# Targeting PIM Kinases Impairs Survival of Hematopoietic Cells Transformed by Kinase Inhibitor–Sensitive and Kinase Inhibitor–Resistant Forms of Fms-Like Tyrosine Kinase 3 and BCR/ABL

Myriam Adam,[1] Vanda Pogacic,[2] Marina Bendit,[2] Richard Chappuis,[1] Martijn C. Nawijn,[3] Justus Duyster,[4] Casey J. Fox,[5] Craig B. Thompson,[5] Jan Cools,[6] and Juerg Schwaller[1,2]

[1]Department of Pathology, Geneva Medical School, Geneva, Switzerland; [2]Department of Research, University Hospital Basel, Basel, Switzerland; [3]Division of Molecular Genetics, the Netherlands Cancer Institute, Amsterdam, the Netherlands; [4]Department of Internal Medicine III, Technical University of Munich, Munich, Germany; [5]Abramson Family Cancer Research Institute, Philadelphia; and the [6]Center for Human Genetics and Flanders Interuniversity Institute for Biotechnology, University of Leuven, Leuven, Belgium

## Abstract

**Previous studies have shown that activation of the signal transducer and activator of transcription 5 (STAT5) plays an essential role in leukemogenesis mediated through constitutive activated protein tyrosine kinases (PTK). Because *PIM-1* is a STAT5 target gene, we analyzed the role of the family of PIM serine/threonine kinases (PIM-1 to PIM-3) in PTK-mediated transformation of hematopoietic cells. Ba/F3 cells transformed to growth factor independence by various oncogenic PTKs (TEL/JAK2, TEL/TRKC, TEL/ABL, BCR/ABL, FLT3-ITD, and H4/PDGFβR) show abundant expression of PIM-1 and PIM-2. Suppression of PIM-1 activity had a negligible effect on transformation. In contrast, expression of kinase-dead PIM-2 mutant (PIM-2KD) led to a rapid decline of survival in Ba/F3 cells transformed by FLT3-ITD but not by other oncogenic PTKs tested. Coexpression of PIM-1KD and PIM-2KD abrogated growth factor–independent growth of Ba/F3 transformed by several PTKs, including BCR/ABL. Targeted down-regulation of PIM-2 by RNA interference (RNAi) selectively abrogated survival of Ba/F3 cells transformed by various Fms-like tyrosine kinase 3 (FLT3)–activating mutants [internal tandem duplication (ITD) and kinase domain] and attenuated growth of human cell lines containing FLT3 mutations. Interestingly, cells transformed by FLT3 and BCR/ABL mutations that confer resistance to small-molecule tyrosine kinase inhibitors were still sensitive to knockdown of PIM-2, or PIM-1 and PIM-2 by RNAi. Our observations indicate that combined inactivation of PIM-1 and PIM-2 interferes with oncogenic PTKs and suggest that PIMs are alternative therapeutic targets in PTK-mediated leukemia. Targeting the PIM kinase family could provide a new avenue to overcome resistance against small-molecule tyrosine kinase inhibitors.** (Cancer Res 2006; 66(7): 3828-35)

## Introduction

A high percentage of acute leukemias express functional class III receptor protein tyrosine kinase (PTK) Fms-like tyrosine kinase 3 (FLT3). Approximately 25% of acute myeloid leukemia (AML) patients have activating FLT3 mutations, either internal tandem duplications (ITD) or mutations in the activation loop of the tyrosine kinase domain (1). Overexpression and/or activating point mutations have also been found in acute lymphoblastic leukemia (ALL) from infants associated with rearrangement of the *MLL* gene. Activating mutations of the FLT3 tyrosine kinase are the most common somatic genetic alterations in acute leukemia, and there is evidence that patients with FLT3-ITD may be associated with poorer prognosis. The biological effects of activating FLT3 mutations have been tested in several *in vitro* and *in vivo* experimental systems, indicating that activating FLT3 mutants provide growth factor–independent proliferation and survival to hematopoietic cells *in vitro* and *in vivo* (2).

Several new compounds with activity against FLT3 by competing with ATP binding have been recently characterized (3). These drugs were shown to be selectively cytotoxic to cells either transfected with FLT3-ITD or to primary AML samples carrying FLT3-ITD. *In vivo* application of three different compounds (CEP-701, PKC-412, and MLN-518) has been shown to prolong survival of mice transplanted either with FLT3-ITD–expressing marrow or FLT3-ITD–transfected Ba/F3 cells (4). However, these compounds show limited activity against activation loop FLT3 mutations (5, 6), and resistance to these inhibitors can develop rapidly (7, 8). The molecular mechanisms of hematopoietic transformation by mutated FLT3 are not well understood. Significant differences have been observed in downstream signaling either induced by wild type (WT) or by constitutive active mutated FLT3. First, restoration of the apoptosis inhibitor Bcl-XL was critical for survival of hematopoietic cells by FLT3-ITD but not WT-FLT3. Second, hematopoietic cells transformed by activating FLT3 mutants showed constitutive activation of the signal transducer and activation of transcription 5 (STAT5), whereas transfectants with WT-FLT3 showed only weak or transient activation of STAT5 when exposed to FLT3 ligand (9). Moreover, STAT5 phosphorylation and activation is inhibited by selective FLT3 PTK inhibitors. These findings suggest that regulation of antiapoptotic proteins like Bcl-XL in conjunction with activation of STAT5 may be critical for the leukemogenic effects of oncogenic FLT3 mutants.

Constitutive activation of STAT5 has been shown to be a critical mediator for transformation by several PTK fusion genes, including *BCR/ABL, FLT3*, or *TEL/JAK2* associated with chronic myeloproliferative disorders (10). Several downstream STAT5 target genes (such as *SOCS1, BCL-XL*, and *PIMs*) were shown to play a role in cancer. The PIM family of serine/threonine kinases

**Note:** J. Cools is a postdoctoral researcher of the "FWO-Vlaanderen."

**Requests for reprints:** Juerg Schwaller, Department of Research, Basel University Hospital, ZLF, Lab 318, Hebelstrasse 20, CH-4031 Basel, Switzerland. Phone: 41-61-265-3504; Fax: 41-22-37-24920; E-mail: J.Schwaller@unibas.ch.



doi:10.1158/0008-5472.CAN-05-2309

(PIM-1 to PIM-3) has been originally identified as proviral integration sites involved in lymphomagenesis induced by murine leukemia virus (11). PIM kinases are highly homologous to each other sharing a unique consensus hinge region sequence, but they have <30% sequence identity to other kinases. Determination of the crystal structure of PIM-1 revealed that PIMs adopt a constitutive active confirmation based on extensive hydrophobic and hydrogen bond interactions (12). PIM-1 has been shown to protect hematopoietic cells from apoptosis but to be dispensable for BCR/ABL–mediated transformation (13, 14). In addition, a recent study has suggested that PIM-1 up-regulation by constitutive activated FLT3 may play a role in FLT-3–mediated survival (15). PIM-2 has been shown to be transcriptionally regulated and to maintain cell size and mitochondrial potential independent of activation of the phosphatidylinositol 3-kinase/AKT/target of rapamycin (TOR) pathway. Its cytoprotective activity was dependent on phosphorylation of the translational repressor 4E-PB1 and the BH3 apoptotic regulator BAD (16). Increased PIM-2 expression has been found in a wide range of malignancies, including leukemia, prostate cancer, mantle cell lymphoma, and multiple myeloma (17, 18). Interestingly, gene expression profiling showed strong up-regulation of PIM-2 expression in hematopoietic cells transformed by FLT3-ITD (19). Moreover, promotion of survival of lymphoid cells by PIM-2 has been shown to be dependent on nuclear factor-κB activation (20).

Here, we have analyzed the role of PIMs in transformation of hematopoietic cells by various oncogenic PTKs and their potential as therapeutic targets in leukemia.

## Material and Methods

**Cells.** WT interleukin-3 (IL-3)–dependent murine Ba/F3 cells and generation of the stably factor-independent growing Ba/F3 cells transformed by PTK fusion genes (*BCR/ABL, TEL/JAK2, TEL/TRKC, FLT3-ITD*, and *H4/PDGFβR*) used in this study have been previously described (2, 21, 22). Cells were kept in RPMI 1640 with 10% FCS (Invitrogen, Carlsbad, CA) and 1 ng/mL recombinant murine IL-3 (WT-Ba/F3 cells, Endogen, Pierce Biotechnology, Woburn, MA) at 37°C in 5% $CO_2$. Ba/F3 cells stably expressing FLT3-D835Y, FLT3-I836del, FLT3-ITD-G697R, and FLT3 WT and the human leukemia cell lines MV4-11, K-562, REH, PL-21, and MOLM-13 have been previously characterized (5–7, 23). In addition, Ba/F3 cells transformed by BCR/ABL (p185) WT and various mutants detected in patients under imatinib therapy have been previously described (24). Cellular growth curves were established as followed: $10^5$ live cells were plated in triplicates and counted daily using a hemacytometer. Cells were diluted appropriately to be $<0.5 \times 10^6$/mL. Viability was analyzed by trypan blue dye exclusion. Apoptotic cell death was determined by using 7-AAD (BD Biosciences, San Diego, CA) according to manufacturer's protocol.

**Gene transfer.** WT and kinase-dead mutants (PIM-KD) murine PIM-1(K67M) and PIM-2(K61M) cDNAs were cloned into the retroviral expression vector *pMSCV-IRES-EGFP/EYFP* (19, 25). Retrovirally delivered small interfering RNAs (siRNA) targeting PIM-2 have been characterized previously (16). Retroviral stocks and transduction of Ba/F3 cells were done as described (21). Synthetic siRNAs targeting mouse PIM-2 (Santa Cruz Biotechnology, Santa Cruz, CA) and human PIM-2 (silencer validated siRNAs, Ambion, Inc., Austin, TX and Qiagen, Hilden, Germany) and synthetic siRNA targeting mouse PIM-1 (Santa Cruz Biotechnology) or human PIM-1 (Qiagen) as well as control siRNAs (Santa Cruz Biotechnology and Qiagen) were transfected using the AMAXA nucleofector following the manufacturer's instruction (AMAXA Biosystems, Cologne, Germany).

**Gene expression analysis.** PIM mRNA expression was quantified by SYBRGreen real-time reverse transcription-PCR (RT-PCR). 1 μg of RNase-free DNase (Qiagen)–treated total RNA (isolated with the RNeasy kit, Qiagen) was used for cDNA synthesis (AMV first-strand cDNA synthesis kit, Roche Diagnostics, Rotkreuz, Switzerland), and 50 ng cDNA was used for RT-PCR in iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) using an *icycler* (Bio-Rad). Expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA ($\Delta C_T$ method). Primer sequences and PCR conditions are available upon request. PIM protein expression was analyzed by Western blot analysis. Lysates (50 μg) were separated on 8% to 12% SDS-PAGE and blotted using conventional methods. Antibodies recognizing PIM-1 (clone 19F7), PIM-2 (clone 1D12), and actin (clone C-11) were purchased from Santa Cruz Biotechnology.

## Results and Discussion

Previous studies have proposed *PIM-1* serine/threonine kinase being a STAT5 downstream target gene (26). Based on our previous work showing that STAT5 is an important mediator of malignant transformation by TEL/JAK2 and being able to induce a lethal myeloproliferative disorder itself, we aimed to learn about the role of its putative targets, the PIM kinases, in PTK-mediated transformation (27).

First, expression levels of the three known PIM serine/threonine kinases (PIM-1, PIM-2, and PIM-3) were determined in Ba/F3 cells stably transformed to IL-3 independent growth by six constitutively active PTKs. High levels of *PIM-1* and *PIM-2* mRNA levels were detected in cells transformed by TEL/JAK2, TEL/TRKC, TEL/ABL, BCR/ABL, H4/PDGFRβ, and FLT3-ITD (Fig. 1*A*). In parental Ba/F3 cells, *PIM-1* mRNA expression was strictly dependent on IL-3 stimulation and decreased rapidly after IL-3 depletion. In contrast, in several experiments using different Ba/F3 cell batches, *PIM-2* mRNA expression unchanged upon removal of IL-3 (Fig. 1*A*). No significant *PIM-3* expression levels could be detected in all cell lines tested (data not shown). Western blot analysis confirmed abundant levels of PIM-1 and low but detectable levels of PIM-2 in all cell lines tested (Fig. 1*B*). In parental Ba/F3 cells, depletion of IL-3 lead to a decrease in PIM-1 and PIM-2 protein expression. These data show abundant expression of PIM-1 and PIM-2 but not PIM-3 in hematopoietic cells transformed by oncogenic PTKs. In addition, it suggests that in Ba/F3 cells, PIM-2 expression, unlike PIM-1 expression, is mainly regulated at the post-transcriptional level.

Overexpression of PIM-1 is sufficient to transform hematopoietic cell lines to growth factor independence (13). Likewise, in our series of experiments, overexpression of PIM-1 or PIM-2 or both together was capable to induce growth factor–independent growth of Ba/F3 (data not shown). We also investigated if overexpression of PIM-1, PIM-2, or coexpression of both in bone marrow of mice could induce a hematologic disorder, but no disease was observed, after 10 months (data not shown). These data suggest that PIM kinases may exert their oncogenic properties *in vivo* only in combination with other genetic alterations.

To address whether expression of PIMs is required for transformation of Ba/F3 cells by oncogenic PTKs, PIM-KD variants were coexpressed in PTK-transformed Ba/F3 cells. PIM-KD mutants have been previously tested *in vitro* to lack any kinase

activity and to be able when overexpressed to reduce kinase activity of the wild-type form in a dominant-negative manner (19, 25, 28). In analogy with previous reports, expression of PIM-1KD alone had no significant effect on the growth of Ba/F3 cells transformed by BCR/ABL or FLT3-ITD (Fig. 2; refs. 13, 14). Expression of PIM-2KD resulted in a decreased growth rate of Ba/F3 cells transformed by TEL/JAK2 and to lesser extent in BCR/ABL cells. In sharp contrast, expression of PIM-2KD in FLT3-ITD cells rapidly induced apoptotic cell death in several experimental settings (Fig. 2*C*). Expression of PIM-1KD and PIM-2KD variants was checked by Western blot analysis and by expression of the green fluorescent protein (GFP) selection marker (EGFP and EYFP) driven from an internal ribosomal entry site (IRES) in the retroviral construct (data not shown). This result confirms and extends previous observations showing that expression of PIM-2KD significantly inhibited growth of murine hematopoietic cells (32-D) transformed to growth factor independence by the FLT3-ITD (19). Expression of PIM-2KD in parental Ba/F3 cells growing in IL-3, or in Ba/F3 expressing a wild-type FLT3, stimulated with FLT3 ligand impaired cellular proliferation but did not show any significant impairment of cell survival (data not shown). This observation could reflect the fact that mice lacking all PIM kinases are viable and show only mildly impaired responses to hematopoietic growth factors (29). Surprisingly, coexpression of PIM-1KD and PIM-2KD significantly impaired growth of Ba/F3 cells transformed by TEL/JAK2 or BCR/ABL and rapidly induced cell death in FLT3-transformed cells (Fig. 2). These experiments suggest that PIM-2 and not PIM-1 may play a critical role in mediating growth factor–independent growth and survival of Ba/F3 cells by FLT3-ITD. Moreover, it suggests that combined activation of PIM-1 and PIM-2 may be critical mediators of survival of Ba/F3 cells by oncogenic PTKs.

Expression of kinase-dead mutants harbors the possibility that the observed effect is the consequence of nonspecific interaction with unknown molecules. In addition, because no isoform-specific PIM substrates have been identified, cross-reactivity with both wild-type PIMs cannot be ruled out. Therefore, we also targeted PIM-2 by using RNA interference (RNAi). In our previous work, we have identified a highly specific siRNA targeting murine PIM-2. Expression of this siRNA from the *pBABE* retrovirus significantly reduced PIM-2 mRNA and protein expression (16). In analogy to expression of a PIM-2KD mutant, retroviral expression of siRNAs targeting PIM-2

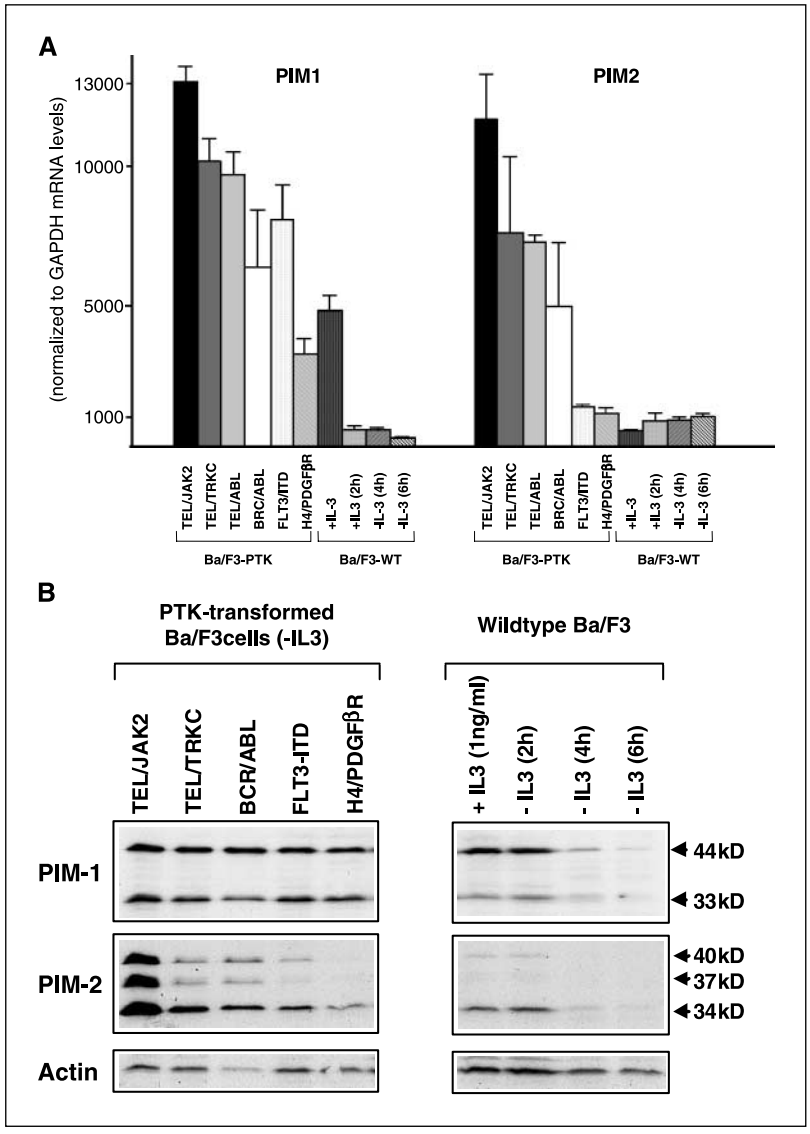


**Figure 1.** Expression of PIM serine/threonine kinase genes in hematopoietic cells (Ba/F3) transformed to growth factor independence by various oncogenic PTKs. *A,* PIM gene expression was analyzed by quantitative RT-PCR analysis in Ba/F3 cells transformed to growth factor independence by stable transfection of various oncogenic PTKs (TEL/JAK2, TEL/TRKC, TEL/ABL, BCR/ABL, FLT3-ITD, and H4/PDGFβR) and wild Ba/F3 growing in the presence or absence (6 hours) of IL-3. Relative PIM expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA expression. *Bars,* SD obtained from two independent experiments, each done in triplicates. *B,* Western blot analysis showing PIM-1 and PIM-2 protein expression. Immunoblots were sequentially probed with antibodies specific for murine PIM-1, PIM-2, and actin. PIM-1 is expressed as two isoforms of 33 and 44 kDa, and PIM-2 as three isoforms of 34, 37, and 40 kDa.

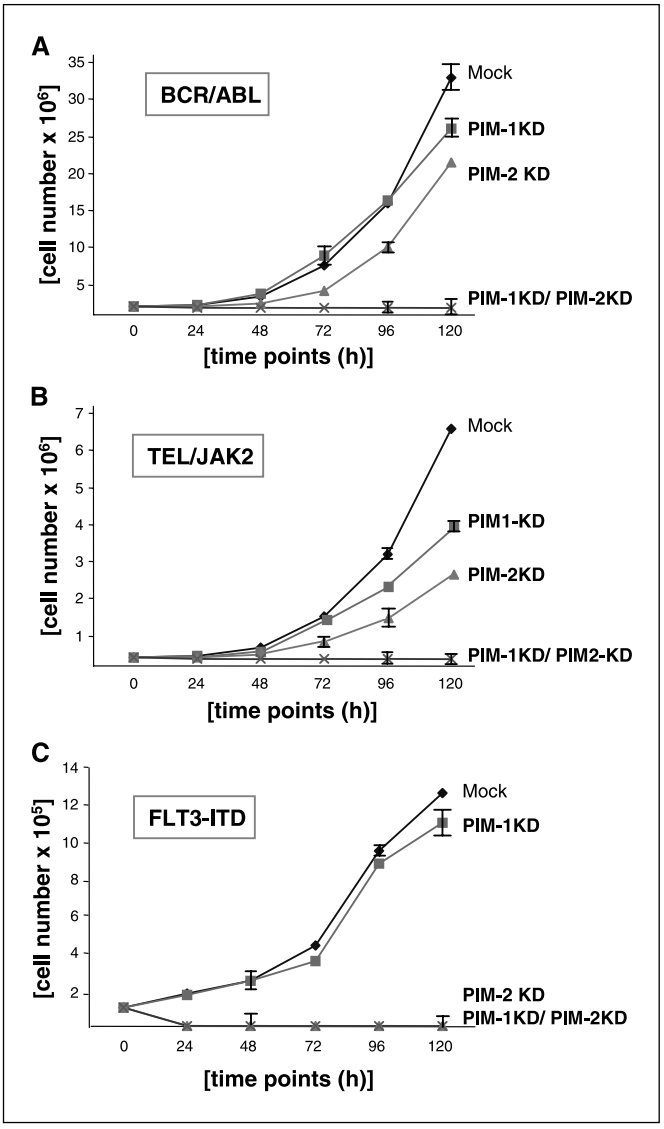


**Figure 2.** Expression of kinase-dead mutants identifies PIM-2 as a critical target for FLT3-ITD–mediated transformation. Ba/F3 cells transformed by BCR/ABL (*A*), TEL/JAK2 (*B*), or FLT3-ITD (*C*) were infected with retrovirus encoding PIM-KD mutants (*pMSCV-PIM-1KD/IRES-EGFP* and *pMSCV-PIM-2KD/IRES-EYFP*) followed by fluorescence-activated cell sorting–assisted selection 48 hours after infection. To generate Ba/F3-overexpressing PIM-1KD and PIM-2KD, cells expressing PIM-1KD were reinfected with the virus encoding PIM-2KD and selected for $EGFP^+/EYFP^+$. Cell growth was followed by plating $10^5$ cells in IL-3–free medium and counted on five consecutive days. No cell survival was observed in FLT3-ITD Ba/F3 cells expressing PIM-2KD after 24 hours.

rapidly induced apoptotic cell death of Ba/F3 cells transformed by FLT3-ITD. In contrast, Ba/F3 cells transformed by BCR/ABL or TEL/JAK2, or overexpressing FLT3 stimulated with FLT3L, as well as parental Ba/F3 growing in IL-3 remained unaffected (Fig. 3*A*; data not shown). Our observation made in Ba/F3 mouse myelolymphoid cells transformed through overexpression of activating FLT3 mutations were confirmed in three human acute leukemia cell lines (Fig. 3*B*): MV4-11, expressing exclusively the mutated allele of FLT3 (FLT3-ITD, homozygous), MOLM-13, and PL-21 (FLT3-ITD, heterozygous; ref. 23). Although attempts to generate MV4-11 cells stably expressing PIM-2 siRNAs by using a viral-based expression system failed (data not shown), nucleofection of synthetic siRNAs targeting human PIM-2 led to induction of apoptosis of a significant fraction of MV4-11 cells. In contrast, the respective differences in cell death were still significant in MOLM-13 and PL-21 cells, consistent with their heterozygous genotype (Fig. 3*B*). No induction of significant apoptosis was detected by expression of PIM-2 siRNAs in REH (pre-B-ALL) and in K-562 cells [BCR/ABL-positive, chronic myeloid leukemia (CML)]. Efficacy of siRNA-mediated PIM-2 down-regulation was in the absence of a specific anti-human PIM-2 antibody only determined by real-time PCR (Fig. 3*B*).

Our observations made by overexpression of dominant-negative acting kinase-dead mutants as well as targeting expression by siRNAs strongly suggests that PIM-2 is a critical target for transformation of hematopoietic cells by FLT3-ITD. These results may somehow contrast a recent report that analyzed the role of PIM kinases in inhibition of FLT3-ITD by a small-molecule inhibitor (15). In their work, treatment of hematopoietic cells harboring FLT3-ITD with the CEP-701 small-molecule tyrosine kinase inhibitor lead to up-regulation of PIM-1 expression. In addition, overexpression of PIM-1 rendered Ba/F3 cells expressing FLT3-ITD more resistant to cytotoxicity induced by the CEP-701 small-molecule tyrosine kinase inhibitor. Although not analyzed, one explanation of their finding could be that CEP-701 may also inhibit one of the PIMs, presumably PIM-2, leading to a compensatory up-regulation of PIM-1. Based on this hypothesis, we were analyzing the expression level of PIMs upon down-regulation by siRNA and overexpression of the KD-mutants. Interestingly, as shown in Fig. 4*A*, down-regulation of PIM-1 by nucleofection of synthetic siRNAs in Ba/F3 transformed by BCR/ABL or FLT3-ITD led to a significant increase of PIM-2 mRNA expression. In contrast, we could not observe a significant change of PIM-2 mRNA expression levels upon down-regulation of PIM-1. Similar results have been obtained in Ba/F3 cells overexpressing the dominant-negative acting PIM-KD mutants (data not shown). Compensatory up-regulation of PIM-2 expression upon down-regulation of PIM-1 may explain why we do not observe a significant effect upon inhibition of PIM-1 in the PTK-transformed cells analyzed here. Upon cotransfection of different siRNAs targeting PIM-1 and PIM-2, a significant reduction in survival of Ba/F3 cells transformed by FLT3-ITD and BCR/ABL was observed confirming the results obtained by coexpression of PIM-KD mutants (Fig. 4*B*; data not shown). Because expression of synthetic siRNAs targeting PIM-2 lead to no significant effect in MV4-11 but BCR/ABL–positive K-562 cells we now asked whether like in the Ba/F3 cell model, interference with both PIMs would be necessary. In several experimental setups using two different siRNA targeting each human PIM, a significant decrease in viability was observed upon conucleofection with siRNAs targeting PIM-1 and PIM-2 in both cell lines (Fig. 4*C*). Keeping in mind that gene transfer rate by nucleofection of human cell lines is limited, this observation suggests that like in the Ba/F3 cell model, combined interference with both PIMs can significantly reduce cell growth of human BCR/ABL–expressing cells. In contrast to the experiments with Ba/F3 cells, we have observed some cross-reactivity with the applied siRNAs (Fig. 4*D*). This is not surprising, because PIM-1 and PIM-2 show >70% sequence homology at the nucleotide level. However, independently of any cross-reactivity, these experiments show that both PIM-1 and PIM-2 have to be targeted to obtain a significant growth reduction.

Two types of mutations lead to constitutive activation of FLT3 in acute leukemia: ITDs in the juxtamembrane region (length mutations) and point mutations in the activation loop of the PTK domain (1). Whereas FLT3 length mutations can be

successfully targeted by several small-molecule inhibitors, the response of FLT3 kinase domain mutants to these compounds is variable. However, similar to cells transformed by FLT3-ITD, Ba/F3 cells transformed by the kinase domain mutations FLT3-D835Y or FLT3-I836del underwent rapidly apoptotic cell death upon expression of the siRNA targeting PIM-2 (Fig. 5*A*). These data further support the idea that PIM-2 is a critical downstream target for cellular transformation by mutant FLT3 either activated by length mutations (ITD) or activation loop mutations.

PTK-transformed hematopoietic cells treated by small-molecule inhibitors targeting the kinase domain can develop resistance by acquiring additional mutations in the kinase domain (30). We and others have recently identified dual mutations in the kinase domain of FLT3 (G697R) combination with FLT3 length (ITD) mutation, which confers resistance to several small-molecule kinase inhibitors, including PKC-412 and SU-5614 (7, 31). Interestingly, down-regulation of PIM-2 by RNAi also induced rapid apoptotic cell death in Ba/F3 cells expressing the dual FLT3 mutation (FLT3-ITD-G697R; Fig. 5*A*). These results show that down-regulation of PIM-2 by RNAi specifically induces cell death in Ba/F3 cells transformed by activating length (FLT3-ITD) or activation loop mutations (FLT3-D835Y and FLT3-I386del), as well as activated FLT3 mutants with additional mutations (FLT3-ITD-G697R) resistant to small-molecule tyrosine kinase inhibitors.

Although PIM-1 and PIM-2 were expressed at high levels in PTK-transformed Ba/F3 cells, only interference of PIM-2 function by either expression of a kinase-dead mutant or by siRNA mediated down-regulation of expression selectively abrogated survival of cells transformed with various activating mutations of FLT3. The correlate of this specificity is currently not understood. Previous

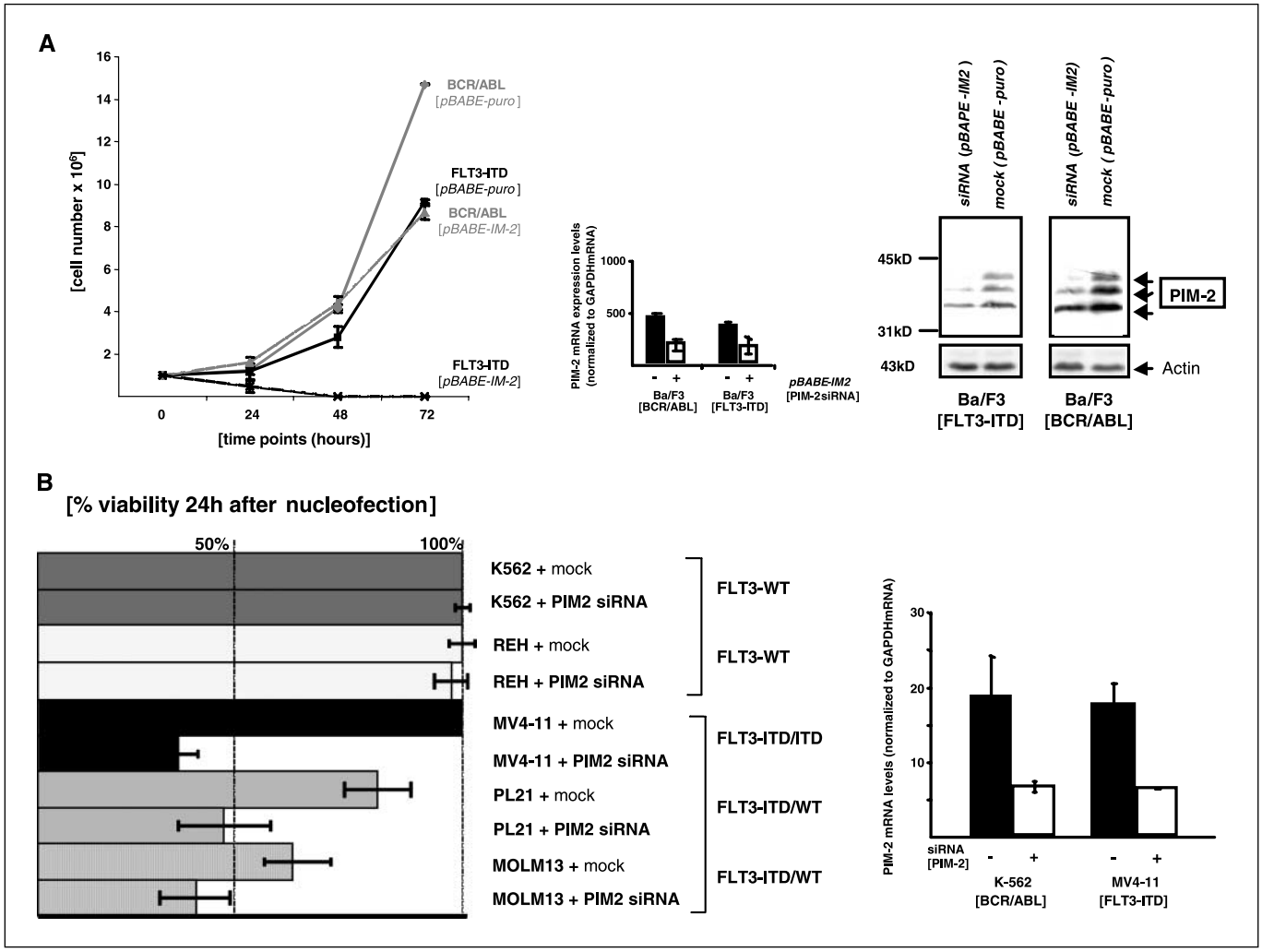


**Figure 3.** Down-regulation of PIM-2 expression by RNAi confirms PIM-2 as a critical downstream target for Ba/F3 cells transformed by mutated FLT3. *A*, Ba/F3 cells transformed by BCR/ABL or FLT3-ITD to IL-3 independence were infected with a retrovirus encoding siRNA-targeting PIM-2 (*pBABE-IM-2*) or the control vector (*pBABE-puro*) followed by puromycin selection (1.0 μg/mL) 24 hours after infection. Cells ($10^5$) were plated in triplicate and followed over three consecutive days. Whereas Ba/F3 BCR/ABL cells were not significantly influenced, no surviving Ba/F3 FLT3-ITD cells expressing *pBABE-IM-2* could be observed 48 to 72 hours after infection under puromycin selection. Efficacy of siRNA-mediated down-regulation of endogenous mPIM-2 was analyzed by real-time PCR assay (*middle*) and by Western blot analysis (*right*). Total cell lysates were harvested as early as 24 hours after infection and separated by 10% SDS-PAGE and immunoblotted with anti-PIM-2 and anti-actin antibodies. *B,* 50 nmol/L synthetic hPIM-2–specific siRNAs were transferred into FLT3-ITD (homozygous)–positive MV4-11, FLT3-ITD (heterozygous), MOLM-13, and PL-21 cells, as well as in K-562 (BCR/ABL–positive CML) and REH (pre-B-ALL) cells by nucleofection (AMAXA). Cell number and viability were determined by counting and 7-AAD staining in triplicate after 24 hours. A low number of viable MV4-11 cells could be observed after 24 hours. Significant reduction of viability was observed in MOLM-13 and PL-21 cells, whereas growth and viability of K-562 or REH cells was not significantly altered (*left*). Efficacy of siRNA-mediated down-regulation of endogenous mPIM-2 was analyzed in all experiments by real-time PCR as shown for K-562 and MV4-11 (*right*).

studies have shown that activation of the AKT signaling pathway as determined by phosphorylation of BAD was critical for mediating survival by WT-FLT3 but not by mutant FLT3 (9). In addition, deletion of AKT or treatment with rapamycin targeting the downstream target mammalian TOR, provided a critical role of PIM-2 (and not PIM-1) for hematopoietic cell growth and survival (32). Based on these data, one could speculate that activation of AKT may not be critical for transformation by FLT3 mutant and therefore leaving PIM-2 for compensatory regulation of critical cellular functions necessary for survival.

Coexpression of PIM-1KD and PIM-2KD mutants or cotransfection of siRNAs targeting PIM-1 and PIM-2 leads to a significant decrease in survival of Ba/F3 cells transformed by (p210)BCR/ABL, the hallmark of CML. Whereas the small-molecule ABL inhibitors like imatinib are highly effective in treatment of chronic-phase CML, patients with pre-B-ALL containing the p185 BCR/ABL isoform seem to develop resistance more rapidly (33). We have therefore analyzed whether combined down-regulation of PIM-1 and PIM-2 through siRNA targeting may be able also to impair survival of hematopoietic cells transformed by BCR/ABL p185 (p185 WT) and several point mutations detected in patients that developed clinical resistance against imatinib-mesylate. As shown in Fig. 5*B*, cotransfection of siRNAs targeting human PIM-1 and PIM-2 led to significant decrease in viability in cells expressing p185-WT, E255Y,

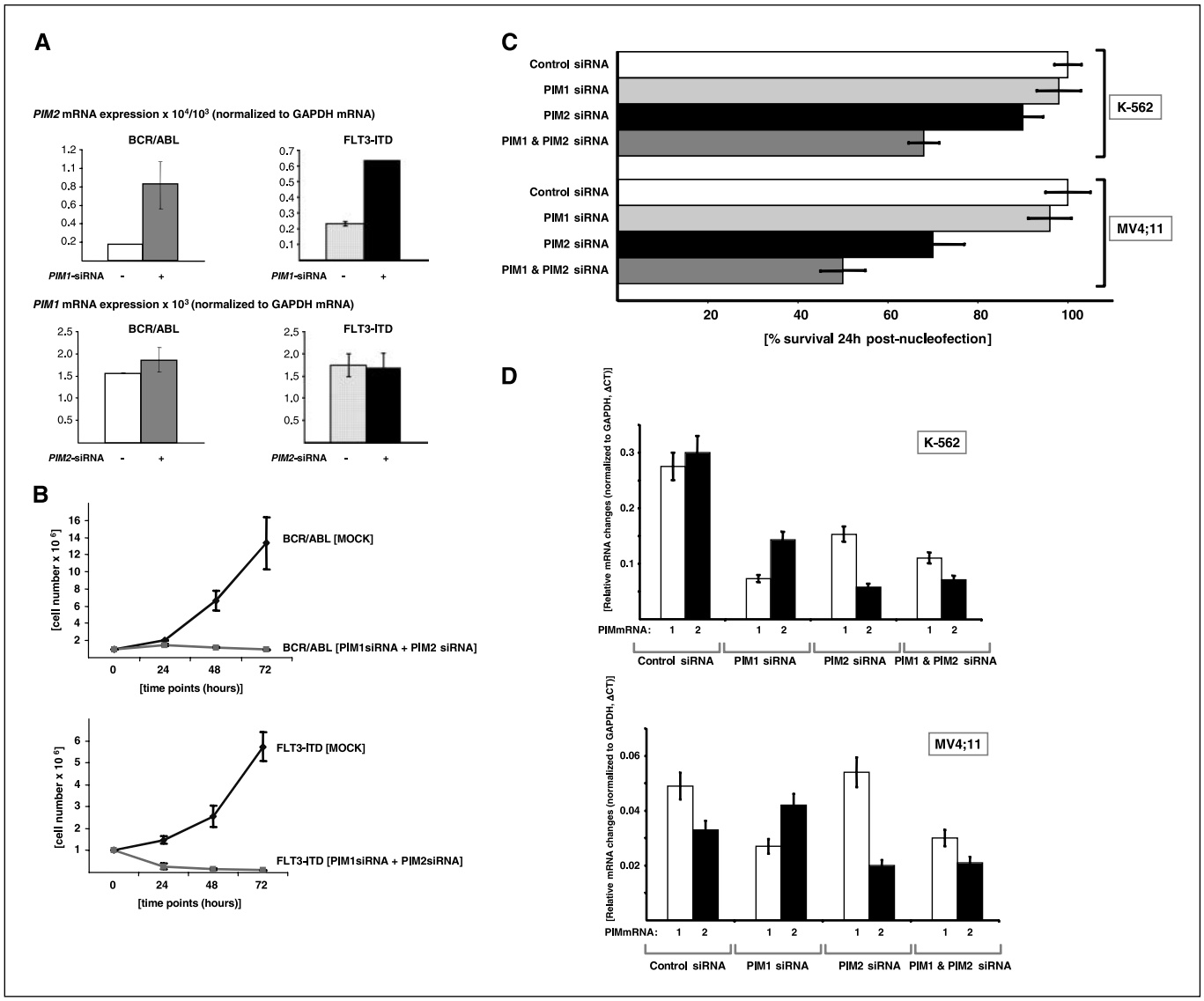


**Figure 4.** Murine and human BCR/ABL-transformed cells are significantly impaired by expression of siRNAs targeting both PIM-1 and PIM-2. *A,* Ba/F3 cells transformed by BCR/ABL or FLT3-ITD were either nucleofected with mouse synthetic PIM-1 (*top*) or PIM-2 (*bottom*) specific siRNAs (AMAXA). Two days after nucleofection or infection PIM gene expression was analyzed by quantitative RT-PCR as described in Fig. 1. *B,* mouse synthetic PIM-1 and PIM-2 siRNAs were transferred by nucleofection (AMAXA) into Ba/F3 cells transformed by BCR/ABL or FLT3-ITD. Survival analysis was carried out as described above. Growth of Ba/F3 FLT3-ITD cells was strongly affected, but coexpression of both siRNAs in Ba/F3 BCR/ABL cells affected growth to a lesser extend compared with the results obtained with overexpression of PIM-KD mutants. *C,* K-562 and MV4-11 cells were nucleofected with 50 nmol/L synthetic siRNAs targeting hPIM-1, hPIM-2, or both, as well as with the control siRNA, and cell number and viability determined by counting after 24 hours. Significant reduction of survival of K-562 and MV4-11 cells was observed in case of siRNA-mediated down-regulation of both hPIM-1 and hPIM-2. *D,* efficacy of siRNA-mediated down-regulation of expression of endogenous hPIM-1 (*1, empty columns*) and hPIM-2 (*2, black columns*) in MV4-11 and K-562 cells nucleofected with nonspecific siRNA (siRNA control), siRNA specific for hPIM-1 (siRNA PIM-1), siRNA specific for hPIM-2 (siRNA PIM-2), and combination of both (siRNA PIM-1 and PIM-2) was analyzed by real-time PCR.

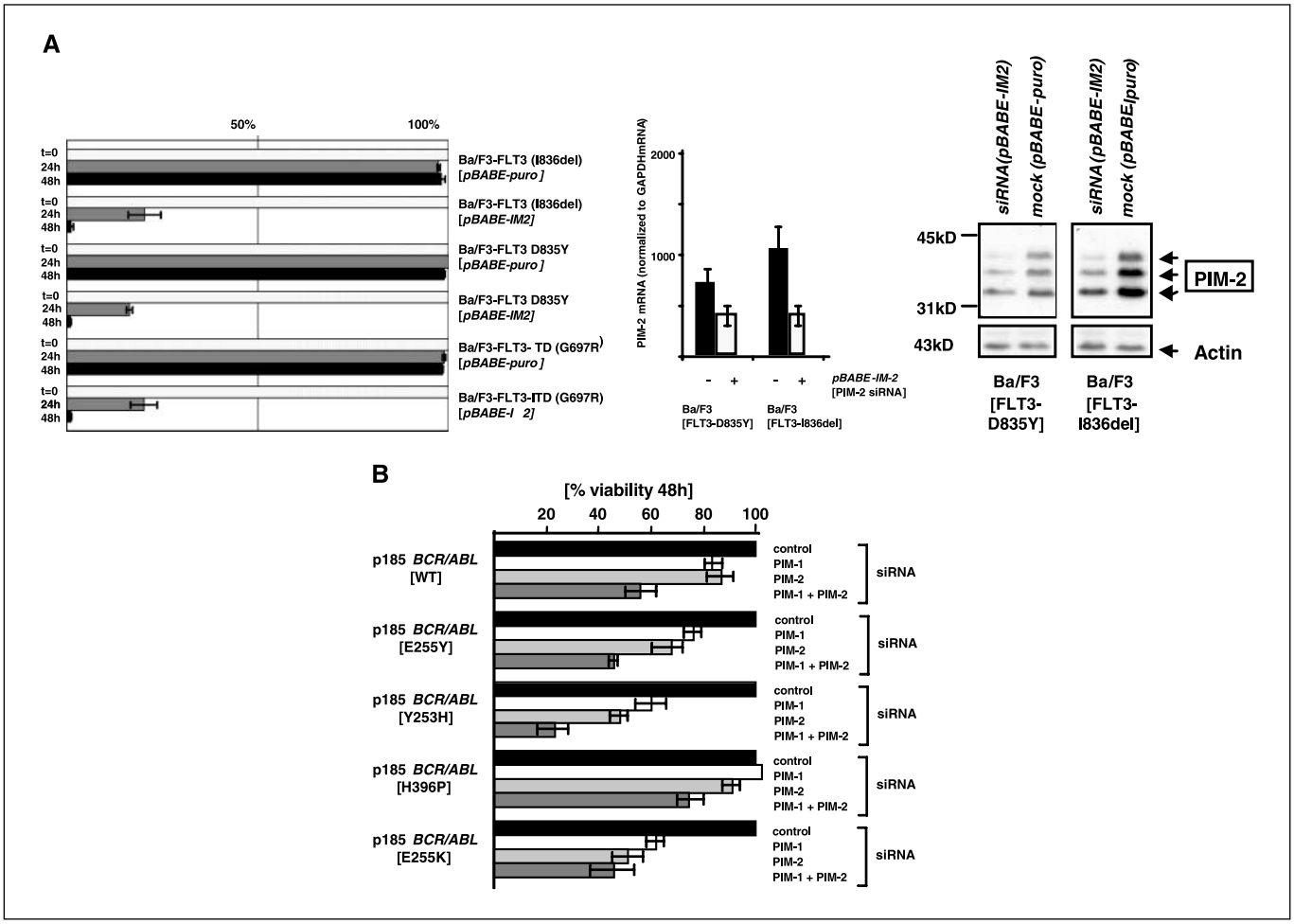


**Figure 5.** Targeting of PIM-2 or PIM-1 and PIM-2 significantly impairs survival of hematopoietic cells transformed by FLT3 or BCR/ABL mutants originating from patients developing clinical resistance against small-molecule tyrosine kinase inhibitors. *A*, Ba/F3 cells transformed to IL-3 independence by various FLT3 activation loop mutations (FLT3-I836del, FLT3-D835Y, and FLT3-ITD-G697R) were infected with a retrovirus encoding siRNA targeting PIM-2 (*pBABE-IM-2*) or the control vector (*pBABE-puro*) followed by puromycin selection by (2.0 μg/mL) 24 hours after infection. Cells ($10^5$) were plated in triplicate and followed over three consecutive days (*left*). Efficacy of siRNA-mediated of endogenous mPIM-2 was again analyzed by real-time PCR (*middle*) and Western blot analysis (*right*). *B,* Ba/F3 cells transformed to IL-3 independence by p185-BCR/ABL (WT) and various mutated variants of p185-BCR-ABL (E255Y, Y253H, H396P, and E255K) were nucleofected with synthetic siRNA targeting PIM-1, PIM-2, or both. Cells ($10^5$) were plated in triplicate and viability was assayed using trypan blue exclusion 24 hours after nucleofection. Viability in % is normalized to cells transfected with a control siRNA. Efficacy of down-regulation of PIM-1, and/or PIM-2 mRNA expression by siRNA was in the range of 50% to 70% as determined by real-time PCR (data not shown).

Y253H, and E255K, whereas cells expressing p185-H396P showed only a limited response rate. These results suggest that targeting PIMs could be of benefit to overcome therapeutic resistance against small-molecule tyrosine kinase inhibitors not only in cells transformed by activating FLT3 mutations but also by BCR/ABL fusion gene variants.

Taken together, our data suggest that inhibition by either blocking PIM-2 kinase activity through a small-molecule inhibitor or through genetic targeting by siRNA could be a new avenue to treat FLT3-mediated leukemia and/or to overcome resistance to small-molecule FLT3 inhibitors. In addition, application of a broad PIM inhibitor targeting PIM-1 and PIM-2 may have a general benefit in the treatment regime against PTK-mediated leukemias resistant to small-molecule tyrosine kinase inhibitors. Although recent determination of the crystal structure of PIM-1 could facilitate the identification of small-molecule inhibitors, it remains to be elucidated whether PIMs may exert any nonenzymatic (e.g., scaffold) function that could support PTK-transformed growth (12, 34). Alternatively, improved delivery technologies like covalent attachment of cholesterol or by protamine-antibody fragment fusions may even allow therapeutic targeting of PIMs by siRNAs (35, 36). Because animals lacking all PIM genes are viable with a mild phenotype, one could expect that this therapeutic approach targeting PIM-1 and PIM-2 could be followed with tolerable side effects (29, 37).

## Acknowledgments

Received 7/11/2005; revised 12/20/2005; accepted 1/27/2006.

**Grant support:** Swiss National Science Foundation grant 3100A0-100758, Swiss Cancer League grant KFS-01077-09-2000, the NOVARTIS Research Foundation, and Gertrude Von Meissner Foundation (J. Schwaller).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Drs. A. Berns (Netherlands Cancer Center, Amsterdam), S. Baker (St. Jude Children's Research Hospital), and P. Rothman (Columbia University, New York) for providing valuable reagents and H. Kohler, M. Berczy, L. Ho, R. Kralovics, and A. Biondi for their input for the presented work.

## References


1. Gilliland DG, Griffin JD. The roles of FLT3 in hematopoiesis and leukemia. Blood 2002;100:1532–42.
2. Kelly LM, Liu Q, Kutok JL, Williams IR, Boulton CL, Gilliland DG. FLT3 internal tandem duplication mutations associated with human acute myeloid leukemias induce myeloproliferative disease in a murine bone marrow transplant model. Blood 2002;99:310–8.
3. Levis M, Small D. Small molecule FLT3 tyrosine kinase inhibitors. Curr Pharm Des 2004;10:1183–93.
4. Kelly LM, Yu JC, Boulton CL, et al. CT53518, a novel selective FLT3 antagonist for the treatment of acute myelogenous leukemia (AML). Cancer Cell 2002;1:421–32.
5. Grundler R, Thiede C, Miething C, Steudel C, Peschel C, Duyster J. Sensitivity toward tyrosine kinase inhibitors varies between different activating mutations of the FLT3 receptor. Blood 2003;102:646–51. Epub 2003 Mar 27.
6. Clark JJ, Cools J, Curley DP, et al. Variable sensitivity of FLT3 activation loop mutations to the small molecule tyrosine kinase inhibitor MLN518. Blood 2004;104: 2867–72. Epub 2004 Jul 15.
7. Cools J, Mentens N, Furet P, et al. Prediction of resistance to small molecule FLT3 inhibitors: implications for molecularly targeted therapy of acute leukemia. Cancer Res 2004;64:6385–9.
8. Cools J, Maertens C, Marynen P. Resistance to tyrosine kinase inhibitors: calling on extra forces. Drug Resist Updat 2005;30:30.
9. Minami Y, Yamamoto K, Kiyoi H, Ueda R, Saito H, Naoe T. Different antiapoptotic pathways between wild-type and mutated FLT3: insights into therapeutic targets in leukemia. Blood 2003;102:2969–75. Epub 2003 Jul 03.
10. Sternberg DW, Gilliland DG. The role of signal transducer and activator of transcription factors in leukemogenesis. J Clin Oncol 2004;22:361–71.
11. Cuypers HT, Selten G, Quint W, et al. Murine leukemia virus-induced T-cell lymphomagenesis: integration of proviruses in a distinct chromosomal region. Cell 1984;37:141–50.
12. Qian KC, Wang L, Hickey ER, et al. Structural basis of constitutive activity and a unique nucleotide binding mode of human Pim-1 kinase. J Biol Chem 2005;280: 6130–7. Epub 2004 Nov 03.
13. Nosaka T, Kitamura T. Pim-1 expression is sufficient to induce cytokine independence in murine hematopoietic cells, but is dispensable for BCR-ABL-mediated transformation. Exp Hematol 2002;30:697–702.
14. Nieborowska-Skorska M, Hoser G, Kossev P, Wasik MA, Skorski T. Complementary functions of the antiapoptotic protein A1 and serine/threonine kinase pim-1 in the BCR/ABL-mediated leukemogenesis. Blood 2002;99:4531–9.
15. Kim KT, Baird K, Ahn JY, et al. Pim-1 is up-regulated by constitutively activated FLT3 and plays a role in FLT3-mediated cell survival. Blood 2005;105:1759–67. Epub 2004 Oct 21.
16. Fox CJ, Hammerman PS, Cinalli RM, Master SR, Chodosh LA, Thompson CB. The serine/threonine kinase Pim-2 is a transcriptionally regulated apoptotic inhibitor. Genes Dev 2003;17:1841–54.
17. Spatz A, Borg C, Feunteun J. X-chromosome genetics and human cancer. Nat Rev Cancer 2004;4:617–29.
18. Cohen AM, Grinblat B, Bessler H, et al. Increased expression of the hPim-2 gene in human chronic lymphocytic leukemia and non-Hodgkin lymphoma. Leuk Lymphoma 2004;45:951–5.
19. Mizuki M, Schwable J, Steur C, et al. Suppression of myeloid transcription factors and induction of STAT response genes by AML-specific Flt3 mutations. Blood 2003;101:3164–73. Epub 2002 Dec 05.
20. Hammerman PS, Fox CJ, Cinalli RM, et al. Lymphocyte transformation by Pim-2 is dependent on nuclear factor-kappaB activation. Cancer Res 2004;64:8341–8.
21. Schwaller J, Frantsve J, Aster J, et al. Transformation of hematopoietic cell lines to growth-factor independence and induction of a fatal myelo- and lymphoproliferative disease in mice by retrovirally transduced TEL/JAK2 fusion genes. EMBO J 1998;17: 5321–33.
22. Schwaller J, Anastasiadou E, Cain D, et al. H4(D10S170), a gene frequently rearranged in papillary thyroid carcinoma, is fused to the platelet-derived growth factor receptor beta gene in atypical chronic myeloid leukemia with t(5;10)(q33;q22). Blood 2001;97: 3910–8.
23. Quentmeier H, Reinhardt J, Zaborski M, Drexler HG. FLT3 mutations in acute myeloid leukemia cell lines. Leukemia 2003;17:120–4.
24. von Bubnoff N, Veach DR, Miller WT, et al. Inhibition of wild-type and mutant Bcr-Abl by pyrido-pyrimidine-type small molecule kinase inhibitors. Cancer Res 2003; 63:6395–404.
25. Chen XP, Losman JA, Cowan S, et al. Pim serine/threonine kinases regulate the stability of Socs-1 protein. Proc Natl Acad Sci U S A 2002;99:2175–80.
26. Mui AL, Wakao H, Kinoshita T, Kitamura T, Miyajima A. Suppression of interleukin-3-induced gene expression by a C-terminal truncated Stat5: role of Stat5 in proliferation. EMBO J 1996;15:2425–33.
27. Schwaller J, Parganas E, Wang D, et al. Stat5 is essential for the myelo- and lymphoproliferative disease induced by TEL/JAK2. Mol Cell 2000;6: 693–704.
28. van der Lugt NM, Domen J, Verhoeven E, et al. Proviral tagging in E mu-myc transgenic mice lacking the Pim-1 proto-oncogene leads to compensatory activation of Pim-2. EMBO J 1995;14:2536–44.
29. Mikkers H, Nawijn M, Allen J, et al. Mice deficient for all PIM kinases display reduced body size and impaired responses to hematopoietic growth factors. Mol Cell Biol 2004;24:6104–15.
30. Nardi V, Azam M, Daley GQ. Mechanisms and implications of imatinib resistance mutations in BCR-ABL. Curr Opin Hematol 2004;11:35–43.
31. Bagrintseva K, Geisenhof S, Kern R, et al. FLT3-ITD-TKD dual mutants associated with acute myeloid leukemia (AML) confer resistance to FLT3 PTK inhibitors and cytotoxic agents by overexpression of Bcl-x(L). Blood 2004;30:30.
32. Hammerman PS, Fox CJ, Birnbaum MJ, Thompson CB. Pim and Akt oncogenes are independent regulators of hematopoietic cell growth and survival. Blood 2005; 105:4477–83. Epub 2005 Feb 10.
33. von Bubnoff N, Schneller F, Peschel C, Duyster J. BCR-ABL gene mutations in relation to clinical resistance of Philadelphia-chromosome-positive leukaemia to STI571: a prospective study. Lancet 2002;359: 487–91.
34. Jacobs MD, Black J, Futer O, et al. Pim-1 ligand-bound structures reveal the mechanism of serine/threonine kinase inhibition by LY294002. J Biol Chem 2005;280:13728–34. Epub 2005 Jan 17.
35. Soutschek J, Akinc A, Bramlage B, et al. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature 2004;432: 173–8.
36. Song E, Zhu P, Lee SK, et al. Antibody mediated *in vivo* delivery of small interfering RNAs via cell-surface receptors. Nat Biotechnol 2005;23:709–17. Epub 2005 May 22.
37. Amaravadi R, Thompson CB. The survival kinases Akt and Pim as potential pharmacological targets. J Clin Invest 2005;115:2618–24.