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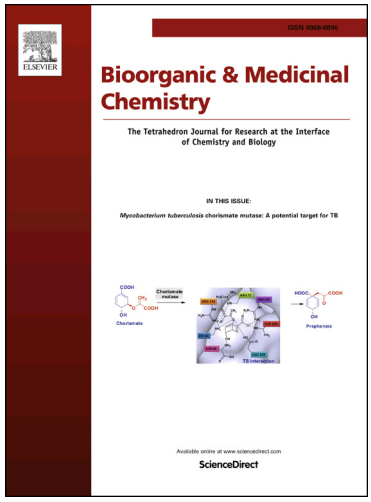

Thiazolidine derivatives as potent and selective inhibitors of the PIM kinase family

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PII: S0968-0896(16)31421-3
DOI: http://dx.doi.org/10.1016/j.bmc.2017.02.056
Reference: BMC 13586

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 20 December 2016
Revised Date: 23 February 2017
Accepted Date: 25 February 2017

Please cite this article as: Bataille, C.J.R., Brennan, M.B., Byrne, S., Davies, S.G., Durbin, M., Fedorov, O., Huber, K.V.M., Jones, A.M., Knapp, S., Liu, G., Nadali, A., Quevedo, C.E., Russell, A.J., Walker, R.G., Westwood, R., Wynne, G.M., Thiazolidine derivatives as potent and selective inhibitors of the PIM kinase family, *Bioorganic & Medicinal Chemistry* (2017), doi: http://dx.doi.org/10.1016/j.bmc.2017.02.056

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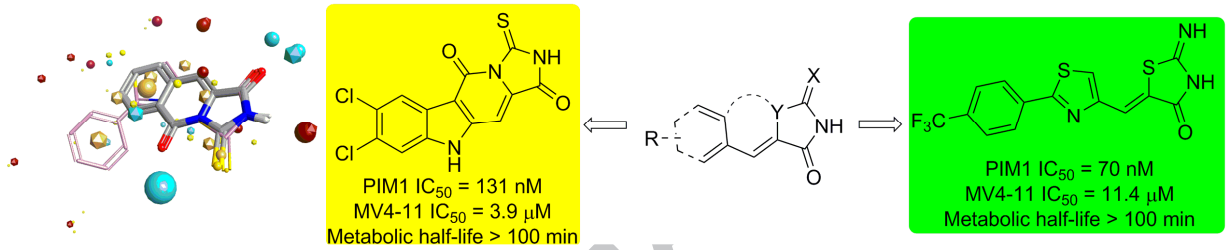

Bioorganic & Medicinal Chemistry
journal homepage: www.elsevier.com

Thiazolidine derivatives as potent and selective inhibitors of the PIM kinase family

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ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
Thiazolidine
PIM kinase
Anti-cancer
High throughput screen
Kinase inhibitor

ABSTRACT

The PIM family of serine/threonine kinases have become an attractive target for anti-cancer drug development, particularly for certain haematological malignancies. Here, we describe the discovery of a series of inhibitors of the PIM kinase family using a high throughput screening strategy. Through a combination of molecular modelling and optimisation studies, the intrinsic potencies and molecular properties of this series of compounds was significantly improved. An excellent pan-PIM isoform inhibition profile was observed across the series, while optimised examples show good selectivity over other kinases. Two PIM-expressing leukaemic cancer cell lines, MV4-11 and K562, were employed to evaluate the *in vitro* anti-proliferative effects of selected inhibitors. Encouraging activities were observed for many examples, with the best example (**44**) giving an IC_{50} of 0.75 μM against the K562 cell line. These data provide a promising starting point for further development of this series as a new cancer therapy through PIM kinase inhibition

1. Introduction

Most eukaryotic cellular processes and cell signaling pathways are regulated by protein phosphorylation mediated by protein kinases and phosphatases.[1] Protein kinases are the largest enzyme superfamily involved in cell signal transduction.[2, 3] It has been established that protein kinases and protein phosphatases represent compelling therapeutic targets for a range of diseases, including leukemias, tumors, cardiovascular diseases, diabetes mellitus and immune/ inflammatory disorders.[4, 5] Kinases are divided into those that phosphorylate serine or threonine residues (388 kinases) and those that phosphorylate tyrosine residues (90 kinases). Atypical kinases are proteins reported to have biochemical kinase activity but lack sequence similarity to the conventional eukaryotic kinases.

The three PIM kinases comprise a small family of serine/threonine kinases regulating several signaling pathways that are fundamental to cancer development and progression. Pim genes were originally identified as oncogenes in the early 1980s.[6] They form an independent branch of the kinase family tree, and are related to the CAMK (calcium/calmodulin kinase) super-family. The *Pim 1* gene was identified initially in 1987,[7] as a pro-viral insertion site for the Moloney Murine Leukemia ~~Virus (~~MoMuLV).[8] Follow-up experiments involving *Pim 1* knock-out models in mice led to the discovery of the two other family members, *Pim 2* and *Pim 3*.[9],[10]

The three family members have six different isoforms from alternate translation initiating sites. All PIM kinases are constitutively active.[11, 12] Their expression is mediated by the JAK/STAT signalling pathway, which is activated by various cytokines and hormones.[13] PIM kinases are broadly expressed in many cell lineages, as well as their corresponding progenitors and also embryonic stem cells.[14, 15]

The expression of *Pim* genes in human cancer has been studied extensively since their discovery as oncogenes in the 1980s;[16, 17] they were first implicated in human acute myeloid leukemia (AML) cases, and have now been found to be over-expressed in many different types of malignancies including hematologic and solid tumors.[13, 18, 19] The PIM kinases have a variety of downstream targets that are thought to contribute to tumour growth and survival. In particular, PIM kinases target the pro-apoptotic Bcl-2-associated death promoter (BAD) family members and inhibit apoptosis.[20] In addition, it has been shown in prostate tumours that PIM1 and c-MYC associate together, resulting in transcriptional upregulation and stabilization of c-MYC; such prostate tumours exhibited higher Gleason scores and are poorly differentiated.[21] Furthermore,

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selective and pan-PIM inhibitors may offer other therapeutic opportunities. In addition to cancer, PIM1 kinase has been described to be over-expressed in an array of diseases;[22-25] it has also been reported to play a role in several autoimmune diseases, mainly in inducing and increasing inflammatory responses.[26] PIM1 kinase has also shown to exert potent cardioprotective effects in the myocardium downstream of AKT, and protect mitochondrial integrity in cardiomyocytes;[27-29] PIM1 could therefore be of high interest in regenerative medicine therapies.

Crystal structures for both PIM1 and PIM2 have been used to understand their unique ATP binding pocket and for computational and medicinal chemistry efforts to develop inhibitors.[30, 31] The hinge region of PIM kinases is unusual as it contains a proline residue, which is a component atypically present in serine/threonine kinase hinges, as well as other unique residues in the ATP binding cleft.[32] The backbone nitrogen required to donate a hydrogen bond to the adenine ring of ATP is therefore absent and conventional ATP mimetic kinase inhibitors are not optimized for this site. This particular feature reduces affinity for ATP, and could therefore lead to the discovery and development of inhibitors selective to PIM against other kinases.[33, 34]

PIM kinases are highly homologous at the amino acid level (PIM1 and PIM2 are 85% identical; PIM1 and PIM3, 93%),[35, 36] yet differ partially in their tissue distribution.[37] Functional redundancy of the three PIM kinases has been shown *in vitro*[38, 39] and *in vivo*[40]. This characteristic can be used advantageously in the development of pan-PIM inhibitors.[41]

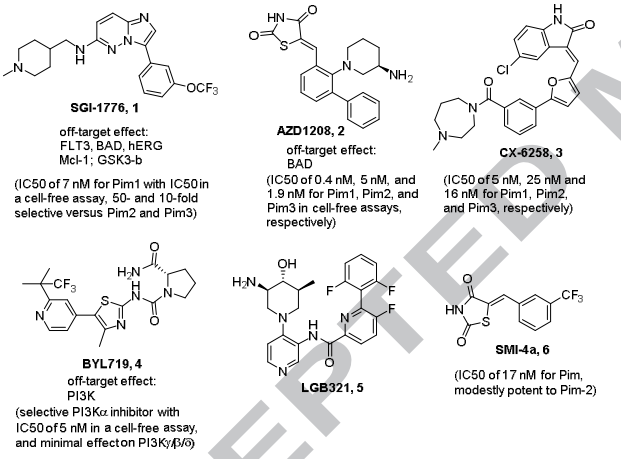


Figure 1. Representative reported PIM inhibitors

Several investigations have reported novel competitive PIM inhibitors, including SGI-1776,[42] AZD1208,[43] (AML) BYL719,[44] CX-6258,[45] SMI-4a,[46] LGB321 (multiple myeloma, MM), which have advanced to clinical trials.[47-56] Those compounds have had various outcomes; SGI-1776 clinical trials were discontinued in phase 1 due to dose limiting toxicity of cardiac QTc prolongation; additional cardiac and pharmacokinetic data evaluation has failed to demonstrate a safe therapeutic window to prudently continue clinical development of this molecule. Phase 1 trials were completed for AZD1208, but the study was terminated whilst the drug was being tested for safety, tolerability, pharmacokinetics and efficacy in AML patients; the reason was not disclosed.[57] BYL719 and LGH447's Phase 1 trials have now finished and those compounds are now ongoing phase 2 trials. Those examples show that non-selective PIM inhibitors likely represent the way forward toward finding the appropriate tool to treat PIM-dependent cancers.

This paper describes efforts towards the identification and preliminary optimization of pan-PIM inhibitors using *in silico* modeling.

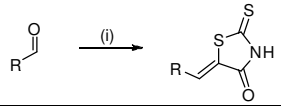
2. Results and Discussion

Our in-house library of ~20000 drug-like compounds was screened using a high-throughput differential scanning fluorimetry (DSF) assay, against the recombinant PIM1 enzyme.[58] Inhibitors were screened at 10 µM and a threshold thermal shift (Tm) value of >3 °C was defined as the minimum. Active compounds were identified and divided into eleven distinct structural classes. DSF assay of the most active 40 compounds against recombinant PIM2 isoenzyme was used to establish their 'pan-inhibitory' properties (Tm value of >2 °C) and multiple families of small molecules were identified. A coupled kinetic assay was used as a secondary orthogonal assay to determine IC_{50} values of the hits compounds against PIM1 and rank their activity.[59] Amongst those, the thioxothiazolidinedione (TTZD) family indicated consistent activities against PIM1 and PIM2 isoenzymes.

Structurally related rhodanine containing compounds have been identified as hits against a diverse range of drug discovery targets in multiple therapeutic areas, and despite the fact that they have attracted considerable attention and commentary relating to their viability as starting points for medicinal chemistry over recent years,[60-62] examples in drug discovery are well precedented (Figure 1 - **SIM-4a, 6**, **AZD1208, 2**).[63] It was therefore decided to use TTZD **7** as a lead compound in our SAR studies. A co-crystal structure of the PIM1 enzyme and a known inhibitor, containing a thioxothiazolidinedione motif (PDB code: 3QF9),[57] was used in *in silico* screening to guide further design and synthesis of our initially identified PIM1 inhibitors.

An additional advantage to this particular structure is the tractable and modular synthesis, based on a Knovenagel condensation[64] between rhodanine and a range of aryl and heteroaryl aldehydes, which allowed rapid access to a wide selection of analogues.

Table 1. Synthetic scheme for the Knoevenagel condensation. Reagents and conditions: (i) rhodanine, cat. piperidine, EtOH, 70 °C, 16 h. Physical properties of various substitution on the R position and biological activities against PIM1. ΔT_m n = 1; IC_{50} n = 3 unless otherwise stated. [a]n = 1, R^2 value > 0.90, values listed in SI.



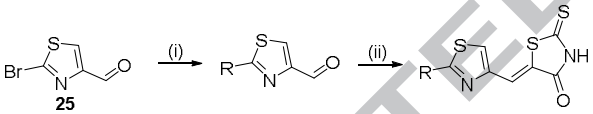
Cpd	Substituent (R)	Yield (%)	PIM1 ΔT_m (°C)	PIM1 IC_{50} (nM)
7	phenyl	64	2.6	-
8	2-Cl phenyl	84	5.5	320±69
9	thien-2-yl	73	4.7	458±40
10	thiazol-2-yl	87	2.8	-
11	thiazol-4-yl	60	-	624[a]
12	thiazol-5-yl	80	3.9	974[a]
13	naphth-1-yl	59	8.3	62±30
14	naphth-2-yl	86	7.6	113[a]
15	benzothien-3-yl	90	10.6	55[a]
16	benzothien-7-yl	72	10.4	68±9
17	benzofuran-2-yl	84	6.8	67[a]
18	benzofuran-3-yl	66	6.4	156±18
19	indol-3-yl	95	10.4	184±69
20	quinolin-2-yl	79	8.0	55[a]
21	quinoxalin-2-yl	81	10.4	126±77
22	7-azaindol-3-yl	73	6.3	175[a]
23	1,8-naphthyridin-2-yl	69	4.7	775±54
24	4-methyl-4H-thieno [3,2-b]pyrrol-5-yl	71	11.0	36[a]

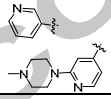
Thus, a range of mono and bicyclic aryl and heteroaryl derivatives were prepared. The (*Z*)-alkene geometry within the products were confirmed by NOE ^{1}H-^{13}C NMR spectroscopy analysis.

While the larger, more demanding bicyclic-bearing TTZDs generally had better activities, as exemplified by the quinolinyl and quinoxalinyl derivatives (**20**, **21**, Table 1), the monocyclic derivatives still displayed good levels of activity. It was therefore decided to base our selection of a starting point on a synthetically versatile low molecular weight exemplar from which analogues could be designed, prepared and developed in a straightforward manner. Compound **11** was selected for optimization based on these criteria, as it demonstrated the best activity compared to its regioisomeric counterparts (**10** and **12**).

Next, additional groups were introduced onto the thiazole moiety at C2 in order to improve the activity. Palladium-catalyzed Suzuki-Miyaura coupling using the appropriate bromo thiazole with an aryl boronic acid, followed by Knoevenagel condensation[64] of the resulting aldehyde with rhodanine afforded the desired tricyclic compounds (Table 2). The inhibitory activity was dramatically improved by addition of a phenyl group; **26** showed approximately a 90-fold increase in activity, (IC_{50} = 624 nM (**11**) to IC_{50} = 6.7±3.1 nM (**26**)). Heterocyclic substituents were also introduced at C2 by the same synthetic method using the corresponding boronic acids. Although good activities against PIM1 were observed with derivatives such as (**27**) and (**28**), due to the relatively narrow chemical scope for further optimization, the synthetic effort focused on improving the aryl substitution pattern.

Table 2. Scheme for the TTZD synthesis *via* a Suzuki reaction and a Knoevenagel condensation. Physical properties of various substitution on the R position and biological activities against PIM1. Reagents and conditions: (i) $RB(OH)_2$, $Pd(PPh_3)_4$, Na_2CO_3, EtOH/DME (1:1), 100 °C, 24 h. (ii) cat. piperidine, rhodanine, EtOH, 70 °C, 16 h. ΔT_m n = 1; IC_{50} n = 3 unless otherwise stated. [a]n = 1, $R^2 > 0.90$, R^{2-} values listed in SI.



Cpd	Substituent (R)	Yield % (i)	Yield % (ii)	PIM1 ΔT_m (°C)	PIM1 IC_{50} (nM)
26		98	14	12.8	6.7±3.1
27		72	55	12.8	25±6
28		73	53	13.4	6.7±3.8
29		58	68	12.1	85±1
30		57	32	16.6	1.4±0.6

An *in silico* study suggested that **26** shared a similar binding mode with **11** against PIM1 (Figure 2, PIM1 crystal structure: PDB code 2C3I),[65] with the oxygen atom on the rhodanine head group of **26** forming an H-bonding interaction with the water molecule which tightly associated with the Lys67 residue.[65] The molecule is predicted to align well within the PIM binding pocket, forming a sandwich-like interaction with the lipophilic area of the pocket; the additional aryl ring is anticipated to serve as a space-filling feature.

Molecular modeling studies were carried out to assist in the design and optimization of **26** analogues. The docking study suggested that the ortho-, meta-, and para- positions of the pendant aryl ring are located in a solvent exposed area of the PIM1 ATP-binding pocket reaching out to the solvent front; therefore substituents ought to be tolerated on this ring. To validate these predictions, a range of groups were systematically introduced onto the 2, 3, and 4 position of the C-ring following the procedure described previously (Table 3).

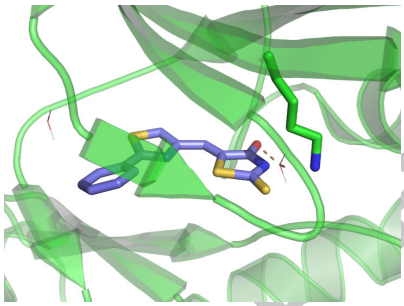
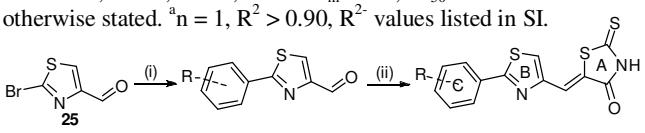


Figure 2. Representative docking picture of **26** in PIM1 active site.

All the synthesized compounds with additional substituents on the phenyl ring showed that they retained similar activity to **26**. Interestingly, as predicted by the docking study, the size of the aryl substituents seemed to play an important role in influencing inhibitory activity against PIM1 (Table 3). Compound **31** (IC_{50} = 9.2±5.4 nM) bearing a strongly electron withdrawing CF_3 group had similar activity to compound **34** (IC_{50} = 11±3 nM) bearing a strongly electron donating methoxy group at the same position, indicating that electronic properties in this region do not appear to play a significant role. The introduction of a hydrogen bonding motif did not lead to a noticeable effect on the enzymatic activity either, with the H-bond donor phenolic group (**39**, IC_{50} = 2.4±0.9 nM) showing comparable activity to an H-bond acceptor SO_2Me (**40**, IC_{50} = 3.6 nM) or halogen bonding group Cl (**41**, IC_{50} = 36±7 nM). Furthermore, in agreement with the performed docking studies, the regiochemistry of substitution on the aryl ring is also well tolerated. It therefore provided a useful handle for further optimization of the physical properties of this series of inhibitors.

Table 3. Scheme for TTZD synthesis *via* a Suzuki reaction and a Knoevenagel condensation. Physical properties of various substitution on the R position and biological activities against PIM1. Reagents and conditions: (i) Ar-$B(OH)_2$, $Pd(PPh_3)_4$, Na_2CO_3, EtOH/DME (1:1), 100 °C, 24 h. (ii) cat. piperidine, rhodanine, EtOH, 70 °C, 16 h. ΔT_m n = 1; IC_{50} n = 3 unless otherwise stated. [a]n = 1, $R^2 > 0.90$, R^{2-} values listed in SI.



Cpd	Substituent (R)	Yield % (i)	Yield % (ii)	PIM1 ΔT_m (°C)	PIM1 IC_{50} (nM)
31	2-CF_3	87	34	9.4	9.2±5.4
32	3-CF_3	63	51	12.3	49±21
33	4-CF_3	71	26	11.7	16±8
34	2-OMe	74	53	9.3	11±3
35	3-OMe	71	69	14.3	21±9
36	4-OMe	55	36	13.2	11±6
37	2-OH	65	31	14.8	8.0±5.7
38	3-OH	24	34	12.7	5.0±0.6
39	4-OH	68	32	14	2.4±0.9
40	4-SO_2Me	74	32	10.3	3.6[a]
41	3-Cl	62	22	13.2	36±7
42	3-$NHSO_2Me$	100	70	9.6	25±4
43	3-piperidine	93	14	13.5	7
44	3-$NHSO_2NMe_2$	28	21	16	2.2±1.8
45	4-OCF_3	91	44	11.7	2.9±1.3
46	4-CH_2-piperidine	100	35	10.4	6.8±4.6

With good enzymatic activities obtained from the SAR studies, a selection of compounds was examined for their broader physicochemical properties and *in vitro* metabolic stability using kinetic solubility, and mouse liver microsome (MLM) stability assays (Table 4).

Table 4. Physicochemical properties and *in vitro* metabolic stability. MLM $t_{1/2}$: half-life measured in mouse liver microsomes, KinSol: kinetic solubility.

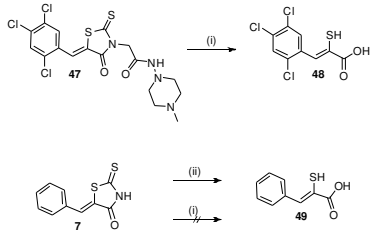
Cpd	Substituent	MLM $t_{1/2}$ (min)	KinSol (µM)
33	4-CF_3	49	<5
41	3-Cl	5	<5
42	3-$NHSO_2Me$	<5	34

Across the series, compound solubility is in the low µM range and half-life in MLMs is < 1 hour. Solubility can be improved by exploiting the tolerance of polar substituents on the phenyl ring, as exemplified by **42** that bears a sulfonamide group and for which the PIM1 inhibitory activity remained excellent (IC_{50} 25±4 nM). Kinetic solubility of this compound was improved from < 5 µM (**33**, **41**) to 34 µM (**42**). Unfortunately, the half-life in MLMs dropped significantly from 49 min (**33**) to less than 5 min (**42**).

The enzymatic activities were excellent throughout this series of inhibitors, but the physical and metabolic properties are suboptimal, which poses a significant challenge in order to progress beyond cellular, to *in vivo* studies. We therefore focused our efforts on improving the physical and metabolic properties of this inhibitor series. We also sought to mitigate any potential issues with chemical instability of this series.

Whilst in our MLM stability studies we observed no degradation in the control experiments in the absence of co-factor, it has been reported in the literature that *N*-substituted rhodanines readily undergo basic hydrolysis to afford α,β-unsaturated-α-mercaptocarboxylic acids (Scheme 1),[66] a functional group that could potentially cause toxicity in biological systems. Furthermore Schofield *et al.* have recently described the cleavage of an *N*-substituted rhodanine-containing compound **47** in Tris buffer pH 7.5 (Scheme 1).[67]

Both set of conditions were carried out on **7**, as the corresponding mercaptocarboxilic acid and hydrolysis are known in the literature. Hydrolysis of **7** with 12M aq. NaOH to afford **49** proceeded with complete conversion in 2 h, and the data were in accordance with the literature.[68] It is interesting to note that the compound did not hydrolyze in Tris buffer. As solubility could be an issue, the reaction was repeated with the addition of DMSO (to reproduce assay conditions), but the compound was recovered in quantitative amount with no trace of the degradation product **49**. It was hypothesized that the presence of an *N*-substituent within rhodanine **47** could increase susceptibility towards hydrolysis, and thus does not present a significant stability issue for analogues such as **7**.



Scheme 1. Reagents and conditions: (i) Tris buffer pH 7.5, 37 °C, 4 h; (ii) NaOH (1M aq, 5 eq), 40 °C, 2 h.

Another possible concern cited for rhodanine derivatives in the literature is the potential for isomerisation of the double bond. While the (*Z*)-isomer of conjugated rhodanines is usually the more thermodynamically stable diastereomer,[69, 70] it can be isomerised under certain conditions, such as photoirradiation, to the alternative geometric isomer. Whilst we did not observe any such photoisomerisation, the combination of the propensity for low photostability and poor solubility / metabolism properties led us to investigate if the introduction of structural and/or steric constraints would improve the overall profile of the series. In order to explore alternative scaffolds to compound 7 we designed a range of linked and ring fused scaffolds which could potential interact in a bioisosteric fashion (Figure 3-A). Using Forge®, a powerful computation suite to understand SAR and design, the 3D electronic and shape of each input scaffold were then aligned with template molecule 7. The tricyclic scaffold **50** provided an effective consensus overlay with a similarity coefficient of 0.79 (see Figure 3-B) A docking study of **50** against PIM1 (PDB code 2C3I) suggested that the carbonyl group in the rhodanine head group has the potential to form similar interaction as **26** through a water molecule-mediated interaction with Lys67 (Figure 3-B-D). The planar conformation of the molecule, suggested to be important to form the lipophilic sandwich within the active site cleft was predicted to be maintained, with the newly formed carbonyl linker pointing towards the solvent exposed surface region of the pocket.

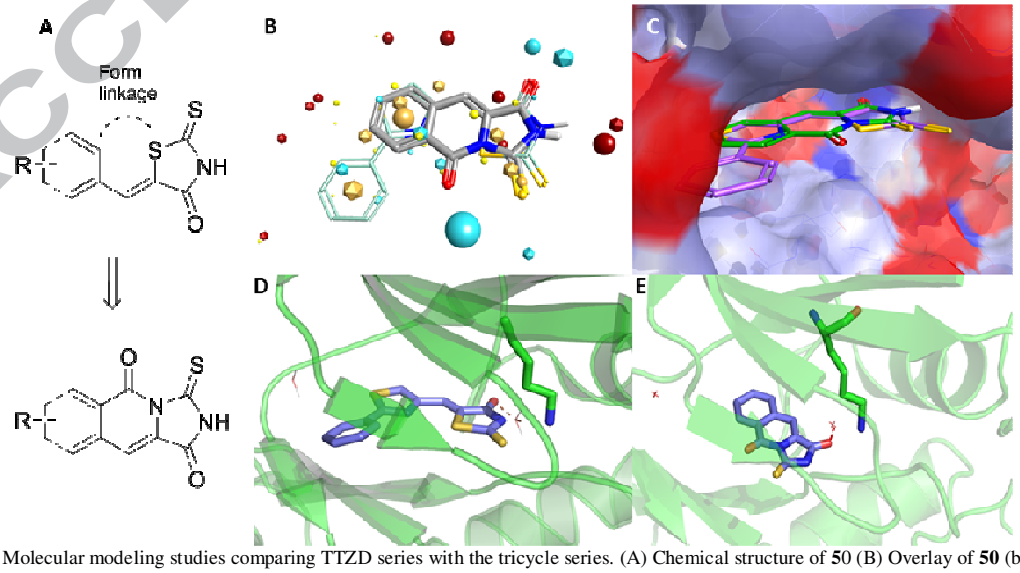
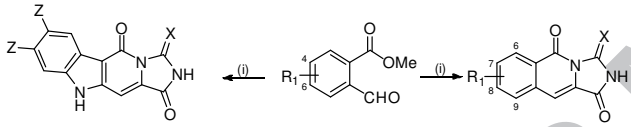


Figure 3. Molecular modeling studies comparing TTZD series with the tricycle series. (A) Chemical structure of **50** (B) Overlay of **50** (ball) with **26** (diamond). Blue represents electron negative field, red represents electron positive field, and yellow represents lipophilic interactions. (C) Overlay of **50** (green) with **26** (purple) in the PIM1 active site. (D) Representative docking picture of **26** in the PIM1 active site. (E) Representative docking picture of **50** in the PIM1 active site; x = H_2O.

A synthesis was designed that allowed the rapid preparation of an array of representative molecules. The requisite ortho-formyl aryl esters, which are commercially available or readily synthetically accessible, were condensed with 2-thiohydantoin to afford the desired tricycle (Table 5).[1] The benzo-fused derivative, **50**, was obtained in good yield and showed moderate activity against PIM1 (IC_{50} = 1341 nM, ΔT_m 3.8 °C). This was comparable to the data obtained for **7** (ΔT_m 2.6 °C) and was therefore a promising start to this series. From the molecular modeling study suggesting that the tricycle series shared the same binding pose with the TTZD series, it was also predicted that the 7- position on the C-ring of the tricycle would be the optimal position for further functionalization. This hypothesis was probed by preparing three regioisomers of the chloro-substituted tricycle, **51**, **52** and **53**. Introducing a chlorine to the 6- and 8- positions (**53** and **51** respectively) led to complete loss of inhibitory activity whilst the 7- position (**52**) showed a modest increase in inhibitory activity (IC_{50} = 784 nM, ΔT_m 4.9 °C). Substituents at this position were next systematically changed to assess their impact on the activity against PIM1.

Table 5. Scheme for polycycle synthesis *via* a Knoevenagel condensation and subsequent ring closure. Physical properties and *in vitro* metabolic stability of various substitutions on the R position and biological activities against PIM1. Reagents and conditions: (i) NaOAc, AcOH, reflux, 4h. ΔT_m n = 1; IC_{50} n = 3 unless otherwise stated. [a]n = 1, $R^2 > 0.90$, R^{2-} values listed in SI. MLM $t_{1/2}$: half-life measured in mouse liver microsomes, KinSol: kinetic solubility

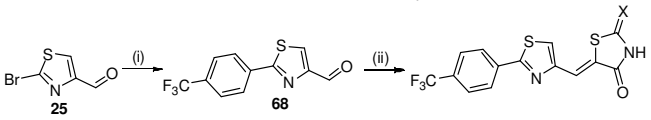


Cpd	R_1	X	PIM1 ΔT_m (°C)	PIM1 IC_{50} (nM)	KinSol (µM)	MLM (min)
50	H	S	3.8	1341[a]		
51	8-Cl	S	5.1	Inact		
52	7-Cl	S	4.9	784[a]		
53	6-Cl	S	1.9	Inact		
54	7-Ph	S	5.5	554±192		
55	7-(pyrid-4-yl)	S	2	Inact		
56	7-(furan-2-yl)	S	1.9	107±10	11.5	14
57	7-O-Ph	S	6.2	267±119	65	28
58	7-NH-Ph	S	1.9	Inact		
59	7-S-Ph	S	3.6	127[a]		
60	7-O-(4-F Ph)	S	0.3	304±9	65	36
61	7-S-(4-tolyl)	S	9.0	335±156		
62	7-O-(4-OMe Ph)	S	2.3	332[a]		
63	4,6-diCl indole	S	7.7	131±13	<5	>100
64	4,6-indole	S	5.5	273±4		
65	*N*-benzyl-4,6-diCl indole	S	ND	100±7	<1	18
66	7-O-Ph	O	ND	804±37		
67	H	H_2	ND	Inact		

Phenyl analogue **54** exhibited similar activity compared to the chloro analogue **52** (IC_{50} = 554 nM vs IC_{50} = 784 nM). Introduction of a pyridyl moiety (**55**) was not well tolerated, whereas a furanyl group (**56**) gave a significant increase in activity (Table 5). Unfortunately, the solubility and metabolic half-life of **56** (11.5 µM and 14 min respectively) were disappointing. Introducing additional *O*- and *S*- linkers between C-ring and 7- position substituents to improve the structural and flexibility also improved the potency (**57**, **59**), whereas an *NH*-linker led to loss of activity (**58**). Substitution on the pendent aryl ring had very little effect on the potency, as shown by the results obtained for the fluoro-, tolyl, and methoxyl analogues (**60**, **61**, **62**). Interestingly, the addition of an *O*- linker gave an increase in solubility, however, the *in vitro* metabolic stability remained modest (**57**, 65 µM and 28 min). The level of activity was dramatically improved by replacing the phenyl moiety by a 4,6-dichloroindole (**63**, IC_{50} = 131 nM). This analogue also gave a noticeably improved in vitro metabolic half-life (>100 min), however, the solubility was poor (<5 µM). The potency increase may be due to the possibility for alternative binding caused by the presence of the two vicinal chloro substituents, as has been reported previously in related systems.[71] With the aim of improving the solubility by addition of rotatable bonds, substituents were introduced to the N8 position of the indole but to no avail (**65**). To eliminate the potential oxidative liability of the C=S group of this series, the corresponding carbonyl derivative was prepared, unfortunately, this led to significant reduction of activity (**66**). Reduction of the thio-carbonyl group to a methylene using $NiCl_2$ and $NaBH_4$[72] resulted in complete loss of activity (**67**). Due to the increased complexity of this series and narrow window for the improvement of physical properties while maintaining the potency, it was decided not to pursue the fused tricyclic scaffold further.

As an alternative strategy it was decided to also investigate circumventing the intrinsic metabolic instability of the rhodanine group; it was suspected that the C=S functionality within the rhodanine could be the cause of metabolic instability.[62] Following the same methodology described to prepare **50**, 1,3-pseudothiohydantoin and thiazolidine-2,4-dione were used as rhodanine mimics to afford **69** and **70**, respectively (Table 6). **70** gave decreased activity against PIM1 (IC_{50} = 171±46 nM), and **69** gave only a modest reduction in activity (IC_{50} = 70±1 nM) compared with **33** (IC_{50} = 16±8 nM). Although the solubility of **69** remained low, the metabolic half-life was dramatically improved (>100 min).

Table 6. Scheme for TTZD synthesis *via* a Suzuki reaction and a Knoevenagel condensation. Physical properties and *in vitro* metabolic stability of various substitution on the R position and biological activities against PIM1. Reagents and conditions: (i) *p*-$CF_3PhB(OH)_2$, $Pd(PPh_3)_4$, Na_2CO_3, EtOH/DME (1:1), 100 °C, 24 h. (ii) cat. piperidine, rhodanine or analogue, EtOH, 70 °C, 16 h. ΔT_m n = 1; IC_{50} n = 3 unless otherwise stated. [a]n = 1, $R^2 > 0.90$, R^{2-} values listed in SI. MLM $t_{1/2}$: half-life measured in mouse liver microsomes, KinSol: kinetic solubility



Cpd	X	PIM1 ΔT_m (°C)	PIM1 IC_{50} (nM)	MLM (min)	KinSol (µM)
33	S	11.7	16±8	49	<5
69	NH	9.4	70±1	>100	<5
70	O	7.5	171±46	ND	ND

The ability to inhibit all members of the PIM family is important for anticancer treatment and representative examples were therefore evaluated against PIM1, 2, and 3 using DSF to establish their PIM isoform selectivity profile (Table 7). Most of the examples showed comparable inhibitory potencies against all of the PIM kinase family members.

[1] see supporting information for synthesis.

Table 7. PIM isoform selectivity.

Cpd	PIM1 ΔT_m (°C)	PIM2 ΔT_m (°C)	PIM3 ΔT_m (°C)
26	12.8	12.0	13.0
31	9.4	8.2	8.8
32	12.3	11.5	9.1
33	11.7	9.7	6.1
35	14.3	13.3	13.4
36	13.2	11.5	12.3
45	11.7	9.7	12
37	14.8	17.0	18.6
38	12.7	12.2	14.9

Representative examples from each series were also selected for inhibitory evaluation in a 24-kinase panel to establish their selectivity profiles (Table 8).[57] Those kinases were of particular interest to measure off target activity as they have been shown to bind polycyclic heteroaromatic structures, and also as some of the kinases play a role in the cell lines utilized (e.g. Flt3 is over expressed in MV4-11 cells) and thus inhibition of such kinases could, in principle confound the cell proliferation read out.[2] All classes showed a high level of selectivity towards the PIM kinase isoforms (both PIM1 and PIM2 were included for reference), the exceptions being off-target effects in **33** and **69** (GSK3β) and in **11** (CK2 and DYRK2). The selectivity profile of **63** was inconclusive and likely due to the poor solubility of the compound. These results are encouraging and suggest that selectivity for PIM isoforms over other kinases is achievable and not compromised by the commonly reported promiscuity of the rhodanine motif.

Table 8. 24-kinase selectivity evaluation (performed using 10 μM of compound). Data is provided as a percentage of residual activity compared to vehicle alone.

kinase	**63**	**11**	**69**	**33**
BTK(h)	94	48	88	90
CHK1(h)	105	108	108	108
CK2(h)	82	-3	117	53
cKit(h)	100	nd	108	101
cSRC(h)	98	74	113	106
DAPK1(h)	90	92	103	102
DYRK2(h)	117	15	87	95
EGFR(h)	110	nd	114	113
Flt3(h)	94	44	94	81
GSK3β(h)	68	28	6	4
IR(h)	99	143	102	101
JAK2(h)	100	89	116	106
JNK2α2(h)	99	95	103	105
KDR(h)	99	114	88	110
PIM1(h)	49	17	0	1
PIM2(h)	43	nd	2	2
PKBα(h)	76	106	101	63
Plk1(h)	81	98	94	103
SAPK2a(h)	103	109	94	102
SIK(h)	106	82	119	103

Compounds were evaluated for antiproliferative activity against two cancer cell lines MV4-11 (human acute monocytic leukemia cell line)[73] and K562 (human immortalised myelogenous leukemia cell line), both previously reported to be PIM sensitive;[74] modest activities were observed in most cases (Table 9). In the TTZD series, antiproliferative activities were observed with representative exemplars across the series, with the most active analogue **44** giving an IC_{50} of 3.4 μM against MV4-11 cells and 0.75 μM against K562 cells. In all cases a significant reduction in potency was observed between the enzymatic inhibitory activities and whole cell activities. This observation may be attributed to a lower free concentration of compound due to binding to serum proteins present in the cell growth media, a phenomenon previously reported for similar molecules. Effective cell permeability may also be a contributory factor. In the tricycle series, the indole motif containing analogues showed the best cellular activities across the series, which is in an agreement with the enzymatic activities discussed previously, with **64** giving an IC_{50} of 3.9 μM. The substitution on the N8 position did not produce a notable increase in cellular activities despite the noticeable improvement in the enzymatic activities. The lack of improvement in cellular activities could be due to cleavage of the weak C-N bond formed between the substituent and indole nitrogen *in situ*.

Table 9. Cellular antiproliferative activity of selected PIM inhibitors determined by an MTT assay. ND= not determined. Inact. = inactive.

Cpd	PIM1 IC_{50} (nM)	Cellular activity IC_{50} (μM)	
		MV4-11	K562
11	624[a]	inact	ND
31	9.2±5.4	ND	16.8
32	49±21	8.1	11.9
33	16±8	3.8	7.1
35	21±9	ND	2.0
36	11±6	5.7	6.1
37	8.0±5.7	2.4	2.4
38	5.0±0.6	ND	4.3
40	3.6[a]	3.2	14.9
42	25±4	9.9	10.9
43	7	8.9	4.3
44	2.2±1.8	3.4	0.75
45	2.9±1.3	8.7	6.9
46	6.8±4.6	8.5	5.1
63	131±13	3.9	ND
65	100±7	ND	2.8
69	70±1	inact	ND
70	171±46	11.4	11.1

Although changing the C=S (**33**) to C=O (**70**) resulted in the notable reduction in the enzymatic activity, it seems to have little impact on the respective cellular activities (**70** against MV4-11 = 11.4 μM, K562 = 11.1 μM).

3. Conclusion

In this paper, a high throughput screen and *in silico* study were described to identify series of PIM kinase inhibitors. SAR studies were carried out on the initial hit molecule and the thiazole core was identified to be a suitable B-ring group with the optimal balance of properties. Extending the pharmacophore of the initial hit to a fused 3-ring system significantly improved the activity of this family. Functionalization of the C-ring not only improved the potency but also provided an additional option for optimization of physical properties. Metabolic stability was highlighted as an issue to address, and two strategies were proposed for improvement. Additional constraints were introduced into the system to minimise the potential reactivity for hydrolysis and conjugate addition, and resulted in the identification of a fused tricycle series. This series of inhibitors had good activity and led

[2] See supporting information for full protocol.

to improvement in both metabolic stability and solubility. The second strategy involved replacement of the rhodanine head group with a pseudothiohydantoin, which resulted in a significant increase in metabolic stability. Additionally, the pseudothiohydantoin head group provided further opportunity for future optimisation. Selected inhibitors were evaluated to establish the selectivity profile against the three PIM isoforms. An excellent PIM pan-inhibition profile was observed across the series. A kinase selectivity profile was established, and few off-target activities were also observed, supporting our initial hypothesis that the activity of this family of compounds was not obtained through the suggested promiscuous nature of the rhodanine, but instead from its specific affinity towards the PIM family. Two leukaemia cancer cell lines, MV4-11 and K562, were employed to evaluate the antiproliferative activities of selected inhibitors. Moderate activities were observed for a number of examples, with the best example showing IC_{50} value of 0.75 μM against K562 cells. Together this data represents a very promising starting point for the development of in vitro and in vivo pharmacological probes and drug candidates. Future efforts will focus on the optimisation of physical properties and cellular activity of this series of compounds for potential *in vivo* evaluation.

Acknowledgments

This work was supported by Cancer Research UK through a Small Molecule Cancer Drug Discovery Award (C17468/A9332, MBB, SB, MD, KVMH, AMJ, GL, AN, CEQ and RGW). We would also like to thank Research Councils United Kingdom for a fellowship (AJR). SK and OF are grateful for support by the SGC, a registered charity (number 1097737) that receives funds from AbbVie, Bayer Pharma AG, Boehringer Ingelheim, Canada Foundation for Innovation, Eshelman Institute for Innovation, Genome Canada, Innovative Medicines Initiative (EU/EFPIA) [ULTRA-DD grant no. 115766], Janssen, Merck & Co., Novartis Pharma AG, Ontario Ministry of Economic Development and Innovation, Pfizer, São Paulo Research Foundation-FAPESP, Takeda, and Wellcome Trust (092809/Z/10/Z).

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