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AEE788 is a vascular endothelial growth factor receptor tyrosine kinase inhibitor with antiproliferative and proapoptotic effects in acute myeloid leukemia

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Objective. **Aberrant activation of tyrosine kinase receptors is frequently observed in acute myelogenous leukemia (AML). Moreover, activating mutations of the fms-like tyrosine kinase 3 (FLT3) receptor can be found in approximately 30% of patients, thereby representing one of the most frequent single genetic alterations in AML. AEE788, a novel dual receptor tyrosine kinase inhibitor of endothelial growth factor and vascular endothelial growth factor (VEGF), is being studied in several solid tumors with remarkable success. It is not known, however, about the efficacy of this inhibitor in the treatment of AML. Therefore, we investigated the effect of AEE788 in the treatment of three human AML cell lines and seven AML patient samples.**

Materials and Methods. **Cell survival in THP-1, MOLM-13, and MV4-11 cell lines (the two last harboring the FLT3/internal tandem duplication mutation) and AML blasts incubated with 0.5 to 15 μM AEE788 were quantified. We also studied the activation of VEGF/VEGF receptors loop, FLT3, and their downstream effectors (Akt, extracellular signal-regulated kinase, signal transducers and activators of transcription 5, and nuclear factor−κB).**

Results. **Our data showed that AEE788 was a tyrosine kinase inhibitor of FLT3 activity and had antiproliferative and proapoptotic activity in AML-derived cell lines and AML blasts that presented phosphorylation of the FLT3 receptor. Consistently, in these cells AEE788 abrogated VEGF/VEGF receptors activation and the survival signaling pathways studied.**

Conclusion. **Taken together, the activity of AEE788 might represent a promising new option of targeting FLT3 for the treatment of AML.** © **2010 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Angiogenesis has been associated with the growth, dissemination, and metastasis of solid tumors and increased levels of angiogenic molecules have been correlated with poor prognosis in several solid tumors. Although the initial studies were performed on this kind of tumor, increasing evidence indicates that angiogenesis also plays an important role in hematologic malignancies. Recent studies have reported an increased microvessel density in acute myelogenous leukemia (AML) [1] and appeared to be independent of cellularity and associated with a significant increase in angiogenic factors [2], suggesting that vascularity in hematologic malignancies is an active and controlled process and may play a role in the development of the leukemogenic process. Among the known angiogenic factors, vascular endothelial growth factor (VEGF) is the most important stimulator of the angiogenic process. Increased VEGF expression levels have been found in the plasma from patients with AML compared with those in the control group, having prognostic significance in these patients [3]. The two primary signaling receptor tyrosine kinases that mediate the biologic effects of VEGF are VEGF receptor (VEGFR)−1 (Flt-1) and VEGFR-2 (Flk-1/KDR) [4]. In addition to the important role of VEGF in

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the initiation of angiogenesis, this proangiogenic molecule may play a role in AML through autocrine and paracrine loops. Recent works provided evidence that AML not only produce VEGF, but also express functional VEGFR-2, resulting in an autocrine loop for tumor growth and propagation [5,6]. Moreover, several studies have suggested a possible paracrine pathway between VEGF and its receptors. The AML-derived VEGF can stimulate the production of growth factors by human endothelial cells, contributing to poor survival in a subset of leukemias and to the progression of the disease [7–9].

In response to VEGF stimulation, KDR and Flt-1 promote cell survival by inhibiting apoptosis through the activation of multiple pathways, including the mitogen-activated protein kinase (MAPK), the phosphatidylinositol 3-kinase (PI3K), the signal transducer activator of transcription (STAT) cascades, and the nuclear factor−κB (NF-κB) [9–13].

In addition, in AML cells, these pathways are also activated by overexpression and/or mutations of other tyrosine kinase receptors, such as fms-like tyrosine kinase 3 (FLT3). FLT3 is involved in the disease progression of AML, and the presence of AML blasts with high expression of the wild-type (WT) FLT3 messenger RNA is associated with lower overall survival [14]. Furthermore, internal tandem duplications (ITDs) in the juxtamembrane domain of FLT3 are one of the most common molecular defects identified in AML and has been shown to be associated with an even worse prognosis [15–17].

The well-established role of the VEGFRs in promoting tumor growth and in the pathogenesis of hematological malignancies has led to the rational design and development of agents that selectively target this pathway. Several small molecular inhibitors are under clinical development. SU11248, a multitarget inhibitor against FLT3, c-kit, platelet-derived growth factor receptor (PDGFR) and Flt-1 and KDR, has been reported to induce clinical response with short duration in AML patients with FLT3 mutant or WT in two phase I studies [18,19]. PTK787/ZK 222584, targeting VEGFR1/2/3, PDGFR, and c-kit, induces complete remission in about 30% of patients with AML when combined with chemotherapy [20,21]. These early clinical trial data suggest that disrupting VEGF/VEGFRs signaling pathways is potentially clinically efficacious.

AEE788 is a novel synthesized, oral small-molecule inhibitor of both epidermal growth factor receptor (EGFR) and VEGFR tyrosine kinases [22]. The dual inhibition of EGFR and VEGFR phosphorylation by AEE788 reduced the growth and induced apoptosis of hepatocellular carcinoma cells by disrupting mitochondrial transmembrane potentials and by inhibiting MAPK phosphorylation. Moreover, in a subcutaneous xenograft model, AEE788 decreased tumor growth by reducing proliferation and vascularization [23].

This drug is being evaluated in phase I studies in patients with advanced solid tumors and with recurrent glioblastoma [24–27]. However, the effects of AEE788 in acute myeloid leukemia are still unknown.

The goal of our study was to test the efficacy of AEE788 in the induction of cell death, and in the inhibition of proliferation in AML. AEE788 was found to have a proapoptotic in vitro activity, through the differential inhibition of the extracellular signal-regulated kinase (ERK), Akt, STAT5, and NF-κB activation in AML cells. In addition to inhibiting the VEGFR/VEGFRs loop, we found that AEE788 also targeted FLT3 receptor. These results might positively encourage the use of AEE788 combined with chemotherapy in AML clinical trials.

Material and methods

AML cell lines and blasts in vitro experiments

The cell lines THP-1 ($Flt3^{WT/WT}$), MOLM-13 ($Flt3^{ITD/WT}$), and MV4-11 ($Flt3^{ITD/-}$) were obtained from DMSZ (Braunschweig, Germany). They were cultivated on fresh Roswell Park Memorial Institute (RPMI)−1640 medium (Cambrex Bio Science, Verviers, Belgium) supplemented with 10% fetal bovine serum, 100 U penicillin/mL, and 100 μg streptomycin/mL, in a humidified atmosphere of 5% CO_2.

Leukemic and control cells from seven AML patients and two healthy donors were obtained from bone marrow aspirates after informed consent, according to ethical standards of the institutional guidelines. In all analyzed AML samples, the percentage of leukemic infiltration exceeded 80%. Leukemia and normal cells were isolated on Ficoll-Hypaque density gradient centrifugation. Cell number was determined by trypan blue dye exclusion.

$CD34^+$ cells from normal bone marrow were selected using the EasySep human $CD34^+$ selection kit according to the manufacturer's recommendations (Stem Cell Technologies, Vancouver, Canada). $CD34^+$ preparations were verified to be >90% $CD34^+$ using a human anti-CD34 fluorescein isothiocyanate−conjugated (Miltenyi Biotec, S.L, Madrid, Spain) by flow cytometry (Fig. 6A). Flow cytometry was performed by fluorescence activated cell sorting (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ, USA).

Cells were cultured in complete RPMI-1640 medium. Details of AML patients examined in the study are found in Table 1. All AML samples presented FLT3 WT.

Cell lines, leukemic and $CD34^+$ normal cells were seeded at 5×10^5/mL in RPMI-1640 complete medium for 48 and 24 hours, respectively, and treated with different doses (0.5−10 μM) of AEE788 (a kind gift from the Novartis Pharmaceutic, Basel, Switzerland). The controls were performed with vehicles of AEE788 solvent. All experiments were carried out in duplicate and repeated at least three times.

Western blot

Cells washed twice in phosphate-buffered saline (PBS) were lysated on ice for 10 minutes in NP-40 lysis buffer, containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM Na2-ethylene glycol tetraacetic acid, 0.1 mM Na2−ethylene diamine tetraacetic acid, 1 mM sodium orthovanadate, 1% NP-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg/mL aprotinin. Samples were pelleted by centrifugation at 15,000*g*

Table 1. Clinical details of acute myeloid leukemia patients

No.	Gender	Age (y)	FAB	Blasts (%)	Karyotype	WBC ($\times 10^9$/L)	Flt-1[a] (%)	KDR[a] (%)	pFLT3[a] (%)
1	M	43	M0	92	45, XY, 7	66.1	25	29.5	35.5
2	F	72	M1	88	47, XX, +6	18.3	39.16	27	42.6
3	M	71	M1	89	N/A	27.1	25.5	23.6	45.4
4	M	46	M2	90	45, XY, del(5), -7, +8	15.5	24.9	34.5	30.2
5	F	69	M3	93	46, XX, t(15;17)	30	28.2	41.04	25.8
6	F	54	M4	85	46, XX, t(1;11)(p32;q23)	10.7	28	56.2	50.3
7	M	63	M5	95	47, XY, +11, del (12)/46	20.5	22.1	63.7	37.5

FAB = acute myeloid leukemia subtype, according to the classification of the French- American-British committee; pFLT3 = phosphorylated fms-like tyrosine kinase 3; WBC = white blood count.
[a]Percent of expression and phosphorylation in untreated AML cells.

for 1 minute at 4°C. The supernatant (cytoplasmic lysates) was recovered and frozen at −80°C.

Pellets were incubated on ice for 15 minutes in a lysis buffer containing 20 mM HEPES (pH 7.9), 0.4 mM NaCl, 1 mM ethylene diamine tetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 25 μg/mL aprotinin. Samples were centrifuged at 14,000*g* for 5 minutes at 4°C. The supernatant (nuclear lysates) was recovered and frozen at −80°C. Protein concentrations were determined using a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA).

Cytoplasmic cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblottings were incubated with the following antibodies: human anti-pSTAT5 a/b, anti−phospho-ERK 1/2, anti−phospho-Akt, anti-FLT3, and anti−FLT3 ligand (FL). The immunoblots were reprobed with human anti-STAT5, anti-Akt, anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-ERK 1/2 (Calbiochem, La Jolla, CA, USA).

Nuclear cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis in 7% and inmunoblots were incubated with human anti-VEGFR2 monoclonal antibody, and then the inmunoblots were reprobed with human anti-TFIIB (Santa-Cruz Biotechnology).

Immunocomplexes were detected with appropriate horseradish peroxidase−conjugated secondary antibodies and detected by enhanced chemiluminescence (GE Healthcare, Little Chalfont, UK).

Inmunoprecipitation

Cytoplasmic cell lysates (1 mg) were incubated with 2 μg antibodies to human anti-VEGFR1 (Santa Cruz Biotechnology) or anti-FLT3 for 4 hours at 4°C, followed by incubation with 20 μL protein A-Agarose overnight at 4°C with continuous mixing. After centrifugation at 12,000*g* during 5 minutes, the inmunoprecipitates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis 7% and immunoblots were incubated with human anti−phospho-Tyr antibody (Santa Cruz Biotechnology). Immunocomplexes were detected with the appropriate horseradish peroxidase-conjugated secondary antibody and detected by enhanced chemiluminescence (GE Healthcare).

Electrophoretic mobility shift assay

Nuclear extracts were tested for NF-κB binding activity, employing consensus oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGGC-3′ and 3′-TCAACTCCCCTGAAAGGGTCCG-5′) from Santa Cruz Biotechnology, using the Digoxigenin electrophoretic mobility shift assay kit from Roche Diagnostics (Basel, Switzerland). A total of 20 μg nuclear protein was incubated with digoxigenin-labeled NF-κB oligonucleotide in the binding buffer supplied by the kit. Complexes were detected with disodium 3-[4-metoxyspiro (1, 2-dioxetane-3, 2′-(5′-chloro) tricycle 3.3.1.1) decan] phenyl phosphate (CSPD) chemiluminescent substrate, and exposed to Hyperfilm from Amersham Biosciences, in a film holder for 4 to 16 hours at room temperature.

Combined Annexin-V/propidium iodide staining

Viability was assessed by using an Annexin-V staining kit (Bender MedSystems, Vienna, Austria), according to manufacturer's recommendations. Briefly, cells were harvested, washed in PBS and finally dissolved in 1× 195 μL binding buffer and 5 μL Annexin-V, mixed and incubated in darkness for 10 minutes at room temperature. Cells were then washed with PBS and dissolved in 190 μL in 1× binding buffer and 10 μL (20 μg/mL) propidium iodide. Subsequently, binding of fluorescein-conjugated Annexin-V and propidium iodide was measured by FACSCalibur (BD).

Cellular proliferation

Cell viability was assessed using an XTT colorimetric assay (Roche Applied Science, Indianapolis, IN, USA). Cells were seeded on 96-well plates at a concentration of 25,000 cells/well. After treatment with AEE788 at indicated times and doses, the XTT assay was performed following the protocol supplied by the manufacturer. In brief, 50 μL XTT reagent were added to each well, and the plates were incubated at 37°C for 4 hours. Plates were then analyzed at a wavelength of 450 nm using a GENios Reader (TECAN, Salzburg, Austria).

Enzyme-linked immunosorbent assay (ELISA)

VEGF ELISAs were performed on culture supernatants at 48 hours of culture. Cultures were centrifuged at 2,000*g* for 5 minutes. Supernatant was separated, aliquoted, and stored at −80°C until assay. VEGF concentrations were determined using a commercially available ELISA designed to measure VEGF levels (Quantikine, R&D Systems Europe, Oxford, UK). The assay employs the quantitative sandwich ELISA technique using Sf21-expressed recombinant human VEGF165 and antibodies raised against the recombinant protein. Optical density was measured at 450 nm, with the correction wavelength set at

540 nm, using a microtiter plate reader. All experiments were assayed in triplicate.

Flow cytometric detection of CD34, VEGFR1, and VEGFR2 expression and FLT3 phosphorylation

CD34, VEGFR1/Flt-1, and VEGFR2/KDR expression and FLT3 phosphorylation were determined by flow cytometry. Cells were incubated with specific monoclonal antibodies to human Flt-1- and KDR-phycoerythrin (R&D Systems) and CD34−fluorescein isothiocyanate conjugated (Miltenyi Biotec) for 20 minutes at 4°C. After washing with PBS, the CD34 and VEGFRs expression was analyzed on a FACS (BD).

For FLT3 phosphorylation, cells were centrifuged and resuspended in PBS containing 2% paraformaldehyde for 10 minutes. Cells were then permeabilized with 90% cold methanol for 30 minutes and resuspended in PBS containing 1% BSA. Cells were incubated with specific monoclonal antibodies to human phospho-FLT3 (Tyr591), Alexa Fluor−conjugated (Cell Signaling Technology Inc, Beverly, MA, USA) for 1 hour at 4°C. After washing with PBS, the phosphorylation of FLT3 was analyzed on a FACS (BD).

Cells were incubated with phycoerythrin- or Alexa Fluor−conjugated nonspecific antibodies from the same manufacturer to set the threshold for fluorescence parameter, such that fraction of cells with positive fluorescence was $<2.5\%$ of total cells. The CD34, Flt-1, KDR expression and FLT3 phosphorylation−positive cells were determined from the fraction of cells in the sample incubated with specific antibodies that had signal intensity exceeding the threshold obtained with nonspecific antibodies immunoglobulin Gs.

Statistical analysis

All data are expressed as mean ± standard error of mean. Statistical analyses were carried out with the SigmaStat software package (Systat, Point Richmond, CA, USA). Before comparing two data groups, a normality test and an equal variance test were performed. If data groups passed both tests, a comparison was made by a parametric approach (paired Student's *t*-test). If the normality and/or equal variance test was violated, a comparison was made by a nonparametric method (Mann-Whitney rank sum test). Differences were considered statistically significant at $p < 0.05$.

Results

AEE788 induced apoptosis and inhibited cell proliferation in AML cell lines

We analyzed the ability of AEE788 to induce apoptosis using Annexin-V and propidium iodide by flow cytometric analysis. THP-1, MOLM-13, and MV4-11 cell lines were treated in vitro with different concentrations of AEE788 for 48 hours. AEE788 induced apoptosis of the AML cells in a dose-dependent manner (Fig. 1A). An apoptosis response of 50% was induced at a concentration of 10 μM for MOLM-13 and MV4-11, and of 15 μM for THP-1 cells.

In addition, AEE788 dramatically inhibited cellular proliferation in a dose-dependent manner, evident at 5 μM. At 15 μM, the three cell lines suffered a total inhibition of cell proliferation (Fig. 1B).

Parallel results were apparent in optical microscopy in which AEE788 inhibited cell growth of THP-1, MOLM-13, and MV4-11 cells in a dose-dependent way (Fig. 1C). As shown, the effects of this inhibitor were more potent in MOLM-13 and MV4-11 cell lines, which harbor the FLT3/ITD mutation.

AEE788 decreased VEGF expression levels and the VEGFRs phosphorylation in AML cells

Increased microvessel density and increased VEGF and KDR expression have been reported in bone marrow of AML patients compared with healthy donors [5,28,29]. Accordingly, the three cell lines studied showed high levels of VEGF. Treatment with different doses of AEE788 for 48 hours inhibited VEGF production in a dose-dependent manner with an IC_{50} of approximately 0.5 μM for MOLM-13 and MV4-11, and 10 μM for THP-1 cells (Fig. 2A). Because VEGF levels were normalized for milligrams total cellular protein, the reduced VEGF levels were not a reflection of decreased cell number induced by AEE788.

Cytometry analysis showed that the three AML cell lines presented elevated KDR and Flt-1 expression levels. Flt-1: THP-1 (92% ± 6.5%), MOLM-13 (25.5% ± 3.6%), MV4-11 (61.5% ± 4.5%). KDR: THP-1 (60.5% ± 5.4%), MOLM-13 (45.8% ± 5.5%), MV4-11 (54.5% ± 3.5%).

Next, we determined whether AEE788 could abrogate the phosphorylation of Flt-1 and KDR in AML cells. Cytoplasmic and nuclear cell lysates were analyzed by Western blotting for tyrosine-phosphorylated Flt-1 and KDR expression, respectively. As shown in Figure 2B, constitutive tyrosine phosphorylation of Flt-1 was inhibited in a dose-dependent manner after AEE788 treatment.

Because KDR phosphorylation implies its translocation to the nucleus [30], to test the KDR activation we analyzed the nuclear KDR presence. Culture with AEE788 decreased the nuclear translocation of KDR in a dose-dependent way. Low doses of AEE788 were required to significantly (0.5 μM) inhibit the Flt-1 and KDR activation in MOLM-13 and MV4-11. THP-1 suffered inhibition of phosphorylation of these receptors, after 5 μM AEE788 treatment.

AEE788 inhibited FLT3-WT and FLT3/ITD mutant phosphorylation

To investigate the AEE788 activity in FLT3, in vitro experiments were performed in one cell line, which showed the FLT3/WT (THP-1) and two cell lines with the FLT3/ITD mutation (MOLM-13 and MV4-11) (Fig. 3A). The Western blot studies showed that all AML cell lines presented high levels of FL protein expression (data not shown). In THP-1 cells (FLT3/WT), the addition of FL was not necessary to stimulate FLT3 phosphorylation because we observed around 30% of phosphorylation of FLT3 in these cells.

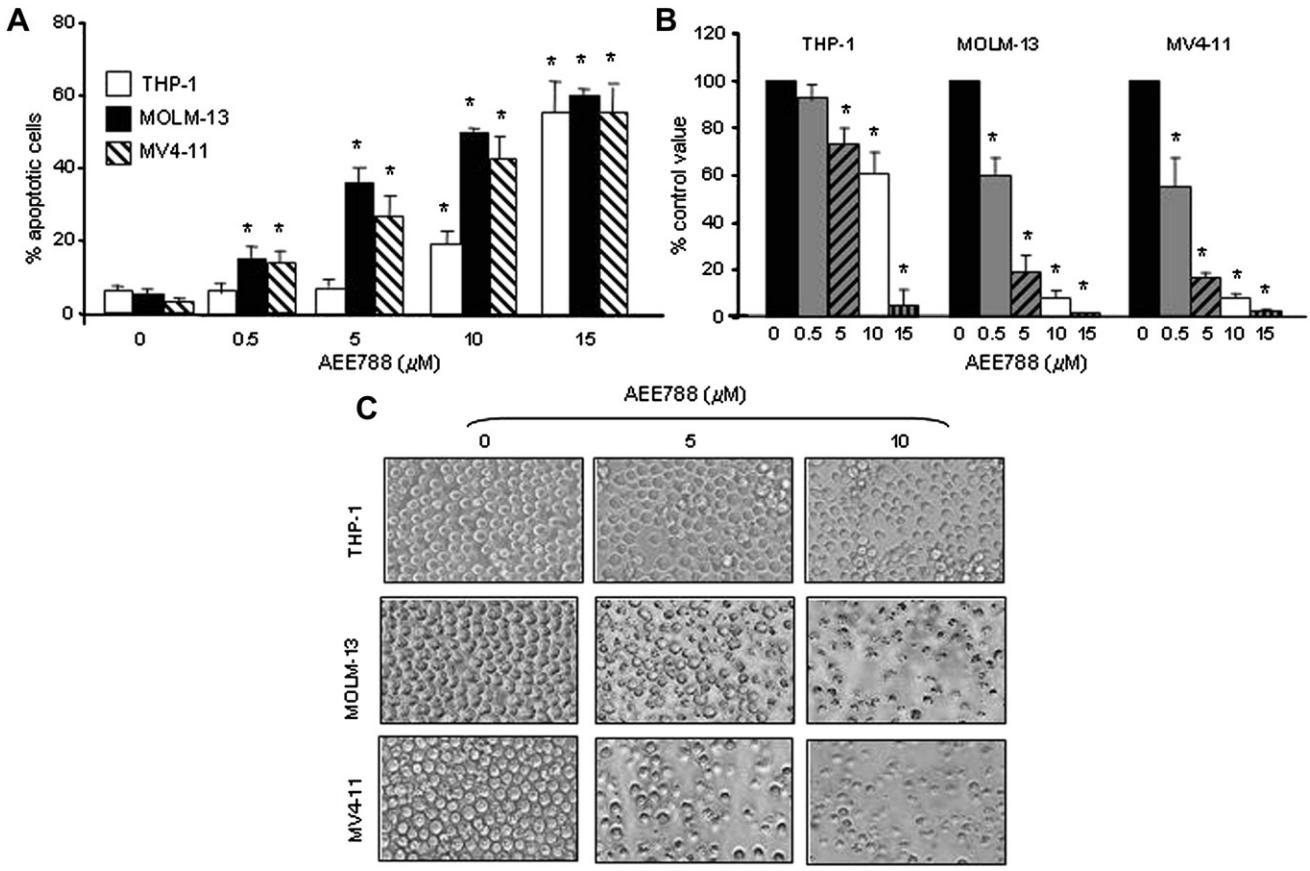

Figure 1. AEE788 induces apoptosis and inhibits cell proliferation in acute myeloid leukemia cell lines. THP-1, MOLM-13, and MV4-11 cells were incubated with AEE788 at different concentrations for 48 hours. (**A**) The apoptosis was evaluated by the presence of Annexin-V on the cell surface. (**B**) Cell proliferation was assessed by the XTT-based assay. The panels show averages mean ± standard error of three experiments performed in triplicate; $*p < 0.05$ vs untreated cells. (**C**) Images were obtained from an optical microscope. The panels show one representative experiment of three independent experiments with similar results.

So, we suggest that FLT3 signaling is constitutively activated in THP-1 cells.

As shown in the Figure 3A and B, treatment with different concentrations of AEE788 inhibited phosphorylation of FLT3 WT in THP-1 cells in a dose-dependent manner with an IC_{50} of approximately 5 μM because AEE788 decreased the FLT3 expression (Fig. 3B).

In MOLM-13 and MV4-11 cells (which express FLT3/ITD), it is noteworthy that both presented high levels of FLT3 phosphorylation (67.6% ± 5.3% and 85.4% ± 5.5%, respectively) in the absence of FLT3 ligand. AEE788 inhibited FLT3 phosphorylation in a dose-dependent manner with an IC_{50} of 0.5 μM in FLT3/ITD cells (Fig. 3B). A similar decrease in FLT3 expression was observed in these cells after higher doses of AEE788.

Downregulation of ERK, Akt, STAT5, and NF-κB activation induced by AEE788

VEGF and FLT3 signaling identified in AML are likely to reflect activation of a number of downstream effector pathways, such as the RAF/MEK/ERK, PI3K/Akt, STAT, and NF-κB [9–13]. Previous reports have shown that AEE788 blocked the phosphorylation of both ERK1/2 and Akt in vitro and in vivo in solid tumors [31,32]. Therefore, we determined the effects of AEE788 on these intracellular signaling in AML cells.

In the two cell lines that harbored the ITD mutation of FLT3 (MOLM-13 and MV4-11), AEE788 treatment resulted in a dramatic decreased phosphorylation of ERK1/2, Akt, and STAT5 in a dose-dependent manner (Figs. 4, 5A). Inhibition of Akt and STAT5 phosphorylation was evident from 0.5 μM doses, at which FLT3/ITD receptor was inhibited by AEE788. All of these kinases (Akt, ERK, and STAT5) were significantly inhibited at 5 μM, in agreement with the significant induction of apoptosis and inhibition of cell proliferation.

In THP-1 cells, which presented constitutively phosphorylated around 30% of FLT3, treatment with AEE788 significantly inhibited the phosphorylation of ERK1/2 and Akt kinases from 5 μM, the dose at which AEE788 inhibited the phosphorylation of FLT3/WT. There was not activation of STAT5 in these cells (Figs. 4, 5B).

Constitutive activation of NF-κB pathway has been identified in many AML patients, and NF-κB has been

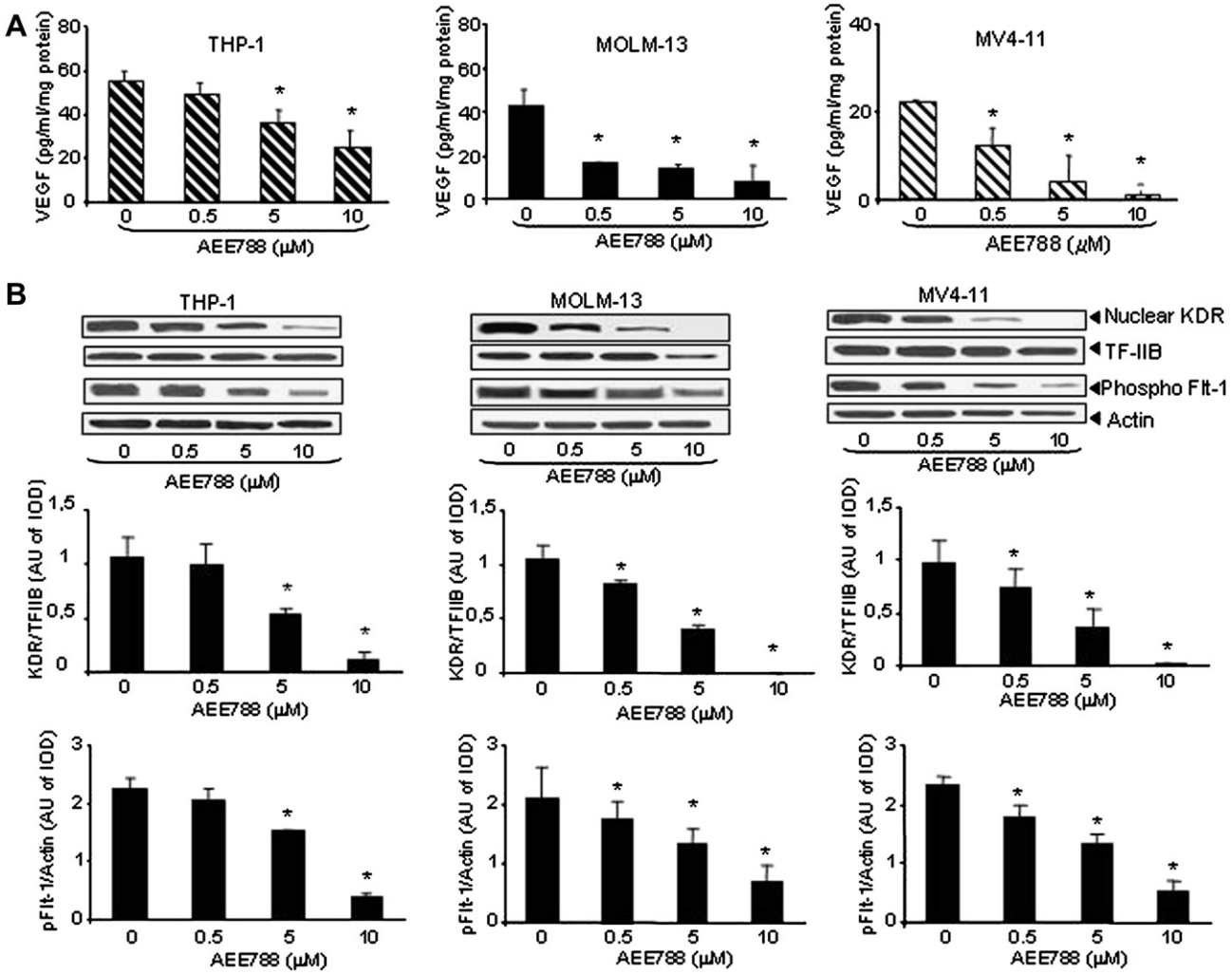

Figure 2. AEE788 decreases vascular endothelial growth factor (VEGF) supernatant levels and inhibits phosphorylation of VEGF receptors in acute myeloid leukemia cell lines. Cells were incubated with different concentrations of AEE788 for 48 hours. (**A**) Supernatant levels of VEGF were analyzed by enzyme-linked immunosorbent assay. Panels show averages mean ± standard error (SE) of three experiments performed in triplicate; $*p < 0.05$ vs untreated cells. (**B**) Nuclear KDR localization: 50 μg nuclear proteins extracts were electrophoresed on a 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred to nitrocellulose membranes. Membranes were probed with human anti-KDR polyclonal antibody. To demonstrate equal protein loading, the immunoblots were stripped and reprobed with human anti−TFII-B. Flt-1 phosphorylation; 1 mg proteins was immunoprecipitated with human 2 μg anti−Flt-1 antibodies, electrophoresed on 7% SDS-PAGE gels, and then transferred to nitrocellulose membranes. Filters were probed with anti−phospho-tyr. The panels show one representative experiment of three independent experiments with similar results. Lower graphs show the relative integrated optical density (IOD) values of the respective blots. Graphs show averages mean ± SE of three independent experiments; $*p < 0.05$ vs untreated cells.

shown to contribute to the uncontrolled proliferation and antiapoptosis in AML cells [33]. In our study, electrophoretic mobility shift assay analysis showed that AEE788 abolished activation of NF-κB in MOLM-13 and MV4-11 at 5 μM. Higher doses (10 μM) were necessary to slightly inhibit the activation of this transcription factor in THP-1 cells (Fig. 5).

AML cells sensitivity to AEE788

We next evaluated the direct effect of AEE788 on the cell survival of AML samples and healthy donors. Seven AML samples and two controls were cultured with or without the VEGFRs kinase inhibitor for 24 hours. The cytometry analysis showed that all samples presented values of KDR and Flt-1 expression between 20% and 65%, and FLT3 phosphorylation between 25% and 50% (Table 1). Lower levels of KDR (3.5% and 5.5%) and Flt-1 (13.5% and 9.5%) cell surface expression were seen in control samples. The amount of VEGF secreted was detected by ELISA in the supernatant of AML and control cells. Variable levels of VEGF were found in the supernatant of the seven AML samples (between 10 and 115 pg/mL) and nondetectable VEGF levels were seen in the supernatant of $CD34^+$ control cells.

As shown in the Figure 6B, AEE788 induced apoptosis in a dose-dependent manner in all AML samples, reaching

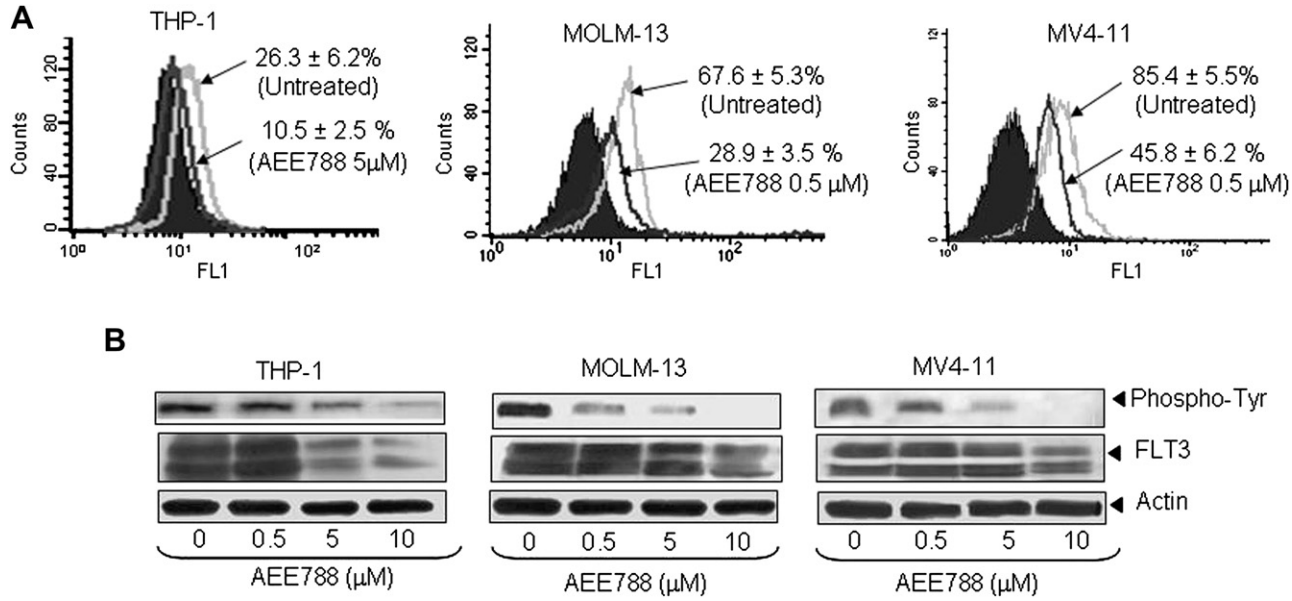

Figure 3. AEE788 inhibits the phosphorylation of fms-like tyrosine kinase 3 (FLT3)/wild-type (WT) and FLT3/internal tandem duplication in the cell line models. Acute myeloid leukemia cell lines were incubated with AEE788 at different concentrations for 48 hours. (**A**) Phosphorylation of FLT3 was analyzed by flow cytometry after staining with anti−phospho FLT3-Alexa antibody. Filled histograms represent isotype control. Open histograms show fluorescence intensity of phospho-FLT3. The panel shows one representative experiment of three independent experiments with similar results. (**B**) FLT3 phosphorylation; 1 mg protein was immunoprecipitated with human 2 μg anti-FLT3 antibody, electrophoresed on 7% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, and then transferred to nitrocellulose membranes. The filters were probed with antiphospho-tyrosine antibody (top). FLT3 expression; cell lysates were prepared and cytoplasmic proteins (50 μg/lane) were electrophoresed on a 6% SDS-PAGE gels and then transferred to nitrocellulose membranes. The membranes were probed with human anti-FLT3 monoclonal antibody. To demonstrate equal protein loading, the immunoblots were stripped and reprobed with human anti-actin antibody. Similar results were obtained in at least 3 independent experiments.

30% ± 6.5% of inhibition at 10 μM. Moreover, this inhibitor significantly decreased AML cell proliferation from 1 μM (Fig. 6C). No significant apoptosis or decrease of cell proliferation was observed in $CD34^+$ bone marrow cells after treatment with AEE788. Regarding VEGF expression, treatment with different doses of AEE788 inhibited production of this proangiogenic molecule in a dose-dependent manner. This inhibition was significant from 0.5 μM (Fig. 6D). Moreover, no detectable expression of VEGF protein was shown in $CD34^+$ nonmalignant cells.

Discussion

In the present study, we have shown that AEE788 inhibited the cell survival of three AML cell lines and blasts from seven AML patients. In addition, we show that AEE788 inhibits phosphorylation of FLT3/ITD, as well as FLT3/WT; however, its effects were more potent in FLT3/ITD cells. Moreover, this inhibitor decreased activation of VEGF/VEGFRs loops and its downstream effectors, such as ERK, Akt, STAT5, and NF-κB.

In cellular assays, AEE788 promoted significant apoptosis in the three AML cell lines studied (THP-1, MOLM-13, and MV4-11) and AML samples. Parallel experiments showed that AEE788 inhibited cell proliferation in a dose-dependent manner in AML cells. Consistent with these data, AEE788 has been shown to block tumor growth by reducing proliferation and inducing apoptosis in hepatocellular carcinoma, thyroid cancer, and colon cancer cells [23,34,35].

Overexpression of FLT3 receptor and FLT3/ITD mutation is one of the most common molecular defects in AML, conferring a poor prognosis. We show that AEE788 inhibited phosphorylation of FLT3/ITD, as well as FLT3/WT. AEE788 was more potent against AML cells with FLT3/ITD mutation compared with those harboring the WT FLT3. In vitro studies showed that AEE788 inhibited the FLT3/ITD phosphorylation with an IC_{50} of 0.5 μM, a concentration 10-fold lower than the dose necessary to inhibit FLT3 phosphorylation in FLT3/WT cells. Similar results were obtained by O'Farrell et al., when they treated AML cells with an FLT3 inhibitor, SU11248 [36]. They exposed several possibilities to account for this effect: the inhibitor may bind to FLT3/ITD with increased affinity due to the differences in conformation between the WT and ITD; alternatively, the accessibility to the drug may be influenced by differences in cellular localization.

We and others have previously reported that myeloblasts from AML patients have demonstrable VEGFR1/Flt-1 and VEGFR2/KDR expression [21,37]. In our conditions, all AML cell lines presented high levels of VEGF and activation of KDR and Flt-1. AEE788 reduced VEGF secretion in AML blasts and phosphorylation of VEGFRs in all AML cells studied. However, in FLT3/ITD cells, AEE788

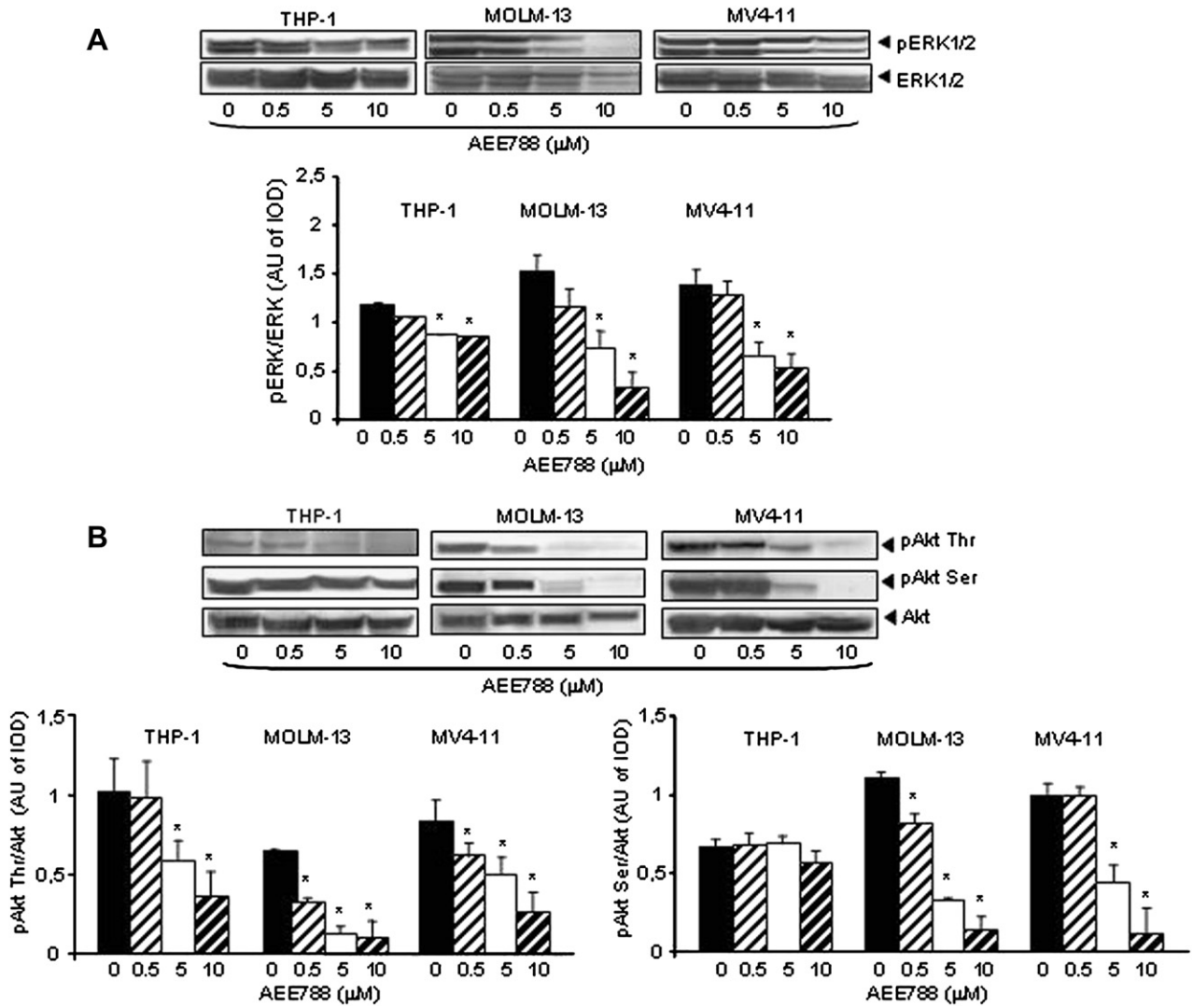

Figure 4. AEE788 downregulates the phosphorylation of (**A**) extracellular signal-regulated kinase (ERK) and (**B**) Akt kinases. Cells were incubated with AEE788 at different concentrations for 48 hours. Cell lysates were prepared and cytoplasmic proteins (50 μg/lane) were electrophoresed on a 10% and 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred to nitrocellulose membranes. The membranes were probed with human anti−phospho-ERK or anti−phospho-Akt monoclonal antibodies. To demonstrate equal protein loading, the immunoblots were stripped and reprobed with human anti-ERK or anti-Akt antibodies. The panels show one representative experiment of three independent experiments with similar results. Graphs show the relative integrated optical density (IOD) values of the respective blots and the averages mean ± standard error of three independent experiments with similar results; $^*p < 0.05$ vs untreated cells.

inhibited significantly the VEGF levels at lower doses than in FLT3/WT cells. This reduction was independent from the expression of VEGF and VEGFRs because these cells (MOLM-13 and MV4-11, FLT3/ITD) presented lower levels of VEGF and VEGFRs than the FLT3/WT cells (THP-1). Thus, a direct relationship between expression levels of VEGFR and sensitivity to the treatment with AEE788 could not be established. Similar results were obtained in recent studies in which we and others did not find a relationship between the level of KDR expression and the response to other VEGFR inhibitor, PTK787/ZK222 485 in AML patient samples [21,38]. Nevertheless, it was found a great difference in the response of AML cell lines to the AEE788 treatment depending on the presence or not of the FLT3/ITD mutation. These data are in agreement with a previous report suggesting that VEGF is a direct target of the FLT3/ITD signal transduction pathway [19].

On the other hand, the treatment with AEE788 reduced significantly the activation of Akt and ERK1/2 in AML cell lines. This reduction is consistent with previous reports in which AEE788 blocked phosphorylated ERK and Akt in vitro studies in solid tumors [23,30,39–41]. A constitutive activation of STAT5 has been also described in the majority of primary AML blast cells. It has been described that STAT5 phosphorylation was part of the FLT3/ITD signaling [42]. In accordance, our results show that

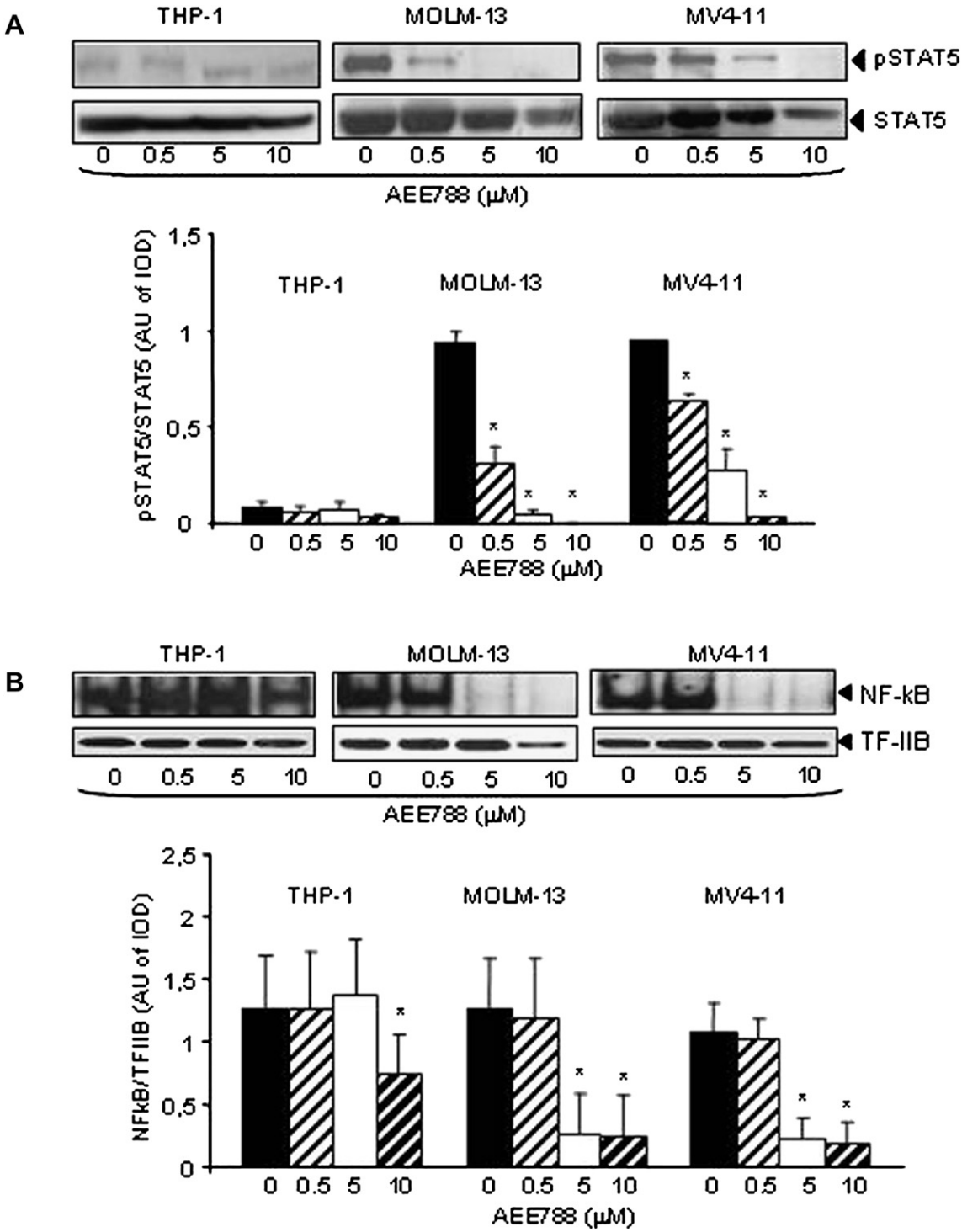

Figure 5. AEE788 inhibits the phosphorylation of signal transducers and activators of transcription (STAT) and nuclear factor−κ (NF-κB) activation. Cells were incubated with AEE788 at different concentrations for 48 hours. (**A**) Cell lysates were prepared and cytoplasmic proteins (50 μg/lane) were electrophoresed on 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred to nitrocellulose membranes. Membranes were probed with human anti−phospho-STAT5 monoclonal antibody. To demonstrate equal protein loading, the immunoblots were stripped and reprobed with human anti-STAT5 antibody. (**B**) Nuclear fractions were incubated with digoxigenin-labeled oligonucleotides, corresponding to a NF-κB consensus sequence. Complexes were subjected to native PAGE (8%), transferred into nylon membranes and incubated with anti-digoxigenin alkaline phosphatase-conjugated antibody. To demonstrate equal protein loading, the immunoblots were probed with human anti−TF-IIB. The panels show one representative experiment of three independent experiments with similar results. Graphics show the relative integrated optical density (IOD) values of the respective blots and the averages mean ± standard error of three independent experiments with similar results; $*p < 0.05$ vs untreated cells.

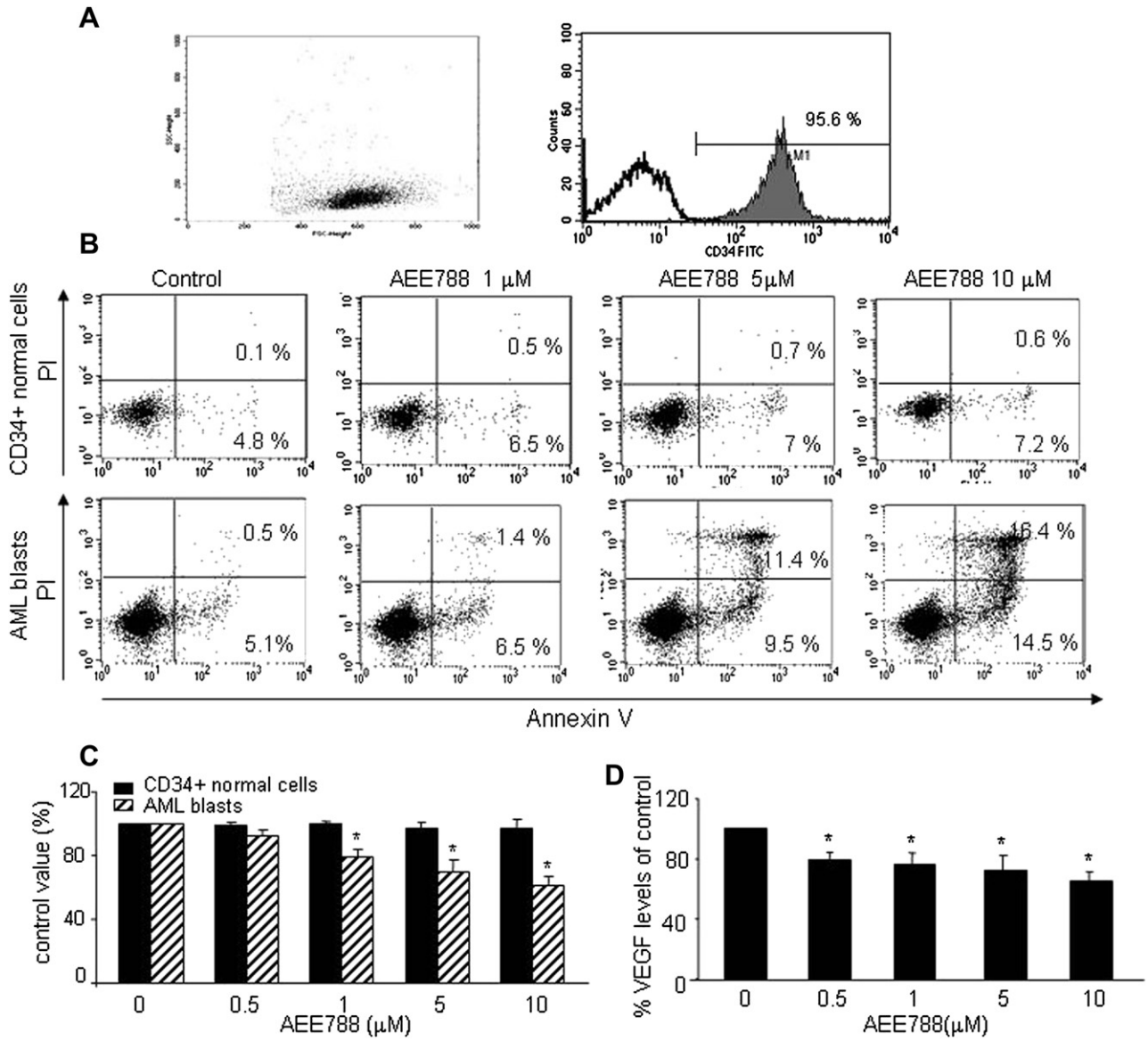

Figure 6. AEE788 causes apoptosis, inhibition of cell proliferation and vascular endothelial growth factor (VEGF) production in acute myelogenous leukemia (AML) blasts. Blasts from seven AML patients and two healthy donors were treated with different doses of AE788 (0.5−10 μM) for 24 hours. (**A**) $CD34^+$ cells were tested by flow cytometry. (**B**) Apoptosis was evaluated by the presence of Annexin-V on the cell surface. The panels show one representative experiment of three independent experiments with similar results. (**C**) Cell proliferation was assessed by the XTT-based assay. (**D**) Supernatant levels of VEGF in AML blasts after AEE788 treatment were analyzed by enzyme-linked immunosorbent assay. Panels show mean ± standard error of three independent experiments performed in seven AML samples and two healthy donors; $*p < 0.05$ vs untreated cells.

AEE788 strongly inhibited activation of STAT5 only in FLT3/ITD cells.

The constitutive activation of NF-κB pathway has been identified in many AML patients, and NF-κB has been identified to contribute to the uncontrolled proliferation and antiapoptosis in AML cells [32]. In our hands, AEE788 abolished the NF-κB activation in those cells that express FLT3/ITD mutation, suggesting that NF-κB is also a key mediator in the FLT3/ITD signaling. In THP-1 cells (FLT3/WT) the slight inhibition of NF-κB occurred at higher doses, indicating that at this concentration the effects of AEE788 are not specific and other signaling pathways might be involved in the inhibition of NF-κB promoted by AEE788.

In a recent study, we tested the effects of PTK787/ZK 222485 in the same AML cell lines and AML patient cohort used in the present study [21]. We showed that PTK787/ZK 222485 decreased VEGF levels and VEGFR phosphorylation in the AML cells showing the FLT3/ITD mutation. It inhibited the cell proliferation and promoted apoptosis in these cells. In addition, combined treatment with PTK787/ZK 222485 and conventional chemotherapy achieved a better therapeutic efficacy than chemotherapy alone in AML cells, especially in those with FLT3/ITD in

which the combination further prevented activation of the angiogenic process. In the present study, we have found that AEE788 has activity in AML cells at four-fold less effective doses than PTK787/ZK 222485. Although the main effects of PTK787/ZK 222485 were found on AML cell lines with the FLT3/ITD mutation, this inhibitor did not abrogate the FLT3 phosphorylation. By contrast, AEE788 were found to inhibit phosphorylation of both FLT3/WT and FLT3/ITD AML cells. In that way, AEE788 seems to be a better candidate to be used as an alternative therapy for AML treatment. Nevertheless, it remains to be tested whether the effect of this drug can be potentiated after combined treatment with conventional chemotherapy.

Small-molecule tyrosine kinase inhibitors have been developed and have been shown to successfully inhibit FLT3 signaling both in vitro and in vivo, including Lestaurtinib (CEP701) [43,44] and midostaurin (PKC412) [45], which are now being investigated in a phase III trial in AML. Some in vitro studies have compared the efficacy of midostaurin and lestaurtinib in AML cells. The results showed that inhibition of FLT3 phosphorylation by midostaurin was similar to that achieved by lestaurtinib. However, the cytotoxic effect induced by midostaurin was more limited, probably due to that lestaurtinib inhibits other cellular targets upstream of STAT5 [46,47]. Nevertheless, phase I and II clinical trials of lestaurtinib and midostaurin monotherapy in similar patient AML groups have demonstrated very similar rates and degrees of clinical response [48,49]. These agents are also being tested in combination with conventional chemotherapy. Most of the responses to these FLT3 inhibitors consisted of clearance of peripheral blasts (30−50% of patients) and, less frequently, major reductions in bone marrow blasts. The responses tended to be transient, lasting weeks to months, followed by progressive disease. In that way, AEE788 can also exert a potent tumor inhibition on blasts with a broad range of targets including FLT3 and its downstream regulators, such as ERK, Akt, STAT5, and NF-κB, and it is also an inhibitor of VEGFRs, which regulate angiogenesis, all of them associated with disease progression. Moreover, to date, no development of resistance has been shown in AEE788 treatment. Thus, the demonstration that AEE788 exhibits potent inhibition and efficacy, in FLT3 activating models, suggests that this drug may have therapeutic utility in AML.

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Conflict of Interest Disclosure

The authors reported no conflict of interest.

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