Bioorganic & Medicinal Chemistry Letters 18 (2008) 5206–5208

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Isoxazolo[3,4-*b*]quinoline-3,4(1H,9H)-diones as unique, potent and selective inhibitors for Pim-1 and Pim-2 kinases: Chemistry, biological activities, and molecular modeling

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ARTICLE INFO

Article history:
Received 11 July 2008
Revised 20 August 2008
Accepted 22 August 2008
Available online 28 August 2008

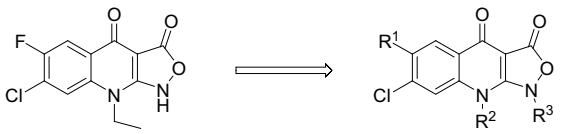
Keywords:
Pim-1 kinase inhibitors
Pim-2 kinase inhibitors

ABSTRACT

A series of isoxazolo[3,4-*b*]quinoline-3,4(1H,9H)-diones were synthesized as potent inhibitors against Pim-1 and Pim-2 kinases. The structure–activity-relationship studies started from a high-throughput screening hit and was guided by molecular modeling of inhibitors in the active site of Pim-1 kinase. Installing a hydroxyl group on the benzene ring of the core has the potential to form a key hydrogen bond interaction to the hinge region of the binding pocket and thus resulted in the most potent inhibitor, **19**, with K_i values at 2.5 and 43.5 nM against Pim-1 and Pim-2, respectively. Compound **19** also exhibited an activity profile with a high degree of kinase selectivity.

Pim-1 was identified as a Myc-cooperating oncogene linked to T cell lymphomagenesis and is preferably activated by proviral insertion in Moloney murine leukemia virus in lymphoblastic T cells.[1] Related studies have led to the discovery of a small family of serine/threonine Pim kinases including Pim-1, Pim-2, and Pim-3 with a high level of sequence homology among them.[2] Pim-1 and Pim-2 kinases have been overexpressed in a variety of human hematopoietic malignances such as myeloid or lymphoblastic leukemia and different forms of lymphomas.[3] In addition, the two kinases function in pathways distinct from/parallel to mTOR (a rapamycin target) in stimulated T cells, demonstrating an immunotherapeutical implication.[4] There is also evidence to show that Pim-1 may be involved in prostate cancers thereby extending its role to solid tumors.[5] These findings suggest a potential role for small molecule Pim-1 and/or Pim-2 inhibitors as anti-cancer agents. Recent reports of X-ray co-crystal structures of an ATP analog and other small molecule Pim-1 inhibitors bound to the active site of the Pim-1 kinase[6] may facilitate the drug discovery process.

Much of the interest in Pim kinases has been focused on biology and structural biology aspects of the targets. Here, we report our medicinal chemistry efforts leading to a novel class of potent and selective inhibitors against both Pim-1 and Pim-2 kinases. High-throughput-screening (HTS) of internal compound collections identified **1**, a molecule containing an isoxazolo[3,4-*b*]quinoline-3,4(1H,9H)-dione core, as a potent inhibitor for Pim-1 (K_i = 21.8 nM) and Pim-2 (K_i = 174 nM) kinases. This paper will disclose the general synthesis, preliminary structure–activity-relationships (SAR) studies, and the molecular modeling of this class of compounds. A key finding was that, as postulated initially, a suitable substitute at R^1 could establish an important hydrogen bond interaction with the hinge region of the active site motif of the Pim-1 kinase and lead to higher potency.



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The synthesis of compounds with R^2 variations (**1**, **6**–**8**) is shown in Scheme 1. The commercially available bis-fluoro compound **2** was treated with NaH and carbonic acid diethyl ester to give **3**. The enolate of **3** underwent nucleophilic addition to an alkyl isothiocyanate followed by methylsulfanylation led to intermediate **4**. Upon deprotonation, the amino group of **4** replaced the neighbouring fluoro on the benzene ring to furnish the quinolinone. Subsequent sulfinylation using mCPBA resulted in **5**. The isoxazolone ring of the final products was formed upon treatment of **5** with hydroxylamine. The analogs with R^3 variations (**9**-**13**, Scheme 2) were synthesized conveniently from **5** (where R^2 = ethyl) in the presence of an *N*-alkyl

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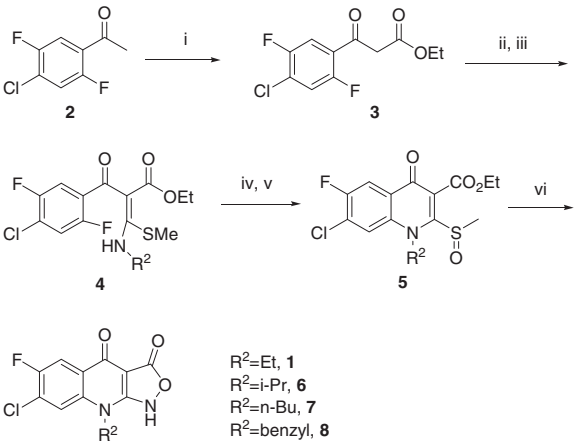
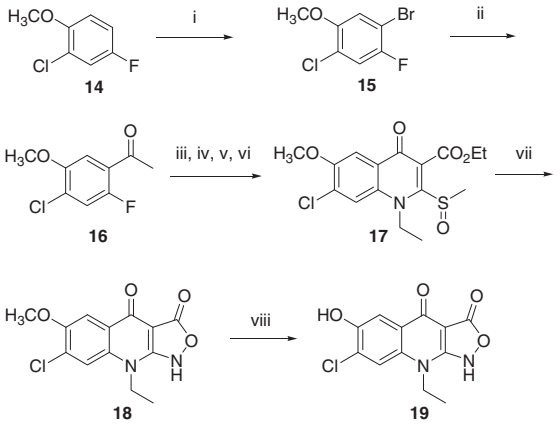
doi:10.1016/j.bmcl.2008.08.079



Scheme 1. Reagents and conditions: (i) NaH, $(EtO)_2CO$, 50%; (ii) NaH, R_2NCS, DMF; (iii) MeI, 42–71% (2 steps); (iv) NaH, THF, 78–94%; (v) mCPBA, CH_2Cl_2, 69–87%; (vi) $NH_2OH{\cdot}HCl$, NaOAc, THF, H_2O, 8–77%.

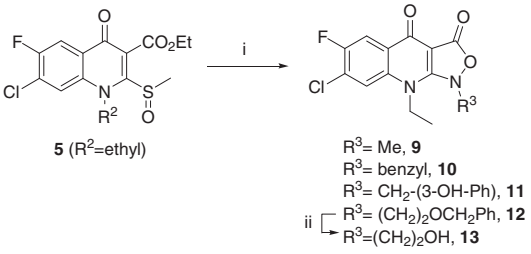


Scheme 3. Reagents and conditions: (i), Br_2, H_2SO_4; (ii) a—$PdCl_2$, PPh_3, CH_2=CHOBu, Na_2CO_3; b—HCl, Ref. 8; (iii) NaH, EtNCS, DMF; (iv) MeI, 48% (2 steps); (iv) NaH, THF; (vi) mCPBA, 63% (2 steps); (vii) $NH_2OH{\cdot}HCl$, NaOAc, THF, H_2O, 41%; (viii) $AlCl_3$, toluene, 43%.

hydroxylamine. It is noteworthy that *N*-methyl hydroxylamine gave a 1:1 mixture of the N–O regio-isomers[7] even though only the structure of the desired isomer **9** is shown in Scheme 2. The mixture was difficult to separate and thus was tested in the assay without separation. Compound **13** was derived from de-benzylation of **12** using iodotrimethylsilane.

Scheme 3 outlines the synthesis of compounds **18** and **19**. 2-Chloro-4-fluoro-1-methoxy-benzene **14** was brominated to give **15**, which was in turn acylated via a Heck coupling to afford **16** following literature procedures.[8] Compound **16** was converted into **18** utilizing the same protocols as described in Scheme 1. De-methylation of **18** in the presence of $AlCl_3$ provided the hydroxy-bearing inhibitor **19**.

X-ray co-crystal structures of the ATP analog AMP-PNP (5′adenylyl-β, γ-imidodiphosphate)[6a] or small molecule Pim-1 inhibitors bound to the active site of Pim-1 kinase have been reported recently.[6] When the HTS hit **1** was docked into the AMP-PNP co-crystal structure (Fig. 1), it was notable that it lacked any strong hydrogen bonding to the backbone carbonyl of Glu121 in the hinge region. The interaction between the ligand and the protein was instead anchored by a hydrogen bond between the carbonyl of the isoxazolone and the charged amino group of Lys67 (distance was about 3.0 Å). The oxygen on the quinolinone moiety of the inhibitor also bound to Lys67 via a water molecule. This phenomenon was observed with other Pim-1 inhibitors.[6b,6e] The region occupied by R^2 is exposed to the solvent front. Consequently, the modifications at R^2 with bulkier and longer alkyl groups did not alter the K_i value significantly (see Table 1, **1** vs **6**, **7**, and **8**). The R^3 group projects toward the Gly-rich loop of the active site and is in close proximity to the sidechain of Phe49 and the mainchain of Gly45. These residues limit the steric size of the R^3 group that can be tol-



Scheme 2. Reagents and conditions: (i) R^3-NHOH, NaOAc, THF, H_2O, 7–26%; (ii) Me_3SiI, $ClCH_2CH_2Cl$, 32%.

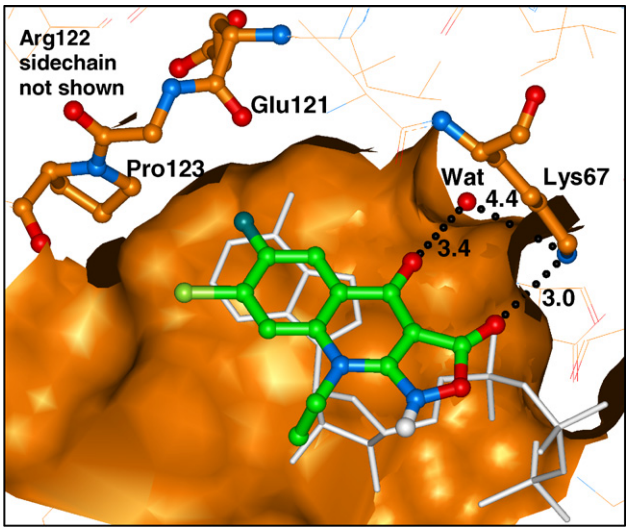


Figure 1. Model of **1** (green carbon atoms) in Pim-1 kinase (orange carbon atoms). The outline of the active site is shown as an orange surface. The 3-D structure of **1** was energy minimized (CFF force field, InsightII software, Accelrys, San Diego, CA) and manually docked into the crystal structure of Pim-1 kinase (PDB code: 1YI3) so as to optimize the Van der Waals contact with the hinge residues Glu 121 and Pro 123 and make H-bonds with Lys 67 and a conserved water molecule (Wat 21 in 1YI3, Ref. 6b; Wat 17 in 1YXV, Ref. 6c; Wat 64 in 2OBJ, Ref. 6e). Other orientations were considered, but were observed to have sub-optimal H-bond or VdW interactions. Hydrogen bond distances between heavy atoms are indicated in Angstroms. The structure of AMP-PNP (1YXT) is shown in white for reference.

Table 1
Inhibitory activities[10] against Pim-1 and Pim-2 kinases

Compound	Pim-1 K_i (nM)[a]	Pim-2 K_i (nM)[a]
1	21.8	174
6	12.3	62.8
7	15.0	165
8	36.5	558
9[b]	50.1	>1424
10	1271	>1424
11	271	>1424
12	4894	>1424
13	1162	>1424
18	2698	>1424
19	2.5	43.5

[a] Values are the mean of at least two measurements (except **12**, $n = 1$).
[b] 1:1 mixture of two regio-isomers.

erated. Replacing the proton at R^3 of compound **1** with an alkyl species larger than a methyl resulted in inhibitors with at least 12-fold less potency against Pim-1 (see Table 1, **9** to **13**) and also with less activity against Pim-2. The methylated inhibitor **9** was assayed as a mixture (~1:1) of two regio-isomers.[7] Since **9** was only slightly less active than **1** (Pim-1 K_i: 50.1 nM vs 21.8 nM) it does not appear that methylation had much impact electronically on the H-bond between the carbonyl of the isoxazolone and Lys67. In addition, neither isomer by itself would be significantly more potent than **1**. However, **9** did suffer a loss of potency against Pim-2.

In order to take advantage of a potential hydrogen bond between the ligand and the hinge region of Pim-1 kinase, the fluoro group on the benzene ring of **1** was replaced with a hydroxyl. As shown in Fig. 2, the hydroxyl group on inhibitor **19** can donate an H-bond to Glu121 O with a desirable distance of 3.0 Å. The impact of this designed hydrogen bond on affinity was uncertain because of the complicated energetics of solvation/desolvation of ligand, protein, and protein-ligand complex.[9] Additionally, any lone-pair repulsion between the fluoro group of **1** and Glu121 O would presumably be eliminated in **19** and could also be a factor in any potency change. Without a clear prediction from this complex interplay of molecular forces, we were able to evaluate compound **19** and found that its hydroxy improved the K_i value (Pim-1) of **19** over **1** by about 8 fold (2.5 nM vs 21.8 nM, Table 1). Compound **19** also displayed higher potency against Pim-2 as compared to **1** (43.5 nM vs 174 nM). The importance of the hydroxyl group on **19** was further demonstrated by inhibitor **18** where the hydroxyl was replaced by a methoxy, showing much reduced activities against both Pim-1 and Pim-2 (2698 nM and >1424 nM, respectively, Table 1). Compound **19** was assayed in a panel of 22 serine/threonine kinases (Table 2) and displayed an activity profile with a high degree of selectivity. As compared to its potent inhibitory activity against Pim-1 (6.3 nM in this assay format), this molecule was weak against all other kinases tested (K_i values at least over 1 μM) except Zipk (K_i = 27.3 nM).

In conclusion, guided by molecular modeling and known X-ray co-crystal structures, we have carried out a concise SAR study following an HTS hit (**1**) and demonstrated that compounds with a novel isoxazolo[3,4-*b*]quinoline-3,4(1H,9H)-dione core can serve as potent inhibitors for Pim-1 and Pim-2 kinases. The R^2 position of the tricyclic core can tolerate various alkyl groups due to the availability of space in the kinase active site. At R^3 only a small group, such as a proton or a methyl, can maintain the inhibitory activity of the molecules. Installing a hydroxyl group on the benzene ring (replacing F on **1**) of the core has the potential to form an optimal H-bond interaction to the hinge region of the binding pocket and thus resulted in a more potent inhibitor, **19**, with K_i values at 2.5 and 43.5 nM against Pim-1 and Pim-2, respectively. Compound **19** also exhibited a high degree of selectivity against other serine/threonine kinases.

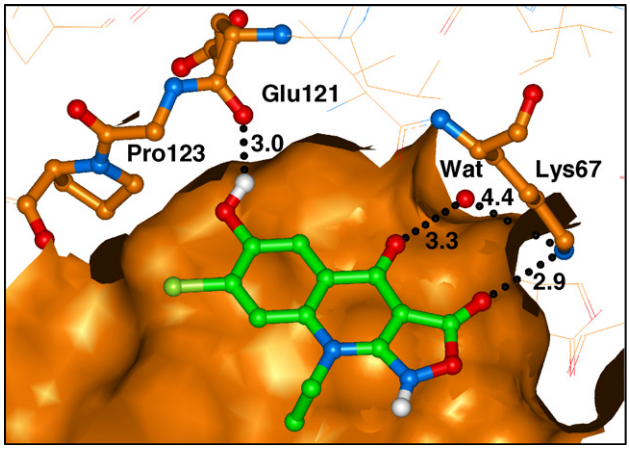


Figure 2. Model of **19** in Pim-1 kinase with similar protein interactions as **1** (Fig. 1) with the designed hydrogen bond to the backbone carbonyl of Glu 123.

Table 2
Kinase selectivity profile of **19**

Kinase	K_i (μM)[a]	Kinase	K_i (μM)
Pim-1	0.0063	CTAK1	>6.67
Src	>1.94	Casein2	1.07
Zipk	0.0273	Gsk3a	1.11
Cdc2	>8.91	MARK	>8.44
Cdk2	>8.57	PKA	>7.50
AMPK	1.73	PKCδ	>8.89
Akt1	>8.21	PKC γ	>8.75
Aurora1	>6.88	PKCξ	>8.33
Aurora2	>6.67	Plk1	>8.89
Chk1	>8.78	Rock1	>9.11
Chk2	>9.52	Rock2	>7.50

[a] 6-point screening format.[11]

Acknowledgment

The authors thank Dr. Daniel Chu for earlier synthetic work.

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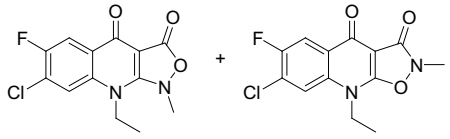
7. **9** is a mixture (~1:1) of the following two compounds:



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10. Pim kinase assays: Assays were conducted as follows with final concentrations as listed. In 384-well v-bottom polypropylene plates, 10 ml compound (2% DMSO), was mixed with 20 ml of Pim-1 (50pM), or Pim-2 (500pM) and peptide substrate (biotin-C_6linker-VRRLRRLTAREAA) (2 mM), followed by immediate initiation with 20 ml λ-[^{33} P]-ATP (5 mM, 2 mCi/mmol) using a reaction buffer comprising 25 mM HEPES, pH 7.5, 0.5 mM DTT, 10 mM $MgCl_2$, 100 mM Na_3VO_4, 0.075 mg/ml Triton X-100. Reactions were quenched after 1hr by the addition of 50 ml stop buffer (50 mM EDTA, 2 M NaCl). 80 mL of the stopped reactions were transferred to 384-well streptavidin-coated plates (FlashPlate Plus, Perkin-Elmer), incubated 30 min at rt and washed 3 times with 0.05% Tween-20/PBS using an ELX-405 automated plate washer (BioTek), and counted on a TopCount Scintillation Plate Reader (Packard).
11. In vitro kinase selectivity assays: Assays were conducted exactly as described for the Pim in vitro assay (Ref. 10) except for the enzyme concentration and substrate used, which were custom to each kinase. Substrates were either chosen from those described in literature/vendor protocols or identified through screening of a 720 peptide Jerini Kinase Substrate Set (Jerinin AG). Kinases specific reagents (phosphotidyl serine, diacyl glycerol, calcium chloride, calmodulin, cGMP) were employed only where required.