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

Ailda C. Weidenaar¹, Arja ter Elst¹, Kim R. Kampen¹, Tiny Meeuwen-de Boer¹, Willem A. Kamps¹, Jan Jacob Schuringa², and Eveline S.J.M. de Bont¹

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.

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], VEGFR1 [fms-related tyrosine kinase 1 (FLT1), IC₅₀ 0.077 µmol/L], and VEGFR3 [fms-related tyrosine kinase 3 (FLT4), IC₅₀ 0.64 µmol/L; c-KIT (KIT, also known as CD117, IC₅₀ 0.300 µmol/L), PDGFR-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, IC₅₀ 0.300 µmol/L), PDGFR-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, IC₅₀ 0.300 µmol/L).
or stem cell factor receptor, IC50 0.73 μmol/L), and colony-stimulating factor-1 receptor (also known as c-FMS, IC50 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 PDGFRβ (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 × 10⁴ 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 (IMC1121b, 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 (FLT3-ITD–positive sample) that the suspension cells harbor the heterozygous FLT3-ITD mutation (86%).

**AML cell lines HL-60, NB4, THP-1, and MOLM13**

The cell lines THP-1, HL-60, NB4, and MOLM13 were obtained from American Type Culture Collection and cultured in RPMI-1640 medium supplemented with 1% penicillin/streptomycin (Sigma Aldrich) and 10% FBS (Hyclone).

**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-I-GFP (empty vector, negative control) and 2 μg packaging plasmid pCLamp into 293T cells using FuGENE HD transfection reagent (Roche). A total of 5 × 10⁴ 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 × 10⁶ 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 11A M L sample 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 (Phizer), × 5 first-strand buffer, RNasin, and reverse transcriptase (Gibco BRL). Real-time PCR (RT-PCR) was conducted using iQ 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-CD34-PE, anti-CD38-PerCP-Cy5.5, 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 LSRII (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.

Phosphokinease 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 are 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<sup>+</sup> 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<sup>−</sup> 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<sup>+</sup> 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&lt;sup&gt;+&lt;/sup&gt; cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML1</td>
<td>BM</td>
<td>M2</td>
<td>NK</td>
<td>10</td>
<td>P/MS</td>
<td>28.7</td>
</tr>
<tr>
<td>AML2</td>
<td>BM</td>
<td>M4</td>
<td>inv(16)</td>
<td>7, then †</td>
<td>P/B</td>
<td>68.8</td>
</tr>
<tr>
<td>AML3</td>
<td>BM</td>
<td>M0</td>
<td>9q-</td>
<td>10</td>
<td>P/MS</td>
<td>83.1</td>
</tr>
<tr>
<td>AML4</td>
<td>BM</td>
<td>M3</td>
<td>t(15;17)</td>
<td>9, then †</td>
<td>P/MS</td>
<td>16.8</td>
</tr>
<tr>
<td>AML5</td>
<td>BM</td>
<td>M3</td>
<td>t(15;17)</td>
<td>10</td>
<td>P/B</td>
<td>2.3</td>
</tr>
<tr>
<td>AML6</td>
<td>PB</td>
<td>M5</td>
<td>Complex</td>
<td>10</td>
<td>P/MK</td>
<td>0.5</td>
</tr>
<tr>
<td>AML7</td>
<td>BM</td>
<td>M0</td>
<td>Complex</td>
<td>10</td>
<td>P/MS</td>
<td>88</td>
</tr>
<tr>
<td>AML8</td>
<td>PB</td>
<td>M1</td>
<td>NK</td>
<td>8, then †</td>
<td>P/MS</td>
<td>15.2</td>
</tr>
<tr>
<td>AML9</td>
<td>BM</td>
<td>M4</td>
<td>NK</td>
<td>10</td>
<td>P/MS</td>
<td>48.4</td>
</tr>
<tr>
<td>AML10</td>
<td>BM</td>
<td>M2</td>
<td>t(6;9)</td>
<td>10</td>
<td>P/B</td>
<td>28.6</td>
</tr>
<tr>
<td>AML11</td>
<td>BM</td>
<td>M2</td>
<td>t(8;21)</td>
<td>10</td>
<td>P/B</td>
<td>77.9</td>
</tr>
<tr>
<td>AML12</td>
<td>BM</td>
<td>x</td>
<td>NK</td>
<td>9, then †</td>
<td>B</td>
<td>61.1</td>
</tr>
<tr>
<td>AML13</td>
<td>BM</td>
<td>M5</td>
<td>Complex</td>
<td>&gt;5</td>
<td>B</td>
<td>0.8</td>
</tr>
<tr>
<td>AML14</td>
<td>BM</td>
<td>M5</td>
<td>Complex</td>
<td>&gt;5</td>
<td>MK</td>
<td>64.0</td>
</tr>
</tbody>
</table>

NOTE: CD34<sup>+</sup> cells were isolated from bone marrow (BM) or peripheral blood (PB); percentage CD34<sup>+</sup> 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.

Figure 2. The effect of PTK/ZK on the outgrowth of pediatric LICs. Growth curves of 11 AMLs. At week 5, the leukemic cells both in suspension and adherent were harvested and replated on new MS5 to initiate secondary cocultures. † indicates stop leukemic expansion.
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 VEGFR2-expressing cells could be detected in 11 of the 13 AML samples, ranging from 3% to 94% VEGFR2-positive cells (Table 2; for 2 patients representative FACS data are shown in Fig. 4B). FACS results for VEGFR1, VEGFR3, c-KIT, c-FMS, and PDGFR-b are shown in Table 2. Interestingly, PTK/ZK responsiveness was not found to be correlated with the expression of one particular receptor (Table 2).

To measure downstream effects of the reduced outgrowth of CD34+ pediatric AML cells induced by PTK/ZK, cell lysates of 3 AML samples treated with or without PTK/ZK were subjected to a Human Phospho-Kinase Array Kit (Fig. 3B). Phosphorylation of PI3K/Akt kinases (AKT, TOR, p70S6K) was clearly reduced in all 3 samples, whereas phosphorylation of focal adhesion kinase (FAK, Paxillin) and Src kinase (Src, Lck, PYK2) signaling pathways were slightly reduced upon treatment with PTK/ZK in all 3 AML samples (Fig. 3C; complete list shown in Supplementary Table S2). Phosphorylation of RAS/RAF/MEK/ERK kinases and STAT kinases was differentially affected upon treatment with PTK/ZK (Fig. 3C). In 2 AML cell lines (MOLM13 and THP-1), we confirmed a downregulation of pAKT independent of total AKT levels after 48 hours of incubation with PTK/ZK compared with control (Supplementary Fig. S1C). pERK was found to be downregulated in MOLM 13, whereas pSrc and total Src levels did not significantly differ between cells treated with PTK/ZK compared with control. In summary, downregulation of signaling via pAKT might be the main downstream effect of PTK/ZK. Therefore, we evaluated whether inhibition of this specific pathway has a similar effect on cell survival and apoptosis in AML cells. In 3 AML cell lines and primary AML samples, treatment with increasing doses of a specific

### Table 2. Protein expression of the tyrosine kinase receptors and response of the AML samples to PTK/ZK.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>% Growth of control</th>
<th>FACS at the start</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wk 5</td>
<td>Wk 10</td>
</tr>
<tr>
<td>AML1</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>AML2</td>
<td>45</td>
<td>† (wk 7)</td>
</tr>
<tr>
<td>AML3</td>
<td>29</td>
<td>7</td>
</tr>
<tr>
<td>AML4</td>
<td>80</td>
<td>† (wk 9)</td>
</tr>
<tr>
<td>AML5</td>
<td>62</td>
<td>40</td>
</tr>
<tr>
<td>AML6</td>
<td>48</td>
<td>12</td>
</tr>
<tr>
<td>AML7</td>
<td>4</td>
<td>69</td>
</tr>
<tr>
<td>AML8</td>
<td>9</td>
<td>† (wk 8)</td>
</tr>
<tr>
<td>AML9</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>AML10</td>
<td>38</td>
<td>17</td>
</tr>
<tr>
<td>AML11</td>
<td>66</td>
<td>90</td>
</tr>
<tr>
<td>AML12</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>AML13</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>AML14</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

**NOTE:** Percentage of CD34+ cells that express protein levels of the tyrosine kinase receptors VEGFR1, VEGFR2, VEGFR3, c-KIT, c-FMS, and PDGFR-b 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.
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, RSK1/2, 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). Together, our results are in agreement with data on LTC-IC cultures of adult AML samples and show that the growth kinetics of pediatric AML samples overlap with the growth features of adult AML cells.

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...
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, VEGF 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/Mapk/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 mRNA VEGFA expression of the AMLs. Together, VEGFA alone does not significantly influence the growth of sorted CD34+/pediatric AML cells. Therefore, VEGF signaling interference does not seem to be the main or only target for the inhibitory effects of PTK/ZK on LICs.

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.

Authors’ Contributions
Conception and design: A.C. Weidenaar, A.T. Elst, W.A. Kamps, E.S.J.M. de Bont
Development of methodology: K.R. Kampen, T.G.J. Meeuwen-de Boer, J.J. Schuringa
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.G.J. Meeuwen-de Boer, E.S.J.M. de Bont
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.C. Weidenaar, E.S.J.M. de Bont
Writing, review, and/or revision of the manuscript: A.C. Weidenaar, A.T. Elst, E.S.J.M. de Bont
Study supervision: A.T. Elst, W.A. Kamps

Acknowledgments
The authors thank Kintin Brewery for providing cytokines, Genentech/Roche for providing bevacizumab, and Novartis Pharmaceuticals/Schering AG for providing PTK/87/ZK 222584, S. Ronai for the microscopic evaluation of the cytospins, Henk Moes, Geert Mesander, and Roelof van der Lei 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).

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

References
11. Tombran-Tink J, Barnstable CJ. Osteoblasts and osteoclasts express PEDF, VEGF-A isoforms, and VEGF receptors: possible mediators of


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


Updated version

Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-12-0113

Supplementary Material

Access the most recent supplemental material at:
http://mcr.aacrjournals.org/content/suppl/2013/02/07/1541-7786.MCR-12-0113.DC1

Cited articles

This article cites 35 articles, 16 of which you can access for free at:
http://mcr.aacrjournals.org/content/11/4/339.full.html#ref-list-1

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.