A Kinase Inhibitor with Anti-Pim Kinase Activity is a Potent and Selective Cytotoxic Agent Toward Acute Myeloid Leukemia

Ronja Bjørnstad1,2, Reidun Aesoy1, Øystein Bruserud3, Annette K. Brenner3, Francis Giraud4, Tara Helen Dowling5, Gro Gausdal6, Pascale Moreau4, Stein Ove Døskeland7, Fabrice Anizon4, and Lars Herfindal1

Abstract

More than 40 years ago, the present standard induction therapy for acute myeloid leukemia (AML) was developed. This consists of the metabolic inhibitor cytarabine (AraC) and the cytostatic topoisomerase 2 inhibitor daunorubicin (DNR). In light of the high chance for relapse, as well as the large heterogeneity, novel therapies are needed to improve patient outcome. We have tested the anti-AML activity of 15 novel compounds based on the scaffolds pyrrolo[2,3-c]carbazole-3-carbaldehyde, pyrazolo[3,4-c]carbazole, pyrazolo[4,3-a]phenanthridine, or pyrrolo[2,3-g]indazole. The compounds were inhibitors of Pim kinases, but could also have inhibitory activity against other protein kinases. Ser/Thr kinases like the Pim kinases have been identified as potential drug targets for AML therapy. The compound VS-II-173 induced AML cell death with EC50 below 5 μmol/L, and was 10 times less potent against nonmalignant cells. It perturbed Pim-kinase–mediated AML cell signaling, such as attenuation of Stat5 or MDM2 phosphorylation, and synergized with DNR to induce AML cell death. VS-II-173 induced cell death also in patients with AML blasts, including blast carrying high-risk FLT3-ITD mutations. Mutation of nucleophosmin-1 was associated with good response to VS-II-173. In conclusion new scaffolds for potential AML drugs have been explored. The selective activity toward patient AML blasts and AML cell lines of the pyrazolo-analogue VS-II-173 make it a promising drug candidate to be further tested in preclinical animal models for AML.

Introduction

Acute myeloid leukemia (AML) is a highly heterogeneous disease characterized by aberrant myeloid differentiation and subsequent accumulation of immature myeloid cells in the bone marrow (BM). The standard chemotherapy regimen is based on the metabolic inhibitor cytarabine (AraC) and the cytostatic topoisomerase 2 inhibitor daunorubicin (DNR), and has changed little over the past 40 years (1). Intense research efforts aim to identify drug targets that can help overcome AML chemotherapy resistance and relapse, as well as the severe toxic side effects of DNR (see ref. 2, for a recent review on potential drug chemotherapy resistance and relapse, as well as the severe toxic side effects of DNR (see ref. 2, for a recent review on potential drug targets). One of the important regulators of AML cell survival are Pim1, 2, and 3 (3). They are involved in the regulation of multiple cellular processes, including cell-cycle progression and cell survival. The Pim kinases mediate signals from Abelson murine leukemia viral oncogene (ABL), Janus kinase 2 (JAK2), and fms like tyrosine kinase 3 (FLT3; refs. 4, 5), all oncogenes. Pim kinases lack regulatory domains, and are constitutively active (6). Their activity is regulated by altering the intracellular levels, that is by transcription and translation, or by degradation (7). Together this makes them a likely Achilles heel for AML cells dependent on a reliable communication of cytokine signals to the nucleus in order to survive and multiply. Mice depleted of all three Pim kinase proteins have a normal life span and are fertile, only exhibiting a slight deficient growth response with a reduced body size (8). Consequently, no severe side effects from inhibition of selective Pim kinases are expected. Several Pim kinase inhibitors have been evaluated for therapeutic potential against hematologic disorders, in particular multiple myeloma and AML, but also CML, ALL, and B-cell lymphoma (9, 10). Previously, we have reported the synthesis of a series of Pim kinase inhibitors exhibiting various N-containing heteroaromatic scaffolds such as pyrrolo[2,3-c]carbazole-3-carbaldehydes, pyrazolo[3,4-c]carbazoles, pyrazolo[4,3-a]phenanthridines, and pyrrolo[2,3-g]indazoles (Fig. 1A; refs. 11–18). A broad selection of molecular scaffolds is important in order to develop the best therapeutic agents against a specific target. All the above-mentioned compound classes showed acceptable IC50 values toward Pim kinases (below 10 nmol/L in some cases, see Table 1). The ATP-binding pocket, along with the substrate-recognition residues are identified as crucial for Pim kinase inhibitors (19).
These compounds were reported to have activity toward AML cells. We wanted to extend our investigation of these compounds as potential drugs for AML, and investigate the possibilities for one or two selected compounds to enter further preclinical investigations.

Materials and Methods

Description of the kinase inhibitors

All compounds described in Fig. 1A are designed to bind in the ATP-recognition site of Pim kinases. Their synthesis has been described elsewhere (11–18). Compound B3 exhibited a selective profile when evaluated toward a panel of 66 protein kinases (11). X-ray co-crystal structure with Pim1 (PDB code: 3JPV) demonstrated that it inserted into the ATP-binding pocket in a manner typical for non-ATP mimetic Pim inhibitors (Fig. 1B; ref. 11). A similar binding mode was observed by molecular modeling for analogues of this series. Compound LG-VII-126 is part of another Pim inhibitor series (Fig. 1A). Molecular modeling showed that the indazole ring inserted into the ATP-binding pocket with high-affinity interactions in Pim1 and Pim3 (16). The synthesis of a homolog series identified VS-II-173 as a nanomolar Pim1 and Pim3 inhibitor (17). The study of its putative binding with Pim1/Pim3 by molecular modeling demonstrated that VS-II-173 inserted deeply into the ATP site of Pim1 and Pim3 (17).

The in vitro protein kinase screening was performed at the International Centre for Kinase Profiling (ICKP; Dundee, Scotland). VS-II-173 was evaluated toward a panel of 50 protein kinases in duplicate assays at a compound concentration of...
1 μmol/L as described previously (20–22). The results were expressed as the percentage of residual kinase activity. The protein kinases were of human origin except Lck (mouse) and ROCK2 (rat).

### Cell culture conditions

The following AML cell lines were utilized in this study: the human acute monocytic leukemia cells Molm-13 (ACC, 554; ref. 23), Molm-13 cells with shRNA mediated silencing of p53 (Molm-13 shp53; ref. 24), MV4-11 human monocytic leukemia cells (ATCC, CRL-9591), MV4-11 cells with silenced p53 (MV4-11 shp53), the rat promyeloid leukemia wild-type cell lines IPC-81 (25), IPC-81 with enforced expression of Bcl-2 (both created by Dr. Michel Lanotte, Hôpital St. Louis, Paris, France, and maintained by prof. Stein Ove Desseland; ref. 26), and the human AML cell line OCI-AML3 (ATCC, ACC-582). The Molm-13 and MV4-11 cells with silenced p53 were created by Prof. Stein Ove Desseland, Drs. Sjur Huseby, and Gro Gausdal by retroviral transfection for stable expression of shRNA against p53 using the preTRO SUPER-p53 vector (27). Selection was by increasing puromycin concentration up to 400 μg/mL as described previously (20–22). The results were expressed as the percentage of residual kinase activity. The protein kinases were of human origin except Lck (mouse) and ROCK2 (rat).

### Assessment of cell death

Cytotoxicity assays were performed in 96-well plates at 0.1 mL medium/well. Suspension cells were seeded at 0.5 × 10^4 cells/mL and used the day of seeding. The NRK and H9c2 cells were seeded at 0.05 × 10^6 cells/mL, and left over night to attach before experiments. The compounds were tested alone, or in combinations with the following cytostatics: DNR (Cerubidine; Sanofi Aventis Lysaker), Geldanamycin, Emetine (both purchased from Sigma-Aldrich), bortezomib (Velcade; Janssen), Etoposide (Eposin, Teva), Cisplatin (Accord Healthcare), and AraC (Cytarabin, Fresenius Kabi). The pan-Pim kinase inhibitor AZD1208 was from Tocris Bioscience, the Bcl-2 inhibitor Venetoclax / ABT-199 from Cayman Chemical, and the tyrosine-kinase inhibitor Midostaurin from Sigma-Aldrich. The viability of the treated cells was assessed using a WST-1 Cell Proliferation Assay (Roche Applied Sciences). After recording metabolic activity, the cells were fixed by adding 2% buffered (PBS, pH 7.4) formaldehyde containing 0.1% of the DNA-specific Hoechst 33342 (0.01 mg/mL; Sigma–Aldrich) to visualize the nuclei. Cell death was assessed by UV-microscopic evaluation of nuclear morphology (28), and the data from the metabolic activity were used to confirm the data from microscopy. EC_{50} values were estimated by nonlinear regression using the statistical software IBM SPSS statistics for Apple, ver. 24.

$$Y = \min + \frac{\max - \min}{1 + \left(\frac{X}{X_h}\right)^h}$$  \hspace{1cm} (1)

where Y is the response (cell death); max and min, the maximum and minimum cell death from the curve; X the concentration of the drug/compound; and h is the hill index. Determination of coefficient of drug interaction (CDI) was done by finding the ratio between the effect of treatment and control (R) and further calculates as shown in Eq. 2.

$$\text{CDI} = \frac{R_{\text{combination}}}{R_{\text{Drug1}} \times R_{\text{Drug2}}}$$ \hspace{1cm} (2)

A value around one indicates additive effect, values below 0.7 demonstrate clear synergy, and higher than one antagonistic effect.

### Membrane permeability assay

The Corning Gentest pre-Coated parallel artificial membrane permeability assay PAMPA Plate System (Corning Discovery Labware) was done according to manufacturer’s protocol. The compounds were tested at 50 μmol/L in PBS at 21°C. After incubation for 5 hours, 50 μL of each sample were added 15 μL acetonitrile, and injected into a reversed phase HPLC column (Kromasil 100-5 C18 150×4.6 mm; Akzo Nobel) connected to a Merck-Hitachi LaChrom HPLC-system (VWR) with a L-7455 diode array detector. Mobile phase A was 0.05% aqueous TFA, and mobile phase B was acetonitrile. The flow rate was 1.6 mL/min and compounds eluted between 1 and 4.5 minutes during a 4.5 minutes gradient from 70:30% mobile phase A:B to 0:100% mobile phase A:B. The ratios of the compound concentrations in the donor and acceptor compartments were calculated after integration of the peaks at 248 nm. P_{eq} was calculated as described in ref. 29.

---

**Table 1. Ability of the novel compounds to inhibit activity of Pim-1, 2, and 3 kinases**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC_{50} (μmol/L) Pim1</th>
<th>IC_{50} (μmol/L) Pim2</th>
<th>IC_{50} (μmol/L) Pim3</th>
</tr>
</thead>
<tbody>
<tr>
<td>B3 (11)</td>
<td>0.12</td>
<td>0.51</td>
<td>0.01</td>
</tr>
<tr>
<td>RAG-II-45 (11)</td>
<td>0.0068</td>
<td>0.131</td>
<td>0.024</td>
</tr>
<tr>
<td>RAG54 (12)</td>
<td>0.57</td>
<td>&gt;30</td>
<td>0.04</td>
</tr>
<tr>
<td>RAG-II-115 (13)</td>
<td>0.075</td>
<td>&gt;1</td>
<td>0.08</td>
</tr>
<tr>
<td>FGS87 (14)</td>
<td>0.009</td>
<td>&gt;1</td>
<td>0.026</td>
</tr>
<tr>
<td>MB032 (14)</td>
<td>0.042</td>
<td>nd</td>
<td>0.05</td>
</tr>
<tr>
<td>MB071 (15)</td>
<td>1.4</td>
<td>nd</td>
<td>0.38</td>
</tr>
<tr>
<td>MB069 (15)</td>
<td>1.56</td>
<td>nd</td>
<td>0.6</td>
</tr>
<tr>
<td>FG644B (15)</td>
<td>0.39</td>
<td>nd</td>
<td>0.19</td>
</tr>
<tr>
<td>EE057 (15)</td>
<td>1.1</td>
<td>nd</td>
<td>0.7</td>
</tr>
<tr>
<td>FG610 (15)</td>
<td>0.74</td>
<td>nd</td>
<td>0.34</td>
</tr>
<tr>
<td>FG773 (15)</td>
<td>&gt;1</td>
<td>nd</td>
<td>2.9</td>
</tr>
<tr>
<td>LG-VI-126 (16)</td>
<td>0.04</td>
<td>49% (1 μmol/L)</td>
<td>0.10</td>
</tr>
<tr>
<td>LS-II-173 (17)</td>
<td>0.07</td>
<td>46% (1 μmol/L)</td>
<td>0.02</td>
</tr>
<tr>
<td>LG-VI-142 (18)</td>
<td>0.46</td>
<td>&gt;30</td>
<td>0.033</td>
</tr>
</tbody>
</table>

nd, not determined.

*The numbers in parentheses refer to the citation in the reference list.

b% residual kinase activity at 1 μmol/L.
Western blot analysis

Western blotting was conducted as described previously (30). To ensure that we studied effects of kinase inhibition, we chose concentrations that gave less than 30% apoptosis. This would avoid loss of proteins due to caspase cleavage or disintegrated cells. After treatment with Pim kinase inhibitors, the cells were washed and lysed, and protein concentrations determined using the Quick Start Bradford protein assay (Bio-Rad Laboratories). SDS-polyacrylamide gels, 10% or 12% were loaded with 30 concentrations that gave less than 30% apoptosis. This would avoid loss of proteins due to caspase cleavage or disintegrated cells. After treatment with Pim kinase inhibitors, the cells were washed and lysed, and protein concentrations determined using the Quick Start Bradford protein assay (Bio-Rad Laboratories). SDS-polyacrylamide gels, 10% or 12% were loaded with 30 to 50 µg protein, and the prelabeled “Precision Plus Protein Standard All Blue” (Bio-Rad Laboratories). Proteins were transferred to polyvinylidene difluoride membranes (GE Healthcare Life Sciences) by electrophoresis. The following primary antibodies were used: Pim1, Pim2, and Pim3 (all from Santa Cruz Biotechnology, except for on OCI-AML3, where Pim2 antibody from Sigma-Aldrich was used). Bad, pBad phospho S112, 4EBP1, p4EBP1 phospho T37/46, STAT5 and pSTAT5 phospho Y694, Akt and Akt phospho S473, p70 S6 kinase and p70 S6 kinase phospho S371 (from Cell Signaling Technology), MDM2 and pMDM2 phospho S166 antibody (Abcam plc), anti-β-actin (Sigma-Aldrich). The secondary antibodies were donkey anti-mouse and anti-rabbit antibodies conjugated to horse-radish peroxidase (Jackson Immunoresearch Laboratory). Membranes were developed using Supersignal West Pico or West Femto Chemiluminescence Substrate from Pierce Biotechnology Inc., according to the manufacturers’ protocols. The immunoblots were imaged by a LAS-4000 image reader (Fujifilm). After imaging, the membranes were stripped using Restore PLUS Western Blot Stripping Buffer (ThermoFisher Scientific) to reprobe the membranes with the antibody for the phosphorylated version of the proteins. Anti-β-actin was used for loading control.

Flow cytometric analysis of AML patient material

The collection of patient cells was approved by the regional research ethics committee (Health Region III, Bergen, Norway, REK approval number: 2012/2247). Peripheral blood mononuclear cells (PBMC) were obtained from freshly drawn peripheral blood samples collected with approval from the patients and stored under liquid nitrogen in biobanks at the Norwegian Directorate for Health and Social Affairs. Blasts from 37 patients were used in this study. The patients were not selected from the donor pool, but low membrane permeability. There were also compounds with high membrane permeability, like RAG-II-45, FG587, RAG54, and MB071. However, VS-II-173 concentrations inducing 100% death in the AML cells. The compounds MB032, LG-VI-142, LG-VII-126, RAG54, and FG587 also showed selectivity toward AML cell lines, but were less potent than VS-II-173. The compound RAG-II-115 was a potent inducer of cell death, but did not discriminate between AML-derived and other cell lines (Fig. 2A). VS-II-173 was able to overcome the pro-survival effect of poor prognostic factors in AML such as FLT3-ITD expression (MV4-11 and Molm-13 cells), p53 silencing (Molm-13 shp53 and MV4-11 shp53) and, to some extent, Bcl-2 overexpression in IPC-81 cells (Fig. 2A). To find if the differences in efficacy was due to the compound’s ability to penetrate the cellular membrane, we tested the membrane permeability using the PAMPA assay. Membrane permeability was classified by the range defined by Bennion and colleagues (29), which divides compounds into four groups: low permeability (LogP<sub>eff</sub> < 5.33), intermediate permeability (LogP<sub>eff</sub> > 5.66 to < 5.33), low permeability (LogP<sub>eff</sub> > 6.14 to < 5.66) impermeable (LogP<sub>eff</sub> < 6.14). The compounds RAG-II-115 and MB032 showed intermediate to high efficacy toward cells (Fig. 2A) and intermediate to high membrane permeability (Fig. 2B), whereas compound VS-II-173 had high efficacy toward cells, but low membrane permeability. There were also compounds with low efficacy toward cells, and low membrane permeability, like RAG-II-45, FG587, RAG54, and MB071. However, the compounds MB069, FG644B, EE057, and FG610 all showed high membrane permeability (Fig. 2B), but low ability to induce cell death (Fig. 2A). We also checked whether there were large differences between microscopic assessment of nuclear morphology and metabolic conversion of the WST-1 reagent (Fig. 2C and D). The WST-1 assay gave a lower EC<sub>50</sub> value compared with microscopic evaluation, presumably due to its ability to detect cell growth inhibition. Still there was a good correlation between the

**Results**

**Characterization of anti-AML activity of kinase inhibitors**

We previously found that pyrrolo[2,3-a]carbazole-3-carboxylic acid kinase inhibitors (15) had promising activity against AML cells. We therefore decided to test the efficacy of an in-house selection of Pim inhibitors containing different hetero-aromatic scaffolds (Fig. 1A) against a panel of cell lines (Fig. 2A). The inhibitor VS-II-173 was found to be a potent and selective inducer of AML cell death. The cardiomyoblast-like epithelial cell lines, and the chronic myeloid leukemia cell line K562 were unaffected at VS-II-173 concentrations inducing 100% death in the AML cells. The compounds MB032, LG-VI-142, LG-VII-126, RAG54, and FG587 also showed selectivity toward AML cell lines, but were less potent than VS-II-173. The compound RAG-II-115 was a potent inducer of cell death, but did not discriminate between AML-derived and other cell lines (Fig. 2A). VS-II-173 was able to overcome the pro-survival effect of poor prognostic factors in AML such as FLT3-ITD expression (MV4-11 and Molm-13 cells), p53 silencing (Molm-13 shp53 and MV4-11 shp53) and, to some extent, Bcl-2 overexpression in IPC-81 cells (Fig. 2A). To find if the differences in efficacy was due to the compound’s ability to penetrate the cellular membrane, we tested the membrane permeability using the PAMPA assay. Membrane permeability was classified by the range defined by Bennion and colleagues (29), which divides compounds into four groups: low permeability (LogP<sub>eff</sub> < 5.33), intermediate permeability (LogP<sub>eff</sub> > 5.66 to < 5.33), low permeability (LogP<sub>eff</sub> > 6.14 to < 5.66) impermeable (LogP<sub>eff</sub> < 6.14). The compounds RAG-II-115 and MB032 showed intermediate to high efficacy toward cells (Fig. 2A) and intermediate to high membrane permeability (Fig. 2B), whereas compound VS-II-173 had high efficacy toward cells, but low membrane permeability. There were also compounds with low efficacy toward cells, and low membrane permeability, like RAG-II-45, FG587, RAG54, and MB071. However, the compounds MB069, FG644B, EE057, and FG610 all showed high membrane permeability (Fig. 2B), but low ability to induce cell death (Fig. 2A). We also checked whether there were large differences between microscopic assessment of nuclear morphology and metabolic conversion of the WST-1 reagent (Fig. 2C and D). The WST-1 assay gave a lower EC<sub>50</sub> value compared with microscopic evaluation, presumably due to its ability to detect cell growth inhibition. Still there was a good correlation between the

**Statistical analysis**

Response to treatments were evaluated by Student t test, correlation of data by two-tailed Pearson correlation test, and patient data by cross-table analyses for chi-square, all by using IBM SPSS statistics for Apple, ver. 24 (IBM Corp.). Significance was defined as P < 0.05. Heat maps were created by MeV (31). Analyses of Pim expression was done using the J-Express 2012 software (32) after normalization and log(10) transformation of data. Kinome tree was created using the Kinome Render software (33).

**Flow cytometric analysis of cells stained with Pacific-Blue-AnnexinV (#640918; BioLegend) and propidium iodide (PI; #421301; BioLegend). Between 10,000 and 30,000 events were collected for each sample using a BD Fortessa flow cytometer. The data were analyzed with the software Flow Jo (Tree Star, Inc.).**

**Mol Cancer Ther; 18(3) March 2019**
Figure 2.
Ability of different compounds to induce cell death in AML cell lines. A, The cells were incubated for 24 hours with the compounds (30 μmol/L) before assessment of metabolic activity using the WST-1 proliferation assay and then fixed in 2% buffered formaldehyde containing the DNA stain Hoechst 33342. Apoptotic cells were identified and quantified by microscopic evaluation of cell nuclei. B, Membrane permeability (LogP_eff) of the different compounds, measured by the Corning Gentest precoated parallel artificial membrane permeability assay PAMPA Plate System. C and D, Dose–response curves of Molm-13 cells with VS-II-173 over 24 hours. The same experiments were assessed by both microscopy (C) and metabolic activity by WST-1 assay (D) and EC50 values determined by nonlinear regression. The inset in C shows surface and nuclear morphology of Molm-13 cells after 24 hours of treatment with either solvent (upper images) or 10 μmol/L VS-II-173. The bars indicate 9 μm. The inset in D shows correlation between the WST-1 signal and apoptotic morphology, using Pearson Correlation statistics (two tailed, P > 0.05). E, Molm-13 cell death kinetics of different kinase inhibitors. The cells were incubated with the different compounds, and aliquots sampled and fixed at the given time-points and apoptotic cells quantified by microscopic evaluation of nuclear morphology. The data in A–E are from three to five separate experiments and SD (B–D). F, Kinase screen of the residual activity of different kinases after treatment with 1 μmol/L of VS-II-173. The size of the circles is inversely proportional to the residual activity of the kinases. The tree was generated using the Kinome Render software, and the illustration reproduced courtesy of Cell Signaling Technology (www.cellsignal.com). A table with the values from F is available as supplementary information (Supplementary Table S1).
but were nontoxic to the OCI-AML3 cells at the concentrations tested. A total of 50 μmol/L of the pan-Pim kinase inhibitor AZD1208 induced around 60% cell death in MV4-11 cells after 24 hours incubation, whereas the other cell lines were mostly unaffected (Fig. 3H). The Bcl-2 inhibitor venetoclax was effective toward Molm-13 and MV4-11 in the low nanomolar range, but were nontoxic to the OCI-AML3 cells at the concentrations tested (Fig. 3I). Finally, the AML drug midostaurin was tested. This multitarget kinase inhibitor was efficient toward Molm-13 and MV4-11 cells, but to a small degree toward OCI-AML3 (Fig. 3J).

VS-II-173 attenuates phosphorylation of Pim kinase substrates

Pim kinases have been shown to act downstream of FLT3 signaling (5), and we compared the efficacy of VS-II-173 on AML cell lines with different FLT3 mutation status. The VS-II-173 had higher efficacy toward cell lines harboring the FLT3-ITD mutation (Molm-13, FLT3-ITD homozygous, and MV4-11 FLT3-ITD homozygous), with EC_{50} values between 2 and 3 μmol/L (Fig. 4A and B, respectively). The OCI-AML3 cells (FLT3 WT) were less sensitive to VS-II-173, with EC_{50} about 16 μmol/L (Fig. 4C).

To study early events (the initiation phase) of the apoptosis process triggered by VS-II-173, we used concentrations that would produce apoptosis after 5 to 10 hours, in line with previous studies on for instance anthracycline and AML (34, 35). When examining the expression of Pim1, 2, and 3 in the AML cell lines treated with VS-II-173, we noted a rapid, but transient downregulation of all three kinases in Molm13 cells (Fig. 4D, see Supplementary Fig. S1 for quantification). The response was less profound in MV4-11 and OCI-AML3 cells (Fig. 4E and F). Furthermore, the Molm13 and MV4-11 cells showed a rapid dephosphorylation of Stat5 (Fig. 4G and H) upon treatment with VS-II-173. The MV4-11 cells showed a decrease of the prosurvival phospho-Bad form of the otherwise apoptogenic BH3 only protein Bad (Fig. 4H) and the OCI-AML3 cells showed a strong decrease in p-4E-BP1 and pMDM2 (Fig. 4I).

Blasts from patients with AML with poor prognostic markers are sensitive to the kinase inhibitor VS-II-173

To better assess the potential of VS-II-173 as a lead compound for the treatment of AML, it was tested on a panel of nonselected AML patient blasts. The blasts were treated with VS-II-173 alone, or in combination with DNR. Our experience with AML patient blasts is that they are generally more resistant to cytostatics compared with most AML cell lines, and in order to ensure an effect, we increased the doses of both VS-II-173 and DNR. The age of the patients were from 19 to 86 years, with a median of 69 years. They were classified using the French–American–British (FAB) classification (36), and cytogenetics analyzed based on the MRC classification (37). Eleven patients had FLT3-ITD (high-risk characteristic), 21 had WT FLT3, and 3 were not tested. Ten patients had insertion mutation of NPM-1 (favorable prognostic factor), and 22 had wild-type NPM-1 (three not tested). See Fig. 5A for further patient information.

The results from the AML patient blast experiments showed one large group that responded well to treatment with VS-II-173, where doses between 6 and 24 μmol/L could kill more than 50% of the blasts (Responders, Fig. 5). Some of the VS-II-173 responding patient blasts also responded well to the DNR (patients 11 and 24). In some cases, even the highest concentration of DNR (100 nmol/L) did not affect the blast viability whereas VS-II-173 showed high potency against these patient blasts (patients 1 and 2). Another group responded to DNR, but not to VS-II-173, for instance patient 24 and to some extent patient 25. There were two groups that did not appear to benefit from VS-II-173. One, which appeared to be resistant to both DNR and VS-II-173, like patients 36 and 37, and one where the combination gave a reduced effect (patients 22 and 23). Despite
New Pim Kinase Inhibitor for AML Therapy

Figure 3.
Drug interactions between VS-II-173 and seven anticancer compounds. A, Molm-13 cells were treated with VS-II-173 (1 μmol/L), MB032 (100 μmol/L) in combination with DNR. After 24 hours, the cells were fixed and viability was assessed by microscopic evaluation of apoptosis using Hoechst 33342 DNA stain. The vertical dotted lines indicate EC50 values. B, Molm-13 cells were treated with 1 μmol/L VS-II-173 in combination with different anticancer compounds for 24 hours before assessing viability as in A. The CDI was calculated as described in the section Materials and Methods. The concentrations of the compounds tested were: Geldanamycin, 10 nmol/L; Etoposide, 0.6 μmol/L; Emetine, 5 mmol/L; DNR, 75 mmol/L; Cisplatin, 6 μmol/L; AraC, 6 μmol/L; Bortezomib, 10 and 5 nmol/L. In B, *** indicates P < 0.001, one-sample T test. a: For bortezomib, the CDI for the high concentration is shown. C–E, Examples of drugs which act synergistically (C, DNR), antagonistically (D, bortezomib), or additively (E, Etoposide). F and G, Synergistic activity between VS-II-173 and DNR in WT Molm-13 AML cells (left) or silenced p53 (shp53, right). The cells were incubated with increasing doses of DNR with or without 2 or 4 μmol/L VS-II-173 for 24 hours. The insets show induction of p53 expression in WT-cells (F), but not in shp53 cells (G). Viability was assessed as in A. The data in A–G are the mean and the SD from 5 to 12 experiments. H–J, Cell death inducing effect of venetoclax (H), midostaurin (I), and AZD1208 (J) on different cell lines after 24 hours incubation. Cell death was scored using the WST-1 assay and is relative to untreated cells. The data are average of two (MolM13, Oci-AML3, and MV4-11) or four (NRK) experiments and the lines are from four-parameter regression analysis as described in the section Materials and Methods.

this, the majority of the patients (24 out of 37) could be characterized as responders to VS-II-173.

Neither sex, age, nor the FAB classification seemed to influence the responsiveness of the patient blasts to VS-II-173 (Fig 5A). The three patients with adverse cytogenetics (patients 20, 30, and 31) showed poor to intermediate response to VS-II-173, whereas the two patients with good risk cytogenetics showed high response. Patients with FLT3-ITD were represented in the responder group (8 of 20, 40%) of the patients with known status, and in the intermediate/non responder group (3 of 13, 23%), but there was no significant difference between the occurrence of FLT3-ITD in the responder and nonresponder groups (Pearson chi-square = 1.55, P = 0.21). Furthermore, the patients with mutated NPM1 were overrepresented in the responder group (9 of 19 patients (47%) in the responder group, and 1 of 14 patients (7%) in the intermediate/non responder group, Pearson chi-square = 6.175, P = 0.013). Three-way chi-square test of our data showed NPM1 status was important for response to VS-II-173 in patients with WT FLT3 (Pearson chi-square = 4.89, P = 0.27), but not in patients with FLT3-ITD (Pearson chi-square = 0.749, P = 0.387).

Compared with the responder group, BM aspirates or PBMC were less affected by VS-II-173 (Fig. 5A), but the AML blasts from
two patients classified as nonresponders had equal or slightly lower response compared with the PBMC or BM cells. We found no correlation between response to VS-II-173 and Pim kinase expression level (Fig. 5B), based on mRNA expression of Pim kinases related to the median expression in patients (no significance using Pearson Chi-Square statistics).

**Discussion**

Of the 15 compounds we tested, VS-II-173 exhibited potent selective activity toward the AML cell lines. This could be explained by the compound selectively targeting rapidly growing cell lines, but the K562 CML cell line has a doubling time similar to that of Molm-13 cells, thus rapid proliferation does not seem to be the reason for the AML-selectivity observed in Fig. 2A. Importantly, VS-II-173 was active toward cells with high-risk characteristics like decreased expression of p53 (Molm-13 shp53 and MV4-11 shp53; Figs. 2A and 3G), and cells overexpressing the survival protein Bcl-2 (IPC-81-Bcl2; Fig. 2A) as well as FLT3-ITD (Figs. 2A, 4A–C, and 5). We could not detect any correlation between the ability to inhibit the different Pim kinases and the ability to selectively induce AML cell death (Table 1; Fig. 2A).
instance, LG-VII-126 and VS-II-173 had similar inhibitory activity toward the three kinases, but VS-II-173 was a far more potent AML cell death inducer than LG-VII-126. This could partly be explained by differences in the ability to cross phospholipid membranes (Fig. 2B). The kinase screen showed that VS-II-173 also inhibited other kinases than the three Pim kinases (Fig. 2F). The level of promiscuity was similar to that shown for the pan-Pim kinase inhibitor SGI-1776 (38), which is active against AML cells (39).

Many chemotherapeutic therapy regimens for AML are based on drug combinations, such as the 7+3 regimen with DNR and AraC or high-dose AraC plus a nucleoside analogue (see refs. 40, 41 for recent reviews on established and emerging therapies for AML). It became apparent that the most potent compound selective for AML cells, VS-II-173 increased the cellular response to DNR with high synergy (Fig. 3A–C). Combination with cisplatin and VS-II-173 gave more modest synergy (Fig. 3B).

Thus, combinations of VS-II-173 with DNR or possibly cisplatin could be clinically relevant. This agrees with a previous study by Doshi and colleagues showing that Pim kinase inhibition sensitized AML cell lines to DNR (42). Furthermore, our results indicate that Ara-C and VS-II-173 have sub-additive action, suggesting they should not be combined simultaneously. Other Pim kinase inhibitors have acted both in a synergistic and antagonistic manner in combination with AraC in AML cell lines and primary AML cells (42, 43).

The transiently reduced level of Pim1, 2, and 3 kinases in the Molm-13 cells in response to VS-II-173 (Fig. 4D) indicates that inhibition of Pim kinases increases degradation or attenuates production of the protein. Pim kinase activity is not regulated by post-translational modifications or allosteric modulators, but by increased or attenuated expression (7). Treatment with VS-II-173 apparently perturbed the ratio between synthesis and degradation of the Pim kinases, but not irreversibly, suggesting that this was not due to cell-death associated protein degradation. The

Figure 5. VS-II-173 induces cell death in blasts from patients with AML. A, Patient blasts and BM cells were suspended at 2 × 10^6 cells/mL in StemSpan culture medium, whereas PBMC were suspended in MEME medium, and plated at 100,000 cells/well in 96-well plates before adding VS-II-173 at 6, 12, and 24 μmol/L alone or in combination with DNR, for 24 hours. Apoptosis was assessed by flow cytometric analysis of cells stained with PacificBlue-AnnexinV and PI. Between 10,000 and 30,000 events were collected for each sample on a BD Fortessa flow cytometer. B, Median levels of Pim1 to 3 mRNA in selected patients compared with their response to VS-II-173. Abbreviations: F, female; M, male; N, normal; I, intermediate; A, adverse; ITD, FLT3 internal tandem duplications; INS, insertion mutation in NPM-I; nt, not tested.
compensation mechanisms and subsequent restoration of the Pim kinase levels appears presumably too late to initiate the necessary survival mechanisms, since the cells died after eight to twelve hours (Fig. 2E).

Cells with the FLT3-ITD mutation mediate aberrant signaling through the Pim kinases (4). Thus, inhibition of Pim represents an attractive approach to target FLT3-ITD cells. Pim kinases promote survival of AML cells via phosphorylation of Bcl-2 antagonist of cell death (Bad), and also 4E-BP1 (4, 39), in accordance with reduced phosphorylation of these proteins seen upon VS-II-173 treatment, and by previous findings by Chen and colleagues (39). It has been demonstrated that Pim1 kinase directly affects the FLT3 receptor by a stabilizing serine phosphorylation (44). This positive feedback loop prompts Stat5 activation and Pim signaling (44). We found abolished phosphorylation of Stat5 in FLT3-ITD cell lines Molm-13 and MV4-11 (Fig. 4G and H). We did not detect Y694 phosphorylated Stat5 in OCI-AML3 (Fig. 4I), which has wild-type FLT3. Elevated levels of Pim1 kinase induce p53 signaling and MDM2 phosphorylation (45). Accordingly, our results showed reduced phosphorylation in MDM2 when using VS-II-173 in Molm-13 cells (Fig. 4G). Furthermore, we found that both VS-II-173 and the pan-Pim kinase inhibitor AZD1208 reduced the phosphorylation of Akt and p70-S6 kinase (see Supplementary Fig. S2); however VS-II-173 had a higher potency toward AML cell lines than AZD1208 in mono-treatment (Fig. 3H and Fig. 4A–C).

It must be noted that some of the perturbations in cell signaling could be caused by inhibition of other kinases. RSK1 was significantly inhibited by VS-II-173 (Fig. 2F). RSK1 is a mediator of the anti-apoptotic function of FLT3-ITD through phosphorylation of Bad at Ser112 in AML cells (46). The reduction of pBad (Fig. 4H) could be due to inhibition of RSK1 along with Pim kinase inhibition. AMPK is a stress sensor in cells, and can contribute to initiation of autophagy, and protects AML-cells from metabolic stress (47). AMPK can thus be an important target for AML therapy, particularly to prevent leukemogenesis, although another study shows that activation of AMPK initiates autophagic cell death (48). DYRK1 is a tumor suppressor, which is heavily down-regulated in AML patients (49), and mutations in the HIPK2 gene is linked to pathogenesis of AML (50). Taken together, the anti-AML activity can be inhibition of Pim kinase activity acting in concert with additional inhibition of some or all of the above-mentioned factors.

The fact that VS-II-173 affects more than one target is in line with several of the so-called precision therapies, like the tyrosine kinase inhibitors. In fact, the second-generation kinase inhibitor Dasatinib has a higher level of promiscuity than its predecessor Imatinib (51). With this in mind, a possible explanation for the potency and selectivity of VS-II-173 can be its activity toward key AML survival factors like RSK1 and AMPK together with its pan-Pim kinase activity. The much more selective Pim kinase inhibitor AZD1208 (52) was less efficient toward our AML cell lines than VS-II-173 (Fig. 3H), perhaps due to the broader range of substrates of the latter. Pan-kinase inhibition is believed to be favorable to the multitargeted kinase inhibitor midostaurin (see ref. 53; Fig. 3I). The mono-selective Bcl-2 inhibitor venetoclax (54), which emerges as therapy against AML (55), failed to induce cell death in OCI-AML3 cells (Fig. 3I) and appears to be effective against different subtypes of AML compared to VS-II-173.

A panel of nonselected AML patient blasts was included and 24 of 37 patient responded well to the treatment with VS-II-173, where doses between 6 and 12 μmol/L could kill more than 50% of the blasts (Fig. 5). Approximately 40% of patients with AML with FLT3-ITD mutation also harbor the NPM1 mutation (56). Our data showed NPM1 status was important for response to VS-II-173 in patients with FLT3-WT, but not in patients with FLT3-ITD. Thus, the presence of the high-risk factor FLT3-ITD is not influenced by the low-risk factor NPM1 mutation when it comes to response to VS-II-173. These findings suggest that this compound can be used irrespective of FLT3 status. There was no correlation between the response to VS-II-173 and the level of Pim kinases in the patient blasts (Fig. 5B), which could be ascribed the effect on other kinases (Fig. 2F). However, the values in Fig. 5B were compared with a pooled average, and AML cells are expected to have higher levels of Pim kinases than normal BM (3).

**Conclusion**

New strategies for AML therapy are urgently needed. Despite this, only one new drug has been developed and approved for AML during the last 15 years. Unfortunately, it appears to yield only modest improvement in survival, even in preselected patients (57). Our data point to pyrazolo[4,3-ά]phenanthridine as a relevant scaffold for compounds useful in the treatment of AML. The lead compound VS-II-173 exhibited crucial properties needed to exert therapeutic effect. It is selective toward AML cells, in which it efficiently dampens prosurvival signals. It is also potent against AML patient blasts, including some that showed high tolerance to DNR, and blasts carrying mutations associated with poor prognosis. The compound and future analogs of pyrazolo[4,3-ά]phenanthridines represent possible candidates for future AML treatment. Future studies will aim to reveal possible biomarkers of the patient AML cells which responded well to treatment with VS-II-173, as well as conducting preclinical studies on human AML xenografts in mice. If the abovementioned issues are resolved, we believe that pyrazolo[4,3-ά]phenanthridines have a future in AML treatment.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

**Conception and design:** R. Bjørnstad, Ø. Bruserud, L. Herdalndal

**Development of methodology:** R. Bjørnstad, G. Gaundal, S.O. Danskeland, L. Herdalndal

**Acquisition of data (acquired and managed patients, provided facilities, etc.):** R. Bjørnstad, G. Gaundal, T.H. Dowling, P. Moreau, F. Anizon, L. Herdalndal

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** R. Bjørnstad, R. Aesoy, Ø. Bruserud, A.K. Brenner, S.O. Danskeland, L. Herdalndal

**Writing, review, and/or revision of the manuscript:** R. Bjørnstad, R. Aesoy, Ø. Bruserud, P. Moreau, S.O. Danskeland, F. Anizon, L. Herdalndal

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** L. Herdalndal

**Study supervision:** L. Herdalndal

**Acknowledgments**

The authors want to thank Ing. Nina Lied Larsen and Kaja Skålnes Knudsen, MSc for technical assistance, and Sjur Huseby, PhD for assistance with generation of the AML cell lines. Prof. Bjørn Tore Gjersten and Mishah Sabet, MSc are thanked for supplying bone marrow samples. Kathrine Åsrud, PhD is thanked for critically reading through the manuscript. This study was supported...
New Pim Kinase Inhibitor for AML Therapy

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.

Received December 13, 2017; revised May 5, 2018; accepted January 14, 2019; published first January 24, 2019.

financially by the Norwegian Western Regional Health Authorities to Lars Herfindal (Grant No. 912052), Ronja Bjornstad (Grant No. 912028), Stein O. Daskeland (Grant No.: 303485), and Øystein Bruserud (Grant No. 911788), by the Norwegian Cancer Society to Lars Herfindal (Grant No. 112502), Stein O. Daskeland (Grant No. 705268), Øystein Bruserud (Grant No. 62370, 62371, and 100933), and by the Norwegian Research council to Lars Herfindal and Reidun Aesoy (Grant No. 254752).

References


Molecular Cancer Therapeutics

A Kinase Inhibitor with Anti-Pim Kinase Activity is a Potent and Selective Cytotoxic Agent Toward Acute Myeloid Leukemia

Ronja Bjørnstad, Reidun Aesoy, Øystein Bruserud, et al.