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Identification and structure–activity relationships of substituted pyridones as inhibitors of Pim-1 kinase

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Abstract—A novel series of highly potent substituted pyridone Pim-1 kinase inhibitors is described. Structural requirements for in vitro activity are outlined as well as a complex crystal structure with the most potent Pim-1 inhibitor reported (IC_{50} = 50 nM). A hydrogen bond matrix involving the Pim-1 inhibitor, two water molecules, and the catalytic core, together with a potential weak hydrogen bond between an aromatic hydrogen on the R^1 phenyl ring and a main-chain carbonyl of Pim-1, accounts for the overall potency of this inhibitor.

The *pim-1* proto-oncogene was initially identified as a frequent site of integration for the slowly transforming Maloney murine leukemia virus in murine T cell lymphomas.[1] Pim-1 is an oncogenic serine/threonine kinase that is a member of a family of proteins containing homologues Pim-2 and Pim-3, and is transcriptionally regulated by cytokines, mitogens, and numerous growth factors.[2–4] Elevated expression of Pim kinases has been described predominantly in murine and human leukemias and lymphomas.[5] The enzymatic substrates reported for Pim kinases are diverse (p100,[6] Bad,[7] NFATc1,[8–10] p21/Cip1,[11] Cdc25a,[12] SOCS,[13,14] and others), suggesting that Pim plays a central regulatory role in numerous processes. Indeed, Pim kinases have defined biological roles in cell survival, proliferation, and differentiation,[4] particularly for cells of hematopoietic lineage. Studies using either *pim* transgenic or *pim* knockout mice suggest that overlapping functions exist for Pim-1 and Pim-2, and that the full transforming potential of Pim kinases cannot be realized in vivo without cooperation from other oncogenes.[15–19] Perhaps the most compelling biological roles described for Pim-1 and Pim-2 include the regulation of rapamycin-resistant T cell growth and survival[18] as well as the recently reported impairment of mutant Fms-like tyrosine kinase 3 (FLT3)[10,20] or drug-resistant BCR/ABL-transformed hematopoietic cell survival,[21] upon functional suppression of Pim kinases. From these perspectives, inhibitors of Pim kinases may have immunomodulatory potential alone or in combination with immunosuppressive drugs such as rapamycin, or as an alternative method to treating patients who have developed resistance to small-molecule protein tyrosine kinase inhibitors for cancer.

X-ray crystallographic studies of Pim-1 support a high degree of main chain similarity to other structurally defined serine/threonine kinases, with characteristic fold-hinge-fold domains.[22–24] However, Pim kinases possess a unique proline residue, Pro123, in the hinge region where other hydrophobic amino acids are more typically found, as well as insertion of one or two residues in the hinge region following Pro123. As Pim-1 adopts a unique structure in the hinge region, which acts in part as a boundary for positioning ATP binding, it may be possible to identify highly selective ATP-competitive Pim kinase inhibitors for potential use as anticancer or immunomodulatory agents. Small molecule Pim-1 kinase inhibitors derived from various chemical scaffolds have been previously described.[23–25] None of these inhibitors have progressed to the clinic. Here we report the identification of a novel series of Pim-1 kinase inhibitors based on a substituted pyridone scaffold and outline the potential mechanism by which this type of inhibitor could compete and interfere with Pim-1 ATP utilization.

Keywords: Pim-1 kinase; Pim-1 kinase inhibitors; Pyridone; Fused-ring pyridone; X-ray complex structure; Pim-1; Pim.
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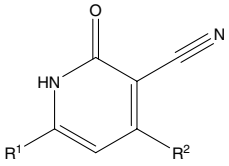
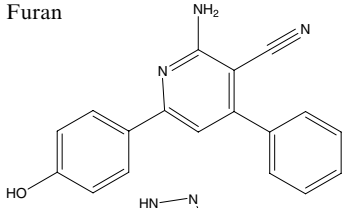
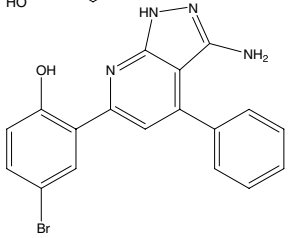
In order to identify alternative scaffolds we screened a compound library of approximately 160,000 molecules using an enzymatic luminescent assay for Pim-1 inhibition.[26] A substantial number of Pim-1-inhibitory hits were identified, many of which had IC_{50} values less than 1 μM. Several potent chemical library hits along with a number of newly synthesized derivatives of these compounds are listed in Table 1. Corresponding IC_{50} values were determined by radioactive filter-plate assay using histone proteins as the substrate for Pim-1 kinase.[27] Compound **1**, a substituted pyridone scaffold, had the lowest IC_{50} at 0.05 μM. This molecule served both as a starting point for SAR chemical syntheses and was used for co-crystallization with Pim-1 protein. A limited number of compounds with highly similar chemical composition were also identified from our library including compounds **4**, **6**, **7**, **8**, and **10**. Although none of these compounds produced IC_{50} values superior to compound **1** they were informative with respect to the SAR. Specifically, modification of R^1 contributed more substantially to the loss of in vitro activity compared to alteration of R^2.

To help comprehend the inhibitory mechanism of action for compound **1**, we took advantage of a highly purified and enzymatically active Pim-1 protein[28] to initiate complex structure studies by X-ray crystallography.[29,30] The structure of Pim-1 co-crystallized with compound **1** was resolved to 2.5 Å. Analysis of the electron density plot for **1** supported the conformation of the complex structure without ambiguity (Fig. 1). Compound **1** appeared to adopt a partially flat conformation with co-planar R^1 phenyl and central pyridone rings, while the R^2 phenyl ring was twisted perpendicularly with respect to the other rings. We contend that the possibility for H-bonding between the 2-OH group on the R^1 phenyl ring and the pyridone N is high and within acceptable H-bonding distance (1.9 Å). This internal H-bonding may serve to fix the conformation of the two rings into a nearly planar relationship.

Compound **1** bound convincingly within the ATP-binding site of Pim-1 suggesting an ATP-competitive inhibitory mechanism (Fig. 2). Perhaps the most significant finding from this complex structure was the prominent interaction of the carbonyl group on the pyridone ring with both the Lys67 side chain (interacts with phosphates from ATP) of Pim-1 together with a hydrogen bond to a water molecule (Wat1) that appeared to play an integral part of a larger H-bonding network in this region. The apparent H-bonding network further consisted of a second conserved water molecule (Wat2) that interacted with both the Pim-1 kinase catalytic residue Phe187 and Wat1 (Fig. 2). Interestingly, the only potential interaction of **1** with the hinge region occurred on the left side of the R^1 phenyl ring where the complex

Table 1. Pyridone-based inhibitors of Pim-1 kinase



Compound	R^1	R^2	IC_{50}[a] (μM)
1	3-Br-6-OH-Ph	Ph	0.05
2	3-Me-6-OH-Ph	Ph	0.34
3	Ph	3-Me-6-OH-Ph	0.37
4	6-OH-Ph	4-OH-Ph	0.43
5	3-Et-6-OH-Ph	3-Cl-Ph	1.03
6	4-OMe-5-Br-Ph	CF_3	1.14
7	Thiophene	Thiophene	0.99
8	CF_3	Thiophene	3.54
9	Furan	CF_3	16.00
10			4.41
11			1.77

[a] Values are means of three experiments.

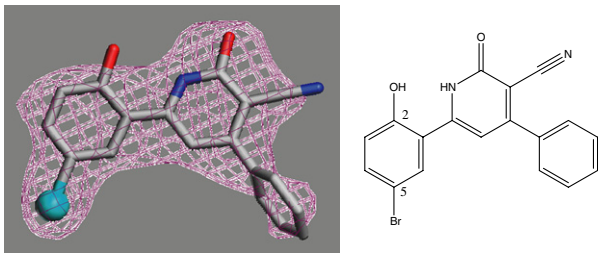


Figure 1. X-ray electron density plot of compound **1** in complex with Pim-1 obtained at a resolution of 2.5 Å. Structure of **1** is shown to the right.

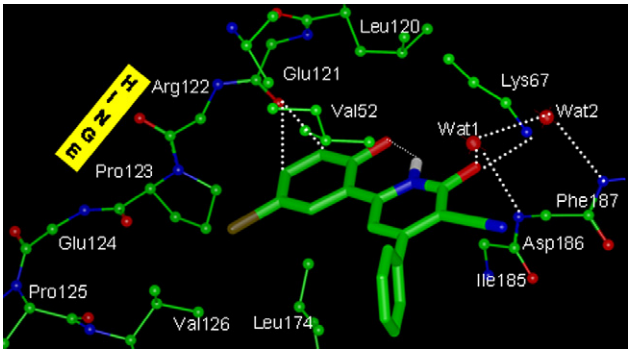
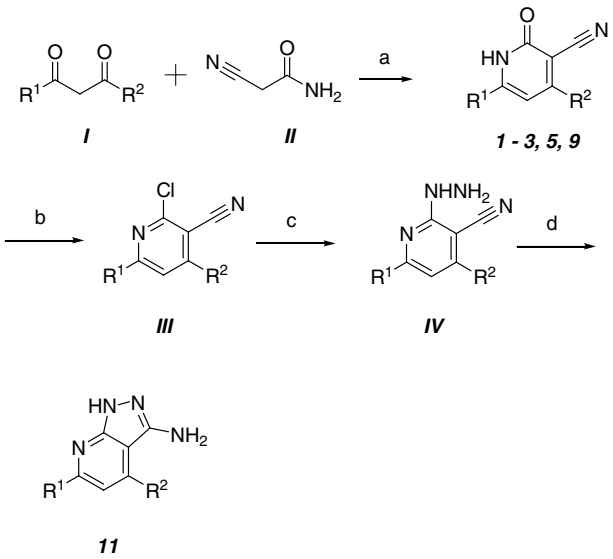


Figure 2. X-ray complex structure of compound **1** with Pim-1 at 2.5 Å resolution. Hydrogen and side-chain atoms are omitted for clarity.

structure defined a weak H-bond between the main-chain carbonyl of Glu121 and an aromatic hydrogen (C-3 position) on R^1 (2.6 Å C═O···H–C). Although significantly less favorable at 3.0 Å, potential H-bonding between the same carbonyl of Glu 121 and the aromatic H at the C-4-position may also contribute to stabilization of **1** with the hinge. Hydrogen bonding of this nature was also reported for interactions between Pim-1 Glu121 with the known phosphatidylinositol 3-kinase inhibitor LY294002.[23] The role of the Br atom on R^1 is less well defined from a SAR perspective although its importance is exemplified by the fact that replacement of the Br as in **2** by a methyl group leads to a 7-fold loss of inhibition. It is our interpretation that stabilization of **1** by a robust H-bonding network involving two water molecules accounts for the majority of the inhibitor's potency. Hence compound **1** binds and competitively inhibits ATP utilization thereby suppressing Pim-1 kinase function.

Newly synthesized compounds based on the planar complex structure of **1**, including compounds **2**, **3**, **5**, **9**, and **11**, are listed in Table 1. However, highly potent compounds were not identified within this sub-series of SAR molecules. Disruption of the pyridone carbonyl by ring closure (**11**) led to a 35-fold reduction in potency compared to **1**, substantiating the fragile nature of the proposed H-bonding network in this area. Contributions to inhibitor activity are less obvious for the cyano group although its strong electron-withdrawing potential may result in modification to the electronics, and hence H-bonding capability, of the pyridone ring. The key reactions necessary to synthesize the SAR derivatives are outlined in Scheme 1.



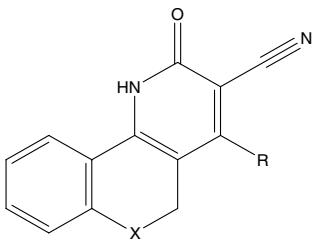
Scheme 1. The synthetic route to **1**–**3**, **5**, **9** and **11**. Reagents and conditions: (a) DBU, toluene, 90 °C, 16 h, 50-70%; (b) $POCl_3$, pyridine, dioxane, 80 °C, 14 h, 16%; (c) NH_2NH_2 in H_2O, DMF, 90 °C, 14 h, 90%; (d) CH_3COOH, EtOH, 90 °C, 14 h. Refer to Table 1 for R^1 and R^2 designations.

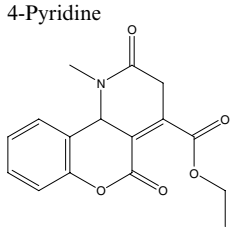
The synthesized pyridone derivatives **1**–**3**, **5**, and **9** were obtained from a cyclo-condensation reaction in one step (Scheme 1). Reaction of commercially available diketone **I** with cyanoacetamide **II**, in the presence of DBU in toluene at 90 °C for 16 h, readily afforded the title compounds which were purified by HPLC and characterized by ^{1}H NMR and MS.

The synthesis of ring-fused product **11** started with **1** combined with three additional steps including sequential chlorination, displacement, and cyclization reactions. Thus, reaction of **1** with $POCl_3$ with pyridine in dioxane at 80 °C for 14 h formed chloro intermediate **III**, which was displaced by hydrazine to give **IV**. Treatment of **IV** with acetic acid in ethanol under reflux for 14 h formed the desired product **11**, which was HPLC purified and confirmed by ^{1}H, 2D NMR and MS.

Rigidification of the R^1 phenyl ring in relation to the central pyridone through cyclization (similar to phenanthrene, although not fully aromatic) (Table 2) was envisioned to mimic the proposed intra-molecularly H-bonded co-planar rings of **1** when complexed with Pim-1, without the R^1 2-OH moiety. Although compounds **12**–**14** produced IC_{50} values less than 1 μM, they failed to reach low nanomolar potencies. Compounds containing an O rather than S at position X were seemingly more potent (**12** vs **19**) when the R group was thiophene. Lack of halogenation on the left-side phenyl ring may account in part for these lower efficacies. Although the R group was never an unsubstituted phenyl ring (as in R^2 of **1**), the probability of significant interaction with the protein in this section of the compounds was likely minimal due to the predominant solvent exposure observed near R^2 of Pim-1/compound **1**.

Table 2. Inhibitors of Pim-1 kinase



Compound	R	X	IC_{50}[a] (μM)
12	Thiophene	O	0.46
13	Furan	O	0.87
14	4-Cl-Ph	O	0.92
15	6-Cl-Ph	O	1.23
16	4-OMe-Ph	O	>20
17	4-Pyridine	O	20.0
18	2-Pyridine	O	>20
19	Thiophene	S	1.49
20	3-Pyridine	S	4.79
21	2-Cl-Ph	S	20.0
22	4-F-Ph	S	>20
23	4-Pyridine	CH_2	>20
24			>20

[a] Values are means of three experiments.

In summary, a series of substituted pyridone molecules was identified from a high throughput screen as potent inhibitors of Pim-1 kinase. Because of its compelling in vitro efficacy, a complex structure and mechanism of action were defined for compound **1**. The pyridone carbonyl group was critically positioned within the Pim-1 ATP binding site effecting kinase inhibition. Preliminary data further suggested that **1** lacked in vitro inhibitory activity toward related serine/threonine kinases Pim-2 and MEK1/2 ($IC_{50} > 20$ μM; data not shown). Hence, small molecules similar to compound **1** may serve as useful starting scaffolds for the development of other improved yet selective Pim-1 inhibitors.

Acknowledgment

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26. High throughput screening assay: A purified recombinant GST–Pim-1 fusion protein together with histone proteins (Sigma) as substrate was used for the Kinase-Glo assay (Promega). Briefly, reaction conditions were 25 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 4% DMSO, 15 μM ATP, 1 μg GST-Pim-1, 5 μg calf thymus histones plus the test compound in a 20 μl total volume. Reactions were performed in a 384-well plate for 1 h at room temperature and were terminated by the addition of the Kinase-Glo reagent then counted on the LJL Analyst plate reader. The Z' for the assay was 0.8.
27. Radioactive filter plate assay: Pim-1 kinase reaction conditions included 25 mM Hepes (pH 7.5), 10 mM $MgCl_2$,

5 mM DTT, 4% DMSO, 25 μM ATP, 0.5 μCi [γ-^{33}P] ATP, 100 nM enzyme, and 2.5 μg calf thymus histone substrate in a 25 μl reaction volume. Reaction mixtures proceeded for 1 h at 23 °C and were terminated by the addition of 20% trichloroacetic acid/1% pyrophosphate. Reactions were transferred to a 96-well filter plate, washed, and dried, then scintillation cocktail was added and the radioactivity counted on a TopCount plate reader (Perkin-Elmer). Compounds were titrated using these conditions and the IC_{50} values were determined.

28. Protein purification: Full-length Pim-1 (residues 1–313) was PCR cloned into the bacterial expression vector pET28a in-frame with the N-terminal hexahistidine tag. Expression was carried out in BL21 (DE3) pLysS *Escherichia coli* in LB medium after induction with IPTG for 4 h at 28 °C. Following cell lysis, the His-tagged Pim-1 was subjected to a sequential purification scheme initially incorporating FPLC using a nickel-chelating Sepharose column followed by a Resource Q ion exchange column and lastly a Superdex 75 gel filtration column. Using this process we recovered milligram quantities of Pim-1 with purity sufficient for attempting crystallization.
29. The Pim-1/inhibitor complex was obtained by combining 25 μl protein (12 mg/ml) with 0.35 μl of 20 mM compound **1** in DMSO. The samples were incubated at 4 °C for 1–2 h. Co-crystals were grown using a sitting-drop vapor diffusion tray with a crystallization solution containing 1.0 M ammonium phosphate (dibasic), 300 mM sodium chloride, and 100 mM citrate buffer (pH 5.0). Crystallization plates were incubated at 23 °C and optimal crystal size was reached in approximately 10 days. Data acquisition was performed on a Rigaku R-Axis IV+ detector mounted on a Rigaku RUH3R generator operating at 50 kV and 100 mA. Measurement intensities were integrated, scaled, and merged using HKL software (Z. Otwinoski and W. Minor).
30. Coordinates for the complex structure of Pim-1/compound **1** have been deposited with the Protein Data Bank (www.rcsb.org) under PDB code 2OBJ.