Letter

Design and Synthesis of Potent and Selective PIM Kinase Inhibitors by Targeting Unique Structure of ATP–binding Pocket

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Design and Synthesis of Potent and Selective PIM Kinase Inhibitors by Targeting Unique Structure of ATP–binding Pocket

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ABSTRACT: In the development of kinase inhibitors, one of the major concerns is selectivity. An effective strategy to achieve high selectivity is to utilize structural differences among kinases to inform inhibitor design. Here, we set out to improve the PIM (proviral integration site for Moloney murine leukemia virus) kinase-inhibitory selectivity of our previously reported 7-azaindole derivative **2**, which has promising ADMET properties, by targeting a unique bulge in the ATP-binding pocket. 6-Substituted 7-azaindoles, especially the 6-chlorinated derivatives, proved to be potent and selective PIM kinase inhibitors, and appear to be promising lead compounds for future drug discovery.

Selective kinase inhibitors are useful both as tools to probe signal cascades and as candidate drugs to treat diseases.[1] Kinases form a large family in the human proteome (>500 members), and regulate cellular events by phosphorylating a wide range of substrates. Over-activation or over-expression of kinases may lead to excessive phosphorylation of their substrates, which in turn may lead to hyper-activation of signal cascades and physiological changes such as tumorigenesis and inflammation.[2] Because kinases share ATP as a common substrate, development of ATP-competitive inhibitors ("type I" inhibitors) is a general strategy to inhibit over-activated kinases. However, the ATP-binding pockets of kinases are generally similar, making the design of selective ATP-competitive kinase inhibitors challenging. To achieve high selectivity, it has been proposed to target the non-ATP-competitive pocket of the inactive form of kinases ("type II" inhibitors) or the allosteric pocket ("type III and IV" inhibitors), because these pockets are not shared among kinases.[3] However, targeting the active conformation is the only available strategy to develop inhibitors of kinases that do not form either inactive conformations or allosteric pockets.

Typically, ATP-competitive type-I kinase inhibitors form a pair of hydrogen bonds in the ATP-binding pocket of kinases, in a similar way to ATP.[4] The hydrogen-bonding partners are a carbonyl O (acceptor) and an amide NH (donor) pair in the hinge region, which connects the N-lobe and the C-lobe of the protein.

PIM (proviral integration site for Moloney murine leukemia virus) kinases are the only kinases that cannot form the canonical bidentate hydrogen bonds with ATP and ATP-competitive inhibitors, because they have a proline residue in the hinge region at the position of the hydrogen donor.[5–7] The lack of this hydrogen donor, a significant structural difference from other kinases, offers an opportunity to design PIM-selective inhibitors.

The PIM kinase family consists of three constitutively active proto-oncogenic serine/threonine kinases.[8–10] PIM kinases share substrates such as BAD, p21, p27 and MYC, and regulate apoptosis, the cell cycle and cell survival. Typically, PIM1 is mainly overexpressed in acute myeloid leukemia and PIM2 plays a major role in multiple myeloma.[11] PIM3 is reported to be overexpressed in endoderm-derived organ cancers, including pancreatic cancer.[12] PIM triple knockout mice are reported to be healthy, although their size is smaller than usual.[13] Various PIM inhibitors have been reported to date,[14–16] however, none of them has been marketed so far. SGI-1776 is a representative first-generation PIM inhibitor, which had been under clinical trials for leukemia and prostate cancer.[17–19] While most of the first-generation PIM inhibitors are PIM1-selective, there is currently great interest in the potential of pan-PIM inhibitors to treat cancer, because the three PIM kinases are reported to function redundantly.[20] Representative pan-PIM inhibitors are AZD1208 from AstraZeneca,[21,22] and PIM447 from Novartis.[23] Clinical trials of PIM447 are underway.

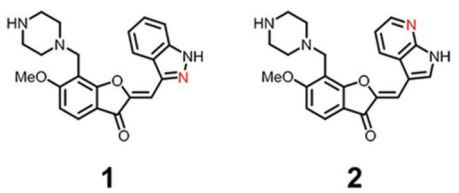


Figure 1. Structures of compounds **1** and **2**.

In previous study, we reported a 2-azaindole (indazole) derivative **1** and a 7-azaindole derivative **2** as potent PIM1 inhibitors (Figure 1).[24] A schematic representation of **1**-PIM1 binding is depicted in Figure 2. Compound **1** was confirmed to be highly PIM1-selective, whereas **2** was poorly selective. The difference between **1** and **2** is the position of the nitrogen atom in the indole ring. In **1**, the highly selective PIM1 inhibitor, there is no hydrogen bond-accepting nitrogen at the 7-position of the ring, which is proximal to the gatekeeper + 3 residue, the canonical hydrogen donor of general kinases (Figure 2).[24] On the other hand, the 7-position nitrogen in **2** can accept hydrogen from off-target kinases, which lowers the selectivity. However, **2** showed more favorable in vitro ADMET properties, such as higher aqueous solubility and membrane permeability, and lower hERG inhibition (Table 3, *vide infra*). Therefore, we set out to improve the kinase selectivity of **2**, which is promising in terms of ADMET properties.

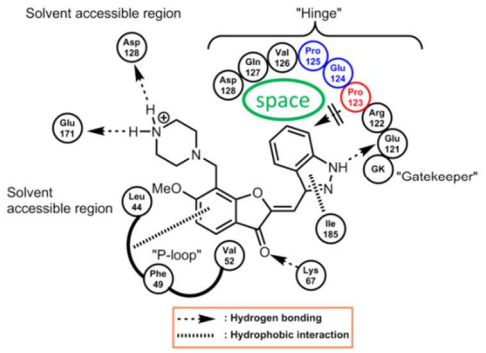


Figure 2. Schematic representation of the complex of compound **1** and PIM1 kinase (PDB ID: 3UMW). Dashed arrows indicate hydrogen bonds between compound **1** and PIM1. Pro123, the non-hydrogen donor in the gatekeeper + 3 position, is colored red. Glu124 and Pro125, two inserted residues, are colored blue.

To improve the kinase selectivity of the 7-azaindole derivative, we targeted structurally distinct features in the ATP-binding pocket of PIM kinases.[5–7] First, PIM kinases are the only kinases that have a proline at the gatekeeper + 3 position and cannot donate hydrogen, as mentioned above. Second, in contrast to most kinases, PIM kinases have one or two extra amino acids inserted downstream of the ATP-binding pocket to create a bulge at the end of the hinge region, which should accommodate substituents at the 6-position of the heterocyclic ring (Figure 2). We hypothesized that introduction of a suitable 6-substituent in the 7-azaindole ring would be sterically tolerated by PIM, but not by off-target kinases, and hydrogen bonding between the 7-position nitrogen in the 7-azaindole ring and off-target kinases would be blocked, thereby increasing the selectivity for PIM kinase. Therefore, we planned to introduce substituents at the 6-position of the 7-azaindole ring in order to improve the poor kinase selectivity of **2** (Figure 3).

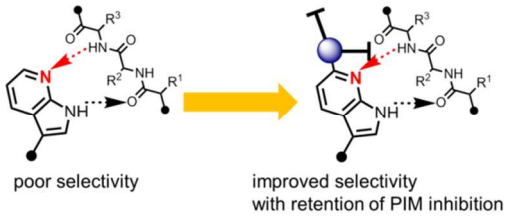
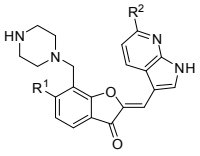


Figure 3. Basis of our plan for improving the kinase selectivity of **2**. Substituents at the 6-position of the 7-azaindole ring would interfere with hydrogen bonding between the inhibitor and off-target kinases.

Table 1. Effect of substituents at the 6-position of the 7-azaindole ring on PIM1-inhibitory potency



Comp.	R^1	R^2	IC_{50} (nM) [a]	cLogP [b]	LLE [c]
2	OMe	H	0.84 [d]	2.8	6.3
3		Me	0.64	3.3	5.9
4		Et	1.1	3.9	5.1
5		*n*-Pr	4.5	4.4	3.9
6		*n*-Bu	2.1	4.9	3.8
7		Ph	16	4.9	2.9
8		F	0.28	3.1	6.4
9		Cl	0.41	3.6	5.8
10	OH	H	2.0	2.5	6.2
11		Me	3.8	3.0	5.4
12		Et	16	3.5	4.3
13		F	1.8	2.7	6.0
14		Cl	1.4	3.3	5.6

[a] All compounds were evaluated in the same experiment (n = 4). [ATP] = 30 μM.

[b] Calculated logarithm of the octanol/water distribution coefficient using ChemBioDraw Ultra Version 13.0.

[c] LLE = $-\log(IC_{50})$ – cLogP. [d] In a previous study (ref. 24), the IC_{50} was reported as 2 nM.

To test our hypothesis, we introduced various substituents at the 6-position of the 7-azaindole ring, and examined whether or not the resulting compounds retained PIM1-inhibitory potency. Initially, we introduced neutral alkyl and aromatic groups at the 6-position of the 7-azaindole ring in **2** as steric shields, because such neutral groups would not form unwanted extra hydrogen bonds and/or polar interactions with off-targets (Table 1, com-

pounds **3**–**7**). As we expected, the introduction of a small methyl group did not affect the potency, while the introduction of a bulky phenyl ring reduced the potency. Unexpectedly, longer alkyl groups such as ethyl, *n*-propyl and *n*-butyl groups also did not reduce the potency, compared with the unsubstituted analogue **2**, although LLE scores[25] were significantly decreased with longer alkyl groups. Because there is only a small hydrophobic space in the pocket, there could be unfavorable steric clash between the longer alkyl chains and the protein. We speculated that the sub-optimal binding modes of the derivatives due to unfavorable steric clash of alkyl chains were compensated by hydrophobic interaction of the flexible P-loop and the methoxy group on the benzofuranone core (Figure 2).[26] Indeed, when the methoxy group was replaced with a hydroxyl group to eliminate the hydrophobic interaction, a 6-methyl substituent was tolerated, but a 6-ethyl substituent, only one carbon atom larger, was not tolerated (compounds **10**–**12**). Further, we introduced small halogen atoms in the same position. Fluorine or chlorine substitution did not affect the PIM1-inhibitory potency, irrespective of the substituents on the benzofuranone core (compounds **8**, **9**, **13** and **14**).

We then confirmed that the poor kinase selectivity of **2** was significantly improved by the introduction of substituents at the 6-position of the azaindole ring, as we had hypothesized (Figure 4 and Table S1, Supporting Information). The selectivity of each inhibitor was evaluated at 200 nM concentration against a panel of 52 kinases at Carna Biosciences. In the case of **2**, nine off-target kinases were inhibited by more than 50%, in addition to PIM1 and 3. Among the nine off-targets, seven were potently inhibited (>75%). In particular, PKCα and ROCK1 were completely inhibited (right-side of the graph) by **2**. Compared with **2**, compounds **3** and **4**, having sterically demanding short-alkyl substituents, showed improved selectivity profiles. Six off-targets were inhibited by more than 50% by the methylated derivative **3**, and only four off-targets were inhibited by more than 50% by the ethylated derivative **4**. One kinase (DAPK1: 91%) was potently inhibited by **3**, and two kinases (DAPK1: 77% and PKD2: 90%) were potently inhibited by **4**. It is noteworthy that PKCα and ROCK1, which are two major off-targets of **2**, were inhibited by **3** and **4** with significantly reduced potency (PKCα: ≈ 10% inhibition and ROCK1: ≈ 50% inhibition). Because halogens are electron-withdrawing atoms, it is possible that they reduce the hydrogen-accepting ability of the 7-nitrogen atom sterically as well as electronically, thereby significantly improving the kinase selectivity. Indeed, **8**, with small but electronegative fluorine substitution, also showed an improved selectivity profile compared with **2**. Six off-targets were inhibited by more than 50%, and three of them were potently inhibited (PDGFRα: 79%; DAPK1: 97%; ROCK1: 94%) by **8**. PKD2, the major off-target of **4**, was only modestly (59%) inhibited by **8**. These results show that the poor kinase selectivity profile of **2** could be greatly improved by introducing sterically demanding substituents (**3** and **4**) or an electronegative substituent (**8**). Gratifyingly, **9**, having the sterically demanding and electronegative chlorine substituent, showed a very clean selectivity profile, as expected. Indeed, compound **9** inhibited only three off-target kinases by more than 50% (PDGFRα: 61%; DAPK1: 80%; PKD2: 62%) while it completely inhibited PIM1 (99%; IC_{50} = 0.49 nM) and PIM3 (>99%; IC_{50} = 0.40 nM). Moreover, **14**, a less lipophilic analog of **9**, showed an even cleaner profile; no off-targets were inhibited potently (>75%), and only two off-target kinases were inhibited by more than 50% by **14** (PDGFRα: 63%; DAPK1: 57%). PKD2 was not inhibited by **14** (30% inhibition). Importantly, **14** completely inhibited PIM1 (99%; IC_{50} = 1.3 nM) and PIM3 (>99%; IC_{50} = 0.88 nM). The hinge of PDGFRα and DAPK1 are one amino acid residue shorter than that of PIM, therefore we could not find a structural rationale why **14** still inhibits the two off-targets. To our knowledge, PDGFRα and DAPK1 are not common off-targets of PIM inhibitors. Further cell–based analysis may clarify whether the two off-targets are inhibited by **14** in cell, and whether inhibition of the two off-targets has any influence on the development of PIM inhibitor. In addition to their clean profiles, **9** and **14** inhibited PIM2 to the extents of 41% and 58%, respectively, whereas **3** and **4**, 6-alkylated derivatives, inhibited PIM2 by only 25% and 23%.

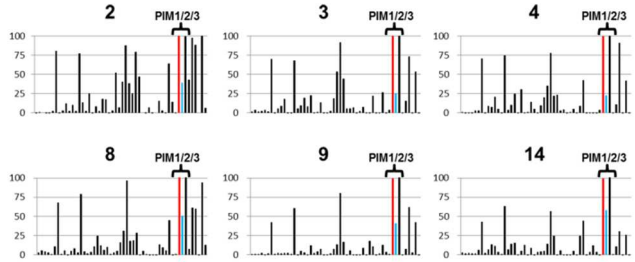


Figure 4. Kinase selectivity profiles of compounds **2**, **3**, **4**, **8**, **9** and **14**. Experiments were carried out at Carna Biosciences. All compounds were tested against a panel of 52 kinases at 200 nM concentration. In each graph, the x-axis shows %inhibition of each kinase. Red bars represent %inhibition of PIM1, and blue bars represent %inhibition of PIM2. Black bars next to the blue bars represent %inhibition of PIM3.

Because it seemed probable that substituents at the 6-position of the 7-azaindole ring would influence not only the kinase selectivity but also the inhibitory potency toward PIM2, we further determined the IC_{50} values of our compounds for PIM2 (Table 2). For comparison, the IC_{50} value of the indazole derivative **1**, a selective PIM1 inhibitor, is also shown in Table 2.[24] Irrespective of the substituents on the benzofuranone core, **9** and **14** (the chlorinated derivatives) achieved a significant increase of PIM2 inhibition, as expected from the results of the kinase panel study. Under the assay conditions used, the IC_{50} values of **9** and **14** for PIM2 were 140 nM. Therefore, **9** and **14** are about three times as potent as the non-substituted analogues **2** and **10**, and about eight times as potent as the indazole derivative **1**. Glu124 and Val126 in PIM1 are re-

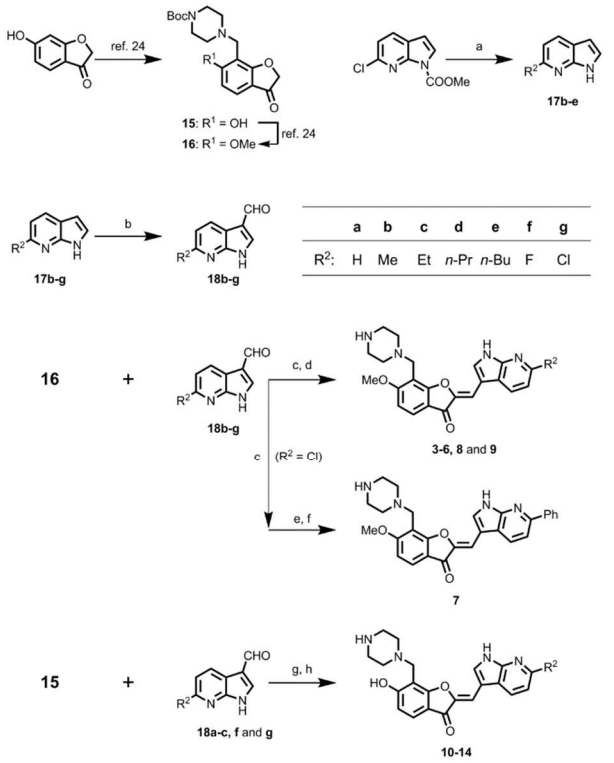
placed by Leu and Ala in PIM2, which may change lipophilicity and steric accessibility of the available space in the pocket.[26–28] However, improvement of PIM2 inhibition by the chlorine substituent cannot be explained simply in terms of lipophilic and steric effects, because the alkylated derivatives **3**, **4** and **11** did not achieve any noticeable gain in PIM2-inhibitory potency compared with their non-substituted analogues **2** and **10**. These results indicate that the space utilized by the 6-chloro moiety of the 7-azaindole ring could be a key to the design of other potent and selective pan-PIM kinase inhibitors in the future.

The in vitro ADMET properties are summarized in Table 3. Compared with indazole analogue **1**, the 7-azaindole analogue **2** shows a better in vitro ADMET profile, with higher aqueous solubility, higher membrane permeability and lower hERG inhibition, although it is a non-selective kinase inhibitor. Compound **3**, the 6-methylated derivative, retained acceptable solubility and a low hERG inhibition profile, but showed poor membrane permeability. At present, we have no idea why the membrane permeability is decreased so severely as a result of simple methylation. Compound **9**, the 6-chloro derivative of **2**, was also scarcely membrane-permeable, and showed very poor aqueous solubility compared with **2**. The lower solubility might be due to higher lipophilicity of **9**. Compound **14**, the less lipophilic phenolic derivative of **9**, showed better aqueous solubility, as well as greater membrane permeability. A plausible explanation of the recovery of permeability is the formation of an intra-molecular hydrogen bond between phenol and piperazine nitrogen. Overall, **14** showed superior ADMET properties to **1**, including lower hERG inhibition and higher membrane permeability in Caco-2 cells, and had better kinase selectivity than **2**.

To further evaluate the potential of **14**, cell–based assay was carried out with a leukemia cell line MOLM–16, which was used to evaluate the representative pan-PIM inhibitor AZD1208 in the literature (Table 4).[21] In MOLM–16 growth inhibition assay, IC_{50} of **14** was 200 nM, which was weaker than that of AZD1208, but stronger than that of SGI-1776, the representative first-generation PIM inhibitor. Reflecting the clean kinase selectivity profile, **14** scarcely inhibited the growth of WI–38, a human diploid lung fibroblast cell line, which was used as a surrogate for general toxicity.

Compounds were synthesized as shown in Scheme 1. Synthesis of **1** and **2** was reported previously by us.[24] Synthesis of two active methylene compounds for Knoevenagel condensation, **15** and **16**, was also reported previously.[24] 7-Azaindole-3-carbaldehydes **18a–g**, coupling partners of **15** and **16**, were prepared as follows, except for commercially available **18a**. Methyl 6-chloro-1*H*-pyrrolo[2,3-*b*]pyridine-1-carboxylate[29] was reacted with trimethylboroxine or alkylboronic acids under Suzuki coupling conditions to yield 6-alkyl-7-azaindoles **17b–e**. Then, **17b–e** were formylated with hexamethylenetetramine to obtain 6-alkyl-7-azaindole-3-carbaldehydes **18b–e**. **18f** and **g** (R = halogen) were also synthesized by formylation of commercially available **17f** and **g**. Then, Knoevenagel condensation of **15**/**16** and **18a–g** was carried out in the presence of piperidine in MeOH at 60°C, and the Boc protective group was removed under acidic conditions to obtain the final products **3–6**, and **8–14**. The Knoevenagel product obtained from 6-chloro-7-azaindole-3-carbaldehyde **18g** was reacted with phenylboronic acid under Suzuki coupling conditions and the Boc protective group was removed to yield 6-phenyl-7-azaindole derivative **7**.

Scheme 1. Synthesis of compounds 3–14[a]



[a]Reagents and conditions: (a) trimethylboroxine or alkylboronic acid, $PdCl_2$(dppf), K_2CO_3, 1,4-dioxane, 140°C or 160°C, microwave, 35–66%; (b) hexamethylenetetramine, AcOH/H_2O, 120°C, 35–99%; (c) piperidine, MeOH, 60°C, 30%–quant.;(d) TFA, CH_2Cl_2, rt, 32–89%; (e) $PdCl_2$(dppf), 2M aq. Na_2CO_3, 1,2-dimethoxyethane, 140°C, microwave, 98%; (f) TFA, CH_2Cl_2, rt, 28%; (g) piperidine, MeOH, 60°C, 37–75%; (h) TFA, CH_2Cl_2, rt, 7–59%.

In this study, we successfully transformed our previous non-selective 7-azaindole derivative **2** into potent and selective PIM inhibitors by means of substitution targeting the unique structural features of PIM. It is noteworthy that chlorine substitution not only reduced unfavorable interactions with off-targets but also improved the inhibitory potency toward PIM2. In addition, the chloro-substituted derivative **14** showed acceptable in vitro ADMET properties, indicating that our PIM inhibitors have potential for further development. These results suggest that the space occupied by the chlorine atom is a promising target for the design of more sophisticated pan-PIM inhibitors, although the effect of altering the chlorine substituent on the improved PIM2 inhibition

remains to be clarified. More detailed analyses, such as crystallographic, calorimetric and kinetic studies, should also be helpful for further development of rationally designed PIM inhibitors.

Table 2. Results of PIM2 inhibition assay [a]

Comp.	1	2	3	4	9	10	11	14
PIM2 IC_{50} (nM)	1200 [b]	390	300	290	140	420	510	140

[a] Carried out at Carna Biosciences. [ATP] = 5 μM. [b] Ref. 24.

Table 3. ADMET profiling data [a]

Comp.	1	2	3	9	14
Aqueous solubility (μM) [b]	190	≧200	130	21 [f]	130
hERG inhibition @ 10 μM (%) [c]	60	19	14	22	30
Caco-2 permeability (x 10^{-6} cm/s) [d]	0.89	2.1	0.13	0.31	1.7
Stability in human liver microsomes (%) [e]	68	90	74	82	82
Stability in mouse liver microsomes (%) [e]	83	72	69	58	63

[a] Carried out at Cerep. [b] Solubility in PBS, pH 7.4. Final DMSO concentration was 2%. [c] A patch-clamp method was used to evaluate hERG inhibition. [d] Compound concentration was 10 μM. [e] %Remaining of the compound. 1 μM compound was incubated with 0.3 mg/mL protein at 37 °C for 1 h. [f] Final DMSO concentration was 10%.

Table 4. Cell growth inhibitory potency of 14, AZD1208 and SGI-1776

leukemia cell line MOLM-16 IC_{50} (nM) [a]			diploid lung fibroblast cell line WI-38 IC_{50} (nM) [b]		
Compound 14	AZD1208	SGI-1776	Compound 14	AZD1208	SGI-1776
200	12	600	>10,000	>10,000	1,700

[a] Incubated for 72 h. [b] Incubated for 48 h.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Raw data of the kinase selectivity studies (compounds **3**, **4**, **8**, **9** and **14**), protocols of biological assays and details of chemistry. (PDF)

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Author Contributions

All authors contributed to writing the manuscript. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

ADMET, absorption, distribution, metabolism, excretion, toxicity; BAD, Bcl–2–associated death promoter; DAPK1, death-associated protein kinase 1; dppf, [1,1'-bis(diphenylphosphino)ferrocene]; hERG, human ether–a–go–go related gene; LLE, ligand lipophilicity efficiency; MYC, myelocytomatosis oncogene; PDGFRα, platelet–derived growth factor receptor alpha; PIM, proviral integration site for Moloney murine leukemia virus; PKCα, protein kinase C alpha; PKD2, protein kinase D2; ROCK1, Rho-associated coiled–coil containing protein kinase 1; TFA, trifluoroacetic acid.

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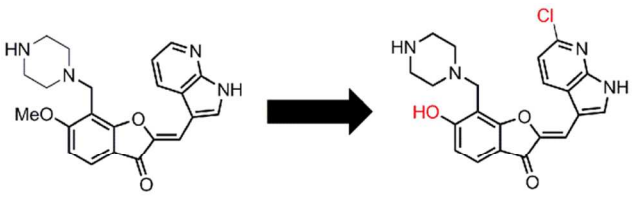

2 poor kinase selectivity

14 improved kinase selectivity
improved PIM2 inhibition