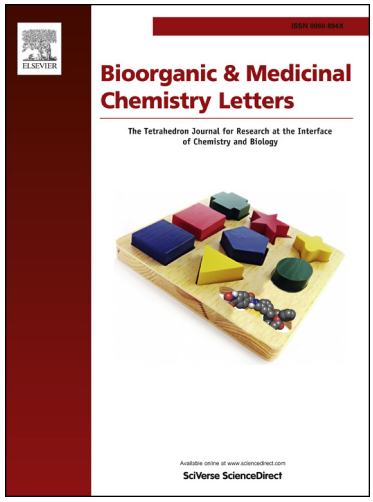

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Design and synthesis of substituted pyrido[3,2-*d*]-1,2,3-triazines as potential Pim-1 inhibitors

Yin-Bo Fan[a], Kun Li[a], Min Huang[a], Yu Cao[a], Ying Li[b], Shu-Yu Jin[a], Wen-Bing Liu[a], Jia-Chen Wen[a], Dan Liu[a*] and Lin-Xiang Zhao[a*]

[a]*Key Laboratory of Structure-Based Drugs Design & Discovery of Ministry of Education, Shenyang Pharmaceutical University, Shenyang 110016, China*

[b] *Department of Pharmacology, Shenyang Pharmaceutical University, Shenyang 110016, China*

[*]Corresponding authors. Tel.: +86 024 2398 6420; Fax: +86 024 2398 6420

E-mail address: sammyld@163.com (Dan Liu); linxiang.zhao@vip.sina.com (Lin-Xiang Zhao)

Abstract

A novel series of substituted pyrido[3,2-*d*]-1,2,3-triazines were designed and synthesized as Pim-1 inhibitors through scaffold hopping. Most of the derivatives showed potent *in vitro* Pim-1 inhibitory activities and anti-proliferative effects towards prostate cancer cells. Among them, **6b**, **6h** and **6m** showed the best Pim-1 inhibitory activity with IC_{50} values of 0.69, 0.60 and 0.80 μM, respectively. Furthermore, compound **6b**, **6i**, **6j** and **6m** showed strong inhibitory activity to human prostate cancer LNcap and PC-3 cell lines with IC_{50} values at low micromolar level. Structure-activity relationship analysis revealed that appropriate substitutions at C-6 positions contributed to the kinase inhibition and antiproliferative effects. Moreover, western blot assay suggested that **6j** could decrease the levels of p-BAD and p-4E-BP1 in a dose-dependent manner in PC-3 cells. Docking studies showed that 3-N of the scaffold formed a hydrogen bond with Lys67, aromatic 4-aniline formed a key π-π stack with Phe49. Taken together, this study might provide the first sight for developing the pyrido[3,2-d]-1,2,3-triazine scaffold as novel Pim-1 inhibitors.

***Keyword*:** Pim-1 kinase inhibitors; Pyrido[3,2-d]-1,2,3-triazines; Anti-proliferative activity; Prostate cancer cells.

Cancer presents to be the most threat to human health, despite of numerous investments, the relentless and lethal nature of cancer persists, with only limited overall improvements in treatment outcomes. The discovery of anticancer drugs is undergoing and remains a highly challenging endeavor.[1] Dysregulation of kinase function has emerged as a robust research area for cancer therapy, as it consists of one of the major mechanisms through which cancer cells escape from normal constraints on growth and proliferation. About 518 kinases have been recognized in human kinome, among which only a few have been successfully targeted for diseases like cancer and inflammation.[2]

Pim-1, a member of serine/threonine kinase, has been greatly involved in complex signaling mechanisms associated with tumorigenesis and drug resistance.[3-6] High expression level of Pim-1 has often been associated with hematological malignancies and solid cancers like prostate cancer.[7-9] Increasing expression of Pim-1 leads to promotion of proliferation and blockade of apoptosis. Pim-1 has been reported to phosphorylate lots of substrate proteins including BAD and 4E-BP1 to enhance cell survival and proliferation.[5] In addition, it synergistically works with c-Myc in tumorigenesis.[10] Thus, Pim-1 serves as a promising therapeutic target to solve numerous unmet medical needs in cancer therapy.

There has been persistent interest for developing Pim-1 inhibitors, and typical inhibitors reported contain rigid fused rings as critical scaffold such as Imidazopyridazines, triazolopyridazines, pyrazolopyrimidines and triazolopyridines (**Figure 1**), which were recently utilized in the design of potent Pim-1 inhibitors.[11-15] Among those inhibitors, SGI-1776 (**1**) was the first generation of Pim-1 inhibitor characterized with imidazopyridazine structure. Though exhibiting excellent potency towards Pim-1 kinase, it was terminated due to

the dose limiting cardiac toxicity in phase Ⅰ clinical trail.[13] Efforts to improve the safety files were undergoing, it also provoked us to discover alternative novel chemotypes to be explored as Pim-1 inhibitors.

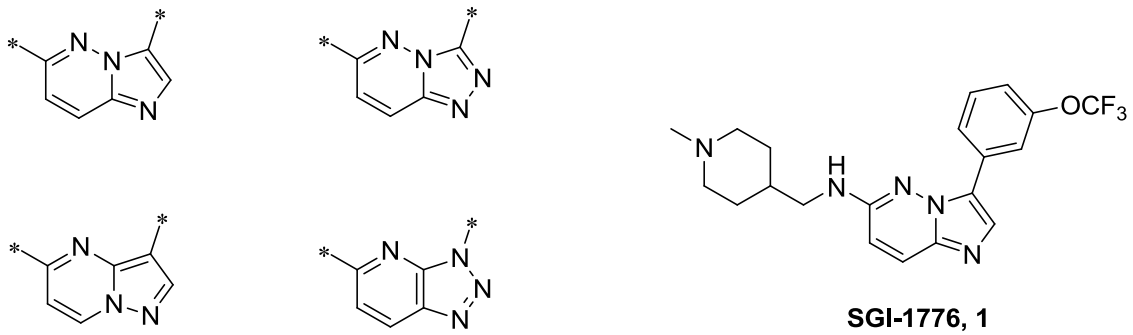


Figure 1. The structures of reported Pim kinase inhibitor cores including Imidazopyridazines, Triazolopyridazines, Pyrazolopyrimidine, Triazolopyridine and the representative lead SGI-1776.

In our efforts to discover novel Pim-1 kinase inhibitors, the five-membered imidazole, pyrazole and triazole ring in the reported structures were replaced with the six-membered ring of triazine to accomplish a novel scaffold and template of pyrido[3,2-*d*]-1,2,3-triazine under the guidance of scaffold hopping strategy in medicinal chemistry, 3-trifluoromethoxyphenyl was changed into corresponding aniline at suitable C-4 position (**Figure 2**). To illuminate the Pim-1 kinase inhibition by this unique and proprietary scaffold in a systemic way, a series of novel pyrido[3,2-d]-1,2,3-triazine derivatives featured with various C-6 substitutions were described, their inhibitory activities towards Pim-1 as well as the *in vitro* antiproliferative activity towards two human prostatic cancer LNcap and PC-3 cell lines were studied. Finally, the inhibition towards the phosphorylation of BAD and 4E-BP1, two well-established Pim-1 kinase substrates, were also investigated with representative compound in PC-3 cells. The

possible binding mode in the ATP pocket of Pim-1 was illustrated to find a unique and tight binding for these compounds.

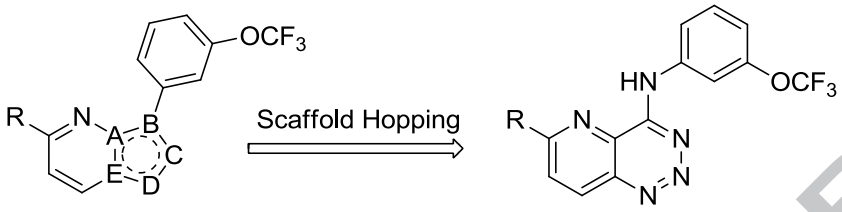
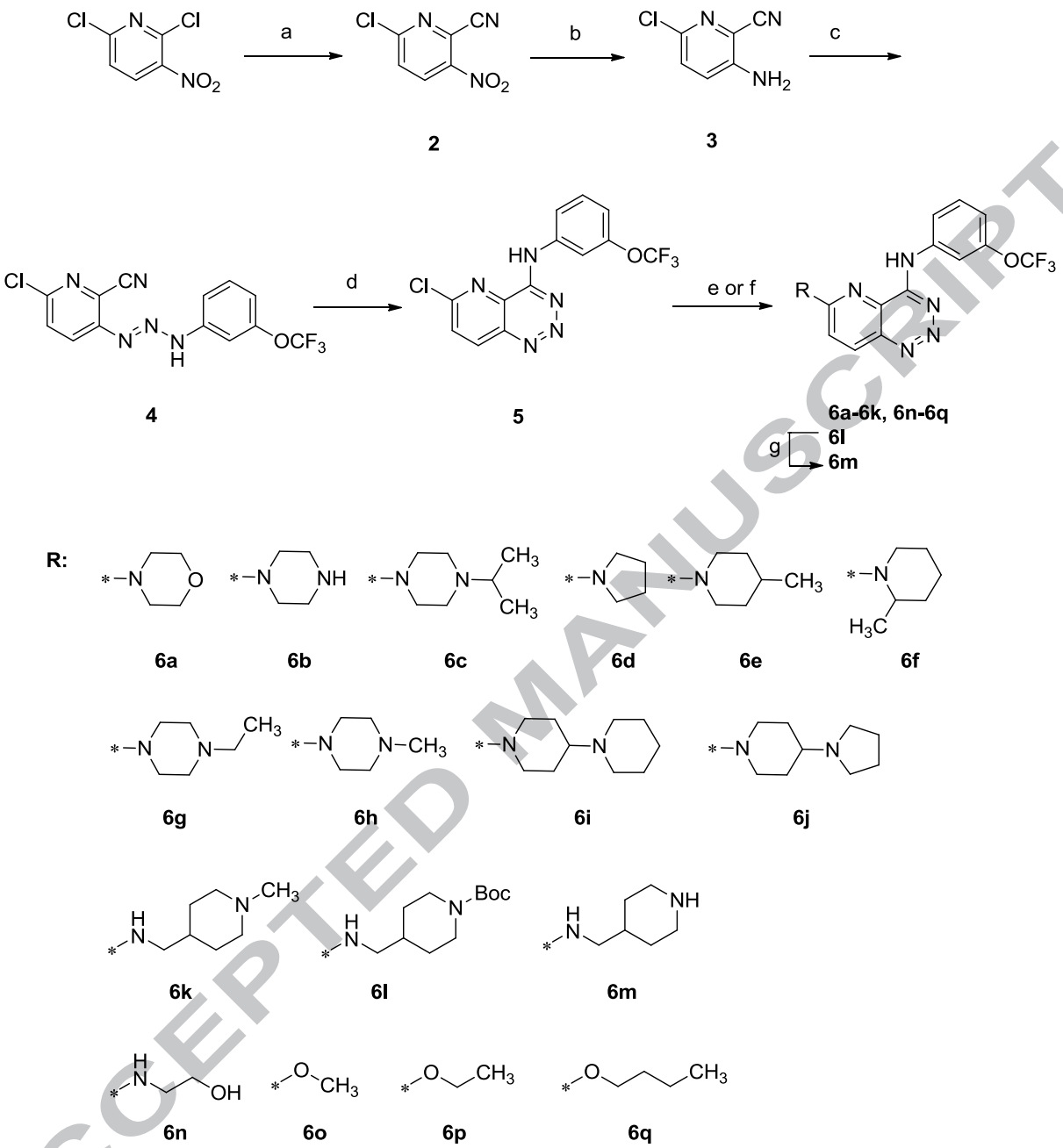


Figure 2. Design of pyrido[3,2-d]-1,2,3-triazines as new scaffold for Pim-1 inhibitors.

The construction of pyrido[3,2-*d*]-1,2,3-triazine scaffold was described in our earlier work (as shown in **Scheme 1**).[16] The requisite intermediate **2** was synthesized in a traditional method by treating commercial available 2,6-dichloro-3-nitropyridine with cuprous cyanide at reflux, sodium thiosulphate was utilized as reductant converting nitro into amino group to give 3-amino-6-chloro-2-cyanpyridine **3**. The synthesis of scaffold **5** was conducted through cyclization and Dimroth rearrangement of **4**, which was prepared by diazotization of **3** and 3-trifluoromethoxyaniline. Scaffold **5** was further reacted with two fold stoichiometric saturated *N*-heterocycles to afford **6a-6l**, de-protection of the Boc group with chlorine hydride in EtOAc from **6l** afforded **6m**. Moreover, in order to evaluate the influence on Pim-1 kinase inhibition of C-6 position substitutions at the pyrido[3,2-*d*]-1,2,3-triazine scaffold, linear alkyl substituted **6n** was also prepared with **5** and monoethanolamine in neutral ethanol under reflux, nucleophilic attack of **5** with particular sodium alkoxide in corresponding alcohol afforded alkyl ethers **6o-6q**.



Scheme 1. Synthesis of compounds **6a-6q**. Reagents and conditions: a) CuCN, NMP, reflux, 31%; b) $Na_2S_2O_3$, CH_2Cl_2, H_2O, 40%; c) 3-trifluoromethoxy-aniline, $NaNO_2$, HCl, 0℃, 50%; d) 70% EtOH(*aq*.), reflux, then acetic acid, reflux, 60%; e) RH, EtOH or *i*-PrOH, reflux, 65-90%; f) RH, correspounding sodium alkoxide, r.t., 71-77%; g) HCl in EtOAc, CH_2Cl_2, r.t., 70%.

To investigate the Pim-1 kinase inhibitory potency of all synthesized compounds **6a-6q**, the IC_{50} values were determined for each compound with an advanced HTRF method at Cisbio. SGI-1776 (**1**) exhibited a significant inhibitory effect with IC_{50} value of 0.048 μM in the tested system (the reported value is 0.007 μM),[8] and the results for all target compounds were shown in **Table 1**. The scaffold **5** with chlorine substituted at C-6 position exhibited little potency (IC_{50}>10 μM). Generally, the substitutions with various saturated *N*-heterocycles at this particular position (**6a-6m**) were found to be more potent than **5** and their ethanolamine (**6n**) or alkyl ether counterparts (**6o-6q**). Morpholine (**6a**), piperazine (**6b**), isopropylpiperazine (**6c**) ethylpiperazine (**6g**) and methylpiperazine (**6h**) substitutions led to strong inhibition with IC_{50} values 1.45, 0.69, 1.08, 2.04 and 0.60 μM, respectively, indicating that a heteroatom is favorable at 4'-position of the heterocycles, compared with non-heteroatom counterparts (**6d** and **6e**: IC_{50}>10 μM). The large substitution of heterocycles were tolerated to the Pim-1 kinase inhibition effects, most strikingly elucidated by di-heterocycle substituted **6i** and **6j** (IC_{50} values were 2.47 and 1.05 μM, respectively). **6k** shares the same substitution with SGI-1776, but improved potency was not observed against Pim-1 with IC_{50} of 1.87 μM. It was noteworthy that **6l**, which has a bulky Boc terminal group compared with **6k**, lost potency completely. Meanwhile, **6m** with 1'-NH uncovered at the piperidine showed improved activity with IC_{50} value of 0.80 μM. Moreover, **6n** exhibited similar potency with **6a**, which could be attributed to the existence of ethanolamine group, the hydroxyl group might be equivalent with the 4'-heteroatom of saturated *N*-heterocycles, as the ethyl linker could help to expand it into a suitable position similar with the six-membered saturated rings. For comparison, compound **6p** and **6q** with linear alkyl ether attached to the

bicyclic core at C-6 was detrimental for Pim-1 inhibitory activity (IC_{50}> 10 μM), as the flexible alkoxyl groups lack terminal heteroatom, sharply decreasing the kinase inhibition activity. All these data confirmed that the substitutions at C-6 position were beneficial to pledge potent Pim-1 kinase inhibitory activities of pyrido[3,2-*d*]-1,2,3-triazine analogues. Piperazine (**6b**), methylpiperazine (**6h**) and piperidin-4-ylmethanamine (**6m**) were the best in the series, with IC_{50} values of 0.69, 0.60 and 0.80 μM, respectively.

Pim-1 is often highly expressed in prostate cancer cells, the antiproliferative activity of the target compounds against human prostate adenocarcinoma LNcap and PC-3 cell lines was further evaluated using MTT assay, and the results fitted well with the Pim-1 kinase inhibition data. SGI-1776 displayed good potency with IC_{50} values of 6.85 and 4.86 μM towards LNcap and PC-3 cells. The target compounds demonstrated no significant difference in inhibiting the growth between two cell lines, regardless of dependence on androgen. Generally, the introduction of suitable saturated *N*-heterocycle in C-6 position of the scaffold leading to potent Pim-1 inhibition also contributed to improved anti-proliferative activities, while alkoxy substituted groups sharply decreased the inhibitory activities towards Pim-1 and intact cancer cells. Di-heterocycle derivatives **6i** and **6j** displayed the most potent inhibitory effects towards both cell lines with IC_{50} ranging from 4.90 to 10.70 μM, similar with the reported Pim-1 inhibitor SGI-1776. Mono-heterocycle derivatives **6b**, **6c**, **6g**, **6h** and **6m** proved to be less potent antiproliferative compounds, which displayed a low micromolar activity between 5.51 and 16.89 μM against both cancer cells. compound **6b** and **6m** were more active than **6a**, suggesting that 4'-NH as a hydrogen bond donor in the heterocycle showed some advantage over 4'-O both in biochemical kinase assay and in cultured cells. Once the 4'-position of the

piperazine in **6a** was alkylated with methyl, ethyl or isopropyl group, its potency decreased, as can be seen from comparing **6b** with **6c**, **6g** and **6h**, even though methylpiperazine gave the most potent compound **6h** towards Pim-1 inhibition. Substituted piperidyl derivatives **6e** and **6f** precipitated in the testing system, while tetrahydropyrrole substituted **6d** showed no potency (IC_{50}> 100 μM), indicating that compounds substituted with mono-heteroatom-containing heterocycle was inferior to two-heteroatom-containing ones, which was consistent with Pim-1 kinase inhibition data. **6n** exhibiting potent Pim-1 inhibitory activity failed to display expected potency in cultured cell. In comparison, the substitution of alkoxy group led to abrogation of activities, ethoxy substituted compound **6p** had no effect towards the tested cells, while **6o** and **6q** precipitated during the cell effect measurement. Though SGI-1776 demonstrated greater potency in Pim-1 inhibition, it displayed equivalent activity with **6i** and **6j** against cancer cells. In conclusion, **6b**, **6i**, **6j** and **6m** with good inhibition of Pim-1 kinase activity showed significant activity toward the two tested cancer cell lines, and were thus regarded as the most promising candidates as potential Pim-1 inhibitors among those target compounds.

Table 1. The inhibitory activities of compounds **5**, **6a-6q** on Pim-1 kinase, LNcap and PC-3 cell lines.

Compd.	IC_{50}(μM)		
	Pim-1[a]	LNcap[b]	PC-3[b]
5	>10	8.62±0.35	5.99±0.16
6a	1.45	30.99±6.49	40.97±2.43
6b	0.69	8.51±0.81	11.50±0.24
6c	1.08	10.67±0.96	13.82±0.33
6d	>10	>100[c]	>100
6e	>10	-[d]	-
6f	1.31	-	-

6g	2.04	14.28±0.46	16.89±0.88
6h	0.60	20.27±0.97	24.04±3.13
6i	2.47	4.90±1.29	5.58±0.30
6j	1.05	10.70±1.33	8.16±0.57
6k	1.87	17.34±0.63	15.76±1.42
6l	>10	>100	>100
6m	0.80	11.52[c]	8.32[c]
6n	2.10	42.88±3.36	>100
6o	2.02	-	-
6p	>10	>100	>100
6q	>10	-	-
SGI-1776	0.048	6.85±0.24	4.86±0.16

[a]All results represent the mean of duplicate experiments. [b]Data shown are means ± SD of three independent experiments. [c]Data shown are the results of single experiment. [d]Precipitated in the testing system.

To gain further insights into the mechanisms of action of these compounds, compound **6j**, which possessed significant Pim-1 and cellular inhibitory effects, was selected to examine the inhibitory effect on the phosphorylation level of BAD at Ser112 with western blot analysis in PC-3 cells. Pim-1 catalytic BAD phosphorylation results in its anti-apoptotic activity, thus the phosphorylation level of BAD could reflect the residual activity of Pim-1 kinase indirectly.[17] PC-3 cells were treated with 10 μM SGI-1776 and **6j** at different concentrations or DMSO as negative control for 24 h. The results (as shown in **Figure 3**) indicated that **6j**, acting exactly like SGI-1776, could dose-dependently block the BAD phosphorylation without affecting Pim-1 expression in PC-3 cells. Meanwhile, Pim-1 regulates protein translation through phosphorylating 4E-BP1, which releases eIF4E, a curial component involved in cap-dependent translation to promote survival for cancer cells.[3,18] Treatment of **6j** for 24 h down-regulated 4E-BP1 phosphorylation at Thr37/46 in PC-3 cells. Both of the dephosphorylating effects of **6j** might be partially caused by Pim-1 kinase inhibition just like SGI-1776. Additionally, the level of c-Myc associated with Pim-1 kinase was observed to

decrease upon SGI-1776 and **6j** treatment, suggesting a potential inhibitory effect on *myc* oncogene driven oncologic events. Taken together, our cellular assays implied that the representative **6j** blocked proliferation in intact prostate cancer cells partly through inhibiting Pim-1 enzymatic activity.

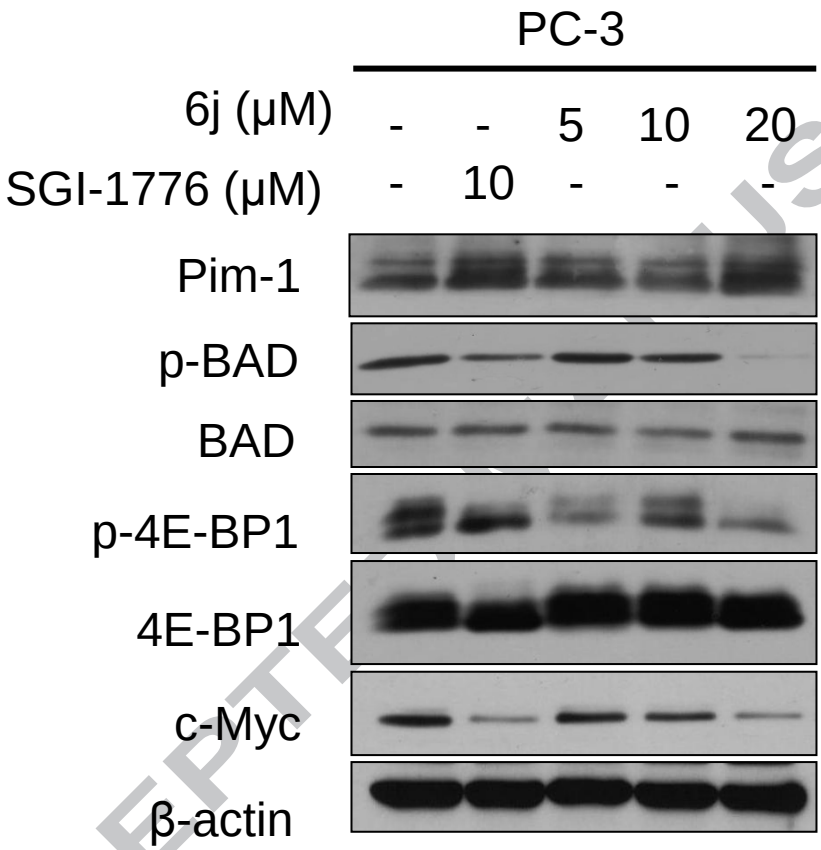


Figure 3. 6j (0, 5, 10, 20 μM) and SGI-1776 (10 μM) decreased the expression of p-BAD, p-4E-BP1 and c-Myc in PC-3 cells.

In order to understand the binding mode of **6j** within Pim-1 kinase, the docking experiments were conducted with Discovery studio software based on the X-ray co-crystal structure of Pim-1 with a 1,2,3-triazolo[4,5-b]pyridine derivative (PDB 4A7C).[14] Supporting the docking results, the original ligand was first docked and fitted well with that in crystal structure, then **6j** was docked with the same parameters. The binding mode (shown in **Figure**

4) demonstrated that **6j** inserted into and occupied the Pim-1 ATP binding cleft completely. Differently, **6j** adopted a 180 degree flip manner comparing to the inherent triazolopyridine ligand in the pocket as shown in the overlapped version (**Figure 5**). Consistent with hydrogen bonds formed with Lys67 in the ligand binding mode, a key hydrogen bonds established between Lys67and 3-N of the triazine scaffold. Despite the loss of interaction with Asp128 due to the flip effect, a π-π stack existed between the substituted aniline of **6j** and Phe49 in return, which further stabilized the binding. The side chain at C-6 position of the scaffold extended to the entrance of the pocket. The hydrophobic segments of **6j** were buried into the hydrophobic pocket formed by Phe49, Ala65, Val52, Ile104, Leu120 and Ile185. Similar to the original ligand, no interactions could be observed between the molecule and the hinge region of Pim-1 (Arg122). The docking study suggested a unique and tight binding within the ATP-binding region, distinguished from the triazolopyridine template.

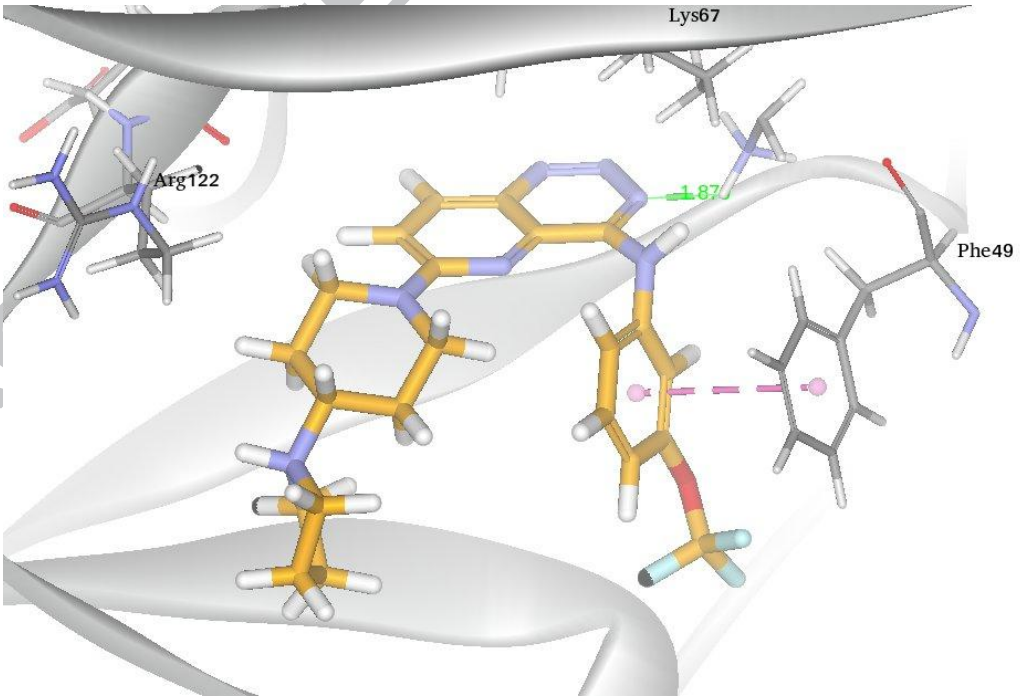


Figure 4. Proposed binding mode of **6j** in the ATP binding pocket of the Pim-1 kinase domain.

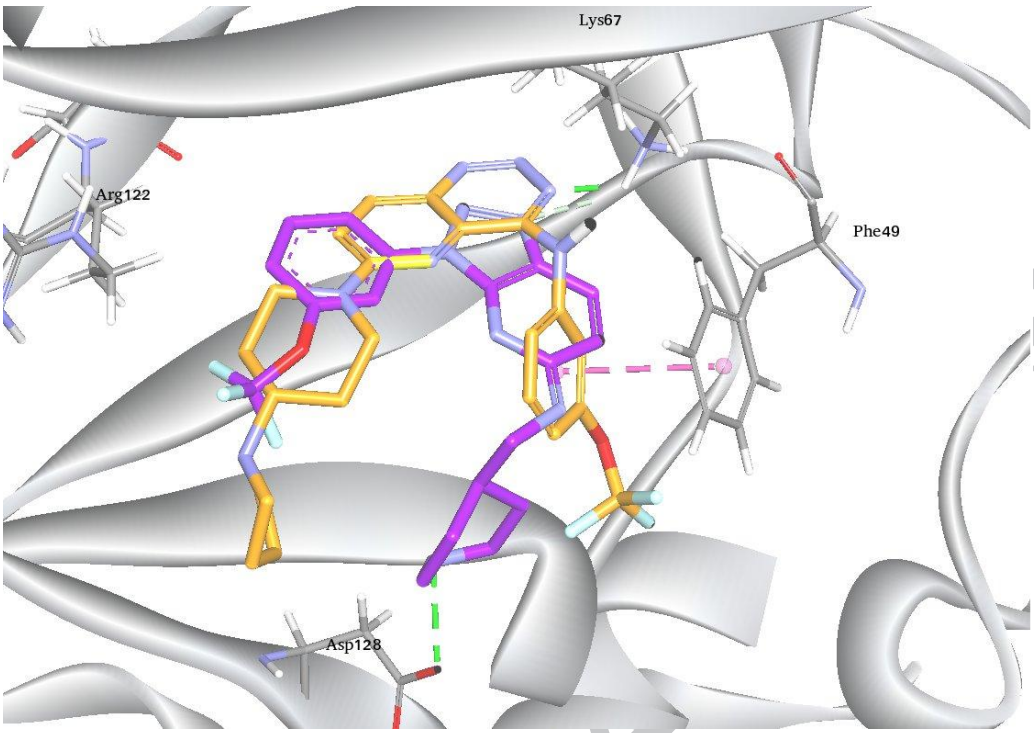


Figure 5. Overlapping spectacle of **6j** (carbon atoms colored in orange) and triazolopyridine ligand (carbon atoms colored in purple) within the ATP binding pocket of the Pim-1 kinase domain.

We report herein the discovery and synthesis of novel pyrido[3,2-*d*]-1,2,3-triazines as potential Pim-1 inhibitors. Compared to the scaffold **5** and its analogues substituted with 6-alkoxyl (**6o**-**6q**), the 6-amine series (**6a**-**6n**) were identified as more potent Pim-1 kinase inhibitors. Among which, **6b**, **6h** and **6m** were the most potent Pim-1 inhibitor with IC_{50} values of 0.69, 0.60 0.80 μM, respectively; **6i** and **6j** exhibited a good potency to inhibit Pim-1, with IC_{50} values 2.47 and 1.05 μM. They all exhibited low micromolar anti-proliferative activity toward prostatic cancer PC-3 and LNcap cell lines. In addition, **6j** could block the phosphorylation of BAD and 4E-BP1 in a dose-dependent manner in PC-3 cells. Molecular modeling illustrated a different but strong binding effect for **6j** within Pim-1 ATP-binding site, compared to the triazolopyridine ligand. Together, these results provide

compelling evidence that the antiproliferative activity of these compounds in PC-3 cells was mediated, at least in part, by the inhibition of Pim-1 kinase, thereby enhanceing the proapoptotic effect of BAD. Therefore, this unique pyrido[3,2-*d*]-1,2,3-triazine scaffold demonstrated great potential to be further explored as potent Pim-1 inhibitors with improved physicochemical property and potency. Efforts are ongoing to further optimize and provide a systematic structure-activity analysis at the proper position and the results will be reported in due course.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http:// .

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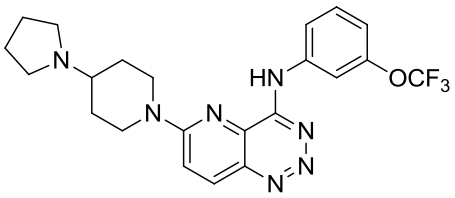
Graphical abstract



6j

Pim-1 IC_{50} = 1.05 μM

PC-3 IC_{50} = 8.16 μM

LNcap IC_{50} = 10.7 μM