Cell Death and Survival

Impaired Long-Term Expansion and Self-Renewal Potential of Pediatric Acute Myeloid Leukemia–Initiating Cells By PTK787/ZK 222584

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Abstract

Although most children with acute myeloid leukemia (AML) achieve complete remission, the relapse rate is 30% to 40%. Because it is thought that leukemia-initiating cells (LIC) are responsible for AML relapses, targeting these cells might improve outcome. Treatment of pediatric AML blasts with the receptor tyrosine kinase (RTK) inhibitor PTK787/ZK 222584 (PTK/ZK) induces cell death in vitro. However, the role of PTK/ZK inhibition on outgrowth of (pediatric) LICs is unknown. In this study, we cultured CD34+ cells from pediatric patients with AML on MS5 stromal cells in long-term cocultures. In analogy to adult AML, long-term expansion of leukemic cells up to 10 weeks could be generated in 9 of 13 pediatric AMLs. Addition of PTK/ZK to long-term cocultures significantly inhibited leukemic expansion in all samples, ranging from 4% to 80% growth inhibition at week 5 compared with untreated samples. In 75% of the samples, the inhibitory effect was more pronounced at week 10. Proteome profiler array analysis of downstream kinases revealed that PTK/ZK reduced activation of PI3K/Akt kinase signaling. Although main targets of PTK/ZK are VEGF receptors (VEGFR), no effect was seen on outgrowth of LICs when cultured with bevacizumab (monoclonal VEGFA-antibody), specific antibodies against VEGFR2 or VEGFR3, or exposed to stroma-derived VEGFA. These data suggest that the effect of PTK/ZK on LICs is not only dependent on inhibition of VEGFA/VEGFR signaling. Taken together, our data elucidated antileukemic properties of PTK/ZK in long-term expansion cultures, and suggest that targeting multiple RTKs by PTK/ZK might be a potential effective approach in eradicating (pediatric) LICs. Mol Cancer Res; 11(4); 339–48. ©2013 AACR.

Introduction

Children with acute myeloid leukemia (AML) have a moderate to poor prognosis. Despite an initial complete remission rate of 90%, 30% to 40% of the pediatric patients with AML relapse, and the 5-year overall survival rate is approximately 60% (1–3). Therefore, certain leukemic cells have to be resistant to current treatment strategies, and persist after therapy. AML is a hematologic disease, which may originate at the level of the pluripotent hematopoietic stem cell (HSC; ref. 4). It was thought that the leukemic blast population is organized as a hierarchy, whereby leukemia-initiating cells (LIC; also known as leukemic stem cells) reside at the top of this hierarchy (5, 6). Because therapy-surviving LICs may be responsible for AML relapses, it seems advantageous to attack and eradicate these cells to improve survival rates.

It has been suggested that LICs depend on bone marrow niches for self-renewal, like HSCs (7). HSCs reside within specialized "stem cell niches" in the bone marrow, and these niches have been defined by their secretion of specific signaling molecules, growth factors, and cytokines including macrophage colony stimulating factor-1 (CSF-1), interleukins (IL), stem cell factor (KIT-ligand), and VEGFA (8–11). Interaction with the cytokines dictates, at least in part, stem cell fate, indicating an important role for the environment-derived cytokines and its receptors (7, 12).

PTK787/ZK 222584 (PTK/ZK) is a small-molecule kinase inhibitor that penetrates into cells and reaches its intracellular target with, as a result, a decrease in phosphorylation of the VEGF RTKs VEGF receptor (VEGFR) 2 [kinase insert domain receptor (KDR), IC50 0.037 µmol/L] and VEGFR1 [fms-related tyrosine kinase 1 (FLT1), IC50 0.277 µmol/L]. At higher concentrations, it also inhibits other receptor tyrosine kinases (RTK) including platelet-derived growth factor receptor-β (PDGFR-β, IC50 0.58 µmol/L), VEGFR3 [fms-related tyrosine kinase 4 (FLT4), IC50 0.64 µmol/L], c-KIT (KIT, also known as CD117...
or stem cell factor receptor, IC_{50} 0.73 μmol/L, and colony-stimulating factor-1 receptor (also known as c-FMS, IC_{50} 1.4 μmol/L; ref. 13). We previously showed that PTK/ZK induces a dose-dependent decrease in cell survival in 3 AML cell lines as well as in 33 primary pediatric AML blasts in short-term cultures (14). The level of VEGF at the time of diagnosis has been shown to be an independent prognostic factor for biologic response in (pediatric) AML, for example, occurrence of relapse (15–18). AML cells can produce VEGF and express its receptors, which make AML cells sensitive for VEGF-dependent proliferation (19). The downstream effects of VEGF are mainly executed by VEGFR2 binding, resulting in increased AML cell survival and proliferation via downstream signaling pathways such as via mitogen-activated protein kinase (MAPK) or the phosphatidylinositol 3-kinase (PI3K) pathway (20, 21). However, these downstream signaling pathways can also be activated by various other RTKs including c-KIT and PDGFR-β (22–24).

In this study, we have investigated the role of the tyrosine kinase inhibitor PTK/ZK on the (out)growth and self-renewal of pediatric LICs. To study the interaction between bone marrow-derived stromal cells and LICs, a previously described leukemic long-term culture-initiating cells (LTC-IC) assay has been used in which long-term leukemic expansion of LICs can be established using MS5 bone marrow stromal cells, thereby mimicking the stem cell niche (25). We cultured sorted CD34+ pediatric AML cells on stroma in the absence or presence of PTK/ZK. Our results indicate that PTK/ZK induced a decrease in long-term AML expansion.

Materials and Methods

Establishing leukemia long-term cultures on stroma

AML blasts from peripheral blood cells or bone marrow cells from untreated pediatric patients with AML were studied after informed consent. AML mononuclear cells were isolated by density gradient centrifugation, and CD34+ cells were selected by MoFlo sorting (DAKO Cytomation). Sort efficiency was measured by fluorescence-activated cell sorting (FACS) analysis and CD34+ percentages of 95% to 99% were found. A total of 40–50 × 10^5 sorted CD34+ cells (i.e., LICs) were plated in 12-well plates precoated with confluent layer of MS5 stromal cells. Cells were expanded in LTC medium [α-MEM supplemented with heat-inactivated 12.5% fetal calf-serum (FCS), heat-inactivated 12.5% horse serum (Sigma), penicillin and streptomycin, 2 mmol/L glutamine, 57.2 μmol/L β-mercaptoethanol (Sigma), and 1 μmol/L hydrocortisone (Sigma)] supplemented with 20 ng/mL IL-3, granulocyte colony-stimulating factor, and thrombopoietin as previously described (25). Cultures were kept at 37°C and 5% CO₂. Cultures were demidepopulated weekly for analysis. Self-renewal was studied in cocultures that generated leukemic cobblestone areas (L-CA) by harvesting suspension and adherent cells at week 5, and CD45+ cells were sorted and replated onto new MS5 stroma to initiate second cocultures. Cells were cultured in the presence of 10 μmol/L PTK787/ZK 222584 (a kind gift from the joint development project between Novartis Pharmaceuticals) or 25 μg/mL bevacizumab (a kind gift from Genentech/Roche). The used concentration of PTK/ZK in this study is based on our previous results. The used concentration of Bevacizumab in this study is based on literature (14, 26). Dimethyl sulfoxide (DMSO) was used as a control. Addition of the above mentioned drugs did not change the macroscopic appearance of MS5 cells. Moreover, one AML sample was cultured with VEGFR2-inhibitor (IMC121b, a kind gift from Imclone) or VEGFR3-inhibitor (IMC3C5, a kind gift from Imclone). All drugs were added during demidepopulation. The fact that cocultures generated L-CA after replating, a feature of self-renewal that has not been shown in normal cord blood CD34+ cells, confirms the leukemic origin of the expanding cells (25). In addition, with FLT3-ITD fragment analysis of the suspension cells in the LTC-IC cocultures at week 2, we showed for AML10 (FLT-ITD–positive sample) that the suspension cells harbor the heterozygous FLT3-ITD mutation (86%).

Transduction of MS5 stromal cells

MS5 cells were cultured in α-MEM medium supplemented with 1% penicillin/streptomycin and 10% FBS (Hyclone). Retroviral supernatants were generated by cotransfection of 2 μg reporter constructs pMSCV-iGFP-VEGFA165 or pMSCV-iGFP (empty vector, negative control) and 2 μg packaging plasmid pCLamp into 293T cells using FuGENE HD transfection reagent (Roche). A total of 5 × 10^4 MS5 cells were incubated with retroviral supernatants, which were filtered through 0.45 μm pore size syringe-mounted filters. Incubation was supplemented with 8 μg/mL polybrene. This procedure was repeated for 2 consecutive days after which stably transduced cells were expanded. Transduction efficiency was measured by fluorescence-activated cell sorting (FACS) analysis, which showed an efficiency of 94% for cells transduced with the empty vector (MS5-control) and 81% for cells transduced with VEGFA165 (MS5-VEGFA). Cells were sorted on a MoFlo.

Cell survival assay

Cell survival assays were conducted for primary AML samples and AML cell lines. A WST-1 colorimetric viability assay protocol was conducted following the procedures recommended by the manufacturer (Roche). Cells were seeded at a density of 1 × 10^5 cells per 100 μL/well in RPMI medium supplemented with 10% FCS. All samples were studied for the dose-dependent effects of the Akt inhibitor (MK2206, Bioconnect). Cells were incubated for 48 hours. After addition of the WST-1 cell survival reagent, the absorbance was measured at 450 nm
in a microplate reader (Benchmark; Bio-Rad). The data are presented as the cell survival percentage relative to DMSO-treated cells.

**RNA extraction and real-time PCR**

Total RNA from MS5-transduced cells or pediatric AML cells (material from 11 samples at start of the culture was available) were extracted with NucleoSpin RNA II Kit according to the manufacturer’s protocol (Macherey-Nagel). cDNA was prepared at 37°C for at least one hour in 20 µL reaction mixture containing 2 µg of total RNA, random hexamers (Promega), × 5 first-strand buffer, RNAsin, and reverse transcriptase (Gibco BRL). Real-time PCR (RT-PCR) was conducted using iTQ SYBR green supermix (Bio-Rad). All PCR and data analysis were conducted on the iCycler iQ Real-Time Detection System (Bio-Rad). Specific primers are shown in Supplementary Table S1. The expression of the RTKs and VEGFA was standardized for expression of β-actin and/or RPL22 (Arbitrary Units).

**ELISA and functional assay of VEGFA**

Secretion of VEGFA was detected in supernatant using commercially available ELISAs (Quantikine immunoassays, R&D systems) following manufacturer’s instructions. The functionality of secreted VEGFA from transduced cells was detected by adding its supernatant to endothelial cells (human umbilical vein endothelial cells) and quantify expression of VEGFA-specific genes EGR3, NUR77, and NOR1 in endothelial cells with RT-PCR, described in detail by Liu and colleagues (27; Supplementary Fig. S1).

**Microscopy and cytospins**

For morphologic analysis, May–Grünwald–Giemsa staining was used to analyze cytospins. Pictures of MS5 cocultures and cytospins were taken at magnification × 400. Morphologic analysis of May–Grünwald–Giemsa stains showed an immature myeloid phenotype after at least 4 weeks of culture.

**Flow cytometry analysis**

Cells were blocked by PBS 1% bovine serum albumin (Sigma), and stained with anti-VEGFR1 (Sigma Aldrich), anti-VEGFR2/KDR antibody (Sigma Aldrich), anti-VEGFR3/FLT4-APC (R&D systems), anti-CD115/ cFMS-biotin (R&D systems), anti-CD117-PerCP, and anti-CD140b/ PDGFRβ-PE (BD Bioscience). Primary VEGFR1 and KDR antibodies were visualized using phycoerythrin (PE)-conjugated secondary antibody (Dako cytomation). Primary CD115 antibodies were visualized using streptavidin fluorescein isothiocyanate (FITC). IgG1-FITC/PE/APC/PerCP were used as a negative isotype controls. Expression percentages of 3% or more were considered as actual protein expression above isotype controls. THP-1 and MOLM13 cells were incubated with different concentrations of PTK for 24 and 48 hours before Annexin V-FITC/propidium iodide (PI) analysis (IQ products). Primary AML cells were incubated with 0.1 µmol/L MK2206 for 96 hours before Annexin V-FITC analysis. Cells were stained with Annexin V-FITC and PI for 15 minutes in staining buffer following manufacturer’s protocol (Annexin-V-FLUOS staining kit, Roche). Necrotic, early apoptotic, and viable populations are distinguished on the basis Annexin V and PI expression. Expression was analyzed using LSR II (BD FACS DIVA software, BD Bioscience). The data were eventually developed using FlowJo software (Tree Star Inc.).

**Western blot analysis**

After 48 hours of incubation with 10 µmol/L PTK787/ZK 222584, 1 × 10⁶ THP-1 and HL-60 cells were lysed in laemmli sample buffer (Bio-Rad laboratories). Proteins were separated by SDS-PAGE and transported to nitrocellulose membranes. First, the membranes were incubated overnight with monoclonal primary antibodies for phospho-extracellular signal-regulated kinase (pERK) and total ERK (tERK), pAkt and tAkt, pSRC and...
tSRC, and actin (Cell Signaling), and thereafter incubated for 1 hour with horseradish peroxidase (HRP)-conjugated secondary antibodies (DAKO). Protein bands were visualized by chemiluminescence, on an X-ray film. Actin was used as a loading control.

### Phosphokinese arrays

Proteome Profiler Human Phospho-Kinase Array Kits from R&D Systems, Inc. were used to measure protein phosphorylation according to protocol. Per patient sample, 50 μg protein was applied to the array. In short, in this method proteins are captured by antibodies spotted on a nitrocellulose membrane. Levels of phosphoprotein were then assessed using an HRP-conjugated antibody followed by chemiluminescence detection. In our experiment, the amount of chemiluminescence was detected and analyzed using array software (ScanAlyze; Eisen Software; http://rana.lbl.gov/eisen).

### Statistical analysis

Statistical differences in leukemic outgrowth between cocultures were determined in a paired Wilcoxon signed rank test.

### Results

#### Long-term culture of pediatric LICs on MS5 bone marrow stroma

Culture of sorted CD34+ pediatric AML cells in an LTC-IC assay showed a long-term expansion of leukemic cells up to 10 weeks in 9 of 13 AMLs (Fig. 1A; patient characteristics listed in Table 1). Expansion of LICs on MS5 stromal cells was associated with the formation of phase-dark L-CAs underneath the stroma appearing after 2 to 5 weeks of culture (Fig. 1B). Self-renewal capacity of the pediatric AML cells could be shown by initiating secondary cocultures on new MS5 stroma after 5 weeks of culture. Secondary L-CAs were formed in 8 of the 9 cases cultured up to 10 weeks. These data show that a long-term culture of the primitive subfraction of pediatric AML cells can be established at least up to 10 weeks. In contrast, sorted CD34+ pediatric AML cells were not able to initiate long-term expansion or secondary cocultures (n = 4; samples AML2, AML5, AML6, and AML10; data not shown).

#### Impaired outgrowth and self-renewal of pediatric LICs by the tyrosine kinase inhibitor PTK/ZK

We previously reported that PTK/ZK induces cell death in leukemic cell lines as well as in primary pediatric AML samples. PTK/ZK increased the percentage of (early) apoptotic cells in a dose-dependent way in THP1 and MOLM13 cell lines (high, respectively, low VEGFR expression; Supplementary Fig. S1A and S1B). In primary AML cells, PTK/ZK at a concentration of 10 μmol/L was effective in reducing AML cell survival (14). To investigate the effect of PTK/ZK on long-term expansion and proliferation, CD34+ sorted pediatric AML cells were cultured in the absence or presence of 10 μmol/L PTK/ZK in the LTC-IC assay (n = 11). Figure 2 represents the growth curves of 11 individual patients and shows that the addition of PTK/ZK led to a decrease in leukemic expansion in all cases. The median value of 11 samples showed a significant (P < 0.05) delay in expansion throughout week 5 to 10. However, variability in sensitivity of the cultured AMLs for PTK/ZK was shown by a decrease in growth, ranging from 4% to 80% at week 5 as compared with

### Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>BM/PB</th>
<th>FAB</th>
<th>Karyotype</th>
<th>Growth up to week</th>
<th>Experiment</th>
<th>%CD34+ cells</th>
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<td>BM</td>
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<td>BM</td>
<td>M0</td>
<td>9q-</td>
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<td>BM</td>
<td>M3</td>
<td>t(15;17)</td>
<td>9, then †</td>
<td>P/MS</td>
<td>16.8</td>
</tr>
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<td>BM</td>
<td>M3</td>
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<td>P/B</td>
<td>2.3</td>
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<td>P/MK</td>
<td>0.5</td>
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<td>M0</td>
<td>Complex</td>
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<td>P/MS</td>
<td>88</td>
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<td>PB</td>
<td>M1</td>
<td>NK</td>
<td>8, then †</td>
<td>P/MS</td>
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<td>t(8;21)</td>
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<td>NK</td>
<td>9, then †</td>
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<td>Complex</td>
<td>&gt;5</td>
<td>B</td>
<td>0.8</td>
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</table>

NOTE: CD34+ cells were isolated from bone marrow (BM) or peripheral blood (PB); percentage CD34+ cells in the total AML mononuclear cell fraction; AMLs were categorized according to the French-American-British (FAB) classification; Karyotype is indicated; experiment carried out with PTK/ZK (P), and/or bevacizumab (B), and/or MK2206 (MK) and/or cultured on MS5-control/MS5-VEGFA stromal cells (MS).

†, stop leukemic expansion.

Abbreviation: x, not available.
the controls. After 10 weeks of culture, the inhibitory effect of PTK/ZK became even more pronounced in 75% of the samples (Table 2). Self-renewal potential was inhibited by PTK/ZK as shown by the reduced capability to initiate secondary cocultures after replating (Table 2). Analysis of May–Grunwald–Giemsa stains showed an enhanced differentiation in PTK/ZK-treated cells compared with the control cells. Control AML cells of AML5 and AML11 showed a less mature phenotype, whereas the treated AML cells displayed a more differentiating phenotype (Fig. 3A), suggesting that treatment with PTK/ZK might contribute to an impaired outgrowth and a more mature phenotype of these cells. These data indicate that PTK/ZK inhibited leukemic outgrowth and self-renewal potential of CD34 \(^+\) sorted pediatric AML blasts.

Outgrowth and self-renewal of pediatric LICs not affected by addition of VEGFA or treatment with bevacizumab

Previously, it was shown that VEGFA levels at diagnosis are an independent prognostic factor for relapse-free survival in (pediatric) AML. We, therefore, specifically determined whether VEGFA has a key role in the effect induced by PTK/ZK. VEGFA mRNA could be detected in all tested patient samples (\(n = 11\); Supplementary Fig. S2A). We investigated the effect of VEGFA signaling on the outgrowth of pediatric LICs by addition of VEGFA or treatment with bevacizumab. To obtain a constant supply of VEGFA in the long-term cocultures, MS5 stromal cells were transduced with a retroviral vector containing VEGFA165. A 30-fold upregulation of VEGFA165 mRNA was achieved in MS5-VEGFA cells compared with MS5 control cells (Supplementary Fig. S2B). Protein VEGFA levels at week 10 were measured and showed that VEGFA production was sustained during the culture (\(n = 2\); Supplementary Fig. S2C). In addition, functionality of the protein was confirmed (Supplementary Fig. S2D). Sorted CD34 \(^+\) cells of 6 pediatric patients with AML were plated onto transduced MS5 stromal cells. No significant effect was seen on the growth of the pediatric LICs when exposed to stroma-derived VEGFA compared with culture on MS5-control cells (Supplementary Fig. S2E; \(n = 6\), median value). Interestingly, PTK/ZK still remained its effect when cultured in an environment with VEGF
overexpression (by MS5-VEGFA cells). The median value of 6 samples cultured on MS5-VEGFA cells showed a significant ($P < 0.05$) inhibited expansion throughout week 2 to 6 and 8 to 9 when treated with PTK/ZK compared with control (data not shown).

To investigate whether selective targeting of the VEGFA-signaling would affect the leukemic outgrowth, we cultured LICs of 6 pediatric patients with bevacizumab, a monoclonal antibody to VEGFA. Treatment with bevacizumab did not result in a significant reduction of leukemic expansion after 10 weeks of culture (Supplementary Fig. S2F; $n = 6$, median value). Together, these data suggest that the effect of the tyrosine kinase inhibitor PTK/ZK is not mainly dependent on blocking VEGFA signaling. Also no response was seen in 3 AML samples when cultured with a specific antibody for VEGFR2 or VEGFR3, ascribing the results (data shown for AML6; Supplementary Fig. S2G).

PTK/ZK exerts its effect via a reduced phosphorylation of downstream targets, though mainly via the PI3K/Akt kinase pathway

To investigate the mechanisms by which PTK/ZK can inhibit long-term expansion of AML cells, we studied the expression of the RTKs inhibited by PTK/ZK. Data on mRNA level showed that expression of most of these RTKs was present in the AML cells although in different expression profiles (Fig. 4A). FACS analysis of the CD34+ sorted AML cells at start of the experiment showed that VEGF-R2-expressing cells could be detected in 11 of the 13 AML samples, ranging from 3% to 94% VEGF-R2-positive cells (Table 2; for 2 patients representative FACS data are shown in Fig. 4B). FACS results for VEGF-R1, VEGF-R3, c-KIT, c-FMS, and PDGFRb at the start of the cultures is shown. The response to PTK/ZK is shown, as compared with the growth of control group (% growth of control).

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Wk 5</th>
<th>Wk 10</th>
<th>VEGFR2</th>
<th>VEGFR1</th>
<th>VEGFR3</th>
<th>PDGFRb</th>
<th>c-KIT</th>
<th>c-FMS</th>
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<td>0</td>
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<td>x</td>
<td>ND</td>
<td>x</td>
<td>ND</td>
<td>x</td>
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<td>† (wk 7)</td>
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<td>34</td>
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<td>80</td>
<td>† (wk 9)</td>
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NOTE: Percentage of CD34+ cells that express protein levels of the tyrosine kinase receptors VEGFR1, VEGFR2, VEGFR3, c-KIT, c-FMS, and PDGFRb at the start of the cultures is shown. The response to PTK/ZK is shown, as compared with the growth of control group (% growth of control).

†, Stop leukemic expansion.

Abbreviations: ND, not detectable; x, not available.

Table 2. Protein expression of the tyrosine kinase receptors and response of the AML samples to PTK/ZK.
Figure 3. The effect of PTK/ZK on phosphorylation of downstream targets in pediatric LICs. A, morphologic analysis of suspension cells of AML5 and AML11 was conducted by May–Grunwald–Giemsa staining of cytopsins. B, representative images of Proteome Profiler Human Phospho-Kinase Array on sorted CD34+ cells treated with PTK/ZK or control (AML11). C, phosphorylation signal intensity of intracellular kinases from 3 pediatric AML samples treated with or without PTK/ZK, applied to the Proteome Profiler Human Phospho-Kinase Array. D, figure summarizing the affected downstream targets of PTK/ZK, identified by Proteome Profiler Human Phospho-Kinase Array. Treatment with PTK/ZK downregulated kinases within the PI3K/Akt signaling pathway (PI3K, Akt, TOR, p70S6), and slightly downregulated kinases involved in focal adhesion kinase pathway proteins (FAK, Paxillin) and Src kinase pathway proteins (Src, PYK2). Phosphorylation of the kinases PLCγ, RKS1/2, and cAMP-responsive element binding protein involved in the Ras/Raf/MEK/ERK pathway were decreased after addition of PTK/ZK, whereas MEK and ERK were differentially phosphorylated. Phosphorylation of kinases that belong to the MAPK pathway (p38, HSP27, JNK) and STAT pathway was differentially effected upon PTK/ZK. A decreased phosphorylation of c-Jun and cAMP-responsive element binding protein should result in reduced proliferation. The colors represent lower expressed (red), differentially expressed (yellow) kinases upon treatment with PTK/ZK; white kinases are not measured on the array. —— indicates stimulatory effect; —— indicates inhibitory effect.
Akt-inhibitor MK2206 resulted in declined percentages of cell survival and in an increase in the number of (early) apoptotic cells (Supplementary Fig. S1D and S2H–S2I).

In conclusion, treatment of pediatric LICs with PTK/ZK showed various differences in signaling networks, with a major role for the PI3K/Akt pathway, all resulting in lower cell proliferation and renewal.

Discussion

In this study, we showed for the first time that pediatric CD34+ sorted AML cells can be cultured in a long-term leukemic stem/progenitor assay. After replating the cultured AML cells onto new MS5, secondary L-CAs were generated underneath the stroma and maintained self-renewing capacity for up to 10 weeks in 9 of the 13 (69%) pediatric AML samples. Our results are in agreement with data on adult LICs, which show that sorted CD34+ cells from adult patients with AML can be cultured on a stromal layer (25).

In contrast with results from van Gosliga and colleagues, we were able to maintain a long-term culture up to 10 weeks in 2 samples from good risk patients (AML5 and AML11) although they were characterized by a slower expansion compared with other AML samples.

In our study, we cultured sorted CD34+ cells of 4 pediatric AML samples (AML2, AML5, AML6, and AML10) and could not initiate long-term expansion cultures and secondary cocultures, similar to data from van Gosliga and colleagues (data not shown; ref. 25).

The level of VEGFA at the time of diagnosis has been shown to be an independent prognostic factor for treatment outcome in (pediatric) AML (e.g., refractory disease or relapse; refs. 15, 17, 18). In addition, increased expression

Figure 4. Protein and mRNA expression of the tyrosine kinase receptors. A, relative mRNA expression of VEGFR1, VEGFR2, VEGFR3, c-KIT, c-FMS, and PDGFRB for 11 AML samples is shown. B, percentage cells positive for protein expression of the tyrosine kinase receptors VEGFR1, VEGFR2, VEGFR3, c-KIT, c-FMS, and PDGFRB at the start of the cultures was measured by FACS analysis. Data of AML6 and AML7 are shown.
PTK/ZK Impairs Pediatric LICs Outgrowth and Self-Renewal

of VEGFA correlated with the enhanced angiogenesis found in AML bone marrow biopsies (28, 29). It had been suggested that LICs reside within "stem cell niches," consisting among others of a vascular niche formed by sinusoidal endothelium lining blood vessels (4, 7, 30, 31). Moreover, these bone marrow niches might secrete growth factors including VEGFA (9–11). Because PTK/ZK was initially designed to block the VEGF/VEGFR signaling pathway with its main effect upon VEGFR2 inhibition, we specifically determined whether VEGFA has a key role in the effect induced by PTK/ZK. No effect was seen on the growth of pediatric samples when exposed to stroma-derived VEGFA or cultured in the presence of bevacizumab, independent on the mRNA VEGFA expression of the AMLs. Together, VEGFA alone does not significantly influence the growth of sorted CD34+/pediatric AML cells. Therefore, VEGFA signaling interference does not seem to be the main or only target for the inhibitory effects of PTK/ZK on LICs.

Activation of multiple signal transduction pathways, such as the Ras/Raf/MEK/ERK, PI3K/Akt/mTOR, and Jak/STAT pathways, have a progressively worse adverse effect on AML outcome (32–35). These downstream intracellular signaling pathways, functioning as an interrelating network, are activated by phosphorylation of RTKs. Our study showed that the addition of PTK/ZK reduces the outgrowth of pediatric LICs, whereas a more specified anti-VEGFA antibody or a specific VEGFR inhibitor has no effect on leukemic outgrowth. We hypothesize that PTK/ZK exerts its effect by blockade of multiple RTKs with a decrease in the network of signal transduction pathways, and therefore blockade of only VEGFA is not sufficient to induce a reduced leukemic expansion. These results are in line with new hypotheses that targeting multiple kinase pathways will be more beneficial for patients with AML than interfering with single growth factors or RTKs (34). Interestingly, pAKT was downregulated in all tested pediatric AML samples. It is known that AKT is a downstream target of multiple tyrosine kinase receptors, including VEGF receptors, c-KIT, c-FMS, and PDGFRβ (22–24). Therefore, pAKT might be the main downstream effect of PTK/ZK. Treatment of AML cells with a specific Akt-inhibitor showed similar results, underscoring the postulation that the impaired outgrowth of primary pediatric AML cells by PTK/ZK occurs via multiple receptor inhibition, resulting in a reduced phosphorylation of the PI3K/Akt pathway.

In conclusion, we showed for the first time that pediatric CD34+/LIC enriched AML cells expand and self-renew in long-term culture assays, and that addition of PTK/ZK to these cultures impairs these processes likely via targeting multiple downstream pathways. Taken together, our work suggests that PTK/ZK might be an effective approach in eradicating the primitive leukemic cell and could be a promising approach for improvement of AML (relapse) therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acknowledgments

The authors thank Kint Brewery for providing cytokines, Genentech/Roche for providing bevacizumab, and Novartis Pharmaceutical/Schering AG for providing PTK/87/2ZK22584S, S. Ronati for the microscopic evaluation of the tyrosins, Henk Moes, Geert Mesander, and Roelof Jan van der Leij for the help with flow cytometry, and ImClone for their generous supply of VEGFR-2 and VEGFR-3 antibodies.

Grant Support

This work was supported by a grant from the "Dutch Cancer Society" to E.S.J.M. de Bont (3661).

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Received February 27, 2012; revised December 7, 2012; accepted December 21, 2012; published OnlineFirst February 7, 2013.

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