Addition of PTK787/ZK 222584 can lower the dosage of amsacrine to achieve equal amounts of acute myeloid leukemia cell death

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Acute myeloid leukemia (AML) is a disease with a poor prognosis. It has been demonstrated that AML cells express the vascular endothelial growth factors, VEGFA and VEGFC, as well as kinase insert domain-containing receptor (VEGFR2), the main receptor for downstream effects, resulting in an autocrine pathway for cell survival. This study investigates the role of the VEGFR inhibitor PTK787/ZK 222584 in leukemic cell death, and the possibility of an additional effect on cell death by a chemotherapeutic drug, amsacrine. In three AML cell lines and 33 pediatric AML patient samples, we performed total cell-kill assays to determine the percentages of cell death achieved by PTK787/ZK 222584 and/or amsacrine. Both drugs induced AML cell death. Using a response surface analysis, we could show that, in cell lines as well as in primary AML blasts, an equal magnitude of leukemic cell death could be obtained when lower doses of the more toxic amsacrine were combined with low dosages of the less toxic VEGFR inhibitor. This study shows that PTK787/ZK 222584 might have more clinical potential in AML when combined with a chemotherapeutic drug such as amsacrine. In future, it will be interesting to study whether the complications and the long-term effects of chemotherapy can be reduced by lowering the dosages of amsacrine, and by replacing it with other drugs with lower toxicity profiles, such as PTK787/ZK 222584. *Anti-Cancer Drugs* 19:45–54 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The outcome of patients with acute myeloid leukemia (AML) has improved owing to intensive chemotherapy combined with a reduction in treatment-related mortality. The long-term disease-free survival, however, varies between 13 and 44%, depending on age and cytogenetics; therefore, new treatment strategies are still warranted [1–3].

The level of vascular endothelial growth factor (VEGF) A at time of diagnosis has been described to be an independent prognostic factor for treatment outcome in pediatric AML: high VEGFA levels in AML cells are related to worse outcomes [4,5]. VEGFA is the prototype of the VEGF family, a group of angiogenic proteins, and it was originally cloned from the HL-60 cell line. It seemed to be a potent stimulator of endothelial cell migration and proliferation [6]. Differential splicing of exon 6 and/or exon 7 results in various isoforms: the 121- and the 165- amino-acid isoforms have the most potent activity on endothelial cells [7]. In the meantime, six VEGFs are described; VEGFA to VEGFF [8–12]. These proteins bind with various binding abilities to two cell-surface receptor families: the tyrosine kinase receptors (VEGFRs) and the neuropilin (NRP) receptors. Three VEGFRs have been identified, namely, VEGF receptor (VEGFR1) (FMS-like tyrosine kinase 1, FLT1), VEGFR2 [kinase insert domain-containing receptor (KDR)] and VEGFR3 (FLT4) [13–15]. Only two neuropilins (NRP1 and NRP2) have been described until now [16,17]. NRP1 seems to act as a coreceptor that enhances VEGF165 binding to KDR on endothelial cells [18].

KDR is thought to be the main receptor for downstream effects of VEGF, and its deletion in mice is lethal [19]. KDR is expressed not only in endothelial cells, in which stimulation enhances the proliferation and differentiation of endothelial cells, but also in AML cells [20]. In response to KDR stimulation, intracellular signals are transmitted, such as signaling via mitogen-activated protein kinase, the phosphatidylinositol 3-kinase (PI3K/Akt) cascades or via the signal transducers and activators

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of transcription pathway, resulting in leukemic cell proliferation and cell survival [21–23]. Antiapoptotic effects of VEGFC have also been described by FLT4 signaling in AML [24].

The production of VEGFA and VEGFC, as well as the VEGFR expression by AML cells, resulted in the knowledge that an autocrine pathway for cell survival exists. Interference with the autocrine VEGF pathway by the blockade of VEGF/VEGFR signaling might, therefore, result in increased apoptosis in leukemic cells.

PTK787/ZK 222584 inhibits the phosphorylation of VEGF receptor tyrosine kinases [25]; it not only exerts its major effect on KDR (IC_{50} 0.037 μmol/l), but it also inhibits FLT1 (IC_{50} 0.077 μmol/l), FLT4 (IC_{50} 0.64 μmol/l) and other tyrosine kinases such as PDGFR-β (IC_{50} 0.58 μmol/l), c-kit (IC_{50} 0.73 μmol/l) and c-Fms (IC_{50} 1.4 μmol/l). The aim of this study was to gain more insight into the effect of PTK787/ZK 222584 on cell death in AML cell lines and primary AML cells. Moreover, the additional effect of VEGFR inhibition on cell death achieved by a conventional cytostatic drug such as amsacrine was investigated.

Methods

Acute myeloid leukemia cell lines and primary acute myeloid leukemia blasts

The cell lines HL-60, TF-1, THP-1 and K562 were obtained from American Type Culture Collection (Manassas, Virginia, USA), cultured in RPMI-1640 medium supplemented with penicillin/streptomycin and 10% fetal bovine serum (Hyclone, Logan, Utah, USA) for HL-60, THP-1 and K562 cells and additionally supplemented with 1 ng/ml granulocyte monocyte colony stimulating factor for TF-1. Before incubation with amsacrine (Pfizer, Capelle aan den IJssel, The Netherlands) and/or PTK787/ZK 222584 (a kind gift from the joint development project between Novartis Pharmaceuticals, Basel, Switzerland and Schering AG, Berlin, Germany), AML cell lines were serum starved overnight in serum-free medium.

After getting written informed consent, primary AML blast samples at diagnosis from pediatric AML patients were obtained from the Dutch Childhood Oncology Group (DCOG), The Hague, The Netherlands, in accordance with the regulations and protocols of the medical ethics committee. Diagnoses of AML were confirmed at the laboratory of the DCOG. Table 1 [26] summarizes the patient characteristics. Mononuclear cells were separated by using Lymphoprep (Nycomed, Oslo, Norway) density gradients, which had been cryopreserved in liquid nitrogen until use. The cryopreserved AML cells were thawed rapidly at 37°C and diluted in a 5 × volume of normal calf serum, as described previously [27]. The remaining blast cell population contained > 95% AML cells, and is referred to hereafter as AML cells.

Table 1 Characteristics of pediatric AML patients

Characteristics	
No.	33
Sex (male/female), *n*	22 : 11
Age at diagnosis, years	9 (0–16)
Leukocytes at diagnosis, $\times 10^9$/l	127.6 (7.5–355.0)
Thrombocytes at diagnosis, $\times 10^9$/l	45.0 (7.0–221.0)
FAB classification, *n*	
M0	5
M1	2
M2	0
M3	0
M4	16
M4eo	2
M5	6
M5A	1
Unknown	1
Died, *n*	16

The characteristics (age, leukocytes and thrombocytes) are given as median (range).
AML, acute myeloid leukemia; FAB, French American British [26].
No., number of patient samples.

RNA extraction and RT-PCR

Total RNA was extracted by Trizol methods, following the description of the manufacturer (Life Technologies, Gibco/BRL, Grand Island, New York, USA). Complementary DNAs (cDNAs) were prepared by reverse transcription at 37°C, for at least 1 h, in a 20-μl reaction mixture containing 2 μg of total RNA, random hexamers (Pfizer), 5 × first-strand buffer, RNAsin and reverse transcriptase (Gibco/BRL). cDNA was amplified in the presence of primers, 10 × buffer, 1.5 mmol/l $MgCl_2$, dNTPs and Taq (Gibco/BRL). The mixture was amplified in a Perkin Elmer apparatus with PCR cycle conditions specific for the PCRs tested. The PCR product was analyzed by electrophoresis in 1.5% agarose gel. Gels were stained with ethidium bromide and photographed. Specific primers for β_2-microglobulin were sense (CCA GCA GAG AAT GGA AAG TC) and antisense (GAT GCT GCT TAC ATG TCT CG); PCR product: 260 bp; 22 cycles and annealing temperature 55°C.

For VEGFA, sense (GAG TGT GTG CCC ACT GAG GAG TCC AAC) and antisense (CTC CTG CCC GGC TCA CCG CCT CGG CTT); PCR product: 177, 312, 384 bp; 35 cycles and annealing temperature of 55°C were used. The primers for VEGFA span the splice junctions, enabling the amplified product of splice variants to be separated electrophoretically.

For VEGFC, sense (AGG CTG GCA ACA TAA CAG AGA A) and antisense (TGT AAT TGG TGG GGC AGG TC), PCR product 479 bp; 30 cycles and annealing temperature of 65°C were used.

Fluorescence-activated cell sorting

KDR protein expression was measured with the monoclonal anti-VEGF receptor 2 mouse immunoglobulin (IgG1) isotype (Sigma, St Louis, Missouri, USA), which recognizes an internal epitope of the KDR protein. Cells (0.5×10^6) were washed and incubated for 15 min at room temperature, in phosphate-buffered saline (PBS). Subsequently, the cells were incubated for 20 min at 4°C with 1.0 μg of monoclonal anti-VEGFR2 mouse IgG1 isotype or with 1.0 μg of IgG1 isotype control (BD Biosciences, San Jose, California, USA). Cells were washed with PBS and incubated for 20 min at 4°C with phycoerythrin-conjugated rabbit-antimouse F(ab)2 fragments (DakoCytomation, Glostrup, Denmark). Phycoerythrin fluorescence was measured on a FACScalibur flow cytometer (BD Biosciences, Erembodegem, Belgium) and expressed as median fluorescence intensity. The control cell line used to standardize the KDR protein expression assay included the KDR-negative cell line K562.

Cellular drug-resistance measurement using a total cell-kill assay

For the cell lines, amsacrine (0.001–1 μg/ml) at different concentrations, and/or the VEGFR inhibitor PTK787/ZK 222584 (5–100 μmol/l) at different concentrations, were tested, both in 96-well microculture plates, in quadruplicate. A total of 33 patient samples were studied for the effect of the VEGFR inhibitor PTK787/ZK 222584 (5–100 μmol/l), amsacrine (1 μg/ml) or a combination of those drugs. In two patient samples, combined testing was impossible owing to the low cell number. In six pediatric AML samples, more concentrations of amsacrine (0.001–2 μg/ml) and/or PTK787/ZK 222584 (5–50 μmol/l) in quadruplicate were studied. The pharmacological profile of PTK787/ZK 222584 shows inhibition of the VEGF receptor tyrosine kinases. The strongest inhibition is found against KDR (IC_{50} 0.037 μmol/l); it exhibits a weaker inhibition of FLT1 (IC_{50} 0.077 μmol/l) and FLT4 (IC_{50} 0.64 μmol/l). At higher concentrations, the VEGFR inhibitor also inhibits other kinases belonging to the same class as the VEGFRs, the PDGFR-β, c-kit and c-Fms [25]. The in-vitro cellular drug resistance of the cell lines and patient samples (100 000 cells/well) were assessed using a 3-day cell-culture assay, on the basis of the principle that only viable cells would be able to reduce MTT (5 mg/ml in PBS), added to each well for 4 h, to a colored formazan product that could be measured spectrophotometrically at 520 nm, as described earlier [28]. The optical density (OD) in the MTT assay was linearly related to the number of viable cells. Control wells contained only leukemic cells, with culture media without drugs, and blank wells contained only culture media. Percentages of cell survival were calculated at each drug concentration by the equation (mean OD treated wells/mean OD control wells) × 100%, after correcting for the background found in the blank wells. The results were considered evaluable if the control wells still contained 70% or more leukemic cells [determined by May–Grunwald–Giemsa (MGG) staining] after a 3-day culture period. The mean OD of the control wells, after correction for the background, at 3 days always exceeded 0.1 arbitrary units for valid results. The LC_{50} value (drug concentration needed to kill 50% of the leukemic cells) was used to compare the differences between patients and/or various drugs combinations. LC_{50} value equation: ([% leukemic cell survival > 50%] – 50)/([% leukemic cell survival > 50%] – [% leukemic cell survival < 50%]) × (drug concentration when leukemic cell survival < 50% – drug concentration when leukemic cell survival > 50%) + (drug concentration when leukemic cell survival > 50%) [28].

Statistical analysis

The purpose of the statistical analysis was to study the relationships between the survival (S) (after correction for background activity of media culture) and the cytostaticum concentration (CC) and VEGFR inhibitor concentration (VC). For this purpose we used a type of a response surface analysis. The survival proportions (S) were transformed into logits $Y = \log[S/(1-S)]$, and mixed-effects models were fitted to the data [29]. Y was considered as being a normally distributed response variable; VC and CC were considered to be variables defining the fixed-effects parameters of the model, and replications entered the model as random intercepts. For patient data, the model also included the random effects due to patients.

The building of a model proceeded as follows. First, we fitted separate models for VC without CC and for CC without VC. These preliminary analyses were used to look for suitable transformations for VC and CC, which were as simple as possible and such that the model assumptions were satisfied. On the basis of these preliminary analyses, VC and CC were transformed into $V = \log(VC + 25)$ and $C = \log(CC + 0.01)$. For the cell lines HL-60, TF-1 and THP-1, datapoints numbering 4, 2 and 4, respectively, seemed to be outliers – with the absolute value of standardized residuals exceeding 3 – and it was decided to exclude these points from the analysis. Fitting mixed-effects models with polynomials in V and C and analyzing their interactions revealed that the data are well described by the following: for the cell line HL-60, the fourth-order polynomial in V, third-order in C and the third-order in product terms (comprising 15 fixed-effects parameters); for the cell line TF-1, the fourth-order polynomial in both variables and in product terms (19 parameters) and for the cell line THP-1, by the third-order polynomial in both variables and in product terms (13 parameters). For patient cells, the best-fitting model included eight fixed-effects parameters.

Next we proceeded to fit joint models in both transformed variables. The best-fitting model was used

to determine isoboles, that is, concentration combinations resulting in the same cell survival. Computations were carried out in S-PLUS 6.2 software (Insightful, Seattle, Washington, USA).

The Wilcoxon signed-rank test and the Spearman's rank-correlation test were used to compare differences between groups.

Results

In leukemic cell lines the vascular endothelial growth factor receptor inhibitor PTK787/ZK 222584 can partially replace amsacrine, resulting in equal extent of cell death

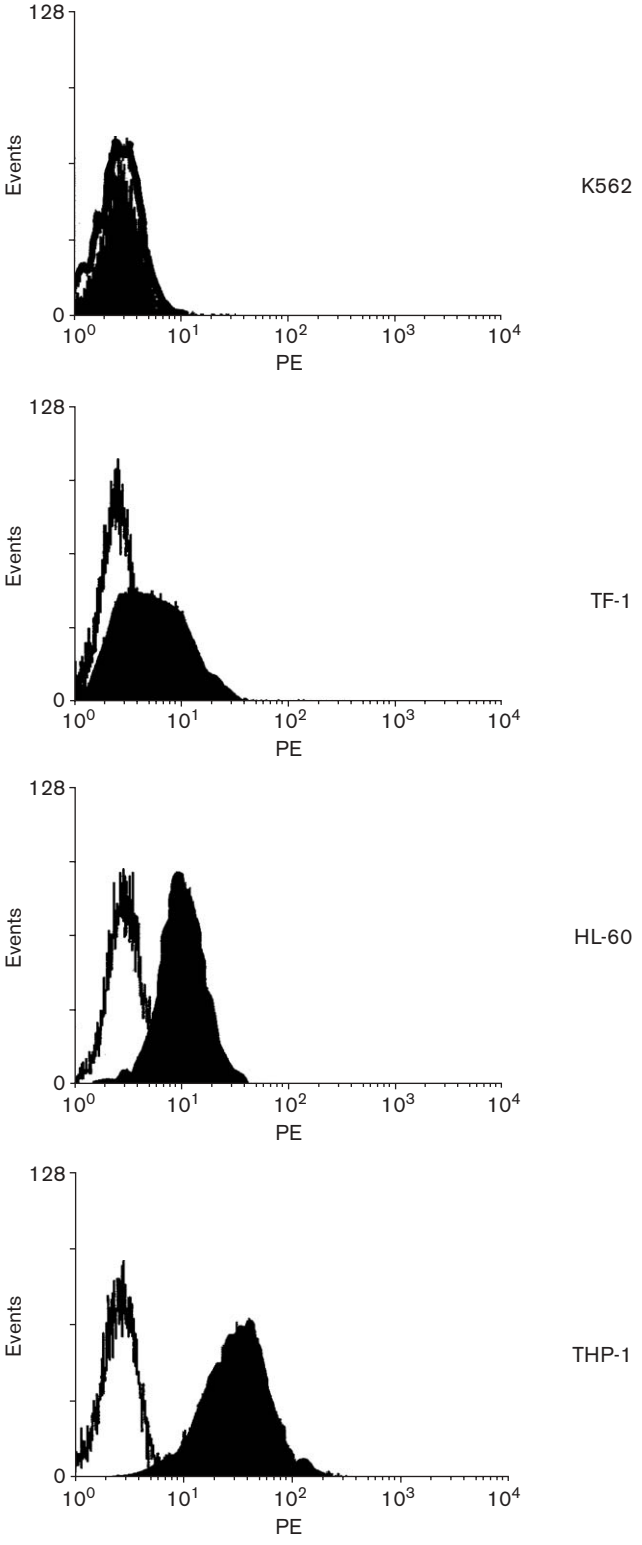
To investigate whether cell lines can be potential targets for the VEGFR inhibitor PTK787/ZK 222584, we first determined the expression of VEGFA, VEGFC and KDR. THP-1, TF-1 and HL-60 expressed three splice variants of VEGFA (121, 165 and 189 amino acids). In contrast to VEGFA, only THP-1 cells showed mRNA production of VEGFC (data not shown). Next we determined KDR protein expression (Fig. 1). THP-1 showed the strongest expression of KDR compared with the cell line K562, whereas TF-1 cells were only slightly positive for KDR. PTK787/ZK 222584 is a potent inhibitor of VEGF receptor tyrosine kinases [25]; it inhibits VEGF-induced phosphorylation, and its action can be abolished by the addition of VEGFA. PTK787/ZK 222584 is most potent against KDR, but it also has an effect on FLT1 and FLT4.

Direct effects of PTK787/ZK 222584 on the four leukemic cell lines were determined. In a total cell-kill assay, we demonstrated that the VEGFR inhibitor induces cell death in all three cell lines, as shown in Fig. 2a (LC_{50} values for HL-60: 27 μmol/l; TF-1: 49 μmol/l; THP-1: 24 μmol/l). Excellent cell survival was seen in the KDR-negative cell line K562, demonstrating that the cell death induced by PTK787/ZK 222584 at identical concentrations was not a toxic effect.

As shown before by others, phosphorylation of KDR was decreased dose-dependently when the inhibitor PTK787/ZK 222584 was used in our system (data not shown) [25].

The topoisomerase inhibitor amsacrine is one of the drugs used in AML treatment. For this experiment amsacrine was chosen because of reproducible and moderate cell-death results in all cell lines; with a drug resulting in hardly 50% of cell death at full dose, as well as with a drug resulting in massive cell death at the lowest concentration, it will be hard to show the additional effects of other agents *in vitro*. Leukemic cell survival after incubation with various concentrations of amsacrine is shown in Fig. 2b. Our results clearly point out that all three cell lines were sensitive to amsacrine, with LC_{50} values of

Fig. 1

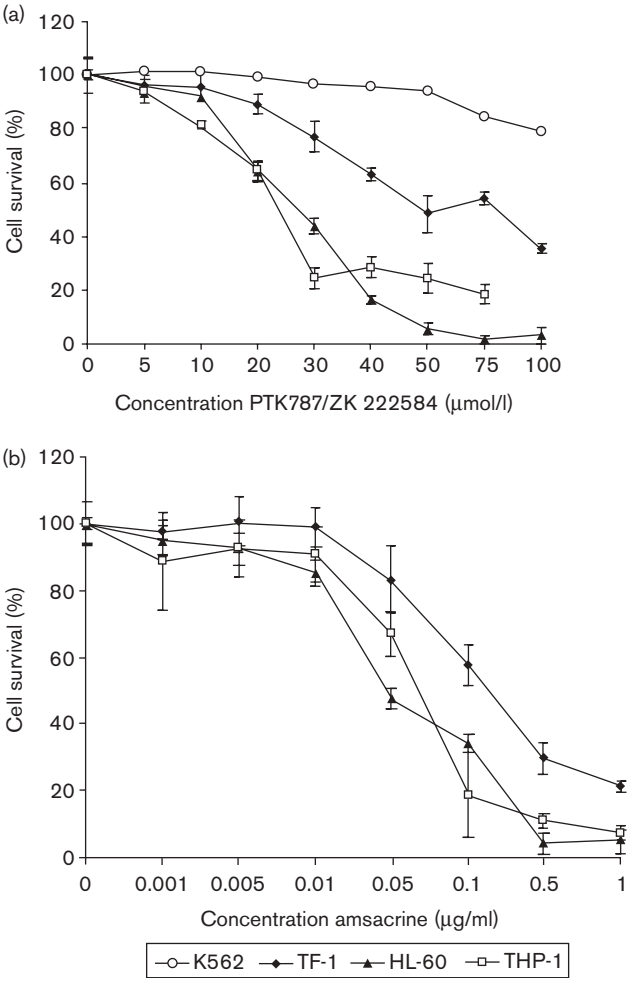


FACS analysis of the kinase insert domain-containing receptor (KDR) expression in the leukemic cell lines TF-1, THP-1, HL-60 and K562. THP-1 showed the strongest expression of KDR compared with the negative control cell line K562. HL-60 showed a weaker expression, whereas TF-1 cells were only slightly positive for KDR. PE, phycoerythrin.

0.047 μg/ml for HL-60, 0.207 μg/ml for TF-1 and 0.067 μg/ml for THP-1.

To investigate the additional effects of VEGFR inhibition on the cell death achieved by amsacrine, we combined both drugs in a total cell-kill assay. A response surface analysis was used, which emphasizes the finding of a particular treatment combination that can evoke maximum or minimum responses. Figure 3 presents plots of cell survival as a function of concentrations of both drugs. For each cell line the raw data are displayed in the first plot (Fig. 3a, d, g). The raw data are transformed into predicted survival models, which are based on the best-fitting models for the polynomials and parameters (Fig. 3b, e, h). The raw data and the model-predicted survival demonstrate, for each cell line, that when increasing the concentration of one or both drugs, the survival percentage of the AML cells will decrease. Next, the best-fitting model was used to determine isoboles (Fig. 3c, f, i). Isoboles estimate drug combinations that result in the same survival; the lines in the figure represent the drug combinations at which 30, 40, 50, 60 or 70% (0.3–0.7) cell survival will be achieved. For example, for TF-1 Fig. 3c suggests that a 50% leukemic cell survival (the line 0.5) can be achieved as follows: this can be the result of amsacrine alone dosed at 0.15 μg/ml or amsacrine at 0.12 μg/ml combined with 20 μmol/l PTK or 0.03 μg/ml amsacrine combined with 80 μmol/l PTK. Interestingly, the isoboles for each cell line point out that the concentration of amsacrine can be lowered by a certain dose of the potentially less toxic VEGFR inhibitor PTK787/ZK 222584, to achieve the same percentage of leukemic cell death.

Fig. 2



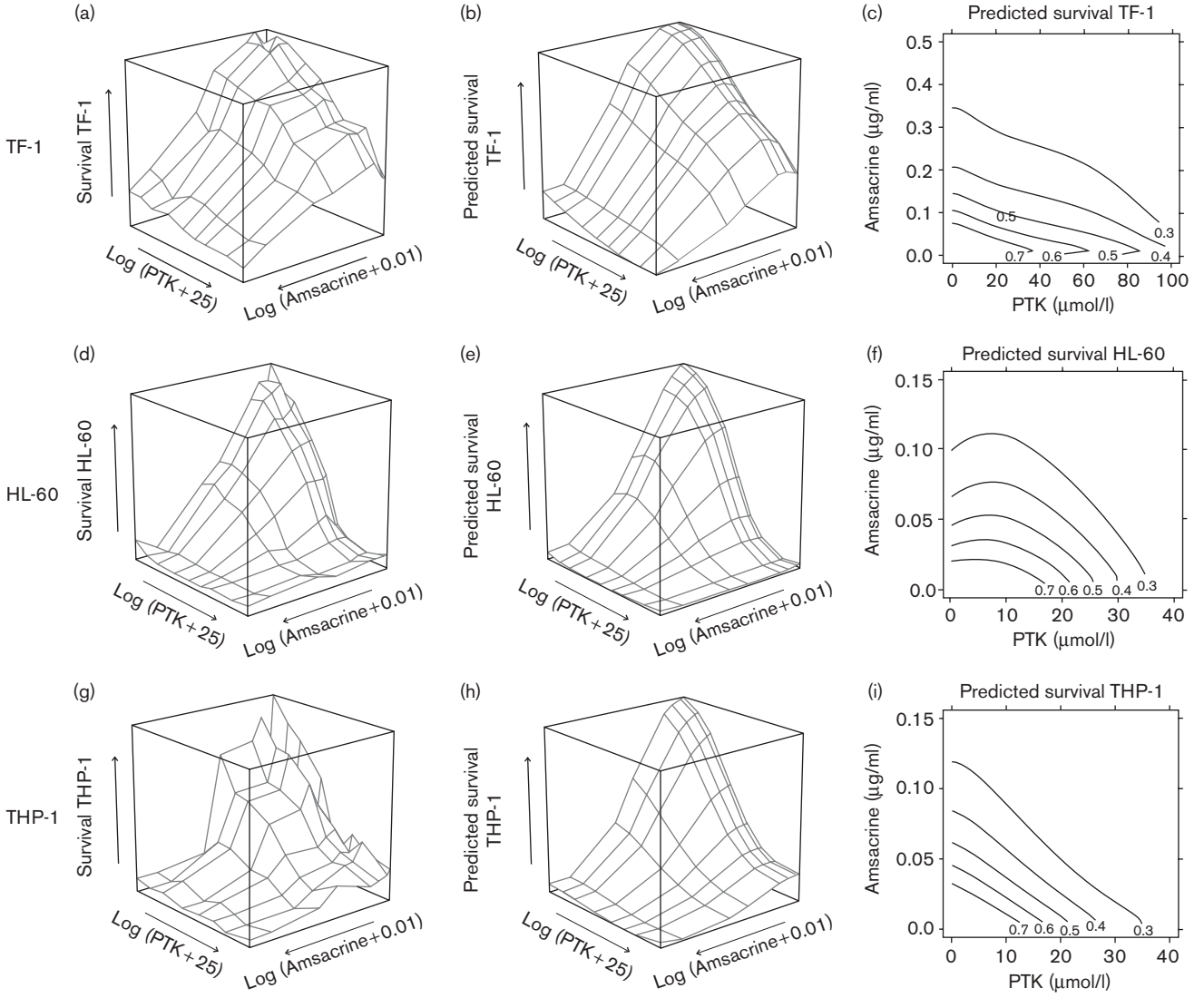
(a) Cell-survival percentages after incubation with the vascular endothelial growth factor receptor (VEGFR) inhibitor PTK787/ZK 222584 were determined by using an MTT assay. The data indicates that the VEGFR inhibitor induces cell death in all three cell lines. HL-60 showed the strongest response, whereas TF-1 is least sensitive to the VEGFR inhibitor. Values are expressed as means; bars, SE. (b) Cell-survival percentages after incubation with amsacrine at different concentrations were determined by using an MTT assay. The data clearly indicates that amsacrine induces cell death in all three cell lines. Values are expressed as means; bars, SE.

PTK787/ZK 222584 increases cell death in primary leukemic blasts when combined with amsacrine

Underscoring the results in the literature, KDR expression was demonstrated in all the AML samples tested [30]. Figure 4 shows the LC_{50} values of the primary AML samples with a median of 5.1 μmol/l ($n = 33$). Primary blast samples are overall 5–10-fold more sensitive to PTK787/ZK 222584 than the leukemic cell lines used in this study. Patients with an LC_{50} value above the median did not differ from patients with an LC_{50} value below it, with regard to age at diagnosis, sex, French American British classification, leukocyte count or thrombocyte count. Moreover, there was no relationship between the expression of KDR in the patients we tested and sensitivity to PTK787/ZK 222584 (Spearman's $\rho = -0.63$, $P = 0.825$).

The next step was to test the addition of the VEGFR inhibitor PTK787/ZK 222584 to amsacrine on the primary AML blasts, and to determine the cell-death percentage induced by both drugs ($n = 31$). We first treated the patient samples with 1 μg/ml amsacrine alone; we chose 1 μg/ml amsacrine because this concentration was near the LC_{50} value of the patient samples. A varying percentage of cell death was seen for 1 μg/ml amsacrine alone, with a median value of 60.6%. Interestingly, we found a correlation between the LC_{50} values of the VEGFR inhibitor PTK787/ZK 222584 and the cell-death percentages induced by 1 μg/ml amsacrine (Spearman's $\rho = -0.386$, $P = 0.032$), which means that samples sensitive to one drug are also sensitive to the other drug. Two samples achieved a cell-death percentage of $> 90\%$ and were excluded from further combination therapy, because it was not possible to show additional effects of the VEGFR inhibitor PTK787/ZK 222584.

Fig. 3

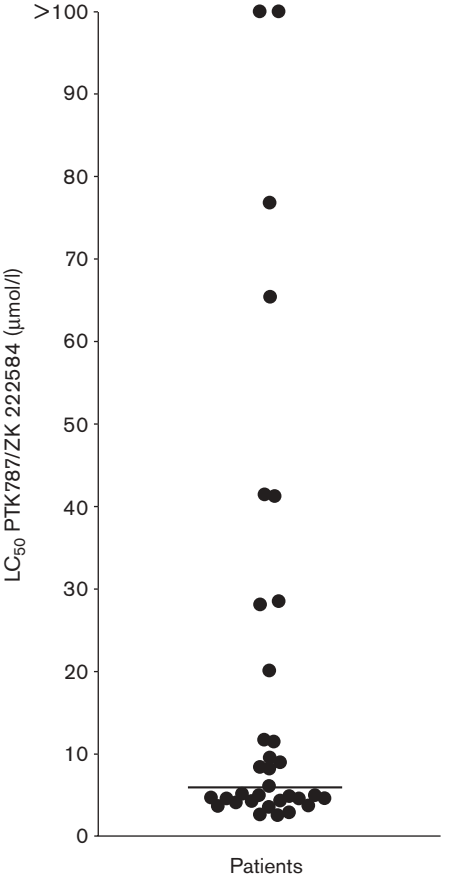


Interaction data between the vascular endothelial growth factor receptor (VEGFR) inhibitor PTK787/ZK 222584 (PTK) and amsacrine for the three acute myeloid leukemia cell lines. (a, d, g) Three-dimensional (3D) plots presenting the raw data for each cell line. (b, e, h) 3D plots presenting a predicted model based on the raw data are demonstrated for all three cell lines. In these six figures, the vertical axis represents the (predicted) survival of the cell line, estimated at different concentrations of both drugs (horizontal axes). These six plots show that increasing the concentration of one or both drugs (in the figures named log amsacrine and log PTK) results in a decrease of the survival. (c, f, i) A model that can be used to calculate drug combinations that result in a predicted survival, the so-called isoboles. The lines in these figures (shown as 0.3–0.7) represent the drug combinations at which 30, 40, 50, 60 or 70% of cell survival will be achieved. All three figures point out that the concentration of amsacrine can be lowered and replaced by a dose of the VEGFR inhibitor PTK787/ZK 222584, to achieve the same cell-death percentage. In the 3D plots, the (transformed) PTK and amsacrine axes extend over the full range of values as used in the experiments, respectively, 0–50 and 0–2. The scale used in the isobole plots differs per cell line.

Next, 29 patient samples were treated with 1 µg/ml amsacrine and 25 µmol/l PTK787/ZK 222584, with a median value of 92.3% cell death. Figure 5 demonstrates the cell-death percentage achieved by 1 µg/ml amsacrine with 25 µmol/l PTK787/ZK 222584 compared with the cell-death percentage achieved by 1 µg/ml amsacrine alone. The addition of 25 µmol/l PTK787/ZK 222584 resulted in an increase in cell death of $> 20\%$ (median increase in cell death 24%) in 18 of the 29 samples (62%), whereas 10 samples showed a moderate increase between 0–20%. No significant additional cell death was demonstrated when PTK787/ZK 222584 dosage was increased up to 100 µmol/l.

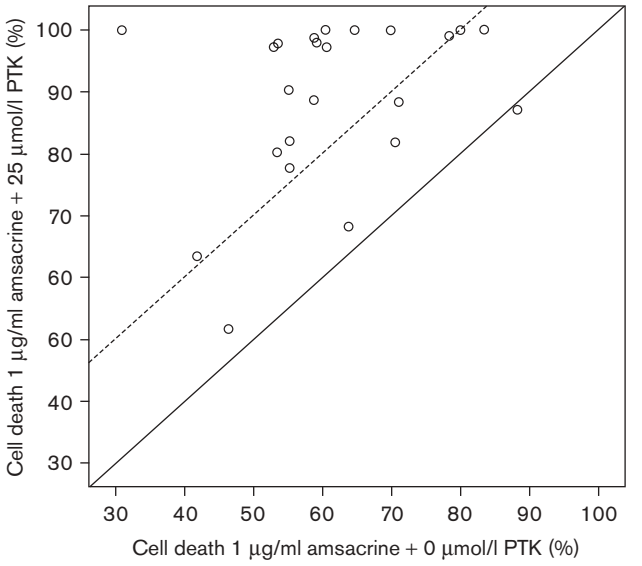
In addition, for six AML patient samples the combination of amsacrine and PTK787/ZK 222584 treatment was investigated more extensively to obtain isoboles for the AML patient samples. The interaction between PTK787/

Fig. 4



The LC_{50} value of 33 pediatric acute myeloid leukemia samples: the concentration needed to kill 50% of the cells after incubation with the vascular endothelial growth factor receptor inhibitor PTK787/ZK 222584. The bar represents the median value of 5.1 μmol/l. In two primary blast samples, the LC_{50} value was above 100 μmol/l PTK787/ZK 222584. Patients with an LC_{50} value above the median did not differ from those with an LC_{50} value below the median with regard to age at diagnosis, sex, French American British classification or white blood cell count.

Fig. 5



The cell-death percentage achieved by 1 μg/ml amsacrine combined with 25 μmol/l PTK787/ZK 222584 (PTK) compared with that achieved by 1 μg/ml amsacrine alone ($P<0.001$, Wilcoxon signed-rank test). The solid line represents equal extent of cell death with amsacrine ± PTK787/ZK 222584, whereas the dashed line represents 20% increase in cell death with addition of PTK787/ZK 222584. The samples above the dashed line achieved an increase in cell death of more than 20% when treated with 25 μmol/l PTK787/ZK 222584 added to 1 μg/ml amsacrine; 18 of the 29 samples showed an increase in cell death of more than 20%.

ZK 222584 and amsacrine for primary AML blasts is given in Fig. 6. Again in Fig. 6a the raw survival data are demonstrated, whereas Fig. 6b represents the model predicted using the raw data. In Fig. 6c the isoboles are shown. Identical to the results from the cell line, in primary patient material also we were able to demonstrate that lowering the concentration of the chemotherapeutic drug amsacrine resulted in identical cell death, by adding a certain dose of the potentially less toxic VEGFR inhibitor PTK787/ZK 222584 to primary AML blasts.

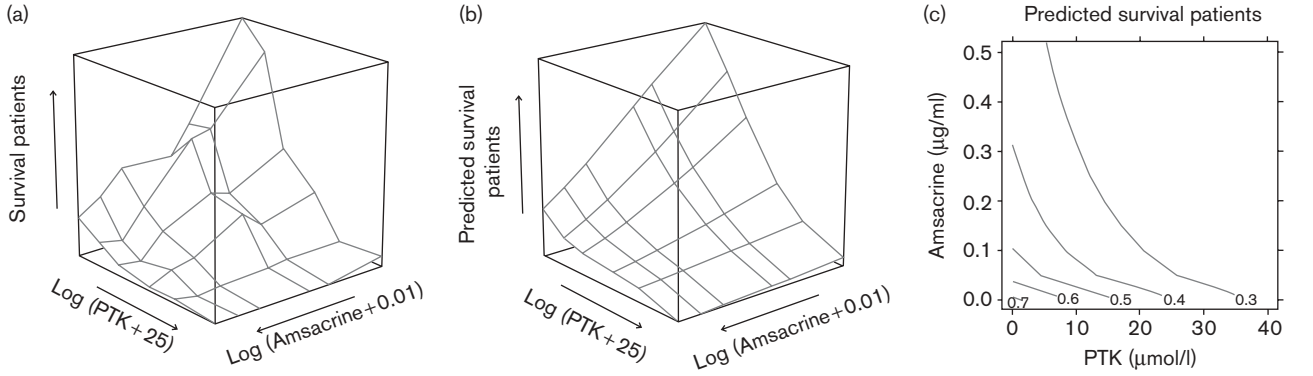
Discussion

The results of this study show that PTK787/ZK 222584, a VEGFR inhibitor, has potent in-vitro cell-death effects against three AML cell lines and primary AML blasts. Interestingly, we show that in a combined strategy, the toxic amsacrine can be partially replaced by a potentially less toxic VEGFR inhibitor, PTK787/ZK 222584, inducing equal amounts of leukemic cell death *in vitro* not only in cell lines but also in primary AML blasts.

VEGF/VEGFR signaling has been described in several tumors such as multiple myeloma [31], melanoma [32] and leukemia [33], resulting in proliferation and survival of tumor cells. In our study we demonstrate that HL-60, THP-1 and TF-1 do show expression of KDR and respond to the VEGFR inhibitor PTK787/ZK 222584. The low sensitivity of TF-1 might be the result of a low KDR expression. The KDR-negative cell line K562 showed no response to the VEGFR inhibitor PTK787/ZK 222584, whereas K562 is positive for FLT1 and FLT4 [34,35]. In all patient samples, we demonstrated high KDR expression, supporting recent results of others [30]. A relationship between the expression of KDR and sensitivity for PTK787/ZK 222584 was not found.

Recently, studies of other VEGFR inhibitors for the treatment of AML were published. SU5416, a receptor tyrosine kinase inhibitor of c-kit, FLT3 and KDR (IC_{50} 0.20 μmol/l), induces growth arrest and apoptosis in AML cells *in vitro* [36,37]. A phase 2 study of SU5416 in refractory AML showed modest clinical activity: in 17 of

Fig. 6



Interaction data between the vascular endothelial growth factor receptor (VEGFR) inhibitor PTK787/ZK 222584 (PTK) and amsacrine for primary acute myeloid leukemia blasts ($n=6$). (a) Three-dimensional (3D) plots presenting the raw data in terms of survival and logits for patient samples. (b) 3D plots presenting a predicted model for logits and survival demonstrated for patient samples. The vertical axes in these figures represent the (predicted) survival of the patient samples, estimated at different concentrations of both drugs (horizontal axes). Increasing the concentration of one or both drugs results in a decrease of survival. (c) An isobole of the patient samples containing lines (shown as 0.3–0.7) that represent the drug combinations at which 30, 40, 50, 60 or 70% of cell survival will be achieved. All three figures point out that the concentration of amsacrine can be lowered and replaced by a dose of the VEGFR inhibitor, PTK787/ZK 222584, to achieve the same cell-death percentage.

42 patients, despite continuous therapy, a strong increased blast count was found [38]. Poor oral bioavailability might be a reason for these results [25]. Interestingly, in this particular study, patients with AML blasts expressing high levels of VEGF had a significantly higher response rate than patients with low VEGF expression.

Furthermore, a phase 1 study of SU11248, a receptor tyrosine kinase inhibitor of c-kit, FLT3, KDR (IC_{50} 10 nmol/l) and PDGFR-β in the treatment of patients with refractory or resistant AML, showed molecular and clinical activity in AML [39]. The inhibitor induced partial remissions of short duration.

Two phase 1 trials with PTK787/ZK 222584 treatment in patients with advanced colorectal cancer show that patients with stable disease for at least 2 months achieve up to 40% tumor regression [40]. The first results from a phase 3 study in patients with metastatic adenocarcinoma of the colon or rectum demonstrate that patients who receive a combination of PTK787/ZK 222584 with a chemotherapy regimen had a 17% reduction in the risk of disease progression, compared with the outcome of chemotherapy treatment alone [41]. Further analyses on the data, including information on the overall survival points, are expected soon.

In a phase 1 study of PTK787/ZK 222584 for the treatment of primary refractory or relapsed AML, no significant responses to treatment with PTK787/ZK 222584 were found. PTK787/ZK 222584 was also tested in patients with secondary AML; monotherapy resulted in stable disease for 10–14 months in two of the 35 patients. Of the 17 patients treated with induction chemotherapy and PTK787/ZK 222584, five achieved complete remission [42]. The potential clinical activity of PTK787/ZK 222584 in AML cell lines warrants further clinical investigation.

It is demonstrated that single-agent VEGFR inhibitor resulted in initial response, but of marginal duration. These results suggest ongoing activation by other signaling pathways. Recently, Kornblau *et al.* [43] showed that activation of multiple signaling transduction pathways in AML patients is common. The prognosis of AML patients worsens if more signaling transduction pathways are activated. Therefore, targeting more signaling pathways byconventional chemotherapeutic drugs and/or specialized tyrosine kinase inhibitors might be beneficial for patients.

Interestingly, another clinical approach for the treatment of AML used bevacizumab, an anti-VEGFA monoclonal antibody, following chemotherapy [44]. Bevacizumab targets extracellular VEGFA; thereby, its administration prevents the stimulation resulting from VEGFA binding to cell-surface receptor tyrosine kinases. In adults with relapsed and refractory AMLs that were resistant to traditional chemotherapy, combining cytotoxic chemotherapy with bevacizumab resulted in 33% complete remission and a median disease-free survival of 7 months in 35% of the patients. In summary, all these clinical studies show that a therapeutic alternative is possible by blocking VEGF/VEGFR signaling in the treatment of AML *in vivo*, in combination with chemotherapy.

An internal and external autocrine VEGF/VEGFR pathway is demonstrated to regulate VEGFR signaling [45,46]. External/paracrine VEGF stimulation of hematopoietic stem cells (HSCs) did not result in survival or proliferation; however, blocking the internal loop with intracellularly acting inhibitors of VEGFR dramatically reduced colony formation by HSCs, demonstrating the importance of a VEGF/VEGFR internal/autocrine loop in these cells. In contrast, in AML patient samples and leukemic cell lines, it has been shown that treatment with an external blocker results in a shift in KDR localization from the nucleus to the surface of the cell, suggesting that VEGF needs to be exported to internalize KDR and activate signaling pathways. Moreover, internal and external inhibitors exerted their effect via distinct mechanisms, the internal having the strongest proapoptotic effects by affecting the mitogen-activated protein kinase/extracellular signal-related kinase (ERK) and the PI3K/protein kinase B (Akt) pathways. Santos and Dias also found that cells treated with both the internal and external VEGF/KDR blockers *in vitro* undergo cell death by apoptosis, to an even more significant extent than those treated with either drug alone. Another interesting difference in these studies is that KDR is thought to be the main receptor for leukemic cell growth and cell survival, whereas stimulation of both FLT1 and KDR can rescue survival of VEGF-deficient HSCs. PTK787/ZK 222584 exerts its effect on KDR and, to a lesser extent, on FLT1, but targeting both the internal and external pathways might induce even more cell death.

In this study, we show that combined use of PTK787/ZK 222584 and amsacrine results in cell death and that, in a combination strategy, a low dose of amsacrine combined with PTK787/ZK 222584 can result in cell-death percentages *in vitro* identical to those from a high dose of amsacrine alone. Whether identical results will be obtained *in vivo* remains to be investigated. *In vivo*, VEGF/VEGFR signaling results in autocrine effects not only in AML cells, but also in the surrounding tissues. For instance, VEGFR inhibitors might inhibit paracrine effects, in which VEGFR stimulation of stromal bone marrow cells results in interleukin-6 and granulocyte colony-stimulating factor known to be potential AML growth factors [47]. Moreover, endocrine effects by which VEGF induces new vessel formation can be inhibited, a crucial prerequisite in tumor growth [48].

Amsacrine is a DNA topoisomerase inhibitor; DNA topoisomerase inhibitors have been shown to transactivate the VEGF promoter, resulting in the expression of VEGF. It is known from literature that topoisomerase inhibitors can cause treatment-related AML because these drugs induce chromosomal breakage [49]. Moreover, a number of chemotherapeutic agents, such as amsacrine, are also known for their cardiotoxicity [50]. To reduce the risk and the complications of long-term effects, it would be better to find a treatment strategy in which the doses of such chemotherapeutic drugs, as for instance amsacrine, can be at least lowered.

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