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Review Article

Modulation of signaling pathways by adaphostin (NSC-680410), an antileukemic tyrosine kinase inhibitor: the past, the present and recommendations for the future

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CONTENTS

Abstract

Angiogenesis is involved in many physiological and pathological conditions including embryonic development, wound healing, menstrual cycle, chronic inflammation and the development of tumors. Vascular endothelial growth factor (VEGF) is a major initiator and regulator of angiogenic processes and is associated with tumor growth, invasion and metastasis. The VEGF family of proteins (VEGF-A, VEGF-B, VEGF-C, VEGF-D and the placenta growth factor [PGF]) share tyrosine kinase receptors (VEGFR-1, VEGFR-2, VEGFR-3) which are expressed on many cell types including endothelial cells, hematopoietic stem cells and many solid tumor and leukemia blast cells. The VEGF receptor signaling pathway activates a tyrosine kinase cascade involving various intracellular proteins (particularly PI-3 and RAS/MAP kinases) and is a potential antiangiogenic target that can be inhibited at various levels. The work presented here deals primarily with the small molecule tyrosine kinase inhibitors such as adaphostin. Adaphostin and congeners have the potential to specifically inhibit tyrosine kinases of the VEGF receptors with an acceptable toxicity profile to the host. These compounds have shown good inhibition of VEGFR-1 and relatively good inhibition of VEGFR expressing human leukemia cells. Furthermore, combination regimens of this class of compounds with cytotoxic drugs can downregulate both angiogenesis and its associated effects in the bone marrow of mammalian systems and provide synergistic combination drug regimens for the treatment of refractory malignancies.

Introduction

Cellular proliferation, differentiation and death are regulated by a number of extracellular molecules, such as cytokines, growth factors and hormones, as well as intracellular signaling mediated by cell surface receptors and their ligands. Understanding how pluripotent blast cells develop into complex differentiated tissues containing multiple cell types is an important goal for developmental biologists.

Growth factor receptor tyrosine kinase signaling pathways

In recent years, the fact that the developmental fate of individual cells is often established by activating transmembrane receptors of the tyrosine kinase (RTK) family has become clear. RTKs, many of which are growth factors, dimerize in response to ligand binding, which leads to autophosphorylation as well as phosphorylation of adjacent signaling proteins on tyrosine residues (1).

VEGF receptors are transmembrane proteins with an extracellular ligand-binding domain, consisting of 7 subunits, a transmembrane domain and highly conserved intracellelular domain that mediates the activation, via two domains of tyrosine kinase activation-phosphorylation, of a number of downstream signaling proteins, which initiate many intracellular signal transduction cascades. When the ligand binds the extracellular domain, the activated ligand-receptor dimerizes and a cascade of signal transduction begins (Fig. 1).

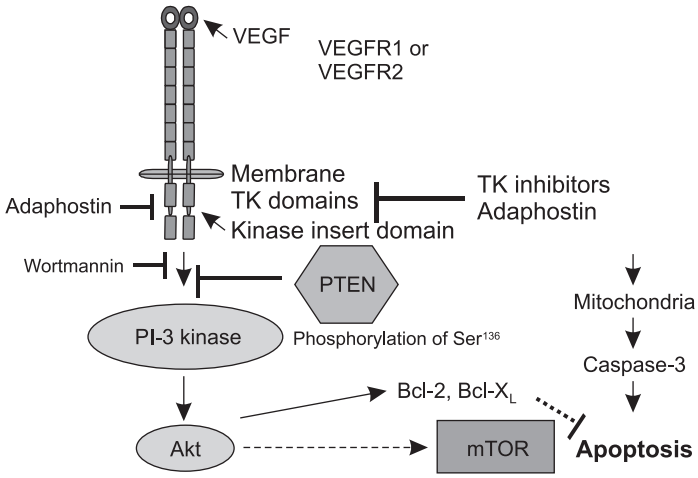

Fig. 1.

Experimental evidence suggests that many growth factors, including hematopoietic growth factors, promote cell survival by suppressing or bypassing apoptosis. Interleukin (IL-3, IL-7, *etc.*), platelet-derived growth factor (PDGF), c-Kit, VEGF and granulocyte-macrophage colony stimulating factor receptors induce tyrosine phosphorylation of a number of downstream proteins, which are involved in the promotion of cell survival and suppression of apoptosis (2-4). Activation of these receptors as well as the Bcr-Abl fusion oncoprotein [Philadelphia (Ph^+) chromosome] activate their intracellular tyrosine kinase domains and have been shown to play a crucial role in a variety of biological responses in hematopoietic and lymphoid malignancies. Phosphorylated tyrosine residues in RTKs serve as initiators of signal transduction to effector proteins. RTKs include a diverse set of proteins dichotomized into distinct classes: Class I includes the epidermal growth factor receptor (EGFR); Class II includes the insulin-like growth factor receptor (IGFR), PDGFR and CSF-1R; Class III includes the stem cell factor receptor (c-Kit) and Flt-3R; and class IV includes the FGFR (5-7).

PI-3 kinase-Akt (PKB) pathway

Activation of growth factor receptor tyrosine kinase mediates the activation of phosphatidylinositol-3 (PI-3) kinase. PI-3 kinase is directly linked with mitogenic responses and is tyrosine phosphorylated after stimulation by growth factor signaling. This in turn can activate Akt, also known as protein kinase B (PKB) (Fig. 1). The growth factor activation of PI-3 kinase leads to the generation of activated membrane phospholipids, which mediate the recruitment of inactive PKB (Akt) to the plasma membrane via PH domain binding of this protein (8, 9). Thousands of papers have been published on this protein and its downstream signaling, eventually leading to activation of the "target of rapamycin" (TOR) proteins that promote glucose metabolism, transcription, apoptosis, protein synthesis, cell growth, angiogenesis and cell motility (10, 11). Despite decade-long studies in this area, additional studies are required to clearly define the link (if any) between the PI-3 kinase-Akt signaling pathway and other proteins.

JAK-STAT kinases

The activated and dimerized subunits of cytokine receptors (IL-2, erythropoietin, *etc.*) are associated with intracellular TKs, such as members of the Src or Jak families of kinases and the signal is propagated to start gene transcription (12, 13). The precise regulation of both the magnitude and the duration of Janus kinase (JAK) catalytic activity is essential for the cytokine orchestration of many biological processes, and the dysregulation of JAK activity has pathological implications (14). Immunosuppressive disease states, such as X-linked severe combined immunodeficiency, arise from inappropriate JAK inhibition. In contrast, a limited number of cancers, primarily leukemias, result from constitutive or enhanced activation of JAK activity. JAKs are no longer implicated only in classic cytokine receptor-mediated signaling pathways, but are also known to integrate indirectly into other receptor-mediated signal transduction processes. Therefore, an increasing number of therapeutic applications exist for biological response modifiers that can restore aberrant JAK activity to normal levels (15). JAK2 is required for BCR-ABL-mediated stimulation of erythropoiesis. JAK2 is essential for normal red cell development and for erythropoietin receptor (EpoR) signaling (16). In addition, activated JAK proteins activate a number of intracellular signaling proteins, among which STATs are the best defined (14, 17, 18). The phosphorylation of STAT proteins leads to their homo- and heterodimeriza-

tion and translocation into the nucleus, followed by gene activation (19). Hence, inhibition of cytokine-JAK signal transducers may result in a selective pharmacological benefit in cancer patients. Such pathways have been theorized and specific TK inhibitors have been used successfully in the treatment of patients with chronic lymphocytic leukemia (CLL), a disease characterized by clonal expansion of relatively mature B-lymphocytes but with considerable heterogeneity, resulting in proliferative action. The t(9;22) (Ph^+ chromosome) translocation associated with chronic myelogenous leukemia (CML) fuses the c-Abl gene on chromosome 9 with the Bcr gene on chromosome 22, resulting in the production of one or more of a family of chimeric oncoproteins, p190, p210 or p230 Bcr/Abl (20). The discovery of "intelligent" molecules targeting CLL cells, the most appealing of which is STI-571 (Gleevec™, Glivec®, imatinib mesylate), has brought hope for new therapies (21).

Angiogenesis and leukemias

There is accumulating evidence that angiogenesis supports the growth of solid tumors *in vitro* and *in vivo*. The effects of antiangiogenesis inhibitors may very well depend on the discrete stages of carcinogenesis and/or tumor metastasis. For example, studies in transgenic animals have demonstrated that antiangiogenesis inhibitors may block the development of neovasculogenesis and angiogenesis at the early stages of hyperplasia/dysplasia of tumor development (*i.e.*, adenomas or adenocarcinomas), but have a limited or no effect on small or large tumors of invasive carcinomas (22). Emerging data suggests that targeting tumor angiogenesis with agents that block endothelial proliferation, such as with neutralizing VEGFR-specific antibodies, results in delayed growth of solid tumor cell lines implanted into mice (23). However, the exact mechanism whereby inhibition of the VEGF-VEGFR axis results in the regression of tumor tissue is not well studied (24).

It has been suggested that as the tumor endothelial mass expands in response to tumor-derived angiogenic factors such as VEGF, it supports tumor growth in a paracrine fashion. However, demonstration that angiogenesis also plays a role in liquid tumors such as leukemia has not been rigorously examined. Nevertheless, there are numerous reports indicating that there is increased bone marrow angiogenesis in patients with different hematological malignancies such as acute lymphoblastic leukemia (25-29). Moreover, leukemic cell release of angiogenic growth factors such as VEGF is a prediction of poor clinical outcome and progression of the disease (28). Similar to solid tumor growth in response to leukemia-derived angiogenic factors such as VEGF, it has been suggested that the proliferating bone marrow endothelial mass may release growth factors that support leukemic cell growth in a paracrine fashion (30). Therefore, blocking VEGF signaling on endothelial cells and on leukemia cells may reduce growth factor production, thereby delaying leukemic growth. However, clinical studies thus far have failed to demonstrate whether blocking angiogenesis results in delayed human leukemic growth *in vivo*. For example, autocrine stimulation of VEGFR-2 activates human leukemic cell growth, and therefore blocking this pathway may provide for a novel antileukemic intervention (31).

Antiangiogenic agents block the effects of tumor-derived angiogenic factors (paracrine factors), such as VEGF on endothelial cells, inhibiting the growth of solid tumors. However, whether inhibition of angiogenesis may also play a role in liquid tumors is not well established. Certain leukemias not only produce VEGF but also selectively express functional VEGFRs, such as VEGFR-2 (Flk-1, KDR) and VEGFR-1 (Flt-1), resulting in the generation of an autocrine loop (24).

Tyrphostins: tyrosine kinase inhibitors

The experience from clinical trials in leukemias has taught us that treatment outcome depends on a complex interaction of host, leukemia cell biology and treatment characteristics. Examination of known molecular targets ($p210^{bcr\text{-}abl}$) which play an important role in specific malignancies has yielded important new clinical avenues for treatment (*e.g.*, STI-571). However, with the development of drug resistance to this agent, additional molecular targets in this area need to be identified in order to develop novel treatments against refractory leukemias.

Recent studies have shown that VEGF-induced angiogenesis may be involved in the pathogenesis of hematological malignancies (32, 33). Leukemia patients seem to have high concentrations of growth factors circulating in their blood (*e.g.*, VEGF, PDGF, EGF and IGF). In addition, leukemic cell survival depends on paracrine and autocrine growth loops. These growth factors, which facilitate the expansion of the vascular network of solid tumors (34-37), possibly play an important role in the survival of leukemic cells in the bone marrow stroma as well (32).

One known mechanism by which VEGF and other growth factors promote angiogenesis is by stimulating the activity of integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on endothelial cells (38). It has been shown that the IL-3-independent expression of the antiapoptotic protein, Bcl-x_L, is induced by Bcr-Abl through activation of signal transducer and activator of transcription 5 (STAT 5). Inhibition of the Bcr-Abl kinase activity in Bcr-Abl-expressing cell lines and $CD34^+$ cells from CML patients induces apoptosis by suppressing the capacity of STAT 5 to interact with the Bcl-x_L promoter (18, 19, 39). The $p210^{bcr/abl}$-initiated survival signaling can be interrupted at several points, the most important being the inhibition of the tyrosine kinase domain of the protein by specific inhibitors (*e.g.*, STI-571, AG-957, *etc.*) or by the use of Wortmannin, a potent inhibitor of PI-3 kinase (40-43).

The VEGF receptor family consists of three transmembrane receptors, VEGFR-1 (Flt-1), VEGFR-2 (Flk-1)

and VEGFR-3 (Fit-4). A soluble form of VEGF-R1 has also been documented. Leukemia patients have high concentrations of growth factors circulating in their blood, *e.g.*, VEGF, PDGF, EGF and IGF (27, 32-34). The VEGF-related receptors contain two tyrosine kinase domains spaced by a kinase insert domain in the intracellular region of the receptor. The activation of tyrosine kinase follows the interaction of ligand-receptor. These receptors can initiate cell signaling pathways and participate in various biological functions, including cell survival, migration, differentiation, vessel sprouting, stabilization and permeability of the new blood vessels. The activation of tyrosine kinases has been shown to be key activating pathways controlling cell proliferation and survival (31, 43-45). This process is facilitated by secretion of growth factors by the tumor or epithelial cells, thus acting in an autocrine and/or paracrine manner in both solid tumors and leukemias (11, 32, 34, 37, 41-45).

Tyrphostins are small ATP-like molecules which have been found in the past decade to induce many desirable effects, including differentiation and apoptosis in leukemic cells (2, 46, 47). Apoptosis inducing tyrphostin-treated leukemic cells are characterized by a diminished stainability of DNA. Tyrosine phosphorylation in general is an essential step in IL-3 and other cytokine signal transduction. Hence, the inhibition of tyrosine kinase by tyrphostins (Fig. 1) suggests that a balance may be shifted from cell survival to apoptosis (2). Pharmacological intervention, therefore, may result in a significant anticancer effect. Numerous tyrphostins inhibit $p210^{Bcr\text{-}Abl}$ tyrosine kinase activity in CML cells and could be used for purging of Ph^+ chromosome blasts in preparation for autologous bone marrow transplantation in CML (40, 46). Another group of tyrpshostins inhibit the growth of K562 Ph^+ CML cells but not $p210^{Bcr\text{-}Abl}$ tyrosine kinase activity.

AG-957 is the most active of these agents, inhibiting both DNA synthesis and $p210^{Bcr\text{-}Abl}$ tyrosine kinase activity within hours after exposure of CML cells, and the inhibition is irreversible after 24 h of continuous exposure (48-50). Of interest is the observation that tyrphostin AG-555, which does not have $p210^{Bcr\text{-}Abl}$ tyrosine kinase activity, did not affect colony formation or proliferation of CML cells. In contrast, AG-957 resulted in a dose-dependent significant inhibition of CML colony formation, strongly indicating that the more active tyrphostin inhibited $p210^{Bcr\text{-}Abl}$ tyrosine kinase activity (43, 51, 52). In addition, AG-957 inhibited mitogen-activated protein (MAP) kinase activity in human Jurkat T lymphoblastic cells (53).

AG-490 blocks IL-7-mediated proliferation in a dose-dependent manner, suggesting a link between early events of PI-3 kinase phosphorylation and activation with IL-7-induced cell growth (3). AG-17 was found to inhibit HL-60 human promyelocytic leukemia and a panel of human tumor cells very efficiently with submolar or micromolar IC_{50} values (54). AG-17 was one of 17 tyrphostins most active in inducing cell arrest at G_1 phase followed by apoptosis with general reduction in the intracellular levels of tyrosine phosphorylated proteins. The mechanism of AG-17 is most likely due to inactivation of cdk2 kinase and Bcl-2 (55).

STI-571 is a 2-phenylaminopyrimidine derivative and a very potent agent for the treatment of Ph^+ acute lymphoblatic leukemia (ALL) and for CML (40, 56, 57). STI-571 has been shown to produce very effective clinical results in CML patients in whom interferon therapy had failed as a single agent. STI-571 induced hematologic complete remission in 96% of patients after 4 weeks of treatment (56, 57). STI-571 is a selective inhibitor of c-Abl, $p210^{bcr\text{-}abl}$, c-Kit and PDGFR tyrosine kinases, which has demonstrated remarkable antileukemic activity in phase II–III clinical studies (58-60). However, these studies, as well as a variety of experimental models, have suggested that clinical resistance could rapidly develop to STI-571 as a single agent. Drug resistance can develop only in cell populations with rapid and continuous cell replication. In the absence of cell replication, as in the chronic phase of CML, there is no probability of a resistant clone developing to STI-571. In contrast, resistance to this drug is rapid in CML patients in blast crisis (57, 60-62). Therefore, after a successful initial response in leukemia patients to drugs targeting signal transduction pathways, drug resistance develops rapidly to STI-571 in most patients (59-64).

Adaphostin

Previous studies demonstrated that AG-957 and its adamantyl congener adaphostin (NSC-680410) inhibited $p210^{bcr/abl}$ tyrosine kinase in immune complex assays (43, 49, 50). Adaphostin has a 5-fold poorer autokinase K_m value for $p210^{bcr/abl}$ tyrosine kinase, but it has approximately 2-fold lower IC_{50} values against a number of cell lines than its parent compound AG-957. Hence, it is postulated that this drug must have other targets (43). On this basis, we hypothesized that antiangiogenesis drugs induce cell cytotoxicity in drug-resistant leukemic clones due to inhibition of tyrosine kinases of growth factor receptors, *i.e.*, VEGFR-1, c-Kit and other tyrosine kinases (37). To this goal, studies in reversing drug resistance in a human leukemia cell and a brain tumor model with VEGF inhibitors are reported here.

Effect of adaphostin and STI-571 on $p210^{bcr/abl}$ tyrosine kinase

The biochemical basis for the potential differences between these two drugs on $p210^{bcr/abl}$ phosphorylation and selected downstream signals in K562 Ph^+ CML cells has been investigated recently (65). Adaphostin-treated K562 cells (10 μM) showed a time-dependent apoptosis and gradual decreases in phosphorylation of $p210^{bcr/abl}$ and other tyrosine phosphorylated polypeptides persisting for at least 6 h. In contrast, 20 μM of STI-571 completely inhibited $p210^{bcr/abl}$ phosphorylation over the same time; however, the degree of cellular apoptosis was

significantly less than that of adaphostin (65). This robust inhibition by STI-571 of $p210^{bcr/abl}$ bcr/abl was followed by the weaker inhibition of Stat5 phosphorylation and the downregulation of Bcl-x_L and Mcl-1; only 7% and 25% of cells were apoptotic after 16 and 24 h, respectively, whereas greater than 90% of cells were in apoptosis at those time points after adaphostin treatment. These two agents also differed in their effects on $p210^{bcr/abl}$ protein levels. There was a gradual decrease of the $p210^{bcr/abl}$ protein after 8 h of adaphostin (10 μM) treatment, whereas STI-571 (20 μM) had no appreciable effect after 24 h, and at 48 h the $p210^{bcr/abl}$ protein levels were only marginally diminished. Additional experiments showed that the two drugs were synergistic in inducing apoptosis and that adaphostin was active in STI-571-resistant K562 cells (65). This data suggests that adaphostin must have different, as yet unidentified, mechanisms of action than STI-571. These results were further supported by the finding that adaphostin-sensitive leukemia cell lines activate distinct pathways in response to the drug, including the negative regulation of activated tyrosine kinase receptors (66). The downregulation of the VEGF receptor has also been demonstrated in our laboratory (37). Taken together, these results suggest that adaphostin has a distinct mechanism of action in CML cells from that of STI-571 and it maintains its antileukemic activity in STI-571-resistant leukemia clones.

Cytotoxicity studies

The cytotoxicity of adaphostin and its analog, NSC-682492, were determined in 9 T-lymphoblastic human leukemia cell lines, which were used as a model for a multitude of relapsed patients. The cells were exposed to 9 different concentrations of the drugs for 48 h and the cytotoxicity was determined by MTT assay (37). The results demonstrated that adaphostin exerts similar cytotoxicity in the 2 wild-type and 7 drug-resistant cell lines. NSC-682492 appeared to be less efficient in inducing cellular apoptosis than adaphostin. Adaphostin is a more potent antileukemic agent as indicated by the lower IC_{50} values (range 2- to 10-fold) than NSC-682492. Furthermore, counting apoptotic cells in 5 fields in digitized micro-photographs confirmed the biological response determined by the MTT assay (37).

DNA, RNA and protein inhibition studies

Since adaphostin demonstrated significant antitumor activity in both wild type and drug-resistant leukemia cell lines, we examined the agent's possible mechanism of action. DNA synthetic capacity was determined by the incorporation of [^{3}H]thymidine in DNA in 3 cell lines (CEM/0, CEM/ara-C/1/ASNase-0.5-2 and CEM/ara-C/I/ASNase-1-1) in triplicate, independent determinations up to 24 h after treatment with adaphostin. Four hours after drug treatment, [^{3}H]thymidine incorporation was one-half in comparison to [^{3}H]thymidine incorporation in DNA of untreated cells; however, synthetic capacity recovered to or above 100% in all cell lines by 24 h (37). RNA synthesis was determined by measuring the amount of 6'-[^{3}H]uridine incorporation into RNA. No RNA synthesis inhibition was detected in the treated cells at any time over 24 h. Similarly, protein synthesis of cells was determined by measuring [^{3}H]leucine incorporation in the protein fraction of these cell lines. By 4 h after treatment with adaphostin (0.1 or 1 μM), protein synthesis was inhibited to approximately 30% of control. By 24 h, protein synthesis was less than 10% of control in CEM/0 and two drug-resistant cell lines (37). These experiments strongly suggested that adaphostin induces cellular apoptosis by inhibiting cellular protein and, to some extent, DNA synthesis but not RNA production.

Inhibition of VEGF

Human leukemia cell lines were treated for 24 h *in vitro* with adaphostin 0.2 or 2 μM, approximately equal to the collective average IC_{50} concentration, to evaluate the possible inhibition of growth factors. VEGF concentrations were determined by the ELISA assay. The Jurkat/E6-1 cell line was used as a negative control and U937 cell line was used as a positive control for VEGF secretion. Two drug-resistant cell lines did not secrete VEGF. The parent line, CEM/0, and 5 drug-resistant clones secreted VEGF into the growth media, with an average of 100 pg/ml. Adaphostin inhibited VEGF secretion in a concentration-dependent manner, with an IC_{50} concentration inhibiting greater than 85% and a 10-fold higher concentration inhibiting 96% of control. In certain cell lines the agent inhibited VEGF to the assay's minimum level of detection (37). To verify this finding, 2 μg/ml Flt-1/Fc chimera inhibited VEGF detection in all cell lines to less than 10 pg/ml, which was the minimum level of the ELISA assay. The contribution of VEGF by the fetal bovine serum in the growth media was below the minimum level of detection of the assay.

Since adaphostin inhibits protein synthesis, the inhibition of VEGF secretion could have been a subsequent event of nonspecific protein synthesis inhibition. To further evaluate this issue, 1 IU/ml of L-asparaginase, a specific T-cell protein inhibitor, was used against CEM/0 and 4 of its drug-resistant clones. Treatment with L-asparaginase for 24 h produced no reduction of VEGF secretion in the media of any of the cell lines tested, including the wild-type CEM/0, which is sensitive to L-asparaginase. After treatment, 2 of the 4 resistant clones secreted greater amounts of VEGF (150% of control) than untreated controls (37). The data suggested that inhibition of VEGF secretion by adaphostin is not a result of inhibition of protein synthesis; hence, it must be by a specific, as yet unknown, mode of action.

VEGFR-1

In order to evaluate whether VEGF could be acting on the leukemic cells in an autocrine and/or paracrine manner, the presence of VEGFR-1 was determined. In addition to the CEM/0 and CEM/ara-C/I/ASNase-0.5-2 cell lines, the human U87 MG (glioblastoma) and DAOY (meduloblastoma) cell lines were analyzed by Western blot analysis (37). The leukemia cell lines were also examined before and after adaphostin treatment with the respective IC_{50} concentrations for 24 h. The results demonstrated that both the wild-type and the drug-resistant clone expressed VEFGR-1, as did the DAOY and U87 MG brain tumor cell lines. The VEGFR-1 protein levels were decreased in both human leukemic cell lines after treatment with adaphostin. This evidence is further supported by similar observations reported recently (66).

Caspase-3 activation

Recent investigations have reported that both adaphostin and STI-571 induce leukemia cell apoptosis through the mitochondrial pathway of effector caspase activation (65). To further evaluate the cytotoxicity of adaphostin, caspase-3 presence was determined by Western blot analysis. Fragmentation of procaspase-3 into active caspase-3 with fragment sizes of 11 and 21 kD was detected in all cell lines except CEM/ara-C/I/ASNase-1-1. Furthermore, caspase-3 activity was quantified using an ELISA assay in these cells before and after treatment with 1 μM of adaphostin for 24 h except for CEM/ara-C/I/ASNase-1-1 cell line. The results demonstrated a significant increase as percent of control in all cell lines after adaphostin treatments. CEM/0 and two drug-resistant clones had the highest increase in caspase-3 activity. When the data of caspase-3 activity was normalized per μg protein, the same result was observed (37). In contrast, NSC-642492 (1 μM) treatment produced limited increase in caspase-3 activity in these cell lines despite its average submicromolar IC_{50} values. When the caspase-3 activity was plotted against the IC_{50} concentrations of these cell lines, no relationship was obtained. The conclusions from these *in vitro* experiments that adaphostin and, to a lesser extent, NSC-642492 are active drugs against p53-null human leukemia lines, with IC_{50} concentrations in the 0.1-0.25 μM and 0.4-1 μM ranges, respectively. Adaphostin, but not NSC-642492, inhibits protein synthesis and VEGF secretion. Lastly, treatment of human drug-resistant leukemia cells with low concentrations of adaphostin (0.1-1 μM) also activated caspase-3 and induced apoptosis *in vitro.*

Effect of adaphostin alone and in combination with Flt-1/Fc chimera on U87 MG glioblastoma cells

Since adaphostin inhibited VEGF secretion, the inhibited downstream proteins (PI-3 kinase, AKT) should block the Bcl-dependent inhibition of cyt-c release from mitochondria, thus initiating cellular apoptosis. To test this possibility, U87 MG human glioblastoma, which express VEGF receptors, were treated with Flt-1/Fc chimera, a specific inhibitor of VEGF and showed a 30-43% fraction affected by MTT assay with an IC_{50} of 17.6 μg/ml. The U87 MG human brain tumor cell line was not affected by adaphostin when used as a single agent. However, in combination with Flt-1/Fc there was an apparent 8-fold drug synergism *in vitro* in this cell line by decreasing the IC_{50} value of the Flt-1/Fc chimera by a similar value. A similar pattern was seen both in the CEM/0 and CEM/ara-C/I/ASNase-0.5-2 cell lines.

To verify this *in vitro* observation, athymic mice inoculated orthotopically with 10^6 U87 MG human glioblastoma brain tumor cells were treated i.p. with 8.33 mg/kg adaphostin alone daily x3 doses x4 weeks or in combination with Flt-1/Fc chimera 5 mg/kg mouse s.c. x1. The control group had a median survival of 23 days with tumors becoming detectable with imaging techniques by day 14 and averaging approximately 1 g on day 23. The control mice had large tumors at the orthotopic site of brain tumor and large extracranial tumors (37). Treatment with adaphostin alone for 4 weeks produced a median percentage of increase in life span (%ILS) of at least 133%, whereas treatment with the combination for 1 week achieved an ILS of 142%. The increase in life span of mice treated with the combination might have been longer, but the animals were euthanized on day 29 or 33 to harvest the tumors for pathology evaluations.

The adaphostin-treated mice euthanized on day 29 had smaller brain tumors at the site of inoculation and no extracranial tumors. On day 33, or 6 days after the last drug treatment, 2 of the 6 mice treated with adaphostin up to day 28 had grown the brain tumor growth extracranially at an average of 1450 mg. The data suggested that when the U87 MG tumors were left untreated for 6 days in mice, tumor growth was no longer inhibited, as expected by a cytostatic/moderately cytotoxic molecule. In contrast, tumors in animals treated with adaphostin + Flt-1/Fc were very small around the orthotopic inoculation and did not grow between day 28 and day 33 (37). These results demonstrated that, along with the synergistic effect, this combination treatment had a longer lasting inhibitory effect on tumor growth than treatment with adaphostin alone.

In addition to the above studies, and since the tyrosine kinase inhibitors most likely will not be used as single agents, we have demonstrated synergism in a human leukemia model, the rationale and results of which are discussed below.

Multiple pathways of tumor drug resistance to STI-571 have been reported to hinder the activity of this drug (57, 59-62, 67, 68). Indeed, tyrosine kinase inhibitors should be administered in combination with other cytotoxic antineoplastic drugs and/or gamma radiation for improved long-term clinical efficacy and possible prevention of drug resistance. Currently, one of the most active combination regimens used in the treatment of pediatric

Table IA: Effect of adaphostin on VEGF secreted in the supernatant in human leukemia cell lines.

Adaphostin treatment, x 24 h	VEGF in supernatant, CEM/0, pg/ml	VEGF % of control CEM/0	VEGF in supernatant, CEM/ara-C/I/ASNase-0.5-2, pg/ml	VEGF % of control CEM/ara-C/I/ ASNase-0.5-2
Control	351.20	100.0%	100.22	100.0%
0.2 μM	46.10	13.1%	9.56	9.5%
2 μM	13.43	3.8%	0.16	0.16%

Table IB: Effect of adaphostin on intracellular caspase-3 in human leukemia cell lines.

Adaphostin treatment, x 24 h	Caspase-3 in CEM/0 pmol/μg protein	Caspase-3 % increase in CEM/0	Caspase-3 in CEM/ara-C/I/ ASNase-0.5-2 pmol/μg protein	Caspase-3 % increase in CEM/ara-C/I/ ASNase-0.5-2
Control	0.233	100.0%	0.138	100.0%
1 μM	0.823	353.2%	0.288	208.7%

leukemias is idarubicin plus the DNA cytotoxic drugs fludarabine and ara-C, providing hematologic (bone marrow) complete remission as high as 70-80% (69). In pediatric patients with ALL, the Ph^+ chromosome phenotype is not easily detected before treatment with induction chemotherapy. Thus, it is important to use regimens which are active against all leukemia blasts. Since the idarubicin/fludarabine/ara-C regimen has demonstrated considerable activity against non-Ph^+ leukemia blasts, we used it as the cornerstone in combination with the novel tyrosine kinase inhibitor (69). We hypothesized that the combination of an antiangiogenesis tyrosine kinase inhibitor with cytotoxic drugs would produce synergistic drug regimens. With this in mind, studies examining synergism between adaphostin or STI-571 and cytotoxic antileukemic drugs in both wild-type (CEM/0) and drug-resistant (CEM/ara-C/I/ASNase-0.5-2) human leukemia cell lines were conducted (37, 70, 71). The results from these studies are reported here.

The drug concentrations used in these evaluations are easily achieved in patients' plasma after administration of the idarubicin/fludarabine/ara-C combination. The peak to trough plasma levels of idarubicin and its active anabolite, idarubicinol, range from 1500 to 4 ng/ml (72, 73). These concentrations correspond to the 0.001-1 μM concentrations used in the combination studies. In addition, the drug concentrations of fludarabine and ara-C are identical to the peak and trough plasma levels from our clinical studies (69, 74).

The Median Effect Principle (MEP) and multiple drug effect equations were used as the basis of the present analyses (37, 74, 75). The multiple drug-effect analysis was performed by a computer program, which utilizes the dose-effect data and computes the median-effect dose (D*m*), sigmoidicity of dose-effect curve (*m*), the dose that is required to produce a given effect (ED_{50}, ED_{70}, ED_{90}, ED_{99}, *etc.*), the effect that can be produced by a given drug dose or drug mixtures, *i.e.*, the fractional effect (*f*a), standard errors of the mean parameters and a combination index (CI) that is calculated from the estimated *m* and D*m* values and from the multiple drug-effect equations.

Antileukemic activity of adaphostin against human T-lymphoblastic leukemia cell lines

The dose-response lines demonstrate that after a 72-h incubation as a single drug, adaphostin achieved over 90% cell kill at the 1 μM concentration used, with higher drug concentrations (2 μM) not improving the drug's cytotoxicity. Adaphostin as a single agent is more effective as an antileukemic drug than the combination of fludarabine/ara-C against human leukemia cells. The average ED_{50} (IC_{50}) concentration of adaphostin was 0.23 ± 0.2 μM for CEM/0 (wild-type) and 0.56 ± 0.58 μM for CEM/ara-C/I/ASNase-0.5-2 (multidrug-resistant) cell lines (37, 71). Adaphostin inhibited VEGF secretion in both cell lines (Table IA). In addition, to verify cellular apoptosis, significant activation of caspase-3 was documented after 24 h of treatment with adaphostin in the same cell lines (Table IB).

Microphotographic evidence further supported the apoptotic mode of cell kill by adaphostin against CEM/0 cell lines (37, 70). These results indicated that the drug inhibits VEGF secretion and interferes with the VEGF/VEGFR-1 activation pathway eventually leading to cellular apoptosis, as indicated by the activation of the terminal caspase-3.

Combination studies between adaphostin with idarubicin and/or fludarabine + ara-C

The combination of fludarabine and ara-C was very active against CEM/0, but not, as expected, against the CEM/ara-C/I/ASNase-0.5-2 drug-resistant clone. This clone was > 10^4-fold resistant to the drug combination in comparison to the wild-type parent cell line. The issue of drug sequence as a possible effector of drug synergism

Table II: Dose effect analyses of adaphostin with cytotoxic drugs in CEM/0 human leukemia cells. Synergistic factors are obtained assuming mutually nonexclusive effects of the drugs.

Drug treatments	Molar ratio of drug combination	Dm*, μM	Drug synergism, additivity or antagonism
Adaphostin	–	0.188	–
Fludarabine + ara-C	10:1	0.158	–
Idarubicin	–	0.01	–
Idarubicin + ara-C	1:10	ED_{50}**	5-fold synergism over idarubicin
Adaphostin + Idarubicin	1:1	ED_{50}**	17.8-fold drug synergism over adaphostin Additive effect over idarubicin alone
Adaphostin + Fludarabine + ara-C	1:10:1	ED_{50}**	Additive effect over adaphostin Additive effect over fludarabine + ara-C
Adaphostin (4 h) → + Fludarabine + ara-C	1:10:1	ED_{50}**	63.4-fold synergism over adaphostin Additive effect over fludarabine + ara-C
Adaphostin + Idarubicin + Fludarabine + ara-C	1:1:10:1	ED_{50}** ED_{70}** ED_{90}**	23.7-fold synergism over fludarabine + ara-c 9.6-fold synergism over fludarabine + ara-C 2.3-fold synergism over fludarabine + ara-C
Adaphostin + Idarubicin + ara-C	1:1:10	ED_{50}** ED_{70}** ED_{90}**	58.8-fold synergism over adaphostin 17.3-fold synergism over adaphostin 2.4-fold synergism over adaphostin Additive effect over idarubicin + ara-C

*Dm value: Dose of a single drug or the combination regimen to achieve 50% cell growth inhibition at the molar ratio shown.
**ED_{50}, ED_{70}, ED_{90}: Effective combined dose of drug combinations at drug ratios described to achieve 50%, 70% and 90% cell growth inhibition over control cultures.

Table III: Dose effect analyses of adaphostin with cytotoxic drugs in CEM/ara-C/I/ASNase-0.5-2 drug-resistant human leukemia cells. Synergistic factors are obtained assuming mutually nonexclusive effects of the drugs.

Drug treatments	Molar ratio of drug combination	Dm*, μM	Drug synergism, additivity or antagonism
Adaphostin	–	0.683	–
Fludarabine + ara-C	10:1	5.1E+04	–
Idarubicin	–	0.004	–
Adaphostin + Idarubicin	1:1	ED_{50}** ED_{50}** ED_{70}** ED_{90}**	Additive effect over idarubicin alone 221.4-fold drug synergism over adaphostin 792.8-fold drug synergism over adaphostin 6.0×10^3-fold drug synergism over adaphostin
Adaphostin + Fludarabine + ara-C	1:10:1	ED_{50}**	534.9-fold drug synergism over fludarabine + ara-C
Adaphostin (4 h) → + Idarubicin + Fludarabine + ara-C	1:1:10:1	ED_{50}** ED_{50}**	2.2×10^7-fold synergism over fludarabine + ara-C Additive effect over adaphostin (4 h) -> idarubicin
Adaphostin + Idarubicin + Fludarabine + ara-C	1:1:10:1	ED_{50}** ED_{70}** ED_{90}**	6.47×10^6-fold synergism over fludarabine + ara-C 6.4×10^8-fold synergism over fludarabine + ara-C 1.0×10^{10}-fold synergism over fludarabine + ara-C Additive effect over idarubicin + adaphostin

*Dm value: Dose of a single drug or the combination regimen to achieve 50% cell growth inhibition at the molar ratio shown.
**ED_{50}, ED_{70}, ED_{90}: Effective combined dose of drug combinations at drug ratios described to achieve 50%, 70% and 90% cell growth inhibition over control cultures.

was examined in the following experiments. In CEM/0 cells, adaphostin was added first at time zero followed by a sequential combination with fludarabine, which was added 4 h later and the cells were cultured at 37 °C. Ara-C was added 24 h later and treatment with the three-drug combination significantly increased the leukemic cell kill, especially in the lower drug concentration ranges, thus yielding ED_{50} values that were 63.4-fold more effective than either of the drug treatments (Table II) (70). However, when fludarabine was added before adaphostin a moderate 2-fold antagonism was obtained in the same cell line.

Adaphostin, when used in combination with idarubicin in CEM/0 cells, produced a 17.8-fold synergistic effect, whereas in CEM/ara-C/I/ASNase-0.5-2 the combination was found to be 221.4-fold synergistic over adaphostin alone. These evaluations were determined under mutually exclusive and mutually nonexclusive analyses, with no statistical difference (Table III). The best analysis of these results was obtained when a plot of the combination index

(CI) of the regimen *vs* the cellular fraction affected (fa) of the cells was made. In the CI *vs* fa plot for adaphostin in combination with fludarabine + ara-C or idarubicin in CEM/0 cells, under mutually exclusive and mutually non-exclusive evaluations, all of the data were in the synergistic range, below CI=1 (70).

Further examination of the median effect analyses at higher percentage of cell kill (*e.g.*, ED_{70} and ED_{90}) produced even greater drug synergism values in both cell lines tested. These analyses suggested that this drug combination becomes more efficacious at higher drug concentrations implying that the regimen is synergistic in a dose-dependent manner (Table III). In CEM/ara-C/I/ASNase-0.5-2, in which fludarabine + ara-C regimen did not maintain its cytotoxicity, the addition of adaphostin for the three drug combination was 535-fold synergistic over fludarabine + ara-C, the least effective component (Table III). In effect, adaphostin when used in combination with fludarabine + ara-C can reverse the drug resistance toward the DNA damaging antileukemic drugs. With the addition of ara-C to the adaphostin + idarubicin combination, adaphostin + idarubicin + ara-C was 58.8-fold synergistic over idarubicin + ara-C in CEM/0. Furthermore, the 4-drug combination, adaphostin + idarubicin + fludarabine + ara-C was 2.4-fold synergistic over fludarabine + ara-C in CEM/0 (Table III). In contrast, the same 4-drug combination was 6.47 $x10^6$-fold synergistic in CEM/ara-C/I/ASNase-0.5-2 multidrug resistant leukemia cell line. Each of the plots showed the CI *vs* fa relationship when the drugs under examination have a mutually exclusive or nonmutually exclusive mechanism of action in the two leukemia cell lines. However, since there is no difference between these lines, these analyses suggest that the drug synergism is independent of the mechanism of action by these drugs against human leukemia cells. However, due to high cytotoxicity of each of the treatments alone, no synergism was detected in the high concentrations of the combination (70). The CI *vs*. fa plot for this 4-drug combination in CEM/ara-C/I/ASNase-0.5-2 cells, under mutually exclusive and mutually nonexclusive evaluations contains all of the data in the synergistic range, whereas in the CEM/0 cells the data crossed the additivity line (CI=1) into a region of low level antagonism at the high fa, which were achieved at very high drug concentrations. The data clearly suggested that adaphostin contributes significantly in augmenting synergism of active cytotoxic drugs in wild-type leukemia cells and is much more effective in eliciting drug synergism in the drug resistant leukemia clone. Lastly, repeated experiments of adaphostin in combination with gamma radiation produced moderate levels of cytotoxic modality synergism against both CEM/0 and CEM/ara-C/I/ASNase-0.5-2 cell lines at low levels of radiation (<200 rad), whereas at high levels of gamma radiation (>1000 rad) an additive effect was obtained, presumably due to the high cytotoxicity caused by the radiation treatments (70).

Discussion and suggestions for future directions

The results of many studies have demonstrated the correlation of high levels of VEGF and poor prognosis of patients with solid tumors and leukemias (11, 32, 33, 37, 70, 76, 77). In one study, the median VEGF levels in CLL samples were 7-fold higher than the median levels in normal peripheral blood mononuclear cells (29). Additional evidence supports the fact that VEGF levels and neovascularization in the bone marrow play an important role in B-cell and promyelocytic leukemias (32, 33). Protein tyrosine kinases have been shown to be key enzymes controlling downstream signals for cell proliferation and survival. This process is initiated by secretion of growth factors acting in an autocrine and/or a paracrine manner in both solid tumors and leukemias (32-37, 78). Laboratory observations demonstrate that 1-20 leukemic cells rarely, if ever, grow into soft agar and provide viable colonies. A minimum of 200-400 cells are required for colony growth yielding a clonogenic percentage of approximately 50% (79). This contrasts with the evidence that a single murine leukemia cell injected into a syngeneic mouse will grow and kill the host. Death after leukemic cell injection can be predicted by the size of the leukemic inoculum (80). Leukemia cells will rarely grow into colonies, if there is no paracrine growth loop, or significant growth factors present in the serum of the animals. This evidence suggests that leukemia cells and their colonies depend on many growth factor secretions, like VEGF, in paracrine and/or autocrine growth loops (11, 76).

Abnormally high concentrations of growth factor ligands lead to persistently enhanced tyrosine kinase activity, which can lead to cell survival. Many studies have demonstrated that inhibition of tyrosine kinases by specific drugs can inhibit the growth of both solid tumors and leukemias (34, 35, 40-42, 44, 57, 81-83). Interestingly, after inhibition by STI-571 of the Bcr-Abl kinase, the expression of Bcl-x_L is downregulated more rapidly in chronic phase than in blast crisis CML cells, suggesting an involvement of this protein in disease progression (41). Similarly, other bcr/abl tyrosine kinase inhibitors (SU-5416, AG-957) seem to be very active against Ph(+) leukemic cells (43, 84). In our recently reported studies we described the antileukemic effects of a novel tyrosine kinase inhibitor, adaphostin against a p53-null and drug-resistant leukemic cell model not expressing Ph(+) chromosome and in human brain tumors (37, 70). The results demonstrated that adaphostin was capable of inducing apoptosis relatively rapidly in the drug-resistant and wild-type human leukemia cells, whereas more prolonged exposures and combination with the anti-VEGF chimera antibody were required to induce a similar effect in human brain tumors (glioblastoma). Despite the apparent lack of selectivity for Bcr-abl expressing leukemia cells (K562) (65), adaphostin is a strong candidate for further clinical evaluations.

Drugs active in the drug-resistant clones used in these studies have successfully predicted the clinical

efficacy of new drugs or new drug regimens in relapsed patients (69, 74, 85). In this cell model, cellular apoptosis is induced within 24 h after adaphostin treatment *in vitro*. The inhibitory effect, expressed as IC_{50} values, in the drug-resistant clones were superimposable to the ones in the wild-type cell lines (37). Since the multidrug-resistant clones have additional point mutations in the p53, this suggested that the mechanism of cytotoxicity is not mediated via the p53 pathway. Despite this fact, caspase-3 activation took place, suggesting that this is a mitochondrial pathway cellular apoptosis (65). Furthermore, synergistic combination regimens of adaphostin or imatinib mesylate with cytotoxic drugs have been designed and testing in patients has begun (70). This is important for clinical trial design because most relapsed patients with leukemias lack a functional p53 gene in their blasts (69, 86, 87), and thus are considered to be refractory to p53-dependent drug treatments.

To confirm that the cytotoxicity of adaphostin is mediated via apoptosis, we examined caspase-3 activity, which is activated before cellular apoptosis takes place. For comparison, we studied its congener NSC-642492 against this leukemic cell model, which has a 3- to 4-fold higher IC_{50} concentration in the non-$p210^{bcr/abl}$ expressing cell lines than adaphostin. These results might be explained by the greater lipophylicity of adaphostin than NSC-642492 due to its adamantyl moiety. The same explanation was provided for the differential cytotoxicity between adaphostin and NSC-642492 against CML cells *in vitro* (43). Furthermore, caspase-3, an affector caspase, was activated after treatment with adaphostin, indicating that the drug treatment had resulted in activation of the terminal caspase, which routinely is activated prior to cellular apoptosis. In subsequent experiments, this investigational drug was found to exert inhibitory activity on DNA synthesis to approximately 50% of control, which reversed to control levels by 24 hours. The initial inhibitory effect on protein synthesis was even greater than DNA inhibition in all the wild type and two drug-resistant clones tested, except that there was no recovery by 24 h (37). Recent verification of similar effects by adaphostin make this pathway much more important than originally thought (66). Of importance is that the protein inhibition was independent of RNA synthesis. This may be a direct effect of the drug's inhibition of tyrosine kinases or the indirect effect of proteolysis during apoptosis.

Using the human brain tumor cell lines DAOY and U87 MG as positive controls for VEGFR-1, which possesses an intracellular tyrosine kinase domain, it was detected in protein extracts of CEM wild-type and drug-resistant clones. This evidence suggests that VEGF, which is secreted by these lines, acts in an autocrine manner as a growth stimulus. These data may explain the poor response to treatment in leukemia patients having high circulating levels of VEGF (27, 29, 76). Treatment with adaphostin resulted in decreased detection of VEGFR-1 in both of the cell lines tested, CEM/0 and a CEM ara-C resistant clone. A possible explanation is that this agent is binding and inhibiting the tyrosine kinase domain of the receptor altering the folding thus, preventing the antibody from recognizing the epitope. An alternative explanation could be that this agent is inhibiting the expression of VEGFR-1 due to its inhibition of protein synthesis or indirectly degrades this receptor possibly *via* proteolysis (37).

To further evaluate whether inhibition of VEGF plays an important role in human leukemic cell growth, the VEGF protein concentrations were determined in the supernatants of CEM wild type and drug-resistant cell cultures. The U837 promyelocytic leukemia cell line was used as a positive control and Jurkat E6-1 as a negative control for VEGF secretion (88). Like Jurkat E6-1, two CEM drug-resistant cell lines (37) did not secrete VEGF in the culture media. Adaphostin inhibited VEGF secretion in a dose-dependent manner in CEM/0 and 5 drug-resistant clones from 50-99.9% inhibition, whereas it had limited inhibition of VEGF secretion in the U937 line. In contrast, NSC-462492 showed limited inhibition of VEGF secretion in the media of all cell lines. Thus, we postulated that the inhibition of VEGF secretion is a critical step for the induction of cellular apoptosis, as shown in Figure 1. The cell growth inhibition occurs by inhibiting the tyrosine kinase domain of VEGFR-1. This, in turn, may inhibit further autokinase action on tyrosine residues thus, preventing the cascade of intracellular signaling, which includes the secretion of VEGF. This interrupts the perpetual cycle of pro-life signaling in malignant cells.

The use of Flt-1/Fc chimera, a specific inhibitor of VEGF, *in vitro* against both U87 MG and CEM cell lines demonstrated cellular apoptosis by 24 h and 48 h, further strengthening this hypothesis. There was a 15-50% inhibition of growth in these cell lines after Flt-1/Fc treatment. These results appear to be in agreement with previously reported observations in leukemic cells (37, 88). Adaphostin as a single agent had minimal to no effect against the U87 MG human glioblastoma brain tumor cells; however, it demonstrated an 8-fold drug synergism in combination with Flt-1/Fc chimera against this cell line. This evidence suggested that the mechanism of cytotoxicity of adaphostin is elicited *via* inhibition of the tyrosine kinase of VEGFR-1 or other growth factor receptors expressed by these cell lines. Hence, a probable mechanism of cytotoxic action of this drug has been identified.

The importance of these findings was demonstrated in the orthotopic brain tumor model using *nu/nu* athymic mice and U87 MG glioblastoma tumor cells. In these *in vivo* studies, the brain tumors in vehicle-treated animals grew in a parabolic manner, similar to previous reports (36, 78). U87 MG cells formed large brain tumors at the site of inoculation, which protruded extracranially. Mice treated with the investigational drug grew smaller brain tumors 1 day after the last drug treatment was administered. However, 6 days later in 2 animals, these tumors had grown extracranially in a similar size as the control brain tumors 10 days earlier. Lastly, the combination of adaphostin and Flt-1/Fc chimera was very successful in not permitting the brain tumor to grow from the perimeter of the needle track (37) even after

discontinuation of adaphostin on day 28. The data suggest that adaphostin in combination with the anti-VEGF chimera is highly synergistic against brain tumor cells *in vitro* and *in vivo* and may be useful in the treatment of unresectable or microscopic brain tumors after surgery. Adaphostin is much more potent than its congener against CML cells from patients (65). These studies demonstrated that adaphostin is an active antileukemic drug in non-Ph(+) chromosome drug-resistant human leukemia (ALL) cell lines and in human glioblastoma brain tumors and is worthy of further investigation.

Recent insights into cancer biology have allowed us to determine the ease with which tumor cells augment their survival functions. Among these processes are the activation of multidrug resistance genes, telomerase, increased activity of DNA repair and the antiapoptotic proteins Bcl-2 and Bcl-x_L. In addition to this classical definition of tumor drug resistance, it is expected that resistance occurs at the tumor microenvironment in the host, such as leukemia blasts forced into cell replication by the growth factors secreted locally by the bone marrow stroma cells. These include the upregulation of angiogenesis-promoting mechanisms of cell survival, such as increased PDGF-, VEGF- and Bcr-abl receptor-related proliferative signals (89). Thus, circumventing or inhibiting these pathways of drug resistance is paramount for a successful treatment outcome in cancer patients. Attempts have been made to overcome or circumvent antiangiogenesis drug resistance by combination regimens (58, 90).

One known mechanism by which VEGF and other growth factors promote angiogenesis is by stimulating the activity of the "co-receptors", such as $\alpha_v\beta_3$, $\alpha_v\beta_5$ integrins, vascular endothelial-cadherin and neuropilin-1 and -2 on endothelial cells (38, 39, 62, 64). It has been shown that the IL-3-independent expression of the antiapoptotic protein, Bcl-x_L, is induced by Bcr-abl through activation of signal transducer and activator of STAT 5. Protein tyrosine kinases activate STATα and STATβ proteins. Activated by dimerization, STATs rapidly translocate into the nucleus and interact with specific regulatory elements to induce gene transcription (17). STAT activation has been studied extensively in leukemias as to the potential role they play in signaling in leukemogenesis (14).

Inhibition of Bcr-abl tyrosine kinase activity in Bcr-abl-expressing cell lines and $CD34^+$ cells from CML patients (Ph^+ chromosome) induces apoptosis by suppressing the capacity of STAT 5 to interact with the Bcl-x_L promoter (39). The $p210^{bcr/abl}$-initiated survival signaling can be interrupted at several points, the most important of which is inhibition of the tyrosine kinase domain of the protein by specific inhibitors, such as STI-571 and AG-957, or by the use of wortmannin, a potent inhibitor of PI-3 kinase (40-43). Following VEGF-VEGFR activation, the downstream activation of PI-3 kinase leads to overexpression of Bcl-2 and Bcl-x_L antiapoptotic proteins and, hence, drug resistance. In contrast, downregulation of Bcl-2 by combinations of cytotoxic antileukemic drugs in human leukemia cell lines not expressing the Ph^+ chromosome, appeared to be a prerequisite for cellular apoptosis, even in the drug-resistant clones (71, 74, 75).

In one study, the median VEGF levels in B-cell CLL samples were 7-fold higher than those in normal peripheral blood mononuclear cells (29). Additional evidence supports the fact that VEGF levels and neovascularization in the bone marrow play an important role in B-cell and promyelocytic leukemias (27, 32, 33). Also, recurrent childhood ALL blasts appears to secrete VEGF (74, 91). Elevated VEGF serum levels adversely correlated with poor molecular response to therapy in children with relapsed ALL (92). More importantly, VEGF-C signaling through VEGFR-3 (FLT-4) protected leukemic cells expressing this receptor from chemotherapy-induced apoptosis in response to treatment with ara-C, etoposide and daunorubicin. The antiapoptotic protection of VEGF-C was mediated via the induction of Bcl-2 and subsequently increased Bcl-2/Bax ratios (45). In addition, VEGF-C by acting in a paracrine fashion may actually contribute to the development of leukemia and the low frequency of apoptotic cells in solid tumors. Therefore, drugs that target the VEGFR or other tyrosine kinase-dependent signaling pathways may have therapeutic potential for certain types of leukemias (31, 45, 56, 57, 63, 93).

In pediatric leukemia patients (ALL) cytogenetic studies determine the presence of the Ph^+ chromosome on day 14 bone marrow aspirate, which is long after the non-Ph^+ leukemia blasts have been eliminated by induction treatment. In addition, most drug refractory human leukemia blasts express c-Kit, PDGF and VEGF receptors. We know that expression of the PDGF and VEGF receptors initiate a signal transduction leading to cell survival via the upregulation of Bcl-2 and Bcl-x_L. Therefore, there is a rationale to use adaphostin in combination with cytotoxic drugs to contribute to the elimination of the Ph^+ chromosome and other drug refractory leukemia blasts (70).

Tyrosine kinase inhibitors (STI-571 or adaphostin and its congeners) most likely will be used in combination regimens in clinical trials. Our studies presented above investigated the appropriate drug combinations and the sequence-specificity of adaphostin or STI-571 with antileukemic agents against 2 human T-lymphoblastic leukemia cell lines. The drug synergism or additivity seen by adaphostin in combination with cytotoxic drugs was further supported by the high number of apoptotic cells seen in the microphotographs after drug treatment (70). To further examine the potential drug synergism, and since the isobologram method in determining additive or synergistic effect in cancer chemotherapy has been controversial, we applied the MEP method. MEP analyses have demonstrated many effective drug synergisms in human T-cell lines *in vitro*, which were proven to be very active in leukemia patients, for example, the fludarabine/ara-C/idarabucin combination (70, 75). The MEP analyses demonstrated that when the CI was plotted against the fa of the cells treated with various combination regimens between adaphostin and fludarabine/ara-C

or fludarabine/ara-C/idarubicin, a significant drug synergism was observed against both wild-type and drug-resistant cell lines. Similarly, MEP analyses demonstrated drug synergism between STI-571 and adaphostin or STI-571 in combination with the same cytotoxic drugs, but at a lower degree than adaphostin and cytotoxic drugs (70).

VEGF protein concentrations have been determined in the supernatants of CEM wild-type and drug-resistant cell cultures (37, 91). The mechanism of action of adaphostin as monotherapy is due to dose-dependent inhibition (50-99.9%) of VEGF secretion in CEM/0 and 5 drug-resistant clones, whereas it had limited inhibition of VEGF secretion in the U937 line (37). This effect has been correlated with the upregulation of caspase-3 together with downregulation of VEGF secretion by these leukemic cells. These results demonstrated that adaphostin treatment interrupts the signal transduction pathway leading to cellular apoptosis. Therefore, we presume that abnormally high concentrations of growth factor ligands, like VEGF, lead to persistently enhanced tyrosine kinase activity, which can lead to upregulation of anti-apoptotic proteins (*e.g.*, Bcl-2) and hence to cell survival. Recent studies have reported that VEGF concentrations below 30-40 pg/ml have been determined to be correlated with good response to chemotherapy treatment in both solid tumors and in pediatric leukemia patients (94). In contrast, VEGF concentrations higher than 100 pg/ml correlate with disease progression and/or poor treatment outcome in standard risk ALL pediatric patients (94, 95). These results are in agreement with our data (Table 1A), where high VEGF levels are dose-dependently reduced by adaphostin (the lower dose is equivalent to ED_{50} value) to less than 50 pg/ml, levels which are not associated with adverse clinical outcome in patients with solid tumors or standard risk ALL leukemia.

Despite the efficacy of STI-571 in treating CML patients, drug resistance specific to this drug has already been noted both *in vitro* and *in vivo* (57, 59, 68, 96). Molecular studies have demonstrated a single point mutation in the ATP-binding site of the kinase activation loop in the $p210^{bcr\text{-}abl}$ protein that is sufficient to confer resistance to STI-571. This mutation occurred at nucleotide 1127 (GàA) resulting in a substitution at codon 255 of lysine (mutant) for a glutamic acid (wild-type or Glu255Lys amino acid substitution) in two-thirds of patients tested after STI-571 treatment, but not in the matched samples from these patients before beginning treatment with the drug (59). Other studies have demonstrated this Bcr-abl mutation at amino acid 255 and also in one patient a Glu255Val amino acid change after treatment with STI-571 (64).

Other clinical studies have recently demonstrated three additional point mutations (T3151, Y253H and F317L) in CML cells from patients treated with STI-571, which abrogated the action of the drug (57, 62, 83, 90). In addition to these point mutations, the serum concentrations of alpha-1 acid glycoprotein, a serum protein which binds to STI-571, are increased after treatment with the drug (57, 61, 62, 64, 67, 68). Moreover, STI-571 potentiates the therapeutic activity of retinoic acid in acute promyelocytic leukemia cells HL60 and U937 (58) and has synergistic or additive activity with daunorubicin and adaphostin (65, 70). The mechanism of the drug synergistic regimens is by increasing the proapoptotic effect of each drug on CML lymphocytes (65, 97). Interestingly, this report also demonstrated that the significant synergistic effect of the combination of daunorubicin plus STI-571 observed in CML lymphocytes was absent in normal lymphocytes (97). STI-571 was also found to have additive or superadditive effects with 4-hydroperoxy-cyclphosphamide and vincristine; however, this report showed that STI-571 had subadditivity (antagonistic) activity with methotrexate in numerous Ph^+ leukemia cell lines (70, 82).

Following these important findings, our investigations demonstrated that adaphostin is synergistic with many antileukemic agents, such as idarubicin, fludarabine and ara-C in both the wild-type and multidrug-resistant T-lymphoblastic leukemia cell lines. There are many similarities between adaphostin and STI-571 in combination with cytotoxic drugs. Both tyrosine kinase inhibitors are highly synergistic with fludarabine plus ara-C against CEM/0 cells with the addition of idarubicin improving very little the 3-drug synergistic regimen. However, in the drug-resistant clone, representing the clinically refractory leukemic blast population in a multi-relapsed leukemia patient, the contribution of idarubicin is of paramount significance in obtaining highly synergistic 4-drug regimens. It is clear that the tyrosine kinase inhibitors must be administered first in sequence followed by fludarabine in order to obtain maximum synergism, whereas treatment of leukemic cells with DNA-damaging drugs negates the synergistic effect. Further studies must follow these observations, preferably in immunosuppressed mice, to elucidate the molecular mechanism of drug synergism or antagonism. Hence, the data strongly suggest that there is sequence specificity between STI-571 and fludarabine + ara-C. Most importantly, drug synergism was observed between the two tyrosine kinase inhibitors, STI-571 and adaphostin (70, 65). The addition of adaphostin to sensitize $p210^{Bcr\text{-}abl}$-expressing leukemia cells to STI-571 (65) suggests that this combination may be worthy of further preclinical testing in experimental animals and/or possible clinical testing.

The results of synergism between adaphostin, a weak inhibitor of Bcr-abl kinase and STI-571, a specific and potent inhibitor of Bcr-abl kinase, could suggest that these ATP-mimicking molecules act on separate tyrosine kinases which are abundantly available in the cells. In effect, the less specific the tyrosine kinase inhibitor is the greater the probability that it will have multiple targets and, hence, a better overall antileukemic drug and the lower likelihood of development of drug resistance. These findings provide further insights into the antileukemic potential of these drugs and should be evaluated further in animal leukemia models. Lastly, the probable clinical demonstration of the benefits of including antiangiogenic agents with DNA cytotoxic drug regimens will have to be

further explored. Such a clinical study using STI-571 and 2 cytotoxic drugs was recently approved as a clinical protocol to be tested against refractory leukemias.

It is important to stress the differences between adaphostin and STI-571 in combination with cytotoxic drugs against human leukemia cell lines. Adaphostin achieves higher synergistic levels with cytotoxic drugs than STI-571, despite the fact that adaphostin induces a much slower decrease in $p210^{Bcr\text{-}abl}$ signaling, which does not lead to an appreciable decrease in STAT 5 phosphorylation, nor does it result in rapid decrease of Bcl-x_L (65). Such mechanistic differences in the actions of the two drugs, as well as data from the CEM cell line model, suggest that adaphostin kills leukemia cells, at least in part, by a mechanism not involving the inhibition of $p210^{Bcr\text{-}abl}$ mediated signaling (37, 65, 66, 70). These pharmacodynamic investigations need to be pusued in future clinical studies.

In conclusion, adaphostin is highly synergistic with idarubicin, idarubicin + ara-C, and idarubicin + fludarabine + ara-C over the respective cytotoxic drug regimens, whereas there was a sequence specificity between adaphostin and the cytotoxic drugs against human leukemia cell lines. Furthermore, idarubicin is not needed for optimal synergistic activity in the CEM/0 cell line. However, it is a necessary component for drug synergism in the drug-resistant clone, probably due to overcoming ara-C resistance. Adaphostin is moderately synergistic with gamma radiation. And finally, the combinations of antiangiogenesis and DNA-damaging cytotoxic drugs are highly synergistic regimens in both wild-type and drug-resistant leukemia and human glioblastoma cell lines and should be examined further.

Therapeutic implications

We have reviewed a wealth of information related to tyrphostins. They are one of the most promising classes of novel anticancer drugs to appear in the last few decades. Some of the best molecules have already been licensed for use in human patients in the U.S. and Europe. However, many questions remain unanswered, such as the precise mechanisms of action, the rapidity of drug resistance and the complete spectrum of host toxicity by these drugs.

Sufficient evidence exists that adaphostin is better than STI-571 in tumor models and it is hoped that the next congener to be synthesized will be even better than adaphostin. There is a need for small molecules with broad-spectrum activity against tyrosine kinases and therefore enhanced antitumor activity against many and diverse types of human tumors. This has been demonstrated in these studies. Drug resistance by the tumor and by the host will always be a significant hindrance to chemotherapy. In order to avoid, circumvent or diminish the probability of drug resistance development, drug combinations have to be applied. These combinations of active cytotoxic regimens with the most promising tyrosine kinase inhibitors have to be used wisely to increase their clinical efficacy and hopefully have no overlapping host toxicities.

The important steps will be to have the preclinical pharmacodynamic models well understood before these efforts are moved into the experimental animal setting for validation. At the same time, animal experimentation will have to be used extensively to better understand the pharmacokinetics, pharmacodynamics and possible drug-drug interactions in the mammalian system *in vivo,* before they are moved into the clinical setting.

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