The Vascular Endothelial Growth Factor Receptor Tyrosine Kinase Inhibitors Vatalanib and Pazopanib Potently Induce Apoptosis in Chronic Lymphocytic Leukemia Cells In vitro and In vivo


Abstract

Purpose: There is evidence that vascular endothelial growth factor (VEGF) is a critical microenvironmental factor that exerts angiogenesis-independent effects on the survival of chronic lymphocytic leukemia (CLL) cells. Vatalanib and pazopanib are potent orally available VEGF receptor tyrosine kinase inhibitors. We investigated the efficacy and selectivity of both compounds in CLL cells, simulated potential combination with conventional cytostatics, and tested the effect of both substances on CLL-like tumor xenografts.

Experimental Design: Primary CLL and normal peripheral blood cells were tested for viability after incubation with varying concentrations of both inhibitors. Further, phosphorylation status of VEGF receptor on treatment, caspase activation, and poly(ADP-ribose) polymerase cleavage were assessed. Combinations of each inhibitor with fludarabine, vincristine, and doxorubicin were analyzed for possible synergistic effects in vitro. For in vivo testing, mice grafted with the CLL-like cell line JVM-3 were treated orally with each inhibitor.

Results: Vatalanib and pazopanib decreased phosphorylation of the VEGF receptor, along with induction of apoptosis in CLL cells in clinically achievable concentrations. Healthy B cells were only mildly affected. Immunoblots showed downregulation of the antiapoptotic proteins XIAP and MCL1, whereas poly(ADP-ribose) polymerase cleavage was increased. Combinations with conventional cytostatic agents resulted in synergistic effects. Treatment of xenografted mice with 100 mg/kg of body weight for 21 days resulted in tumor inhibition rates of 76% (vatalanib) and 77% (pazopanib). In two mice, a total tumor eradication could be observed. No gross systemic toxicity occurred.

Conclusion: We conclude that VEGF inhibition is a promising new therapeutic approach in CLL. Vatalanib and pazopanib seem to be effective and safe candidates to be further evaluated for this purpose.

Chronic lymphocytic leukemia (CLL) is the most frequent type of leukemia in adults in North America and Western Europe. Biologically, CLL is characterized by an accumulation of mature but dysfunctional CD5+, CD19+, and CD23+ B cells with poor self-regeneration (1). Rather, their accumulation results from a defect in the initiation of their regular cell death through apoptosis (2). Accumulating neoplastic CLL cells replace other healthy blood components, which results in symptoms of this disease. CLL patients show a very diverse clinical course. The reasons may be differences in genetic predisposition or molecular causes, and continue to be a subject of scientific research. The molecular background of the resistance of CLL cells to apoptosis, the so-called apoptotic block, is a major focus of this research as it may reveal target structures for specific therapies.

Vascular endothelial growth factor-A (VEGF-A) has been discussed to play an angiogenic-independent role in CLL cells by preventing apoptosis in these cells. VEGF-A, commonly referred to as VEGF, belongs to the superfamily of platelet-derived growth factors and exhibits several isoforms through alternative splicing, with VEGF121,
VEGF	extsubscript{165}, VEGF	extsubscript{186}, VEGF	extsubscript{189}, and VEGF	extsubscript{206} being the most common ones (3–5). VEGF is regarded as the most important proangiogenic factor; however, its biological functions range from the regulation of embryonic vascular development to the enhancement of vascular permeability (6, 7). Further, with respect to a nonangiogenic role, VEGF was shown to be a substantial factor for the survival of hematopoietic stem cells (8).

Thus far, three VEGF receptors were described: VEGF-R1 or fms-like receptor tyrosine kinase (Flt-1), VEGF-R2 or fetal liver kinase-1 (Flk-1), or kinase-insert domain containing receptor (KDR) and VEGF-R3 or Flt-4. VEGF receptors homodimerize on ligand binding, which results in transphosphorylation of their intracellular kinase domains and subsequently to activation of signaling pathways (9). Under physiologic conditions, VEGF receptors are mainly located on vascular, lymphatic, and endothelial cells, but have also been shown in several nonendothelial cells. VEGF receptors were further detected on several cancer cells, such as breast, colon, and pancreatic cancer cells. Under functional aspects, VEGF-R1 is mainly known for cell differentiation and migration; VEGF-R2 regulates cell proliferation, growth, and survival; whereas VEGF-R3 is associated with lymphangiogenesis (2, 10–14).

CLL cells spontaneously secrete VEGF, primarily the isoforms VEGF	extsubscript{165} and VEGF	extsubscript{211} (15, 16). Further, they constitutively express activated VEGF-R1 and VEGF-R2 (16). Several correlations have been described between VEGF levels in serum or plasma and CLL progression or disease stage. In early stages, a positive association was found between VEGF levels in serum and disease progression (17). Although the majority of investiga-

tions showed a positive correlation between the level of VEGF receptors on the cell surface and shorter survival in CLL patients (18) there is a single study reporting the duration of progression-free survival tending to be shorter in patients with low intracellular VEGF levels; however, this was not statistically significant (19). It could further be shown in biopsies that the density of vessels in the bone marrow of CLL patients was greater than that in healthy individuals, and vessel density in bone marrow was positively correlated with disease progression (20). There is also evidence for angiogenesis-independent effects of VEGF in CLL. Thus, it was shown that exogenic recombinant VEGF	extsubscript{165} can reduce the rate of spontaneous as well as chemotherapy-induced apoptosis in CLL cells in vitro (21). Simultaneously, a significant upregulation of antiapoptotic proteins such as myeloid cell leukemia-1 (MCL1) and X-linked inhibitor of apoptosis (XIAP) was found on VEGF stimulation (21). SU112657, a receptor tyrosine kinase inhibitor (RTKI), was found to reduce the phosphorylation of VEGF-R1 and VEGFR-R2 as well as trigger apoptosis in CLL cells in vitro (21, 22). However, the authors speculated that this compound does not have sufficient activity in vivo to work in clinical trials for CLL patients (21).

Vatalanib (an anilino phthalazine) and pazopanib (an indazolyl pyrimidine) are potent inhibitors of VEGF-R. Both substances inhibit the VEGF receptor tyrosine kinase in the submicromolar range (IC	extsubscript{50}, VEGF-R1, 54 and 10 nmol/L, respectively; VEGF-R2, 39 and 30 nmol/L, respectively). At higher concentrations, they also inhibit other tyrosine kinases such as platelet-derived growth factor and c-Kit (9, 23). For pazopanib, a steady-state concentration of ≥40 μmol/L is required for optimal in vitro activity against VEGF-R2. This in vivo-in vitro discrepancy can be attributed at least in part to a high protein binding of the substances (24). Vatalanib has a half-life of 5.3 hours. However, its area under the curve (AUC) and half-life are reduced along with treatment duration (AUC ~50% on day 14), most likely through enzyme induction (9). The half-life of pazopanib (35 hours) is markedly longer (9).

Vatalanib is currently being tested in various clinical studies for malignant diseases. In a phase III trial, pazopanib showed promising results in metastatic renal cell carcinoma and is currently being tested in a number of phase II trials. Side effects observed with either substance were generally mild, including nausea and vomiting, diarrhea, hypertension, weakness, and dizziness (9, 25). In mouse models, with daily doses of 100 mg/kg of body weight, chronic serum levels of ≥10 μmol/L vatalanib were achieved and were well tolerated (26). Both compounds have the advantage of being orally available, strongly increasing their applicability.

Based on the above-mentioned findings, we speculated that vatalanib and pazopanib can induce apoptosis in CLL cells at clinically achievable concentrations. We further intended to investigate both compounds as...
additional drugs for combination with conventional cytostatics.

Materials and Methods

Patient samples and purification of lymphocytes
All patients had been informed about the use of their blood for the purposes of the study and had provided their consent. Patients were either untreated or did not receive treatment for the last 3 months before blood sampling. The study was conducted in accordance with the Declaration of Helsinki (6th revision, Seoul/South Korea, 2008) and was approved by the local ethics committee. B cells were purified by negative selection using RosetteSep (StemCell Technologies, Inc.). This cocktail contains antibodies directed against surface markers of non–B cells (CD2, CD3, CD16, CD36, CD56, CD66b, and glycophorinA). The antibodies cross-link non–B cells to multiple red blood cells, which are pelleted along with free red blood cells during density gradient centrifugation. CLL or healthy B cells were assimilated along with free red blood cells during density gradient centrifugation. CLL or healthy B cells were assimilated along with free red blood cells during density gradient centrifugation. 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Xenograft mouse model

Four-week-old female BALB/c nu/nu mice (Charles River Laboratories, Inc.) were irradiated with 3 Gy and inoculated s.c. with $1 \times 10^7$ JVM-3 cells in the posterior flank. When the tumor volume reached about 100 mm$^3$, mice were divided into three groups of 10 mice each. Animals were treated with 100 mg/kg of body weight vatalanib, pazopanib, or aqueous 2% carboxymethylcellulose solution with 0.1% polysorbate 20 (vehicle control) per day orally through a gastric tube. Tumor volume (TV) was determined every 2nd day with a caliper using the formula $TV = 1/2 \times \text{length (mm)} \times \text{height (mm)} \times \text{breadth (mm)}$. Mice were inspected visually for occurrence of obvious side effects. As part of the inspection of their general condition, mice were weighed once every week. After the last treatment, the liver, kidneys, and spleen were removed for the purpose of investigation.

Statistical analysis

Statistical analysis was done using the program GraphPad Prism (GraphPad Software). If not mentioned otherwise, unpaired two-sided Student’s $t$ test was used. Differences of $P < 0.05$ were considered to be significant.

Results

Vatalanib and pazopanib induce dose-dependent and selective apoptosis in CLL cells

To test the effect of vatalanib and pazopanib on the survival of CLL cells, CLL cells were incubated for 24 hours with each substance at concentrations of 1 to 200 μmol/L. The percentage of living cells was determined using flow cytometry. A dose-dependent reduction in the number of living CLL cells was observed in all samples. The determined 50% lethal concentration (LC50) was 48.4 μmol/L for vatalanib and 32.7 μmol/L for pazopanib. To determine the selectivity of the substances toward CLL cells, B cells of healthy donors ($n = 5$) and CLL cells were similarly treated with vatalanib or pazopanib at concentrations of 10, 50, and 100 μmol/L for 24 hours. Although both RTKI had a significant cell death–inducing effect on CLL cells, normal B cells were significantly less affected (Fig. 1B). Even at a concentration of 100 μmol/L, 80.5 ± 4.5% of B cells were alive, whereas that was the case for only 35.1 ± 4.3% of CLL cells ($P \leq 0.0001$). Treatment with pazopanib yielded similar results: Only 26.5 ± 3.4% of CLL cells survived 24-hour treatment with 100 μmol/L pazopanib; however, for healthy B cells, the values were still as high as 89.1 ± 2.2% ($P \leq 0.0001$).

Fig. 1. Vatalanib and pazopanib induce dose-dependent and selective apoptosis in CLL cells. A, primary CLL samples were cultured in the presence of 1, 10, 25, 50, 75, 100, and 200 μmol/L vatalanib (left) or pazopanib (right) for 24 hours. Survival was measured via Annexin/PI–based fluorescence-activated cell sorting (FACS) assay. The determined LC50 of vatalanib is 48.4 μmol/L while that of pazopanib is 32.7 μmol/L ($n = 24$ and $n = 19$, respectively). B, primary CLL cells ($n = 24$ for vatalanib/$n = 19$ for pazopanib) and healthy B cells ($n = 5$) were treated for 24 hours with 10, 50, and 100 μmol/L vatalanib (left) and pazopanib (right). White columns, CLL samples; black columns, healthy B cells. Survival was assessed by Annexin/PI–based FACS assay. Data are shown as mean ± SEM. $P$ values indicate significance and were calculated using Student’s $t$ test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. 

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VEGF receptor phosphorylation status decreases after RTKI treatment

To check whether vatalanib and pazopanib actually act through inhibition of VEGF signaling, we determined the phosphorylation status of the VEGF receptor in CLL samples after 24 hours of treatment with 10 μmol/L vatalanib and pazopanib compared with a DMSO-treated control group (n = 5). Figure 2 shows a clear shift toward a decreased phosphorylation of VEGF-R2 (Tyr951) in the treated samples. In concordance to previously published data, healthy B cells do not exhibit VEGF receptors (data not shown, ref. 24).

VEGF RTKI induce downregulation of antiapoptotic proteins

To further determine the mechanism of apoptosis induction by the VEGF RTKIs, CLL cells were incubated for 24 hours with vatalanib (Fig. 3A) or pazopanib (Fig. 3B) at concentrations ranging between 0.1 and 100 μmol/L. Further, untreated and DMSO (1%)–treated controls were added. Lysates were analyzed through immunoblotting for apoptotic markers. β-Actin served as loading control. Vatalanib and pazopanib induce concentration-dependent PARP cleavage along with reduced levels of the antiapoptotic proteins XIAP and MCL1.

Vatalanib- and pazopanib-mediated apoptosis is caspase dependent

To determine the role of caspases in vatalanib- and pazopanib-induced apoptosis, we incubated CLL cells with both substances at high concentrations of 100 μmol/L alone or in combination with the pan-caspase inhibitor Z-VAD-FMK at 50 μmol/L (n = 3) for 24 hours. Pretreatment with the pan-caspase inhibitor resulted in a significantly reduced apoptosis rate on RTKI treatment. In Fig. 4, a representative sample is depicted. Although vatalanib treatment alone resulted in a survival rate of only 6.3% of CLL cells (75.8% survival in DMSO treated control), caspase inhibition protected CLL cells from vatalanib-mediated cell death, as seen by a survival rate of 65.6%. The same effect could be observed with pazopanib, where RTKI treatment alone lead to 17.4% surviving CLL cells, whereas pretreatment with V-VAD-FMK significantly increased CLL cell survival (54.2%).

Combination of VEGF RTKI with conventional cytostatics shows synergistic effects

To investigate potential synergistic effects, CLL cells were treated for 24 hours with lower doses of vatalanib or pazopanib (10 μmol/L) alone or in combination with classic chemotherapeutic agents such as fludarabine (10 μmol/L), vincristine (0.1 μmol/L), and doxorubicin (10 μmol/L). The combinations of vatalanib with fludarabine, vincristine, and doxorubicin as well as the combination of pazopanib with fludarabine were associated with marked and significantly higher rates of apoptosis (Fig. 5).

Combined treatment of vatalanib together with cytostatics decreased the survival rate an average of 26.2% compared with fludarabine, vincristine, and doxorubicin alone (Fig. 5A).

The analogous experiment with pazopanib showed significant additive effects when pazopanib was combined with fludarabine and vincristine.

**Fig. 2.** Phosphorylation of the VEGF receptor (VEGFR) decreases after RTKI treatment. CLL cells were treated for 24 hours with 10 μmol/L of vatalanib and pazopanib, and the phosphorylation status of VEGFR-2 (Tyr951) was determined by flow cytometry. This representative sample demonstrates the decrease in phosphorylated VEGF receptor after treatment with both RTKIs (n = 5).

**Fig. 3.** Vatalanib and pazopanib induce concentration-dependent cleavage of PARP and downregulation of antiapoptotic proteins. Whole-cell lysates were analyzed after 24-hour incubation of CLL cells with 0.1, 1, 10, and 100 μmol/L vatalanib (A) or pazopanib (B) by immunoblotting. Untreated samples (UTC) and samples treated with 1% DMSO served as control. The blots illustrate the concentration-dependent decrease of the antiapoptotic proteins XIAP and MCL1 and the increase of cleaved PARP in CLL cells after exposure to the RTKIs. One representative blot out of three experiments is shown.
with fludarabine, with a survival rate of 75.2% after pazopanib treatment alone and 81.3% after fludarabine treatment alone, and only 52.7% survival after combined treatment ($P = 0.042$; Fig. 5B). Vincristine and doxorubicin-induced cell death were slightly increased when pazopanib was added: 53.0 ± 9.3% survival after vincristine alone versus 35.2 ± 5.5% survival after combined treatment with vincristine and pazopanib; 59.1 ± 8.0% survival after doxorubicin alone versus 45.0 ± 7.4% survival after combined treatment with doxorubicin and pazopanib. Nevertheless, no statistical significance could be achieved by this combination.

**Vatalanib and pazopanib inhibit tumor growth in a xenograft mouse model**

Both substances were further tested in the JVM-3 xenograft nude mouse model. Mice were generated as explained earlier. Before treatment, mice were split into three groups with the following mean tumor volumes: 112.0 ± 15.8 mm$^3$ in the control group, 112.9 ± 13.3 mm$^3$ in the group to be treated with vatalanib, and 113.3 ± 18.7 mm$^3$ in the pazopanib group. Mice were treated daily through gastric tube. As early as day 12, mean tumor volume was significantly reduced in the pazopanib-treated group compared with the vehicle-treated group, with mean tumor volumes of 446.4 ± 144.5 mm$^3$ versus 663.9 ± 180.1 mm$^3$ ($P = 0.0098$), respectively. On day 14, the tumor volume was also significantly reduced in the pazopanib-treated group compared with the vehicle-treated group, with mean tumor volumes of 446.4 ± 144.5 mm$^3$ versus 663.9 ± 180.1 mm$^3$ ($P = 0.0098$), respectively. On day 14, the tumor volume was also significantly reduced in the group treated with vatalanib with a mean tumor volume of 684.9 ± 167.6 mm$^3$ compared with 1,003.1 ± 226.8 mm$^3$ ($P = 0.027$) in control-treated animals (Fig. 6).

After completion of the treatment period of 21 days, the mean tumor volume was 2,458.3 ± 782.5 mm$^3$ in the control group, 671.8 ± 208.7 mm$^3$ ($P = 0.002$) in the group
treated with vatalanib, and $645.0 \pm 252.3$ mm$^3$ ($P = 0.002$) in the group treated with pazopanib. During the treatment period of 21 days mice showed no signs of pain, disability, or lethargy. Tumor inhibition rates were calculated according to the following formula (27): % inhibition = $1 - \frac{\text{mean tumor growth in the treated group}}{\text{mean tumor growth in the control group}}$.

The tumor inhibition rate was 36% for vatalanib and 49% for pazopanib as early as day 14 (Supplementary Table S1). At the last day of treatment (day 21), tumor inhibition rates were increased to 76% in the vatalanib-treated group and 77% in the pazopanib-treated group. After 3 weeks of treatment, two animals of the vatalanib-treated group did not show a tumor anymore.

Discussion

It has previously been shown that exogenous VEGF can substantially reduce the spontaneous apoptosis rate in CLL cells (22). In our studies we registered that VEGF originated from the bone marrow-derived stromal cell line HS5 improved CLL cell survival in culture. This effect could be reversed by adding a VEGF-neutralizing antibody. Furthermore, it was found that model substances with an inhibitory effect on VEGF receptor tyrosine kinases could trigger apoptosis in CLL cells in vitro (22). To what extent these compounds are tolerated by healthy B cells remains unknown. For a potential therapeutic approach, however, the selectivity of the substances to tumor cells is of crucial importance. In our in vitro experiments, we were able to show that the VEGF RTKIs vatalanib and pazopanib, whose pharmacokinetics in solid tumors have been well described, trigger apoptosis in CLL cells in a concentration-dependent manner. Healthy B cells were much less influenced by these orally available drugs that are highly selective for VEGF-R1 and VEGF-R2. This is in agreement with the results achieved by Podar et al.: Healthy peripheral blood mononuclear cells tolerated the treatment with pazopanib at concentrations of up to 20 $\mu$g/mL ($\approx 43$ $\mu$mol/L) much better than multiple myeloma cell lines (25).

The fact that the effect of the substances on cell survival is dependent on the induction of apoptosis and is caspase dependent was shown by \((a)\) flow cytometric Annexin/PI staining, \((b)\) immunobots for PARP cleavage as a direct target of caspase 3, and \((c)\) the effect of the pan-caspase inhibitor Z-VAD-FMK. Downregulation of antiapoptotic proteins such as XIAP and MCL1 after treatment with VEGF RTKIs observed here is in agreement with the observations of other authors who used multikinase inhibitors (21, 22).

The published literature provides examples of clinically achievable serum concentrations of both VEGF RTKIs used here: $\geq 40$ $\mu$mol/L for pazopanib after once-daily administration of $\geq 800$ mg (28) and $15.8 \pm 9.5$ $\mu$mol/L for vatalanib after once-daily administration of 1,000 mg (29).

From our observations, comparable concentrations were sufficient to markedly reduce the percentage of living CLL cells, whereas healthy B cells were significantly less affected in this concentration range. These data and the LC$_{50}$ values obtained by flow cytometry could be confirmed by...
another ATP-based cell viability assay (ATP Glow, Promega; data not shown). In multiple myeloma cell lines, Podar et al. (25) achieved comparable LC50 values of 5 to 15 μg/mL (≈10–30 μmol/L) for pazopanib. Antiproliferative effects were registered for vatalanib in multiple myeloma cell lines somewhat earlier in concentrations ranging from 1 to 5 μmol/L (25).

Thus far in the treatment of solid tumors, the use of VEGF RTKIs alone was moderately successful. Thus, combination therapies with classic chemotherapeutic agents have been established in this field. It is assumed that VEGF RTKIs remodel the vascular supply of tumors and thus make them more accessible to chemotherapeutic agents. According to our hypothesis, VEGF RTKIs exert an angiogenesis-independent effect in CLL; however, in a study focusing on the potential effect of pazopanib for the treatment of multiple myeloma, Podar et al. showed that combining pazopanib with chemotherapeutic agents may potentiate the effects in hematologic diseases as well (25). We registered a synergistic effect when vatalanib was administered with classic chemotherapeutic agents such as fludarabine, vincristine, and doxorubicin. Pazopanib showed a statistically significant synergistic effect in combination with fludarabine.

To confirm the in vitro data in an in vivo model, the CLL-like cell line JVM-3 was xenografted into irradiated nude mice. The JVM-3 cell line was originally derived from a patient with B-prolymphocytic leukemia and might therefore at first not be considered a convincing CLL mouse model. However, these cells exhibit a Matutes score of 3, which, although not sufficient for the diagnosis of CLL, suggests a certain biological similarity to this disease, and due to a lacking alternative are regarded as a model at least related to CLL (27, 30). We tested both inhibitors, vatalanib and pazopanib, in these xenograft mouse models. After oral administration of vatalanib and pazopanib, the mean tumor volume was reduced to only 26% and 27% of the tumor volume in the control group after 21 days of continuous treatment, respectively. According to the formula (28) % inhibition = 1 - (mean tumor growth in the treated group/mean tumor growth in the control group), this equals a tumor inhibition rate of 76% for vatalanib and 77% for pazopanib. Similar inhibition rates were achieved with pazopanib, administered twice daily at 100 mg/kg of body weight for 21 days in xenografts of other tumor cell lines: 82%, 64%, and 53% for HT29 colon, A375P melanoma, and PC3 prostate, respectively, as shown by Kumar et al. (28). In the same study, tumor inhibition rates of 64%, 95%, and 99% could be achieved by once-daily administration of 100 mg/kg of body weight pazopanib for 21 days in Caki-2 renal, BT474 breast, H322 lung xenografts, respectively. Thus, the inhibition rates we registered after once-daily administration of the drugs were in the mid range. It has to be highlighted that in two animals, complete tumor eradication could be observed. However, because CLL usually presents as leukemic rather than as a tumor disease, it cannot be excluded that in addition to the direct cytotoxic effects an antiangiogenic mechanism might have contributed to the treatment response. Certainly, it would also be desirable to combine VEGF RTKI with conventional cytostatics in our animal model. However, due to the i.p. toxicity of vincristine and doxorubicin, this cannot be done.
In conclusion, vatalanib and pazopanib were well tolerated in our CLL xenograft mouse model. Both compounds exerted a selective effect in vitro and in vivo, and justify the expectation of a good risk-benefit ratio in humans as well. The availability of oral pharmaceutical forms and the long terminal half-life of the drugs are probably associated with good compliance and permit economic outpatient treatment.

Summing up, our observations provide evidence that selective inhibition of the VEGF signaling pathway by vatalanib or pazopanib, either alone or in combination with existing and future therapies, could be a promising approach for further improvement of CLL therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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