PIM Kinase Inhibitors Downregulate STAT3\textsuperscript{Tyr705} Phosphorylation

Marisa Chang\textsuperscript{1,2}, Nisha Kanwar\textsuperscript{1,2}, Eric Feng\textsuperscript{1,2}, Allan Siu\textsuperscript{1,2}, Xiujie Liu\textsuperscript{3}, Dawei Ma\textsuperscript{3}, and Jan Jongstra\textsuperscript{1,2}

Abstract

Using a cell-based high-throughput screen designed to detect small chemical compounds that inhibit cell growth and survival, we identified three structurally related compounds, 21A8, 21H7, and 65D4, with differential activity on cancer versus normal cells. Introduction of structural modifications yielded compound M-110, which inhibits the proliferation of prostate cancer cell lines with IC\textsubscript{50}s of 0.6 to 0.9 \textmu mol/L, with no activity on normal human peripheral blood mononuclear cells up to 40 \textmu mol/L. Screening of 261 recombinant kinases and subsequent analysis revealed that M-110 is a selective inhibitor of the PIM kinase family, with preference for PIM-3. The prostate cancer cell line DU-145 and the pancreatic cancer cell line MiaPaCa2 constitutively express activated STAT3 (pSTAT3\textsuperscript{Tyr705}). Treatment of DU-145 cells with M-110 or with a structurally unrelated PIM inhibitor, SGI-1776, significantly reduces pSTAT3\textsuperscript{Tyr705} expression without affecting the expression of STAT3. Furthermore, treatment of DU-145 cells with M-110 attenuates the interleukin-6–induced increase in pSTAT3\textsuperscript{Tyr705}. To determine which of the three PIM kinases is most likely to inhibit expression of pSTAT3\textsuperscript{Tyr705}, we used PIM-1–, PIM-2–, or PIM-3–specific siRNA and showed that knockdown of PIM-3, but not of PIM-1 or PIM-2, in DU-145 cells results in a significant downregulation of pSTAT3\textsuperscript{Tyr705}. The phosphorylation of STAT3 on Tyr694 in 22Rv1 cells is not affected by M-110 or SGI-1776, suggesting specificity for pSTAT3\textsuperscript{Tyr705}. These results identify a novel role for PIM-3 kinase as a positive regulator of STAT3 signaling and suggest that PIM-3 inhibitors cause growth inhibition of cancer cells by downregulating the expression of pSTAT3\textsuperscript{Tyr705}. Mol Cancer Ther; 9(9); 2478–87. ©2010 AACR.

Introduction

The PIM family of oncogenic serine/threonine kinases consists of three members, PIM-1, PIM-2, and PIM-3. The \textit{pim}-1 proto-oncogene was first identified as a locus frequently activated by proviral integration in Moloney murine leukemia virus–induced mouse T-cell lymphomas (1, 2), and \textit{pim}-2 was identified as a gene frequently activated in secondary transplants of virus-induced lymphomas. PIM-3 was identified as a PIM-1– and PIM-2–related kinase (3). The oncogenic nature of PIM-1 and PIM-2 was confirmed by the observation that transgenic mice overexpressing these kinases in the lymphoid system develop lymphomas. Simultaneous overexpression of c-myc further increases the frequency of lymphoma-genesis (4, 5). PIM kinases are also implicated in the development of solid tumors. For instance, PIM-1 and PIM-2 are implicated in prostate cancer development (6, 7), PIM-1 is overexpressed in head and neck squamous cell carcinoma (8), and PIM-3 is overexpressed in colorectal, pancreatic, and hepatocellular carcinomas (9–11). PIM-1 and PIM-2 overexpression in prostate cancer correlates with tumor progression (6) and overexpression of exogenous PIM-1 or PIM-2 in the prostate cancer cell line PC3 increases cell proliferation (12). The molecular mechanisms by which PIM kinases regulate cell proliferation may include the activation of cell cycle inhibitors p27\textsuperscript{kip1} (13) or p21\textsuperscript{cip1} (14) or the activation of molecules that positively regulate cell cycle progression, such as CDC25A, CDC25C, or the kinase C-TAK1 (15). PIM kinases also regulate cell viability by phosphorylating the apoptotic proteins BAD and ASK1 (16, 17). Regulation of gene transcription by PIM kinases through interactions with c-myc, c-myb, and NFATc (15, 18) may also play a role in the regulation of cell proliferation and viability. However, the involvement of PIM kinases in specific signaling pathways important for cell proliferation and viability is not well understood.

STAT3 is a latent transcription factor that is activated by tyrosine phosphorylation on residue 705. STAT3 activation by cytokines such as interleukin-6 (IL-6) is mediated by Janus-activated kinase (JAK) family kinases that associate with cytokine receptors that lack intrinsic tyrosine...
kinase activity or by growth factor receptors such as epidermal growth factor or by a number of cytoplasmic tyrosine kinases that can phosphorylate STAT3 on Tyr705. Activated STAT3 (pSTAT3^{Tyr705}) then dimerizes and enters the nucleus where it regulates transcription by binding to consensus DNA sequences in the promoter regions of target genes, many of which encode proteins involved in cell proliferation and cell survival (19–21). Active STAT3 is expressed in many types of cancer. For instance, several studies have shown that up to 82% of late-stage prostate cancers express pSTAT3^{Tyr705} and that increased expression of pSTAT3^{Tyr705} in prostate cancer patients correlates with increased severity of the disease and shorter survival times (22–24). The overexpression of pSTAT3^{Tyr705} in prostate cancer is thought to be, in large part, due to increased levels of the cytokine IL-6 found in prostate cancer patients (25). The persistent expression of pSTAT3^{Tyr705} found in patient tumors is maintained in the prostate cancer–derived cell line DU-145 that produces IL-6 (26). Downregulation of STAT3 (23, 26–29) inhibits DU-145 cell proliferation, which makes DU-145 cells a good model system to study the importance of STAT3 in regulating prostate cancer cell growth.

Because of the role of PIM kinases as regulators of cell proliferation, small-molecule inhibitors of PIM are being developed for use as anticancer agents. Recently, a selective small-molecule PIM inhibitor, SGI-1776, was identified and shown to inhibit the survival of leukemia cells and the proliferation of prostate cancer cell lines (30). Here, we describe compound M-110, a novel and highly selective inhibitor of PIM kinases with a preference for PIM-3. M-110 is a potent inhibitor of prostate cancer cell growth, and treatment of DU-145 cells with M-110 inhibits constitutive pSTAT3^{Tyr705} expression. Knockdown experiments using PIM isoform–specific siRNA established a role of PIM-3 as a positive regulator of STAT3 activation, suggesting that PIM-3 inhibitors cause growth inhibition of prostate cancer cells that express active STAT3 by downregulating STAT3 signaling.

Materials and Methods

Identification of initial compounds

We used a high-throughput screen to identify small chemical compounds that inhibit cell survival. The high-throughput screening was done at the Samuel Lunenfeld Research Institute Robotics Facility and was designed as a homogeneous cell-based assay using the mouse B-lymphoma cell line W10 that was sensitized to cell death by treatment with immunoglobulin M (IgM) antibody (31, 32). For compound screening, each well of a 96-well plate received 25,000 W10 cells in 50 μL of culture medium. Different test compounds from the Maybridge Screening Collection (Maybridge PLC) dissolved in DMSO (200 nL) were added to each of the central 80 wells of the plate (columns 2–11) to a final concentration of 2 μmol/L. Negative (DMSO) and positive (the PKC inhibitor Gö6976) controls were present on each plate. After 48 hours, 10 μL of the viable dye Alamar Blue were added to each well and the plates were incubated for an additional 24 hours. Reduction of Alamar Blue by viable cells was quantitated by measuring the decrease in optical density at 630 nm (OD_{630}) and the increase in fluorescence at 590 nm. Compounds were identified as positive if both measurements were >3.5 SD away from the mean of the 80 test wells.

Cell culture

W10 cells are a clonal derivative of WEHI-231 cells (from M.M. Davis, Stanford University, Stanford, CA) and were grown in RPMI 1640 with 10% heat-inactivated FCS (Hyclone) as described (31, 32). Cell identity was not authenticated by the authors. DU-145, 22Rv1, PC3, MiaPaCa2, and SW480 cells were purchased from the American Type Culture Collection and grown in MEM supplemented with 10% FCS. All media were supplemented with 100 units/mL penicillin, 0.1 mg/mL streptomycin, 2 mmol/L glutamine, and 1× MEM non-essential amino acids (Sigma-Aldrich). Cells were used at low passage number (up to passage 20 or less than 3 months in culture). No authentication of the cells other than that by the American Type Culture Collection using short tandem repeat analysis was done on the cells used in this study.

Reagents and antibodies

Compounds 21A8, 21H7, and 65D4 were purchased from Rya Scientific and dissolved at 20 mmol/L in DMSO. Compound M-110 was synthesized by Sundia MediTech Company and M-142 and SGI-1776 were synthesized at the Ontario Institute for Cancer Research. Recombinant human IL-6 was from Peprotech. Sulfurhodamine B (SRB), DMSO, and trichloroacetic acid were from Sigma-Aldrich. Antibodies specific for tyrosine-phosphorylated STAT3 (Tyr705), tyrosine-phosphorylated STAT5 (Tyr694), and STAT5 were from Cell Signaling Technology. STAT3, survivin, and cyclin D1 antibodies were from Santa Cruz Biotechnology. Anti-actin was from Sigma-Aldrich.

Cell proliferation assays

Growth inhibition of prostate cancer–derived cell lines with the test compounds was determined using the SRB assay as described (33) except that 0.1% acetic acid was used for staining cells with SRB and removing unbound SRB.

Cell viability assays

Cell death assays for W10 cells and human peripheral blood mononuclear cells (PBMC) were done essentially as described (32) except that no IgM antibodies were present. Each compound was tested three times in triplicate on PBMC preparations from three independent healthy human donors. The results for treated samples were standardized to 100% of the viability of the untreated PBMC preparations after 72 hours, which ranged from 56% to 72%. To determine the viability of prostate cancer
cell lines, cells were plated in six-well plates in MEM containing 10% FCS and left to adhere overnight and then incubated in medium containing 0.2% FCS for 48 hours. One well was trypsinized and the number of cells was set at 100%. Compound was added to the remaining wells and the cells were counted after an additional 48 hours. The number of viable cells is expressed as a percentage of the cells present at the time of compound addition. Control experiments showed that untreated cells remained viable but did not proliferate during the last 48 hours of incubation in 0.2% FCS–containing medium.

Kinome screen and kinase assays

The 261 kinases were screened for inhibition at 5 μmol/L M-110 by the kinase profiling service of Reaction Biology Corp. using HotSpot technology (http://www.reactionbiology.com). Kinase reactions were carried out in 20 mmol/L HEPES (pH 7.5), 10 mmol/L MgCl2, 1 mmol/L EGTA, 0.02% Brij 35, 0.02 mg/mL bovine serum albumin, 2 mmol/L DTT, and 1% DMSO. The final concentration of ATP was 10 μmol/L. Kinase assays to determine IC50 values were performed by Reaction Biology Corp. Purified recombinant kinases were incubated with serial 3-fold dilutions of test compounds starting at a concentration of 100 μmol/L. ATP concentration was 10 μmol/L unless otherwise indicated. Dose-response curves were fitted using Prism 5.0 from GraphPad Software.

siRNA transfections

DU-145 cells (2.5 × 105) were transfected in six-well plates with PIM-1, PIM-2, PIM-3 siRNA, STAT3, or control siRNA (FlexiTube siRNAs Hs_PIM1_6, Hs_PIM2_5, Hs_PIM3_1, Hs_STAT3_7, or Ctrl_AllStars siRNA, respectively; Qiagen) using the HiPerFect transfection reagent (Qiagen). RNA expression was analyzed by quantitative real-time PCR 40 hours after the addition of siRNAs. Protein expression was analyzed 64 hours after the addition of siRNAs.

Quantitative real-time PCR

RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) and cDNA was generated using the High-Capacity cDNA Synthesis Kit (Applied Biosystems). Real-time PCR was done using SYBR Green PCR Master Mix (Applied Biosystems). Quantitation was calculated using the ΔΔCt method normalized to hypoxanthine phosphoribosyltransferase.

Results

Selection of 21A8, 21H7, and 65D4

To isolate novel cytotoxic small molecules, we developed a homogenous cell-based high-throughput screen using anti-IgM–treated W10 B-lymphoma cells as the cellular target (Fig. 1A). Treatment of W10 cells with anti-IgM, an antibody against the antigen receptor expressed on the surface of W10 cells, induces growth arrest in the G1 phase of the cell cycle, and we have previously shown that this treatment sensitizes W10 cells to cell death (31, 34). For the primary screen, we tested a total of 11,400 compounds from the Maybridge Screening Collection. This screen resulted in 32 positive hits, three of which, 21A8, 21H7, and 65D4, were structurally related (Fig. 1B) and were selected for further characterization.

The screen was designed to preferentially identify compounds that are cytotoxic to W10 cells. To confirm this expectation, we treated W10 cells with different concentrations of 21A8, 21H7, and 65D4 and determined the viability of the cells after 72 hours by fluorescence-activated cell sorting analysis of propidium iodide–stained cells. Dose-response curves show that 21A8, 21H7, and 65D4 decrease the viability of W10 cells, with LD50 values of 0.4 to 1.0 μmol/L, with 65D4 being the most potent inhibitor of cell survival (Fig. 1C). Interestingly, 65D4, 21A8, and 21H7 are significantly less cytotoxic to normal cells, as 20% to 35% of normal human PBMC remained viable after a 72-hour treatment with 40 μmol/L, the highest dose of compound tested (Fig. 1D), suggesting differential activity on tumor versus normal cells. To determine whether these compounds were also active on epithelial tumor–derived cells, we tested the effect of 65D4 on the survival of the human prostate cancer cell line DU-145. Cells were seeded in six-well plates at 104 per well and treated with 5 μmol/L 65D4 for 6 days and counted. Untreated cells grew to 50 × 104 in number (not shown), but only 0.15 × 104 DU-145 cells treated with 65D4 were recovered (Fig. 1E), showing significant cytotoxicity of 65D4 to DU-145 cells. To investigate the growth inhibitory properties of 65D4, prostate cancer cells were treated with different concentrations of 65D4 for 3 days and the IC50 for growth was then determined using the SRB assay. Figure 1F shows that 65D4 inhibits the growth of DU-145 cells, with an IC50 of 2 μmol/L. These data show that growth-arrested W10 B-lymphoma cells are efficient targets in cell-based screens aimed at identifying novel compounds with promising anticancer activities on lymphoma cells as well as on cells derived from solid tumors.

Structural changes in 65D4 result in M-110, a potent and selective PIM kinase inhibitor

Given the promising anticancer activities of 65D4, we wanted to determine whether this compound could be used as a starting compound for further development into new anticancer agents. We therefore made a preliminary determination of whether 65D4 is amenable to structural changes without losing the ability to inhibit cancer cell growth. For this, a small collection of 21 structural variants of 65D4 was synthesized. We divided the structure of 65D4 into three zones (Fig. 2A) and made modifications in each zone. Several modifications were aimed at improving the aqueous solubility.
of 65D4 (~0.1 mg/mL) significantly to ~25 mg/mL for M-110. We also varied the linker length or the site of attachment of the morpholine group in zone A and probed the influence of halogen and hydroxyl substitutions in the ortho- and para-positions in zone C and the importance of substitutions in zone B. Testing each of these 21 compounds for their effects on the growth of prostate cancer cells showed that compound M-110 was the most potent, inhibiting the growth of DU-145 cells with an IC50 value of 0.9 μmol/L, whereas compound M-37 had little growth inhibitory activity, with IC50s of >20 μmol/L (Fig. 2B and C). To determine whether M-110 had retained the ability to induce cell death, we treated DU-145 cells with 5 μmol/L M-110 or the inactive compound M-37 and counted the cells after 6 days. This showed that after treatment with M-110, <5% of the input cells was recovered, whereas treatment with M-37 had only a small effect on the recovery of viable cells (Fig. 2D). Interestingly, treatment of normal human PBMC with up to 40 μmol/L 65D4 did not result in significant cytotoxicity (Fig. 2E). Thus, although limited in scope, these preliminary structural studies show that the anticancer characteristics of 65D4 can be improved significantly by introducing structural alterations that result in increased aqueous solubility and significantly less activity on normal human PBMC. This identifies 65D4 and M-110 as promising candidates for the development of new clinically useful chemotherapeutic agents.

Because of the multitude of important biological processes that are regulated by phosphorylation events, protein kinases are among the most attractive therapeutic targets. Therefore, we next examined whether the M-series compounds might act as kinase inhibitors. Because of its action as a potent growth inhibitor of prostate cancer cells in the absence of measurable activity on normal cells, we selected M-110 to screen a collection of recombinant kinases (Supplementary Table S1) for possible inhibition by M-110 at a fixed concentration of 5 μmol/L. Of the 261 kinases tested, only the oncogenic protein kinase PIM-1 was strongly inhibited (85.2% inhibition) by M-110 (Fig. 3A). The second most inhibited kinase was CK2α2 (49% inhibition). Dose-response curves for inhibition of PIM-1 and CK2α2 by M-110 and its inactive derivative M-37 showed a significant difference in the activity of these two compounds on PIM-1 but not on CK2α2 (Fig. 3B and C), suggesting that the inhibition of CK2α2 does not contribute to growth inhibition. The IC50 of M-110 for PIM-1 inhibition increased with increasing concentrations of ATP, a characteristic typical of ATP competitive inhibition (Supplementary Fig. S1).
reciprocal plot of 1/velocity against 1/[ATP] confirmed
the ATP-competitive nature of M-110, as curves repre-
senting assays using different concentrations of M-110
showed different slopes intersecting the y-axis (1/velocity)
at similar intercepts. This indicates that increasing
M-110 concentrations increases the apparent $K_a$ of PIM-1
for ATP without affecting the $V_{max}$. From the increase in
slope, a $K_i$ of 0.3 μmol/L can be calculated.

PIM-2 was present in the collection of 261 recombinant
kinases screened for inhibition by M-110, but was not in-
hibited by M-110 under the conditions used for the ki-
nome screen. However, in the kinome screen, the
inhibitory activity of M-110 on PIM-1 and PIM-2 was mea-
sured using different substrates, the S6 kinase-derived
peptide KKRNRRTLTK and the BAD protein-derived pep-
tide RSRHSSYPAGT (PIM2tide), respectively. Thus, to
facilitate comparisons of inhibitory activity for all PIM
kinase family members, we next determined the effect of
M-110 on PIM-1 and PIM-2, as well as PIM-3, which was
absent in the kinome screen, using the PIMtide peptide
ARKRRRHPSGPPTA, which is an efficient substrate for
each of the three PIM kinase family members (35). These
results show that using PIMtide as a substrate, M-110 in-
hibits PIM-1 and PIM-2 with similar IC$_{50}$s of 2.5 μmol/L
and is a potent inhibitor of PIM-3 with an IC$_{50}$ of 0.047
μmol/L (Fig. 3D). Thus, M-110 can inhibit all three mem-
bers of the PIM kinase family but is most active on PIM-3.

**M-110 treatment or PIM-3 knockdown inhibits
STAT3$^{\text{Y705}}$ phosphorylation**

DU-145 cells are an appropriate model system to study
the involvement of active STAT3 signaling in the regula-
tion of prostate cancer cell proliferation. To determine if
the growth inhibitory activity of M-110 on DU-145 cells
may involve inhibition of STAT3 signaling, we used, in
addition to M-110, a second highly selective PIM kinase
inhibitor, SGI-1776 (30), and M-142, a compound that is
identical to M-110 but lacks the hydroxyl group in zone
C (Fig. 2A). M-142 has little activity against PIM-1 and
PIM-3 and does not inhibit DU-145 cell growth up to
10 μmol/L (Table 1). Figure 4A and B shows that treat-
ment of DU-145 cells with 10 μmol/L M-110 or SGI-1776
for 18 hours reduces the expression of p-STAT3$^{\text{Y705}}$ to
23.5% and 35.9%, respectively, compared with untreated
cells without affecting the expression of STAT3. The effect
of treatment with 10 μmol/L M-142 on pSTAT3$^{\text{Y705}}$ is
not significantly different from that of treatment with
DMSO alone. Additional experiments showed that sig-
nificant pSTAT3$^{\text{Y705}}$ inhibition with 10 μmol/L of
both PIM inhibitors also occurs after 8 hours of treatment
(Supplementary Fig. S2). To determine whether the inhib-
itory effect of PIM inhibitors on pSTAT3$^{\text{Y705}}$ expression
is restricted to DU-145 cells, we determined the expres-
sion of pSTAT3$^{\text{Y705}}$ in the prostate cancer cell lines
22Rv1 and PC3, the pancreatic cancer–derived cell line
MiaPaCa2, and the colon cancer–derived cell line
SW480. Only MiaPaCa2 cells expressed detectable levels
of pSTAT3$^{\text{Y705}}$ that were comparable to those found
in DU-145 cells. Incubation of MiaPaCa2 cells with
10 μmol/L M-110 or SGI-1776, but not with M-142, for
18 hours resulted in an inhibition of pSTAT3$^{\text{Y705}}$ similar
to that measured in DU-145 cells (Supplementary Fig. S3),
showing that the effect of M-110 on pSTAT3$^{\text{Y705}}$ is not restricted to DU-145 cells. Proliferation

![Figure 2](image-url)
of DU-145 and MiaPaCa2 cells was also inhibited by M-110 or SGI-1776 but not by M-142 (Table 1), with M-110 being more potent than SGI-1776. Furthermore, we also determined whether M-110 inhibits the expression of pSTAT5Tyr694. Among the five cell lines tested for expression of pSTAT3Tyr705, only 22RV1 cells expressed a detectable amount of pSTAT5Tyr694 that was not significantly affected by an 18-hour treatment with 10 \( \mu \text{mol/L} \) M-110, suggesting the specificity of M-110 for pSTAT3Tyr705 (Supplementary Fig. S4).

STAT3 is activated by cytokines such as IL-6, which stimulates the growth of DU-145 cells. To determine whether M-110 may interfere with IL-6-induced STAT3 phosphorylation of Tyr705, we treated DU-145 cells with M-110 followed by addition of IL-6. Figure 4C and D shows that pretreatment of DU-145 cells with M-110 significantly attenuates the induction of pSTAT3Tyr705 by IL-6, suggesting that M-110 interferes with IL-6/STAT3 signaling, which may contribute to growth inhibition.

To determine whether M-110 inhibits the expression of known STAT3-regulated genes, we measured the expression of cyclin D1, c-myc, and survivin (20, 36, 37). Because STAT3 genes are frequently coregulated by the NF\(\kappa\)B or Wnt/\(\beta\)-catenin signaling pathways and may display cell type–specific regulation (20, 36), we first determined the effect of STAT3 knockdown on the expression of the selected genes in DU-145 cells. In addition, we also determined

![Image](https://example.com/image.png)

**Figure 3.** A kinome screen identifies M-110 as a highly selective inhibitor of PIM-1. A, a collection of 261 recombinant kinases were tested for possible inhibition by 5 \( \mu \text{mol/L} \) M-110. Results are expressed as the percent kinase activity of DMSO-treated samples. B and C, dose-response curves of PIM-1 or CK2a2 inhibition by M-110 and M-37. D, dose-response curves of PIM-1, PIM-2, and PIM-3 inhibition by M-110 using PIMtide as substrate.

**Table 1.** IC\(_{50}\)s for the growth of DU-145 and MiaPaCa2 cells and IC\(_{50}\)s for PIM kinases using PIMtide

<table>
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<tr>
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<th>IC(_{50}) for growth (( \mu \text{mol/L} ))</th>
<th>IC(_{50}) for PIM (( \mu \text{mol/L} ))</th>
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<tr>
<td></td>
<td>DU-145 MiaPaCa2 PIM-1 PIM-2 PIM-3</td>
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<tr>
<td>M-110</td>
<td>0.9 3.9 2.5 2.5 0.05</td>
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<tr>
<td>SGI-1776</td>
<td>4.3 7 0.0007 0.36 0.07</td>
<td></td>
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<tr>
<td>M-142</td>
<td>&gt;10 &gt;10 &gt;33.3 nd &gt;33.3</td>
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whether the expression of the selected genes was inhibited by the JAK2 inhibitor WP-1066 (38). This analysis showed that only survivin gene expression is affected by STAT3 knockdown and JAK2 inhibition in DU-145 cells (Fig. 5A and B). No changes in cyclin D1 (Fig. 5A) or c-myc protein and/or RNA were detected after STAT3 knockdown or JAK2 inhibition (not shown), suggesting that signaling pathways other than the STAT3 pathway are important for the regulation of cyclin D1 and c-myc genes in DU-145 cells. Treatment of DU-145 cells with M-110 resulted in a significant inhibition of survivin protein expression (Fig. 5C and D) and survivin RNA (Fig. 5E). These results further support our data that M-110 interferes with STAT3 signaling.

Our experiments show that both M-110 and SGI-1776, two structurally unrelated but highly selective PIM

Figure 4. Inhibition of pSTAT3 Tyr705. A, DU-145 cells were incubated with 10 μmol/L of M-110, SGI-1776, or M-142 for 18 h. An equal number of untreated (lane 1) and treated cell equivalents (lanes 4–6) were analyzed by Western blotting for the expression of pSTAT3 Tyr705 or STAT3. Lanes 2 and 3 contain 2-fold and 4-fold dilutions of the untreated sample in lane 1 to provide a scale for quantitation of the expression levels in lanes 4 to 6. B, the expression of pSTAT3 Tyr705 is presented as a percentage of expression in untreated cells and is normalized for the expression of STAT3. Columns, mean of three experiments; bars, SEM. Significance is calculated compared with DMSO control samples. **, P ≤ 0.01; *, P ≤ 0.05. C, DU-145 cells were treated with 10 μmol/L M-110 for 18 h and then incubated with 50 ng/mL recombinant IL-6 for 30 min. The signal in lane 2 could not be accurately quantitated but is included in the figure to show that M-110 was active. Lanes 4 and 5 contain 2- and 4-fold dilutions of the IL-6–treated sample analyzed in lane 3. D, results are presented as a percentage of IL-6–treated cells and are normalized for the expression of STAT3. Columns, mean of three experiments; bars, SEM.

Figure 5. M-110 inhibits survivin gene expression. A, treatment with STAT3 siRNA reduces the expression of STAT3 and survivin, but not cyclin D1, proteins. B, treatment of DU-145 cells with 2.5 μmol/L WP-1066 for 8 h inhibits the expression of survivin RNA. C to E, treatment of DU-145 cells with 10 μmol/L M-110 for 18 h inhibits survivin protein and RNA expression. Quantitation in D was as described in the legend to Fig. 4. Columns, mean of three experiments; bars, SEM.
inhibitors, inhibit the constitutive STAT3 Tyr705 phosphorylation in DU-145 cells, suggesting that the effects of M-110 and SGI-1776 on STAT3 phosphorylation are mediated through PIM. SGI-1776 is a significantly more potent inhibitor of PIM-1 and PIM-2 than M-110, with IC_{50} values for PIM-1 and PIM-2 of 7 and 363 nmol/L, respectively. M-110 inhibits these two enzymes equally with IC_{50} of 2.5 μmol/L. However, M-110 and SGI-1776 are both potent inhibitors of PIM-3, with IC_{50} of 50 and 69 nmol/L, respectively (Table 1), suggesting that the M-110– and SGI-1776–mediated inhibition of pSTAT3 Tyr705 expression is due to inhibition of PIM-3 kinase activity. To support this hypothesis, we determined the effect of PIM-1, PIM-2, and PIM-3 knockdown on pSTAT3 Tyr705 expression. Figure 6A shows that transfection of DU-145 cells with PIM-1, PIM-2, or PIM-3–specific siRNAs significantly inhibited the expression of the respective RNAs. Treatment of DU-145 cells with control siRNA did not affect the expression of PIM-1, PIM-2, or PIM-3 RNA. Interestingly, knockdown of PIM-1 leads to an upregulation of PIM-2 RNA, suggesting that PIM-1 may regulate the expression of PIM-2. Only knockdown of PIM-3 resulted in a significantly lower expression of pSTAT3 Tyr705 (Fig. 6B and C). Thus, both the use of selective PIM inhibitors and PIM isoform–specific knockdown experiments showed that PIM-3 kinase is a positive regulator of STAT3 activation.

Discussion

It is estimated that in North America and Europe, approximately 120,000 men will die of androgen-independent prostate cancer this year (39, 40). The current standard of care for treatment of androgen-independent disease is a combination chemotherapy treatment with docetaxel and prednisone. This treatment is essentially palliative but affords a modest survival advantage and an improvement in the quality of life (41). Thus, the characterization of M-110 as a compound with potent growth inhibitory activity on prostate cancer–derived cell lines and with a well-defined molecular target is an important and exciting contribution to the search for more efficacious chemotherapeutic agents for late-stage prostate cancer patients. PIM kinases are attractive anticancer targets because they are positive regulators of cell growth and cell survival and function as oncogenes. Interestingly, the oncogenic action of PIM kinases is significantly enhanced by coexpression of c-myc. Because c-myc is overexpressed in many human tumors, the increased oncogenicity of PIM kinases in the presence of overexpressed c-myc makes PIM kinases de facto tumor-specific targets. Through the use of two highly selective PIM kinase inhibitors and PIM isoform–specific siRNA, we showed that PIM-3 is a positive regulator of STAT3 phosphorylation on Tyr705. Knockdown of PIM-1 or PIM-2 did not result in inhibition of pSTAT3 Tyr705, suggesting a unique function for PIM-3 as a positive regulator of STAT3 signaling. Studies in HEK293 cells identified PIM-1 and PIM-2 as negative regulators of pSTAT3 Tyr705 phosphorylation through the stabilization of SOCS-1, a negative regulator of JAK/STAT signaling. PIM-1 was also shown to be a negative regulator of STAT5 phosphorylation on Tyr694 in myeloid cells through interactions with SOCS-1 and SOCS-3 (42, 43). Some of these results have been duplicated in prostate cancer cell lines, as downregulation of SOCS-1 by siRNA increases the proliferation of DU-145 cells. However, SOCS-3 may have an opposite function in DU-145 cells, as downregulation of SOCS-3 in DU-145 cells decreases cell proliferation (44, 45). A role for PIM-3 in the regulation of SOCS proteins has not
yet been described, but it is tempting to speculate that PIM-3, similar to PIM-1 and PIM-2, acts as a positive regulator of SOCS-3. More studies to investigate the possible interactions between PIM-3 and SOCS-3 and the effect of such interactions on SOCS-3 and pSTAT3 \( \text{Tyr}^{705} \) expression are needed.

Many tumor types including prostate adenocarcinomas express pSTAT3 \( \text{Tyr}^{705} \), which is thought to be secondary to autocrine or paracrine stimulation by IL-6. DU-145 cells express constitutively active NF\( \kappa \)B, leading to IL-6 production and the subsequent stimulation of the IL-6 receptor and STAT3 phosphorylation on Tyr705. Thus, interference in NF\( \kappa \)B-mediated IL-6 expression may be one way in which M-110 treatment, through inhibition of PIM-3, leads to downregulation of pSTAT3 \( \text{Tyr}^{705} \) and growth inhibition. Several mechanisms have been described to explain the constitutive activation of NF\( \kappa \)B, including constitutive activation of I\( \kappa \)B kinase and increased turnover of I\( \kappa \)Bo (46) and sequestration of the NF\( \kappa \)B component p65/RelA in the nucleus through an interaction with nuclear STAT3 (47). It remains to be determined whether PIM-3 kinase plays a role in any of these mechanisms in DU-145 or MiaPaCa2 cells. Alternatively, M-110, through inhibition of PIM-3, may affect the functions of protein tyrosine phosphatases or tyrosine kinases that regulate the phosphorylation of STAT3 at Tyr705 (48), as has been described for PIM-1 (49, 50), but whether PIM-3 similarly regulates the activity of tyrosine phosphatases or tyrosine kinases is not known.

Our finding that M-110 inhibits the expression of active STAT3 through inhibition of PIM-3 strongly supports the further development of PIM inhibitors into clinically useful chemotherapeutic agents for the treatment of patients with androgen-independent prostate cancer or other types of cancer that display constitutive expression of active STAT3. In addition, the use of PIM inhibitors is not restricted to cancers that express pSTAT3 \( \text{Tyr}^{705} \) because M-110 also inhibits the proliferation of 22Rv1, PC3, and SW480 cells, with IC\( _{50} \) values of 0.6 to 0.8 \( \mu \text{mol/L} \) (not shown). These cells do not express activated STAT3 and the growth inhibitory action of M-110 may be due to the direct action of PIM kinases on a number of regulators of cell cycle progression, such as CDC25, p21, or p27. Nevertheless, because many patient tumors express activated STAT3, the inhibition of STAT3 signaling is likely to be an important attribute of the anticancer activity of any of the M-110 derivatives when tested in a clinical setting.

**Disclosure of Potential Conflicts of Interest**

A provisional patent application describing M-110 and related structures has been filed.

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**References**

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Marisa Chang, Nisha Kanwar, Eric Feng, et al.


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