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Why target PIM1 for cancer diagnosis and treatment?

Nancy S Magnuson^{†,1}, Zeping Wang¹, Gang Ding², and Raymond Reeves¹

¹School of Molecular Biosciences, Washington State University, Pullman, WA 99164-7520, USA

²Medical College, Shanghai Jiaotong University, Shanghai, China

Abstract

The highly conserved proto-oncogenic protein PIM1 is an unusual serine or threonine kinase, in part because it is constitutively active. Overexpression of PIM1 experimentally leads to tumor formation in mice, while complete knockout of the protein has no observable phenotype. It appears to contribute to cancer development in three major ways when it is overexpressed; by inhibiting apoptosis, by promoting cell proliferation and by promoting genomic instability. Expression in normal tissues is nearly undetectable. However, in hematopoietic malignancies and in a variety of solid tumors, increased PIM1 expression has been shown to correlate with the stage of disease. This characteristic suggests it can serve as a useful biomarker for cancer diagnosis and prognosis. Several specific and potent inhibitors of PIM1's kinase activity have also been shown to induce apoptotic death of cancer cells, to sensitize cancer cells to chemotherapy and to synergize with other anti-tumor agents, thus making it an attractive therapeutic target.

Keywords

antiapoptotic; biomarker; cell cycle arrest; DNA repair; drug target; genomic instability; PIM1; serine/threonine kinase; SGI-1776

PIM1 kinase overexpression promotes tumor formation

The PIM1 protein belongs to a small group of three closely related mammalian serine or threonine kinases that are evolutionarily highly conserved and naturally constitutively active [1]. This latter characteristic is distinctive and means that, rather than having to be 'activated', as with most kinases, the level of PIM1 enzymatic activity in a cell is dependent on the absolute amount of protein present. PIM1 was originally identified from mouse lymphoma samples as a frequently activated gene resulting from the preferential integration of Moloney leukemia virus into the 3'-untranslated region of the *pim-1* gene [2]. This integration resulted in the insertion of a premature stop codon in front of a destabilizing A/U-rich motif in the *pim-1* transcript. The consequence was an unusually long-lived *pim-1* transcript, which allowed an increased level of translation of the transcript and hence higher levels of PIM1 protein in the affected cells [3]. The *pim-1* gene was later found to function as a proto-oncogene because, when overexpressed, it induced lymphomas in transgenic mice, albeit at a low frequency [2,4]. However, when *pim-1* was coexpressed in mice with

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[†]Author for correspondence: Tel.: +86 509 335 0966, Fax: +86 509 335 4159, magnuson@wsu.edu.

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the oncogene *c-myc*, 100% of the transgenic mice died of lymphomas *in utero* [5]. Thus, by itself, PIM1 is not a strong oncoprotein but when expressed with other proteins such as c-MYC, it exerts a potent synergistic transforming effect on cells, particularly when the functions of those proteins are involved in proliferation and cell survival [6].

Synergistic activity of PIM1 & c-MYC in prostate cancer development

While the synergistic interaction of PIM1 and c-MYC was determined early on as being a driving factor in lymphomagenesis, it was only much later that PIM1 was discovered to be highly expressed in a significant fraction of human prostate tumors in which c-MYC was also overexpressed [7,8]. PIM1 was also found to be overexpressed in c-MYC-driven transgenic mouse prostate tumors [9]. PIM1 overexpression also increased the tumorigenicity of human prostate cancer cell lines [10,11]. Most recently, using a tissue recombination model coupled with lentiviral-mediated gene transfer, it was found that neither PIM1 nor c-MYC alone was very oncogenic, but when overexpressed together, tumor development in mice was dramatic [12]. Importantly, it was determined that this synergistic effect is critically dependent on PIM1 kinase activity, which further underscores the idea that PIM1 could serve as an effective target in cancer treatment of those cancers in which *pim-1* is overexpressed.

Although the precise molecular mechanism for the oncogenic activity resulting from the coexpression of PIM1 and c-MYC is not completely understood, it has been shown that PIM1 interacts with and phosphorylates c-MYC and increases its half-life [13]. In addition, it has been demonstrated that overexpression of PIM1 enhances the transcriptional activity of c-MYC by acting as a cofactor for c-MYC [14].

PIM1 knockouts only have subtle effects

One of the attractive features of PIM1 as a drug target is that the knockout of *pim-1* in mice is not lethal nor does its absence induce any immediately obvious phenotype [15]. Loss of *pim-1* may be compensated for by *pim-2*, another member of the *pim* gene family, although not in all cases [16,17]. In one case where the PIM2 kinase did compensate, it appeared to contribute to cell survival, indicating that in some instances it functions similarly to PIM1 [18]. Other candidate compensatory kinases include PIM3, and some unrelated kinases such as PKA, PKB/Akt and PKC, all of which phosphorylate many of the same substrates as PIM1 because they recognize similar amino acid consensus sequences (Table 1). Although these kinases may share preferred phosphorylation consensus sequences with PIM1, their modes of regulation are dissimilar, their expression patterns differ in various cell types, and they are often activated by separate signal transduction pathways. The most important difference between these kinases and PIM1 is that they are constitutively expressed and require some sort of post-translational modification for activity. For example, PKC requires lipid second messengers (diacylglycerol) and phosphorylation for kinase activity [19]. PKA requires the second messenger cAMP and A-kinase anchor proteins for activity [20]. Akt requires the lipid phosphatidylinositol 3,4,5-triphosphate and multiple phosphorylations from an upstream kinase, PDK1 [21–23]. Therefore, the level of these kinases may be relatively high in cells without exhibiting a correspondingly high enzymatic activity. PIM1, on the other hand, is constitutively expressed at low levels but increased levels are rapidly induced in response to various stimuli including cytokines, hormones and a variety of stresses [24]. Unlike PKC, PKA and Akt, PIM1 is constitutively active. Therefore, the level of PIM1 kinase activity is dependent on the absolute amount of protein present in the cell [1].

Upon closer examination, the *pim-1*-knockout mice do exhibit one subtle but interesting phenotype: erythrocyte microcytosis [25]. Abnormally small red blood cells may suggest

that PIM1 has something to do with the development of the hematopoietic system, and the regulation of the cell cycle in these cells. This phenotype also suggests that in *pim-1* null cells the signal to go through mitosis may supersede the need to pause for growth signals and the doubling of the cytoplasmic volume. Therefore, the emerging picture of PIM1's role in the cell cycle is that it helps coordinate timing events through phosphorylation of regulatory proteins such as Cdc25A phosphatase [26], the cell cycle inhibitor, p21^{Cip1}/WAF1 [27] and p27^{Kip1} [28].

A second effect of knocking out *pim-1* is that it results in a lack of responsiveness of cells to growth-stimulating cytokines such as IL-3 and IL-7 [29]. After engaging their receptors, IL-3 and IL-7 induce a signal transduction cascade that promotes cell proliferation and/or survival. Murine myeloid cells in culture that are dependent on IL-3 for growth will die if this cytokine is withdrawn from the medium. One consequence of exposure of cells to IL-3 is an immediate (within 15–30 min) induction of *pim-1* transcription and message translation [30]. These seminal observations led Michael Lilly to suggest that one of the major functions of PIM1 is to act as a survival factor for cells exposed to adverse conditions. Later evidence strongly supporting this notion came from experiments demonstrating that forced expression of PIM1 allows factor-dependent cells to survive and proliferate in the absence of IL-3 [31].

In addition to promoting cell survival, analysis of mice with various combinations of all three family members of the PIM family knocked out revealed that both PIM1 and PIM3 make significant contributions to body size determination; mice lacking these two kinases have decreased cell numbers compared with their normal littermates [32]. Nevertheless, these small, double knockout mice are viable, healthy and fertile (although impaired cytokine signaling can be detected in their hematopoietic cells), indicating that PIM2 alone can compensate for most, but not all, of the activities of PIM1 and PIM3.

PIM1 is a cell survival factor

PIM1 phosphorylates, and regulates the activity of many proteins involved with cellular division, differentiation and apoptosis [33]. However, in terms of cancer a preponderance of evidence points to PIM1 playing a major role in the inhibition of apoptosis and the regulation of cell proliferation [34,35]. Owing to its link to cancer, the specific molecular mechanism by which PIM1 influences cell survival and proliferation have been the focus of much attention and research. The findings from these investigations have led to a concerted effort to develop therapeutics that directly target the kinase activity of PIM1 for cancer treatment. This aspect will be discussed in more detail below.

Available evidence indicates that high endogenous levels of PIM1 promote cell survival (Figure 1). From a historical perspective, it was observed more than 15 years ago that PIM1 overexpression protected cells from undergoing apoptosis whether induced by exposure to stress factors such as dexamethasone [35] or resulting from the removal of essential growth factors from cell culture medium [36]. The molecular basis responsible for PIM1's survival function remained elusive, however, until a key observation was made using the IL-3-dependent myeloid cells as mentioned above [30]. When these factor-dependent cells were deprived of IL-3, it was noticed that PIM1 overexpressing cells survived far longer than non-PIM1 expressing cells. Furthermore, this increased survival was found to be correlated with the maintenance of both mitochondrial transmembrane potential ($\Delta\psi_m$) and *bcl-2* mRNA levels. In the absence of PIM1 expression, not only did the cells exhibit reduced $\Delta\psi_m$ upon IL-3 deprivation, but they also exhibited lower *bcl-2* transcript levels and elevated production of harmful reactive oxygen species (ROS). The relative levels of Bcl-2 and PIM1 are important in helping maintain normal mitochondrial membrane potential and

low ROS levels was similarly demonstrated in experiments with IL-6 dependent B-cell hybridomas that were deprived of this cytokine growth factor [36]. It had long been known that apoptotic cell death induced by radiation, growth factor withdrawal, glucocorticoids and various kinds of chemotherapeutic agents is inhibited by increased levels of Bcl-2 [37–39]. What was not appreciated, until these experiments with IL-3- and IL-6-dependent cells, was that PIM1-promoted cell survival by influencing the Bcl-2 antiapoptotic pathway.

PIM1, Bcl-2/BAD & apoptosis

The first molecular mechanism proposed for how PIM1 contributes to cell survival was based on experiments demonstrating that PIM1 physically interacts with B-cell lymphoma-extra large (Bcl-xl)/Bcl-2-associated death promoter (BAD), a proapoptotic member of the Bcl-2 family. PIM1 phosphorylates BAD on Ser112, a residue that is known as the gatekeeper site for BAD inactivation [40]. Phosphorylation of BAD on Ser112 promotes its binding to 14-3-3 proteins and sequestration of the resulting phosphorylated BAD-14-3-3 complexes in the cytoplasm [41]. When BAD is unphosphorylated, it can heterodimerize with both Bcl-2 and Bcl-xl, members of an antiapoptotic protein family located in the mitochondrial outer membrane [42]. Under normal antiapoptotic conditions, Bcl-2 and Bcl-xl form heterodimeric complexes with BAX, a proapoptotic protein also found in the mitochondrial membrane, neutralizing its pro-apoptotic activity [43]. Unphosphorylated/unsequestered BAD can bind to Bcl-xl and Bcl-2 and displace BAX from these membrane complexes. The released BAX protein forms homodimers that create proapoptotic pores in the membrane that allow the release of cytochrome c from the mitochondria into the cytoplasm and the triggering of apoptosis [44]. Thus, as a consequence of residue-specific phosphorylation of BAD, PIM1 can promote cell survival by interfering with mitochondrial membrane-mediated apoptosis.

PIM1 expression is controlled by the JAK-STAT signaling pathway

Further evidence for PIM1 playing a role in promoting cell survival via the Bcl-2-BAD pathway comes from studies that evaluated the role of a novel small molecule inhibitor of JAK2. The study was initiated to understand how a constitutively active mutant of JAK2 promotes deregulated hematopoiesis [45,46]. JAK kinases phosphorylate the signal transducers and activators of transcription (STAT) family of proteins [47,48]. PIM1 is known to be induced at the transcriptional level by both STAT3 and STAT5 [49]. JAK2 inhibition with a specific drug results in decreased mRNA and protein levels of both Bcl-xl and PIM1 and also, as might be expected, inhibition of BAD phosphorylation at Ser112 [50]. These results suggest that malignant hematopoietic cells expressing PIM1 might be successfully treated with specific inhibitors of PIM1 and JAK2. It should also be noted that besides the increases mediated by activation of the JAK-STAT pathway, PIM1 levels can also be increased in cells by binding of the protein to the heat shock protein 90 (Hsp90), which increases PIM's half-life by preventing rapid degradation by the 26S proteasome [51,52]. Thus, as will be discussed, drugs that specifically inhibit PIM1 may also ameliorate some of the deleterious effects of stresses that activate heat shock proteins and possibly contribute to the malignant transformation of cells.

PIM1 levels can be increased by mutations in a number of proteins

FLT3-internal tandem duplication

As previously mentioned, and recently confirmed by a number of groups, PIM1 is important in hematopoietic tissue where its induction correlates with cytokine-enhanced cell survival [49,53,54]. Nevertheless, there is one noticeable example where cytokines do not appear to be involved in the upregulation of PIM1. Acute myeloid leukemia (AML) patients who have

point mutations within the internal tandem duplication (ITD) of the Fms-like tyrosine kinase (FLT3) receptor gene have constitutively active receptors and poor prognosis for survival. Stimulation of the wild-type FLT3 receptor results in the phosphorylation of STAT5, which, in turn, induces expression of PIM1 in cells. Constitutive signaling by the mutant FLT3-ITD receptor therefore strongly upregulates cellular PIM1 levels in the absence of cytokine stimulation, an increase that is further enhanced by the binding to, and stabilization of, FLT3-ITD by the stress-induced protein Hsp90 [55]. Consistent with these findings, it has been demonstrated *in vivo* that inhibition of the FLT3-ITD receptor by specific drugs results in a marked decrease in PIM1 levels in cells [56]. Since it has been demonstrated that FLT3-ITD, but not FLT3-wild type, is phosphorylated by PIM1 *in vitro* and because specific PIM1 inhibitors such as SGI-1776 (Supergene, Inc., CA, USA) are now entering clinical trials, experiments were recently performed to determine the consequences of drug inhibition of PIM1 on FLT3-ITD [57]. The results of these investigations demonstrated that inhibition of PIM1's enzymatic activity (or its expression) resulted in both decreased expression and stability of the FLT3-ITD receptor. Thus, PIM1 appears to help maintain FLT3-ITD by phosphorylating the mutant receptor protein and enhancing its binding to chaperones such as Hsp90, thus protecting it from proteasomal degradation. Inhibition of PIM1 had no effect on FLT3 kinase activity but decreased FLT3-ITD binding to, and activation of, STAT5 and, hence, the expression of PIM1 itself. From a clinical standpoint, it is also significant that a combination of the PIM1 kinase inhibitor quercetagenin and the FLT3 inhibitor PKC412 together has been demonstrated to have a synergistic effect in inducing apoptosis of FLT3-ITD positive cells [57].

BCR-Abl

In a similar scenario involving chronic myelogenous leukemia (CML), it was shown that the expression of PIM1 is induced by breakpoint cluster region (BCR)-Abl-activated STAT5 [58,59]. Although the precise mechanism by which *Abl* oncogenes transform hematopoietic cells has not been elucidated, it is known that PIM1 is highly expressed in *v-Abl* transformed cells and that PIM1 deficiency has a marked negative effect on the ability of *v-Abl* to transform bone marrow cells [60]. It has also been found that when PIM1 levels are high in cells expressing *v-Abl*, that the level of Bcl-x_s (a splice variant of the proapoptotic factor Bcl) is decreased as is the proapoptotic function of BAD. This situation contributes to apoptotic resistance of *v-Abl* cells even when they are treated with imatinib, a potent Abl inhibitor [61]. In fact, there have been several reports that PIM1 can enhance drug resistance and prolong the survival of cancer cells treated with chemotherapeutic drugs besides the examples discussed above [62,63]. This again strengthens the case for PIM1 being a useful target for cancer therapy. For example, T cells from *pim1*-knockout mice are very sensitive to the immunosuppressant rapamycin, whereas T cells from normal littermates, when treated with cytokines (e.g., IL-4 or IL-7) that induced PIM1, are resistant to rapamycin-induced apoptosis [64].

Survival induced by stress

Survival pathways are often activated by cellular stresses such as hypoxia and oxidative stress [65,66]. Indeed, it has been shown that hypoxia induces PIM1 expression, which promotes solid tumor growth [67,68]. In addition to hypoxia and oxidative stress, anti-tumor drugs can sometimes induce stress that results in enhanced tumor survival. A mechanism underlying such a response was determined for the anticancer drug docetaxel [69]. Docetaxel induced a survival response in the prostate epithelial cell line RWPE-2 by the activation of STAT3 that upregulated PIM1, which, in turn, mediated activation of nuclear factor (NF)-κB [70]. It is known that docetaxel induces an increase in ROS in cells treated with the drug [71]. Although a precise mechanism responsible for the increased cell survival was not elucidated, it is known that NF-κB regulates the expression of antiapoptotic

effectors such as Bcl-2, Bcl-xl, X-linked inhibitor of apoptosis protein (XIAP) and c-IAP2 [72]. In addition, apoptosis signaling kinase 1 (ASK1) is also activated by a variety of stress-related stimuli, including ROS [73]. In light of this, our laboratory has recently demonstrated that PIM1 phosphorylates ASK1 both *in vitro* and *in vivo* and inactivates its enzymatic activity [74]. Therefore, the fact that STAT3 induces PIM1 which, in turn, can phosphorylate both BAD and ASK1 and inhibit their proapoptotic activities, combined with the fact that PIM1 also leads to the induction of NF- κ B and the expression of a number of antiapoptotic proteins, further underscores PIM1 as a focal point for therapy to decrease the survivability of cancer cells.

Other PIM1 substrate proteins associated with cell survival

There are two other substrate proteins that PIM1 phosphorylates, which have the potential to promote survival of cancer cells. One is the proapoptotic transcription factor FOXO3a. It upregulates proapoptotic proteins such as BIM and FAS ligand to induce apoptosis [75]. Phosphorylation by Akt has been reported to trigger FOXO3a translocation from the nucleus to the cytosol resulting in its degradation via the double-minute chromosome 2 (MDM2)-dependent ubiquitin-proteasome pathway. Thus, removal of FOXO3a promotes cell survival when phosphorylated by Akt [76,77]. Recently, PIM1 has also been shown to phosphorylate FOXO3a on the same residues as Akt, which downregulates its transcriptional function [28]. Therefore, it is reasonable to assume that this phosphorylation event by PIM1 would also promote cell survival.

The other protein that PIM1 phosphorylates is MDM2, which functions as an E3 ligase [78,79]. MDM2 is overexpressed in many tumor types, including leukemia [80], lymphoma [81], breast carcinoma [82], non-small-cell-lung carcinoma [83], and colorectal cancer [84]. High levels of MDM2 have been linked to advanced and progressive cancer, poor response to chemotherapy and poor prognosis [85,86]. MDM2 targets multiple cellular protein for degradation, including pRB, HIF-1, p73, p53, NF- κ B, E3F1 and FOXO3a [76,87]. The tight association between endogenous MDM2 and PIM1 suggests a physiological role for this interaction [79]. Future investigation should clarify the significance of this observation.

Regulation of the cell cycle & proliferation

pim-1 induction correlates with initiation of proliferation

In addition to playing a substantial role in regulating cell survival, PIM1 also has been demonstrated to influence cell proliferation (Figure 2). The earliest indication that PIM1 might play a role in proliferation came from the analysis of the kinetics of transcriptional activation of PIM1 in factor-dependent myeloid cells [88]. This analysis suggested a role in the progression of the cell cycle from G₀ to S phase. When primary murine or bovine T cells were stimulated with either IL-2 or the mitogen concanavalin A, a marked increase in the synthesis of *pim-1* message was observed as the cells began to proliferate [89]. Not only have cytokines such as IL-2 been found to induce *pim-1* following stimulation of cells to proliferate, but also hormones such as prolactin [90,91]. A closer analysis of the various phases of the cell cycle using a synchronized myeloid cell line revealed that the levels of PIM1 increased during the progression of the cell cycle from early to late G1 and remained high throughout the G1/S boundary and G2 phases [92]. Therefore, PIM1 protein levels change with the stages of the cell cycle and appear to be related to the onset of DNA synthesis and commitment to proliferation.

Signaling pathways associated with *pim-1* induction & cell proliferation

Cross-linking the T-cell receptors on mature T lymphocytes induces signaling pathways that activate T cells to proliferate and produce cytokines [88]. One of the first signaling pathways

linking *pim-1* expression and proliferation involved the activation of the tyrosine kinase JAK2 when factor-dependent cells were stimulated to proliferate with erythropoietin [93]. It was later demonstrated that activation of STAT3, which is downstream of activated JAK2 in cytokine signaling pathways, also promotes *pim-1* upregulation and cell proliferation [94]. As with cytokine stimulation of hematopoietic cells, *pim-1* mRNA expression is rapidly induced by cross-linking the T-cell receptor with antibodies to CD3 [95]. Using PKC inhibitors and cells that were deficient in one or more of the PKC isoforms, it was demonstrated that T-cell antigen receptor activation results in *pim-1* expression, which is coupled to the PKC component of T-cell activation. Although PKC activation has been shown to activate Raf-1 activity and increase IL-2 expression [96], Raf-1 activation had no effect on *pim-1* expression eliminating that pathway in T cells for induction of *pim-1*. Interestingly, it appears likely that *pim-1* upregulation via T-cell receptor engagement results from the activation of the ϵ , but not the β , isoform of PKC [95]. Regardless of which isoform is involved, it is known that PKC activation is responsible for the activation of NF- κ B, which is required for cell proliferation and the production of cytokines [97,98]. In B cells, NF- κ B also plays a role in upregulating *pim-1*. CD40 engagement is known to regulate growth, survival, differentiation and immunoglobulin class switching in B cells and was demonstrated to upregulate PIM1 expression primarily via the activation of NF- κ B [99]. Inducing growth arrest of IgM expressing WEHI-231 cells by treatment with anti-IgM, also correlated with a substantial decrease in PIM1 levels, providing further evidence that PIM1 is involved not only in the regulation of survival, but also with the proliferation of B cells.

PIM1 target proteins involved with the cell cycle

Unraveling the molecular mechanism(s) contributing to PIM1's role in proliferation began when the first confirmed PIM1 substrate was identified (Figure 2). This was CDC25A, a cell cycle phosphatase [26]. Phosphorylation by PIM1 enhances its phosphatase activity and promotes transit of cells through the G1 and S phases of the cell cycle. The protein kinase CDC25C-associated kinase 1 (C-TAK1) was also found to be phosphorylated by PIM1 [100]. This phosphorylation event results in decreased C-TAK1 kinase activity, thereby reducing its ability to phosphorylate and inactivate CDC25C, a second phosphatase that actively promotes cell cycle progression at the G2/M phase. It was subsequently found that PIM1 also phosphorylates CDC25C, which enhances its phosphatase activity and promotes cell cycle progression [101].

High levels of PIM1 have been shown to mediate the phosphorylation and inactivation of the cell cycle inhibitors p21^{Cip1/WAF1} and p27^{Kip1}, thereby promoting progression through G1 and G2/M phases of the cell cycle [27,28,102]. This occurs in part by inhibiting the binding of proliferating cell nuclear antigen (PCNA) to p21^{Cip1/WAF1} [102]. PCNA is the processivity factor for DNA polymerase δ and ϵ . It is best known for its involvement in DNA synthesis and DNA repair [103]. The dissociation of PCNA from p21^{Cip1/WAF1} in the nucleus restores the function of the DNA synthesis machinery but also reduces the accuracy of DNA repair (Figure 3) [104]. In addition, PIM1 also binds to and phosphorylates the cyclin-dependent kinase inhibitor, p27^{Kip1} [28]. This promotes its binding to the 14-3-3 protein and the resulting complex is then exported to the cytoplasm where it undergoes proteasome-dependent degradation. Not only does PIM1 phosphorylation reduce the level of p27^{Kip1}, it also phosphorylates and inactivates the forkhead transcription factors, FOXO1a and FOXO3a, that upregulate the expression of p27^{Kip1} expression. Thus, PIM1 has many functions in promoting proliferation by regulating cell cycle progression at multiple points and through various target proteins.

PIM1 targets that influence mitosis

Besides influencing the cell cycle, PIM1 also regulates other facets of cell proliferation. One involves facilitating the function of the mitotic apparatus. In this connection, PIM1 displays a dynamic pattern of redistribution within the cell at different stages of the cycle, first being located in both the nucleus and cytoplasm during interphase and then rapidly translocating to the developing spindle poles during late G2 and metaphase [105]. Furthermore, PIM1 has been shown to phosphorylate the nuclear mitotic apparatus (NuMA) protein and associate in a complex with the motor proteins dynein, dynactin and heterochromatin protein (HP)1 β . An earlier report demonstrated that HP1 γ , an isoform of HP1, is phosphorylated by PIM1 [106]. Although members of the HP1 family of proteins are structurally similar, it is not known if PIM1 directly phosphorylates HP1 β [107]. A dominant negative form of PIM1 hinders the completion of mitosis indicating that PIM1 plays a vital role in mitosis. However, the precise role(s) that PIM1 might be playing in the mitotic complex has yet to be established.

Nevertheless, the consequences of overexpression of PIM1 in human prostate epithelial cells is an interference with the mitotic spindle checkpoint leading to polyploidy and chromosome mis-segregation [108]. It was also observed that PIM1-induced polyploidy occurs in a passage-dependent manner under *in vitro* culture conditions of prostate cell lines [109,110]. It appears that overexpression of PIM1 affects spindle dynamics by disrupting the normal function of the spindle complex, thereby contributing to genomic instability. Overexpression of PIM1 has since been shown to induce genomic instability in other cell lines as well, indicating that this is in part how PIM1 contributes to tumorigenesis [111].

Additional PIM1 targets that influence proliferation

PIM1 and PIM2, both members of the PIM kinase family, are highly homologous to one another and also share a unique hinge region sequence that provides constitutive enzymatic activity [32]. However, they do differ in their tissue expression [112]. Nevertheless, until recently, it was not clear to what extent these two kinases differ in terms of their substrate specificity. Evidence has now been obtained that suggests that both PIM1 and PIM2 have the same function when expressed in identical genetic backgrounds [113]. This finding is important because PIM2 has been shown to phosphorylate the ribosomal protein 4E-binding protein-1 (4EBP-1), causing it to dissociate from the eukaryotic translation initiation factor 4E (eIF-4E), thereby influencing the rate of protein synthesis [18]. This suggests that PIM1 most likely has the same function and effect on protein synthesis. With this notion in mind, in studies focused on determining why the prostate cancer cell line, PC3, transfected with PIM1 grew twice as fast as cells transfected with vector only, it was hypothesized that PIM1 might in part be phosphorylating proteins that were involved with protein synthesis such as 4E-BP1 [114]. What was found was that PIM1 transfected cells maintained their ability to phosphorylate 4E-BP1, thereby releasing eIF-4E to initiate translation of all available mRNAs. This finding suggests that PIM1 also has a significant positive impact on both protein synthesis and tumor cell growth.

With regards to cancer cell proliferation, under certain conditions PIM1 may play a role by negatively regulating transcription of the androgen receptor gene. For example, transfection experiments have demonstrated that androgen receptor is attenuated by PIM1 in a dose-dependent manner in PC3, HeLa and COS-1 cell lines [115]. In addition, in primary human prostate tumors, PIM1 is highest in hormone refractory tumors, which have reduced levels of androgen receptor, suggesting that PIM1 might be playing a role in creating an androgen-independent phenotype. These observations suggest that increased expression of PIM1 may be one of the determinants that leads to the transition of prostate carcinoma cells from an androgen-dependent to an androgen-independent state. However, arguing somewhat against this idea is the fact that overexpression of PIM1 induces increased transcription of the

androgen receptor gene in the PC3 and LNCap prostate cancer cell lines when they are grown in the presence of low levels of dihydrotestosterone [116].

PIM1 targets that negatively regulate its own gene expression

The family of suppressor of cytokine signaling (SOCS) proteins are inhibitory molecules whose expression is rapidly induced by cytokines. These proteins, in turn, downregulate cytokine signaling and STAT activation by interacting with active JAKs preventing the tyrosine phosphorylation of STAT proteins [117]. Interestingly, PIM1 is induced via STAT signaling pathways by several related cytokines including IL-2, IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) [88,118,119]. However, the induction of PIM1 also has a negative effect on cytokine signaling because PIM1 phosphorylates and stabilizes SOCS1 and SOCS3 making them more effective suppressors [120,121]. This negative feedback loop is thought to finely regulate STAT activity in response to cytokines during normal cell growth. It is hypothesized that constitutive expression of PIM1 together with other oncoproteins (e.g., c-MYC or BCR-Abl) might interfere with or impair this fine tuning regulation and therefore, contribute to cellular hyperproliferation and tumor formation.

PIM1: a potential diagnostic & prognostic marker

Soon after the discovery of the link between PIM1 and murine lymphoma, it was observed that overexpression of PIM1 also occurs in a significant fraction of human myeloid and lymphoid leukemias, as well as in lymphomas [122,123]. Investigators therefore began to look more closely at other types of cancers for evidence of increased expression of PIM1. Thus, over the past decade, data have been accumulating, which suggest that PIM1 might be a useful marker to distinguish between many normal and disease conditions. For example, the first identification of PIM1 as a potential biomarker in prostate cancer was made in 2001 when its level of expression assessed from 810 tissue microarray elements was shown to correlate significantly with the clinical outcome of cancer patients [7,124]. Clinically, tumor grade correlated with increased levels of PIM1 expression except at more advanced metastatic stages when some tumors did not have detectable PIM1 protein. The absence of PIM1 expression in these tumors correlated with poor prognosis. In another study, moderate-to-strong staining for PIM1 was observed in 68% of 121 prostate cancer specimens. When the Gleason score was 7 or higher, strong expression was observed in 76% of the tumors [8]. While staining was also found to be moderately strong in some prostatic intraepithelial neoplastic growths (107 out of 110), suggesting that presence of the protein was a potential early event in the development of prostate cancer, expression in normal tissue was not detected or, when it was, it was very weak [8,125]. Results from a more recent evaluation of *pim-1* mRNA levels in 145 cases of prostate cancer by quantitative real-time PCR (RT-PCR) and immunohistochemistry were consistent with these original observations [126,127].

Similar to prostate cancer, upregulation of PIM1 as assessed by RT-PCR in 51 cases of pancreatic intraepithelial neoplasia and pancreatic ductal carcinoma correlated with severity of disease [128]. In a recent study, both *pim-1* mRNA and protein were evaluated with quantitative RT-PCR, immunofluorescence and immunocytochemistry [68]. Compared with benign, inflammatory and premalignant conditions (0 of 10 specimens), expression of *pim-1* mRNA and protein was increased significantly in 22 of 35 pancreatic malignancies. Interestingly, similar to prostate cancer, absence of PIM1 in more advanced pancreatic tumors was also found to strongly correlate with a poor prognosis.

Head and neck squamous cell carcinomas are difficult to treat. In a study to determine if particular genes were being differentially expressed during tumor progression of head and

neck cancers, microarray analyses revealed that *pim-1* mRNA was expressed in such tumors [129]. Immunohistochemical analyses in this same study revealed that PIM1 protein was expressed in 98% (41 out of 42) of tumors of different grades and stages, but not in normal tissue; however, the pattern of expression did not appear to have any correlation with the stage or histological grade of the tumor. As the classic prognostic markers such as tumor size, nodal status or histological grade do not predict response of head and neck cancers to radiotherapy, and there are currently no generally accepted clinical markers predicting radiosensitivity, there was interest to re-examine PIM1 expression in such carcinomas. In a later study of head and neck cancers it was found that moderate-to-high PIM1 expression levels, 32 of 71 tumors, do actually tend to be associated with poor prognosis in comparison with tumors with low-to-negative PIM1 expression levels 29 of 71 tumors [130]. Importantly, PIM1 overexpression was significantly associated with a poor response to radiotherapy and, therefore, it was proposed that measuring its level in tumors could be of use in predicting prognosis. The most recent evaluation of PIM1 expression in 77 cases of squamous cell carcinoma demonstrated that its expression is increased in the more advanced and nodal stage tumors, further underscoring the possible use of PIM1 as a prognostic indicator [131].

A survival prediction model for surgery-related outcome for gastric cancer has been developed by selecting genes from gene expression array profiles [124]. The three genes selected were *pim-1*, *SLAM* and *CD36* and their expression measured in tumor tissue by semiquantitative RT-PCR. After comparing 18 pairs of cancerous and noncancerous tissue from patients undergoing surgery that survived greater than 30 months with patients who survived less than 12 months, it was found that expression patterns of these three genes could accurately predict surgery-related outcome in gastric cancer patients. More recently, cancer tissues from 117 patients with gastric cancer were evaluated immunohistochemically for PIM1 [132]. In this study, it was found that PIM1 immunoreactivity increased as the grade of malignancy increased, further emphasizing that PIM1 levels might serve as a tumor marker in gastric cancer.

It is also known that *Helicobacter pylori* is a major risk factor of gastric cancer and mucosa-associated lymphoid tissue lymphoma. A potential connection was made between *H. pylori* and gastric cancer when it was shown that the bacteria could induce *pim-1* in gastric epithelial cells [133]. This connection was made stronger when strains of *H. pylori* isolated from patients with gastric cancer were found to specifically upregulate the expression of *pim-1* when cocultured with gastric epithelial cells as determined by cDNA microarray analysis [134]. This suggests that *H. pylori*-induced *pim-1* might be involved in cancer production.

Another example of using PIM1 levels as a prognostic indicator involved an attempt to discriminate between two types of primary cutaneous large B-cell lymphomas, one of which has a 5-year survival of more than 95% and the other which has a 5-year survival of approximately 52%. From the expression profiles of lymphoma cells of 21 patients, it was found that the type of lymphoma with the poorer prognosis consistently had a high expression of *pim-1* mRNA [135]. In mantle cell lymphoma, gene expression profiling has suggested that the proliferation signature, which included PIM1, is an important predictor of clinical outcome. In a prospective Phase II cooperative group study of 102 patients, PIM1 protein expression was semiquantitatively assessed by immunohistochemistry [136] and found to be elevated in 72% of mantle cell cancer cases with higher levels correlating with poor patient prognosis.

There are many noninvasive diagnostic tests for colorectal cancer such as the serum tumor markers (e.g., CEA, TPA and CA199), and the fecal occult blood test. However,

approximately 50% of these tests were either false positives or false negatives [137]. A recent study demonstrated that it may be possible to use a simple serum screening test for autoantibodies to PIM1 that are produced during the development and progression of colorectal cancer [138]. From screening 8000 human proteins on commercial arrays with 20 sera from either colorectal cancer patients or healthy subjects, five immunoreactive antigens, PIM1, MAPKAPK3, STK4, SRC and FGFR4, showed the highest prevalence in cancer samples, whereas ACVR2B was more abundant in normal sera. Three of the antigens, PIM1, MAPKAPK3 and ACVR2B, were used for further validation in an ELISA-based test. These markers were confirmed for disease and the tests also strongly suggested that they could be used to diagnose colorectal cancer with high specificity and sensitivity.

PIM1 inhibitors

The potential for development of PIM1 selective inhibitors is enhanced by the crystal structure of PIM1, which has been recently solved by multiple groups [1,139–141]. These structural studies revealed that the PIM1 protein has a classical bilobal kinase domain architecture and, apart from a unique N-terminal peptide sequence, has all of the conserved secondary structure elements of typical protein kinases. However, the hinge region that contains the ATP-binding site has a novel architecture, containing an additional amino acid residue not found in other protein kinases. This residue, proline-123, is incapable of making hydrogen bonds with ATP owing to a lack of a key amide H-bond donor. This unique hinge architecture of PIM1 kinase suggests that very selective inhibitors can be identified using this structural difference. Indeed, over 50 potential PIM1 inhibitors have been selected but the proteome-wide specificity of these inhibitors is largely unknown [123].

Several different classes of PIM1 inhibitors have recently been reported, including ruthenium-containing organometallic complexes [142,143], bisindolylmaleimides [141], imidazo[1,2-*b*]pyridazines [144], pyridones [145], flavonoids [146,147], benzoisoxazoles [148], isoxazoloquinoline-3,4(1*H*,9*H*)-diones [149], 5-arylidene-2,4-thiazolidinediones [150], Cinnamic acids [151] and 3*H*-benzo[4,5]thieno[3,2-*d*]pyrimidin-4-ones [152]. Most PIM1 inhibitors work either as ATP competitors or ATP mimetic compounds (Table 2). In addition, several inhibitors, such as isoxazoloquinoline-3,4(1*H*,9*H*)-diones, interact via halogen atoms with the PIM1 hinge region. Most of the identified inhibitors are potent with IC₅₀ (K_i; half maximal inhibitory concentration) values in the submicromolar to nanomolar range. Several of the inhibitors have also been shown to impair the growth of cancer cell lines *in vitro* [144,153,154]. Importantly, it has recently been demonstrated that SGI-1776 (an imidazo[1,2-*b*] pyridazine compound; see below) both induces both apoptosis in chronic lymphocytic leukemia cells [57] and resensitizes chemoresistant cancer cells to taxanes [154]. Furthermore SGI-1776 was shown to be a fairly selective inhibitor of the PIM kinases in a broad scale kinase screen, although it is also noteworthy that SGI-1776 shows the most potent activity against PIM1 (IC₅₀ of 7 ± 1.8 nmol/l) compared with PIM2 (IC₅₀ of 364 ± 2.6 nmol/l) and PIM3 (IC₅₀ of 69 ± 9.2 nmol/l) [154].

SGI-1776 inhibition of PIM1 in overexpressing cells induces apoptosis

In vitro studies demonstrated that overexpression of PIM1 enhances tumor growth and protection from drug-induced apoptosis of cancer cells [155]. Therefore, considerable effort has gone into trying to find small molecules that specifically inhibit PIM1 activity at low concentrations in order to render PIM1 overexpressing cancer cells more susceptible to apoptotic cell death. In a recent preclinical study, one such inhibitor, SGI-1776 (SuperGen, Inc., CA, USA), was identified, which caused depletion of both pBAD (Ser112) and c-MYC, while inducing p27^{Kip1} and p21^{Cip1/WAF1} expression, in two cell lines (K562, a human chronic myeloid leukemia cell line; and HEL92.1.7, an erythro-leukemia cell line)

that contain high levels of PIM1 [155]. Treatment with SGI-1776-induced apoptosis in both of these lines as well as in cells that had been stably transfected with a constitutively active *JAK2* mutant gene, but not in cells with the wild-type *JAK2*. Similar effects of SGI-1776 were observed with primary cells derived from human CML patients and cells from individuals with myeloproliferative neoplasms. Treatment of the K562 and HEL92.1.7 cells with either AUY922 (a nongeldanamycin inhibitor of Hsp90; Novartis Pharma, Switzerland), or with the pan-histone deacetylase inhibitor panobinostat (Novartis Pharma) resulted in the disruption of the PIM1–Hsp90 association and, at the same time, promoted increased PIM1 binding to Hsp70, which led to proteasomal degradation of PIM1. Of particular importance for drug-targeting of PIM1, simultaneous treatment of overexpressing cells with both SGI-1776 and AUY922 caused greater depletion of pBAD (Ser112), c-MYC and Akt than either of the inhibitors alone and synergistically induced apoptosis in both K562 and HEL92.1.7 cells. Taken together, these preclinical findings indicate that simultaneous drug-induced depletion of both the level and activity of PIM1 acts synergistically to induce apoptosis in a number of different cancer cell types while leaving normal, PIM1 non-overexpressing cells unaffected. Clinical trials to use SGI-1776 for the treatment of refractory non-Hodgkin lymphoma and prostate cancer patients are currently underway.

Role of PIM1 kinase in therapeutic resistance

PIM1 has been demonstrated to be frequently upregulated in human prostate cancers strongly suggesting that it plays a role in the development and progression these malignancies. Previous studies have shown that the 44-kDa isoform of PIM1, in contrast to the 33-kDa isoform, can protect prostate cancer cells from apoptosis induced by chemotherapeutic drugs [156]. A subsequent study demonstrated that a member of the multidrug resistance protein family, ATP-binding cassette transporter BCRP/ABCG2, directly interacts with, and is phosphorylated by, the 44-kDa PIM1 isoform [157]. The dimerization or oligomerization of BCRP on the plasma membrane plays a critical role in its drug efflux activity [158]. Knocking down the level of expression of the 44-kDa protein in drug-resistant prostate cancer cells abolished multimer formation of endogenous BCRP and resensitized the resistant cells to chemotherapeutic drugs. This suggests that BCRP-mediated multidrug-resistant tumors may, in some cases, be treatable by drugs targeting the 44-kDa PIM1 isoform protein.

More recently, SGI-1776 was found to sensitize a number of drug-resistant human prostate cancer cell lines to chemotherapeutic agents by inducing G₁ cell cycle arrest and promoting apoptosis [154]. To determine if SGI-1776 could sensitize prostate cancer cells to therapy with taxane, the parental androgen-independent cell line 22Rv1 and the taxane-resistant cell line 22Rv1-T were tested. First, 22Rv1 was treated with a combination of SGI-1776 and paclitaxel. This resulted in a minor enhancement of taxane-sensitization of 22Rv1. However, treatment of the taxane-resistant cell line, 22Rv1-T, which overexpresses the multidrug resistance associated protein 1 (MDR1) with SGI-1776, resulted in restoring the sensitivity of the cells to paclitaxel. While it is not known what role PIM1 plays in this event, it was found that SGI-1776 does not alter MDR1 mRNA expression, nor does it inhibit MDR1 activity. The ability to overcome drug resistance mediated by MDR1 through pharmacologic inhibition of PIM1 is a novel finding. This further underscores the utility of PIM1 kinase inhibitors in sensitization of chemotherapeutic agents.

In addition to these chemical inhibitors, recent work from our group demonstrated that an anti-PIM1 monoclonal antibody (mAb) can function as an effective inhibitor of PIM1. Treatment of prostate cancer cell lines with this anti-PIM1 mAb lead to disruption of PIM1–Hsp90 complexes, reduced BAD phosphorylation and activated the mitochondria apoptotic

pathway [159]. The molecular mechanisms that underlie the anti-tumor effects of this anti-PIM1 mAb remain to be explored. Nevertheless, the substantial growth inhibition exhibited by this mAb on both prostate cancer xenografts and leukemia cells *in vivo* not only confirmed PIM1 as a valuable therapeutic target but also suggested new avenues for cancer treatment [160].

Conclusion

The overexpression of PIM1 in a variety of cancers indicates it can be employed as a reliable diagnostic marker for several types of tumors and, in some cases, can even serve as a prognostic indicator of clinical outcomes. In recent years, a variety of molecular mechanisms have been elucidated explaining how PIM1 overexpression contributes to tumor development and progression. The finding that elimination of PIM1 activity has no apparent effect on normal cells and yet is lethal to overexpressing cancer cells, makes PIM1 a potentially useful target for chemotherapy without the detrimental side effects that occur with most conventional treatments. With the identification of several small molecule inhibitors of PIM1 that are very potent and highly specific, there is a possibility that in the near future inhibitor cocktails can be formulated that target PIM1 and other oncogenes found to be upregulated in particular cancers as the basis for customized therapy for each individual patient.

Future perspective

Within the next 5–10 years, patients will receive customized treatment for cancer. Suspected malignancies will be analyzed by protein arrays to determine the protein profile of that tumor. Patients will then be given a cocktail of inhibitors that will target the proteins that are in elevated levels and known to be involved in tumor development. In a majority of cases, PIM1 will be one of the main targets, and other targets will likely include proteins such as Hsp90. Depending on the type of tumor, treatment may also include a low level, less damaging amount of chemotherapy because by reducing PIM1 activity tumors become far more sensitive to such therapy. The end result should be complete control of, if not elimination of, the malignancy without undue or debilitating side effects.

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■ of interest

■ ■ of considerable interest

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Figure 1. PIM1 is involved in a variety of mechanisms that result in the inhibition of apoptosis

Increased PIM1 expression in cells promotes cell survival and the increases of PIM1 can result from a variety of factors. These include cytokine and hormone stimulation, exposure to hypoxia and stress (e.g., heat shock and reactive oxygen species) and constitutively activated tyrosine kinases that result from translocations (e.g., BCR-Abl and FLT3-ITD) that cause continuous activation of STAT5. The high levels of PIM1 facilitate the phosphorylation of BAD on Ser112, which promotes its binding to 14-3-3 and sequesters it away from the mitochondria. PIM1 can also phosphorylate and inactivate the proapoptotic proteins ASK1 and FOX3a, and phosphorylate and stabilize MDM2, which maintains its E3 ligase activity on targets such as FOX3a.

BAD: Bcl-2-associated death promoter; BCR: Breakpoint cluster region; FLT: Fms-like tyrosine kinase; ITD: Internal tandem duplication; MDM: Double-minute chromosome; STAT: Signal transducers and activators of transcription.

Figure 2. PIM1 is involved in regulating the activity of a number of proteins associated with cell cycle progression

The proteins that PIM1 is known to phosphorylate are indicated in the diagram at the various stages of the cell cycle. Starting at the G₁ phase of the cell cycle, CDC25A is a dual specificity phosphatase that is required for progression from G₁ to the S phase of the cell cycle. PIM1 phosphorylates CDC25A, which promotes this activity. p21^{Cip1/WAF1} is a cell cycle inhibitor and inhibits the activity of cyclin E/CDK2 and cyclin D/CDK4 complexes, and thus functions as a regulator of cell cycle progression at G₁ and G₂/M phases. In some cells, PIM1 phosphorylation of p21^{Cip1/WAF1} results in the inactivation of p21^{Cip1/WAF1} by promoting its translocation to the cytoplasm. p27^{Kip1}, also a cell cycle inhibitor, binds to cyclin D either alone, or when complexed to its catalytic subunit CDK4. PIM1 phosphorylation of p27^{Kip1} inactivates its activity. CDC25C is a phosphatase that directs dephosphorylation of cyclin B-bound CDC2 and triggers entry into mitosis. CDC25C-associated kinase 1 (C-TAK1) contributes to the regulation of CDC25C phosphatase and the MAPK scaffold protein KSR1 *in vivo* through the generation of 14-3-3-binding sites. PIM1 phosphorylation of C-TAK1 reduces its kinase activity thereby reducing its ability to phosphorylate and inactivate CDC25C. NuMA is essential for mitotic spindle formation and changes its cellular localization with the progression of the cell cycle. NuMA is required for stabilizing and tethering the spindles to the poles and aligning them in a dynein–dynactin-dependent manner. PIM1 phosphorylates NuMA and associates with it and HP1 β , dynein and dynactin in a complex. However, when a kinase-dead form of PIM1 is introduced into cells, fragmentation of chromosomes is observed. HP1 β is a member of a family of small proteins thought to contribute to the transcriptional silencing of heterochromatin. PIM1 phosphorylation of HP1 γ , an isoform of HP1 β , was demonstrated to contribute to heterochromatin silencing. NuMA: Nuclear mitotic apparatus protein.

Figure 3. Potential contribution of PIM1 towards introducing mutations mediated by proliferating cell nuclear antigen in translesion DNA synthesis

High levels of PIM1 have been shown to mediate the phosphorylation and inactivation of p21^{Cip1/WAF1}, thereby promoting the progression through G1 and G2/M phases of the cell cycle. This occurs in part by inhibiting the binding of PCNA to p21^{Cip1/WAF1}. PCNA is the processivity factor for DNA polymerase δ and ϵ . It is best known for its involvement in DNA synthesis and DNA repair. The dissociation of PCNA from p21^{Cip1/WAF1} following phosphorylation of p21^{Cip1/WAF1} by PIM1 in the nucleus restores the function of the DNA synthesis machinery. However, it also reduces the accuracy of translesion DNA synthesis potentially leading to the introduction of mutations. This may be a contributing factor to chromosome instability that is observed in cell lines overexpressing PIM1.

PCNA: Proliferating cell nuclear antigen.

Table 1

Comparison of the preferred phosphorylation consensus sequences of kinases.

Kinase	Preferred consensus sequence	Ref.
PIM1	RXRHX-S/T-X	[140,161–163]
PKC	R/K-X-R/K-R/K-X-S/T-F-R/K-R/K	[164]
PKA	RX-S/T RRX-S/T	[20]
Akt	KRXX-S/T RXX-S/T RXXRXX-S/T	[21,165]

Table 2

Characteristics of PIM1 inhibitors.

Name of inhibitors	Mechanism of inhibition	IC ₅₀ (or K _i)	Ref.
Orgametallic complexes	ATP mimetic	10–25 nM	[142,143]
Bisindolylmaleimide	ATP mimetic	27 nM	[141]
Imidazole[1,2-b]pyridazines	ATP competitive	40 nM	[144]
Pyridones	ATP competitive	50 nM	[145]
Flavonoids	ATP competitive	340 nM	[146,147]
Benzoisoxazoles	ATP competitive	11 nM	[148]
Isoxazoloquinoline-3,4-diones	ATP competitive	2.5 nM	[149]
5-arylidene-2,4-thiazolidinediones	ATP competitive	13 nM	[150]
Cinnamic acids	ATP competitive	11 nM	[151]
Benzo-thieno-pyrimidin-4-ones	ATP competitive	0.5 nM	[152]
Anti-PIM1 mAb P9	Ab/Ag interaction	2.5–5 µg/ml	[159,160]

K_i: Binding affinity of inhibitor; mAb: Monoclonal antibody.