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Experimental Hematology

Experimental Hematology 2009;37:679–691

Additive effect of PTK787/ZK 222584, a potent inhibitor of VEGFR phosphorylation, with Idarubicin in the treatment of acute myeloid leukemia

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(Received 10 December 2008; revised 1 March 2009; accepted 2 March 2009)

Acute myeloid leukemia (AML) is a disease with a poor prognosis. It has been demonstrated that AML cells express vascular endothelial growth factor (VEGF) as well as Flt-1 and KDR, resulting in an autocrine pathway for cell survival. PTK787/ZK 222584 is a new oral antiangiogenic molecule that inhibits tyrosine kinase activity of all known VEGF receptors. The present study aimed to investigate the therapeutic efficacy of combining PTK787/ZK 222584 with a chemotherapeutic agent, such as Idarubicin, for treatment of AML. We have analyzed in four AML cell lines and seven AML patient samples, cell proliferation, apoptosis, angiogenesis. and activation of several related intracellular pathways after treatment with PTK787/ZK 222584 alone or combined with Idarubicin. PTK787/ZK 222584 decreased VEGF levels and VEGF receptor phosphorylation in the AML cells showing Fms-like tyrosine kinase 3/internal tandem duplication mutation (Flt3/ITD). Both drugs, given separately, inhibited cell proliferation and promoted apoptosis. Moreover, combined treatment promoted more apoptosis and inhibition of cell proliferation than each compound administered separately in all AML cells. In conclusion, PTK787/ZK 222584 combined with Idarubicin 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. © 2009 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.

Angiogenesis plays a crucial role in facilitating tumor growth and the metastatic process. Although this process first seemed to be related to solid tumors, there is emerging evidence that angiogenesis also contributes to pathogenesis of hematological malignancies. Several reports confirmed that patients with acute myeloid leukemia (AML) have increased bone marrow vascularization. In most cases, leukemia progression acute phase appears to be accompanied by an increase in bone marrow microvessel density and elevated local and circulating proangiogenic growth factors [1–3]. Among the angiogenic regulators, vascular endothelial growth factor (VEGF) is one of the major proangiogenic molecules involved in the angiogenic process. Because of that, the VEGF/VEGF receptor (VEGFR) pathways have been intensively studied in basic and clinical cancer research [4]. It has been demonstrated that circulating VEGF levels are significantly increased in approximately 90% of AML patients, and appear to accompany disease progression. Moreover, high levels of plasma/serum VEGF correlated with increased number of circulating blasts and the decrease of complete remission achievements and reduced survival in AML patients untreated [5].

Leukemic blasts may promote their own development via both paracrine and autocrine interactions, through expression of VEGF and its tyrosine kinase receptors: VEGFR1/Flt-1 and VEGFR2/KDR [6]. Inhibition of both paracrine and autocrine VEGF/KDR pathways is necessary to induce long-term remission in mice bearing acute

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0301-472X/09 $–see front matter.
doi: 10.1016/j.exphem.2009.03.001

leukemia [7]. Moreover, it has been shown that in bone marrow, expression of VEGFR2 is restored to normal expression levels in patients with complete remission after chemotherapy [8].

In response to VEGF stimulation, KDR and Flt-1 have been shown to transmit intracellular signals leading to cell proliferation and survival. The signaling pathways activated by VEGF, downstream of its receptors and linked to these particular functions, include the mitogen-activated protein kinase, phosphatidylinositol 3 kinase, and signal transducer activator of transcription (STAT) cascades [9–11].

In addition, in AML cells, these pathways are also activated by overexpression and/or mutations of others tyrosine kinase receptors, such as Fms-like tyrosine kinase 3 (Flt3). Flt3 is involved in disease progression of AML and the presence of AML blasts with high expression of the wild-type Flt3 messenger RNA (mRNA) is associated with lower overall survival [12]. Furthermore, internal tandem duplications (ITDs) in the juxtamembrane domain of Flt3 are the most common molecular defects identified in AML and have been shown to be associated with an even worse prognosis [13–15].

The well-established role of both VEGFR and Flt3 in promoting tumor growth and pathogenesis of hematological malignancies has led to the rational design and development of agents that selectively target this pathway. Several small molecule tyrosine kinase inhibitors that target the tyrosine kinase receptors are already being studied in clinical trials. These include ZD6474, a VEGFR2 inhibitor, SU11248, a small molecule that potently inhibits platelet-derived growth factor receptors (PDGFR), VEGFR1, VEGFR2, c-KIT, and Flt3 and PTK787/ZK 222584, which inhibits VEGFR1 and VEGFR2 [16].

PTK787/ZK 222584 (PTK/ZK) is an oral angiogenesis inhibitor targeting VEGFR tyrosine kinases, including VEGFR1/Flt-1, VEGFR2/KDR, VEGFR3/Flt-4, PDGFR, and c-KIT [17,18]. It has no known effects against other tyrosine or serine/threonine kinases. PTK/ZK is currently being investigated in patients with different solid tumor types for its therapeutic utility [17–19]. In those preclinical models, PTK/ZK caused a significant decrease in vessel permeability, reduction in tumor vessel density and repression of tumor growth with high efficacy.

Different clinical studies with inhibitors of VEGFR signaling, namely tyrosine kinase inhibitors, have also shown promising results in treatment of AML patients [20,21]. However, to date, only two studies have analyzed the biological activity of PTK/ZK in AML [22,23]. Thus, the aim of the present study was to gain more insight into the effects of PTK/ZK on both growth and angiogenic process in AML cells with or without Flt3/ITD mutation.

We and others have previously reported a constitutive and simultaneous activation of the main signaling pathways (extracellular signal-regulated kinase [ERK], Akt, and STAT) involved in survival and differentiation of AML [24–29]. Thus, if multiple independent pathways are abnormally expressed in AML cells, reversion of leukemogenesis might be enhanced through the simultaneous inhibition of different cellular targets. Moreover, an increasing number of studies have suggested that tyrosine kinase inhibitors need to be combined with other antileukemic treatments to obtain the best clinical results. Theoretically, it would be advantageous to add chemotherapy agents that act synergistically with tyrosine kinase inhibitors for antileukemic effects. Thus, we also sought to profile the activity of PTK787/ZK in combination with a traditional antileukemic agent, Idarubicin.

In this study, we show that PTK/ZK monotherapy has in vitro proapoptotic and antiangiogenic effects against four AML cell lines and primary AML blasts, causing greater effect in AML cell lines harboring the Flt3/ITD mutation. Moreover, the combined strategy with Idarubicin achieved a better therapeutic efficacy than the chemotherapeutic agent given alone in AML cells, especially in those showing Flt3/ITD mutations, in which the angiogenic process was further abrogated.

Materials and methods

AML cell lines and blasts in vitro experiments

The cell lines NB4 (Flt3 $^{WT/WT}$), 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 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.

AML blasts cells were obtained from bone marrow aspirates after informed consent, according to ethical standards of the institutional guidelines. In all analyzed samples, the percentage of leukemic infiltration exceeded 80%. Leukemia cells were isolated on Ficoll-Hypaque density gradient centrifugation. Cell number was determined by trypan blue dye exclusion. AML blasts were cultured in complete RPMI-1640 medium. Details of patients examined in the study are indicated in Table 1. All AML samples presented Flt3 wild-type.

Cell lines and AML blast cells were seeded at 5×10^5/mL in RPMI-1640 complete medium for 24 and 48 hours and treated with different doses of PTK/ZK (a kind gift from the Novartis Pharmaceutic, Basel, Switzerland) or Idarubicin alone or combined. PTK/ZK was added 30 minutes prior to the addition of Idarubicin. Controls were performed with vehicles of PTK/ZK solvent. All experiments were carried out in duplicate and repeated at least three times.

Western blot

Cells washed twice in phosphate-buffered saline 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 (EGTA), 0.1 mM Na2−ethylenediamine 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* for

Table 1. Clinical details of acute myeloid leukemia patients

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

FAB = acute myeloid leukemia subtype, according to the classification of the French-American-British committee; WBC = white blood count.

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 EGTA, 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. Supernatant (nuclear proteins) was recovered and frozen at −80°C. Protein concentrations were determined using a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA).

Whole-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. The immunoblots were reprobed with human anti-STAT5, anti−ERK 1/2, anti-Akt, and anti-Actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Nuclear cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis 7% and immunoblottings were incubated with anti−phospho-VEGFR2 monoclonal antibody, then the immunoblots were reprobed with human anti-TFII-B (Santa Cruz Biotechnology).

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

Immunoprecipitation

Cytoplasmic cell lysates (1 mg) were incubated with 2 μg antibodies to human anti-VEGFR1 (Santa Cruz Biotechnology) 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 immunoprecipitates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis 7% and immunoblottings 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).

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 fluorescein-activated cell sorting (BD, Franklin Lakes, NJ, USA).

RNA extraction and quantitative real-time reverse transcriptase-polymerase chain reaction

Total RNA was extracted by Tri-Reagent (Sigma, St Louis, MO, USA), according to manufacturer's recommendations. RNA concentration was determined spectrophotometrically at 260 and 280 nm and its integrity was verified following separation by electrophoresis into a 0.8% agarose gel containing ethidium bromide. RNA samples were stored at −80°C until use. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on one-step using the QuantiTect SYBR Green RT-PCR kit (QIAGEN GmbH, Hilden, Germany) following manufacturer's protocol.

Expression levels of VEGF gene and glyceraldehyde phosphate dehydrogenase (GAPDH) as housekeeping gene, were measured by quantitative real-time RT-PCR using the LightCycler thermal cycler system (Roche Diagnostics, Indianapolis, IN, USA). Sequence of primers used for study, theoretic size of PCR products, and efficiency were: VEGF: (470 bp), forward primer: 5′-CGAAGTGGTGAAGTTCATGGATG 3′; reverse primer: 5′-TTCTGTATCAGTCTTTCCTGGTGA-3′, efficiency, 1.89; GAPDH: (240 bp), forward primer: 5′-TGATGACATCAAGAAGGTGGTGAAG-3′; reverse primer: 5′-TCCTTGGAGGCCATGTAGGCCAT-3′, efficiency, 1.9.

Quantification of relative expression was determined by standard curve method according to manufacturer's instructions. Target amount was normalized to GAPDH gene and relative expression was calculated by ratio of normalized target values and calibrator normalized target values.

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 PTK/ZK and Idarubicin 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 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

VEGF enzyme-linked immunosorbent assay were performed on culture supernatants at 48 hours of culture. Cultures were centrifuged at 2000*g* for 5 minutes. Supernatant was separated, aliquoted, and stored at −80°C until assay. VEGF concentrations were determined using a commercially available enzyme-linked immunosorbent assay designed to measure VEGF levels (Quantikine; R&D Systems Europe, UK). The assay employs the quantitative sandwich enzyme immunoassay 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 Flt3 phosphorylation, VEGFR1, and VEGFR2 expression

Flt3 phosphorylation, VEGFR1/Flt-1, and VEGFR2/KDR expression were determined by flow cytometry. To analyze Flt3 phosphorylation, cells were centrifuged and resuspended in PBS containing 2% paraformaldehyde to fix for 10 minutes. Cells were permeabilized with 90% cold methanol for 30 minutes and resuspended in PBS containing 1% bovine serum albumin. The cells were incubated with specific monoclonal antibodies to human phospho-Flt3 (Tyr591) Alexa Fluor-conjugated (Cell Signalling Technology Inc, Beverly, MA, USA) for 1 hour at 4°C. To asses the VEGFR1/Flt-1 and VEGFR2/KDR expression, cells were incubated with specific monoclonal antibodies to human Flt-1 and KDR (R&D Systems) for 20 minutes at 4°C. After washing with PBS, the phosphorylation of Flt3 and VEGFR expression were analyzed on a FACScan (BD).

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

PTK/ZK inhibits cell proliferation and induced apoptosis in AML cell lines

We investigated the effects of inhibition of the VEGF kinase domain containing receptors by PTK/ZK in four AML cell lines in the induction of apoptosis and the inhibition of cell proliferation. PTK/ZK-induced apoptosis in a dose- and time-dependent manner and inhibited cell proliferation in a dose-dependent way in the four AML cell lines. Effects were significant at 48 hours and 40 μM concentration in all cell lines and stronger in MOLM-13 and MV4-11cell lines (Fig. 1).

PTK/ZK inhibits VEGF/VEGFR activation in AML cell lines showing Flt3/ITD mutation

Cytometry analysis showed that the four cell lines expressed high levels of VEGFR: Flt-1: NB4 (85% ± 5%), THP-1 (92% ± 6.5%), MOLM-13 (25.5% ± 3.6%), MV4-11 (61.5% ± 4.5%). KDR: NB4 (70% ± 7.2%), THP-1 (60.5% ± 5.4%), MOLM-13 (45.8% ± 5.5%), MV4-11 (54.5% ± 3.5%).

The treatment with different doses of PTK/ZK (2.5−40 μM) showed no change in VEGFR expression levels seen in AML cell lines (data not shown).

PTK/ZK did not inhibit the levels of VEGF mRNA and protein in NB4 and THP-1 cells (data not shown). However, PTK/ZK decreased those levels in a dose-dependent way in MOLM-13 and MV4-11 cells (Fig. 2). When VEGF supernatant levels were normalized for viable cell number, the inhibitory effect was still significant, suggesting that the reduced VEGF levels are not a reflection of decreased cell number in the presence of PTK/ZK (Fig. 2A).

On the other hand, complete inhibition of the expression of VEGF protein was seen in these two cell lines after 40 μM PTK/ZK treatment (Fig. 2B).

We analyzed activation of the VEGF receptors after treatment with PTK/ZK (5−40 μM) in the four cell lines. Western blot results showed that PTK/ZK inhibited Flt-1 phosphorylation and the nuclear phosphorylated KDR form (KDR internalization requires phosphorylation of the receptor) from a dose of 5 μM in MOLM-13 and MV4-11 cells. The effects of PTK/ZK on inhibition of the VEGFR phosphorylation were slight at 40 μM in NB4 and THP-1 cells (Fig. 3A). This fact might be correlated to the modest activity of this inhibitor on both inhibition of cell proliferation and induction of apoptosis in these cells.

To evaluate the significance of the VEGFR signal blockade in the activation of several intracellular pathways, we studied the phosphorylation of Akt, ERK, and STAT5 kinases. Parallel downregulation of Akt, ERK, and STAT5 kinases in a dose-dependent manner was observed in MOLM-13 and MV4-11 cells after treatment with 20 to 40 μM PTK/ZK. The stronger effects were seen after treatment with 40 μM. A low inhibition of Akt Ser phosphorylation was seen in THP-1 cells. In contrast, no changes were found in the constitutive activation of these kinases in NB4 cells (Fig. 3B).

On the other hand, inhibition of Bcl-2 expression after PTK/ZK 40 μM treatment, as measured by densitometric analysis of Western blot (NB4: control 4582 ± 411, PTK/ZK treatment 3830 ± 255; THP-1: control 3981 ± 403, PTK/ZK treatment 3078 ± 190; MOLM-13: control 4525 ± 216, PTK/ZK treatment 3252 ± 205; MV4-11: 4100 ± 199, 2740 ± 256 AU of IOD; all $p < 0.05$) (Fig. 3B) showed a parallel behavior with the apoptosis levels induced by such treatment (Fig. 1).

PTK/ZK reduced the Bcl-2 expression in a different grade in the cell lines studied according to the percentage of apoptosis induced by the inhibitor.

The effects of PTK/ZK in cell death and inhibition of intracellular pathways activation were stronger in the MOLM-13 and MV4-11 cells, which carried the Flt3/ITD mutation. These data suggested that this mutation could be responsible for the sensitivity of these cells to treatment with PTK/ZK. Therefore, we examined the Flt3 phosphorylation levels by

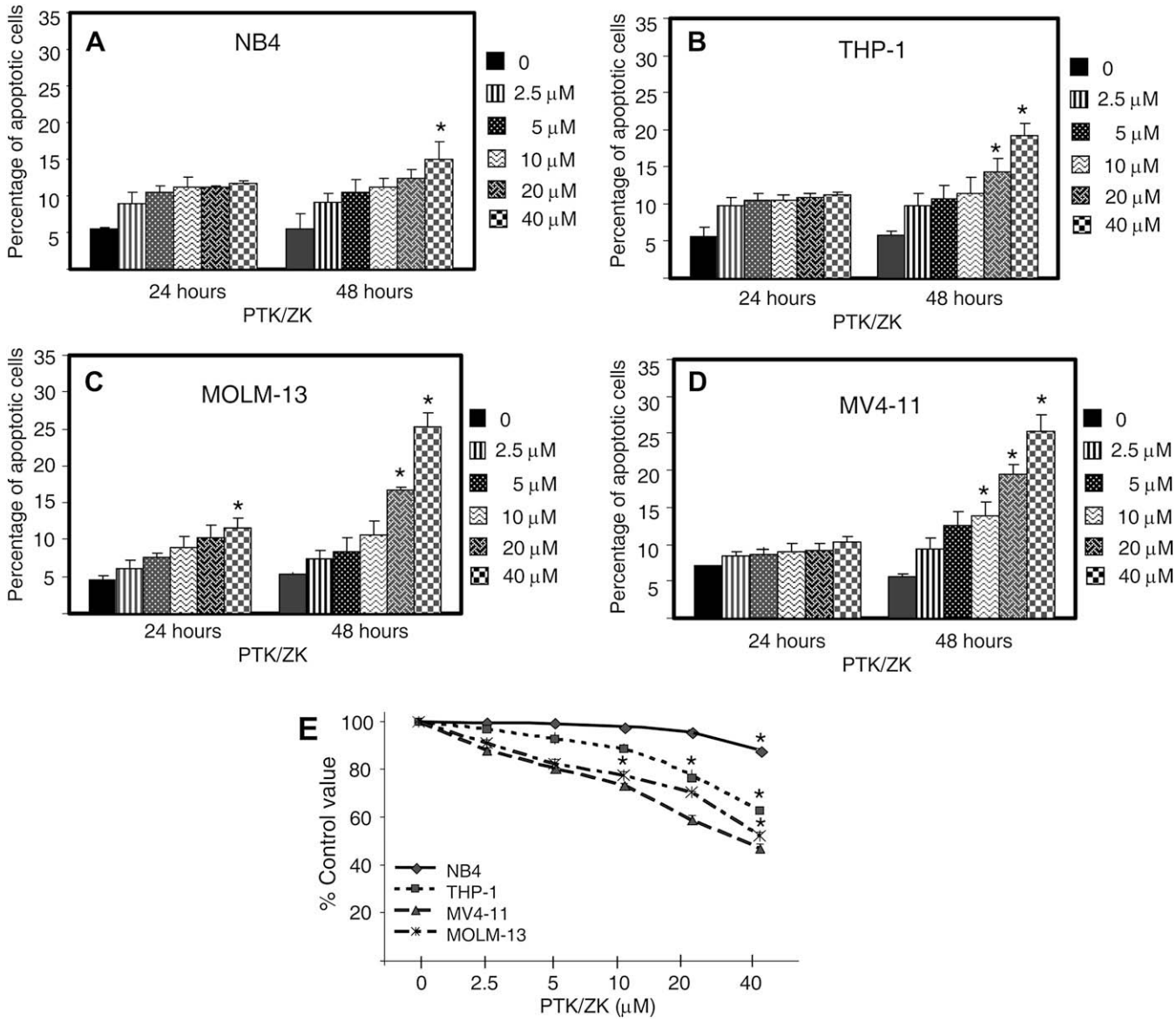

Figure 1. PTK/ZK induces apoptosis and inhibits the cell proliferation in acute myeloid leukemia (AML) cell lines. Cells were incubated with PTK/ZK at different concentrations for 24 and 48 hours. (**A**,**B**,**C**,**D**) Apoptosis was evaluated by the presence of Annexin-V on the cell surface. (**E**) Cell proliferation was assessed by the XTT-based assay. Panels show average (mean ± standard error) of one representative experiment of three experiments performed in triplicate; $^*p < 0.05$ vs untreated cells.

flow cytometry after treatment with different doses of PTK/ZK (20 – 40 μM). Results showed that this inhibitor did not affect the Flt3 activation status seen in MOLM-13 and MV4-11 cells (62.5% ± 3.6% and 92.7% ± 4.5%, respectively), indicating that the mutation in Flt3 was not a direct target for PTK787/ZK in these cell lines.

PTK/ZK enhances AML cells chemosensitivity to Idarubicin

Anthracyclines are a class of chemotherapeutic agents commonly used in the treatment of AML [30]. Therefore, the next step was to test the addition of Idarubicin to the VEGFR inhibitor PTK787/ZK treatment of the AML cells, and to determine the cell death and inhibition of the cell proliferation percentage induced by both drugs.

We first treated the AML cell lines with different doses of Idarubicin alone (0.5 – 10 ng/mL) (data not shown); we chose 2 ng/mL Idarubicin because this concentration was near the apoptosis and proliferation values induced by PTK/ZK 20 μM administered alone.

The combined treatment with PTK/ZK and Idarubicin promoted more apoptosis and inhibition of the cell proliferation than each compound administered separately in all cell lines studied (Fig. 4A and B).

We next evaluated the direct effect of PTK/ZK on the cell growth of AML samples. Seven AML samples were

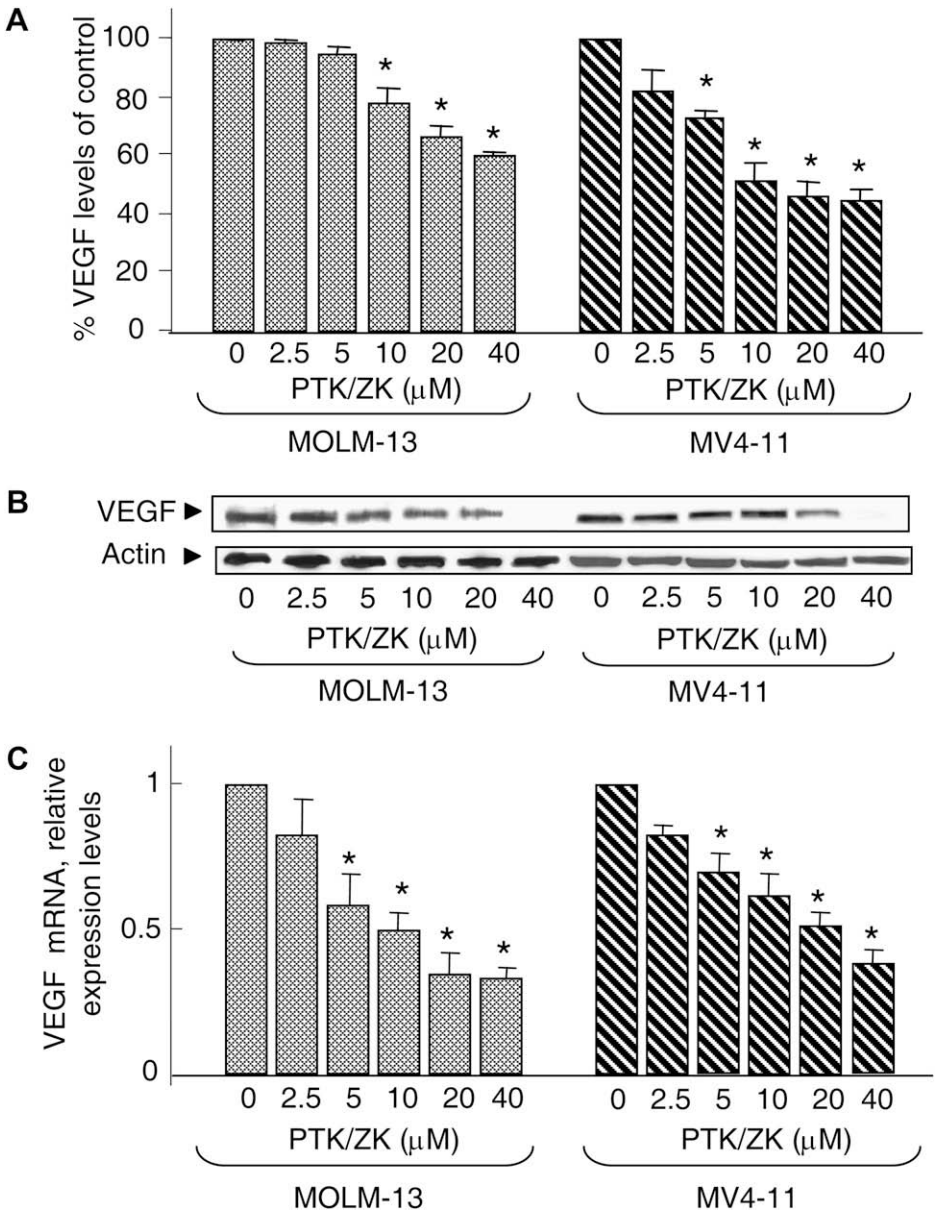

Figure 2. PTK/ZK inhibits vascular endothelial growth factor (VEGF) expression and RNA levels in acute myeloid leukemia (AML) cell lines showing Fms-like tyrosine kinase 3/internal tandem duplication (Flt3/ITD) mutation. Cells were treated with PTK/ZK at indicated concentrations for 48 hours. (**A**) Supernatant levels of VEGF were analyzed by enzyme-linked immunosorbent assay. The panels show average (mean ± standard error) of one representative experiment of three experiments performed in triplicate; $*p < 0.05$ vs untreated cells. (**B**) Total proteins (70 μg/lane) were electrophoresed on a 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and then transferred to nitrocellulose membranes. Membranes were probed with human anti-VEGF polyclonal antibody. To demonstrate equal protein loading, the immunoblots were stripped and reprobed with human anti-Actin. Panels show one representative experiment of three independent experiments with similar results. (**C**) Total cellular RNA was extracted from AML cell lines and subjected to reverse transcription polymerase chain reaction real-time amplification using primer pairs to detect VEGF. The panels show average (mean ± standard error) of one representative experiment of three experiments performed in triplicate; $*p < 0.05$ vs untreated cells.

cultured with or without the VEGFR kinase inhibitor, PTK/ZK given alone or combined with Idarubicin for 24 hours.

The cytometry analysis showed that all samples presented values of KDR and Flt-1 expression between 20% and 65%. PTK/ZK decreased in a dose-dependent manner the cell proliferation in all AML samples, reaching a 70% ± 14.5% of inhibition at 40 μM (data not shown). Primary blast samples were overall more sensitive to PTK/ZK than the leukemic cell lines used in this study.

Exposure of AML samples to the combined treatment with PTK/ZK plus Idarubicin increased both the percentages of Annexin-V−positive cells and inhibition of cell proliferation induced by each treatment administered alone (Fig. 5).

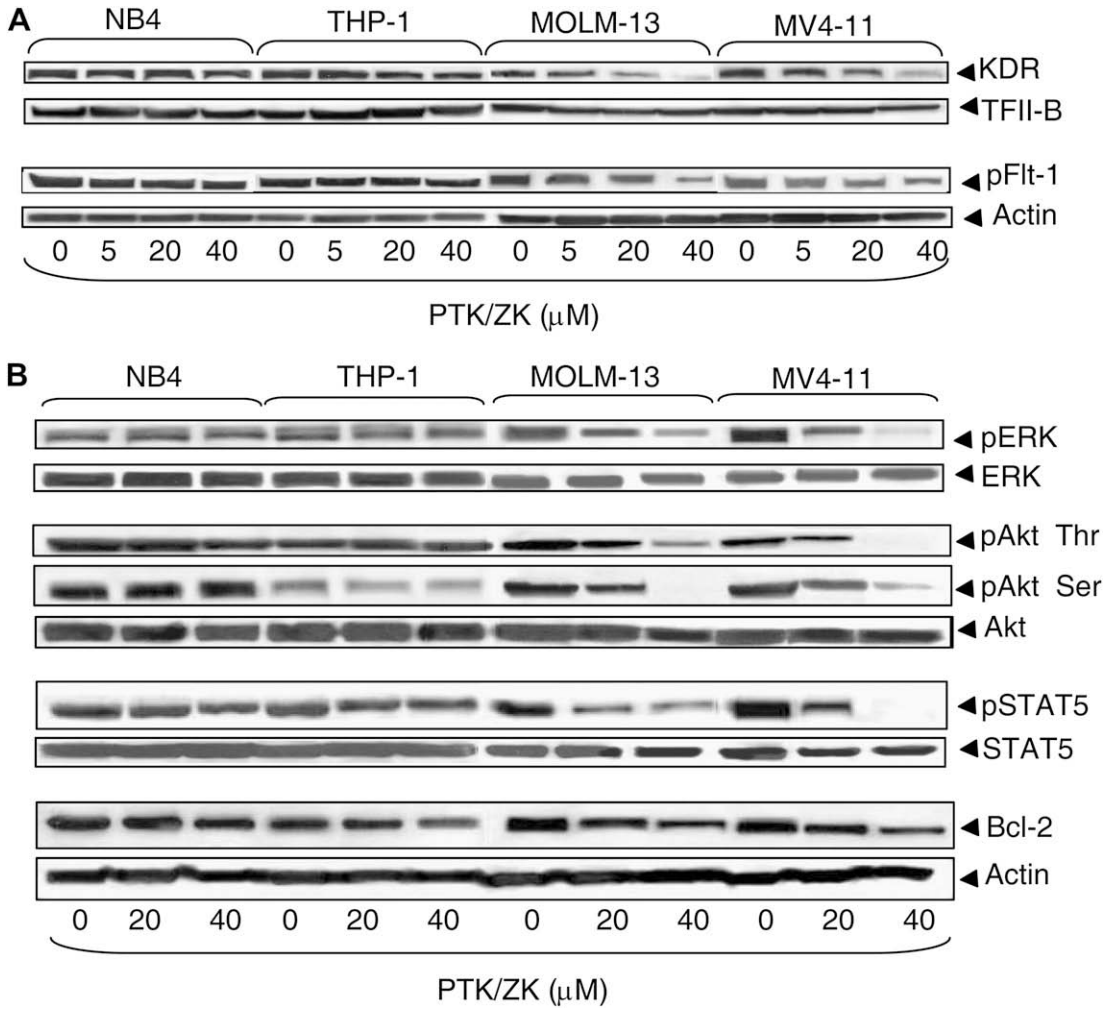

Figure 3. PTK/ZK blocks vascular endothelial growth factor receptors (VEGFR) and intracellular pathways activation in acute myeloid leukemia (AML) cell lines showing Fms-like tyrosine kinase 3/internal tandem duplication (FLT3/ITD) mutation. Cells were treated with PTK/ZK at indicated concentrations for 48 hours. (**A**) Nuclear KDR localization: 50 μg of nuclear proteins extracts were electrophoresed on a 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred to nitrocellulose membranes. The 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 protein 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. Panels show one representative experiment of three independent experiments with similar results. (**B**) Cell lysates were prepared and total proteins (50 μg/lane) were electrophoresed on 10% and 6% SDS-PAGE gels and then transferred to nitrocellulose membranes. Membranes were probed with anti−phospho-extracellular signal-regulated kinase (ERK), anti−phospho-signal transducer activator of transcription 5 (STAT5), anti−phospho-Akt, and anti − Bcl2 polyclonal antibodies. To demonstrate equal protein loading, the immunoblots were stripped and reprobed with anti-ERK, anti-STAT5, anti-Akt, and anti-Actin antibodies. Panels show one representative experiment of three independent experiments with similar results.

Addition of Idarubicin does not alter the decrease of VEGF/VEGFR activation induced by PTK/ZK

To determine if the combined treatment with PTK/ZK and Idarubicin triggers an increased inhibition of the activated intracellular pathways promoted by the VEGFR inhibitor alone; we investigated the phosphorylation of VEGFR and activation of these intracellular pathways: Akt, ERK, and STAT5.

The addition of the chemotherapeutic agent, Idarubicin, to the PTK/ZK treatment did not significantly change the inhibition of VEGF mRNA and protein levels promoted by PTK/ZK given alone in MOLM-13 and MV4-11 cells (Fig. 6A and B). In the same way, Idarubicin did not alter inhibition of the Akt, ERK, and STAT5 phosphorylation caused by PTK/ZK administered separately in these cells (Fig. 6C).

Although the addition of Idarubicin did not produce any effects in the KDR and Flt-1 phosphorylation levels, or in the intracellular pathways activated by them, it caused more inhibition of Bcl-2 protein expression than each compound alone in all cell lines (NB4 and THP-1, data not shown). Thus, the addition of Idarubicin to the PTK/ZK treatment promoted an additive inhibitory effect in

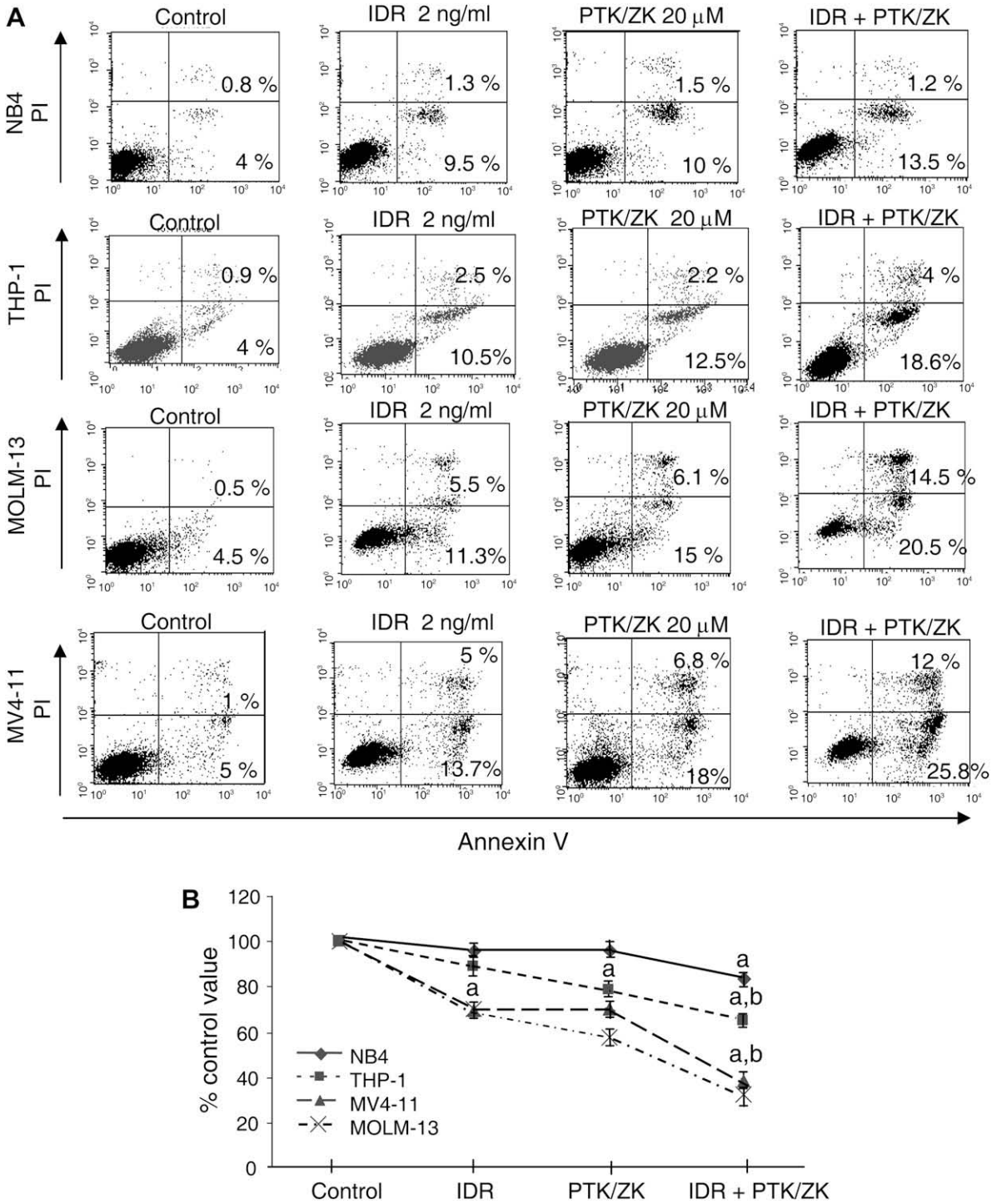

Figure 4. PTK/ZK plus Idarubicin (IDR) increases induction of apoptosis and inhibition of cell proliferation induced by each compound administered alone. The four cell lines were treated with PTK/ZK 20 μM combined with IDR 2 ng/mL for 48 hours. PTK/ZK was added 30 minutes prior to the addition of IDR. (**A**) Apoptosis was evaluated by presence of Annexin-V on the cell surface. Panels show one representative experiment of three independent experiments with similar results. (**B**) Cell proliferation was assessed by XTT-based assay. Panels show average (mean ± standard error) of one representative experiment of three experiments performed in triplicate; $p < 0.05$ (**a**) vs untreated cells (**b**) vs cells treated with each compound alone.

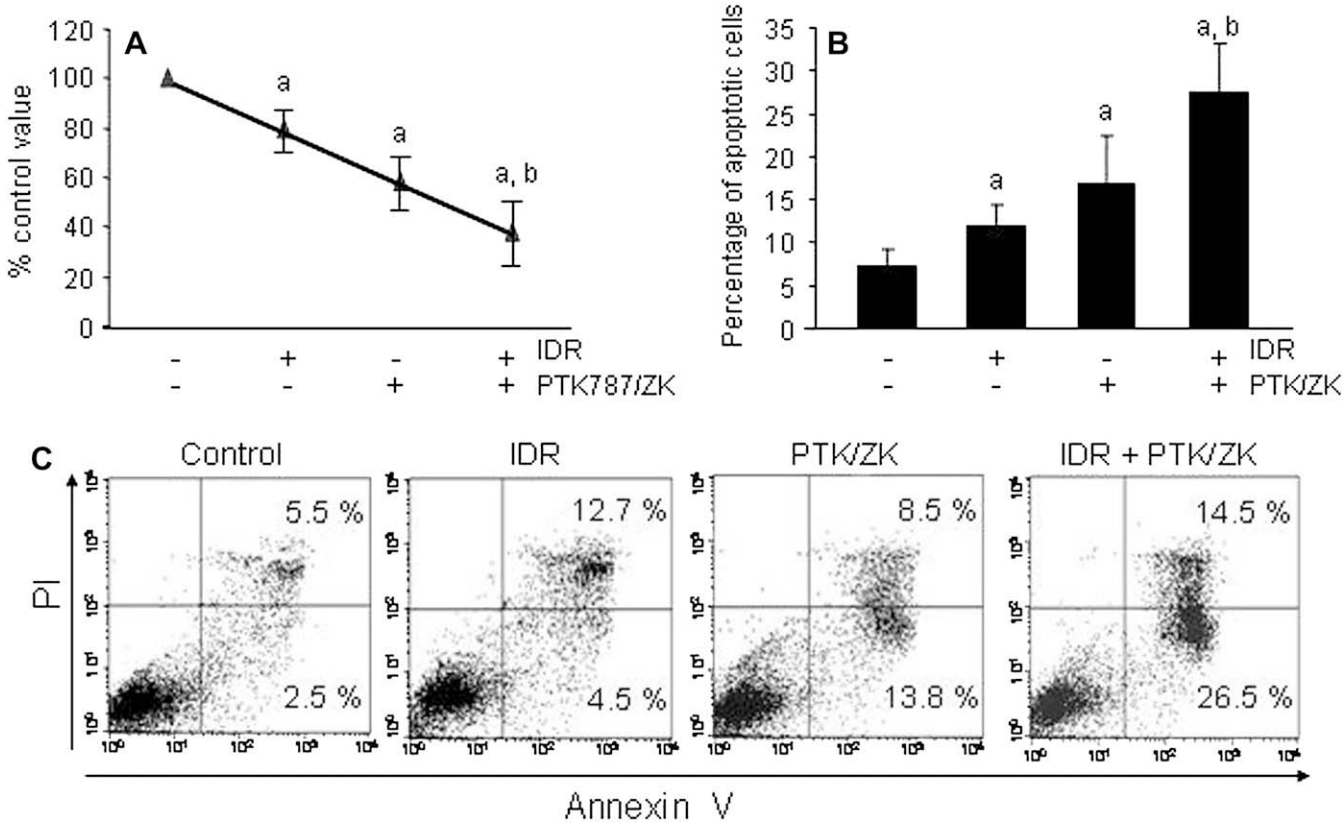

Figure 5. Combined treatment with PTK/ZK and Idarubicin (IDR) causes more apoptosis and inhibition of cell proliferation than each compound administered alone in blasts from acute myeloid leukemia (AML) patients. AML blasts were treated with PTK/ZK 20 μM combined with IDR 2 ng/mL for 24 hours. PTK/ZK was added 30 minutes prior to the addition of IDR. (**A**) Cell proliferation was assessed by XTT-based assay. (**B**) Apoptosis was evaluated by presence of Annexin-V on the cell surface. Panels represent average (mean ± standard error of mean) of treatment on the seven different clinical patients; $p < 0.05$ (**a**) vs untreated cells, (**b**) vs cells treated with each compound alone. (**C**) Apoptosis was evaluated by presence of Annexin-V on the cell surface. Panels show one representative experiment of seven independent experiments with similar results.

the AML proliferation and death cell, but did not affect the inhibition of the angiogenic process produced by PTK/ZK given alone.

Discussion

By analyzing the in vitro activity of PTK787/ZK 222584 combined with Idarubicin in four AML cell lines and blasts from AML patients, the present study has shown an effective inhibitory effect of this combined set of antitumoral drugs on cell proliferation, apoptosis, and angiogenesis.

AML is the most common type of acute leukemia in adults. Patients typically respond to initial treatment with anthracycline and cytosine arabinoside–based induction chemotherapy, but most patients ultimately relapse and die of refractory disease. This fact has led to a search for newer therapies that might target fundamental molecular abnormalities in AML cells [31,32]. Signaling via receptor tyrosine kinases is frequently dysregulated in this disease. We and others have previously reported that myeloblasts from AML patients have demonstrable VEGFR1/Flt-1 and VEGFR2/KDR expression [28,33]. Clinical studies have evaluated the biological activity of PTK/ZK in several advanced solid tumors known to overexpress VEGF and its receptors, such as metastatic gastrointestinal tumors, renal cell carcinoma, prostate cancer, glioblastoma multiforme, and AML or myelodysplastic syndrome [22,34–37]. However, to date, there is only one work that evaluates just the in vitro effects of this inhibitor on AML cell death [23]. Our study further analyzed the effect of PTK/ZK in the angiogenic process, including its effects on the intracellular pathways regulated by the VEGF/VEGFR system.

Mitogenic autocrine loops between VEGF and VEGFR have been described in several tumors, including AML [8,38]. In the present study, we found that all AML cell lines constitutively expressed high levels of both VEGF and phosphorylated VEGFR. However, PTK/ZK decreased significantly those levels in only two cell lines (e.g., MOLM-13 and MV4-11) that presented low levels of VEGFR expression. Thus, a direct relationship between the levels of VEGFR expression and the sensitivity for

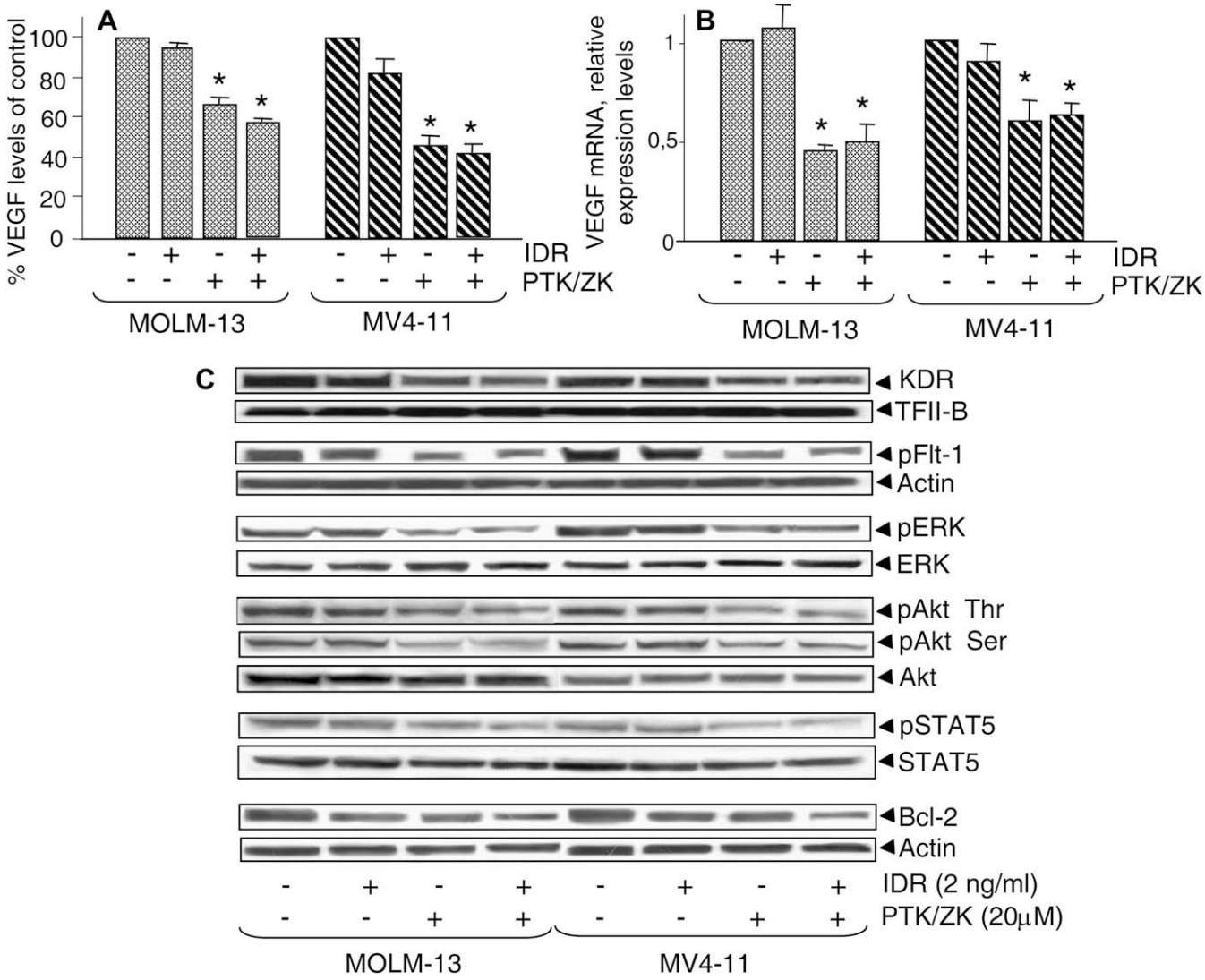

Figure 6. Addition of Idarubicin (IDR) does not affect inhibition of vascular endothelial growth factor (VEGF) expression and intracellular pathways activation induced by PTK/ZK treatment. Cell lines were treated with PTK/ZK 20 μM combined with IDR 2 ng/mL for 48 hours. PTK/ZK was added 30 minutes prior to the addition of IDR. (**A**) Supernatant levels of VEGF were analyzed by enzyme-linked immunosorbent assay. (**B**) Total cellular RNA was extracted from acute myeloid leukemia (AML) cell lines and subjected to reverse transcription polymerase chain reaction real-time amplification using primer pairs to detect VEGF. Panels show average (mean ± standard error) of one representative experiment of three experiments performed in triplicate; $*p < 0.05$ vs untreated cells. (**C**) 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. Cell lysates were prepared and total proteins (50 μg/lane) were electrophoresed on 10% and 6% SDS-PAGE gels and then transferred to nitrocellulose membranes. Membranes were probed with anti−phospho-ERK, anti−phospho-STAT5, anti−phospho-Akt, and anti−Bcl2 polyclonal antibodies. To demonstrate equal protein loading, immunoblots were stripped and reprobed with anti-ERK, anti-STAT5, anti-Akt, and anti-Actin antibodies. Panels show one representative experiment of three independent experiments with similar results.

treatment with PTK/ZK could not be established. These results are in agreement with a recent work in which the authors did not find a relationship between the level of KDR expression and the response to PTK/ZK in AML patient samples [23]. Moreover, the cells that presented more sensitivity for the treatment with PTK/ZK in the inhibition of VEGF expression also showed the autophosphorylation of the mutated tyrosine kinase receptor Flt3. These data are consistent with a previous report that indicated that Flt3 signaling led to secretion of VEGF in vitro, most notably in Flt3/ITD cell lines, suggesting that VEGF is a direct target of Flt3/ITD signal transduction pathway [39].

The antiapoptotic effects of ERK, Akt, and JAK/STAT pathways, activated in response to the stimulation of different receptor tyrosine kinases, are well described in AML cells [28,40–42]. Accordingly, PTK/ZK caused more apoptosis

and inhibition of the cell proliferation in the cell lines showing simultaneously activated various receptor tyrosine kinases (MOLM-13 and MV4-11). Moreover, PTK/ZK also downregulated the VEGFR and Flt3 downstream effectors, ERK, Akt, and STAT, in these cell lines.

To date, Flt3 mutations are attractive AML targets, as these mutations are one of the most common molecular abnormalities found in cases of AML, conferring a poor prognosis [13,15]. In our work conditions, although the greater effect of PTK/ZK took place in AML cell lines with the Flt3/ITD mutation, this inhibitor did not abrogate the Flt3 phosphorylation. Flt3/ITD receptor may not be a direct target of PTK/ZK, but it does act directly against the Flt3 downstream effectors such as STAT5. As described previously, the signals downstream from wild-type and mutant Flt3 receptors are not identical. Contrary to the Flt3/wild-type cells, the survival of Flt3/ITD cells depends on the constitute activation of several intracellular pathways, including Akt and STAT5 [43–45], and the blockade of additional signaling pathways would be necessary to elicit a significant apoptotic and antiproliferative response in Flt3/WT cells. In our results, PTK/ZK potently inhibited the STAT5, ERK, and Akt activation in Flt3/ITD cells. This data might indicate that PTK/ZK may inhibit VEGF expression through suppression of these signaling molecules downstream of Flt3/ITD, but not directly affecting Flt3/ITD phosphorylation. Signaling downstream of Flt3/ITD seems to converge in some point with the VEGFR signaling pathway.

In a recent work, treatment with PTK/ZK administered twice daily was generally well-tolerated in AML patients with a favorable safety profile. Yet, monotherapy with PTK/ZK resulted in no significant clinical efficacy in patients with relapsed, refractory, or newly diagnosed poor-prognosis AML, although two patients did have prolonged stabilization of disease, suggesting some biological activity [22]. It would be interesting to know if these patients presented Flt3/ITD-activating mutations, which might explain the sensitivity of those patients to the monotherapy with PTK/ZK. The relationship between the cell signaling induced by the simultaneous presence of the Flt3/ITD mutation and overexpression of VEGFR needs further investigation in AML patients.

Several inhibitors of the tyrosine kinase activity are currently being studied in phase I and II clinical trials of patients with AML, but no agent, given as monotherapy, has demonstrated an obvious clinical benefit over the traditional cytotoxic chemotherapy [22,46]. In a recent review, Gasparini et al. [47] described the advantages of combining antiangiogenic agents with others anticancer therapies, which include antiangiogenic and cytotoxic agents that work independently on different cellular targets; angiogenesis inhibitors that reduce interstitial pressure and favor tissue diffusion of chemotherapy; anti-VEGF compounds that increase vessel permeability and extravasation of cytotoxic agents, and combination therapy that can result in reduced secretion of both soluble endothelial growth factors and soluble tumor growth factors.

Idarubicin is one of the most effective but also toxic drugs in the treatment of AML [48]. In our study, we showed that the addition of low doses of Idarubicin to the treatment with PTK/ZK resulted in an increased cell death rate and inhibition of cell proliferation in the four cell lines studied, even in those that presented modest response to PTK/ZK (NB4 and THP-1 cells). In addition, an additive effect on apoptosis induction was shown by the treatment with the drug combination in all AML primary blasts.

Our data agreed with a previous report in which, in a phase I study of PTK/ZK for the treatment of primary refractory or relapsed AML, 5 of 17 patients treated with induction chemotherapy and PTK/ZK achieved complete remission [22]. Furthermore, a recent in vitro study showed that the combined treatment with amsacrine and PTK/ZK might lower the dosage of chemotherapy necessary to achieve equal levels of AML cell death [23].

Taken together, our work suggest that targeting VEGFR tyrosine kinase activity by PTK/ZK combined with low dose of Idarubicin might be an effective therapeutic approach in AML, reducing the risk and complications of long-term effects of the chemotherapy treatment.

Acknowledgments

This work was supported by a by grants from the “Junta de Andalucía” (Sevilla, Spain) (Exp. 0030/07), the “Fondo de Investigación Sanitaria” (Madrid, Spain) (FIS PI050910) and The Josep Carreras International Leukaemia Foundation (FIJC-06/ ESP) of Spain (Barcelona, Spain). The authors thank Novartis Pharmaceuticals (Basel, Switzerland) or Schering AG (Basel, Switzerland) for allowing the use of PTK787/ ZK 222584 in this study. The authors reported no conflict of interest.

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