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Vandetanib mediates anti-leukemia activity by multiple mechanisms and interacts synergistically with DNA damaging agents

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Summary

Vandetanib is an orally active small molecule tyrosine kinase inhibitor (TKI) with activity against several pathways implicated in malignancy including the vascular endothelial growth factor receptor pathway, the epidermal growth factor receptor pathway, the platelet derived growth factor receptor β pathway, and REarranged during Transfection pathway. To determine if vandetanib-mediated inhibition of receptor tyrosine kinases is a potential therapeutic strategy for pediatric acute leukemia, these studies aimed to characterize the activity of vandetanib against acute leukemia in vitro. Treatment of leukemia cell lines with vandetanib resulted in a dose-dependent decrease in proliferation and survival. Vandetanib's anti-leukemic activity appeared mediated by multiple mechanisms including accumulation in G1 phase at lower concentrations and apoptosis at higher concentrations. Alterations in cell surface markers also occurred with vandetanib treatment, suggesting induction of differentiation. In combination with DNA damaging agents (etoposide and doxorubicin) vandetanib demonstrated synergistic induction of cell death. However in combination with the anti-metabolite methotrexate, vandetanib had an antagonistic effect on cell death. Although several targets of vandetanib are expressed on acute leukemia cell lines, expression of vandetanib targets did not predict vandetanib sensitivity and alone are therefore not likely candidate biomarkers in patients with acute leukemia. Interactions between vandetanib and standard chemotherapy agents in vitro may help guide choice of combination regimens for further evaluation in the clinical setting for patients with relapsed/refractory acute leukemia. Taken together, these preclinical data support clinical evaluation of vandetanib, in combination with cytotoxic chemotherapy, for pediatric leukemia.

Keywords

Acute leukemia; Vandetanib; Vascular endothelial growth factor; Tyrosine kinase inhibitor; Combination therapy

Introduction

Acute leukemia accounts for approximately 5% of all malignancies with an event free survival of 40% for acute lymphoblastic leukemia (ALL) and approximately 20% for acute myeloid leukemia (AML) [1, 2]. Despite advances in therapy the overall cure rate for either type of acute leukemia in adults remains relatively poor. While acute leukemia is a relatively uncommon disease in adults, it is the most common pediatric malignancy, with over 3,000 new cases diagnosed in U.S. children each year. While more than 75% of pediatric patients will be cured with current intensive therapy, patients with acute lymphoblastic leukemia (ALL) with early relapse or acute myeloid leukemia (AML) with any relapse, or those with specific cytogenetic or molecular abnormalities including mixed-lineage leukemia (MLL) gene rearrangements have a dismal outcome [3–9]. These pediatric patients and the adult population still desperately need new therapies.

With the recent advent of biologically-based approaches to the treatment of human malignancies, new agents against a wide variety of molecular targets are currently in clinical development. These agents target specific genes or proteins that are mutated or dysregulated in cancers. As standard chemotherapeutic agents have significant toxicity, agents that are tailored to cancer-specific abnormalities are particularly appealing. The role of vascular endothelial growth factor (VEGF) in tumor angiogenesis is an active area of cancer research. Neoangiogenesis is required for adequate oxygen and nutrition to support a growing tumor mass. Stimulation of VEGF receptors results in activation of signaling pathways that promote endothelial cell survival, migration, differentiation, and increase vascular permeability [10, 11]. VEGF is over-expressed in multiple solid tumors and is associated with poor outcome [12–15]. As a result, VEGFR signaling has been targeted as an anti-angiogenic strategy in cancer.

Vandetanib (ZD6474) is an orally active small molecule tyrosine kinase inhibitor with activity against the VEGF receptor 2 (VEGFR2), also known as kinase insert domain containing receptor (KDR) [16]. It possesses inhibitory activity at submicromolar concentrations against VEGF receptor3 (VEGFR3), the epidermal growth factor receptor (EGFR), Rearranged during Transfection (RET) tyrosine kinase, and at micromolar concentrations activity against VEGF receptor 1 (VEGFR1) and platelet-derived growth factor receptor beta (PDGFR β) [17–19]. In Phase I clinical trials in patients with solid malignancies vandetanib was well tolerated at doses up to 300 mg once daily [20, 21]. Common dose related side effects included rash, diarrhea, hypertension and asymptomatic QTc prolongation. Pharmacokinetic analyses demonstrate a long half-life of approximately 120 h [20]. Vandetanib is currently being studied in Phase II and III trials. Phase II studies in patients with non-small cell lung cancer, breast cancer, or medullary thyroid cancer have shown stable disease or prolonged time to progression in patients treated with vandetanib [22–26]. Newer studies with vandetanib plus gemcitabine and capecitabine in biliary cancers have shown significant prolongation of survival and clinical responses [27]. Vandetanib was granted orphan drug status by the FDA for treatment of medullary thyroid carcinoma where its activity is mediated in part by inhibition of RET [28, 29].

While the role of vandetanib as an angiogenesis inhibitor is being further elucidated in multiple clinical studies, its activity against hematopoietic tumors is less well defined.

Hematopoietic malignancies express VEGF and VEGF signaling plays an important role in hematopoiesis, mediating hematopoietic stem cell survival and repopulation via an autocrine loop and regulating angiogenesis via a paracrine loop [30]. Paracrine stimulation of other cells in the tumor microenvironment can also result in production of growth factors that stimulate leukemia proliferation and/or survival [30]. All three VEGFRs are expressed on ALL and AML patient cells and cell lines [30]. Activation of VEGFR2 in leukemia cells promotes survival through the nuclear factor κ B (NF- κ B), mitogen-activated protein kinase (MAPK)/Extracellular signal-regulated kinase (ERK), and phosphatidylinositol-3 kinase/Akt pathways [31]. VEGFR1 and VEGFR3 signaling have been shown to promote leukemia cell migration, survival, proliferation and chemoresistance [30]. VEGF expression in leukemias is associated with poorer prognosis and decreased relapse-free survival [32, 33]. In addition, pediatric patients with leukemia have increased bone marrow microvessel density suggesting that leukemia may induce and be dependent upon angiogenesis within the bone marrow environment itself [30, 34]. These observations indicate that VEGF and angiogenesis may be desirable targets in leukemia. Additionally, other vandetanib targets may also play important roles in leukemia. The RET proto-oncogene is expressed on normal human CD34⁺ progenitor cells and leukemic blasts from AML patients and RET expression is increased in more differentiated leukemia subtypes [35, 36]. Of note, EGFR is not felt to have a significant role in leukemia survival or proliferation as its expression is rarely detected in patient samples, and activating EGFR mutations are not observed in leukemia [37, 38].

In a previous preclinical report, vandetanib induced growth arrest and apoptosis in 3 of 14 leukemia cell lines and 10 of 13 patient samples with AML [39]. The goal of the studies presented here was to characterize the activity and mechanisms of vandetanib against acute leukemia in vitro, thereby investigating the role of autocrine signaling through the pathways inhibited. Additionally, we evaluated the interactions between vandetanib and selected chemotherapeutic agents. This is particularly important in pediatric oncology where preclinical studies can facilitate rational design of clinical trials and thereby maximize the amount and relevance of information obtained from the limited number of patients available for clinical studies.

Methods and materials

Cell culture—The Molt-4, REH, RCH-AcV, RS4;11, Nalm-6, and HAL-01 cell lines were obtained from Dr. Stephen Hunger (University of Colorado Denver) in 2001. Jurkat, Molt-3, CCRF-HSB-2, and HL-60 were obtained from Dr. Douglas Graham (University of Colorado Denver) in 2004. HEL, Eo1-1, Molm-13, and Molm-14 were obtained from Dr. Robert Arceci (Johns Hopkins University) in 2005. THP-1 was obtained from Dr. Terzah Horton (Texas Children's Hospital) in 2005. CCRF-CEM, Kasumi-1 and MV4-11 were obtained from the American Type Culture Collection (ATCC; Manassas, VA) in 2008. NOMO-1 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) in 2007. All cell lines were received frozen, and passaged to confluent growth. Experiments were performed on established passages less than 3 months of age. The identity and purity of the THP-1, REH, CCRF-CEM, MV4-11, RS4;11, CCRF-HSB-2, Eo1-1, and Nalm-6 cell lines, the purity of the REH cell line and the purity and common source of the Molm-13 and Molm-14 cell lines were confirmed by multiplex polymerase chain reaction DNA profiling using the ABI Identifiler kit (Applied Biosystems, Carlsbad, CA) followed by comparison with the ATCC database. Continuous cultures were verified by DNA profiling and used for experiments within 3 months. Cell lines were maintained at 37°C in 5% CO₂ in RPMI medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin.

Treatment with vandetanib and other chemotherapeutic agents—Vandetanib was kindly provided by AstraZeneca (Macclesfield, UK). Vandetanib and methotrexate (Sigma Aldrich, St Louis MO) stocks were prepared at 10 mM in dimethylsulfoxide (DMSO). Etoposide (EMD Biosciences, Gibbstown, NJ) stocks were prepared at 20 mM in DMSO and doxorubicin (Sigma Aldrich, St Louis, MO) stocks were prepared at 5 mM in distilled, deionized water. Preliminary experiments were performed to determine the maximum density for each cell line such that nutrients would not be limiting for proliferation. Cells were plated and cultured overnight prior to addition of therapeutic agent(s) or vehicle for an additional 48 h.

Assessment of anti-tumor activity

For determination of relative number of metabolically active cells, cells were cultured in 96-well dishes and treated in triplicate with a therapeutic agent(s) and/or vehicle. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma Aldrich, St. Louis, MO) in PBS was added to a final concentration of 0.65 mg/ml after 48 h of treatment with vandetanib and/or chemotherapeutic agents and cells were cultured for an additional 4 h. Solubilization solution (2×, 10% SDS in 0.01 M HCl) was added and plates were incubated at 37°C overnight. Optical density was determined at 562 nm with a reference wavelength of 650 nm. Relative numbers of metabolically active cells were calculated by subtraction of background absorbance and normalization to untreated controls. IC₅₀ values were determined by non-linear regression using Graphpad Prism v4.0 software (Graphpad Software, La Jolla, CA).

Determination of cell death and cell cycle distribution—Cells were cultured in 24-well dishes and collected by centrifugation at 240 g for 5 min after treatment with therapeutic agent(s) for 48 h. For assessment of cell death, cell pellets were resuspended in PBS containing 1 μM YO-PRO®-1 (Invitrogen, Carlsbad, CA) and 1.5 μM propidium iodide (Invitrogen, Carlsbad, CA) and incubated on ice for 20–30 min. Fluorescence was detected and analyzed using an FC500 flow cytometer and CXP data analysis software (Beckman Coulter, Miami, FL). For assessment of cell cycle distribution, cell pellets were resuspended in PBS and ethanol was gradually added with vortexing to a final concentration of 70% to permeabilize membranes. Cells were incubated at 4°C overnight, collected by centrifugation, resuspended in PBS containing 20 μg/ml propidium iodide and 2 μg/ml RNase A, and incubated again at 4°C overnight prior to analysis by flow cytometry as above.

Determination of Differentiation Marker Expression—Cells were cultured in 6-well dishes or 10 cm tissue culture plates and collected by centrifugation at 240 g for 5 min after treatment with vandetanib for 48 h. For assessment of cell surface marker expression, aliquots of 10⁵ cells were resuspended directly in 5% FBS/PBS + 40 μg/ml human IgG (Sigma Aldrich, St. Louis, MO). Cells were incubated on ice for 30 min to block non-specific antibody binding. An equal volume of fluorochrome-linked or biotin-linked antibody in 5% FBS/PBS + 40 μg/ml human IgG was added and samples were incubated on ice for an additional 60 min. Where indicated, cells were washed with 0.75 ml 5% FBS/PBS and resuspended in 50 μL of fluorochrome-linked streptavidin in 5% FBS/PBS + human IgG for an additional 30 min on ice. After staining, all samples were washed with and resuspended in 5% FBS/PBS, and fluorescence was quantitated by flow cytometry as above. Aliquots of cells were stained with fluorochrome-linked isotype control antibodies to define background fluorescence and positive-staining cells. Antibodies were obtained from BD Biosciences (San Jose, CA). 2X solutions were prepared at the following dilutions: IgG1-FITC (#556028) 1:5; CD15-FITC (#555401); IgG1-PE (#559320) 1:5; CD69-PE (#555531); IgG1-Biotin (#555747) 1:5; Streptavidin-FITC (#554060) 1:100; CD28-Biotin (#555727)

1:5; CD2-FITC (#347593); CD11b-PE (#347557) 1:5; CD4-FITC (#340133) 1:5; CD25-FITC (#347643) 1:5.

Interaction models and statistical analysis—Interactions between vandetanib and cytotoxic chemotherapies were assessed by the Bliss independence model [40]. The frequency of affected (F_a) expected for an additive interaction between 2 agents was calculated based on the Bliss independence model using the following formula:

$$F_a(1+2)=F_{a1}+(1-F_{a1})\times F_{a2}$$

where F_{a1} and F_{a2} are the effects for the individual drugs (1 and 2) when used as single agents at the concentrations of interest.

Statistically significant differences ($p<0.05$) were determined using the student's paired test as indicated. All statistical analyses were performed using Graphpad Prism v4 software (Graphpad Software, Inc., La Jolla, CA).

Results

Vandetanib exhibits anti-tumor activity against established acute leukemia cell lines in vitro—We utilized the MTT assay to determine the effects of treatment with vandetanib on an extensive panel of acute leukemia cell lines including ALLs, AMLs, and bi-phenotypic mixed lineage leukemias (MLLs) with predominantly lymphoid or myeloid features. Vandetanib treatment resulted in a dose-dependent reduction in viable cell number in several acute leukemia cell lines, demonstrating anti-tumor activity at clinically achievable serum levels (Fig. 1) [20, 41]. Cell lines were determined to be sensitive if the respective IC₅₀ was at or below the clinically-achievable serum level of 2.5 μ M. Based on this criterium, 6 cell lines were sensitive and 13 cell lines were resistant. The 6 sensitive lines included the ALL line HSB-2, the AML lines Kasumi-1 and Eo1-1, MLL lines with predominate myeloid features (Molm-13 and Molm-14), and an MLL line with predominant lymphoid features (MV4-11).

Vandetanib mediates anti-tumor activity by multiple mechanisms

Cytotoxicity assays—To elucidate the mechanism(s) by which vandetanib exerts its anti-leukemia effect, we evaluated the cytotoxic potential of vandetanib. Six vandetanib-sensitive leukemia cell lines were treated with various concentrations of vandetanib for 48 h and then stained with propidium iodide and YoPro®-1-iodide in order to quantitate the fraction of viable, apoptotic and dead cells using flow cytometry. Vandetanib induced dose dependent cell death (Fig. 2a). However, induction of significant cell death required treatment with relatively high concentrations of vandetanib (IC₇₅ concentrations or higher). In all cases, reduction in cell number was observed at lower concentrations than those required to induce apoptosis (Figs. 1 and 2a).

Cell Cycle Analysis—To investigate other potential mechanisms of vandetanib-mediated anti-leukemia activity, we characterized the cell cycle profile of vandetanib-sensitive leukemia cell lines. When treated with vandetanib, the vandetanib-sensitive cell lines exhibited alterations in cell cycle distribution, with all lines but Eo1-1, demonstrating significant accumulation in the G1 phase (Fig. 2b). These data demonstrate that vandetanib mediates alterations in cell cycle progression.

Selectivity of tyrosine kinase inhibition—We characterized expression of known vandetanib targets in a subset of both sensitive and resistant leukemia cell lines to

investigate the possible biochemical mechanism of vandetanib-mediated anti-leukemic activity. All of the cell lines expressed one or more of the known targets of vandetanib (VEGFR1, VEGFR3, PDGFR β and/or RET) (Supplementary Figure 1). Only one vandetanib-resistant line (THP-1) expressed VEGFR2 or EGFR. No correlation was observed between expression of receptors and sensitivity to vandetanib.

Treatment with vandetanib alters expression of differentiation markers on leukemia cell lines—The observed accumulation of leukemia cells in G1 phase of the cell cycle following treatment with vandetanib is consistent with the possibility that vandetanib mediates differentiation of leukemic blasts. To investigate this possibility, a subset of the vandetanib-sensitive cell lines were treated with IC₅₀ concentrations of vandetanib and cell surface expression of differentiation markers was determined. Both ALL and AML cell lines demonstrated alterations in expression of hematopoietic differentiation markers (Fig. 3). HSB-2, a T-lineage ALL, down-regulated expression of CD15 (a myeloid marker) and CD69 (a T-cell activation marker that is also expressed on immature myeloid precursors). Expression of CD28, which is expressed on mature T-cells was up-regulated in response to treatment with vandetanib. Both AML cell lines, Eol-1 and Molm-14, exhibited down-regulation of the lymphoid markers including CD25 (an activated T-cell marker, also expressed on monocytes), CD4 (a T-cell co-receptor) on Eol-1 cells and CD2 (T-cell marker involved in interactions with antigen presenting cells) on Molm-14 cells. Molm-14 also demonstrated decreased expression of CD11b, a myeloid marker. These cell surface alterations suggest that exposure to vandetanib promotes altered cell surface characteristics of leukemic blasts.

Vandetanib interacts synergistically with topoisomerase II inhibitors and antagonistically with an anti-metabolite agent—As vandetanib mediates anti-leukemia activity by multiple mechanisms, we investigated the interaction of vandetanib with standard chemotherapeutic agents used in the treatment of leukemia. A vandetanib-resistant cell line (Nalm6), the most vandetanib-sensitive cell line (HSB-2), and a moderately vandetanib-sensitive cell line (Molm-13) were treated with IC₅₀ concentrations of vandetanib and/or a standard chemotherapeutic agent (doxorubicin, etoposide, or methotrexate), alone and in combination for 48 h and cell death was assessed by flow cytometric analysis following staining with YoPro-1-iodide and propidium iodide. Interactions between agents were assessed using the Bliss additivity model [40]. Upon concurrent treatment with vandetanib and a topoisomerase II inhibitor (doxorubicin or etoposide), all three cell lines exhibited statistically significant increases in the fraction of dead or apoptotic cells relative to the predicted values for additive interactions indicating a synergistic interaction (Fig. 4a and b). In contrast, treatment with the anti-metabolite methotrexate in combination with vandetanib resulted in significantly less cell death than the predicted additive values for all three cell lines, indicating an antagonistic interaction (Fig. 5). Synergistic and antagonistic interactions with topoisomerase II inhibitors and methotrexate respectively, were also observed when the Molm-13 cell line was treated with higher concentrations (IC₇₅) of vandetanib, indicating that these interactions are not concentration dependent (Fig. 6).

Discussion

In this study, we investigated the anti-tumor effect of vandetanib on acute leukemia cell lines in vitro. We describe the anti-leukemic activity of this agent and elucidate mechanisms by which it has its effect, both alone and in combination with cytotoxic chemotherapies. While the effect of vandetanib on various solid tumors is well documented, the role of this multi-targeted tyrosine kinase inhibitor with effects on VEGFR2, RET, EGFR and to a lesser extent, VEGFR1, VEGFR3, and PDGFR has been much less studied in acute

leukemia. In addition, it is likely that there are off target effects that remain undefined at this time. Our results demonstrate that vandetanib exhibits anti-leukemic activity against several leukemia cell lines in vitro, including both ALL and AMLs, three of which have not been previously described as vandetanib-sensitive (HSB-2, Molm-13 and Molm-14). The anti-leukemia activity of vandetanib does not appear to be specific to a particular lineage or subtype. Of note, three of the sensitive cell lines possess MLL translocations (Molm-13, Molm-14, and MV4-11) [42, 43]. This is of potential clinical interest, as MLL rearranged leukemias are typically more refractory and associated with poorer outcomes [6, 44, 45]. Similarly a large majority of MLL-rearranged leukemias demonstrate over-expression of *fms*-like tyrosine kinase 3 (*flt-3*), a known negative risk factor [46–48].

The concentrations at which vandetanib exerts its anti-tumor effect on sensitive leukemia cell lines in this study are within the range of concentrations expected to affect the known selective targets of the drug and are clinically achievable. However, vandetanib sensitivity does not appear to correlate with expression of any one specific receptor. While this observation does not preclude inhibition of known vandetanib targets as a mechanism of vandetanib-mediated anti-leukemia activity, it suggests that receptor expression will not be a useful clinical marker of vandetanib sensitivity. These data are also consistent with the possibility that vandetanib's anti-tumor activity occurs through different signaling pathways in different cell lines, by inhibition of multiple pathways in individual cell lines, or by off-target effects which have not yet been elucidated. The kinase inhibitor profile of vandetanib and the broad range of IC_{50} values for sensitive cell lines are consistent with the possibility that inhibition of different receptors results in anti-leukemia activity in different cell lines. Notably, anti-leukemia activity against cell lines containing MLL translocations requires higher concentrations of vandetanib compared to other sensitive cell lines. This observation is consistent with the possibility that anti-leukemia activity is mediated by inhibition of FLT3 in these cell lines as they all express internal tandem duplications of FLT3 and vandetanib is known to inhibit FLT3 phosphorylation at concentrations greater than or equal to 1 μ M [39]. In contrast, vandetanib's anti-leukemia activity against the Eol-1 and HSB-2 cell lines occurred at much lower concentrations and may therefore be mediated by more specific inhibition of known target receptors, such as VEGFR1 and VEGFR3. Thus, it is possible that a subset of the sensitive cell lines are dependent on VEGFR signaling for proliferation and/or survival. VEGFR signaling may also play roles in proliferation and survival of leukemia cell lines that are not sensitive to vandetanib where other signaling pathways function redundantly such that VEGFR signaling is not absolutely required.

We also expanded on previous studies by investigating multiple cellular mechanisms involved in the anti-leukemic effect of vandetanib. Our data indicate that the anti-tumor activity mediated by vandetanib occurs through multiple mechanisms and is concentration dependent with induction of apoptosis and cell death occurring at relatively high concentrations, well above the IC_{50} concentrations. At lower concentrations, little to no apoptosis and cell death are observed, but alterations in cell cycle progression do occur. The increased fraction of cells in G1 phase suggests a delay or arrest in cell cycle progression induced by vandetanib. An similar increase in the fraction of tumor cells in G1 phase has been described in response to vandetanib and other EGFR antagonists and this accumulation in G1 phase in solid tumor cell lines has been shown to be due to decreased cyclin D expression [49, 50], suggesting a mechanism by which cell cycle effects may be mediated. However, the relevance of this mechanism in leukemia cell lines is questionable as none of the sensitive cell lines expressed EGFR.

The observed accumulation of leukemia cells in G1 phase is consistent with the possibility that the cells are undergoing differentiation in response to treatment with vandetanib. Indeed, treatment with vandetanib led to alterations in the expression of hematopoietic

differentiation markers. These alterations generally suggest a more mature phenotype and/or down-regulation of aberrantly expressed markers. HSB-2 is an immature T-cell leukemia cell line [51]. When treated with vandetanib, HSB-2 cells demonstrated increased expression of the mature T-cell marker CD28, decreased expression of CD69, an immature T-cell marker and a marker of mature T cell activation, and decreased levels of CD15, an aberrantly expressed myeloid marker. These changes could be consistent with differentiation towards a less proliferative, more mature phenotype. Similarly, Eol-1 and Molm-14 exhibit down-regulation of CD25, CD4, and/or CD2, markers that are expressed on T cells and on immature myeloid precursors, again suggesting differentiation to a more mature phenotype [52]. To our knowledge, this is the first description of a small molecule inhibitor with VEGFR inhibition properties altering expression of differentiation markers as a single agent. Consistent with our data, down-regulation of cellular VEGF levels has been associated with induction of leukemia cell differentiation into functional leukemic dendritic cells in AML patient samples [53]. Taken together, these data suggest that inhibition of the VEGF pathway can induce differentiation of leukemia cells. These observations are particularly relevant given that abrogation of differentiation is a common mechanism of leukemogenesis, particularly in AMLs. Differentiating agents are also used to treat other cancers that demonstrate an undifferentiated phenotype, such as neuroblastoma, and vandetanib may also be therapeutic in this context. Interestingly, vandetanib does mediate synergistic anti-tumor activity in combination with retinoic acid, a differentiating agent, in neuroblastoma cell lines. In this case, retinoic acid mediates increased activation of vandetanib's target RET and may thereby render neuroblastoma cells more susceptible to apoptosis in response to treatment with vandetanib [54]. Similarly, vandetanib-mediated differentiation may play a role in increasing sensitivity to apoptosis in response to normal cell stressors or treatment with chemotherapy agents. Studies investigating changes in differentiation mediated by vandetanib or other VEGF inhibitors in combination with other agents have not been described.

Additionally, and perhaps more relevant clinically, we evaluated interactions with other therapeutic agents that are currently in clinical use. Vandetanib demonstrates synergy with topoisomerase II inhibitors, even in cell lines in which it does not demonstrate single agent activity, and thus may potentiate certain conventional chemotherapy. These data suggest combination strategies which may be clinically relevant particularly for AML, as more of the sensitive lines were AMLs and topoisomerase II inhibitors are commonly used in AML therapy. The synergy observed when vandetanib is combined with a DNA-damaging agent has been previously described in solid tumors and is thought to be mediated in part by effects on pro-survival pathways [49, 55, 56]. Interestingly, when vandetanib is combined with oxaliplatin in human colon cancer cell lines, there was a marked synergistic decrease in the expression in VEGF-A secretion by the tumor cells [56]. This may be part of vandetanib's anti-leukemic effect, as the leukemia cell lines all express high levels of VEGF as part of an autocrine stimulatory loop (data not shown) [30, 57–61]. With the addition of chemotherapy, VEGF secretion may be further impeded compared with vandetanib alone. Vandetanib can also affect multi-drug resistance through inhibition of the transport functions of both the p-glycoprotein and ABCG2 proteins [62, 63], suggesting another rational reason for clinical evaluation of the synergistic combinations. However, while this mechanism may play a role in some of the synergistic interactions we observed, this is not the only role for vandetanib as the Nalm-6 cell line does not express pGP [64].

The antagonism seen with methotrexate is not surprising, as methotrexate is an anti-metabolite that affects purine metabolism, and therefore DNA synthesis. As vandetanib induces a G1 phase arrest, it likely prevents leukemia cell progression into S phase, where methotrexate exerts its effect. Similarly, we would expect antagonistic interactions between vandetanib and other cell cycle specific agents, including other anti-metabolite agents

affecting DNA synthesis and therapies that exert their effects in G2/M phase, such as the vinca alkaloids and taxanes. In contrast, vandetanib may be particularly effective in combination with G1 phase specific agents such as L-asparaginase. It is also possible that a more favorable interaction between vandetanib and methotrexate could be achieved if the treatments were applied sequentially. Although studies have demonstrated that the sequence in which receptor tyrosine kinase inhibitors and chemotherapeutic agents are administered can affect the interaction between agents, we evaluated only concurrent therapy because the long half-life of vandetanib (120 h in human serum), makes sequencing less clinically feasible [20, 41, 49, 50, 65].

The data presented here focus on the direct anti-tumor effect of vandetanib, however it is also important to note that the bone marrow microenvironment is not represented in our model system. It is well known that the bone marrow stroma abundantly expresses VEGF and its receptors. In addition, leukemic bone marrow has a higher density of microvessels than normal bone marrow, suggesting a role for increased angiogenesis. The studies presented here assess autocrine inhibition but do not account for the paracrine involvement of the endothelial cells and other hematopoietic cells found in the bone marrow microenvironment or the contribution of increased blood supply to leukemogenesis in this environment, both of which can be affected by VEGFR inhibition. As such, further in vivo and clinical studies are warranted to investigate potential anti-leukemic effects that are dependent upon the milieu of the bone marrow microenvironment.

Our results demonstrate that vandetanib has direct anti-leukemic activity in vitro. Its activity is mediated by multiple mechanisms and probably through different receptors or pathways depending on the cell line. Further evaluation of the specific signaling pathways affected by vandetanib will be necessary to elucidate the exact biochemical mechanisms of this drug's anti-leukemic activity and to facilitate development of robust pharmacodynamic markers for clinical trials. This study also provides preclinical data to facilitate rational clinical development of a therapeutic regimen containing vandetanib for pediatric acute leukemias, based on its synergistic interactions with doxorubicin and etoposide. For this combination, the sensitivity of leukemia cells to vandetanib may not be important, as treatment with vandetanib increased sensitivity to cytotoxic chemotherapies independent of whether the cell line was sensitive or resistant to vandetanib. Taken together, our data support further evaluation of vandetanib, both in animal models and in clinical trials. Further evaluation in an in vivo setting will be necessary to determine whether the autocrine effects we demonstrate here are clinically relevant and to evaluate the contribution of paracrine signaling inhibition and anti-angiogenic effects mediated by vandetanib, both as a single agent and in combination with DNA damaging agents. However, vandetanib provides an exciting new molecularly-targeted option for treatment of acute leukemia.

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Abbreviation

ALL acute lymphoblastic leukemia

AML	acute myelogenous leukemia
DMSO	dimethyl sulfoxide
EGFR	epidermal growth factor receptor
FBS	fetal Bovine Serum
Flt-3	fms-like tyrosine kinase 3
KDR	kinase-insert domain containing region
MLL	mixed lineage leukemia
PDGFRβ	platelet derived growth factor β
RET	Rearranged during transfection
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

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Fig. 1.

Vandetanib exhibits anti-tumor activity against acute leukemia cell lines. Acute leukemia cell lines (AML and ALL) were treated with a range of concentrations of vandetanib for 48 h. Numbers of viable cells relative to untreated cultures were determined by MTT assay. Dose-response curves were generated and IC₅₀ values were determined using non-linear regression. **a.** IC₅₀ values for leukemia cell lines treated with vandetanib. The clinically achievable serum level is indicated by the dashed line. Mean values $\pm$ SEM were derived from 4 to 13 independent experiments. (*MLL* Mixed Lineage Leukemia) **b.** Dose-response curves for sensitive cell lines treated with vandetanib. Mean values and standard errors were derived from 4 to 13 independent experiments

Fig. 2.

Vandetanib induces dose-dependent cell death and accumulation in G1 phase in leukemia cell lines. Vandetanib-sensitive acute leukemia cell lines were treated with the indicated concentrations of vandetanib for 48 h. **a.** Dead and apoptotic cells were quantitated by flow cytometric analysis of cells stained with YoPro-1 iodide and propidium iodide. Mean values $\pm$ SEM derived from 3 independent experiments are shown. Statistically-significant differences in the fraction of dead and apoptotic cells in vandetanib-treated cultures relative to control cultures were determined using the student's paired t-test. **b.** Cell cycle profiles were determined by flow cytometric analysis of permeabilized cells stained with propidium iodide. Mean values $\pm$ SEM were derived from 3 independent experiments. Statistically-significant differences in the fraction of cells in G1 phase in vandetanib-treated cultures relative to control cultures were determined using the student's paired t-test. * $p < 0.05$, ** $p < 0.005$

Fig. 3.

Treatment with vandetanib alters cell surface expression of differentiation markers on leukemia cell lines. Vandetanib-sensitive leukemia cell lines were treated with IC₅₀ concentrations of vandetanib (*grey bars*) or with an equal volume of media alone (*white bars*) for 48 h. Cells were then stained with fluorochrome-conjugated antibodies against the indicated cell surface differentiation markers and analyzed by flow cytometry. Mean values $\pm$ SEM were derived from 4 to 5 independent experiments. Statistically-significant differences in the fraction of cells expressing the relevant marker in vandetanib-treated cultures relative to control cultures were determined using the student's paired t-test. * $p < 0.05$, ** $p < 0.005$

Fig. 4.

Concurrent treatment with vandetanib and topoisomerase II inhibitors results in synergistic induction of leukemia cell death. Cultures of the indicated leukemia cell lines were treated with IC_{50} concentrations (determined by MTT assay) of vandetanib (Vand) alone, chemotherapy alone or both drugs concurrently for 48 h as indicated. Apoptotic and dead cells were quantified by flow cytometric analysis of cells stained with YoPro-1 iodide and propidium iodide dyes and normalized to untreated. Interactions between the two drugs were assessed using the Bliss Independence Model. The predicted response for an additive interaction (Bliss Additivity) is determined based on the single agent data. The observed % dead cells for the combination therapies are greater than the % dead cells predicted for an additive interaction, indicating synergy. Mean values and standard errors were derived from 4 to 7 independent experiments. Statistically-significant differences in the fraction of apoptotic or dead cells observed relative to the fraction predicted for an additive interaction were determined using the student's paired t-test. * $p < 0.05$, ** $p < 0.005$. **a** Cultures were treated with vandetanib and/or doxorubicin (Doxo). **b** Cultures were treated with vandetanib and/or etoposide (VP-16)

Fig. 5.

Vandetanib antagonizes methotrexate mediated cell death. Cultures of the indicated leukemia cell lines were treated with IC_{50} concentrations of vandetanib (Vand) or methotrexate (MTX) alone and in combination and interactions were determined using the Bliss additivity model as described in Fig. 4. Mean values and standard errors were derived from 4 to 9 independent experiments. Statistically-significant differences in the fraction of apoptotic or dead cells observed relative to the fraction predicted for an additive interaction were determined using the student's paired t-test. * $p < 0.05$, ** $p < 0.005$

Fig. 6.

The interactions between vandetanib and standard chemotherapeutic agents are not concentration dependent. Cultures of the Molm-13 cell line were treated with IC_{75} concentrations of vandetanib and/or IC_{50} concentrations of doxorubicin (Doxo), etoposide (VP-16), or methotrexate (MTX) and interactions were determined as described in Fig. 4. Mean values and standard errors were derived from 7 independent experiments. Statistically-significant differences in the fraction of apoptotic or dead cells observed relative to the fraction predicted for an additive interaction were determined using the student's paired t-test. * $p < 0.05$, ** $p < 0.005$