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ORIGINAL ARTICLE

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Determination of drug synergism between the tyrosine kinase inhibitors NSC 680410 (adaphostin) and/or STI571 (imatinib mesylate, Gleevec) with cytotoxic drugs against human leukemia cell lines

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Abstract The primary growth factor receptors involved in angiogenesis and lymphomagenesis can be grouped into the vascular endothelial growth factor (VEGF) receptors and related families. Inhibition of VEGF and other growth factors, including c-Abl, c-Kit, platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and insulin-like growth factor (IGF), or their receptors containing tyrosine kinase domains by antiangiogenesis drugs disrupts cell survival signal transduction pathways and may contribute to the proapoptotic pathways in malignant cells. However, clinical trials suggest that signal transduction inhibitors have considerable antitumor activity when used as single agents only for a short time, most likely due to the development of drug resistance by the host or by the tumor cells. In order to prevent this problem and to augment their antitumor efficacy, these agents could be administered in combination with cytotoxic antineoplastic drugs. We hypothesized that the combination of the antiangiogenesis tyrosine kinase inhibitors with cytotoxic drugs would produce synergistic drug regimens. Two human T-lymphoblastic leukemia cell lines that express VEGF-R1, CEM/0 (wild-type, WT) and the drug-resistant clone CEM/ara-C/I/ASNase-0.5-2, were utilized in the drug combination studies. NSC 680410, a tyrosine kinase inhibitor given at 0.1 to 1 μM for 72 h, inhibited VEGF secretion and leukemic cell growth at 90% of vehicle-treated control cultures with an IC_{50} value of less than 1 μM. The cytotoxic drugs idarubicin (IDA), fludarabine (Fludara), and cytosine arabinoside (ara-C) were used for the various drug combinations. One-, two-, three-, and four-drug treatments were tested. Cell viability was documented by the MTT assay and photomicrographic estimation of apoptotic cells. Both the combination index (CI) and isobologram evaluations demonstrated strong synergism between these drugs and the tyrosine kinase inhibitor. NSC 680410 was highly synergistic with IDA, IDA + ara-C, and IDA + Fludara + ara-C, over the respective cytotoxic drug regimens at concentrations easily achieved in patient plasma. NSC 680410 potentiated the activity of IDA in both leukemia cell lines by 17.8- and 221.4-fold in the WT and drug-resistant line, respectively. The activity of NSC 680410 + IDA + ara-C was also potentiated by 58.8-fold in the WT line, and the activity of NSC 680410 + IDA + Fludara + ara-C by 2.4- and 6.47×10^6-fold in the WT and drug-resistant lines, respectively. The results suggest that IDA was not needed for optimal synergistic activity in the CEM/0 cells, but IDA was a necessary component to obtain drug synergism in the drug-resistant clone. Similarly, STI571 (imatinib mesylate, Gleevec), the $p210^{bcr/abl}$ tyrosine kinase inhibitor, demonstrated synergism with Fludara + ara-C or IDA + ara-C. Most importantly STI571 showed synergism with NSC 680410, suggesting that these drugs inhibit different tyrosine kinase domains in human leukemia cells. Lastly, pretreatment of leukemic cells with NSC 680410 showed additivity with gamma radiation in comparison to either treatment modality alone. The data, taken together, suggest that by inhibiting the pro-survival signal transduction pathway (VEGF-R1) and DNA replication by cytotoxic drugs, leukemic cells undergo apoptosis in a synergistic manner. In conclusion, the combinations of antiangiogenesis and DNA-damaging cytotoxic drugs are highly synergistic regimens in both WT and drug-resistant leukemic cell lines and they should be examined further.

Keywords Drug synergism · Tyrosine kinase · Leukemia · Apoptosis · VEGF · NSC 680410 (adaphostin) · STI-571 (imatinib mesylate, Gleevec)

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Introduction

The experience from major 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 examinations of known molecular targets have 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 [15, 28, 29, 36]. In contrast, there are only a few reports of angiogenesis playing a role in hematologic neoplasms [28, 29]. These and more recent studies have shown that VEGF-induced angiogenesis may be involved in the pathogenesis or treatment outcome of hematologic malignancies [36, 43].

The VEGF receptor family consists of three transmembrane receptors, VEGF-R1 (Flt-1), VEGF-R2 (Flk-1), and VEGF-R3 (Flt-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, platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and insulin-like growth factor (IGF) [15, 28, 29, 36]. The VEGF and 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 a key activating pathway controlling cell proliferation and survival [6, 11, 12]. 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 [5, 6, 10, 15, 28]. These growth factors facilitate the expansion of the vascular network of solid tumors [5, 15, 32] and possibly play an important role in the survival and engraftment of leukemic cells in the bone marrow stroma [16, 28].

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 (VE) cadherin and neuropilin-1 and -2 (NRP-1, -2) on endothelial cells [24]. 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 tyrosine kinase activity in Bcr-abl-expressing cell lines and $CD34^+$ cells from chronic myelogenous leukemia (CML) patients (Philadelphia chromosome-positive, Ph^+) induces apoptosis by suppressing the capacity of STAT 5 to interact with the Bcl-xL promoter [23]. 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 (imatinib mesylate, Gleevec, Glivec) and AG957 or by the use of wortmannin, a potent inhibitor of PI3 kinase [9, 10, 39, 40]. Following VEGF-VEGF-R activation, the downstream activation of PI3 kinase leads to overexpression of Bcl-2 and Bcl-xL 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 Philadelphia chromosome, appears to be a prerequisite for cellular apoptosis, even in the drug-resistant clones [2, 34].

Previous studies have demonstrated that AG957 and, its adamantyl congener, NSC 680410, inhibits $p210^{bcr/abl}$ tyrosine kinase in immune complex assays [27, 40]. NSC 680410 has a fivefold poorer autokinase K_m value for $p210^{bcr/abl}$ tyrosine kinase, but it has approximately twofold lower IC_{50} values against a number of tumor cell lines than its parent compound. Hence, it is postulated that this drug must inhibit other tyrosine kinases [5, 40]. In contrast, the tyrosine kinase inhibitor STI571 is a 2-phenylaminopyrimidine derivative and a very potent agent for the treatment of Ph^+ acute lymphoblastic leukemia (ALL) and for CML [9, 14]. STI571 has been shown to produce very effective clinical results in CML patients in whom IFN therapy had failed as a single agent. STI571 induced hematologic complete remission in 96% of patients after 4 weeks of treatment due to its specific inhibition of $p210^{bcr\text{-}abl}$ tyrosine kinase [14]. STI571 is a selective inhibitor of c-Abl, $p210^{bcr\text{-}abl}$, c-Kit, and PDGF-R tyrosine kinases, which has demonstrated remarkable antileukemic activity in phase II and III clinical studies [18, 22]. However, these studies, as well as a variety of experimental models, have suggested that clinical resistance could rapidly develop to STI571 as a single agent [21]. 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 developing a resistant clone to imatinib mesylate. Hence, after a successful initial response in leukemia patients to drugs targeting signal transduction pathways, drug resistance develops rapidly to STI571 in most patients [20, 21, 22, 25].

At least two pathways of tumor resistance to STI571 have been reported to hinder its activity [17, 22, 37]. In reality the tyrosine kinase agents must be administered in combination with other cytotoxic antineoplastic drugs and/or gamma radiation for improved long-term clinical efficacy and possible prevention of the reported drug resistance. Currently, one of the most active combination regimens used in the treatment of pediatric leukemias is the fludarabine (Fludara) + cytosine arabinoside (ara-C) plus idarubicin (IDA) regimen which provides hematologic (bone marrow) complete remissions as high

as 70%–80% [13]. In pediatric patients with ALL, the Ph^+ phenotype is not easily detected pretreatment with induction chemotherapy, so it is important to use regimens which are active against all leukemia blasts. Since this regimen has demonstrated considerable activity against non-Ph^+ leukemia blasts, and we used the IDA + Fludara + ara-C combination, which is DNA cytotoxic, as the cornerstone regimen in combination with the novel tyrosine kinase inhibitors [13]. We hypothesized that the combination of the antiangiogenesis tyrosine kinase inhibitors with cytotoxic drugs will produce synergistic drug regimens. To this goal, studies examining the synergism between NSC680410 or STI571 and cytotoxic antileukemic drugs in human leukemia lines, both the wild-type (CEM/0) and the drug-resistant (CEM/ara-C/I/ASNase-0.5-2) human leukemia cell lines were conducted [4, 5, 34]. The results from these studies are reported here.

Materials and methods

Cell lines and culture conditions

The cell lines used in this study were CCRF/CEM (hereafter called CEM/0), a human T-lymphoblastic leukemia cell line isolated from a pediatric patient with ALL obtained from the NCI-DTC tumor bank, NIH (Frederick, MD). Multiple CEM/ara-C-resistant clones were developed in the laboratory by consecutive treatment with three concentrations of ara-C. 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 that were shown to be resistant to both ara-C and ASNase were used in these studies [34]. The cell lines (CEM/0 and CEM/ara-C/I/ASNase-0.5-2) 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).

Drugs

NSC 680410, the primary investigational drug, provided by the Drug Development Branch, NCI, NIH (Rockville, MD), is the adamantyl derivative of AG957 tyrosine kinase inhibitor of $p210^{bcr/abl}$, the Ph^+ oncoprotein [5, 40]. STI571 was kindly provided by Dr. Elisabeth Buchdunger (Novartis, Basel, Switzerland) [9]. Drug solutions were made in 50% DMSO in RPMI 1640 solution, and were sterilized and diluted to the required drug concentrations. Control cells received the highest percentage of DMSO, which did not exceed 1% in the final culture medium. Other drugs were IDA, Fludara, and ara-C, and were purchased from the hospital pharmacy as the formulations administered to patients. The drug concentrations used in these evaluations are easily achieved in patient plasma after administration of drug combinations (IDA/Fludara/ara-C). The peak to trough plasma levels of IDA and its active anabolite, idarubicinol, range from 1500 to 4 ng/ml [7, 31]. These concentrations correspond to the 0.001 to 1 μM concentrations used in the combination studies. In addition, the drug concentrations of Fludara and ara-C were identical to the peak and trough plasma levels from our clinical studies [3, 13].

Cell cytotoxic assay

The MTT assay was performed as described previously [5, 34].

Photomicrography

Cell cultures were photographed via a Nikon invertoscope with a digital Nikon Coolpix 950 camera. Apoptotic cells were counted in each optical field (six to ten fields per drug treatment) over the total number of cells and compared to values obtained from control cell cultures. The calculations were performed as described previously [5, 34].

VEGF and caspase-3 ELISA determinations

VEGF and caspase-3 were determined by ELISA using a methodology described previously [5, 34].

Drug synergism studies

Two-, three-, and four-drug combinations were tested against the leukemic cell lines. CEM/0 or CEM/ara-C/I/ASNase-0.5-2 cells (3×10^5 cells/ml) were treated with six different drug concentrations of each drug at 37°C for 72 h from initiation of drug treatment in all cases except ara-C, which was added 24 hours after the first drug treatment. The concentrations of NSC 680410, IDA, and ara-C ranged from 0.001 to 1 μM, and the concentrations of Fludara and STI571 ranged from 0.01 to 10 μM. In drug sequence experiments, the first drug was added 4 h before the second drug. Constant ratios of 1:1, 10:1, 1:10:1, 10:1:1:1, and 10:10:1:1 were maintained in these combination regimens. All possible drug sequences were evaluated. All combinations were also tested in a non-sequential manner, i.e., adding all drugs concurrently except ara-C. At the end of the incubation period, cell viability was determined by the MTT assay. Drug synergism between combinations of two or more drugs was estimated using the median effect principle (MEP) [4, 5, 35].

Evaluation of drug combination regimen efficacy

The MEP and multiple drug effect equations were used as the basis of the present analyses [2, 4, 35]. The multiple drug-effect analysis was performed by a computer program which utilized the dose-effect data and computed:

1. The median-effect dose; the Dm value.
2. Sigmoidicity of the dose-effect curve; i.e., the m values.
3. The dose that was required to produce a given effect (effective dose, ED); e.g., ED_{50}, ED_{70}, ED_{90}, ED_{99}, etc.
4. The effect that could be produced by a given drug dose or drug mixture; fractional effect (Fa) = 0.05, 0.5, 0.7, 0.90, etc.
5. Standard errors of the mean parameters.
6. A combination index (CI) from the m and Dm values and from the multiple drug-effect equations.

Results

Antileukemic activity of NSC 680410 against CEM/0 and CEM/ara-C/I/ASNase-0.5-2 human T-lymphoblastic leukemia cell lines

The dose-response lines demonstrated that NSC 680410 after a 72-h incubation as a single drug achieved over 90% cell kill at a concentration of 1 μM used with higher concentrations (2 μM) not improving the cytotoxicity of the drug. NSC 680410 as a single agent was more effective as an antileukemic drug than the combination of Fludara + ara-C against human leukemia

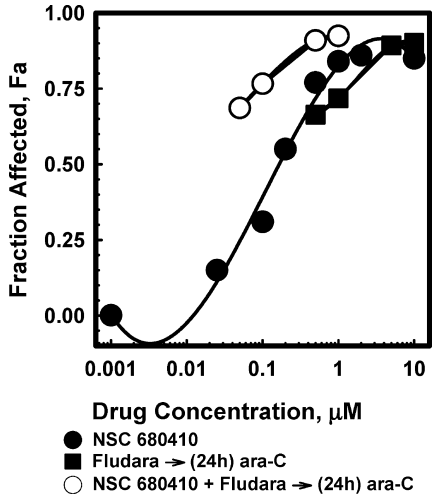

Fig. 1 Cell cytotoxicity (Fa) vs drug concentration of NSC 680410 as a single agent (●), Fludara followed by ara-C (10:1 molar ratio) at 24 h (■), or the three-drug combination (○) in CEM/0 human lymphoblastic leukemic cells. The data from the three-drug treatment is on the left of the other two lines suggesting that this combination regimen was more cytotoxic than either NSC 680410 or Fludara + ara-C treatments

cells (Fig. 1). The average ED_{50} (IC_{50}) of NSC 680410 was 0.23 ± 0.2 μ*M* for the CEM/0 cell line and 0.56 ± 0.58 μ*M* for the CEM/ara-C/I/ASNase-0.5-2 cell line (means ± SD of four independent experiments; Fig. 1). NSC 680410 inhibited VEGF secretion in both CEM/0 and CEM/ara-C/I/ASNase-0.5-2 cell lines (Table 1). In addition, to verify cellular apoptosis, significant activation of caspase-3 was documented after 24 h of treatment with this agent in these cell lines (Table 2). Photomicrographic evidence further supported the apoptotic mode of cell kill by NSC 680410 against CEM/0 cells (Fig. 2). These results indicate that the drug inhibits VEGF secretion and interferes with the VEGF/VEGF-R1 activation pathway, eventually leading to cellular apoptosis indicated by the activation of the terminal caspase-3.

Combination studies with NSC 680410, IDA and/or Fludara + ara-C

The combination of the cytotoxic drugs Fludara and ara-C was very active against CEM/0 cells, 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 this drug combination in comparison to the wild-type parent cell line. The issue of drug sequence as a possible effector of drug synergism was examined in the following experiments. In CEM/0 cells, NSC 680410 was added first at time zero followed by a sequential combination with Fludara, which was added 4 h later and the cells were cultured at 37°C. Ara-C was added 24 h later, and the three-drug combination regimen increased the leukemic cell kill significantly, especially in the lower drug concentration ranges, thus yielding ED_{50} values that were 63.2-fold more effective than either of the drug treatments (Table 3, Fig. 2). However, when Fludara was added before NSC 680410 a moderate twofold antagonism was obtained in the same cell line.

NSC 680410 in combination with IDA in CEM/0 cells produced a 17.8-fold synergistic effect, whereas in CEM/ara-C/I/ASNase-0.5-2 cells the combination was found to be 221.4-fold synergistic over NSC 680410 alone at the ED_{50}. These evaluations were determined in mutually exclusive and mutually non-exclusive analyses, and showed no statistically significant difference (Table 3, Figs. 2 and 3). The best analysis of these results was obtained when the CI of the regimen was plotted against the cellular Fa of the cells. In the CI vs Fa plot for NSC 680410 in combination with Fludara + ara-C or IDA in CEM/0 cells, in mutually exclusive and mutually non-exclusive evaluations, all of the data were

Table 1 Effect of NSC 680410 on VEGF secreted into the supernatant of human leukemia cells

	CEM/0		CEM/ara-C/I/ASNase-0.5-2	
	VEGF in supernatant (pg/ml)	VEGF (% of control)	VEGF in supernatant (pg/ml)	VEGF (% of control)
Control	351.20	100.0	100.22	100.0
NSC 680410 treatment for 24 h				
0.2 μ*M*	46.10	13.1	9.56	9.5
2 μ*M*	13.43	3.8	0.16	0.16

Table 2 Effect of NSC 680410 on intracellular caspase-3 in human leukemia cells

	CEM/0		CEM/ara-C/I/ASNase-0.5-2	
	Caspase-3 (pmol/μg protein)	Caspase-3 (% of control)	Caspase-3 (pmol/μg protein)	Caspase-3 (% of control)
Control	0.233	100.0	0.138	100.0
NSC 680410 1 μ*M* for 24 h	0.823	353.2	0.288	208.7

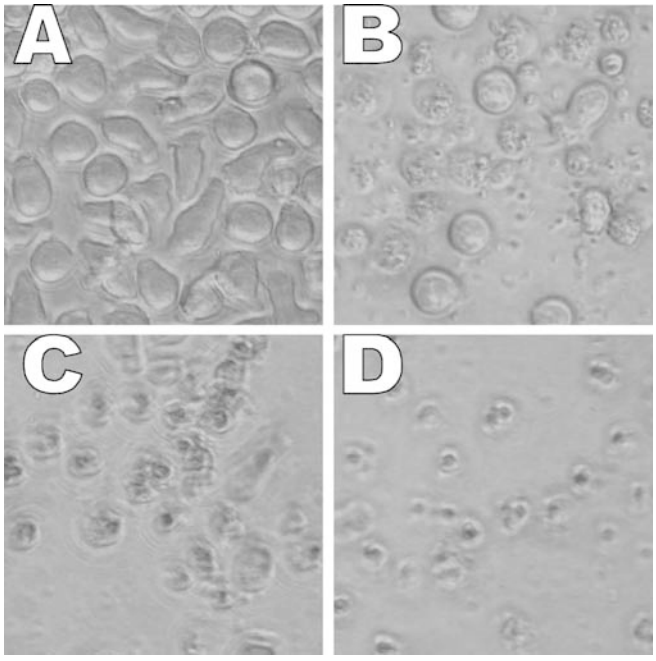

Fig. 2A–D Photomicrography of CEM/0 leukemia cells treated with vehicle (control) (**A**), NSC 680410 as a single agent (**C**), NSC 680410 + Fludara + ara-C at 0.05:0.5:0.05 μ*M* for 72 h (low drug concentrations) (**B**), and the same drug combination at 1:10:1 μ*M* for 72 h (higher concentrations) (**D**), indicating greater cellular apoptosis

in the synergistic range, i.e., CI = < 1 (Fig. 4A). Further examination of the median effect analyses at higher a percentage of cell kill (e.g., ED_{70} and ED_{90}) showed even greater drug synergism in both cell lines. These analyses suggest that this drug combination became more efficacious at higher drug concentrations, implying that the regimen is synergistic in a dose-dependent manner (Table 3). In CEM/ara-C/I/ASNase-0.5-2 cells, in which the Fludara + ara-C regimen did not maintain its cytotoxicity, the addition of NSC 680410 for the three-drug combination was 535-fold synergistic over Fludara + ara-C, the least-effective component (Table 4). In effect, NSC 680410 when used in combination with Fludara + ara-C was able to reverse the resistance toward the DNA-damaging antileukemic drugs.

The three-drug combination, NSC 680410 + IDA + ara-C, was 58.75-fold synergistic over IDA + ara-C in CEM/0 cells. Furthermore, the four-drug combination, NSC 680410 + IDA + Fludara + ara-C, was 2.4-fold synergistic over Fludara + ara-C in CEM/0 cells (Table 3). In contrast, the same four-drug combination was 6.47 $x10^6$-fold synergistic in the CEM/ara-C/I/ASNase-0.5-2 cell line.

Figure 4A, B shows the CI vs Fa relationship when the drugs under examination had a mutually exclusive or non-mutually exclusive mechanism of action in the two leukemia cell lines. However, since there was 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 with the high concentrations of the combination (Fig. 4C). In the CI vs Fa plot of this four-drug combination for CEM/ara-C/I/ASNase-0.5-2 cells, in mutually exclusive and mutually non-exclusive evaluations, all the data were contained in the synergistic range (Fig. 4B), whereas in the plot for the CEM/0 cells at very high drug concentrations the data crossed the additivity line (CI = 1) into the region of low-level antagonism at high Fa (Fig. 4C). The data clearly suggest that NSC 680410 contributed significantly in augmenting synergism between active cytotoxic drugs in the wild-type leukemia cells and was much more effective in eliciting drug synergism in the drug-resistant leukemia clone. Lastly, repeated experi-

Table 3 Dose effect analyses of NSC 680410 with cytotoxic drugs in CEM/0 human leukemia cells. Assuming mutually non-exclusive effects of the drugs, the following synergistic factors were obtained (*Dm* 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 the drug combinations at the ratios indicated to achieve 50%, 70%, and 90% cell growth inhibition over control cultures)

Drug treatment	Molar ratio of drug combination	Dm (μ*M*)	Drug synergism, additivity, or antagonism
NSC 680410	–	0.188	–
Fludara + ara-C	10:1	0.158	–
IDA	–	0.01	–
IDA + ara-C	1:10	ED_{50}	5-fold synergism over IDA
NSC 680410 + IDA	1:1	ED_{50}	17.84-fold synergism over NSC 680410 Additive effect over IDA alone
NSC 680410 + Fludara + ara-C	1:10:1	ED_{50}	Additive effect over NSC 680410 Additive effect over Fludara + ara-C
NSC 680410 (4 h) → + Fludara + ara-C	1:10:1	ED_{50}	63.4-fold synergism over NSC 680410 Additive effect over Fludara + ara-C
NSC 680410 + IDA + Fludara + ara-C	1:1:10:1	ED_{50} ED_{70} ED_{90}	23.7-fold synergism over Fludara + ara-C 9.66-fold synergism over Fludara + ara-C 2.31-fold synergism over Fludara + ara-C
NSC 680410 + IDA + ara-C	1:1:10	ED_{50} ED_{70} ED_{90}	58.8-fold synergism over NSC 680410 17.3-fold synergism over NSC 680410 2.48-fold synergism over NSC 680410 Additive effect over IDA + ara

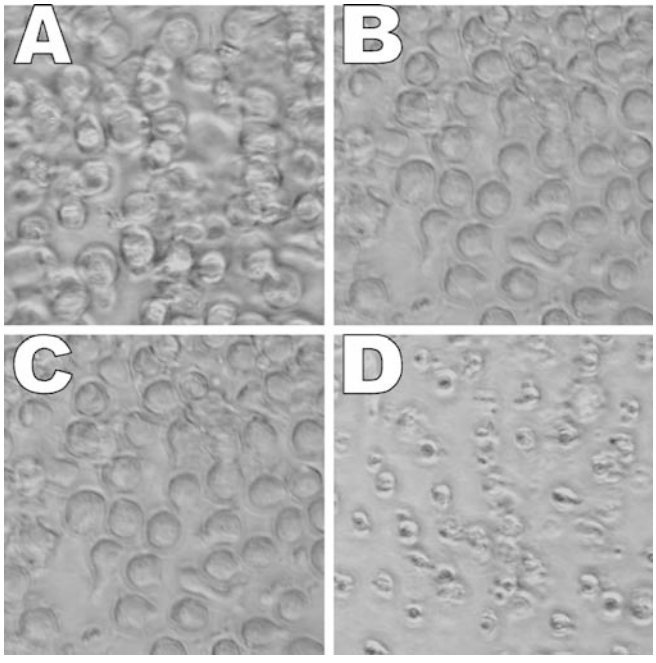

Fig. 3A–D Photomicrography of CEM/ara-C/I/ASNase-0.5-2 drug-resistant leukemic cells treated with vehicle (control) (**A**), Fludara + ara-C at 10:1 μ*M* for 72 h (**B**), NSC 680410 followed by Fludara + ara-C at 0.1:1:0.1 μ*M* for 72 h (**C**), and the same drug combination at 1:10:1 μ*M* for 72 h (**D**), indicating massive cellular apoptosis in the drug-resistant clone

ments with NSC 680410 in combination with gamma radiation produced moderate levels of cytotoxic synergism against both CEM/0 cells and CEM/ara-C/I/ASNase-0.5-2 cells at low levels of radiation (< 200 rad), whereas at high levels of radiation (> 1000 rad) an additive effect was obtained, presumably due to the high cytotoxicity caused by the radiation treatments (data not shown).

STI571 in combination with cytotoxic drugs: cytotoxic interaction between STI571 and IDA or Fludara + ara-C

STI571 as a single drug for 72 h achieved a limited inhibition (10%–25%) of the growth of CEM/0 cells and CEM/ara-C/I/ASNase-0.5-2 cells compared with vehicle-treated control cultures. The results were as expected since these leukemia cell lines were not Ph^+. When STI571 was combined with the cytotoxic drugs IDA, Fludara, and ara-C, the cell kill was increased significantly, achieving ED_{50} values of less than 1 μ*M*. When STI571 was administered first followed by Fludara 4 h and ara-C 24 h later, the regimen was 148-fold synergistic over Fludara + ara-C in CEM/0 cells at ED_{50}. The degree of synergism decreased at ED_{70} and was the lowest at ED_{90} probably due to the high cell kill by Fludara + ara-C in this cell line. The CI vs Fa plots indicated that the drug combination was not synergistic at the higher drug concentrations needed to achieve high

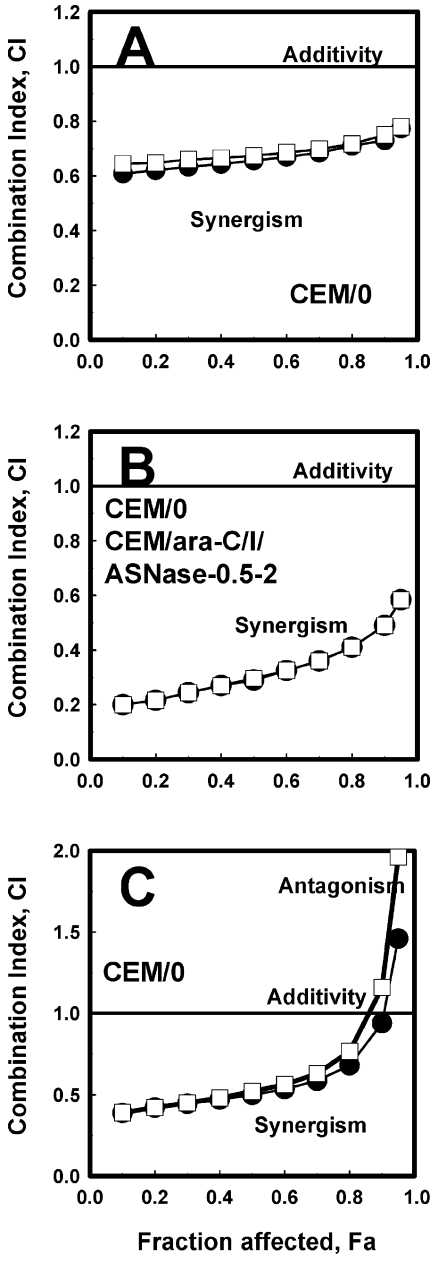

Fig. 4A–C Drug synergism studies of NSC 680410 with cytotoxic drugs in human leukemia cells. **A** Fa vs CI plots of IDA + NSC 680410 (1:1 molar ratio) against CEM/0 cells are shown after mutually exclusive (□) and mutually non-exclusive analyses (●). The analyses demonstrate drug synergism throughout the range of cell cytotoxicity (Fa). **B** Fa vs CI plots of IDA + NSC 680410 plus Fludara + ara-C (1:1:10:1 molar ratio) against CEM/0 (●) or CEM/ara-C/I/ASNase-0.5-2 cells (□) are shown after mutually exclusive analyses. The results demonstrate drug synergism throughout the range of cell cytotoxicity (Fa) in both leukemia cell lines. **C** Fa vs CI plots of IDA + NSC 680410 plus Fludara + ara-C (1:1:10:1 molar ratio) against CEM/ara-C/I/ASNase-0.5-2 cells are shown after mutually exclusive (●) and mutually non-exclusive analyses (□). The analyses demonstrate drug synergism throughout most of the range of cell cytotoxicity (Fa)

Fa values in these cells (Fig. 5). These data indicate that there was a synergistic interaction between STI571 and Fludara + ara-C at ED_{50} and ED_{70}, but not at ED_{90}

Table 4 Dose effect analyses of NSC 680410 with cytotoxic drugs in CEM/ara-C/I/ASNase-0.5-2 drug-resistant human leukemia cells. Assuming mutually non-exclusive effects of the drugs, the following synergistic factors were obtained (*Dm* 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 the drug combinations at the ratios indicated to achieve 50%, 70%, and 90% cell growth inhibition over control cultures)

Drug treatment	Molar ratio of drug combination	Dm (μM)	Drug synergism, additivity, or antagonism
NSC 680410	–	0.683	–
Fludara + ara-C	10:1	5.1×10^4	–
IDA	–	0.004	–
NSC 680410 + IDA	1:1	ED_{50}	Additive effect over IDA alone
		ED_{50}	221.4-fold synergism over NSC 680410
		ED_{70}	792.8-fold synergism over NSC 680410
		ED_{90}	6×10^3-fold synergism over NSC 680410
NSC 680410 + Fludara + ara-C	1:10:1	ED_{50}	534.9-fold synergism over Fludara + ara-C
NSC 680410 (4 h) → + IDA + Fludara + ara-C	1:1:10:1	ED_{50}	2.2×10^7-fold synergism over Fludara + ara-C
		ED_{50}	Additive effect over NSC 680410 (4 h) → IDA
NSC 680410 + IDA + Fludara + ara-C	1:1:10:1	ED_{50}	6.47×10^6-fold synergism over Fludara + ara-C
		ED_{70}	6.4×10^8-fold synergism over Fludara + ara-C
		ED_{90}	1×10^{10}-fold synergism over Fludara + ara-C
			Additive effect over IDA + NSC 680410

(Table 5). The opposite sequence, Fludara administered first, followed 4 h later by STI571 and 24 h later by ara-C was 10.6-fold synergistic in the same cell line. When compared with the previous drug sequence, less synergism was found when Fludara preceded STI571 treatment at ED_{50} and ED_{70}, whereas at ED_{90} no synergism occurred. Hence, the data strongly suggest that there was sequence specificity between STI571 and Fludara + ara-C against leukemia cells.

In addition, the combination of STI571 and IDA + ara-C showed considerable synergism in CEM/0 cells (Table 5). Thus, the highly synergistic regimen of IDA + ara-C potentiated the effect of STI571 with a remarkable increase in synergism with increasing cellular apoptosis, especially at ED_{90}. Both the CI vs Fa and isobologram evaluations demonstrated strong synergism between STI571 and the cytotoxic drugs (isobologram figures were produced by MEP program analysis, data not shown). Lastly, the combination of STI571 and NSC 680410 showed an additive effect over NSC 680410 as a single-drug treatment in CEM/0 and CEM/ara-C/I/ASNase-05-2 cells. This was due to the fact that these drugs have multiple and different target tyrosine kinases among the multiple tyrosine kinases in the refractory leukemia blasts. However, these drugs added together, were 4.5-fold synergistic over NSC 680410 alone in both CEM/0 cells and CEM/ara-C/I/ASNase-0.5-2 cells (Table 5). We conclude that combinations of tyrosine kinase inhibitors with cytotoxic drugs are highly synergistic regimens against both wild-type and drug-resistant leukemia lines.

Discussion

Strong evidence exists supporting the correlation of high levels of VEGF in serum and poor prognosis of patients with solid tumors and myeloid and lymphoblastic leukemias [15, 16, 28, 29, 30, 36, 43]. In one study, the median VEGF levels in B-cell CLL samples was sevenfold higher than the median level in normal peripheral blood mononuclear cells [1]. Additional evidence supports the fact that VEGF levels and neovascularization in the bone marrow play important roles in B-cell and promyelocytic leukemias [1, 28, 29]. Also, recurrent childhood ALL blasts appear to secrete VEGF [5, 28]. Elevated VEGF serum levels are adversely correlated with poor molecular response to therapy in children with relapsed ALL [43]. More importantly, VEGF-C signaling through VEGFR-3 (Flt-4) protects leukemic cells expressing this receptor from chemotherapy-induced apoptosis in response to treatment with ara-C, etoposide, and daunorubicin (DNR). The antiapoptotic protection of VEGF-C is mediated via the induction of Bcl-2 and subsequently increases Bcl-2/Bax ratios [10]. 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 [12, 14]. Therefore, drugs that target the VEGF-R or other tyrosine kinase-dependent signaling pathways may have therapeutic potential for certain types of leukemias [9, 10, 11, 12, 14, 20, 23].

Recent enhanced 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 (MDR), telomerase, increased activity of DNA repair and the antiapoptotic proteins Bcl-2 and Bcl-xL. In addition to this classical definition of tumor drug resistance, it is expected that resistance occurs in the tumor microenvironment in the host, such as leukemia blasts forced into cell replication by the growth factors secreted locally by 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 [30, 33]. Thus, circumventing or inhibiting these pathways of drug resistance is paramount for a successful treatment outcome in

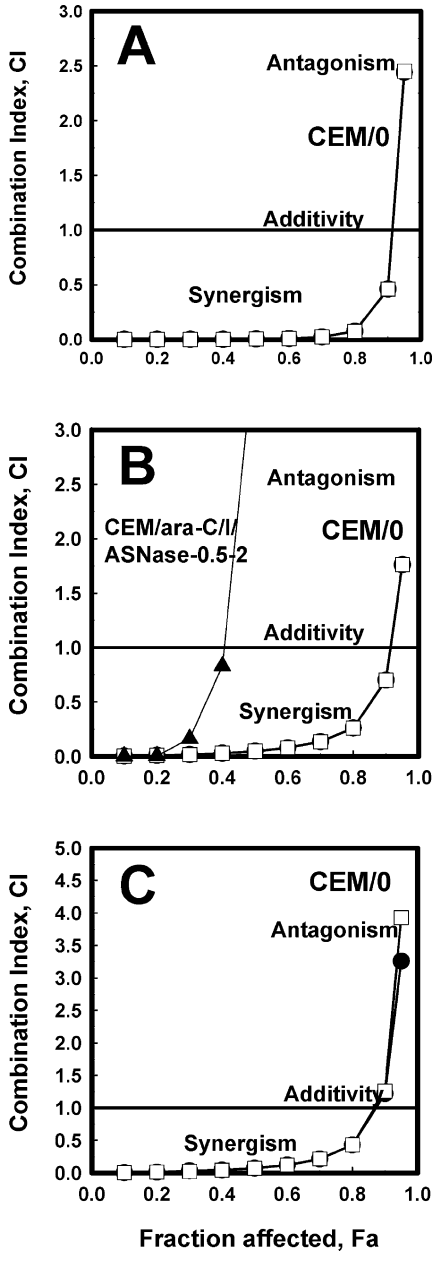

Fig. 5A–C Drug synergism studies of STI571 with cytotoxic drugs in human leukemia cells. **A** Fa vs CI plots of STI571 and Fludara (added together) + ara-C (10:10:1 molar ratio) against CEM/0 cells are shown after mutually exclusive (●) and mutually non-exclusive analyses (□). The analyses demonstrate drug synergism throughout the majority of the range of cell cytotoxicity (Fa). **B** Fa vs CI plots of Fludara added 4 h before STI571 + ara-C (10:10:1 molar ratio) against CEM/0 cells are shown after mutually exclusive (●) and mutually non-exclusive analyses (□). The analyses demonstrate drug synergism throughout most of the range of cell cytotoxicity (Fa) (▲ same experimental design in CEM/ara-C/I/ASNase-0.5-2 cells demonstrating an additive effect at low Fa and a highly antagonistic effect at higher Fa with this drug combination). **C** Fa vs CI plots of STI571 and IDA (added together) + ara-C (1:10:1 molar ratio) against CEM/0 cells are shown after mutually exclusive (●) and mutually non-exclusive analyses (□). The analyses demonstrate drug synergism throughout most of the range of cell cytotoxicity (Fa)

cancer patients. Attempts have been made to overcome or circumvent antiangiogenesis drug resistance [18].

Two tyrosine kinase inhibitors were examined in these studies. NSC 680410 and STI571 can be regarded as the first members of a new family of drugs termed signal transduction inhibitors [9, 27, 40]. They were designed based on the structure of the ATP binding site of the tyrosine kinase domain of $p210^{bcr\text{-}abl}$, PDGF and c-Kit receptor kinases. In the present study, the antileukemic activities of NSC 680410 and STI571 as single agents and in combination with cytotoxic antineoplastic drugs against a p53-null and drug-resistant leukemic cell line model not expressing the Philadelphia chromosome were investigated [4].

STI571 is a highly specific inhibitor of the p210bcr-abl tyrosine kinase domain. However, no inhibitor is as specific as investigators would wish. STI571 is also a potent inhibitor of c-Kit and PDGF receptor tyrosine kinases [9]. We have evidence that STI571 also inhibits the tyrosine kinase domain of VEGF R3, but this is the subject of new investigations. In pediatric leukemia patients, patients with ALL, cytogenetic studies cannot determine until the day-14 bone marrow aspirate the presence of the Philadelphia chromosome, which is long after the non-Ph^+ leukemic blasts have been eliminated by the induction drug treatment. Most 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-xL. Therefore, there is a rationale for using this agent in combination with cytotoxic drugs to contribute to the elimination of the Philadelphia chromosome and other drug-refractory leukemia blasts.

The purpose of the present study was to investigate the appropriate drug combinations and the sequence-specificity of NSC 680410 or STI571 with antileukemic agents against two human T-lymphoblastic leukemia cell lines. When the dose-response curves are to the left of the single drug or drug combination, as in Figs 1, 4 and 5, drug additivity or synergism is considered. The drug synergism or additivity seen with NSC 680410 in combination with cytotoxic drugs was further supported by the high number of apoptotic cells seen in the microphotographs after drug treatment (Figs. 2 and 3). To further examine the potential drug synergism, and since the isobologram method for determining an 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 have been proven to be very active in leukemia patients, such as Fludara + ara-C + IDA [2, 11, 32]. The MEP analyses of the plots of CI against the Fa of the cells treated with various combination regimens of NSC 680410 and Fludara + ara-C or Fludara + ara-C plus IDA demonstrated significant drug synergism against both the wild-type and the drug-resistant cell lines (Tables 3 and 4). Similarly, MEP analyses demon-

Table 5 Dose effect analyses of STI571 with cytotoxic drugs in CEM/0 human leukemia cells. Assuming mutually non-exclusive effects of the drugs, the following synergistic factors are obtained (*Dm* 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 the drug combinations at the ratios indicated to achieve 50%, 70%, and 90% cell growth inhibition over control cultures)

Drug treatment	Molar ratio of drug combination	Dm (μM)	Drug synergism, additivity, or antagonism
STI571	–	4.83	–
Fludara + ara-C	10:1	1.59	–
NSC 680410		0.11	
IDA	–	0.01	–
NSC 680410 + STI571	1:10	ED_{50}	4.9-fold synergism over STI571[a]
		ED_{70}	Additive effect over NSC 680410
		ED_{90}	6.2-fold synergism over STI571
			9.95-fold synergism over STI571
STI571 + Fludara + ara-C[b]	10:10:1	ED_{50}	148-fold synergism over Fludara + ara-C
		ED_{70}	22.8-fold synergism over Fludara + ara-C
		ED_{90}	Additive effect over Fludara + ara-C
Fludara (4 h) $\rightarrow$ + STI571 + ara-C[b]	10:10:1	ED_{50}	10.6-fold synergism over Fludara + ara-C
		ED_{70}	3.8-fold synergism over Fludara + ara-C
		ED_{90}	Antagonistic effect over Fludara + ara-C
STI571 + IDA + ara-C	10:1:1	ED_{50}	4.9-fold synergism over Fludara + ara-C
		ED_{70}	6.3-fold synergism over Fludara + ara-C
		ED_{90}	10-fold synergism over Fludara + ara-C

[a]NSC 680410 + STI571 was 4.5-fold synergistic or additive to moderately antagonistic at various Fa values against CEM/ara-C/I/ASNase-0.5-2 cells

[b]The three-drug regimen was additive at low Fa and antagonistic at high Fa in CEM/ara-C/I/ASNase-0.5-2 cells

strated drug synergism between STI571 and NSC 680410 or STI571 in combination with the same cytotoxic drugs, but at a lower degree than NSC 680410 and cytotoxic drugs (Table 5).

VEGF protein concentrations have been determined in the supernatants of CEM wild-type and drug-resistant cell cultures [4, 5]. NSC 680410 as a single treatment inhibits VEGF secretion in a dose-dependent manner in CEM/0 and five drug-resistant clones from 99.9% to 50% inhibition, whereas it inhibits VEGF secretion in the U937 line to a limited extent [5]. This effect has been correlated with upregulation of caspase-3, a terminal caspase, commensurate with downregulation of VEGF secretion by these leukemic cells. These results indicate that NSC 680410 treatment interrupts the signal transduction pathway leading to apoptosis. Therefore, we presume that abnormally high concentrations of growth factor ligands, such as VEGF, lead to persistently enhanced tyrosine kinase activity, which can lead to upregulation of antiapoptotic proteins, such as Bcl-2, and hence to cell survival. Recent studies have shown that VEGF concentrations below 30–40 pg/ml are correlated with good response to chemotherapy treatment in both solid tumors and in pediatric leukemia [41]. In contrast, VEGF concentrations higher than 100 pg/ml are correlated with disease progression and/or poor treatment outcome in front-line standard-risk ALL pediatric patients (laboratory studies presented at the ASCO Meeting 2003[1]; manuscript submitted to J Clin Oncol, June 2003). These results are in agreement with the data presented in Table 1, showing that high VEGF levels were reduced in the presence of NSC 680410 in a dose-dependent manner (the lower dose is equivalent to ED_{50} value) to less than 50 pg/ml, which levels are not associated with adverse clinical outcome in patients with solid tumors or standard-risk ALL leukemia.

Many studies have demonstrated that inhibition of tyrosine kinases by specific drugs can inhibit the growth of both solid tumors and leukemias [4, 5, 9, 14]. Interestingly, after inhibition of the Bcr-Abl kinase by STI571, the expression of Bcl-xL is downregulated more rapidly in chronic phase than in blast crisis CML cells, suggesting the involvement of this protein in disease progression [10, 23]. Similarly, other bcr/abl tyrosine kinase inhibitors (SU5416, AG957, NSC 680410) seem to be very active against leukemic cells with Philadelphia chromosome abnormalities [4, 24]. Thus, inhibition of tyrosine kinase moieties of various growth factor receptors is a critical step in inhibiting leukemic cell replication.

Despite the efficacy of STI571 in treating CML patients, drug resistance specific to this drug has already been noted both in vitro and in vivo [20, 22, 37, 38]. Molecular studies have demonstrated that a single point mutation in the ATP-binding site by STI571 of the kinase activation loop in the $p210^{bcr\text{-}abl}$ protein is sufficient to confer resistance to this drug. This mutation occurred at nucleotide 1127 (G $\rightarrow$ A) resulting in a substitution at codon 255 of lysine (mutant) for a glutamic acid (wild-type, or Glu255Lys amino acid substitution) in two of three of patients tested after STI571 treatment, but not in matched samples from these patients taken before beginning treatment with this drug [20, 22]. Other studies have demonstrated this Bcr-abl mutation at

[1]Avramis IA, Panosyan EH, Grigoryan RS, Sather H, Gaynon PS, Siegel SE, Holcenberg JS, Avramis VI (2003) Correlation of vascular endothelial growth factor (VEGF) serum levels and outcome in SR ALL patients (pts).: A case-control study (CG-1962). Proc Am Soc Clin Oncol 22: p.808 (Abstr. #3247) Chicago, III

amino acid 255 and also in one patient a Glu255Val amino acid change after treatment with STI571 [20].

Recently, two clinical studies have demonstrated three additional point mutations (T3151, Y253H, and F317L) in CML cells from patients treated with STI571, which abrogate the action of this drug [8, 19]. In addition to these point mutations, the serum concentrations of alpha-1 acid glycoprotein, a serum protein which binds to STI571, are increased after treatment with the drug [17, 25, 37]. Moreover, STI571 potentiates the therapeutic activity of retinoic acid in acute promyelocytic leukemia cells HL60 and U937 [18] and has synergistic or additive activity with DNR. The mechanism of the synergism between these drugs is by increasing the proapoptotic effect of each drug on CML lymphocytes [42]. Interestingly, this study also demonstrated that the significant synergistic effect the combination of DNR plus STI571 observed in CML lymphocytes was absent in normal lymphocytes [42]. Furthermore, STI571 was found to have additive or superadditive effects with 4-hydroperoxy-cyclophosphamide and vincristine, but a subadditive effect (antagonistic activity) with methotrexate in numerous Ph^+ leukemia cell lines [26].

Following these important findings, our investigations demonstrated that STI571 was synergistic with many antileukemic agents, such as IDA, Fludara and ara-C, in both the wild-type and multidrug-resistant T-lymphoblastic leukemic cell lines. There were many similarities between NSC 680410 and STI571 tyrosine kinase inhibitors in combination with cytotoxic drugs. Both tyrosine kinase inhibitors were highly synergistic with Fludara + ara-C against CEM/0 cells with the addition of IDA improving very little the three-drug synergistic regimens. However, in the drug-resistant clone, representing the clinically refractory leukemic blast population in a multirelapsed leukemia patient, the contribution of IDA was of paramount significance in obtaining high synergism in four-drug regimens. It is clear that the tyrosine kinase inhibitors must be administered first followed by Fludara in order to obtain maximum synergism, whereas treatment of the leukemic cells with the DNA-damaging drug negates the synergistic effect. Further studies must follow these observations, preferably in immunosuppressed mice, to elucidate the molecular mechanism of the drug synergism or antagonism. Hence, the data strongly suggest that there is sequence specificity between STI571 and Fludara + ara-C.

Most importantly, drug synergism was observed between the two tyrosine kinase inhibitors, STI571 and NSC 680410. The results of synergism between NSC 680410, a weak inhibitor of Bcr-abl kinase, and STI571, a specific and potent inhibitor of Bcr-abl kinase, could suggest that these ATP-mimic 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 effect. These findings provide further insights into the antileukemic potential of these drugs and should be evaluated further in animal leukemia models. Lastly, the probable benefits of including antiangiogenic agents with DNA-damaging cytotoxic drug regimens will have to be demonstrated clinically. Such a clinical study between STI571 and two cytotoxic drugs was recently approved as a clinical protocol to be tested against refractory leukemias.

In conclusion, NSC 680410 is highly synergistic with IDA, IDA + ara-C, and IDA + Fludara + ara-C over the respective cytotoxic drug regimen, whereas there was no sequence specificity between NSC 680410 and the cytotoxic drugs against human leukemia cell lines. Further, IDA was not needed for optimal synergistic activity in the CEM/0 cell line. However, IDA was a necessary component for drug synergism in the drug-resistant clone, probably due to overcoming ara-C resistance. NSC 680410 is moderately synergistic with gamma radiation. Finally, the combinations of antiangiogenesis and DNA-damaging cytotoxic drugs are highly synergistic regimens in both wild-type and drug-resistant leukemia cell lines and should be examined further.

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