators of apoptosis, as well as homing and migration through modification of the CXCR4 chemokine receptor.

tion of typical lympho-myeloproliferative disease.[78] In contrast, PIM1$^{-/-}$ bone marrow cells were not able to reconstitute lethally irradiated recipients and showed a significant defect for homing to the bone marrow and spleen. Grafting of hematopoietic stem cells is a complex process regulated by several signaling pathways of which the CXCL12/CXCR4 ligand/receptor system plays a predominant role.[79,80] Interestingly, PIM1$^{-/-}$, but not PIM2$^{-/-}$ bone marrow cells expressed significantly lower amounts of surface CXCR4 and were impaired in migration towards a CXCL12 gradient. Blocking PIM1 activity by expression of a dominant-negative acting mutant, siRNAs or by a small molecule inhibitor resulted in impaired CXCR4 surface re-expression after ligand-induced receptor internalization. Site directed mutagenesis experiments and *in vitro* kinase assays suggested that PIM1 might regulate CXCR4 by direct phosphorylation of the S339 residue in the intracellular domain, known for proper receptor internalization and surface re-expression. Surface expression of CXCR4 is a known prognostic factor in acute myeloid leukemia.[81] It is worth noting that a correlation between PIM1 overexpression and surface CXCR4 expression was found in fresh blasts from acute myeloid leukemia patients. Treatment of the cells with a small molecule PIM inhibitor resulted in *ex vivo* downregulation of CXCR4 surface expression in 4 out of 6 patients tested. These observations suggested that PIM1 (but not PIM2) regulate homing and migration of leukemic cells through modification of surface CXCR4 expression.[82]

Several B-cell lymphoproliferative disorders have been associated with latent infections of Epstein-Barr virus or Kaposi sarcoma-associated herpesvirus (KSHV). Interestingly, Epstein-Barr virus infection of primary B lymphocytes has been associated with an increase of PIM mRNA expression, and over-expressed PIM kinases enhanced the activity of the viral transactivator EBNA2.[83] Significantly elevated PIM expression levels were also found in malignant B cells that express the KSHV latency associated nuclear antigen (LANA). LANA has been shown to be a substrate of PIM1 that phosphorylates LANA within the N-terminal domain.[84] In addition, a kinome wide expression library study identified activation of PIM1/PIM3 as a critical element for reactivation of a latent KSHV infection.[85]

B-cell non-Hodgkin's lymphoma (NHLs) is characterized by chromosomal translocations leading to deregulation of several proto-oncogenes controlled by the immunoglobulin gene promoter and enhancer elements. Similar to the immunoglobulin variable region genes in normal B-cell development, aberrant somatic hypermutation of multiple loci, including the proto-oncogenes C-MYC, RhoH, PAX5 and PIM1, have been found in over 50% of diffuse large-cell lymphomas (DLBCL).[86] Typically, these mutations are localized in the 5' untranslated or coding region of the genes, are independent of chromosomal translocations and share features of typical variable-region associated somatic hypermutations. The lack of such mutations in normal germinal-center B cells suggests a direct role for the pathogenesis of malignant lymphomas; however, the molecular mechanisms are currently not understood. Strikingly, several somatic hypermutations affecting PIM1 have been found in cases of other subtypes of B-cell non-Hodgkin's lymphoma including follicular cell lymphoma, AIDS-NHLs, and MALT lymphomas.[87] Rather surprisingly, several PIM1 variants showed a significantly decreased *in vitro* kinase activity, suggesting a so far unknown kinase-independent oncogenic function of PIM1.[88] Recent observations made in a cancer xenograft model, in which overexpression of a kinase-dead PIM1 (K67M) mutant resulted in the formation of larger tumors, supports the hypothesis of an oncogenic function of PIMs independent of catalytic activity.[89] Gene expression profil-

ing identified frequent upregulation of PIM1 expression in aggressive mantle B-cell lymphoma. As PIM1 expression levels seem to be a poor prognostic marker in intensively treated aggressive mantle cell lymphoma, further studies for its role as therapeutic target for this aggressive disease are warranted.[90]

PIM2. Similar to PIM1, significant levels of PIM2 have been found in primary blasts from acute myeloid leukemia patients.[69,91] Interestingly, recent work identified PIM2 (but not PIM1) as the main kinase that phosphorylates 4E-BP1 resulting in mTOR-independent translational control in acute myeloid leukemia cells. This study suggests that a potent PIM2 inhibitor might be able to block rapamycin-resistant translation of oncogenic proteins.[91] PIM2 is also highly expressed in progenitor cells of the B-cell lineage and critically involved in signaling pathways regulating B-cell homeostasis.[92] Moreover, PIM2 has been reported being over-expressed and associated with progression of several malignancies that originate from the B-cell lineage such as chronic lymphocytic leukemia (CLL), diffuse large B-cell lymphoma (DLBCL), mantle cell lymphoma (MCL) or myeloma.[93,94] The ability of PIM2 to promote survival of lymphoid cells seems to be dependent on activation of nuclear factor (NF)-κB through the serine/threonine kinase Cot/Tpl2.[95] As PIM2 is possibly a downstream target of NF-κB signaling, high levels of PIM2 might be the result of a feedback mechanism.[96]

Solid tumors

PIM1. Biomarker delineation for prostate cancer by using gene expression profiling identified the PIM1 serine/threonine kinase being deregulated upon cancer progression. Further validation in over 700 clinical patients' samples showed no or weak PIM1 expression in benign lesions, and moderate to strong PIM1 expression in over 50% of prostate cancer samples. PIM1 expression correlated significantly with a poor therapy outcome in prostate cancer.[97] This study also revealed remarkably similar transcriptional co-regulation of PIM1 and *c-myc*, possibly mediating synergistic oncogenic effects. Subsequently, this hypothesis has been experimentally validated *in vivo* by transgenic mice that express human *c-myc* in the mouse prostate. Cross-species gene expression comparison revealed that "MYC-like" human cancers are characterized by significant upregulation of PIM1.[98] Further studies found increased PIM1 expression in high-grade prostatic neoplasia. This finding suggests that PIM1 overexpression is an early event in prostate carcinogenesis.[99] *In vitro* studies demonstrated enhanced tumor growth and protection from drug-induced apoptosis of prostate cancer cells upon overexpression of PIM kinases.[100]

Retroviral overexpression of PIM1 in immortalized, non-tumorigenic prostate or mammary epithelial cell lines or the LNCaP prostate carcinoma cell line has been shown to induce genomic instability characterized by a defect in the mitotic spindle checkpoint, abnormal mitotic spindles, centrosome amplification and chromosome missegregation resulting in poly- and aneuploidy.[101,102] PIM1 induced chromosomal instability is not restricted to prostate cells but has also been observed in telomerase-immortalized human mammary epithelial cells and associated with dysregulation of cyclin B1.[102] However, further validation of these *in vitro* observations by a single team is needed. It would be interesting to see if expression levels of PIM1 correlate *in vivo* with the degree of genomic instability observed in human malignancies.

Less is known about the role of PIM1 in other solid cancers. While studying PIM1 expression during mammary development, Gapter and colleagues found elevated levels of PIM1 in most mammary carcinoma cell lines. In addition, progesterone increased PIM1 protein levels to some extent in non-tumorigenic mammary epithelia.[103] Elevated PIM1 levels in prostate and breast cancer could be also the consequence of aberrant STAT5 activity that has been associated with disease progression in both tumor types.[104] Peltola and colleagues found that elevated PIM1 expression might be predictive for radiation response in squamous cell carcinoma of the head and neck.[105] In addition, increased PIM1 expression was proposed to be a prognostic marker for pancreatic ductal adenocarcinoma.[106] Tumor-associated hypoxia seems to increase PIM1 expression and to support chemoresistance shown in several solid cancer cell lines.[107] These observations suggest that targeting of PIM1 might be beneficial in combination with chemotherapeutics for the therapy of solid cancers.

PIM2. Perineural invasion, a major mechanism that leads to the spread of prostate cancer cells, has been found to be associated with elevated PIM2 expression.[108] Increased PIM2 levels in prostate cancer correlated with higher proliferation, a decreased rate of apoptosis and many established prognostic factors.[109] *In vitro* studies using HepG2 cells suggested that PIM2 may act as pro-survival kinase in liver cancer.[110]

PIM3. A search for target genes of common fusion proteins associated with human Ewing's sarcoma revealed upregulation of PIM3. Overexpression of PIM3 in rodent fibroblasts (NIH3T3) showed a stronger transforming activity than the EWS-FLI fusion. Furthermore, co-expression of EWS-FLI with a dominant-negative acting PIM3 mutant prolonged survival of mice after subcutaneously injecting transduced NIH-3T3 cells suggesting that PIM3 might be important for transformation by EWS-fusion genes.[111] PIM3 is also highly expressed in human hepatocellular carcinoma but not in normal hepatocytes.[112] Likewise, high PIM3 expression has also been observed in malignant lesions of the pancreas but not in normal pancreatic tissue.[113] Furthermore, the same group found elevated PIM3 expression upon progression of gastric adenocarcinoma.[114] Interestingly, whereas growth and survival of certain solid tumor cells (e.g. prostate or liver cancer cell lines) has been severely affected by inactivation of a single PIM isoform, in leukemia cell lines knockdown of more than one PIM is required. The large number of expression and functional studies have suggested that inhibition of aberrant PIM activity by small molecules may open a new promising avenue for cancer therapy.

PIM kinases as targets for cancer therapy

Insights from the structure

The crystal structure of PIM1 has been reported by a number of laboratories.[22,88,115,116] These structural studies revealed a classic bilobal protein kinase domain architecture and apart from the unique beta hairpin insert located N-terminal to helix αC, all conserved secondary structure elements of typical protein kinases were present (Figure 4). In protein kinases, the binding site for ATP is located in a deep cavity formed by the two kinase lobes and the connecting hinge region. However, ATP binding to this site differs in PIM kinases. The presence of a proline residue at

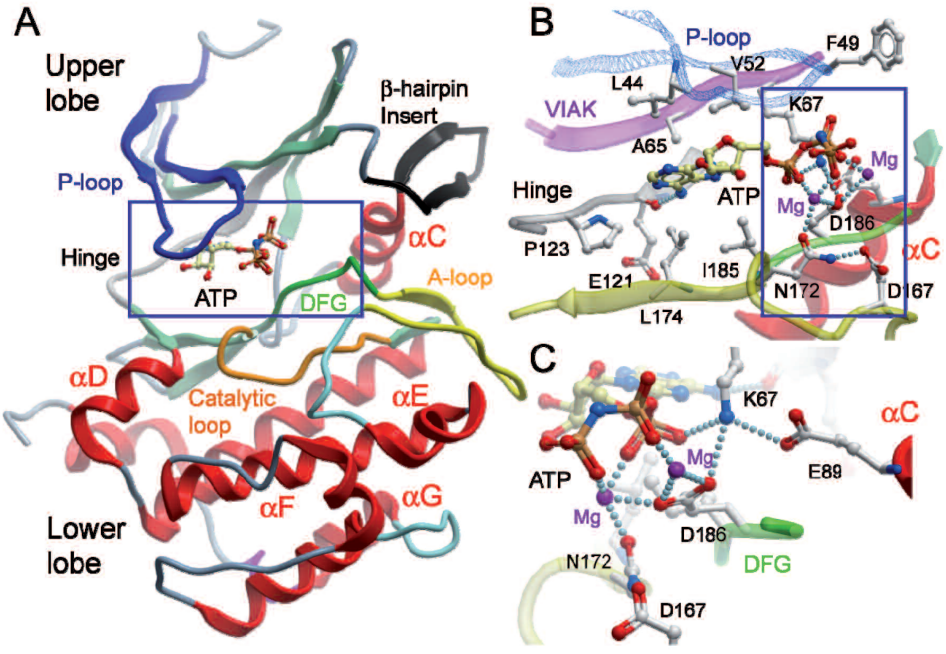

Figure 4. Structural aspects of PIM1. (A) Structural overview of the PIM1 crystal structure in complex with the non-hydrolysable ATP analogue AMPPNP. Regulatory elements (the phosphate binding (P)-loop, the magnesium binding motif DFG, the hinge region, the catalytic loop and the activation segment (A)-loop and the β-hairpin insert) are labelled and highlighted using different colours. Some conserved secondary structure elements are labelled. (B) Details of the ATP binding site (boxed in A) showing the main interactions formed by the co-factor with PIM1. The unusual presence of a proline residue (P123) prevents formation of a second hydrogen bond with ATP. (C) Details of the interaction of the phosphate moieties of AMPP-NP and Mg2+ with the D186FG motif, the conserved lysine (K67) and glutamate (E89) and the catalytic aspartate (D167). The enlarged region corresponds to the boxed area in B.

position 123 does not allow formation of a second hydrogen bond of the hinge backbone to the adenine ring. In addition, the insertion of an additional residue in the kinase hinge results in structural changes in the hinge region due to the unique hinge sequence ERPXPX. The structure of PIM2 has been reported recently.[117] However, so far no crystal structure has been reported for PIM3. Both PIM1 and PIM2 assume an active conformation in the reported crystal structures. The active state of kinases is characterized by the presence of the conserved lysine – glutamate salt bridge (Lys67 and Glu89 in PIM1), a closed lobe conformation and a well-structured activation segment. The activation segment is often unstructured in kinases that require phosphorylation for catalytic activity. Upon phosphorylation, the activation segment folds onto the lower lobe and structures the peptide-binding site leading to enzymatic activation. In contrast, PIM kinases are catalytically active in the absence of phosphorylation and the crystal structures of PIM provide an explanation for this constitutive activity. The unphosphorylated activation segment forms a large number of polar interactions with the lower kinase lobe stabilizing the observed active conformation. Nevertheless, PIM kinases do autophosphorylate, but the functional consequences of these post-translational modifications are currently unknown.[22]

Small molecule PIM kinase inhibitors

The unique hinge architecture of PIM kinases suggests that very selective inhibitors can be identified using this structural difference. Indeed, over 50 potential PIM inhibitors have become public but the kinome wide specificity of these inhibitors is largely unknown.

These inhibitors can be grouped into two main classes based on their binding mode (Figure 5). The first class of inhibitors represents typical ATP-mimetic compounds that form, similar to the adenine ring of the cofactor, a hydrogen bond with the hinge backbone oxygen of PIM1 (Glu121). These compounds comprise the broad-spectrum kinase inhibitor staurosporine and its analog K252, bisindoyl maleinimides (BIM) and the related PKC inhibitor LY333531 (Figure 5, compound 1) as well as a number of extremely potent organometallic inhibitors with sub-nanomolar inhibitor potencies *in vitro* (compound 2).[118-121] Also flavonoids form multiple polar interactions with the hinge backbone and are potent inhibitors of PIM kinases (compound 3).[118,122]

The second class of PIM inhibitors does not interact with the hinge region by forming classical hydrogen bonds and can therefore be considered as ATP competitive but not ATP mimetic inhibitors. This non-canonical binding mode has been first identified for pyrazolo[1,5-a]pyrimidines and the related imidazo[1,2-b]pyridazine (compound 4) and LY294002 (*data not shown*), initially described as a potent phosphatidylinositol 3-kinase inhibitor.[116,118] One important aspect of this binding mode is the formation of polar interactions with the active site lysine (K67) and a conserved water molecule present in most co-crystal structures. These polar interactions anchor the inhibitor to the back of the ATP binding pocket. Typically inhibitor binding is additionally stabilized by a number of hydrophobic interactions. Imidazo[1,2-b]pyridazines have low nanomolar potency *in vitro* and display dose-dependently impaired survival of murine Ba/F3 cells that were made cytokine independent by overexpression of human PIMs.[75] Optimization of a promising imidazo[1,2-b]pyridazine lead compound ("sc-47") resulted in the generation of a highly potent compound (SGI-1776) with *in vitro* activity against PIM1, PIM2 and PIM3 at nanomolar concentrations. SGI-1776 impaired the growth of human leukemic cell lines (KG1, MV4;11 and MOLM13) at a sub-micromolar concentration *in vitro* and had biological activity in MV4;11 xenografts *in vivo*.[123] The most recent studies demonstrated that SGI-1776 induced apoptosis in chronic lymphocytic leukemia (CLL) cells as well as in prostate cancer cell lines.[124,125] Encouraging experimental results initiated clinical trials to explore the

safety of SGI-1776 for the treatment of refractory non-Hodgkin's lymphoma and prostate cancer patients (ClinicalTrials.gov Identifier: NCT00848601).

The promising results generated on imidazo[1,2-b]pyridazines led to the development of a number of other bicyclic scaffolds with nitrogen atoms present at different positions in the aromatic ring system such as N-substituted-3-aryl-[1,2,4]triazolo[4,3-b]pyridazin-6-amine inhibitors and triazolo-pyridazines (compounds 5 and 6).[126,127] Interestingly, Pierce *et al.* used a filter during the docking of compounds that searched for unusual aromatic CH – O bonds to the kinase hinge region instead of the typical ATP mimetic classical hydrogen bonds and identified a number of diverse inhibitors with Ki values between 0.091 and 4.5 μM. Docking studies also revealed indolyl-pyrrolones and pyridones (compound 7) as potent PIM inhibitors.[128,129] Another interesting scaffold that has been reported to inhibit PIM kinases and for which activity in cell based assays could be demonstrated are benzylidene-thiazolidine-2,4-diones (compound 8).[130]

In addition, a number of inhibitors have been identified that interact via halogen atoms with the PIM hinge region. These inhibitors include a series of pyrazine compounds (compound 9) as well as a series of isoxazolo[3,4-b]quinoline-3,4(1H,9H)-diones (compound 6).[131,132] Similar halogen hinge interactions have been reported for 4,5,6,7-tetrabromo-1*H*-benzimidazole, which also strongly inhibit CK2 and di-chloro-substituted β-carbolines (S Knapp, unpublished data, 2009 and pdb code: 3CXW).[133, 134]

An interesting aspect of many PIM inhibitors is the often observed selectivity of many inhibitors for PIM1 and PIM3 over PIM2, the latter usually being inhibited with much lower potency. Structural models are unable to explain this phenomenon that is likely to be related to differences in dynamic properties of the different PIM isoforms.[118]

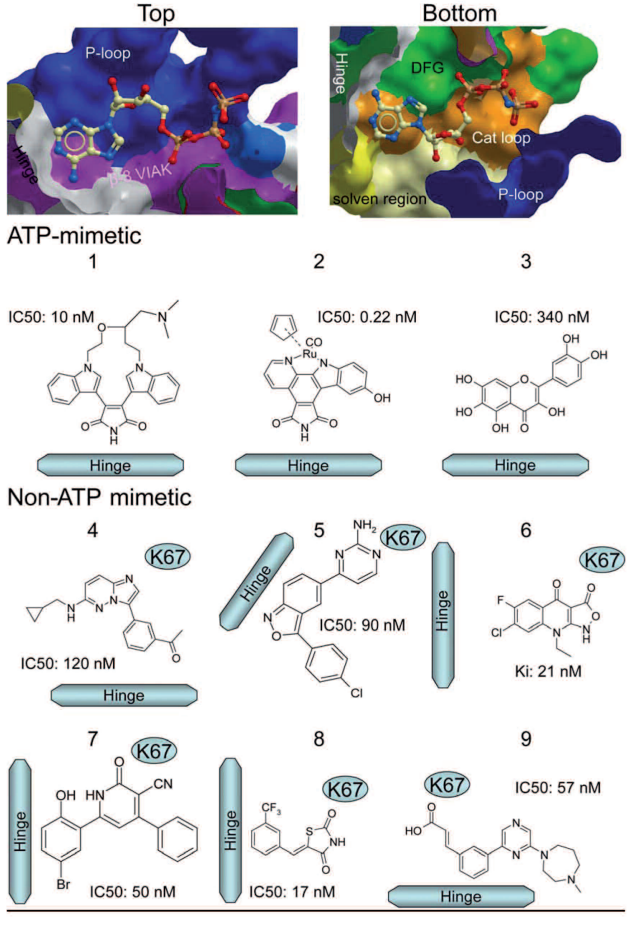

Figure 5. Classes of identified small molecule PIM kinase inhibitors. Structural elements contributing to the PIM1 ATP binding site are shown in the upper 2 panels. The binding surface of the upper lobe (top) and lower lobe (bottom) are shown. Contributions to the binding surface are colour coded: hinge (gray), P-loop (blue), beta 3 and VIAK motif (magenta), solvent region (yellow) and catalytic loop (orange). Examples of the main chemical classes of inhibitors are shown in the lower panel (see text for details). Inhibitors may interact with the kinase hinge region in an ATP mimetic (forming hydrogen bonds with the hinge backbone) or non-ATP mimetic way. The binding mode of each inhibitor is indicated by showing its orientation towards the kinase hinge region and the active site lysine (K67) which offers an alternative anchor point for non-ATP mimetic inhibitors. No experimental structures are available for the compounds 6, 7 and 8 and the shown binding mode has been determined by comparison with known PIM inhibitor complexes, so far unpublished structural data and docking.

Open questions and perspectives

Over 20 years have passed since the identification of PIM serine/threonine kinases as cooperating oncogenes. Several recent studies demonstrated that functional interference with PIM kinases impaired growth and survival of cancer cells. These findings initiated studies that aim to delineate the role of PIM kinases in cancer and its role as potential therapeutic targets. However, the functional redundancy of the three PIM kinases in cancer biology remains unclear. Do all three PIM kinases have proto-oncogenic activity in any cell type? Are PIMs important for maintenance of cancer-initiating cells (also referred to as cancer stem cells) escaping current chemotherapeutic strategies? A hallmark of normal and cancer-initiating cells is the capability of adapting to hypoxia.[135] Expression of PIM kinases is up-regulated by hypoxia and mediates hypoxic-induced drug resistance in cancer cells.[107] As PIM1 regulates the CXCL12/CXCR4 axis, inhibiting PIMs would not only affect survival but could also mobilize leukemic stem cells from their sanctuary niche.[82] Nevertheless, it is not known whether long-term inhibition of one PIM isoform might lead to a compensatory upregulation of other PIM family members. A potential compensatory mechanism suggests that inhibitors of PIM kinases should block all PIM kinases for efficient cancer therapy. Despite the high homology in the ATP-binding pocket, several proposed small molecule PIM inhibitors preferentially inhibit the activity of PIM1 and PIM 3 rather than PIM2. A potential limiting factor for chemical targeting of PIM kinases is the presence of mutations currently known to be present in B-cell lymphomas and Diamond-Blackfan anemia. Interestingly, a point mutation in PIM1 has recently been identified in a case of acute myeloid leukemia.[136] Massive parallel sequencing will help to identify genetic alterations of the kinome in all cancers. A recent study proposed that PIM kinases might be therapeutically targeted by monoclonal antibodies that recognized PIM1 located in the cytosol and nucleus as well as on the surface of human and murine cancer cells.[137] Interestingly, treatment of prostate cancer cell lines with this PIM1-specific antibody resulted in disruption of PIM1/Hsp90 complexes, reduced BAD phosphorylation

and activation of the mitochondrial apoptotic pathway. Although the exact molecular mechanisms of these effects of PIM antibodies remain to be elucidated, the substantial *in vivo* growth inhibition of prostate cancer xenografts and leukemia cells by application of the anti-PIM1 antibody not only confirmed PIM1 as therapeutic target but also suggested new avenues for therapeutic intervention.[137,138]

Heat shock protein (Hsp) mediates protection of PIMs from proteasomal degradation. This suggests a potential therapeutic interest of targeting aberrantly expressed PIM kinases by small molecule Hsp inhibitors such as 17-AAG/Geldanamycin-derivates.[9,10] However, whether the reported anti-leukemic activity of such compounds is also mediated by destabilization and degradation of PIM kinases has not been experimentally demonstrated.[139]

Functional characterization of signaling pathways that result up- and downstream of PIM kinases need to be studied in more detail in order to delineate combined therapeutic approaches. A complicating factor is that kinases important for the evasion of apoptosis such as PIMs, AKT/PKB, or SGK1, regulate cell death through modification of common substrates that affect the apoptotic machinery (e.g. BAD, NF-κB, FKHRL) and cellular metabolism (e.g. 4E-BP1, TSC2-mTOR, GSK3β).[140] PIM kinases have been shown to control mTOR inhibition (rapamycin) resistant proliferation and survival. Therefore, a combination of small molecule inhibitors targeting several survival kinases might be essential for a powerful cancer therapeutic platform.[91,140] Oncogenic protein synthesis through eIF4E binding protein 1 (4E-BP1) seems to be mTOR-independent and controlled by PIM2. These observations suggest that the oncogenic eIF4F translation-initiating complex could be blocked with small molecule PIM2 inhibitors.[91]

Transformation by leukemogenic oncogenes such as constitutive active tyrosine kinases is often mediated by parallel activation of several signaling pathways like JAK-STAT and PI3K-AKT/PKB. Interestingly, both PIM and AKT/PKB kinases show a selectivity for arginine at the -5 and -3 positions within substrates.[22] AKT/PKB and PIM kinases seem to play partly redundant roles in mediating growth and survival of hematopoietic cells most probably due to overlapping substrates like BAD, $p21^{WAF1/CIP1}$, $p27^{KIP1}$, or Cot/Tpl-2.[140,141] Using an unbiased phospho-proteomic approach, Choudhary and colleagues have recently not only identified 21 new potential PIM substrates, but also 8 novel proteins targeted by PIM and AKT/PKB.[142] Whether PIM1 acts up- or downstream of AKT/PKB might be context and/or cell type specific.[34,143] Nevertheless, the exact molecular relationship between PIMs and AKT/PKB remains to be elucidated.

Although transcriptional activation of PIM kinases is quite well understood, we know very little about upstream regulators that functionally modify PIM through posttranslational modifications. Identification and functional characterization of such pathways might also help understand how different isoforms of a PIM kinase (e.g. the long (p44) versus the short (p33) PIM1 isoform) meet their substrates that are located on the cell membrane, in the cytosol as well as in the nucleus. Interestingly, hypoxia seems to induce nuclear localization of PIM1.[144] Despite the many questions and obstacles which remain, we hope that collaborative research efforts will lead to the successful introduction of small molecule PIM inhibitors to the arsenal of targeted pharmacological weapons against human cancer.

Authorship and Disclosures

JS initiated this work and wrote the report. SK provided the section on structural features of PIM kinase inhibitors. All authors read, gave comments, and approved the final version of the manuscript.

The authors reported no potential conflicts of interest.

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