Regular Article

2-Thioxothiazolidin-4-one Analogs as Pan-PIM Kinase Inhibitors

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Proviral integration site for Moloney murine leukemia virus (PIM) kinases are proto-oncogenic kinases involved in the regulation of several cellular processes. PIM kinases are promising targets for new drug development because they play a major role in many cancer-specific pathways, such as survival, apoptosis, proliferation, cell cycle regulation, and migration. Here, 2-thioxothiazolidin-4-one derivatives were synthesized and evaluated as potent pan-PIM kinase inhibitors. Optimized compounds showed single-digit nanomolar IC₅₀ values against all three PIM kinases with high selectivity over 14 other kinases. Compound 17 inhibited the growth of Molm-16 cell lines (EC₅₀ = 14 nM) and modulated the expression of pBAD and p4EBP1 in a dose-dependent manner.

Key words rhodanine; inhibitor; leukemia; proviral integration site for Moloney murine leukemia virus (PIM) kinase; cancer

Introduction

Proviral integration site for Moloney murine leukemia virus (PIM) kinases are serine/threonine protein kinases that phosphorylate substrates involved in the regulation of several cellular processes.¹⁾ The PIM family comprises the isoforms PIM-1, PIM-2, and PIM-3, which are highly homologous and share similar oncogenic functions.^{2–4)} Elevated expression of PIM kinases has been reported in hematological cancers such as acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), and multiple myeloma (MM).⁵⁾ PIM-2 expression is significantly higher in MM than in ALL and AML, whereas PIM-1 expression is higher in ALL and AML than in MM. PIM kinases are promising targets for new drug development because they play a major role in many cancer-specific pathways, such as survival, apoptosis, proliferation, cell cycle regulation, and migration.^{6,7)}

Various small molecules have been reported as ATP-competitive inhibitors of PIM kinases.⁸⁾ Pan-PIM kinase inhibitors are expected to have a higher therapeutic value than PIM-selective inhibitors because the three isoforms have overlapping functions and their expression levels depend on the tumor type. After the first clinical trial of SGI-1776,^{9,10)} several compounds such as AZD1208,^{11,12)} PIM447,¹³⁾ and INCB053914¹⁴⁾ have been studied or are currently underway in clinical studies in patients with hematological tumors (Fig. 1).

A substituted benzylidene-1,3-thiazolidine-2,4-dione (TZD) was found to be an ATP-competitive PIM kinase inhibitor and became a key structural motif for the discovery of AZD1208.¹⁵⁾ Previously, we also synthesized TZD derivatives and identified them as potent inhibitors of all three PIM kinase isoforms.¹⁶⁾ In this study, 2-thioxothiazolidin-4-one (rhodanine) derivatives were synthesized and their inhibitory activities on PIM kinases were evaluated and compared with that of TZD.

Results and Discussion

Rhodanine derivatives were synthesized according to reported procedures¹⁶⁾ with some modifications, as shown in Chart 1. Substituted pyrazine was obtained from the reaction of 2,6-dichloropyrazine with either an amine or alcohol and was then attached to the 3-position of benzaldehyde using a Suzuki cross-coupling reaction under microwave irradiation. The obtained aldehyde was then reacted with 2-thioxothiazolidin-4-one to produce the 5-benzylidene-2-thioxothiazolidin-4-one analog via Knoevenagel condensation.

Among the five-membered heterocycles, which have been considered as privileged scaffolds in drug discovery,¹⁷⁾ TZD has been used as an inhibitor of PIM kinases.^{15,18,19)} Rhodanine, which differs from TZD only in the exocyclic heteroatom

Fig. 1. Structures of PIM Kinase Inhibitors: SGI-1776, AZD1208, PIM447, and INCB053914

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(S vs. O), is known to form more intermolecular interactions with target proteins than TZD.²⁰⁾ PIM kinase inhibitory activities of rhodanine analogs were studied and compared with those of TZD analogs. According to ChemAxon²¹⁾ calculations, 5-benzylidene-2-thioxothiazolidin-4-one was found to be more acidic and lipophilic than 5-benzylidenethiazolidine-2,4-dione ($pK_a = 4.93$ and $Clogp = 2.26$ vs. $pK_a = 6.20$ and $Clogp = 1.52$, respectively). The reported TZD analogs bind to the ATP binding pocket of PIM-1 kinase *via* hydrogen bonding with the amino acid residue Lys67.¹⁵⁾ Rhodanine was expected to interact with the PIM enzyme in the same manner as TZD. Some rhodanine-containing PIM kinase inhibitors with potencies comparable to those of TZD derivatives have been reported previously, but the examples are limited.^{22–24)}

As 3-bromobenzylidene derivatives, both TZD and rhodanine showed inhibitory activity against PIM-1 kinase (Table 1). Substitution of the bromo group by an aromatic ring at the

3-position of the benzene increased the inhibitory activity of rhodanine more than two-fold. The pyridinyl and pyrazinyl analogs of rhodanine exhibited a higher inhibitory effect on PIM kinases than the phenyl analog, and the pyrazinyl analog showed the highest potency in inhibiting the growth of the human myeloid (eosinophilic) leukemia cell line EOL-1.

Various substituents were attached to the introduced pyrazine ring to improve the pan-PIM kinase inhibitory activity of the compounds (Table 2). Five- or six-membered cycloalkyl groups were attached *via* an amine or ether linkage (compounds **4**, **5**, **6**, and **7**). Cycloalkyl groups did not affect the inhibitory activity against PIM-1 and PIM-3. However, ether-linked cycloalkyl groups substantially decreased the inhibitory activity against PIM-2 by more than 6-fold compared to that of amine-linked cycloalkyl groups. In contrast, cycloalkyl ring systems that include an amino group improved the inhibitory activity regardless of the linkage type (compounds **13**, **14**, **15**, and **16**). While the direct attachment of a heterocycle, such as pyrrolidine and piperidine, to the pyrazine ring (compounds **8** and **9**) reduced the inhibitory activity against all three PIM kinases, the addition of a hydrophilic group, either present in the cycloalkyl ring or as a substituent on the ring, restored the inhibitory activity (compounds **10**, **11**, and **12**). Specifically, morpholine restored the inhibitory activity of the compound, while piperazine and 3-aminopiperidine even improved it.

Next, a few alkylamines were attached to the pyrazine ring using a nitrogen linkage. Introduction of an acyclic alkylamine provided a highly potent inhibitor. Ethylene and propylene chains between the pyrazine ring and alkylamine were well tolerated by secondary and tertiary amine linker (compounds **17–21**). In addition, there were no noticeable differences in the activities between the alkylamine-containing compounds with a dimethylamino or diethylamino terminal group. The inhibitory activities of compounds **7**, **17**, and **20** were similar to those of the TZD analogs.

Cell growth inhibitory activities were studied using human

(a) Bis(pinacolato)diboron, $\text{PdCl}_2(\text{dppf})$, KOAc, 1,4-dioxane–ethanol (5:1), microwave, (b) Ar-Cl , $\text{PdCl}_2(\text{PPh}_3)_2$, 2M K_2CO_3 , 1,4-dioxane–ethanol (5:1), microwave, (c) 2-thioxothiazolidin-4-one, sodium acetate, acetic acid, *N,N*-dimethylformamide (DMF), microwave.

Chart 1. Reagents and Conditions for the Synthesis of the Rhodanine Compounds

Table 1. PIM-1, 2, 3 and Cell Growth Inhibition by TZD and Rhodanine Derivatives

Cpd	R	X	IC ₅₀ (nM) ^a			EC ₅₀ (nM) ^b	
			PIM-1	PIM-2	PIM-3	EOL-1	Molm-16
1	Bromo	O	220	N.D. ^c	N.D.	N.D.	N.D.
2	Bromo	S	160	1600	280	N.D.	N.D.
3a		S	73	500	19	>10,000	>10,000
3b		S	19	89	4	1000	3300
3c		S	11	48	4	12	>10,000

^aBiochemical enzyme assays ($n = 2$) were conducted as described previously,¹⁶⁾ and the ATP concentration was set to the K_m value of each PIM kinase isoform. ^bThe values reported are an average of four independent data points. ^cN.D., not determined.

Table 2. PIM-1, 2, 3 Inhibition by TZD and Rhodanine Derivatives^a

X = O: TZD
X = S: Rhodanine

Cpd	R	X	IC ₅₀ (nM) ^b		
			PIM-1	PIM-2	PIM-3
4		S	5	52	3
5		S	3	69	2
6		S	15	340	6
7		S	9	690	7
7a^c		O	6	97	3
8		S	45	910	20
9		S	30	300	22
10		S	4	62	2
11		S	1	10	1
12		S	1	9	1
13		S	2	8	1

Cpd	R	X	IC ₅₀ (nM) ^b		
			PIM-1	PIM-2	PIM-3
14		S	1	6	<1
15		S	2	16	2
16		S	1	13	1
17		S	1	7	1
17a^c		O	<1	6	<1
18		S	<1	3	<1
19		S	<1	5	<1
20		S	<1	2	<1
20a^c		O	3	17	<1
21		S	1	16	1

^aIC₅₀ value of compounds **4–7**, **17–20** as reported in reference 24. ^bBiochemical enzyme assays ($n=2$) were conducted as described previously, and the ATP concentration was set to the K_m value of each PIM kinase isoform. ^cIC₅₀ value of the TZD analog as reported in reference 16.

Table 3. Cell Growth Inhibition (EC₅₀ (nM))^{a)} by TZD and Rhodanine Derivatives

Cpd	Rhodanine analogs		Cpd	TZD analogs	
	EOL-1	Molm-16		EOL-1	Molm-16
17	20	14	17a	87	28
18	37	10			
20	42	21	20a	290	61
21	31	2			

^{a)} The values reported are an average of four independent data points.

myeloid (eosinophilic) leukemia (EOL-1) and acute myeloid leukemia (Molm-16) cell lines (Table 3). The TZD analog compound **17a** inhibited the growth of EOL-1 and Molm-16

cells more markedly than that of biphenotypic B myelomonocytic leukemia (MV-4–11) cells by one order of magnitude (EC₅₀ values = 0.087, 0.028, and 0.8 μ M, respectively).¹⁶⁾ Converting TZD to rhodanine improved the compound's inhibitory activity against EOL-1 and Molm-16 by 2- to 6-fold (compounds **17** and **20**).

The physiological downstream signaling of PIM kinases is mediated through the phosphorylation of a large number of substrates, including regulators of protein translation (4EBP1, S6 ribosomal protein) and a regulator of cellular apoptosis (BAD).⁴⁾ Western blot analysis of the myeloma cell line Molm-16 treated with different concentrations of compound **17** revealed a constellation of PIM downstream signaling events consistent with the inhibition of PIM kinases (Fig. 2). These

Fig. 2. Compound **17** Modulates the Expression of Known PIM Substrates

Western blot analysis of Molm-16 cells after 4h treatment with vehicle control (DMSO) or increasing concentrations of compound **17**. PIM447 (EC_{50} = 17 nM) and AZD1208 (EC_{50} = 20 nM) compounds were used as references.

Table 4. Kinase Activity of 15 Protein Kinases upon Treatment with 1 μ M Compound **17**

Enzyme	Activity in % ^{a)}	Enzyme	Activity in % ^{a)}
Aurora-A	89	JNK3	94
CDK2/cyclinA	61	KDR	77
CK2	–2	MAPK2	97
cSRC	93	Met	124
Flt3	91	Plk1	86
GSK3 β	56	SAPK2a	107
IRAK4	101	TAK1	111
JAK2	85		

a) Values were obtained from the KinaseProfiler™ project of Eurofins.

data demonstrate that compound **17** modulates the phosphorylation of phospho-Bad (pBad, phosphorylated at Ser112) and phospho-4EBP1 (p4EBP1, phosphorylated at Thr70) in a dose-dependent manner.

The selectivity of the rhodanine analog compound **17** was evaluated over 15 kinases (Table 4). Compound **17** exhibited strong inhibition against casein kinase 2 (CK2) and weak to moderate inhibition against cyclin-dependent kinase 2 (CDK2), glycogen synthase kinase 3 beta (GSK3 β), and vascular endothelial growth factor receptor 2 (KDR) at a concentration of 1 μ M. Since the IC_{50} values of compound **17** against all three PIM kinases are in the single-digit nanomolar range, it is over 100-fold more selective for PIM kinases than for all other kinases tested, except CK2. According to a previous report, TZD analog, at 1 μ M concentration, inhibited only weakly interleukin-1 receptor-associated kinase 4 (IRAK4), c-Jun N-terminal kinase 3 (JNK3), KDR, and Met (inhibition was less than 22%). In contrast, the rhodanine analog compound **17** showed 11–54% inhibition of Aurora-A, CDK2, GSK3 β , janus kinase 2 (JAK2), KDR, and polo-like kinase 1

(PLK1) and complete inhibition of CK2. Kinase-panel studies showed that the kinase selectivity of TZD is higher than that of rhodanine.

Rhodanine-based compounds have been considered as pan assay interference compounds (PAINS) in high-throughput screening and virtual high-throughput screening.²⁵⁾ Many of them showed biological activities by non-specific target modulations such as the formation of aggregates, covalently modification of target proteins, and interaction with transition metals. However, the possibility that the biological effects of our compounds are related to the non-specific target modulation can be excluded by several experimental results. Both enzymatic and cellular inhibitory activities of compounds showed the dependency on the structural features of compounds (Tables 1, 2). Cellular activities of compounds varied according to structural features and cell lines (Tables 1, 3). Compound **17** showed a constellation of PIM downstream signaling events consistent with the inhibition of PIM kinases (Fig. 2). Compound **17** behaved as selective kinase inhibitor (Table 4).

Conclusion

We have identified a series of rhodanines as potent and selective inhibitors of PIM kinases. We have shown that rhodanines and TZDs exhibit similar effects on the biochemical activities of all three PIM kinases; however, the antiproliferative activity of rhodanine against EOL-1 and Molm-16 cells was higher than that of TZD. We also demonstrated *in vitro* on-target effects of a rhodanine analog that blocked the phosphorylation of 4EBP1 and BAD. Furthermore, the same rhodanine analog showed excellent kinase selectivity over 15 kinases, although there were subtle differences in the selectivity profile between rhodanine and TZD.

Experimental

Chemistry ¹H-NMR and ¹³C-NMR spectra were recorded on a Bruker Spectrospin 400 (400MHz) or JEOL ECA500 (500MHz) spectrometer. Chemical shifts (δ) are reported in ppm using tetramethylsilane as an internal standard. Mass spectra were obtained using Waters ACQUITY Quattro micro™ API. Microwave-assisted reactions were performed with CEM Discover BenchMate. Silica gel column chromatography was performed using Merck silica gel 60 (230–400 mesh). Unless otherwise noted, all starting materials were obtained from commercially available sources and they were used without further purification. All reactions were performed under a nitrogen atmosphere.

General Method for Synthesis of 2-Thioxothiazolidin-4-one Analogs

3-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)benzaldehyde (R1)

A microwave vessel was filled with 3-bromobenzaldehyde (1.1 mmol, 0.20 g), 1,4-dioxane (2.0 mL) and EtOH (0.40 mL). After the addition of bis(pinacolato)diboron (1.6 mmol, 0.41 g), KOAc (3.2 mmol, 0.32 g), the mixture was purged with N₂ gas for 5 min. To the reaction mixture was added [1,1'-bis(diphenylphosphino)ferrocene]dichloropalladium(II) (PdCl₂(dppf)) (0.032 mmol, 0.024 g), and the mixture was irradiated for 10 min at 110 °C by applying 100 W. After the removal of the solvent *in vacuo*, the residue was treated with DCM and filtered with aid of celite. The solvent was removed under reduced pressure, and the residue was purified by col-

umn chromatography over silica gel with 12:1 hexane/EA to give 0.25 g (95%) of the title compound. $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ : 10.06 (s, 1H), 8.32 (s, 1H), 8.07 (d, $J = 7.5$ Hz, 1H), 8.00 (d, $J = 8.0$ Hz, 1H), 7.54 (t, $J = 7.7$ Hz, 1H), 1.38 (s, 12H).

N-(6-Chloropyrazin-2-yl)-*N'*,*N'*-dimethylethane-1,2-diamine (**R2**)

N,N-Dimethylethylenediamine (1.6 mmol, 0.17 mL) and K_2CO_3 (2.7 mmol, 0.37 g) were added to 5.0 mL of DMF, and the mixture was stirred for 30 min at room temperature. After the addition of 2,6-dichloropyrazine (1.3 mmol, 0.20 g), the reaction mixture was stirred for overnight at room temperature. After the solvent was removed *in vacuo*, the residue was treated with EA and washed with water. The residue was extracted with EA and the extract was washed with water and brine. The organic layer was dried over anhydrous MgSO_4 . The residue was purified by column chromatography over silica gel with 1:9 MeOH/dichloromethane (DCM) to give 0.20 g (74%) of the title compound. $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ : 7.77 (s, 1H), 7.76 (s, 1H), 5.49 (s, 1H), 3.39 (q, $J = 5.5$ Hz, 2H), 2.54 (t, $J = 5.7$ Hz, 2H), 2.26 (s, 6H).

3-(6-((2-(Dimethylamino)ethyl)amino)pyrazin-2-yl)-benzaldehyde (**R3**)

A microwave vessel was filled with compound **R1** (0.82 mmol, 0.19 g), compound **R2** (0.82 mmol, 0.16 g), 1,4-dioxane (2.0 mL), EtOH (0.40 mL), and 2 M K_2CO_3 aq. (2.5 mmol, 1.2 mL). After the addition of bis(triphenylphosphine)palladium dichloride ($\text{PdCl}_2(\text{PPh}_3)_2$) (0.025 mmol, 0.018 g) in N_2 atmosphere, the mixture was irradiated for 10 min at 110 °C by applying 100 W. The solvent was removed *in vacuo*. After the residue was treated with DCM, it was filtered with aid of celite. The filtrate was collected and the solvent was removed *in vacuo*. The residue was purified by two consecutive column chromatography over silica gel with 1:9 MeOH/DCM and 1:10:100 conc. NH_4OH /MeOH/ CHCl_3 to give 0.09 g (41%) of the title compound. $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ : 10.09 (s, 1H), 8.50 (s, 1H), 8.30 (s, 1H), 8.25 (d, $J = 8.0$ Hz, 1H), 7.91 (d, $J = 7.5$ Hz, 1H), 7.88 (s, 1H), 7.61 (t, $J = 7.7$ Hz, 1H), 3.51 (q, $J = 5.5$ Hz, 2H), 2.59 (t, $J = 5.7$ Hz, 2H), 2.28 (s, 6H).

5-(3-(6-((2-(Dimethylamino)ethyl)amino)pyrazin-2-yl)-benzylidene)-2-thioxothiazolidin-4-one (**17**)

To acetic acid (1.0 mL) in a microwave vessel were added compound **R3** (0.15 mmol, 0.041 g), rhodanine (0.18 mmol, 0.024 g), NaOAc (0.61 mmol, 0.050 g). The mixture was irradiated for 10 min at 150 °C by applying 100 W. After the solvent was removed *in vacuo*, the residue was treated with saturated K_2CO_3 solution at 0 °C and extracted with EA. The extract was washed with water and the organic layer was dried over anhydrous MgSO_4 . The residue was purified by column chromatography over silica gel with 1:10:80 NH_4OH /MeOH/ CHCl_3 to give 0.028 g (24%) of the title compound. $^1\text{H-NMR}$ (500 MHz, $\text{DMSO}-d_6$) δ : 8.41 (s, 1H), 8.28 (s, 1H), 8.04 (d, $J = 7.5$ Hz, 1H), 8.00 (s, 1H), 7.61 (d, $J = 8.0$ Hz, 1H), 7.58 (t, $J = 7.5$ Hz, 1H), 7.46 (t, $J = 5.5$ Hz, 1H), 7.35 (s, 1H), 3.80 (q, $J = 5.7$ Hz, 2H), 3.39 (t, $J = 6.3$ Hz, 2H), 2.87 (s, 6H); $^{13}\text{C-NMR}$ (125 MHz, dimethyl sulfoxide (DMSO)- d_6) δ : 202.4, 182.2, 154.3, 147.8, 137.9, 136.0, 135.4, 133.5, 131.4, 130.0, 129.1, 127.8, 127.0, 124.8, 56.4, 43.4, 36.2; electrospray ionization (ESI)-MS m/z : 386 $[\text{M} + \text{H}]^+$.

5-(3-Bromobenzylidene)-2-thioxothiazolidin-4-one (**2**)

Yield: 73%, $^1\text{H-NMR}$ (500 MHz, $\text{DMSO}-d_6$) δ : 7.84 (s, 1H), 7.70 (d, $J = 8.0$ Hz, 1H), 7.64 (s, 1H), 7.57 (d, $J = 8.0$ Hz, 1H),

7.51 (t, $J = 7.8$ Hz, 1H); $^{13}\text{C-NMR}$ (125 MHz, $\text{DMSO}-d_6$) δ : 195.9, 169.7, 135.9, 133.7, 132.0, 130.4, 129.0, 127.8, 123.1; ESI-MS m/z : 298, 300 $[\text{M} - \text{H}]^-$.

5-((3'-Methoxy-[1,1'-biphenyl]-3-yl)methylene)-2-thioxothiazolidin-4-one (**3a**)

Yield: 73%, $^1\text{H-NMR}$ (500 MHz, $\text{DMSO}-d_6$) δ : 7.92 (s, 1H), 7.82 (d, $J = 8.0$ Hz, 1H), 7.76 (s, 1H), 7.64 (t, $J = 7.7$ Hz, 1H), 7.57 (d, $J = 8.0$ Hz, 1H), 7.43 (t, $J = 8.0$ Hz, 1H), 7.29 (d, $J = 7.5$ Hz, 1H), 7.26 (s, 1H), 6.99 (dd, $J = 8.5$, 1.5 Hz, 1H), 3.84 (s, 3H); $^{13}\text{C-NMR}$ (125 MHz, $\text{DMSO}-d_6$) δ : 196.1, 169.9, 160.3, 141.5, 141.0, 134.2, 132.2, 130.7, 130.6, 129.9, 129.5, 129.3, 126.4, 119.6, 114.1, 112.8, 55.7; ESI-MS m/z : 328 $[\text{M} + \text{H}]^+$.

5-(3-(6-Methoxypyridin-2-yl)benzylidene)-2-thioxothiazolidin-4-one (**3b**)

Yield: 54%, $^1\text{H-NMR}$ (500 MHz, $\text{DMSO}-d_6$) δ : 8.40 (s, 1H), 8.21 (d, $J = 7.5$ Hz, 1H), 7.84 (t, $J = 7.7$ Hz, 1H), 7.76 (s, 1H), 7.70–7.61 (m, 3H), 6.85 (d, $J = 8.0$ Hz, 1H), 4.02 (s, 3H); $^{13}\text{C-NMR}$ (125 MHz, $\text{DMSO}-d_6$) δ : 196.1, 169.9, 163.7, 152.7, 140.7, 139.5, 133.9, 132.0, 131.7, 130.3, 128.8, 128.5, 126.4, 113.6, 110.6, 53.4; ESI-MS m/z : 329 $[\text{M} + \text{H}]^+$.

5-(3-(6-Methoxypyrazin-2-yl)benzylidene)-2-thioxothiazolidin-4-one (**3c**)

Yield: 55%, $^1\text{H-NMR}$ (500 MHz, $\text{DMSO}-d_6$) δ : 8.90 (s, 1H), 8.41 (s, 1H), 8.33 (s, 1H), 8.26 (d, $J = 6.0$ Hz, 1H), 7.76 (s, 1H), 7.74–7.67 (m, 2H), 4.07 (s, 3H); $^{13}\text{C-NMR}$ (125 MHz, $\text{DMSO}-d_6$) δ : 196.0, 169.9, 159.7, 147.0, 136.9, 134.8, 134.1, 133.7, 132.0, 131.6, 130.6, 129.0, 128.9, 126.7, 53.7; ESI-MS m/z : 330 $[\text{M} + \text{H}]^+$.

5-(3-(6-(Cyclopentylamino)pyrazin-2-yl)benzylidene)-2-thioxothiazolidin-4-one (**4**)

Yield: 54%, $^1\text{H-NMR}$ (500 MHz, $\text{DMSO}-d_6$) δ : 8.39 (s, 1H), 8.34 (s, 1H), 8.16 (d, $J = 8.0$ Hz, 1H), 7.91 (s, 1H), 7.74–7.70 (m, 2H), 7.62 (t, $J = 7.8$ Hz, 1H), 7.31 (d, $J = 6.5$ Hz, 1H), 4.37–4.30 (m, 1H), 2.14–2.08 (m, 2H), 1.78–1.62 (m, 4H), 1.54–1.48 (m, 2H); $^{13}\text{C-NMR}$ (125 MHz, $\text{DMSO}-d_6$) δ : 196.2, 170.0, 154.5, 146.9, 138.4, 134.0, 133.6, 132.9, 131.8, 130.3, 128.5, 127.6, 127.4, 126.5, 52.3, 32.9, 24.0; ESI-MS m/z : 383 $[\text{M} + \text{H}]^+$.

5-(3-(6-(Cyclohexylamino)pyrazin-2-yl)benzylidene)-2-thioxothiazolidin-4-one (**5**)

Yield: 66%, $^1\text{H-NMR}$ (500 MHz, $\text{DMSO}-d_6$) δ : 8.35 (s, 1H), 8.32 (s, 1H), 8.15 (d, $J = 8.0$ Hz, 1H), 7.91 (s, 1H), 7.75 (d, $J = 8.0$ Hz, 1H), 7.73 (s, 1H), 7.62 (t, $J = 7.8$ Hz, 1H), 7.18 (d, $J = 7.5$ Hz, 1H), 4.01–3.94 (m, 1H), 2.05–2.02 (m, 2H), 1.77–1.74 (m, 2H), 1.64–1.62 (m, 1H), 1.56–1.47 (m, 2H), 1.31–1.17 (m, 3H); $^{13}\text{C-NMR}$ (125 MHz, $\text{DMSO}-d_6$) δ : 195.5, 169.4, 153.4, 146.2, 137.8, 133.3, 133.1, 132.7, 131.2, 129.7, 127.9, 126.9, 126.4, 125.7, 48.4, 32.3, 25.4, 24.6; ESI-MS m/z : 397 $[\text{M} + \text{H}]^+$.

5-(3-(6-(Cyclopentyloxy)pyrazin-2-yl)benzylidene)-2-thioxothiazolidin-4-one (**6**)

Yield: 49%, $^1\text{H-NMR}$ (500 MHz, $\text{DMSO}-d_6$) δ : 8.90 (s, 1H), 8.42 (s, 1H), 8.29–8.24 (m, 2H), 7.79–7.73 (m, 2H), 7.69 (t, $J = 7.8$ Hz, 1H), 5.61–5.58 (m, 1H), 2.18–2.13 (m, 2H), 1.83–1.76 (m, 4H), 1.70–1.67 (m, 2H); $^{13}\text{C-NMR}$ (125 MHz, $\text{DMSO}-d_6$) δ : 159.3, 146.9, 137.1, 135.3, 134.3, 133.4, 132.9, 131.4, 130.6, 128.8, 128.1, 78.8, 32.9, 24.0; ESI-MS m/z : 384 $[\text{M} + \text{H}]^+$.

5-(3-(6-(Cyclohexyloxy)pyrazin-2-yl)benzylidene)-2-thioxothiazolidin-4-one (**7**)

Yield: 49%, $^1\text{H-NMR}$ (500 MHz, $\text{DMSO}-d_6$) δ : 8.88 (s, 1H),

8.34 (s, 1H), 8.27–8.22 (m, 2H), 7.78 (d, $J = 8.0$ Hz, 1H), 7.73 (s, 1H), 7.67 (t, $J = 7.8$ Hz, 1H), 5.28–5.24 (m, 1H), 2.12–2.09 (m, 2H), 1.81–1.79 (m, 2H), 1.62–1.51 (m, 5H), 1.30–1.26 (m, 1H); ^{13}C -NMR (125 MHz, DMSO- d_6) δ : 195.4, 169.5, 158.3, 146.2, 136.4, 134.7, 133.6, 133.0, 132.8, 130.8, 130.0, 128.2, 126.7, 126.1, 73.6, 31.2, 25.0, 23.6; ESI-MS m/z : 398 $[\text{M} + \text{H}]^+$.

5-(3-(6-(Pyrrolidin-1-yl)pyrazin-2-yl)benzylidene)-2-thioxothiazolidin-4-one (**8**)

Yield: 37%, ^1H -NMR (500 MHz, DMSO- d_6) δ : 8.44 (s, 1H), 8.39 (s, 1H), 8.19 (d, $J = 7.5$ Hz, 1H), 7.98 (s, 1H), 7.74 (s, 1H), 7.68 (d, $J = 8.0$ Hz, 1H), 7.64 (t, $J = 7.8$ Hz, 1H), 3.56 (t, $J = 6.0$ Hz, 4H), 2.02–1.98 (m, 4H); ^{13}C -NMR (125 MHz, DMSO- d_6) δ : 196.2, 158.1, 152.4, 150.2, 147.5, 138.3, 137.5, 134.0, 131.9, 130.6, 130.1, 128.6, 128.0, 126.6, 55.5, 46.6, 31.2, 25.4; ESI-MS m/z : 369 $[\text{M} + \text{H}]^+$.

5-(3-(6-(Piperidin-1-yl)pyrazin-2-yl)benzylidene)-2-thioxothiazolidin-4-one (**9**)

Yield: 70%, ^1H -NMR (500 MHz, DMSO- d_6) δ : 8.46 (s, 1H), 8.37 (s, 1H), 8.33 (s, 1H), 8.18 (d, $J = 8.0$ Hz, 1H), 7.74 (s, 1H), 7.70 (d, $J = 8.0$ Hz, 1H), 7.64 (t, $J = 7.7$ Hz, 1H), 3.81–3.66 (m, 4H), 1.73–1.65 (m, 2H), 1.65–1.56 (m, 4H); ^{13}C -NMR (125 MHz, DMSO- d_6) δ : 195.5, 169.4, 153.5, 146.2, 137.4, 133.4, 131.7, 131.1, 130.4, 129.8, 128.0, 127.9, 127.6, 126.0, 44.8, 24.9, 24.1; ESI-MS m/z : 383 $[\text{M} + \text{H}]^+$.

5-(3-(6-(Morpholinopyrazin-2-yl)benzylidene)-2-thioxothiazolidin-4-one (**10**)

Yield: 75%, ^1H -NMR (500 MHz, DMSO- d_6) δ : 8.57 (s, 1H), 8.38 (s, 1H), 8.35 (s, 1H), 8.20 (d, $J = 7.0$ Hz, 1H), 7.75 (s, 1H), 7.68 (d, $J = 8.0$ Hz, 1H), 7.65 (t, $J = 7.3$ Hz, 1H), 3.77 (t, $J = 4.5$ Hz, 4H), 3.68 (t, $J = 4.5$ Hz, 4H); ^{13}C -NMR (125 MHz, DMSO- d_6) δ : 196.2, 169.9, 154.4, 146.9, 137.9, 134.1, 131.8, 131.1, 130.5, 123.0, 129.9, 128.9, 128.8, 126.7, 66.3, 44.8; ESI-MS m/z : 385 $[\text{M} + \text{H}]^+$.

5-(3-(6-(Piperazin-1-yl)pyrazin-2-yl)benzylidene)-2-thioxothiazolidin-4-one (**11**)

After the preparation of compound **R3** series following a similar procedure for compound **17**, compound **11** was needed the procedure of the removal of *tert*-butoxycarbonyl (Boc). To the mixture of DCM (4.0 mL) and trifluoroacetic acid (1.0 mL) was added the compound of preceding step, and the reaction mixture was stirred for 1 h at room temperature. The solvent was removed *in vacuo*. The precipitate obtained by addition of ammonium hydroxide (NH_4OH)/MeOH/chloroform (CHCl_3) mixture (2.5:25:80) was filter and the resulting solid was washed with distilled water. The solid was purified by silica gel chromatography with 1:10:100 conc. NH_4OH /MeOH/ CHCl_3 to yield 95% of the title compound. ^1H -NMR (500 MHz, DMSO- d_6) δ : 8.62 (s, 1H), 8.43 (s, 1H), 8.38 (s, 1H), 8.14 (d, $J = 7.0$ Hz, 1H), 7.65 (d, $J = 8.0$ Hz, 1H), 7.62 (t, $J = 7.8$ Hz, 1H), 7.52 (s, 1H), 3.97 (t, $J = 5.3$ Hz, 4H), 3.29 (t, $J = 5.3$ Hz, 4H); ^{13}C -NMR (125 MHz, DMSO- d_6) δ : 158.7, 158.5, 153.5, 147.2, 137.5, 134.9, 131.6, 131.3, 130.4, 128.7, 128.1, 121.4, 119.0, 116.6, 42.9, 41.6; ESI-MS m/z : 384 $[\text{M} + \text{H}]^+$.

5-(3-(6-(3-Aminopiperidin-1-yl)pyrazin-2-yl)benzylidene)-2-thioxothiazolidin-4-one (**12**)

Yield: 93%, ^1H -NMR (500 MHz, DMSO- d_6) δ : 8.53 (s, 1H), 8.35 (s, 1H), 8.30 (s, 1H), 8.10 (t, $J = 3.5$ Hz, 1H), 7.61 (d, $J = 5.5$ Hz, 2H), 7.40 (s, 1H), 4.38 (d, $J = 10.0$ Hz, 1H), 4.05 (d, $J = 13.5$ Hz, 1H), 3.42–3.28 (m, 5H), 2.08–2.00 (m, 1H), 1.90–1.82 (m, 1H), 1.73–1.59 (m, 2H); ^{13}C -NMR (125 MHz,

DMSO- d_6) δ : 196.4, 158.6, 158.4, 154.0, 147.5, 137.7, 135.5, 131.0, 130.2, 129.6, 128.7, 127.6, 119.0, 116.6, 46.9, 46.7, 44.6, 28.54, 22.3; ESI-MS m/z : 398 $[\text{M} + \text{H}]^+$.

5-(3-(6-(Pyrrolidin-3-ylamino)pyrazin-2-yl)benzylidene)-2-thioxothiazolidin-4-one (**13**)

Yield: 71%, ^1H -NMR (500 MHz, DMSO- d_6) δ : 8.41 (s, 1H), 8.31 (s, 1H), 8.14 (d, $J = 6.0$ Hz, 1H), 7.97 (s, 1H), 7.76 (d, $J = 5.0$ Hz, 1H), 7.68 (s, 1H), 7.65–7.59 (m, 2H), 4.75–4.54 (m, 1H), 3.76–3.60 (m, 1H), 3.34–3.26 (m, 1H), 3.30–3.16 (m, 1H), 2.57–2.48 (m, 1H), 2.47–2.34 (m, 1H), 2.11–1.97 (m, 1H); ^{13}C -NMR (125 MHz, DMSO- d_6) δ : 203.3, 183.6, 153.9, 147.7, 137.7, 136.5, 136.2, 133.3, 131.2, 130.0, 129.1, 127.8, 126.7, 124.1, 50.3, 44.6, 30.6; ESI-MS m/z : 384 $[\text{M} + \text{H}]^+$.

5-(3-(6-(Piperidin-3-ylamino)pyrazin-2-yl)benzylidene)-2-thioxothiazolidin-4-one (**14**)

Yield: 65%, ^1H -NMR (500 MHz, DMSO- d_6) δ : 8.40 (s, 1H), 8.26 (s, 1H), 8.05 (t, $J = 4.3$ Hz, 1H), 7.98 (s, 1H), 7.59 (d, $J = 4.5$ Hz, 2H), 7.37 (d, $J = 6.0$ Hz, 1H), 7.32 (s, 1H), 4.32–4.22 (m, 1H), 3.52 (dd, $J = 11.5$, 3.0 Hz, 1H), 3.27–3.20 (m, 1H), 3.03–2.91 (m, 2H), 2.16–2.07 (m, 1H), 2.05–1.96 (m, 1H), 1.89–1.79 (m, 1H), 1.70–1.60 (m, 1H); ^{13}C -NMR (125 MHz, DMSO- d_6) δ : 202.4, 153.1, 147.2, 137.2, 135.6, 135.5, 132.6, 130.1, 129.3, 128.3, 127.6, 126.3, 123.7, 46.2, 44.1, 43.1, 27.9, 20.6; ESI-MS m/z : 398 $[\text{M} + \text{H}]^+$.

5-(3-(6-(Pyrrolidin-3-yloxy)pyrazin-2-yl)benzylidene)-2-thioxothiazolidin-4-one (**15**)

Yield: 63%, ^1H -NMR (500 MHz, DMSO- d_6) δ : 8.95 (s, 1H), 8.36 (s, 1H), 8.31 (s, 1H), 8.12 (d, $J = 6.5$ Hz, 1H), 7.67–7.57 (m, 2H), 7.31 (s, 1H), 5.87–5.82 (m, 1H), 3.67 (dd, $J = 13.2$, 5.3 Hz, 1H), 3.56 (d, $J = 13.0$ Hz, 1H), 3.47–3.39 (m, 2H), 2.49–2.43 (m, 1H), 2.34–2.27 (m, 1H); ^{13}C -NMR (125 MHz, DMSO- d_6) δ : 202.2, 158.3, 147.5, 136.4, 136.2, 135.4, 134.8, 134.4, 131.5, 130.3, 128.5, 127.3, 124.9, 75.1, 50.7, 44.4, 31.1; ESI-MS m/z : 385 $[\text{M} + \text{H}]^+$.

5-(3-(6-(Piperidin-3-yloxy)pyrazin-2-yl)benzylidene)-2-thioxothiazolidin-4-one (**16**)

Yield: 46%, ^1H -NMR (500 MHz, DMSO- d_6) δ : 8.93 (s, 1H), 8.33 (s, 1H), 8.31 (s, 1H), 8.13 (m, 1H), 7.63 (m, 2H), 7.33 (s, 1H), 5.60–5.52 (m, 1H), 3.49–3.40 (m, 2H), 3.20–3.05 (m, 2H), 2.14–2.07 (m, 1H), 2.06–1.98 (m, 2H), 1.87–1.78 (m, 1H), 1.52–1.38 (m, 2H); ^{13}C -NMR (125 MHz, DMSO- d_6) δ : 202.8, 183.6, 157.5, 147.0, 136.7, 135.9, 135.8, 134.3, 133.6, 130.8, 129.6, 127.6, 126.2, 122.7, 67.0, 45.4, 43.1, 26.4, 18.4; ESI-MS m/z : 399 $[\text{M} + \text{H}]^+$.

5-(3-(6-((2-(Dimethylamino)ethyl)(methyl)amino)pyrazin-2-yl)benzylidene)-2-thioxothiazolidin-4-one (**18**)

Yield: 23%, ^1H -NMR (500 MHz, DMSO- d_6) δ : 8.51 (s, 1H), 8.27 (s, 1H), 8.21 (s, 1H), 8.05 (d, $J = 6.0$ Hz, 1H), 7.63–7.56 (m, 2H), 7.32 (s, 1H), 4.04 (t, $J = 6.5$ Hz, 2H), 3.32 (t, $J = 6.5$ Hz, 2H), 3.18 (s, 3H), 2.81 (s, 6H); ^{13}C -NMR (125 MHz, DMSO- d_6) δ : 202.6, 153.6, 147.7, 137.7, 136.2, 135.8, 131.2, 130.1, 130.0, 129.2, 128.1, 127.1, 124.6, 54.6, 44.6, 43.7, 36.2; ESI-MS m/z : 399 $[\text{M} + \text{H}]^+$.

5-(3-(6-((2-(Diethylamino)ethyl)amino)pyrazin-2-yl)benzylidene)-2-thioxothiazolidin-4-one (**19**)

Yield: 71%, ^1H -NMR (500 MHz, DMSO- d_6) δ : 8.41 (s, 1H), 8.28 (s, 1H), 8.01 (m, 2H), 7.59 (d, $J = 7.5$ Hz, 1H), 7.56 (t, $J = 7.8$ Hz, 1H), 7.41 (t, $J = 6.0$ Hz, 1H), 7.27 (s, 1H), 3.81–3.69 (m, 2H), 3.34 (br, 2H), 3.18 (br, 4H), 1.18 (t, $J = 6.8$ Hz, 6H); ^{13}C -NMR (125 MHz, DMSO- d_6) δ : 202.4, 153.7, 147.2, 137.1, 135.7, 135.6, 132.8, 130.6, 129.3, 128.4, 127.2, 126.1, 123.4,

49.7, 46.6, 35.3, 8.6; ESI-MS m/z : 414 $[M + H]^+$.

5-(3-(6-((3-(Dimethylamino)propyl)amino)pyrazin-2-yl)-benzylidene)-2-thioxothiazolidin-4-one (**20**)

Yield: 36%, $^1\text{H-NMR}$ (500 MHz, $\text{DMSO-}d_6$) δ : 8.36 (s, 1H), 8.36 (s, 1H), 8.02 (d, $J = 7.0\text{ Hz}$, 1H), 7.93 (s, 1H), 7.59 (d, $J = 8.0\text{ Hz}$, 1H), 7.54 (t, $J = 7.8\text{ Hz}$, 1H), 7.39 (t, $J = 5.8\text{ Hz}$, 1H), 7.27 (s, 1H), 3.54 (q, $J = 6.5\text{ Hz}$, 2H), 3.21 (t, $J = 8.0\text{ Hz}$, 2H), 2.79 (s, 6H), 2.10–1.99 (m, 2H); $^{13}\text{C-NMR}$ (125 MHz, $\text{DMSO-}d_6$) δ : 202.9, 154.5, 147.6, 137.8, 136.0, 133.2, 131.8, 129.9, 128.2, 127.3, 126.6, 124.4, 55.6, 43.0, 37.8, 24.7; ESI-MS m/z : 400 $[M + H]^+$.

5-(3-(6-((3-(Dimethylamino)propyl)(methyl)amino)-pyrazin-2-yl)benzylidene)-2-thioxothiazolidin-4-one (**21**)

Yield: 71%, $^1\text{H-NMR}$ (500 MHz, $\text{DMSO-}d_6$) δ : 8.49 (s, 1H), 8.35 (s, 1H), 8.17 (s, 1H), 8.06 (d, $J = 7.0\text{ Hz}$, 1H), 7.65–7.52 (m, 2H), 7.30 (s, 1H), 3.79 (t, $J = 7.0\text{ Hz}$, 2H), 3.17 (s, 3H), 2.77 (s, 6H), 2.05 (t, $J = 8.0\text{ Hz}$, 2H), 1.28–1.20 (m, 2H); $^{13}\text{C-NMR}$ (125 MHz, $\text{DMSO-}d_6$) δ : 153.6, 147.5, 137.7, 136.2, 131.8, 129.9, 129.7, 128.5, 127.4, 126.7, 124.1, 55.2, 46.5, 43.0, 36.2, 22.8; ESI-MS m/z : 414 $[M + H]^+$.

Biology

Biochemical Assay The PIM-1, 2, and 3 kinase reactions were carried out by using a fluorescence polarization assay method in 384-well plate format. All reagents such as enzyme, peptide substrate and ATP were prepared in assay buffer consisting of 10 mM Tris-HCl (pH 7.2), 10 mM MgCl_2 , 0.05% NaN_3 , 0.01% Triton X-100 and 2 mM dithiothreitol (DTT). Inhibitors were dissolved in 100% DMSO and then serially diluted 3-fold in a 10-point dose-response curve. The DMSO dilutions were further diluted in the assay buffer to give a final starting assay concentration of 10 μM in 1% DMSO. A 2.5 μL PIM-1 (1 nM) or PIM-2 (1 nM) or PIM-3 (1 nM) was added and pre-incubated inhibitor for 20 min. Five microliters of the mixture of ATP (the final concentrations of ATP were 30, 5 and 20 μM for PIM-1, PIM-2 and PIM-3, respectively) and 5-FAM-labeled BAD peptide (the final concentration of fluorescent peptide was 100 nM) were added to the assay plate. Kinase reactions were incubated at room temperature for 90 min and stopped by addition of IMAP binding reagent (Molecular Devices, solution containing 75% Buffer A: 25% Buffer B and a 1 in 600 dilution of beads). After incubation for 2 h at room temperature, the fluorescence polarization was measured in excitation/emission wavelengths of 485/530 nm on an Infinity F200 plate reader (Tecan). The IC_{50} value for each compound was calculated using a 4-parameter logistic equation in GraphPad Prism 6 software (San Diego, CA, U.S.A.).

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(\log(\text{IC}_{50} - X) * \text{Hillslope})}}$$

Cell Cultures and Materials EOL-1 and Molm-16 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). EOL-1 and Molm-16 were cultured in RPMI 1640 (Welgene Inc., Gyeongsan, Korea) with 10% and 20% heat-inactivated fetal bovine serum (Welgene Inc.), respectively, under condition of 37.5 °C, 5% CO_2 , and fully humidified air. PIM447 and AZD1208 were purchased from Selleck Chemicals (Houston, TX, U.S.A.). The primary antibodies against PIM-1, PIM-2, PIM-3, p-Bad (Ser112), and Bad were purchased from Abcam (Cambridge, U.K.). Anti- β -actin antibody was purchased from Sigma-

Aldrich (St. Louise, MO, U.S.A.). Anti-horse anti-mouse immunoglobulin G (IgG)-horseradish peroxidase (HRP) and anti-goat anti-rabbit IgG-HRP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). p-p70, p70, p-4EBP1, and 4EBP1 were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.).

Cell Viability Assay To assess the cell viability quantitatively, CellTiter-Blue[®] (Promega Corporation, Madison, WI, U.S.A.), was used for evaluating cell viability. The cell viability assay was performed according to manufacturer's protocol. The percentage of inhibition was calculated for each compound concentration and the data were fitted to the equation of a 4-parameter logistic curve. IC_{50} values were obtained using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, U.S.A.).

Western Blotting After treatment, total cells were harvested, washed with phosphate buffered saline (PBS) buffer, and then the total protein was extracted with a radio immunoprecipitation assay (RIPA) buffer (0.1 mM sodium orthovanadate, 137 mM NaCl, 15 mM ethylene glycol bis (2-aminoethyl ether) -N,N,N',N'-tetraacetic acid (EGTA), 0.1% Triton X-100, 25 mM MOPS, 15 mM MgCl_2 , 100 μM phenylmethylsulfonyl fluoride, and 20 μM leupeptin, adjusted to pH 7.2). The cell culture media supernatants were precipitated using TCA (trichloroacetic acid, protocol by Luis Sanchez 2001). A bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Wilmington, U.S.A.) was used for the determination of the protein concentration. Cell lysates of equal protein concentrations were prepared in 5 $\times$ sample buffer (ELPIS Bio Technology, Daejeon, Korea). Lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto Immobilon-P membranes (Millipore Corporation, Bedford, MA, U.S.A.). The membranes were rinsed and incubated with blocking buffer (5% non-fat milk in 1 $\times$ Tris-buffered saline (TBS)/0.1% Tween[®]20) for 1 h. Then, the membranes were incubated with primary antibodies overnight at 4 °C and then with horseradish peroxidase-conjugated secondary antibodies for 1 h. Immunoreactive proteins were detected using the ELC Western blot detection system kit (Millipore). Signal intensity was measured using the Chemi Image documentation system (Fusion Fx7, VILBER LOUTMAT).

Conflict of Interest The authors declare no conflict of interest.

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