Kit Inhibitor APcK110 Induces Apoptosis and Inhibits Proliferation of Acute Myeloid Leukemia Cells

Stefan Faderl, Ashutosh Pal, William Bornmann, Maher Albitar, David Maxwell, Quin Van, Zhenghong Peng, David Harris, Zhiming Liu, Inbal Hazan-Haley, Hagop M. Kantarjian, and Zeev Estrov

Abstract

Kit is a membrane-bound tyrosine kinase and receptor for stem cell factor (SCF) with a crucial role in hematopoiesis. Mutations of KIT occur in almost half of patients with core-binding factor leukemias, in which they have been associated with worse outcome. Development of new compounds targeting Kit may therefore hold promise for therapy. We investigated the activity and mechanism of action of APcK110, a novel Kit inhibitor, in the mastocytosis cell line HMC1.2 (KITV560G and KITD816V), acute myeloid leukemia (AML) lines OCI/AML2 and OCI/AML3 (both wild-type), and primary samples from patients with AML. We show that (a) APcK110 inhibits proliferation of the mastocytosis cell line HMC1.2 and the SCF-responsive cell line OCI/AML3 in a dose-dependent manner; (b) APcK110 is a more potent inhibitor of OCI/AML3 proliferation than the clinically used Kit inhibitors imatinib and dasatinib and at least as potent as cytarabine; (c) APcK110 inhibits the phosphorylation of Kit, Stat3, Stat5, and Akt in a dose-dependent fashion, showing activity of APcK110 on Kit and its downstream signaling pathways; (d) APcK110 induces apoptosis by cleavage of caspase-3 and poly(ADP-ribose) polymerase; and (e) APcK110 inhibits proliferation of primary AML blasts in a clonogenic assay but does not affect proliferation of normal colony-forming cells. Although APcK110 activity may partly depend on cytokine responsiveness (e.g., SCF) and not exclusively KIT mutation status, it remains a potent inhibitor of AML and mastocytosis cell lines and primary AML samples. APcK110 and similar compounds should be evaluated in clinical trials of patients with AML.

Introduction

Acute myeloid leukemia (AML) is a group of clonal diseases of hematopoietic progenitor cells. Nonrandom cytogenetic abnormalities occur in >50% of diagnostic samples of patients with adult AML and several molecular abnormalities have been identified that have shed further light on AML subtypes (1–3). Besides providing insights into the biology and pathophysiology of AML, cytogenetic and molecular genetic aberrations provide important independent prognostic information and, in some cases, determine treatment decisions (e.g., responsiveness of acute promyelocytic leukemia to all-trans retinoic acid and sensitivity of core-binding factor leukemias to cytarabine; ref. 4). Despite progress in this area, possibilities at treating AML patients in a subtype-specific manner remain limited, as molecular profiling is still inadequate and targeted drug therapies are often elusive.

Protein kinases play a crucial role in both normal and malignant cells and require tight control and regulation. Kit is a type III receptor protein tyrosine kinase and the transmembrane receptor for stem cell factor (SCF; refs. 5, 6). Binding of SCF to Kit leads to receptor dimerization, phosphorylation, and activation of downstream intracellular signaling pathways involved in proliferation and survival (7). Gain-of-function mutations of KIT leading to ligand-independent activation have been described in some solid tumors as well as in patients with AML (8–11). Although activating KIT mutations are rare in unslected patients with AML, they have been reported with frequencies of between 17% and 46.1% in core-binding factor leukemias such as those carrying translocations t(8;21), t(16;16)(p13;q22), and inv(16)(p13q22) (12, 13).

Several recent studies of adults with inv(16) and t(8;21) AML have shown a negative prognostic effect of KIT mutations at codon 816 with respect to relapse rate and overall survival (14–16). Identification of KIT mutations, however, is important not only for prediction of outcome but also for therapeutic decisions. Responses with receptor tyrosine kinase inhibitors in clinical studies have been reported and more specific Kit inhibitors may hold further promise for AML therapy. In the present study, we have been evaluating the activity and possible mechanism of action of APcK110, a novel Kit inhibitor (17).

Materials and Methods

Drug. APcK110 [4-[4-{7-(3,5-dimethyloxyphenyl)-1H-pyrazolo[3,4-]pyridin-6-yl}-4-fluoro]benzene (Fig. 1A) and imatinib mesylate were synthesized in Dr. Bornmann's laboratory (Experimental Diagnostic Imaging, The University of Texas M.D. Anderson Cancer Center, Houston, Texas; ref. 17). Dasatinib was kindly provided by Dr. Francis Lee (Bristol-Myers Squibb). All drugs were dissolved in PBS (Life Technologies) to stock solutions at a final concentration of 50 mmol/L.

APcK110 kinase screening. A primary high-throughput screening of APcK110 at 10 μmol/L was conducted by Ambit Biosciences against a T7-bacteriophage library displaying 240 human kinases using imatinib screening as control. A rough estimation of the binding constant (Kd_1) for each assay was provided by the single-hit value in the primary screen at a single compound concentration. Kinase profiling was done using a bacteriophage library displaying fused human kinases that may attach at the ATP site to a fixed-ligand matrix, which, in turn, may be competitively displaced from binding by the tested compound. As expected, APcK110 showed inhibition of Kit and KITD816V mutant. In addition, a small number of kinases were also inhibited, which included TGFβR2, MEK2, p38, RET, MEK4, MAP4K5, MAP4K4, Aurora kinase B, and PKCβ.
Cell lines. The AML cell lines OCIM2 and OCI/AML3 were provided by M.D. Minden (Ontario Cancer Institute). OCI/AML3 was established from an AML patient and OCIM2 from a patient with erythroleukemia. Both cell lines proliferate in the presence of culture medium and FCS without exogenous growth factors. Neither OCIM2 nor OCI/AML3 cells harbor KIT mutations or mutations of FLT3.4 The mastocytosis cell line HMC1.2 and the murine, interleukin (IL)-3-dependent cell line BaF3 were obtained from the American Type Culture Collection. HMC1.2 cells are harboring KIT V560G as well as KIT D816V mutations (18). The BaF3 system included KIT D816V-expressing cells. Cells were maintained in RPMI 1640 (Life Technologies) supplemented with 10% FCS (Flow Laboratories), grown in plastic tissue culture flasks (Falcon Plastics; Becton Dickinson), and split twice weekly. BaF3 flasks also contained murine IL-3 (R&D Systems).

Patient samples. Fresh marrow samples were collected and analyzed from 12 patients with AML and 5 healthy volunteers (Table 1). Samples were obtained and studies were done with the approval of the Institutional Review Board at The University of Texas M.D. Anderson Cancer Center. All patients signed the informed consent before sample collection.

Cell surface marker analysis. Standard flow cytometric analysis was used to detect cell membrane-bound Kit (CD117) on OCIM2 and OCI/AML3 cells. Flow cytometry analysis was done using FACS Calibur (Becton Dickinson Immunocytometry Systems). Data analysis was done using CellQuest software (Becton Dickinson Immunocytometry Systems) and ModFit LT V2.0 (Verity Software House).

Cell proliferation and survival assay. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT) assay was done using a MTT-based cell proliferation/cytotoxicity assay system from Promega. OCIM2 and OCI/AML3 cells were washed twice in RPMI 1640 containing 10% FCS by centrifugation at 300 g for 5 min. Cell concentration and viability were determined by hemocytometer counts of cells with 0.1% trypan blue dye. Cells were suspended in medium and plated immediately at 5 x 10^4 per well in a total volume of 100 A.L in 96-well flat-bottomed plates (Linbro; Flow Laboratories) and incubated for 48 to 72 h at 37°C in a humidified 5% CO_2 atmosphere. Following incubation, 20 μL CellTiter96 One Solution Reagent (Promega) was added per well. The plates were then incubated for an additional 60 min at 37°C in a humidified 5% CO_2 atmosphere. Immediately after incubation, absorbance was read using a 96-well plate reader at a wavelength of 490 nm. Each data point was determined six times before analysis.

Cell line clonogenic assay. The cell line clonogenic assay was done as described previously (19). Colonies were counted after 7 days by using an inverted microscope. A colony was defined as a cluster of >40 cells.

KIT mutation analysis. The total nucleic acid was extracted from cells using NucliSens (BioMérieux) extraction kit. For KIT mutation analysis, two primer pairs were designed to amplify the juxtamembrane and tyrosine kinase domains of Kit (Kit R1-F ATTGTAGAGCAAATCCATCCCC and c-Kit R1-R GCCCCTGTTTCATACTGACCA and Kit R2-F C TTC CCAACCTAAATGTGATTCACAG and c-Kit R2-R TCA CATGCCCTCCTGAAAAATACA). A 351 bp PCR product (Kit-R1) was amplified from DNA sample spans exon 10, intron 10, and exon 11 containing codon 560 and a 380 bp PCR product (Kit-R2) was amplified spans exon 17 and intron 17 containing codon 816

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1 M. Albitar, unpublished data.
mutation. The PCR products were then filtration purified and sequenced in both forward and reverse directions using ABI PRISM 3100 Genetic Analyzer. Sequencing data were base-called by Sequencing Analysis software and assembled and analyzed by ABI Prism Seqscape software using GenBank accession no. U63834 as reference. For NPM1 testing, the NPM gene PCR amplification was done using NPM intron 11 forward primer (5’-TAAACTCTCTGTTGTTAGATGAA-3’) and 6-FAM-labeled NPM exon 12 reverse primer (5’-FAM-TCGTTCAAGAGGAACTAGC-3’). The NPM mutated or wild-type products were verified by determining the size of PCR products using ABI3100 Genetic Analyzer. The wild-type displayed a 212 bp peak, whereas the NPM mutant display an extra 216 bp peak in addition to the NPM wild-type peak. The percent of mutant to total (peak height) was calculated.

ELISA. ELISA was done with the SCF ELISA kit (Amersham Life Science) in accordance with the manufacturer’s instructions. In brief, cell lysates and standard dilutions of SCF were added to test wells in duplicate and incubated for 2 h at 37° C. The test wells were then washed three times with PBS, incubated with mouse SCF antiserum for 2 h, washed, and incubated for 30 min with goat anti-mouse IgG conjugated to horseradish peroxidase. The test wells were washed, o-phenylenediamine was dissolved in 3% hydrogen peroxide solution, and 4 N sulfuric acid were added. The color intensity was read within 15 min at a wavelength of 490 nm with a microplate autoreader (model EL-309, Biotec). The average net absorbance was calculated.

Western immunoblotting. Cell lysates (from 5×10⁵ cells) were used as described. The following antibodies were used to detect the relevant proteins: mouse anti-human Stat3, Stat5, pStat3, and pStat5 antibodies (Transduction Laboratories) for detection of pro-caspase-3, rabbit anti-proteins: mouse anti-human Stat3, Stat5, pStat3, and pStat5 antibodies (Transduction Laboratories) for detection of pro-caspase-3, rabbit anti-phosphorylated phosphor-di-ATP polymerase (PARP; Pharmingen), and mouse anti-human poly(ADP-ribose) polymerase (PARP; Pharmingen), and mouse anti-human poly(ADP-ribose) polymerase (PARP; Pharmingen), and mouse anti-human KIT and pKIT (Pharmingen). Normal mouse IgG and rabbit IgG (Sigma) were used as controls.

Cell cycle analysis. Cell cycle analysis was done according to standard protocols. Briefly, 5×10⁵ cells were incubated with APcK110 and pelleted. The cell pellets were washed and resuspended in 2 mL of 1% paraformaldehyde in PBS (Life Technologies). Cells were incubated for 15 min at 4° C and then washed again in PBS, resuspended in 2 mL absolute ethanol, and stored at −20° C until staining. The stored cells were washed twice in PBS, resuspended in 0.5 mL propidium iodide staining buffer (50 mg/mL propidium iodide, 10 μg/mL RNase in PBS), and then incubated for 1 h at room temperature in the dark. Flow cytometric analysis was done as described above.

Annexin V assay. The Annexin V-FITC assay (Pharmingen) was used as described previously (20). Cells were analyzed using Annexin V-FITC (BD Pharmingen) and flow cytometry with a FACSCalibur flow cytometer and the CellQuest software program. Data analysis was done using the CellQuest and ModFit LT (version 2.0) software programs. To determine whether OCI/AML3-induced apoptosis was caspase-dependent, OCI/AML3 cells were preincubated with 20 μmol/L of the pan-caspase inhibitor Z-VAD-FMK (BD Pharmingen).

AML blast colony assay. The AML blast colony assay was done as described previously (21, 22). APcK110 was added at the initiation of the cultures at concentrations ranging from 50 to 500 nmol/L. The cultures were incubated in 35 mm Petri dishes in duplicate or triplicate for 7 days at 37° C in a humidified atmosphere of 5% CO₂ in air. AML blast colonies were microscopically evaluated on day 7 of culture. A blast colony was defined as a cluster of >20 cells. Individual colonies were plucked, smeared on glass slides, and stained to confirm their leukemic cell composition.

Colony-forming unit granulocyte-macrophage culture assay. The colony-forming unit granulocyte-macrophage clonogenic assay was done as follows: 2×10⁵ low-density bone marrow cells were cultured in 0.8% methylcellulose in Iscove’s modified Dulbecco’s medium (Life Technologies) supplemented with 10% FCS, 50 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor (Bectax) with or without 50 nmol/L recombinant human SCF (Amgen) in the presence or absence of APcK110. All cultures were evaluated after 14 days for the number of colony-forming unit granulocyte-macrophage defined as clusters of >40 granulocytes and/or monocyte/macrophage cells.

Results

APcK110 inhibits AML cell viability and proliferation: differential response of SCF-responsive and nonresponsive AML cells. We first performed a cell surface marker analysis to establish whether OCIM2 and OCI/AML3 lines express Kit. Using flow cytometry, we found expression of CD117 (Kit) in 21% of OCIM2 and 15% of OCI/AML3 cells, respectively. We then examined the antiproliferative effect of APcK110 on OCIM2 and OCI/AML3 cells and on HMC1.2 cells that harbor an activating KIT mutation. Cells were incubated for 72 h without or in the presence of APcK110 at concentrations of 100, 200, 300, 400, and 500 nmol/L and their viability and rate of multiplication were determined by

### Table 1. Patient sample characteristics

<table>
<thead>
<tr>
<th>AML</th>
<th>Bone marrow blasts (%)</th>
<th>CD117/CD34 (%)*</th>
<th>Cytogenetics</th>
<th>FLT3</th>
<th>KIT</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>43</td>
<td>NA</td>
<td>t(3;21)(q26;q22), CBF+</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>72</td>
<td>98.6</td>
<td>Diploid</td>
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<td>NA</td>
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<td>3</td>
<td>92</td>
<td>56.7</td>
<td>t(3;17)(q21q25)</td>
<td>ITD</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>86</td>
<td>53.3</td>
<td>t(9;21)(q31q22); del(4)(q12); add(21q)(q22)</td>
<td>ITD</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>92</td>
<td>0.3</td>
<td>t(9;11)(q22q23)</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>81</td>
<td>77.3</td>
<td>t(3;3)(q21q26)</td>
<td>ITD</td>
<td>NA</td>
</tr>
<tr>
<td>7</td>
<td>58</td>
<td>89</td>
<td>t(4;12)(q13p13); der(13;15)(q10q10); del(20)(q11.2q13.3)</td>
<td>NA</td>
<td>ITD</td>
</tr>
<tr>
<td>8</td>
<td>85</td>
<td>51</td>
<td>Diploid</td>
<td>ITD</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>94</td>
<td>57</td>
<td>+8, +10, +18, +22, +13</td>
<td>0</td>
<td>D816V</td>
</tr>
<tr>
<td>10</td>
<td>84</td>
<td>97.8</td>
<td>+8, inv(16)(p13.1q22)</td>
<td>0</td>
<td>D816V</td>
</tr>
<tr>
<td>11</td>
<td>53</td>
<td>83.3</td>
<td>t(8;21)(q22q22)</td>
<td>0</td>
<td>D816V</td>
</tr>
<tr>
<td>12</td>
<td>65</td>
<td>95.2</td>
<td>t(8;21)(q22q22); del(7q)(q34)</td>
<td>0</td>
<td>D816V</td>
</tr>
</tbody>
</table>

Abbreviations: NA, not available; 0, no FLT3/KIT abnormalities detected; ITD, FLT3 internal tandem duplication; CBF, core-binding factor.

*As measured by flow cytometry.
the MTT assay. As shown in Fig. 1B, growth inhibition was most significant in OCI/AML3 and HMC1.2 cells. In both cell lines, 80% inhibition of proliferation was achieved at APcK110 concentrations of 500 nmol/L. In contrast, proliferation of OCIM2 cells was far less affected with inhibition of proliferation not exceeding 25% at APcK110 concentrations of 500 nmol/L.

Because OCIM2 responded poorly to APcK110 and OCI/AML3 cells were as sensitive to APcK110 as HMC1.2 cells, we asked whether the sensitivity of OCI/AML3 to APcK110 resulted from an activating mutation(s) similar to that of HMC1.2 cells (in which KIT V560G and KIT D816V mutations were found; ref.18). To look for mutations in OCIM2 and OCI/AML3 lines, we performed a mutation analysis using a 351 bp PCR product amplified from patient DNA samples spanning exons 10, 11, and 17 as well as introns 10 and 17. We did not detect KIT mutations in either of the two AML cell lines including at codons V560D, N822K, and D816V.

Although neither OCIM2 nor OCI/AML3 cells have detectable KIT mutations, their proliferation response to APcK110 was significantly different. Furthermore, inhibition of proliferation of OCI/AML3, a cell line without KIT mutations, was similar to inhibition of HMC1.2, a cell line with mutated KIT. To delineate these differences, we measured SCF levels in both cell line lysates using ELISA. We found that both OCIM2 and OCI/AML3 produce 10 pg SCF/10^7 cells. Then, we investigated whether these AML cell lines proliferate in response to the endogenously produced SCF. We incubated OCIM2 and OCI/AML3 cells with SCF neutralizing antibodies (R&D Systems) at concentrations of 0.01, 0.05, 0.1 and 1 µg/mL in the presence or absence of 50 ng/mL SCF and evaluated colony-forming cell growth using a clonogenic assay. Whereas SCF neutralizing antibodies significantly inhibited OCI/AML3 colony proliferation, the inhibitory effect was completely reversed by exogenous SCF. Furthermore, SCF stimulated OCI/AML3 colony-forming cell proliferation (Fig. 1C). In contrast, no change in colony numbers of OCIM2 cells occurred in the presence of SCF neutralizing antibodies, and there was no further effect to addition of exogenous SCF (Fig. 1D). The results therefore suggest that OCI/AML3 is a SCF-responsive cell line whose growth is stimulated by SCF in an autocrine manner, whereas OCIM2 cells produce SCF and express Kit but do not proliferate in response to SCF.

**APcK110 is a more potent inhibitor of the proliferation of OCI/AML3 cells than imatinib and dasatinib and is at least equally potent an inhibitor as cytarabine.** Imatinib and dasatinib are potent tyrosine kinase inhibitors with broad activity against BCR-ABL, PDGFR, and Kit kinases and, in the case of dasatinib, against SRC kinases. We incubated OCI/AML3 cells for 72 h in the presence of increasing concentrations of imatinib, dasatinib, and APcK110 and, using the MTT assay, compared the antiproliferative effect of these kinase inhibitors. As shown in Fig. 2A, we show stronger inhibition with APcK110. At 72 h and at concentrations of 250 nmol/L for imatinib, dasatinib, and APcK110,
respectively, cell viability was 52%, 48%, and 35% of the control sample. Average IC₅₀ values at 72 h were 175 nmol/L for APcK110.

As cytarabine is the backbone nucleoside analogue for treatment of AML, we also compared the antiproliferative effects of cytarabine with APcK110 in OCI/AML3 cells. As shown in Fig. 2B, we show at least equal, if not stronger, inhibition with increasing concentrations of APcK110 when compared with cytarabine. Maximum inhibition of OCI/AML3 proliferation was reached at concentrations of 500 nmol/L APcK110 (17% of control), 2 mmol/L cytarabine (31% of control), and 250 nmol/L APcK110 with 2 mmol/L cytarabine (12% of control).

**APcK110 preferentially inhibits KIT mutated BaF3 cell lines.** To further explore the sensitivity of APcK110 toward its putative target Kit, we incubated growth factor (IL-3)-dependent murine BaF3 cells with increasing concentrations of APcK110 and compared the proliferative activity by MTT assay with that in mutant KIT-expressing BaF3 cells (Fig. 2C). We show that although APcK110 interferes with IL-3-mediated signaling (wild-type BaF3 cells), inhibition of cell proliferation is stronger in the mutated KIT-expressing cells especially at APcK110 concentrations of 500 to 500 nmol/L. Addition of IL-3 to the mutated BaF3 cell line did not reverse inhibition, supporting selective targeting of KIT mutated cells compared with BaF3 wild-type cells. It should, however, be noted that the dose-response curves are close and therefore suggest a possibly narrow therapeutic window regarding APcK110 and its affinity to mutated KIT.

**APcK110 inhibits the phosphorylation of Kit, Stat3, Stat5, and Akt.** Kit signaling is transmitted through several pathways including signal transducer and activator of transcription, phosphatidylinositol 3-kinase, SRC, and RAS (7). Due to its presumed relevance, we chose the Stat and phosphatidylinositol 3-kinase pathways to determine the effect of APcK110 on Kit downstream phosphorylation and activation events (Fig. 3A). Using Western immunoblotting, we found a dose-dependent inhibition of the phosphorylation of Kit, Stat3, Stat5, and Akt, a downstream effector of phosphatidylinositol 3-kinase. The decrease in levels of the respective phosphoproteins in both OCI/AML3 and HMC1.2 cell lines started at doses of 50 to 100 nmol/L. We show the same inhibition of phosphorylation of Kit, Stat3, Stat5, and Akt in the primary AML sample from patient UPN 8 (see Table 1), suggesting that the findings of APcK110 are not isolated on cell lines (Fig. 3B).

**APcK110 induces caspase-dependent apoptosis in OCI/AML3 cells.** As shown in Fig. 3B, we show at least equal, if not stronger, inhibition with increasing concentrations of APcK110 when compared with cytarabine. Maximum inhibition of OCI/AML3 proliferation was reached at concentrations of 500 nmol/L APcK110 (17% of control), 2 mmol/L cytarabine (31% of control), and 250 nmol/L APcK110 with 2 mmol/L cytarabine (12% of control).

**APcK110 inhibits AML blast colony-forming cell proliferation.** To investigate the effect of APcK110 on AML primary samples, we incubated diagnostic marrow cells from 12 patients with AML and 5 normal controls (see Table 1 for details of AML patients) with increasing doses of the Kit inhibitor APcK110. We studied the activity of APcK110 with or without SCF (50 nmol/L).
using the AML and the colony-forming unit granulocyte-macrophage (for the control normal marrow) colony culture assays. As shown in Fig. 5A, we found that APcK110 did not reduce granulocyte-macrophage colony-stimulating factor-induced colony proliferation unless SCF was absent from the medium. Sample “Normal 1” did not show any response to either SCF or APcK110 (including at the highest concentration of 500 nmol/L). Interestingly, this was very similar to the sample of AML patient 5 in Fig. 5B whose blasts were characterized by absence of SCF receptor (CD117/CD34 negative by flow cytometry; Table 1). In both cases, absence of SCF from the medium did not reduce the granulocyte-macrophage colony-stimulating factor-induced colony proliferation. In all remaining AML samples, a concentration-dependent inhibition of AML colony-forming cell proliferation occurred. In all of these cases, lack of exogenous SCF in the medium also resulted in inhibition of colony growth, supporting the notion that the proliferation of these cells is SCF-responsive, similar to what we found in our previous experiments with OCI/AML3 cells.

Discussion

Molecular profiling of AML blasts has identified several genes, which have been associated with prognosis (23). These observations will ultimately lead to clues about their function and biological significance in leukemogenesis and thus open the possibility of new therapeutic avenues. Mutations of KIT are among those molecular markers that are increasingly recognized in AML and hence are being pursued for their relevance as therapeutic targets (14–16). In addition, the receptor is expressed on >10% blasts in 64% of de novo AML and 95% of patients with relapsed AML (24). In an in vitro study using KG1, a chemotherapy-resistant AML cell line, the combination of anti-SCF with low doses of cytarabine and daunorubicin resulted in a synergistic increase of chemotherapy-induced apoptosis (25). A combination of low-dose cytarabine with imatinib was investigated in a study including 34 patients with Kit-positive AML (26). In 6 of these 38 patients, a “blast response” was observed and 8 more patients had stable disease. Among mutations of several exons within KIT, those at exon 17 affecting codon D816 were crucial for expression of an adverse prognostic phenotype of patients with AML harboring inv(16) and translocations t(8;21). Anecdotal responses with the tyrosine kinase inhibitor imatinib have been reported in patients with core-binding factor AML as well (27, 28). However, imatinib remained ineffective for those patients with D816 mutations, whereas several newer compounds (e.g., PKC412 and dasatinib) have been identified as potent inhibitors of wild-type Kit and KIT D816V with reported clinical activity in patients with mast cell leukemias harboring KIT D816V mutations (29–31).

APcK110 is the result of a structure-based drug design of Kit inhibitors based on the available crystal structure of Kit. The design resulted from in silico screening of several targeted libraries via docking to the crystal structure of Kit followed by aggressive postfiltering by several criteria to determine the most prominent candidates for synthesis (17). Five compounds were selected for further in vitro testing, of which we chose APcK110 for its favorable IC50 value in Kit kinase assays and in AML cell proliferation assays. We investigated the activity of APcK110 in two AML cell lines lacking KIT mutations and one mastocytosis line harboring KIT V560G and KIT D816V mutations. We found growth inhibition in all cell lines, suggesting activity against both wild-type and mutated KIT. However, inhibition of proliferation was most marked...
in OCI/AML3 cells with wild-type *KIT* when compared with both OCIM2 (wild-type) and HMC1.2 (mutated) cells. When compared with growth inhibition of HMC1.2, activity of APcK110 may indeed be slightly higher in cells with wild-type *KIT* than those with *KITV*560G or *KITD*816V mutations. The difference of activity between OCIM2 and OCI/AML3 still raises the question as to why activity differs in these two wild-type *KIT* lines. What may explain this difference is the responsiveness to SCF (Fig. 1). Whereas both lines express surface CD117 (Kit) and produce SCF, only OCI/AML3 cells proliferate in response to SCF (hence SCF-responsive) likely in an autocrine manner. On the other hand, OCIM2 cells could not be stimulated by increasing concentrations of exogenous SCF and the growth of these cells was not inhibited by SCF neutralizing antibodies. Thus, either endogenously produced SCF by OCIM2 cells is surface-bound and could already stimulate cellular proliferation to its maximum without being affected by exogenous SCF or SCF neutralizing antibodies (in that case, OCIM2 cells should have responded to the intracellular Kit inhibitor APcK110), or OCIM2 cells respond poorly to SCF rendering it SCF-non-responsive.

In both OCI/AML3 (wild-type) and HMC1.2 (mutated) cell lines, we show downstream decrease of the levels of pKit, Stat3, Stat5, and Akt. The decrease in the levels of pKit in particular confirms activity of APcK110 on its target with subsequent down-regulation of downstream components of the Stat and phosphatidylinositol 3-kinase/Akt pathways. Down-regulation of pKit requires a higher dose of APcK110 in HMC1.2 cells than OCI/AML3 cells, underlining the results from the MTT assay that APcK110 may be more active against SCF-responsive cells with wild-type Kit. We observed the same results in the marrow of one patient with AML, findings that require extended evaluation in a large number of patient samples.

We extended our observations from cell lines to primary samples from patients with AML (see Table 1 for characteristics). An interesting pattern emerged. Normal marrow cells from healthy volunteers remained unaffected including by the highest concentration of APcK110 used unless SCF was deprived from the serum. The one exception to this observation were the leukemia blasts from patient AML5 where there was no expression of SCF receptor (Kit) and which was also the only sample that was not SCF-responsive. The proliferation rate was unaffected by absence of SCF in the growth medium nor was there any decrease in AML blast colony numbers irrespective of the concentration of APcK110. In all other samples, absence of SCF caused a decrease in growth, whereas addition of SCF in the absence of APcK110 stimulated growth, which in turn was again inhibited in a dose-dependent manner by APcK110 although with a great amount of heterogeneity between samples. The observation in patient samples thus confirms the observation in the cell line models in that those blasts whose proliferation responds to SCF are also the most amenable to inhibition by APcK110.

Figure 5. A, effect of APcK110 and SCF on normal marrow colony-forming unit granulocyte-macrophage. B and C, effect of APcK110 and SCF on AML marrow leukemia blast colony-forming cells. B, experiments without SCF in the presence and absence of APcK110. C, AML cells are incubated with 50 nmol/L SCF and increasing concentrations of APcK110. Colony-forming unit granulocyte-macrophage and AML-CFU are presented as percent of control (50 nmol/L SCF and no APcK110).
Given the narrowness of the dose-response curve in Fig. 2C, questions remain as to the target specificity of APcK110. Although our data suggest preferential inhibition of KIT, it is likely that other kinases are affected as well. Whether this is clinically relevant or not remains to be seen. In general, inhibition of additional kinases can also provide a therapeutic advantage, especially in an as complex and heterogeneous disease as AML.

In conclusion, Kit inhibitors such as APcK110 should be incorporated into future trials of patients with AML.

References


Disclosure of Potential Conflicts of Interest


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APcK110 Inhibits Proliferation of AML Cells

Kit Inhibitor APcK110 Induces Apoptosis and Inhibits Proliferation of Acute Myeloid Leukemia Cells


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