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Review

Potential roles for the PIM1 kinase in human cancer – A molecular and therapeutic appraisal

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ABSTRACT

In vitro experiments have shown the PIM1 kinase to have diverse biological roles in cell survival, proliferation and differentiation. In humans, PIM1 is often expressed in both normal and transformed cells. The PIM1 kinase is a true oncogene implicated in early transformation and tumour progression in haematopoietic malignancies and prostate carcinomas. It is associated with aggressive subgroups of lymphoma, is a marker of poor prognosis in prostate carcinomas and has been suggested to have a role in hormone insensitivity of prostate malignancies. PIM1 has a possible role in other carcinomas with 6p21 genomic alterations. On one hand, PIM1 (due to its role in malignancy) appears to be a promising target for drug development programmes but, on the other hand, the complexity of its molecular structure has posed challenges in the development of PIM1 inhibitors. In this review we discuss PIM1 expression in human tissues (including some new data from our laboratory), its role in human malignancies, as well as the possibilities and challenges in the development of target therapy for PIM1.

1. Introduction

The human *pim-1* (proviral integration site for Molony murine leukaemia virus, or MuLV) oncogene is localised on chromosome 6p21.2, a fragile site involved in certain leukaemias.[1] Its cDNA contains an open reading frame of 313 codons with 94% homology to the mouse counterpart. The RNA transcript is 2.9 kilobases (kb) long.[2]

The PIM1 protein is a serine/ threonine kinase.[3,4] Two ubiquitously expressed isoforms of human PIM1 protein (35 and 34 kda) have been identified.[5] *In vitro* human PIM1 autophosphorylates and exhibits phosphotransferase activity towards various exogenous substrates.[6] The crystal structure of PIM1 reveals that it is a constitutively active kinase; phosphorylation of PIM1 is not necessary for its kinase activity regulation but contributes to its stability.[7] Immunoperoxidase staining using

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monoclonal antibiodies have shown that PIM1 protein is predominantly located in the cytoplasm, although nuclear or nucleo-cytoplasmic patterns of localisation have been described.[8]

PIM1 is often expressed in both normal and transformed cells to different degrees. It is expressed in many cell lines derived from human lymphoid and myeloid malignancies, as well as in several human solid tumour cells. In humans, the *pim-1* oncogene is expressed in lymphoid and haematopoietic malignancies, in prostate malignancies, squamous cell carcinomas of the head and neck region, gastric carcinomas and colorectal carcinomas. PIM1 is a stress-response kinase which is regulated by cytokines,[9–11] growth factors,[12] hormones,[13] by conditions like ischaemia[14] and cellular hypoxia,[15] as well as by infective agents such as the Epstein–Barr virus[16] and *Helicobacter Pylori*.[17]

2. Background: Molecular functions of PIM1

PIM1 has been implicated in signal transduction and transcriptional regulation, as well as cell cycle regulation and survival. These biological functions have been well and extensively reviewed elsewhere, and are not within the scope of this review.[18,19] Here, we provide a very general biological framework, which we briefly expand upon if necessary when addressing the expression of PIM1 in individual tissue types.

The effect of PIM1 in signal transduction is mediated by several players. Adapter proteins SOC1 and SOC3 are involved in negative regulation of cytokine induced JAK-STAT signalling.[20] The nuclear adapter protein p100 (a PIM1 binding partner) is an activation factor of transcription factor c-Myb.[21] In addition, the NFATc protein is involved in relaying signals from T-cell receptors.[22]

Furthermore, several PIM1 substrates have been identified, adding to the evidence that PIM1 can regulate nuclear transcription. Indeed, HP1 (heterochromatin-associated protein 1) and PAP1 (PIM1 associated protein) function in transcriptional repression by the silencing of chromatin and regulation of mRNA splicing, respectively.[23,24]

The PIM1 positive activity in cell cycle regulation is driven by phosphorylation, which enhances the activity of cell cycle promoters such as Cdc25A (a G-1 regulator) and Cdc25c (a G2/M regulator), as well as the inactivation of cell cycle inhibitors such as $p21^{Waf}$ and C-TAK1.[25–27] Further associations with the nuclear mitotic apparatus protein complexes facilitate the progression through mitosis.[28] As a whole, this has a net result in accelerating the passage of cells from G2 into M-phase.

The role of PIM1 in apoptosis and survival is exercised by directly linking with bcl2 at different levels: enhancing the synergy of bcl2 with c-Myc in gp130 activated and STAT3 mediated pathways, as well as by phosphorylation and inhibition of the pro-apoptotic protein BAD.[29,30]

Overall, the molecular functions of the PIM1 are varied and fundamental in the biology of the cell, which may explain its diversified role in human neoplasia.

3. Human PIM1 and malignancy

Human PIM1 has multiple roles in tumourigenesis. It promotes early transformation,[12,16] cell proliferation,[5,31] and cell survival.[8,32,33] In addition, it may have a role in angiogenesis and vasculogenesis as a downstream effector of the VEGF-A/Flk1 pathway.[34] PIM1 expression is correlated with tumour aggressiveness[35,36] and is a marker of poor prognosis.[37] PIM1 expression can be predictive of tumour outcome following chemotherapy[38] and surgery,[39] and has been correlated with the enhanced metastatic potential of the tumour.[15] The following discussion delineates the role of PIM1 in specific tumour types.

3.1. *B-cell non-Hodgkin lymphoma and human PIM1*

Historically, the *pim-1* protooncogene was first identified as a common insertion site in Molony murine leukaemia virus (MuLV)-induced T cell lymphomas,[40] though in the human being it is predominantly associated with aggressive B cell lymphomas. It has been demonstrated that in B-cell, non-Hodgkin's lymphoma (NHL), BCL6 translocations involve immunoglobulin (*IG*) genes but also a number of non-*IG* loci as partners. PIM1 gene is one of the partner genes involved in non-*IG/BCL6* translocations.[41] Furthermore, gain of 6p21 is a genomic aberration frequently associated with B-cell NHL (occurs more frequently in diffuse large B-cell lymphoma (DLBCL) types). This gain has been shown to be an independent, negative prognostic factor in B-cell NHL. Sivertsen and colleagues reported that PIM1 is an amplification target, reflected with higher expression of PIM1 protein, and thus plays a role in the tumourigenesis of some cases.[42]

3.1.1. *Diffuse large B-cell lymphomas and human PIM1*

The *pim-1* oncogene is one of the target loci for an aberrant somatic hypermutation (ASHM) in DLBCL. PIM1 mutations have been detected in up to 50% of DLBCL cases in non-immunocompromised DLBCL patients.[43] Mutations have also been detected in cases of AIDS-associated non-Hodgkin lymphoma,[44] HCV-infected B-cell NHL patients,[45] primary central nervous system lymphomas (PCNSLs),[46] and extranodal DLBCL cases (Table 1).[36]

ASHM is a frequent and specific feature of aggressive lymphoma and it is rare or absent in indolent lymphomas. On comparative evaluation of the aberrant somatic hypermutation of PIM1 (ASHM PIM1) involving extranodal marginal zone B-cell lymphoma (mucosa associated lymphoid tissue - MALT - lymphoma) and extranodal DLBCL, mutations were found to be similar in both the groups i.e. affecting coding exons and leading to amino acid exchanges. However, the extranodal DLBCL revealed a higher frequency of ASHM.[36] Overall, this evidence implicates PIM1 in the pathogenesis of both the diseases and the evolution of MALT lymphoma into overt DLBCL. In addition, ASHM PIM1 has been observed in AIDS-negative PCNSL, but it is absent in AIDS-associated PCNSL. Though the sample size was small (n=4 in both the groups), the observation indicates a divergent role of PIM1 between CNS lymphoma of immunocompetent patients and AIDS-associated cases.

The majority of DLBCLs exhibit evidence of ongoing, high frequency ASHM of the coding sequence or the 5′ untranslated region (UTR) of the *pim-1* oncogene Those mutations involving the coding exons are predictive of change in the structure and, in some cases, the function of the PIM1 protein. Kumar and colleagues described the crystal structures and

Table 1 – SHM PIM1 in B-cell NHL

Study group	No. of cases studied	No. of cases with ASHM PIM1	NHL subtypes with ASHM PIM1				Ongoing activity of ASHM	Mutations affecting coding exons	Amino acid substitution
			DLBCL m/n (%)	BL m/n (%)	OTHERS[a] m/n (%)	MZL m/n (%)			
B-cell NHL[43]	89	12	12/39 (30.7)	0/10	0/10	NS	NS	Present	Present
AIDSassociated NHL[44]	39[b]	5	4/18 (22.2)	1/11 (9)	NS	NS	Detected	Present	Present
HCV infected NHL patients[45]	32	4	4/20 (20)	NS	0/12	0/3	NS	Present	Present
PCNSLs.[46]	10	5	5/10 (50)	NS	NS	NS	NS	Present	Present
Extranodal NHL[36]	35	15	10/18 (55.5)	NS	NS	5/17 (29.4)	NS	Present	Present
Spleenic & Nodal MZL[75]	50	2	NS	NS	NS	2/50 (4)	NS	NS	NS

NHL, non-Hodgkin lymphoma; ASHM, aberrant somatic hypermutation; DLBCL, diffuse large B-cell lymphoma; BL, Burkitt's lymphoma; MZL, marginal zone B-cell lymphoma; AIDS, acquired immunodeficiency syndrome; HCV, hepatitis C virus; PCNSLs, primary central nervous system lymphomas.

a Includes mantle cell Lymphoma; chronic lymphocytic leukaemia/small lymphocytic lymphomas; follicular lymphomas; extra-osseous plasmacytoma; multiple myeloma.

b Includes four AIDS-PCNSL (all primary samples), and six AIDS-PEL (primary effusion lymphoma) cell lines which were negative for ASHM PIM1. The rest were systemic AIDS-NHL.

kinase activity of mutant PIM1 kinases in DLBCL. The amino acid mutations occur on the surface of PIM1 and could potentially participate in protein–protein interactions. The observed effects on kinase activity were consistent with the predicted consequences of the mutation on the PIM1 structure.[47]

PIM1 is of predictive and prognostic importance as it is overexpressed in clinically aggressive subgroups of large B cell lymphoma. The activated B cell-like (ABC) nodal DLBCL (ABC DLBCL), a subgroup identified by gene expression profiling with a predominant expression of a plasma cell-like subset of the genes, shows high expression of PIM1.[42] Following multiagent chemotherapy, ABC DLBCL is known to have a significantly poorer 5 year survival than other subgroups of DBLCL.[48] Similarly, the gene expression profiles of 21 primary cutaneous large B-cell lymphomas (PCLBCLs) identified a PCLBCLs-leg group, characterised by ABC DLBCL-like phenotype, increased *pim-1* oncogene expression and frequent extra-cutaneous metastasis and poor survival.[37]

3.1.2. *Burkitt's lymphoma and human PIM1*

PIM1 is associated with Burkitt's lymphoma by its role in immortalisation and transformation of EBV infected B-cell lymphocytes and promoting survival of transformed cells. EBV infection upregulates PIM kinases which remains elevated in latently infected B cells. PIM1 enhances the activity of the viral transcriptional activator EBNA2[16] which, in turn, is able to activate transcription of several viral and cellular target genes through its acidic transactivation domain. PIM1 expression is absolutely essential for the EBV-induced immortalisation and transformation of primary B-cells.[49] In Burkitt lymphoma cells, PIM1-mediated modulation of apoptosis is dependent on the expression level and the nuclear localisation, regulating the nuclear protein HMDM2 in a concentration dependent fashion.[8] Mdm2 levels affect p53 mediated proapoptosis.[50]

3.1.3. *Mantle cell lymphoma and human PIM1*

PIM1 has a role in the transformation and progression of blastoid variant mantle cell lymphoma (MCL-BV) as it is more frequently upregulated in MCL-BV than in the common MCL.[35] The profile of genes upregulated along with PIM1 (CMYC, BCL2) indicates that the gp130-mediated signal transducer and activator of transcription 3 (STAT3) signalling pathway is involved in the transformation.[51]

3.2. *Leukaemia and human PIM1*

Initial claims for a PIM1 role in leukaemogenesis (as a possible target of the (6,9)(p21,q.33) translocation) are doubtful since the elevated levels of PIM1 mRNA in leukaemias carrying a t(6;9) are not a direct result of this gene rearrangement.[52] Nevertheless, levels of *pim-1* oncogene protein are elevated in many human clinical leukaemias, as well as in myeloid and lymphocytic cell lines.[53] Furthermore, PIM1 has recently been implicated in the regulation of normal haematopoiesis as well as in playing a role in leukaemogenesis in cooperation with Runx family genes. The Runx gene has a role in cell proliferation and differentiation, and its aberrant expression could be leukaemogenic. PIM1 kinase phosphorylates RUNX pro-

teins and enhances their transactivation activity, as well as Runx family transcription factors being novel binding partners and substrates for PIM1 kinase. The Runx1 gene is involved in *t*(12;21) and aberration in 6p21-23 (where PIM gene is located) has been demonstrated in *t*(12;21)-positive ALL.[54]

An immunoblotting analysis of chronic myelogenous leukaemia cell lines revealed that PIM1 increases during the progression from early to late G1 and G2 phases of the cell cycle,[5] suggesting a key role of PIM1 in the progression of the cell cycle. Furthermore, PIM1 has a role in leukaemia clinical progression; *FLT3* mutations are identified as one of the most frequent genetic abnormalities in acute myeloid leukaemia and are also observed in other leukaemias. Interestingly, constitutively activated FLT3 signalling upregulates PIM1 expression in leukaemia cells, and PIM1 plays a role in FLT3 mediated cell survival.[32,33] PIM1 has been suggested to work in a complementary fashion with the antiapoptotic protein A1 in the BCR/ABL-dependent leukaemogenesis, by promoting the proliferation of the BCR/ABL-transformed cells and the stimulation of cell cycle progression, as well as playing a key role in the BCR/ABL-mediated cell protection from apoptosis.[31]

3.3. *Prostate and human PIM1*

PIM1 is overexpressed in high grade prostatic intraepithelial neoplasia (HGPIN) compared to prostate cancer, suggesting PIM1 overexpression in HGPIN may be an early event in the development of prostate malignancy.[55] The overexpression of PIM1 in human prostate epithelial cells induces genomic instability by subverting the mitotic spindle checkpoint. Cells overexpressing PIM1 have abnormal mitotic spindles, centrosome amplification and chromosome misaggregation.[56] Furthermore, *in vitro* PIM1 represses androgen receptor activity in prostate cancer cells.[57] Together, this evidence has led to the postulation that the overexpression of PIM1 may be one of the determinants governing the transition of prostate carcinoma cells from an androgen-dependent to an androgen independent state, by attenuating androgen response.

Clinically, PIM1 expression in the prostate could be useful in distinguishing benign from malignant glands.[58] Other studies using monoclonal antibodies and applying immunohistochemical analysis on radical prostatectomy specimens have reported strong PIM1 expression in high grade prostatic intraepitelial neoplasia,[55,59] thus demonstrating its potential to be used in distinguishing benign prostate epithelium from HGPIN in pathological prostate tissue specimens.

The predictive importance of PIM1 has been highlighted in prostate cancer as decreased PIM1 expression correlated significantly with measures of poor outcome and was found to be associated with a higher cumulative rate of PSA failure and a strong predictor of PSA recurrence.[58] However, the prognostic significance of PIM1 in prostate cancer is still controversial, as one study has reported a higher PIM1 expression level in prostate carcinomas with higher Gleason score[55] while others failed to observe this difference.[59]

3.4. *Squamous cell carcinoma and human PIM1*

One of the integration sites for the HPV16 virus, as detected in a squamous cell carcinoma cell line from a tongue-base tumour, is 6p21.2. The 6p21.2 junction sequence mapped 21 kb upstream from the *pim-1* oncogene[60]

PIM1 expression is correlated with increased differentiation of epidermal keratinocytes as evidenced by its higher expression in well differentiated squamous cell carcinoma cell lines than the one with less differentiation in culture.[61] Thus, poor differentiation, a feature of most head and neck squamous cell carcinoma (HNSCC), also correlates with PIM1 downregulation in cell lines and in human oral squamous cell carcinoma tissue by comparative gene profiling.[62,63]

3.5. *Gastro-intestinal tumours and human PIM1*

Sepulvada and colleagues studied gene expression profiles of *H. pylori* infected gastric epithelial cells. PIM1 was up-regulated by *H. pylori* infection of the AGS gastric cancer cell line in coculture experiments. As PIM1 is also expressed in normal gastric mucosa and in the AGS gastric carcinoma cell lines, it is suggested that PIM1 may be implicated in *H. pylori* pathogenesis, in *H. pylori* gastritis and potentially, as an oncogene, in gastric carcinogenesis.[17]

PIM1 has a predictive value in patients with surgically curable gastric cancer. Chen and colleagues developed a survival prediction model on the basis of three genes extracted from full-genome array studies, namely those encoding PIM1, CD36 and SLAM (signalling lymphocytic activation molecule) where the semiquantitative RT-PCR gene expression profiling of these three genes could accurately predict surgery-related outcome in gastric cancer patients.[39]

3.6. *Pancreas and human PIM1*

PIM1 has a role in the development of pancreatic ductal adenocarcinoma as evidenced by *in vitro* experiments with cell lines and the gene expression profiling of clinical samples of pancreatic cancer. PIM1 is upregulated in KRAS-expressing human pancreatic duct epithelial cells.[12] Oncogenic activation of the *KRAS* gene occurs in >90% of pancreatic ductal carcinoma (PDCA) and is found early in the Pancreatic Intraepithelial Neoplasia (PanIN) - carcinoma sequence. Buchholz and colleagues reported upregulation of PIM1 in PanIN and PDCA in a progressive manner.[64]

3.7. *Gain of 6p in solid tumours and PIM1*

Gain of 6p with a minimum overlapping region at 6p21 (containing *pim-1*) has been observed in intrahepatic cholangiocarcinoma (ICC)[65] and non-small cell lung carcinomas (NSCLC).[66] The gain of 6p21 in NSCLC is associated with poor survival and has been shown to be an independent predictor of poor outcome, suggesting a role of PIM1 as a prognostic marker which needs to be validated further (Table 2).

3.8. *Protein expression in solid tumours and PIM1*

In an effort to explore the involvement of PIM1 in solid tumours further, we performed immunohistochemistry in two main cancer types, namely colon and breast adenocarcinomas. The results, with a newly developed monoclonal

Table 2 – Genomic alterations involving 6p21

Study group	Gain	Significance
t(12;21)-positive ALL patients.[54]	Aberration in 6p21-23	PIM1 role in leukaemogenesis in association with Runx1 gene involved in t(12;21)
B-cell lymphoma cell line U698; 93 cases of B-cell NHL; another set of 35 DLBCL.[42]	PIM1 included in amplified region of 6p	PIM1 gain reflected as high expression in U698 cells but not in DLBCL cases
94 cases of B cell NHL.[76]	Gain of 6p21 more frequent in DLBCL	Independent negative prognostic factor
33 cases of ICC.[65]	Gain of 6p with minimum overlapping region at 6p21	Possible role of PIM1 in ICC oncogenesis
HPV infected squamous cell carcinoma (SCC) cell line of tongue base tumour[60]	Viral integration site at 6p21	Junction sequence mapped 21 kb upstream from *pim-1* oncogene
50 cases of NSCLC[66]	Gain of 6p21 in seven cases	Poor survival and independent predictor of poor outcome

Table 3 – Immunohistochemistry expression of PIM1 monoclonal antibody

Type of tissue	Number samples	PIM1 expression	Localisation	Percentage of expression
Normal colon	47	+	Goblet cells	100%
Colorectal carcinoma (CRC)	90	+	Cytoplasmic	12 % (11/90 CRC)
Normal breast with ductal hyperplasia (DH) and/or apocrine proliferations	6 sections showing cancer and adjacent breast tissue	+/-	Cytoplasmic expression in all lesions	6/6 DH1/1 Apocrine cyst1/1 Apocrine
Invasive ductal carcinoma	11 sections that included all possible permutations of ER/PR status	-	Nil	0/11

antibody (clone 19F7; MBL) and a standard protocol involving microwave-based antigen retrieval in Tris-EDTA at 98 °C for 40 min, are shown in Table 3 and Fig. 1. Prostate cancer in a TURP section with background nodular hyperplasia was used as both positive and negative control to optimise the antibody staining, as recommended elsewhere.[59]

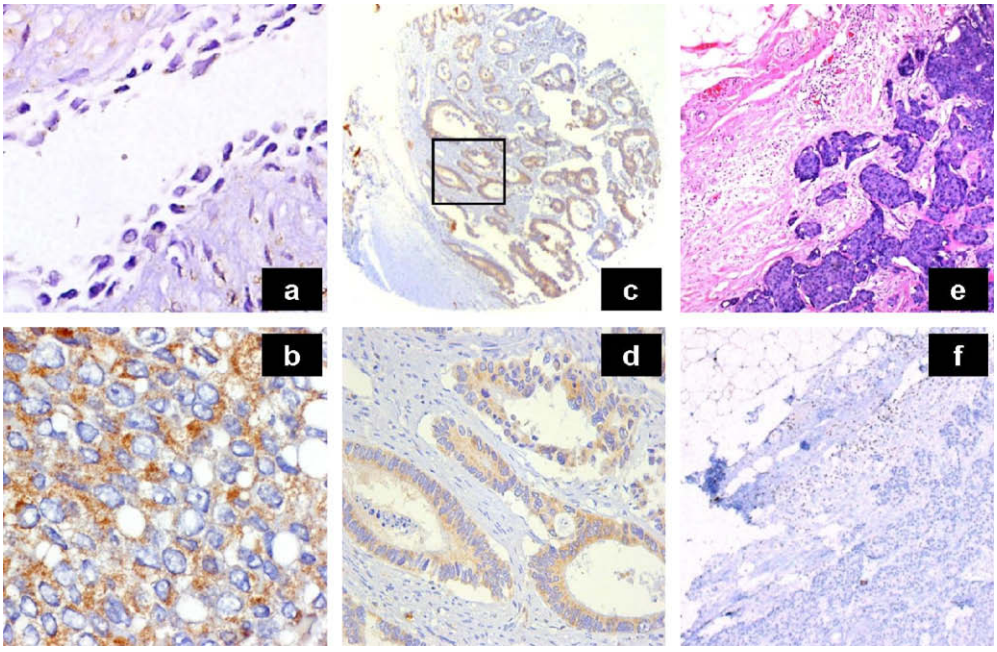

Fig. 1 – Immunohistochemistry expression of PIM1 monoclonal antibody. (a) Negative PIM1 expression in normal prostatic epithelium (IHC, ×600); (b) Positive PIM1 expression in prostatic adenocarcinoma (IHC, ×600); (c) View of colorectal cancer TMA punch with positive PIM1 expression (IHC, ×20); (d) Highlighted area in 1c (IHC, ×600); (e) View of high-grade, invasive breast ductal carcinoma (HE, ×40); (f) Same view, with negative PIM1 expression (IHC, ×40).

This preliminary analysis shows a lack of PIM1 expression in breast cancer and, interestingly, clear PIM1 expression in a subset of colorectal carcinomas. These are findings with potential clinical implications in view of the role of PIM1 inhibitors, briefly reviewed below.

4. PIM1 inhibitors

4.1. *Significance of PIM1 inhibitors*

It is debatable whether PIM1 is a key gene or a downstream player in cancer development and progression. In addition, a full action over the main members of the family (PIM1 and PIM2) may be necessary in order to achieve a strong therapeutic action. In any case, both the serine/threonine kinase nature of PIM1 and the compelling evidence of its involvement in various cancers have made it a promising new drug target.

DLBCL is one of the commonest lymphomas and in up to 50% of these lymphomas PIM1 is affected by ASHM. However, it must be highlighted that DLBCL with ASHM PIM1 are etiologically diverse (AIDS-associated, HCV infected patients etc.) and occur at different locations (nodal, GI lymphomas, PCNSLs). Thus, it is important to characterise the downstream molecular effects of each ASHM PIM1 subtype to delineate the exact role of PIM1 in the pathogenesis of DLBCL, and to help formulate the molecular basis of a more specific role of PIM1 inhibitors in this type of lymphoma. As mentioned before, the ABC subtype of DLBCL carries a poor outcome to treatment and has a high expression of PIM1.[42] Inhibitors of IkappaB kinase (IKK) are toxic to ABC DLBCL cell lines and also downregulate the PIM1 gene.[67] These observations raise the possibility that combination of PIM1 inhibitors with inhibitors of IkappaB kinase may yield interesting results and should be further explored.

PIM1 may also represent a new candidate for inhibition of angiogenesis as it is a downstream effector of VEGF-A/Flk-1 mediated angiogenesis and vasculogenesis.[34] The inhibition of VEGF-A/Flk-1 signalling has proved to be effective in limiting the angiogenesis implicated in tumour progression.[68] Related to this effect is the fact that PIM1 kinase activity has been shown to be important in the pathogenesis of vascular smooth muscle cell (VSMC) proliferation in vessel injury models and in proliferation of VSMC under oxidative stress. PIM1 expression was observed in the smooth muscle cells of thickened intima of human aorta in patients with atherosclerosis.[69] Thus, PIM1 kinase inhibitor molecules could be useful for suppressing VSMC proliferation in atherosclerosis which eventually leads to occlusive vascular diseases.

4.2. *Challenges and progress for PIM1 specific kinase inhibitors*

PIM1 is unique among protein kinases due to the absence of the canonical hydrogen bond donor in the hinge region that is a key element for the binding of many kinase inhibitors. Due to this structural peculiarity, other kinase inhibitor classes may bind only weakly to the PIM family of kinases.[47] The earliest reported inhibitor of PIM1 function is LY294002, which was originally identified as a specific PI3K inhibitor.[70] Seventy co-crystal structures of low molecular mass, low-affinity compounds with PIM1, have been solved in order to identify novel chemical classes as potential PIM1 inhibitors.[47] Recently, ATP competitive small-molecule inhibitors of the PIM1 kinase with growth inhibitory activity against leukaemia and prostate cancer cells have been reported.[71,72] Pogacic and colleagues reported a family of imidazo[1,2-b]pyridazines which specifically interact with and inhibit PIM kinases with low nanomolar potency. This family of small molecules significantly suppressed *in vitro* growth of leukaemic blast cells.[71] LY333′531 (ruboxistaurin), a PKCβ inhibitor being used in trials to treat diabetic complications, has been found to efficiently inhibit PIM1 and *in vitro* experiments suggest its potential use in acute myeloid leukaemia with *FLT3* mutations.[72] Quercetagetin has been reported to be a moderately potent and selective cell permeable inhibitor of PIM1 kinase which is able to inhibit PIM1 activity in prostate cancer cells in a dose dependent fashion.[73] A series of substituted pyridone molecules was identified from a high throughput screen as potent inhibitors of PIM1 kinase; they may serve as a useful starting scaffold for the development of other improved, yet selective, PIM1 inhibitors.[74]

Conflicts of interest statement

None declared.

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