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ORIGINAL ARTICLE

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In vitro and in vivo evaluations of the tyrosine kinase inhibitor NSC 680410 against human leukemia and glioblastoma cell lines

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Abstract *Purpose*: NSC 680410, the novel adamantyl ester of AG957, an inhibitor of the p210bcr/abl tyrosine kinase (CML, Ph^+) and possibly other kinases, was tested for antitumor activity in ten human leukemia and human glioblastoma cell lines. *Methods*: CEM/0, seven ara-C- and/or ASNase-resistant clones, Jurkat/0, the myelomonocytic line U937 and U87 MG glioblastoma cell lines were used for these studies. The drug-resistant leukemic clones lack p53, express bcl-2 and VEGF-R1, and thus are refractory to apoptosis. Since tyrosine kinases drive many proliferative pathways and these activities are increased in many leukemic cells, we hypothesized that NSC 680410 may induce cytotoxicity in drug-resistant leukemia clones, independently of p210bcr/abl expression. *Results*: NSC 680410 exhibited significant antileukemic activity in CEM/0, Jurkat E6-1, and in the drug-resistant leukemic cell lines. The IC_{50} values in nine leukemia lines ranged from 17 to 216 n*M*. Western blot analyses after NSC 680410 treatment demonstrated caspase-3 cleavage and ELISAs showed a fivefold upregulation of its activity in cellular extracts. In addition, U87 MG human glioblastoma cells, which express VEGF-R1, were treated with the Flt-1/Fc chimera, a specific inhibitor of VEGF, and showed 30–43% cell kill in the MTT assay. Furthermore, the combination of NSC 680410 plus Flt-1/Fc chimera demonstrated an eightfold synergism against U87 MG cells in vitro. To verify this observation in vivo, athymic mice were inoculated orthotopically into the caudate putamen with 10^6 U87 MG cells. On day 3, five mice per group were treated i.p. with either 8.3 mg/kg NSC 680410 daily for three doses per week for 4 weeks alone or in combination with one dose of Flt-1/Fc chimera 100 mg/kg subcutaneously. Treatment with NSC 680410 alone produced no weight changes and increased the median survival to 133%, whereas treatment with NSC680410 plus Flt-1/Fc chimera increased survival to 142% over control. Control animals had large intra- and extracranial tumors while the NSC 680410-treated mice had small, only intracranial tumors with necrotic centers. The combination treatment resulted in small residual tumors around the needle track, indicating significant inhibition of tumor growth. *Conclusions*: These studies demonstrate that the tyrosine kinase inhibitor NSC 680410 has significant antileukemic activity in p53-null, drug-resistant human leukemia cell lines, as well as significant antitumor activity in combination with Flt-1/Fc chimera against U87 MG glioblastoma brain tumors implanted in situ in athymic mice.

Keywords Tyrosine kinase · Leukemia · Brain tumors · Apoptosis · VEGF

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Introduction

The experience from clinical trials in leukemias has taught us that treatment outcome depends on a complex interaction of host, leukemic cell biology, and treatment characteristics. Since the examination of known

molecular targets has not yielded important new clinical avenues, additional molecular targets need to be identified in order to develop novel treatments against refractory leukemias. The importance of angiogenesis for the progressive growth of solid tumors is well established [10]. In contrast, only a few studies of angiogenesis in hematological neoplasms have been reported [16, 17, 29]. Recent studies have shown that VEGF-induced angiogenesis may be involved in the pathogenesis of hematological malignancies [16, 17]. 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 facilitate the expansion of the vascular network of solid tumors [10, 21] and possibly play an important role in the survival of leukemic cells in the bone marrow stroma [16].

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 [14]. It has been shown that the interleukin 3-independent expression of the antiapoptotic protein, Bcl-xL, 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 chronic myelogenous leukemia (CML) patients induces apoptosis by suppressing the capacity of STAT 5 to interact with the Bcl-xL promoter [13]. The $p210^{bcr/abl}$-initiated survival signaling can be interrupted at several points, the most important of which is the inhibition of the tyrosine kinase domain of the protein by specific inhibitors, such as STI571, AG957 and others, or by the use of wortmannin, a potent inhibitor of PI3 kinase [6, 7, 33, 35].

Previous studies have demonstrated that AG957 and its adamantyl congener NSC 680410 inhibit $p210^{bcr/abl}$ tyrosine kinase in immune complex assays [15, 35]. NSC 680410 has a fivefold poorer autokinase K_m value for $p210^{bcr/abl}$ than tyrosine kinase, but it has approximately twofold lower IC_{50} values against a number of cell lines than its parent compound. Hence it is postulated that this drug must have other targets [35]. On this basis, we hypothesized that antiangiogenic drugs induce cytotoxicity in drug-resistant leukemic clones by inhibition of tyrosine kinases of growth factor receptors, i.e. VEGF receptor 1 (VEGF-R1), c-kit, and other tyrosine kinases; however, no such evidence has been reported for either drug [25, 35]. With the aim of providing such evidence, the studies reported here of the reversal of drug resistance in a human leukemia cell and a brain tumor model with VEGF inhibitors were conducted.

Materials and methods

Cell lines and culture conditions

The cell lines used in this study were CCRF/CEM, a human T-lymphoblastic leukemia cell line isolated from a pediatric patient with ALL, hereafter called CEM/0, obtained from the NCI-DTC tumor bank (NIH, Frederick, Md.). The CEM/ara-C-resistant clones were developed in the laboratory by consecutive treatment with three concentrations of ara-C [24]. Two of these ara-C-resistant clones were treated with L-asparaginase (ASNase) 0.5 or 1 IU/ml for 24 h, washed and plated in soft agar. The isolated colonies were shown to be resistant to both ara-C and ASNase [23] and four clones along with Jurkat/E6-1, a T-lymphoblastic leukemia cell line, and U937, a myeloid cell line, were used in these studies. The cell lines were cultured in RPMI-1640 medium enriched with 10% fetal bovine serum (FBS), 1% non-essential amino acids, and 1% HEPES buffer (pH 7.4). U87 MG human glioblastoma and DAOY human medulloblastoma brain tumor cell lines were cultured as described previously [21]. The U87 MG brain tumor cells express green fluorescent protein (GFP) [21].

P53 mutations in CEM clones

The CEM cell lines have been reported to have at least one point mutation in the p53 gene [27]. The labeled cellular cDNA was analyzed by DNA array technology (Affymetrix, Santa Clara, Calif.) using the p53 DNA array (catalog no. 100133) by the DNA core facility at the Childrens Hospital Los Angeles [30]. The parent CEM/0 cell line and the drug-resistant clones were characterized as follows: TCRα/β, $CD4^+$, p16/INK $4a^{-/-}$, Scl/Sil (wt) transcription factors, GR-$L753F^+$, $p53^{-/-}$. The p53 gene mutations were examined in greater detail. CEM/0 expresses with high probability the exon 7 R248Q/cgg→cag codon change. The ara-C resistant lines, CEM/ara-C/I and CEM/ara-C/G have an additional point mutation in exon 5, R175H/cgc→cac codon change. All the CEM/ara-C and ASNase double-resistant clones also have the same mutations, exon 7 R248Q and exon 5 R175H. In addition, cell line no. 4, CEM/ara-C/I/ASNase-1-1, has two exon 8 point mutations, S261R/agt→aga and E286V/gag→gat codon changes. Cell line no. 9, CEM/ara-C/G/ASNase-1-1, has one additional mutation in exon 6, Y205H/tat→cat codon change. Jurkat/E6-1 has a point mutation in exon 6 R196*/cga→tga codon change. Finally, the myeloid line U937 has a deletion in exon 5 (adjacent to intron 5) g→deleted and in intron 5 (adjacent to exon 5) g→a codon change with high confidence [11]. Hence, the drug-resistant clones have additional point mutations over the wild-type.

Drugs

NSC 680410, the primary investigational drug, is the adamantyl derivative of AG957 tyrosine kinase inhibitor of $p210^{bcr/abl}$, the Philadelphia-positive (Ph^+) chromosome oncoprotein [35]. The investigational drug NSC 682494 was used for comparative assessments of drug effectiveness in these studies. Both compounds were provided by the Drug Development Branch, NCI, NIH (Rockville, Md.). Drug solutions were made in 50% DMSO in RPMI-1640 solution, which was diluted further to the required drug concentrations. Control cells received the highest percentage of DMSO, which did not exceed 1% in the final culture medium. Flt1/Fc chimera, a VEGF inhibitor, was purchased from R&D Systems (Minneapolis, Minn.) and L-asparaginase (ELSPAR, MSD, West Point, Pa.) was purchased from the hospital pharmacy. All other chemicals were of analytical grade.

Cytotoxicity assay

The MTT assay was used to determine the in vitro cytotoxicity of the investigational drugs used in this study as reported previously [26]. Cells were seeded in 24-well plates using 2×10^5 cells/ml medium. Aliquots of the cells were treated with multiple drug concentrations for 48 h and after the color development, aliquots were measured at OD_{495}. The results were used to calculate IC_{50} values

using the computer program Drug Effect Analysis as described elsewhere [14].

Inhibition of DNA, RNA and protein synthesis

DNA, RNA or protein synthesis inhibition studies were examined by pulse and chase experiments with 5 μCi [^{3}H]thymidine, [^{3}H]uridine, or [^{3}H]leucine, respectively, as described previously [2]. Briefly, cells were treated with a drug concentration and at a selected time, and the radiolabeled material was added for 30 min. At the end of the incubation, the cells were centrifuged, washed three times with PBS and the cell pellets lysed. The macromolecules were extracted, suspended in scintillation liquid, and the radioactivity counted. Control cultures were used for comparisons.

Microphotography

Cell cultures were photographed via a Nikon invertoscope with a digital Nikon Coolpix 950 camera. Apoptotic cells, identified by the nuclear condensation and formation of apoptotic bodies, were counted per field and expressed as percent of the total number of cells per field.

Protein extraction

Leukemic cell pellets were resuspended in protein extraction buffer containing 50 m*M* NaF, 20 m*M* HEPES, pH 7.8, 450 m*M* NaCl, 25% glycerol, 0.2 m*M* EDTA, 0.5 m*M* dithiothreitol in the presence of proteinase inhibitors (0.5 m*M* phenyl methylsulfonylfluoride, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin and 1.5 μg/ml pepstatin), then three times snap-frozen in liquid nitrogen and thawed at 30°C [9]. After centrifugation for 30 min at 4°C, supernatants were obtained and stored at –80°C. Protein determination was done with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif.).

Western blot analysis

Western blot analysis was performed by standard methods [32]. Protein (50 μg) was separated by SDS-PAGE (30% acrylamide/bisacrylamide) with a 5% stacking gel and a 10% resolving gel. Proteins were then transferred to nitrocellulose by electroblot. Nonspecific binding sites were blocked by incubation in 5% BLOTTO (5% skimmed milk in TBS-T) for 1 h at room temperature. The blot was then hybridized overnight at 4°C to the primary antibody appropriately diluted in 5% BLOTTO. After repeated washes, the blot was incubated with the secondary antibody for 1 h, followed by additional washes. The bound antibody was visualized using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Piscataway, N.J.).

Antibodies

The antibodies used for these analyses were the mouse monoclonal IgG2a for caspase-3 (E-8; Santa Cruz Biotechnology, Santa Cruz, Calif.), which reacts with caspase-3 on an epitope corresponding to amino acids 1–277 representing the full length precursor form of caspase-3 (also designated CPP32). The secondary antibody was anti-mouse IgG-HRP (Santa Cruz Biotechnology). Both antibodies were used at 1:1000 dilution. For normalization purposes, goat polyclonal antibody IgG actin (I-19) was used at 1:1000 dilution with a secondary anti-goat IgG-HRP at the same dilution.

Flt-1, also designated VEGF-R1, antibody (rabbit), was purchased from Santa Cruz Biotechnology. The primary antibody was used at the recommended 1:100 dilution and the secondary goat anti-rabbit IgG antibody was used at 1:200 dilution [4].

Caspase-3 ELISA

The CaspACE Assay System (Promega, Madison, Wis.), a colorimetric assay, was used to measure the caspase-3 concentrations in cell extracts. Approximately 10^7 treated or control cells, were centrifuged at 15,000 *g* for 15 min. The supernatants were saved for VEGF assay and the cell pellets were washed once with 1 ml PBS. Cell pellets were drained after centrifugation and resuspended in 100 μl cell lysis buffer provided in the CaspACE kit, followed by vortexing and freezing at –80°C. The tubes were then thawed, vortexed and sonicated and then incubated on ice for 15 min. The protein extracts were collected after centrifugation at 15,000 *g* for 20 min at 4°C and analyzed as described previously [34]. A volume of 20 μl cell extract, equivalent to 2×10^6 cells, was run in each test well in duplicate. All other volumes in the test solutions were prepared as per the assay kit instructions. The plate was incubated at 37°C for 4 h and then read with a Dynatech Laboratories plate reader at 405 nm. The amount of caspase-3 was determined from a standard curve.

VEGF ELISA

The VEGF concentration in cell supernatants containing 10% FBS was determined using a Quantikine Human VEGF ELISA kit from R&D Systems (Minneapolis, Minn.). Tests were done as per the manufacturer's instructions and as described previously [4].

Animal experimentation: orthotopic brain tumor model

The human glioblastoma cell line U87 MG expressing VEGF-R1 was used to test the antitumor activity of NSC 680410 alone and in combination with Flt-1/Fc chimera. Details of the xenotransplant model in female *nu/nu* mice have been described previously [21, 22]. Briefly, 10^6 U87 MG tumor cells in 10 μl PBS were inoculated orthotopically into the caudate putamen. The tumor cells were deposited 1.8 mm to the right of the bregma, 0.4 mm anterior to the coronal suture and 3.0 mm from the skull. This tumor cell inoculum resulted in the growth of tumors in all experimental animals with a highly reproducible growth rate. Intraperitoneal (i.p.) treatment with NSC 680410 (8.33 mg/kg daily for three doses) was initiated on day 3 after tumor implantation into two groups of five mice each. The drug was dissolved in 50% DMSO and sequentially diluted with sterile PBS to 1% DMSO. One of the two treated groups received on day 3 a single subcutaneous (s.c.) injection of 5 mg/kg of Flt-1/Fc chimera. The body weight of each animal was recorded daily. The control group received vehicle i.p. (PBS containing 1% DMSO). Treatment was repeated for 3 weeks, for a total of 4 weeks treatment (ending on day 28). All control animals were killed by day 17 due to symptoms from tumor progression. Treated animals were killed between days 29 and 34. Brains and tumors were fixed in buffered formalin, embedded in paraffin and sections stained with hematoxylin-eosin.

Results

Cytotoxicity studies

The cytotoxicity of NSC 680410 and NSC 682492 were determined in nine T-lymphoblastic human leukemia cell lines. The cells were exposed to nine different concentrations of these drugs for 48 h and the cytotoxicity was determined by the MTT assay (Fig. 1). The results suggest that NSC 680410 exerted similar cytotoxicity in the two wild-type and seven drug-resistant cell lines. NSC 692492 appeared to be less efficient in inducing

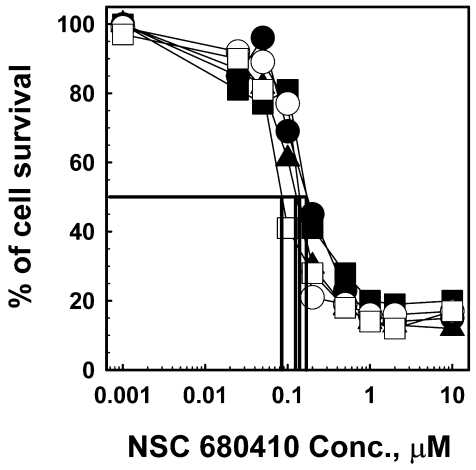

Fig. 1 Cytotoxic effect of NSC 680410 on cell survival in five T-lymphoblastic leukemia cell lines. The cells were treated in triplicate with each concentration for 48 h and the cell viability was determined by the MTT assay as described in Materials and methods. There is similarity between the cytotoxic profiles of this agent in wild-type (*closed circles*, *open squares*) and drug-resistant clones (*closed squares*, *closed triangles*, *open circles*). The symbols represent means, $n = 6$ (the SDs are very small and contained within the size of the symbols). Cell lines: CEM/0 (*closed circles*), CEM/ara-C/I/ASNase-0.5-2 (*closed squares*), CEM/ara-C/G/ASNase-0.5-1 (*closed triangles*), CEM/ara-C/I/ASNase-1-1 (*open circles*), Jurkat E6-1 (*open squares*)

cellular apoptosis than NSC 680410. Table 1 shows the IC_{50} values of NSC 680410 and NSC 642492 in these cell lines. NSC 680410 was a more potent antileukemic agent, as indicated by the lower IC_{50} values (range two- to tenfold), than NSC 682492. Furthermore, counting apoptotic cells in five fields in digital microphotographs confirmed the biological response determined by the MTT assay (Fig. 2).

DNA, RNA, and protein inhibition studies

Since NSC 680410 demonstrated significant antitumor activity in both wild-type and drug-resistant leukemia lines, we proceeded to examine this agent's possible mechanism of action. DNA synthetic capacity (DSC) was determined by the incorporation of [^{3}H]thymidine into DNA in three 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 NSC 680410. [^{3}H]Thymidine incorporation 4 h after treatment with NSC 680410 was one-half that of untreated cells. However, DSC had recovered to 100% or above in all cell lines by 24 h (data not shown). 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 (data not shown). Similarly, protein synthesis of cells was determined by measuring [^{3}H]leucine incorporation into the protein fraction of these cell lines. By 4 h after treatment with 0.1 or 1 μ*M* NSC 680410, 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 (Fig. 3). These experiments strongly suggest that NSC 680410 induces cellular apoptosis by inhibiting cellular protein and to some extent, DNA synthesis, but not RNA production.

Table 1 Cytotoxicity of NSC 680410 and NSC 642492 against human T-lymphoblastic leukemia cell lines

Cell line no.	Cell line	IC_{50} (n*M*)	
		NSC 680410	NSC 642492
1	CEM/0	97.8	367.4
2	CEM/ara-C/I/ASNase-0.5-2	216.3	571.9
3	CEM/ara-C/G/ASNase-0.5-1	34.4	234.4
4	CEM/ara-C/I/ASNase-1-1	34.9	151.2
5	Jurkat E6-1	16.8	630.7
6	CEM/ASNase-1-3	45.6	460.0
7	CEM/ara-C/I	171.2	1021.4
8	CEM/ara-C/G	75.1	225.8
9	CEM/ara-C/G/ASNase-1–1	92.7	464.0

Inhibition of VEGF

Cell lines were treated for 24 h in vitro with 0.1 or 1 μ*M* NSC 680410, approximately equal to the collective average IC_{50} concentration, to evaluate the possible inhibition of growth factors. VEGF concentrations were determined by the ELISA described in Materials and methods. The Jurkat/E6-1 cell line was used as a negative control, and the U937 cell line was used as a positive control for VEGF secretion. Two drug-resistant cell lines (nos. 3 and 9) did not secrete VEGF. The parent line, CEM/0, and five drug-resistant clones secreted VEGF into the growth medium, with an average of 100 pg/ml. NSC 680410 inhibited VEGF secretion in a concentration-dependent manner: inhibition was more than 85% at the IC_{50} and 96% at a tenfold higher concentration. In certain cell lines (nos. 2 and 4), this agent inhibited VEGF to the assay's minimum level of detection (Fig. 4). To verify this finding, 2 μg/ml Flt-1/chimera, inhibited VEGF detection in all cell lines to less than 10 pg/ml, which was the minimum level of detection of the ELISA (Fig. 4). The VEGF contributed by the FBS in the growth medium was below the minimum level of detection of the assay.

Since NSC 680410 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 four of its drug-resistant clones. Treatment with L-asparaginase for 24 h produced no reduction in VEGF secretion in the medium of any of the cell lines tested, including the wild-type CEM/0, which is sensitive to L-asparaginase. After treatment, two of the four

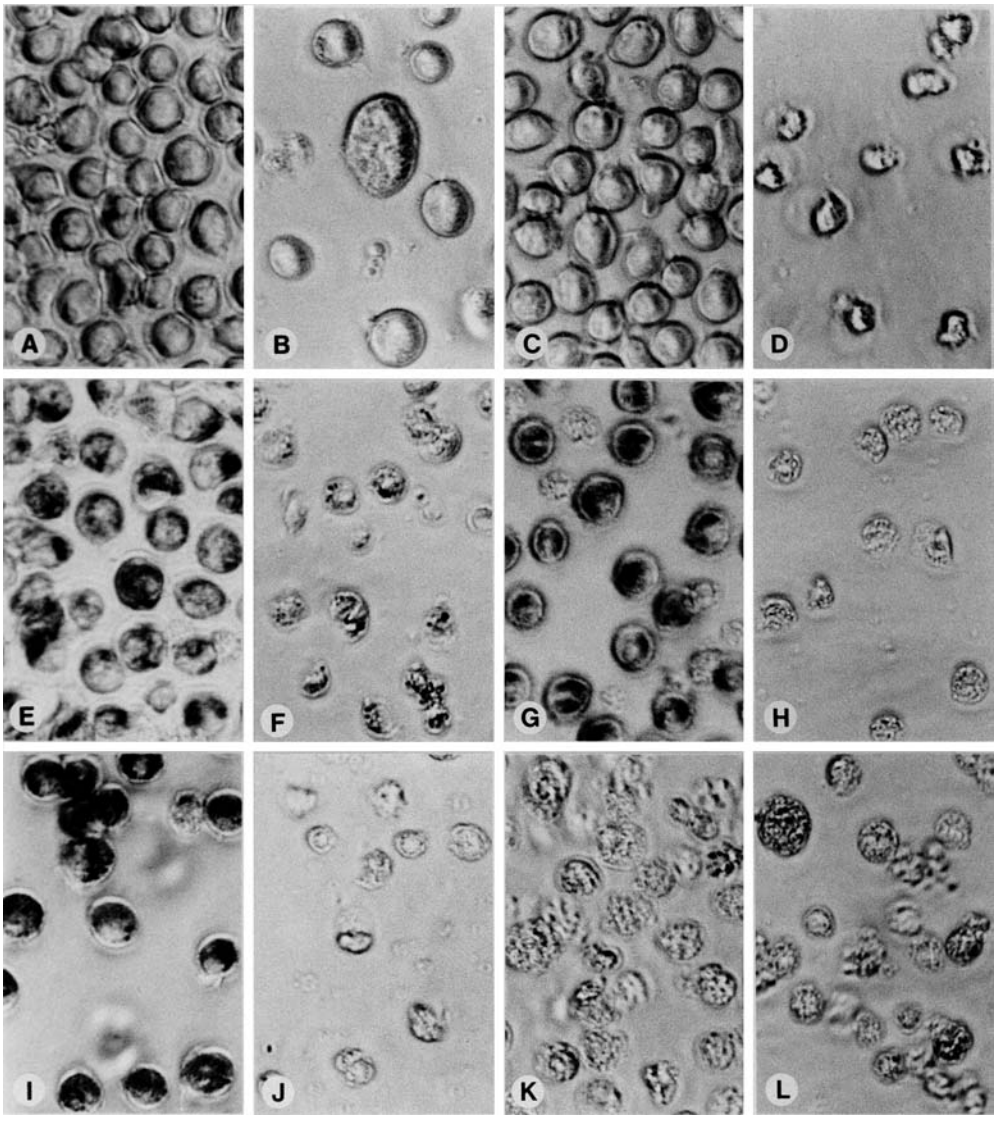

Fig. 2A–L Phase-contrast images (×40) of human leukemic cell lines untreated and treated with NSC 680410 and NSC 682492 showing cellular apoptosis. **A** CEM/ASNase-1-3, control; **B** CEM/ASNase-1-3 treated with 0.1 μ*M* NSC 682492 for 24 h; **C** CEM/ASNase-1-3, control; **D** CEM/ASNase-1-3 treated with 0.1 μM NSC 680410 for 24 h; **E** CEM/ara-C/I, control; **F** CEM/ara-C/I treated with 1 μ*M* NSC 680410 for 24 h; **G** CEM/ara-C/G, control; **H** CEM/ara-C/G treated with 1 μ*M* NSC 680410 for 24 h; **I** CEM/ara-C/I/ASNase-1-1, control; **J** CEM/ara-C/I/ASNase-1-1 treated with 1 μ*M* NSC 680410 for 24 h; **K** U937 myeloid cells, control; **L** U937 cells treated with 0.1 μ*M* NSC 680410 for 24 h

resistant clones secreted greater amounts of VEGF (150% of control) than untreated controls. These findings suggest that inhibition of VEGF secretion is not a result of inhibition of protein synthesis; hence, it must be by a specific, as-yet-unknown, mode of action of NSC 680410.

VEGF-R1

In order to evaluate whether VEGF could be acting on the leukemic cells in an autocrine and/or a paracrine manner, the presence of VEGF-R1 was determined. In addition to the CEM/0 and CEMara-C/I/ASNase-0.5-2 cell lines, the human U87 MG (glioblastoma) and DAOY (medulloblastoma) cell lines were analyzed by Western blot analysis (Fig. 5). The leukemia cell lines were also examined before and after NSC 680410 treatment at the respective IC_{50} values for 24 h. The results demonstrated that both the wild-type cell line and the drug-resistant clone expressed VEFG-R1, as did the DAOY and U87 MG brain tumor cell lines. The level of VEGF-R1 protein was decreased in both human leukemic cell lines after treatment with NSC 680410.

Caspase-3 activation

To further evaluate the cytotoxicity of NSC 680410, the presence caspase-3 was determined by Western blot analysis. Fragmentation of procaspase-3 into active caspase-3 with fragment sizes of 11 and 21 kDa was detected in all cell lines except cell line no. 4. Furthermore, caspase-3 activity was quantified using an ELISA in these cells before and after treatment with 1 μ*M* of NSC 680410 for 24 h, except for cell line no. 4. The results demonstrated a significant increase as percent of control in all cell lines after NSC 680410 treatment. CEM/0 and two drug-resistant clones (nos. 8 and 9) had the highest increase in caspase-3 activity. When the data

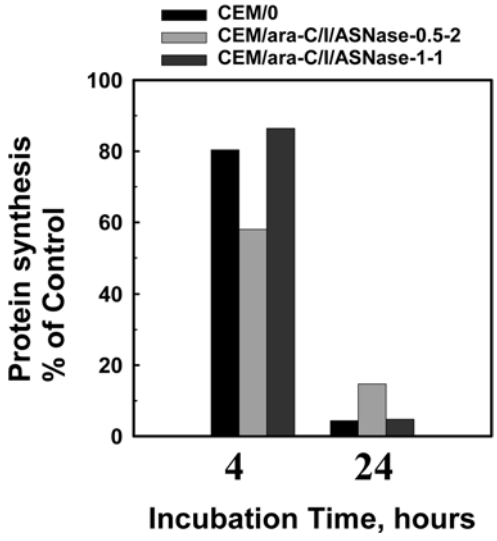

Fig. 3 Protein inhibition studies in CEM/0, CEM/ara-C/I/ASNase-0.5-2, and CEM/ara-C/I/ASNase-1-1 lines 4 and 24 h after treatment with 1 μ*M* NSC 680410 as described in Materials and methods. The [^{3}H]leucine incorporation after 24 hours with this agent was inhibited by 90–97% in comparison with control cultures for 24 h with vehicle

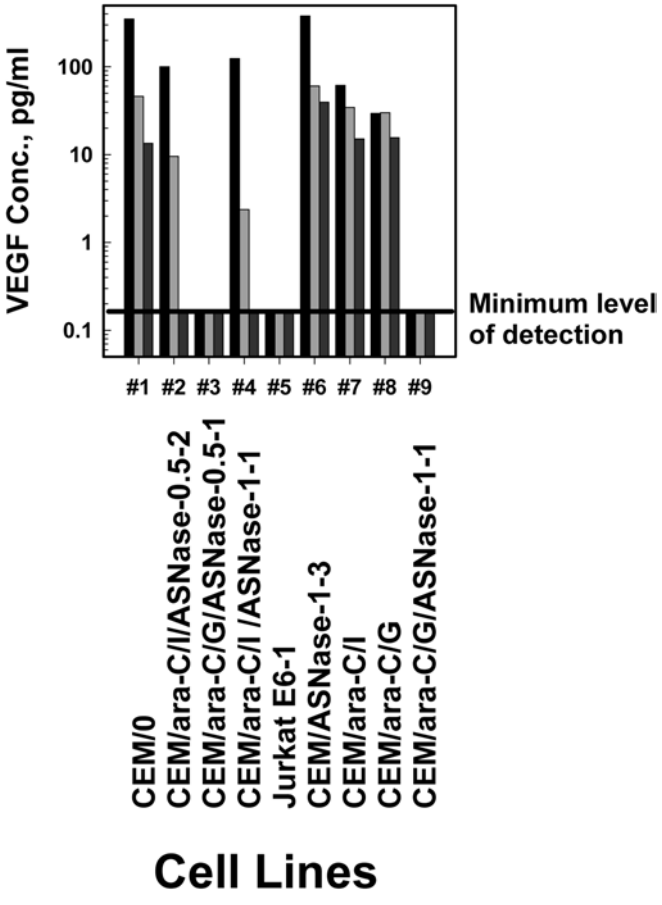

Fig. 4 Inhibition of VEGF secretion in the supernatant after incubation with 0.1 and 1 μ*M* NSC 680410 for 24 h in cell lines 1–9 (see Fig. 2). Control cultures of cell lines 3, 5, and 9 did not secrete VEGF. The contribution of VEGF from the serum was below the detection limit of the assay (0.18 pg/ml). There was a dose-dependent inhibition of VEGF secretion in the supernatants in cell lines 1, 2, 4, 6, and 7 (*solid black bars* control, *light gray bars* 0.1 μ*M* treatment for 24 h, *charcoal gray bars* 1 μ*M* treatment for 24 h)

of caspase-3 activity were normalized per microgram protein, the same result was observed (Fig. 6). In contrast, treatment with 1 μ*M* NSC 642492 produced only a limited increase in caspase-3 activity in these cell lines despite its average submicromolar IC_{50} values (data not shown). When the caspase-3 activities were plotted against the IC_{50} values of these cell lines, no relationship was seen. We conclude from the in vitro experiments that NSC 680410 and, to a lesser extent, NSC 642492 are active drugs against p53-null human leukemia lines, with IC_{50} values in the 0.1 to 0.25 μM and 0.4 to 1 μM ranges, respectively. NSC 680410, but not NSC 642492, inhibited protein synthesis and VEGF secretion. NSC 680410 also activated caspase-3 and induced apoptosis in vitro.

Effect of NSC 680410 alone and in combination with Flt-1/Fc chimera on U87 MG glioblastoma cells, in vitro and in vivo

Since NSC 680410 inhibited VEGF secretion, the inhibited downstream proteins (PI3 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 cells, which express VEGF receptors, were treated with Flt-1/Fc chimera, a specific inhibitor of VEGF, and showed a 30% to 43% fraction affected (Fa) by the MTT assay with an IC_{50} of 17.6 μg/ml. The NSC 680410 plus Flt-1/Fc combination demonstrated an apparent synergism of eightfold in vitro in this cell line by decreasing the IC_{50} value of the chimera. A similar pattern was seen in both the CEM/0 and CEM ara-C/I/ASNase-0.5-2 cell lines.

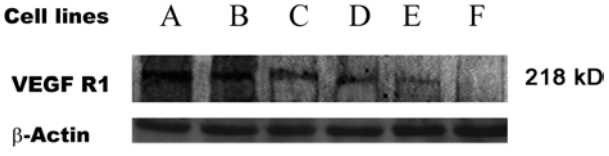

Fig. 5 Western blot analysis of VEGF-R1 in human cancer cell lines (*lane A* DAOY, medulloblastoma, control; *lane B* U87 MG glioblastoma, control; *lane C* CEM/0, control; CEM/ara-C/I/ASNase-0.5-2, control; *lane E* CEM/0 treated with 1 μ*M* NSC 680410 for 24 h; *lane F* CEM/ara-C/I/ASNase-0.5-2 treated with 1 μ*M* NSC 680410 for 24 h). The VEGF-R1 (Flt-1) signal is decreasing in both treated cell lines

To verify this in vitro observation, athymic mice inoculated orthotopically with 10^6 U87 MG glioblastoma cells were treated i.p. with 8.33 mg/kg NSC 680410 alone daily for three doses per week for 4 weeks or in combination with one s.c. dose of Flt-1/Fc chimera 5 mg/kg. The control group had a median survival of

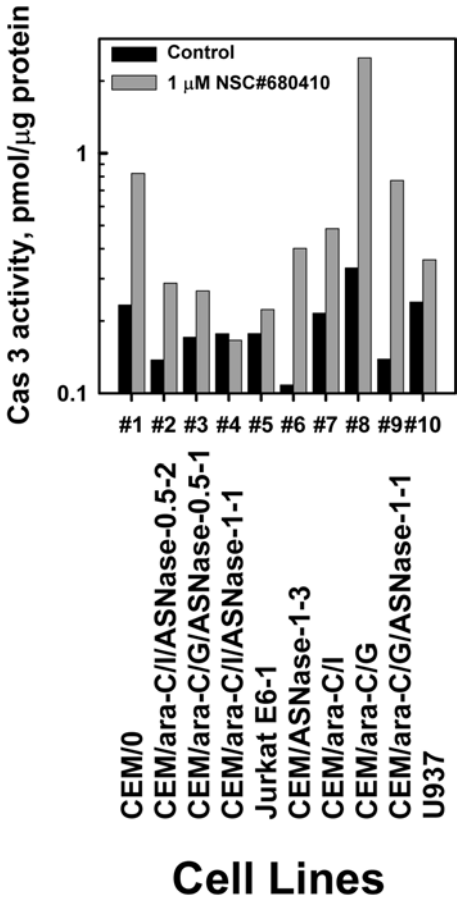

Fig. 6 Caspase-3 activation after treatment with 1 μ*M* NSC 680410 for 24 h in human leukemia cell lines 1–10. Cell lines 1–9 are identified in Table 1 and line 10 is U937

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 and large extracranial tumors (Fig. 7A, B). Treatment with NSC 680410 for 4 weeks produced a median percentage of increase in life span (%ILS) of at least 133%, whereas NSC 680410 plus Flt-1/Fc chimera achieved a %ILS of 142%. The ILS of the combination-treated mice could have been longer, but the animals were killed on day 29 or 33 to harvest the tumors for pathology evaluation.

The NSC 680410-treated mice killed on day 29 had smaller brain tumors at the site of inoculation and no extracranial tumors (Fig. 7C, D). On day 33, or 6 days after the last drug treatment, two of the six mice treated with NSC 680410 up to day 28 showed an average of 1450 mg extracranial tumor growth. These findings suggest that when U87 MG tumors were left untreated for 6 days, tumor growth was no longer inhibited. In contrast, tumors in animals treated with NSC 680410 plus Flt-1/Fc were very small around the orthotopic inoculation site and they did not grow between day 28 and day 33 (Fig. 7E, F). This demonstrates that, along with the synergistic effect, this combination treatment had a longer-lasting inhibitory effect on tumor growth than NSC 680410 treatment alone.

Discussion

Strong evidence exists supporting the correlation of high levels of VEGF and poor prognosis of patients with solid tumors and leukemias [10, 16, 17, 18, 29]. In one study, the median VEGF levels in chronic lymphocytic leukemia (CLL) samples were sevenfold higher than the median levels in normal peripheral blood mononuclear cells (PBMC) [1]. Additional evidence supports the fact that VEGF levels and neovascularization in the bone marrow play an important role in B-cell and promyelocytic leukemias [16, 17]. 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 from the environment, in a paracrine manner, in both solid tumors and leukemias [10, 16, 22]. Laboratory observations demonstrate that 1 to 20 leukemic cells per plate rarely, if ever, grow into soft agar and provide viable colonies. A minimum of 200 to 400 cells are required for colony growth, yielding a clonogenic percentage of approximately 50% [2]. This contrasts with the evidence that a single murine leukemic cell injected into a syngenic mouse will grow and kill the host. Death of a group of mice after leukemic cell injection can be predicted by the size of the leukemic inoculum [31]. Leukemia cells will rarely grow into colonies, if there is no paracrine growth loop, or significant levels of growth factors are present in the serum of the syngenic animals. This evidence strongly suggests that leukemic cells and their colonies depend on the secretion of many growth factors, such as VEGF, in paracrine and/or autocrine growth loops [18].

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 [6, 7, 8, 9, 10, 11, 27]. Interestingly, after inhibition by STI571 of the Bcr-Abl kinase, the expression of Bcl-xL is downregulated more rapidly in chronic phase than in blast crisis CML cells, suggesting an involvement of this protein in disease progression [7]. Similarly, other Bcr/abl tyrosine kinase inhibitors (SU5416, AG957) seem to be very active against leukemic cells with Ph^+ chromosome abnormalities [19, 35]. The present study indicated the anti-leukemic activity of a novel tyrosine kinase inhibitor, NSC 680410, against a p53-null and drug-resistant leukemic cell model not expressing Ph chromosome and brain tumors [3, 21, 22].

Studies using this in vitro drug-resistant leukemia model to test drug or drug regimen activity have been predictive of the clinical efficacy of these treatments in clinically refractory relapsed leukemia patients [5, 8, 11].

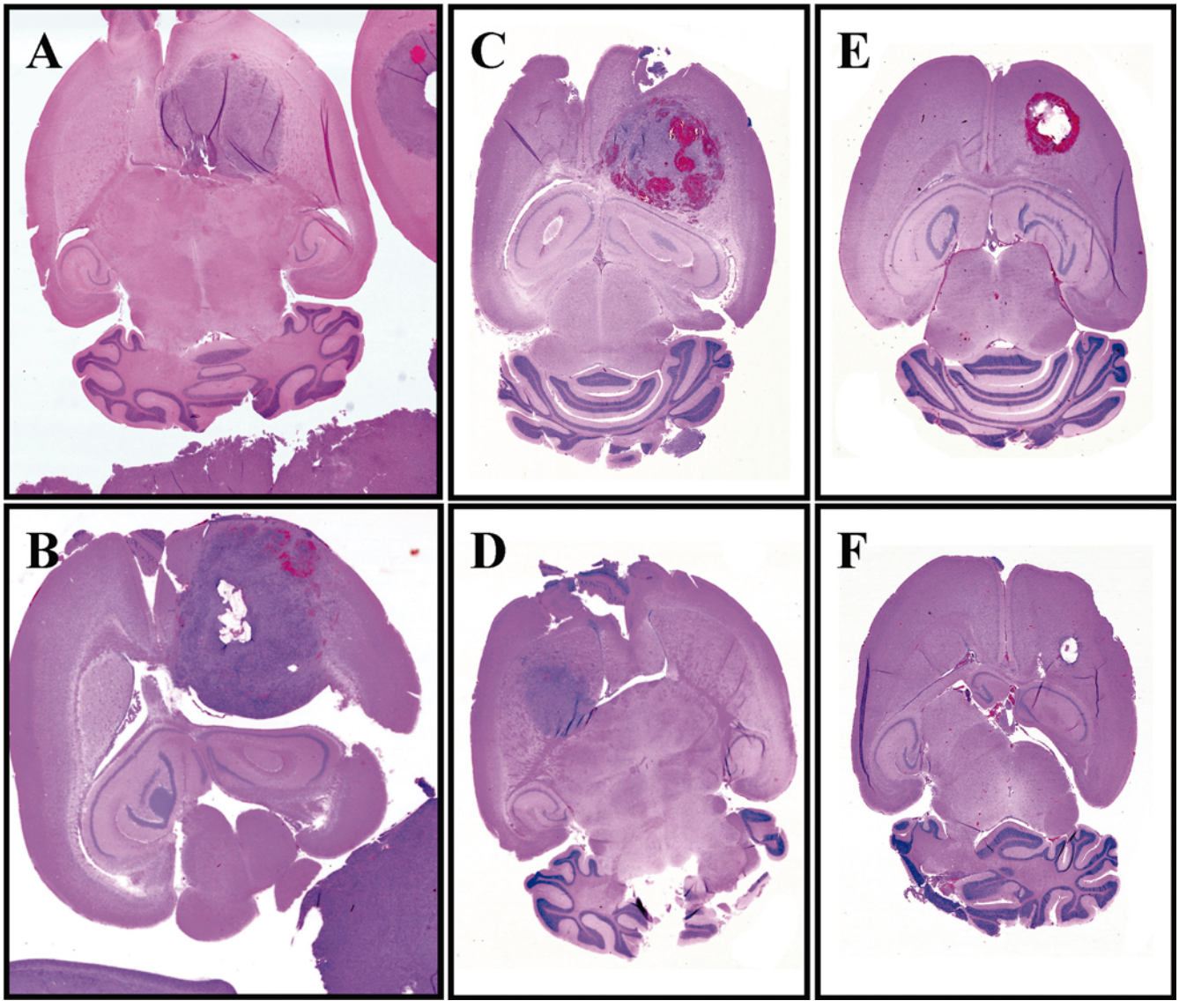

Fig. 7A–F Microphotographs of pathology slides from U87 MG glioblastoma brain tumors in athymic mice inoculated stereotactically and treated with vehicle (control, **A**, **B**). with NSC 680410 8.33 mg/kg three times per week for 4 weeks (**C**, **D**), and with NSC 680410 plus Flt-1/Fc chimera (**E**, **F**), as described in Materials and methods. The pathology slides were converted to digital images and are printed at magnifications of ×5 (**C**, **D**, **F**), ×5.5 (**A**, **E**), and ×7 (**B**)

In this cell model, cellular apoptosis was induced within 24 h after NSC 680410 treatment in vitro (Fig. 2). The inhibitory effects, expressed as IC_{50} values, in the drug-resistant clones were superimposable on those in the wild-type cell lines (Table 1). Since the multidrug-resistant clones have additional point mutations in the p53 gene, this suggests that the mechanism of cytotoxicity is not mediated via the p53 pathway. This is important for clinical trial design because most relapsed patients with leukemias lack a functional p53 gene in their blasts [11, 20], and thus are considered to be refractory to p53-dependent drug treatments.

To confirm that the cytotoxicity of the drug is mediated via apoptosis, we examined its congener NSC 642492 against this leukemic cell model. This agent has a three- to fourfold higher IC_{50} value in the non-$p210^{bcr/abl}$-expressing cell lines than NSC 680410 (Table 1). These results might be explained by the greater lipophilicity of NSC 680410 due to its adamantyl moiety. The same explanation has been suggested for the differential cytotoxicity between NSC 680410 and AG957 against CML cells in vitro [35]. Furthermore, caspase-3, an effector caspase, was activated after treatment with NSC 680410, 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 reverted to control levels by 24 h. 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. 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, the indirect effect of proteolysis during apoptosis, or the inhibition of post-translational protein modifications.

Using the human brain tumor cell lines DAOY and U87 MG as positive controls for VEGF-R1, which possesses an intracellular tyrosine kinase domain, VEGF-R1 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 findings may explain the poor response to treatment in leukemia patients having high circulating levels of VEGF [1, 18, 29]. Treatment with NSC 680410 resulted in a decrease in the levels of VEGF-R1 detected 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 and thus, preventing the antibody from recognizing the epitope. An alternative explanation could be that this agent is inhibiting the expression of VEGF-R1 due to its inhibition of protein synthesis (Fig. 3).

Since tyrosine kinase inhibitors have only just begun to be evaluated preclinically, a search of recent literature showed no additional examples of NSC 680410's effect on other tyrosine kinase systems other than the already mentioned Bcr/abl tyrosine kinase, and there are no other sources of data. However, with the knowledge that glioblastoma brain tumor cells express a multitude of receptors with tyrosine kinase moieties, such as VEGFR, PDGFR and EGFR, and that NSC 680410 downregulates expression of VEGF-R1 and causes apoptosis in vivo and in vitro (Figs. 5 and 7), we believe that this supports the theory that NSC 680410 is inhibiting tyrosine kinases other than the p210 Bcr/abl.

Even though we cannot exclude the possibility that the reduction in the VEGFR levels observed in the NSC 680410-treated glioblastoma and leukemic cell lines reflects the cytotoxic effect of the drug, we presume that the cytotoxicity is the result of the inhibition of the VEGF receptor tyrosine kinase moiety by NSC 680410 for the following reasons. Decrease in VEGF-R1 levels occurred at around 24 h while cytotoxic effects were not maximal until 48 h (MTT assay), suggesting that the VEGF-R1 decrease occurred before VEGF-R1 levels were effected by cell death. In addition, evidence from the synergistic action of the combination of STI571, a specific inhibitor of the p210 Bcr/abl tyrosine kinase moiety, and NSC 680410 against K562 cells and in CML CFU-G suggest that NSC 680410 is acting on other tyrosine kinases in addition to Bcr/abl [25]. The results of the synergism studies and decrease in VEGF-R1 levels prior to apoptosis, along with the finding of NSC 680410's lower affinity for the Bcr/abl tyrosine kinase than AG957 yet higher cytotoxicity, strongly suggest that NSC 680410 is inhibiting other tyrosine kinases in addition to Bcr/abl [25, 35].

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. This assay as well as the caspase-3 assay was not performed with the U87 MG glioblastoma brain tumor cells because, unlike the human leukemic cells which grow in suspension, U87 MG glioblastoma brain tumor cells adhere to the flask surface. Within 48 h after NSC 680410 was added, the U87 MG cells affected by the treatment were detached from the flask and thus they were nonviable. Since viable cells cannot be detached from the flask, assays such as the caspase-3 activation and VEGF ELISAs cannot be performed on a representative cell population, and thus cannot produce data that is representative of the scenario taking place as was done in the leukemia cell lines.

The U937 promyelocytic leukemia cell line was used as a positive control and Jurkat E6-1 as a negative control for VEGF secretion [12]. Like Jurkat E6-1, two CEM drug-resistant cell lines (nos. 3 and 9) did not secrete VEGF in the culture medium. NSC 680410 inhibited VEGF secretion in a dose-dependent manner in CEM/0 and five drug-resistant clones from 99.9% to 50% inhibition, whereas it showed limited inhibition of VEGF secretion in the U937 line. For comparison, NSC 462492 showed limited inhibition of VEGF secretion in the medium of all cell lines. Thus, we postulate that the inhibition of VEGF secretion is a critical step for the induction of cellular apoptosis, as illustrated schematically in Fig. 8. The cell inhibition occurs by inhibiting the tyrosine kinase domain of VEGF-R1. 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 48 h thus further strengthening this hypothesis. There was a 15% to 50% inhibition of growth in these cell lines after Flt-1/Fc treatment. These results appear to be in agreement with previously reported similar observations in leukemic cells [12]. NSC 680410 as a single agent had minimal to no effect against the U87 MG glioblastoma cells; however, it demonstrated an eightfold drug synergism in combination with Flt-1/Fc chimera against this cell line. This evidence suggests that the mechanism of cytotoxicity of NSC 680410 is elicited via inhibition of the tyrosine kinase of VEGF-R1 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 utilizing the *nu*/*nu*

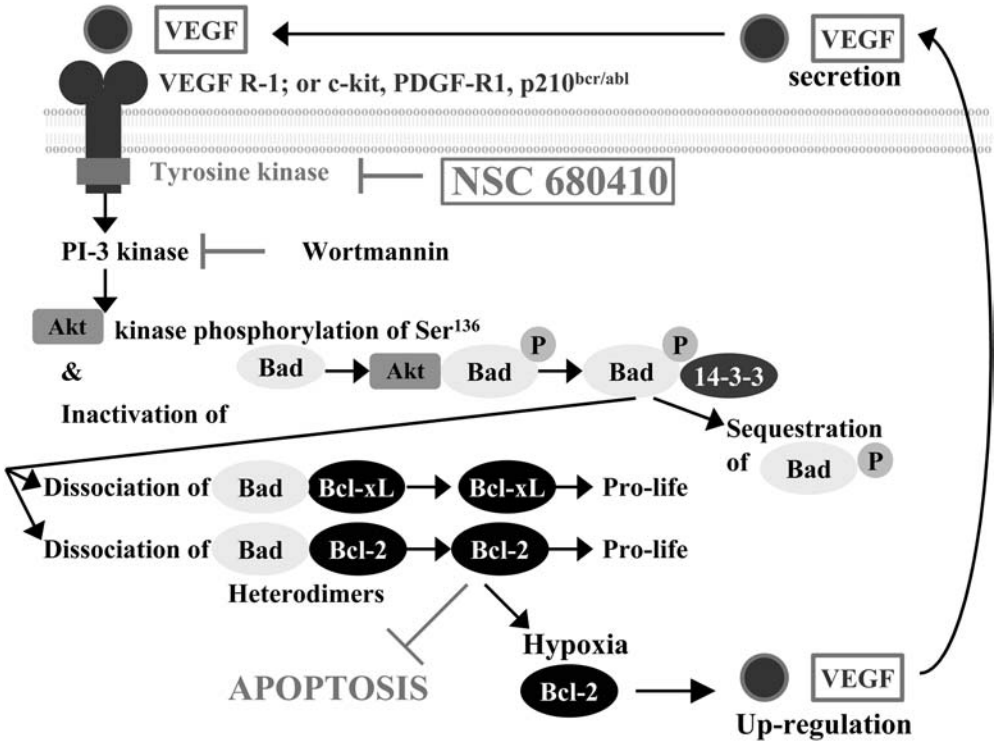

Fig. 8 Inhibition of the autocrine cell growth loop of VEGF and VEGF receptor by NSC 680410

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 that reported previously [21, 22]. U87 MG cells formed large brain tumors at the site of inoculation, which protruded extracranially. Pathology evaluations showed that the mice treated with the investigational drug had smaller tumors than control mice 1 day after administration of the last drug treatment at the end of week 4. However, in two animals that had pathology evaluations performed 6 days after administration of the last drug treatment at the end of week 4 had tumors which had grown to a larger size in the brain as well as extracranially with an apparent growth rate similar to the control brain tumors.

Lastly, the combination of NSC 680410 and Flt-1-Fc chimera was very successful in not permitting the brain tumor to grow from the perimeter of the needle track (Fig. 7) even after the discontinuation of the NSC 6800410 treatment on day 28. The limitations of these experiments, the low number of animals used and the need to kill them for pathology evaluation, is at the core of the lack of significant difference in the ILS between the two regimens, as is demonstrated in Fig. 7. The data suggest that NSC 680410 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. NSC 680410 is much more potent than the similarly structured molecule AG957 against CML cells from patients [35]. These studies demonstrated that NSC 680410 is an active antileukemic drug in non-Ph^+ chromosome drug-resistant human leukemic (ALL) cell lines and human glioblastoma brain tumor and is worthy of further investigation.

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