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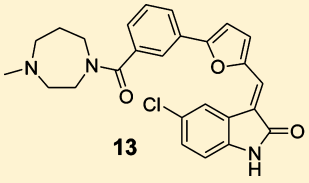
Discovery of CX-6258. A Potent, Selective, and Orally Efficacious pan-Pim Kinases Inhibitor

Mustapha Haddach,* Jerome Michaux, Michael K. Schwaebe, Fabrice Pierre, Sean E. O'Brien, Cosmin Borsan, Joe Tran, Nicholas Raffaele, Suchitra Ravula, Denis Drygin, Adam Siddiqui-Jain, Levan Darjania, Ryan Stansfield, Chris Proffitt, Diwata Macalino, Nicole Streiner, Joshua Bliesath, May Omori, Jeffrey P. Whitten, Kenna Anderes, William G. Rice, and David M. Ryckman

Cylene Pharmaceuticals Inc., 5820 Nancy Ridge Drive, Suite 200, San Diego, California 92121, United States

Supporting Information

ABSTRACT: Structure–activity relationship analysis in a series of 3-(5-((2-oxoindolin-3-ylidene)methyl)furan-2-yl)amides identified compound **13**, a *pan*-Pim kinases inhibitor with excellent biochemical potency and kinase selectivity. Compound **13** exhibited in vitro synergy with chemotherapeutics and robust in vivo efficacy in two Pim kinases driven tumor models.



KEYWORDS: *CX-6258, Pim-1, Pim-2, Pim-3, MV-4-11, PC3*

Pim kinases (Provirus Integration site for Moloney murine leukemia virus) are a family of serine/threonine kinases that regulate cell survival.[1,2] This family of kinases is composed of three different isoforms (Pim-1, Pim-2, and Pim-3) that share 60–70% sequence identity in their kinase domains.[3,4] The Pim kinases are tightly regulated at the level of transcription and translation,[5] and their expression is mediated by the JAK/STAT signaling pathway, which is activated by various cytokines and hormones.[6] In addition, several oncogenes, including Flt3-ITD and Bcr/Abl, were shown to upregulate the expression of Pims.[7] The Pim kinase family members are considered oncogenes and have been implicated in tumorigenesis either alone or operating synergistically with c-Myc.[8−10] Pim kinases are known to suppress apoptosis by the direct phosphorylation and inhibition of pro-apoptotic Bcl-2 antagonist of cell death (BAD).[11−13] Overexpression of Pim kinases has been reported in leukemia and lymphoma tumors.[3,14] They were also found to be overexpressed in solid tumors, including pancreatic cancer and prostate cancer.[8−10,13,15−20]

Recent evidence reveals the overlapping and compensatory nature of Pim-1, Pim-2, and Pim-3 functions, highlighting the importance of inhibiting all three Pim kinase isoforms.[21] Mice mutants for all three Pim kinases are viable and have minimal phenotype differences,[21,22] suggesting that pan-Pim kinase inhibitors should possess favorable toxicity profiles. Moreover, the distinct ATP binding pocket in the active site of Pim kinases should allow for the design of specific and selective inhibitors. In light of these findings, the simultaneous inhibition of Pim-1, Pim-2, and Pim-3 kinases is emerging as a promising strategy for anticancer drug development.

Several structurally distinct chemotypes of inhibitors have been described.[23−31] However, the majority of these molecules were reported to be only potent inhibitors of Pim-1 kinase. The development of Pim-2 selective inhibitors still lags behind despite the high sequence similarity of the ATP-binding sites of Pim-1 and Pim-2. Only a small number of Pim-2 kinase inhibitors have been reported, and they inhibit the Pim-2 kinase at double-digit nanomolar IC_{50} levels. No selective Pim-3 kinase inhibitors have been described in the patent or open literature. To date, only two reports[32,33] have described inhibitors of all three Pim kinases; however, their in vivo anticancer activities remain to be disclosed. Herein, we report our findings of a novel class of oxindole-based derivatives that selectively inhibit all three Pim kinases and show robust in vivo anticancer activity in Pim kinase related xenograft models.

High-throughput screening (HTS) of an internal compound collection identified **5** (Table 1), a molecule containing an oxindole group, as an inhibitor of Pim-1 kinase (IC_{50} = 386 nM). From the same collection of compounds, we discovered that replacing the hydrogen bond donor in the lactam NH of compound **5** with a methyl group led to loss of Pim-1 inhibitory activity (compound **6**, 21% inhibition at 500 nM ATP concentration, Table 1), indicating the importance of this hydrogen bond interaction with the Pim-1 enzyme. To determine the orientation of compound **5** within the Pim-1 ATP active site, we hypothesized that the lactam NH and carbonyl of compound **5** might be interacting with Lys67 and Asp186 in Pim-1 (Figure1). In this proposed binding model of compound **5**, the carboxylate moiety was deemed an appropriate position to incorporate polar groups, such as aliphatic basic amines. This change was predicted to improve the kinase potency due to the possible additional interactions of the polar groups with Asp128 and/or Glu171.

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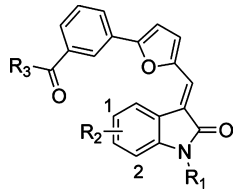
Table 1. SAR around Regions R1, R2, and R3[a]



compd	R1	R2	R3	Pim-1, IC_{50} (μM)	Pim-2, IC_{50} (μM)
5	H	H	OH	0.386	ND
6	Me	H	OH	*	ND
7	H	H	2-(pyrrolidin-1-yl)ethanamine	0.043	ND
8	H	1-Cl	2-(pyrrolidin-1-yl)ethanamine	0.021	0.062
9	H	1-F	2-(pyrrolidin-1-yl)ethanamine	0.0744	0.127
10	H	2-Cl	2-(pyrrolidin-1-yl)ethanamine	0.045	0.445
11	H	1-F	cyclopropylamine	0.506	0.109
12	H	1-Cl	3-aminopropan-1-ol	0.025	0.038
13	H	1-Cl	1-methyl-1,4-diazepane	0.005	0.025
14	H	1-Cl	1,4-diazepane	0.005	0.022

[a]ND = not determined. *21% inhibition at 500 nM conc ATP.

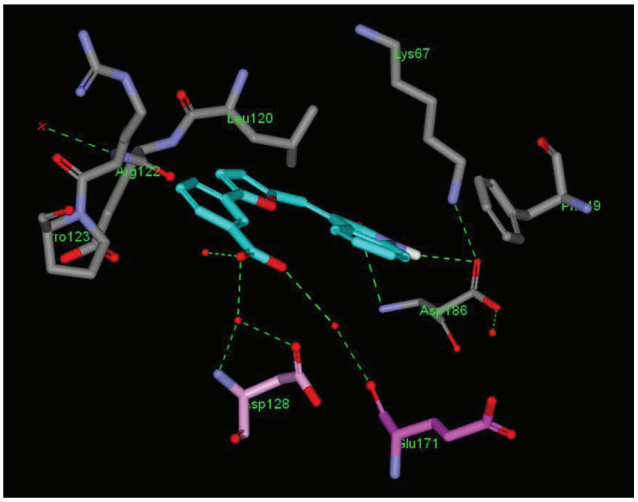


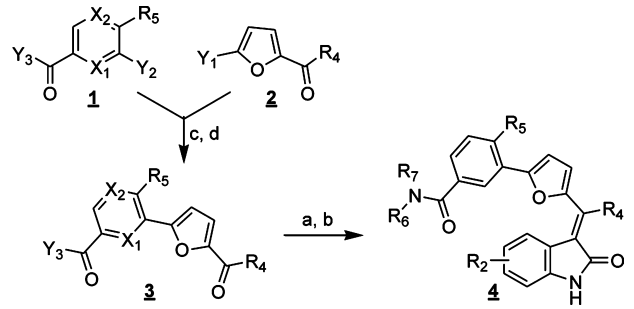
Figure 1. Proposed molecular model of analogue **5**. GLU171 and ASP128 are represented in magenta and pink, respectively.

To probe this hypothesis, we synthesized several compounds via amide coupling between the carboxylate in compound **5** and amine reagents. Analogues in Table 1 and analogue 17 were synthesized by condensing a commercially available intermediate **3** (3-(5-formylfuran-2-yl)benzoic acid or 4-chloro-3-(5-formylfuran-2-yl)benzoic acid) with indolin-2-one derivatives, followed by attachment of various amines to the carboxylate under a standard amide coupling reaction to give the desired analogues (Scheme 1).

The intermediate **3** used in the synthesis of the analogues in Table 2 was prepared by applying the palladium-catalyzed cross coupling reaction to intermediates **1** and **2** (Scheme 1). The exocyclic double bond is exclusively in the *E* configuration for the majority of analogues, as exemplified by the crystal structure of analogue **13** (Figure 2) and as described previously.[34] Analogues **18** and **19** are mixtures of *E* and *Z* configurations with 3 to 1 ratio by ^{1}H NMR, respectively (data not shown).

The analogues described in Scheme 1 were tested in a radiometric enzyme assay using recombinant human Pim-1 and Pim-2, and the results are shown in Tables 1 and 2.

Scheme 1. Synthesis of Analogues[a]



[a]Conditions: Analogues **7**–**17**; (a) Piperidine, oxindole, EtOH; (b) CDI, DMF, NHR_6R_7. Intermediate **3**; Y^1 = $B(OH)_2$; (c) Oxalyl chloride, CH_2Cl_2 and then Et_3N, *N*-methylhomopiperazine; (d) 5-formylfuran-2-ylboronic acid, Na_2CO_3, $PdCl_2(dppf)_2$, DMF, 80 °C. Analogues **18** and **19**; intermediate **2**; Y^1 = Br; *N*,*O*-dimethylhydroxylamine hydrochloride, amide coupling reagents, CH_2Cl_2 and then Me- or Et-MgBr, ether; (c) 3-Methoxycarbonylphenylboronic acid, Na_2CO_3, $PdCl_2(dppf)_2$, DME, 80 °C; (d) 6 M NaOH, EtOH, rt. (a) Amide coupling with methyl homopiperazine; (b) Titanium tetrachloride, THF, 5-chloroxindole, diisopropylethylamine, −78 °C to rt.

Addition of various aliphatic basic amines to compound **5** via amide coupling with the carboxylate in compound **5** led to an increase in potency at inhibiting Pim-1 kinase. As shown by representative examples in Table 1, analogue **7** resulting from the addition of 2-(pyrrolidin-1-yl)ethanamine led to a 9-fold increase in potency at inhibiting Pim-1 versus analogue **5**.

Addition of a chloro group to the C-1 position (analogue **8**) improved further the Pim-1 potency and showed an IC_{50} of 0.062 μM at inhibiting Pim-2. Replacement of the chloro group with a fluoro group (analogue **9**) at the C-1 position led to a decrease of potency against both Pim-1 and Pim-2. However, a chloro group at the C-2 position (analogue **10**) resulted in a significant loss of Pim-2 potency, whereas the Pim-1 potency loss was only 2-fold, indicating that the chloro group at the C-2 position caused a steric clash within the Pim-2 binding site. This representative SAR clearly showed the importance of the basicity of the side chain for Pim-1 potency. Indeed, replacement of the basic side chain in analogue **9** with

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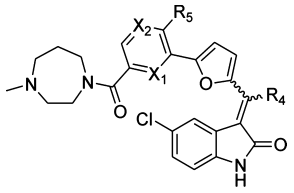
Table 2. SAR of the Core Structure



compd	R4	R5	X1	X2	Pim-1 IC_{50} (μM)	Pim-2 IC_{50} (μM)
15	H	H	N	N	0.067	0.225
16	H	Cl	CH	CH	0.021	0.217
17	H	F	CH	CH	0.003	0.005
18	Me	H	CH	CH	0.057	0.038
19	Et	H	CH	CH	0.131	0.082

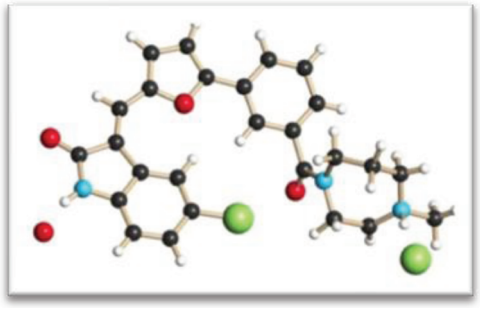


Figure 2. Crystal structure of **13** as the HCl salt coordinated to a water molecule, demonstrating the exocyclic double bond is exclusively in the *E* configuration.

cyclopropylmethanamine (analogue **11**) resulted in a 6.8-fold decrease of Pim-1 potency, whereas Pim-2 potency was not affected. Introduction of a hydroxy group to the side chain (analogue **12**) restored the Pim-1 potency, providing further support for the proposed binding hypothesis that polar or basic groups might be interacting favorably with Asp128 and/or Glu171 in Pim-1 enzyme. Addition of rigidity to the side chain resulted in potent inhibition of both Pim-1 and Pim-2 (analogues **13** and **14**).

We next examined the SAR of the core structure (Table 2) and found that pyrazine analogue **15** exhibited lesser potency than **13** against both enzymes. Introduction of a chloro group at the R5 position (analogue **16**) caused a loss of potency against both enzymes, signifying the importance of coplanarity between the phenyl and furan rings of the core structure for potency in both enzymes. Unexpectedly, introduction of a fluoro group to the R5 position led to the most potent inhibitor of Pim-1 and Pim-2. Finally, the SAR around the alkene region revealed that addition of methyl (analogue **18**) led to loss of potency in Pim-1 while maintaining most of the potency in Pim-2. However, the addition of an ethyl moiety (analogue **19**) led to a more pronounced loss of potency against both Pim-1 and Pim-2, indicting a steric interaction with both enzymes.

Although analogues **14** and **17** exhibited promising in vitro activity profiles (data not shown), both analogues displayed poor oral absorptions. Surprisingly, analogue **13** demonstrated not only a good in vitro activity profile but also a moderate PK profile (Table 3). In addition, analogue **14** was identified as one of the metabolites of **13** (data not shown) and it was expected to contribute positively to the in vivo activity of **13**.

Since Flt-3 regulates the Pim kinases in leukemia,[35] its level of inhibition is important information for the interpretation of

Table 3. Mouse PK Profile of 13 as an Anticancer Agent[a]

IV dosing			oral dosing	
Cls	V_d	$T_{1/2}$	AUC	%F
2.1	85	27	1016	23

[a]Units: Cls, L/h/kg; V_d, L/kg; $T_{1/2}$, h; and AUC(0−24 h), (ng h)/mL; vehicle, D5W; data reported as an average of four animals.

cellular activity performed with Pim kinases inhibitors. The Flt-3 IC_{50} values of analogues **13** and **14** were therefore determined, and it was found that **13** and **14** inhibited Flt-3 with IC_{50} values of 0.134 μM and 0.150 μM, respectively. In addition, the Pim-3 IC_{50} values of both analogues were measured. Analogues **13** and **14** inhibited Pim-3 with IC_{50} values of 0.016 μM and greater than 1 μM, respectively. Since the Pim-3 isoform is overexpressed in pancreatic, gastric, and colon cancer,[15] analogue **13** could be beneficial as an anticancer agent against these malignancies.

Taken together, these studies led to the identification of analogue **13** as an *E*-isomer (Figure 2) for further investigation. To assess its selectivity profile, analogue **13** was screened against 107 kinases (Supporting Information). Using a concentration of 0.5 μM, only Pim-1, Pim-2, Pim-3, and Flt-3 of the 107 kinases were inhibited by more than 80%, demonstrating excellent selectivity of **13**. This analogue was also shown to be a reversible inhibitor of Pim-1 with a K_i of 0.005 μM.

The antiproliferative activity of **13** was examined against a panel of cell lines derived from human solid tumors and hematological malignancies. Analogue **13** demonstrated robust antiproliferative potencies against all cell lines tested. Cell lines derived from acute leukemias were the most sensitive to **13** (Supporting Information).

In mechanistic cellular assays with MV-4-11 human AML cells, **13** caused dose dependent inhibition of the phosphorylation of two pro-survival proteins, Bad and 4E-BP1, at the Pim kinase specific sites S112 and S65 and T37/46, respectively (Figure 3). Using ELISA against phospho-Flt3 (Y591), we demonstrated that **13** does not inhibit Flt3 activity in MV-4-11 cells at relevant concentrations (IC_{50} > 10 μM). These data indicate that the effect of **13** on phosphorylation of Bad and 4E-BP1 results from direct inhibition of Pim kinases rather than being a secondary consequence of Flt3 inhibition.

The Pim kinase family offers a great opportunity in combination therapy, since Pim kinases can modulate chemotherapy resistance through suppression of apoptosis and enhancing the expression of Pgp pumps.[39−41] To explore the

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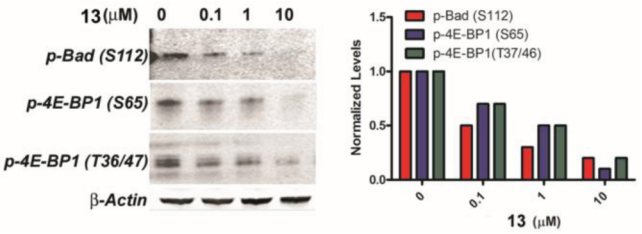

Figure 3. Effect of **13** on phosphorylation of Pim kinase substrates in MV-4-11 cells. MV-4-11 cells were treated for 2 h with various doses of **13**. Cells were lysed, and the relative levels of phospho-proteins were measured using Western hybridization.

potential of using **13** in combination with other drugs, analogue **13** was evaluated for its ability to enhance the activity of chemotherapeutics against prostate adenocarcinoma PC3 cells. As single agents, **13**, doxorubicin, and paclitaxel inhibited the viability of PC3 cells with the IC_{50} values equal to 452 nM, 114 nM, and 2.5 nM, respectively. Combinations of **13** with doxorubicin (10:1 molar ratio) and **13** with paclitaxel (100:1 molar ratio) produced synergistic cell killing with combination index (CI_{50}) values equal to 0.4 and 0.56, respectively, supporting the role of Pim kinases in modulating the chemosensitivity of cancer cells. To our knowledge, this the first report that described the use of a pan-Pim kinase inhibitor in combination with chemotherapeutics.

In an in vivo setting, analogue **13** was evaluated in two human tumor xenograft growth efficacy models, acute myeloid leukemia MV-4-11, and prostate adenocarcinoma PC3. These particular models were selected because of the important roles that Pim kinases were shown to play in both diseases. Mice carrying MV-4-11 xenografts were administered a single oral dose daily. The drug exhibited dose dependent efficacy, with a 50 mg/kg dose producing 45% tumor growth inhibition (TGI) and a 100 mg/kg dose producing 75% TGI (Figure 4).

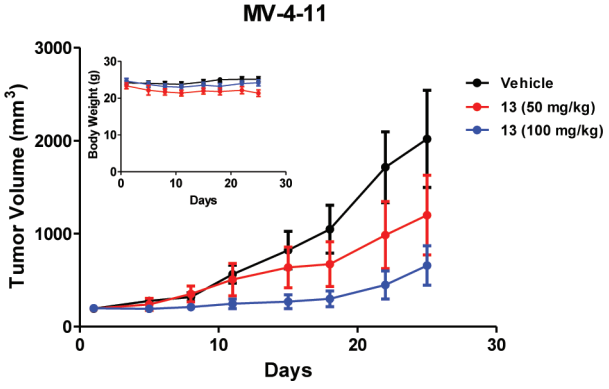

Figure 4. Antitumor efficacy of **13** in MV-4-11 xenograft models. Nude mice were administered vehicle, 50 or 100 mg/kg dose PO once daily over a period of 21 days. Also represented is the animal body weight. Error bars denote the standard error of the mean (SEM).

Compound **13** was well tolerated throughout the study. Treatment of mice carrying PC3 xenografts once daily with a 50 mg/kg oral dose of **13** was also well tolerated and produced 51% TGI (Supporting Information).

In conclusion, we report the discovery of **13**, a novel pan-Pim kinases inhibitor. Compound **13** displays excellent potency in mechanistic and antiproliferative cellular assays. In combination with chemotherapeutics, analogue **13** also exhibited synergistic antiproliferative activity. In addition, **13** displays significant in vivo efficacy in two xenograft models representing the diseases where Pim kinases have been shown to play an important role. Inhibition of Pim kinases is expected to have a beneficial effect as cancer therapy, and compound **13** is undergoing further preclinical studies to determine its viability for human clinical trials.

■ ASSOCIATED CONTENT

Supporting Information

Descriptions of the biological assays, table of the antiproliferative activity of **13**, graph of the in vivo efficacy of **13**, and details of the preparation and characterization of CX-6258. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: mhaddach@san.rr.com or mhaddach@cylenepharma.com.

Notes

The authors declare no competing financial interest.

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