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In Vitro Effects of STI 571-Containing Drug Combinations on the Growth of Philadelphia-Positive Chronic Myelogenous Leukemia Cells

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BACKGROUND. Chronic myelogenous leukemia (CML) is characterized by a molecular aberration, a fusion *BCR-ABL* gene encoding for aberrant tyrosine kinase activity, which is crucial in the pathogenesis of CML. In vitro, inhibition of BCR-ABL protein tyrosine kinase activity by a tyrosine kinase inhibitor, Imatinib mesylate (STI571; formerly CGP57148B), successfully suppressed proliferation/survival of the BCR-ABL positive clones. In clinical studies, hematologic and cytogenetic remissions have been achieved in most patients with chronic phase CML; in accelerated and blastic phases of CML, STI571 appeared less effective. In the current study, the authors tested combinations of STI571 and cytarabine and homoharringtonine (HHT), drugs with documented activity in CML.

METHODS. The single agents and their combinations were studied for in vitro effect on proliferation of BCR-ABL positive cell lines KBM5 and KBM7 by 3(4,5-dimethylthiazol-2yl)-2,5 diphenyl-tetrazolium bromide assay and on primary patient-derived BCR-ABL cells by clonogenic assays. The in vitro additive, synergistic, or antagonistic effects of cytarabine and HHT with STI571 were then investigated by computer-assisted analysis using the CalcuSyn software.

RESULTS. STI571 consistently suppressed BCR-ABL positive cell proliferation with a dose-effect correlation. In the model system used, STI571/cytarabine and STI571/HHT combinations were more effective in inhibiting KBM5 and KBM7 cell growth than each drug as single agent. These results were also verified in primary CML-derived clonogenic cells in semisolid cultures.

CONCLUSIONS. In this experimental system, our studies documented additive or synergistic effects with STI571 plus cytarabine or HHT, supporting the future use of STI571 combinations in clinical trials in patients with Philadelphia chromosome-positive leukemias. ***Cancer* 2002;94:2653–62.**

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KEYWORDS: chronic myelogenous leukemia, tyrosine kinase inhibitor, STI571, Gleevec, cytarabine, homoharringtonine.

Chronic myeloid leukemia (CML) is characterized by a specific cytogenetic abnormality, the Philadelphia chromosome (Ph), which describes a reciprocal translocation between chromosomes 9 and 22.[1] This alteration is also present in 10–25% of cases of adult acute lymphoblastic leukemia[2] and in less than 2% of cases of acute myelogenous leukemia[3] and is associated with poor prognosis. The translocation generates the fusion gene *BCR-ABL*, which encodes a protein that has altered tyrosine kinase activity.[4] A specific pathogenic role of this abnormal Bcl-Abl tyrosine kinase in CML has been shown in vitro and in vivo.[5]

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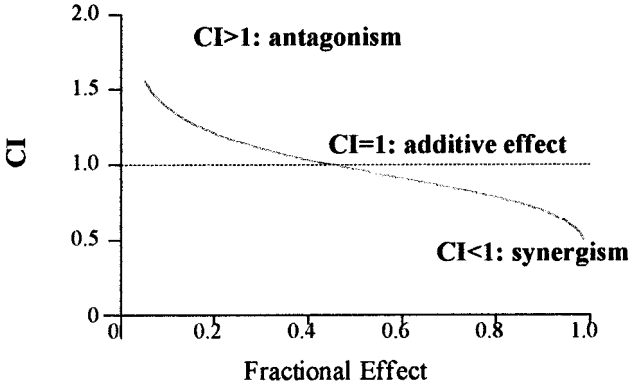


FIGURE 1. Schematic representation of the multiple drug combined effect as plotted by CalcuSyn software. CI: combination index.

STI571 (Gleevec; CGP 57148B) is a phenylalaminopyrimidine derivative which selectively inhibits the tyrosine-kinase activity of c-abl,[6] *BCR-ABL*,[7,8] platelet-derived growth factor receptor,[9] and c-kit.[10] In vitro, STI571 blocked proliferation and induced apoptosis of BCR-ABL positive cell lines[11,12] and leukemic cells isolated from patients with CML.[13] STI571 is currently under evaluation in clinical trials for the treatment of CML and has produced lasting hematologic and cytogenetic remissions in chronic phase CML.[14] However, STI571 appears less effective in the accelerated or blastic phases of CML, inducing only temporary disease control.[15,16]

The efficacy of STI571 might be improved by combining it with other agents that have documented activity in CML. Two such drugs are cytarabine and homoharringtonine (HHT). Cytarabine in vitro selectively suppressed the growth of colony forming unit–granulocyte macrophage (CFU-GM) of marrow cells from patients with CML.[17] This drug is currently used in low doses for the treatment of CML, either as a single agent or in combination with interferon (IFN)-α.[18–21] Recent observations from BCR-ABL positive leukemia models indicated that the antineoplastic activity of cytarabine could be potentiated by combining it with STI571.[22]

Studies in China showed that HHT, a cephalotaxine alkaloid, was effective in leukemias.[23–26] In vitro, HHT inhibited cell proliferation and induced apoptosis[27] of Ph-positive CML cells. In clinical trials, HHT given as a single agent produced hematologic and cytogenetic responses in patients with chronic phase CML.[28]

A combination regimen of HHT and low-dose cytarabine was also effective and safe in patients with CML in whom treatment with interferon had failed.[29]

In the current study, we evaluated whether the combination of STI571 with cytarabine or HHT could enhance the ability of STI571 to inhibit the growth of CML-derived cells in vitro. In two BCR-ABL positive cell lines, we found a synergistic effect between STI571 and cytarabine or HHT in the low dose range, suppressing 90% of cells. These results were verified in primary CML-derived cells in semisolid cultures. Our data confirm the synergism of STI571 and cytarabine, suggest a synergism with HHT in vitro, and support the future use of these drugs in combinations in clinical trials in BCR-ABL–positive leukemias.

MATERIALS AND METHODS

Drugs

STI571 was obtained from Ciba-Geigy, now Novartis (Basel, Switzerland), and stored as a powder and as a 10 mM stock solution in dimethylsulfoxide at −20 °C. Cytarabine was initially dissolved in phosphate-buffered saline (PBS). Homoharringtonine was obtained from Oncopharm (Houston, TX) and dissolved in normal saline. The stability of stored drug solutions was investigated prior to initiation of experiments. Fresh working solutions were prepared in PBS before each experiment.

Cell Lines

The KBM5 and KBM7 cell lines were derived from patients with CML in myeloid blast phase seen at the M. D. Anderson Cancer Center. The KBM5 cell line is positive for Ph chromosome and $p210^{bcr\text{-}abl}$ and does not express normal c-abl;[30,31] the KBM7 cell line is a near-haploid and is Ph-positive and $p210^{bcr\text{-}abl}$ positive.[32] Both cell lines were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS).

Patient Samples

Peripheral blood samples were obtained from five CML chronic phase patients. All patients signed informed consent forms according to the institutional guidelines. Mononuclear cells were collected after Ficoll-Paque (Amersham, Arlington Heights, IL) and resuspended in IMDM.

Short-term Cell Cultures

Triplicate cultures of KBM5 and KBM7 cells were plated in 96-well plates at concentrations of 4×10^5 cells/mL. Drugs were added singly or in combinations at increasing concentrations. When drugs were studied in combinations, different doses and ratios were employed to determinate the most appropriate dose-response curves and to identify drug interactions under various experimental conditions. After 72 hours of exposure, cell growth was evaluated by the 3(4,5-dimethylthiazol-2yl)-2,5 diphenyl-tetrazolium bromide (MTT) (Sigma, St. Louis, MO) colorimetric dye reduc-

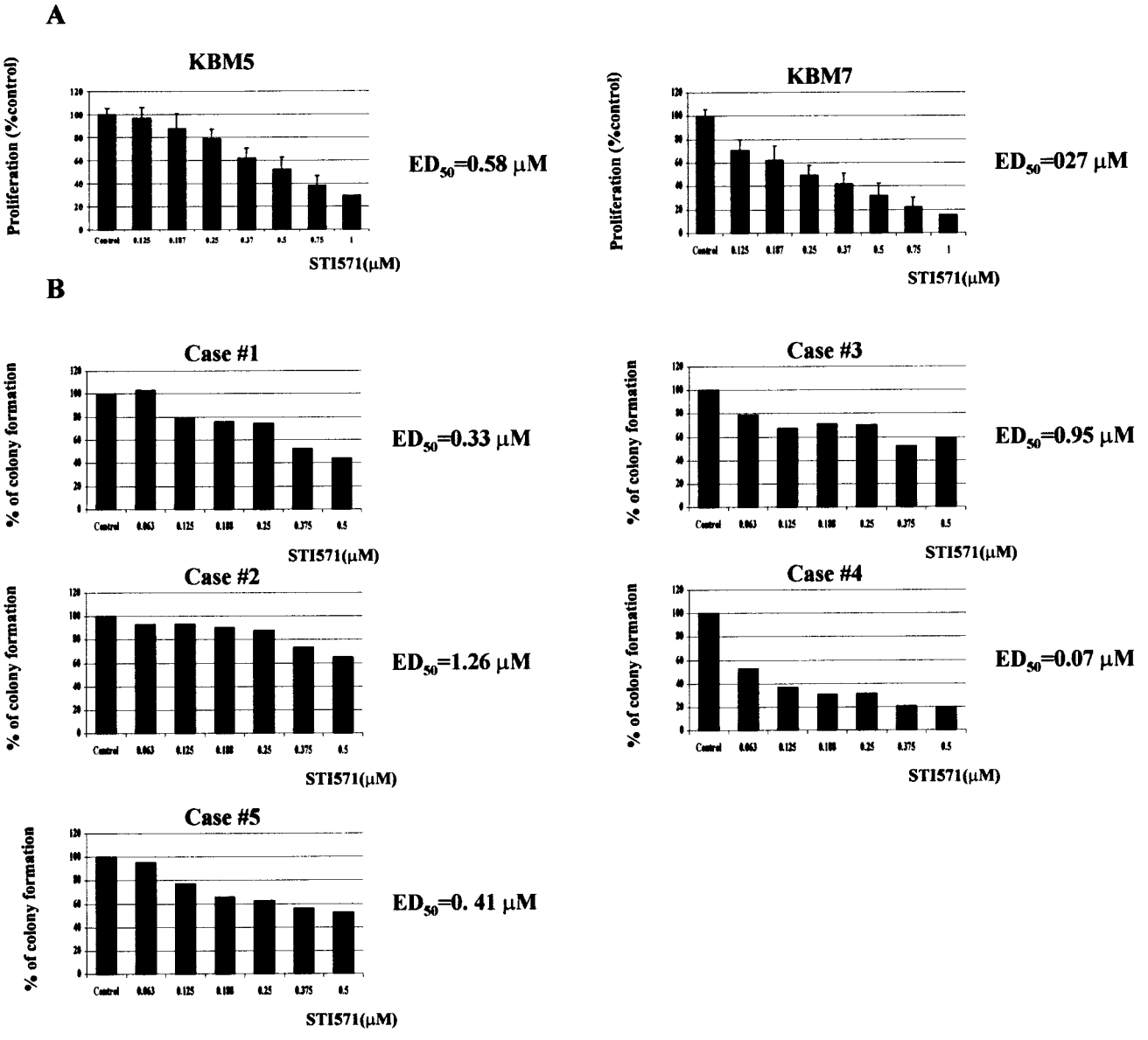


FIGURE 2. STI571 inhibition of proliferation of A) Philadelphia chromosome-positive KBM5 and KBM7 leukemic cell lines and B) colony formation by mononuclear white blood cells (MWBC) derived from five patients with untreated chronic phase of chronic myelogenous leukemia. KBM5 and KBM7 cells were plated at 4×10^5 cells/well in Iscove's modified Dulbecco's medium (IMDM) with 10% fetal calf serum (FCS). STI571 was added at the concentrations shown. Proliferation at 72 hours was measured by 3(4,5-dimethylthiazol-2yl)-2,5 diphenyl-tetrazolium bromide assay. Results are expressed as the percentage of maximal proliferation (control without STI571). In colony formation assays MWBC (1×10^5 cells/mL/dish, duplicate cultures) were plated in IMDM containing 0.8% methylcellulose, 30% FCS, and 5% phytohemoagglutinine-stimulated lymphocyte-conditioned medium, and STI571 was added at the concentrations shown. Colony forming unit–granulocyte macrophage (aggregates of more than 50 cells) were counted after 8 days of culture. Growth inhibition was evaluated as a percentage of colony growth in the control (no-drug) sample. The ED_{50} for each case is shown at the right of the corresponding graph.

tion method. Inhibition of proliferation was evaluated as a percentage of proliferation of cells in the control condition (no drugs added).

Clonogenic Assays

Mononuclear cells were cultured for colony-forming assays as described elsewhere.[33] Briefly, cells were plated in 35×10 mm dishes at 1×10^5 cells/mL in IMDM containing 0.8% methylcellulose, 30% FCS (Stem Cell, Vancouver, CA), and 5% phytohemoagglutinine-stimulated lymphocyte-conditioned medium (PHA-LCM; Stem Cell, Vancouver, CA). Drugs were added singly or in combinations. Numbers of colony-forming units (aggregates composed of more than 50

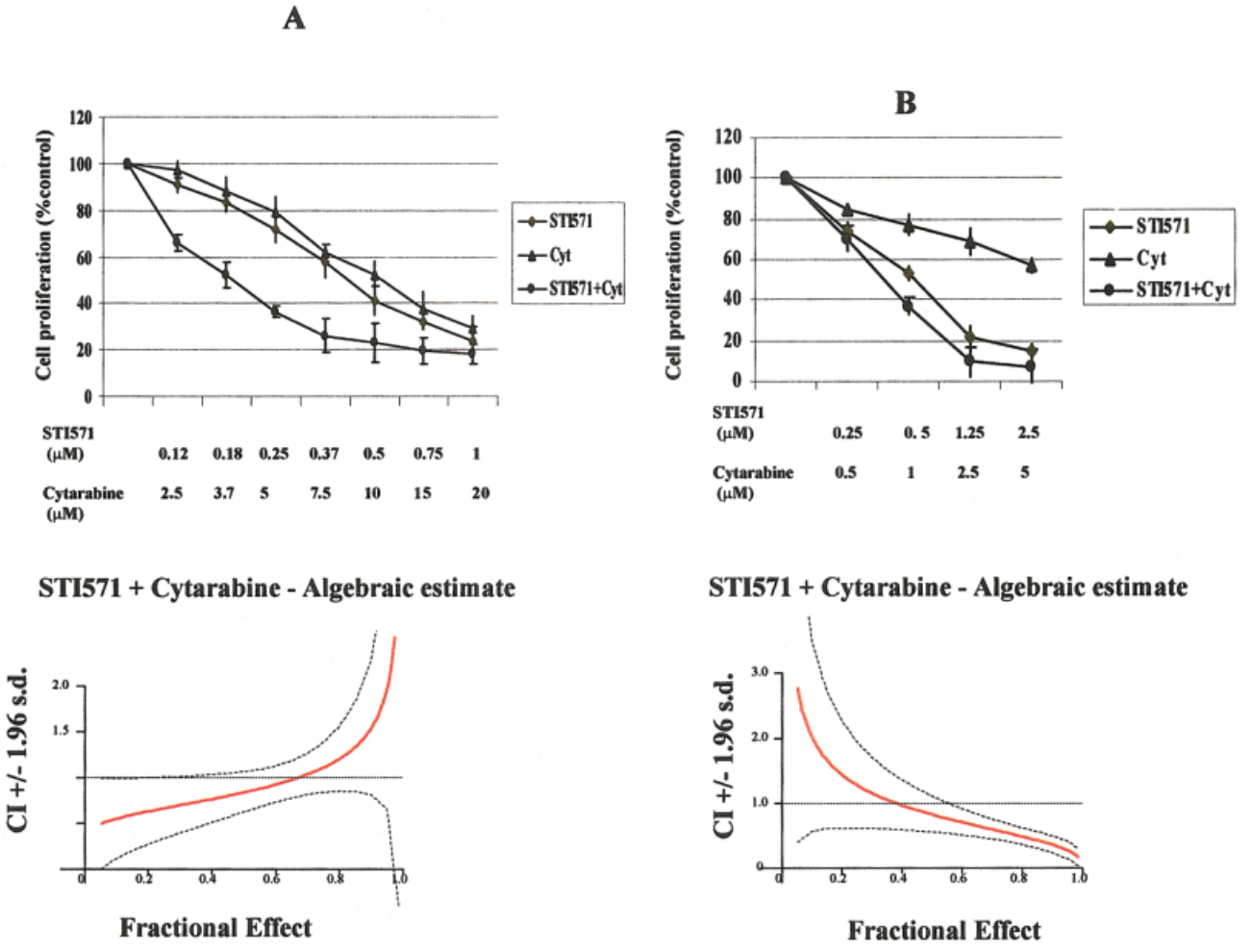


FIGURE 3. Effect of combinations of STI571 and cytarabine in KBM5 cells. A) Effect of combination STI571-cytarabine at a ratio of 1:20. B) Effect of combination STI571-cytarabine at a ratio of 1:2. Proliferation at 72 hours was measured by 3(4,5-dimethylthiazol-2yl)-2,5 diphenyl-tetrazolium bromide assay. Individual values for each condition are mean of triplicate cultures. Upper figures: results are expressed as percentage of maximal proliferation (control without STI571). Lower figures: plot of the combination index (CI) versus cytotoxicity calculated by CalcuSyn software.

cells) were counted after 8 days of culture at 37 °C in a fully humidified atmosphere of 5% CO_2 in air. All clonogenic assays were done in duplicate. Growth inhibition was evaluated as a percentage of growth of control colonies (no drugs added).

Drug Effects

The activity of the drugs, used singly or in combination, was estimated with the CalcuSyn software program (Biosoft, Ferguson, MO). Briefly, this program calculates ED_{50}, does isobologram analyses, and determines the combination index (CI), a quantitative measure of the degree of drug interactions. For each given endpoint, a CI of 1 indicates an additive effect, a CI of less than 1 a synergistic effect, and a CI of more than 1 an antagonistic effect (Fig. 1).[34] Calculations of the CI were made under the assumption that the mechanisms of action of the three drugs (STI571, cytarabine, and HHT) were not mutually exclusive.

RESULTS

STI571

STI571, given as a single agent, inhibited the growth of both KBM5 and KBM7 cells in a dose-dependent manner (Fig. 2A). The cell lines differed in their natural sensitivity to STI571: the ED_{50} for KBM5 cells was 0.55 μM, while the ED_{50} of the more sensitive KBM7 cell line was 0.27 μM.

Colony formation at Day 8, expressed as a percentage of the colony number in untreated cultures, was reduced in the presence of STI571 in all five sam-

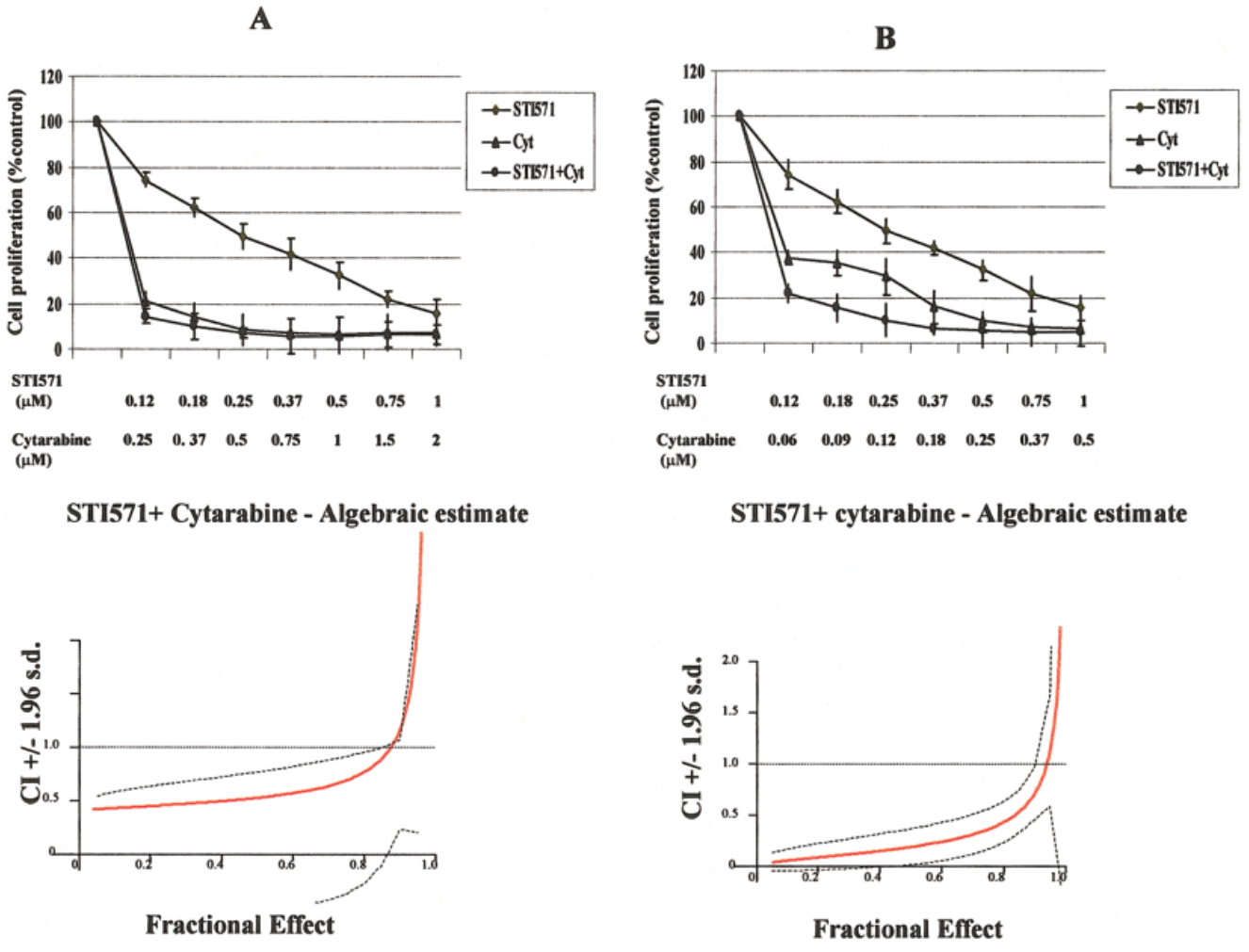


FIGURE 4. Effect of combinations of STI571 and cytarabine in KBM7 cells. A) Effect of combination STI571-cytarabine at a ratio of 1:2. B) Effect of combination STI571-cytarabine at a ratio of 1:0.5. Proliferation at 72 hours was measured by 3(4,5-dimethylthiazol-2yl)-2,5 diphenyl-tetrazolium bromide assay. Individual values for each condition are mean of triplicate cultures. Upper figures: results are expressed as percentage of maximal proliferation (control without STI571). Lower figures: plot of the combination index (CI) versus cytotoxicity calculated by CalcuSyn software.

ples from patients in chronic-phase CML (Fig. 2B); the ED_{50} ranged from 0.07 μM (Case 4) to 1.26 μM (Case 2).

STI571 Plus Cytarabine

Initial experiments involved testing different concentrations of cytarabine with 0.125 1 μM doses of STI571 to determine the appropriate dose response curves. Further experiments assessed whether STI571 plus cytarabine combinations in various proportions produced synergistic, additive, or antagonistic effects. Results in KBM5 cells are shown in Fig. 3: using STI571-cytarabine combination at ratios of 1:20 (Fig. 3A) and 1:2 (Fig. 3B), we found a synergistic effect, more consistent in the latter proportion, and within a dose range that affected more than 90% of the cells.

KBM7 cells were much more sensitive to cytarabine than were KBM5 cells; 79% of the KBM7 cells were affected at a cytarabine concentration of 0.25 μM when we tested the combination at a 1:2 ratio (Fig. 4A). A strong synergism was found between these drugs either at this dose ratio or at a dose ratio of 1:0.5 of STI571:cytarabine (Fig. 4B), and within a dose-range that affected 90% of the cells.

Mononuclear cells from patients with chronic-phase CML were considerably more sensitive to both STI571 and cytarabine. Hence, we tested these drugs at lower concentrations in all five cases, at a ratio of 1:100. In four of the five specimens, the two drugs were essentially additive, with a mild antagonistic effect found at the lowest concentrations in Case 4 (data not shown). Figure 5 shows results in Cases 1 and 5. Only in Case 5 were they strongly synergistic.

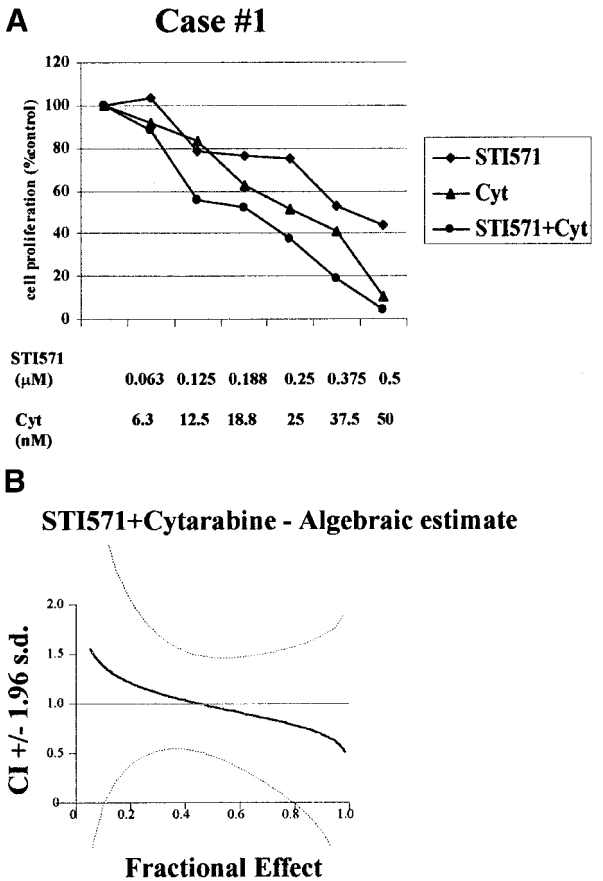


FIGURE 5.

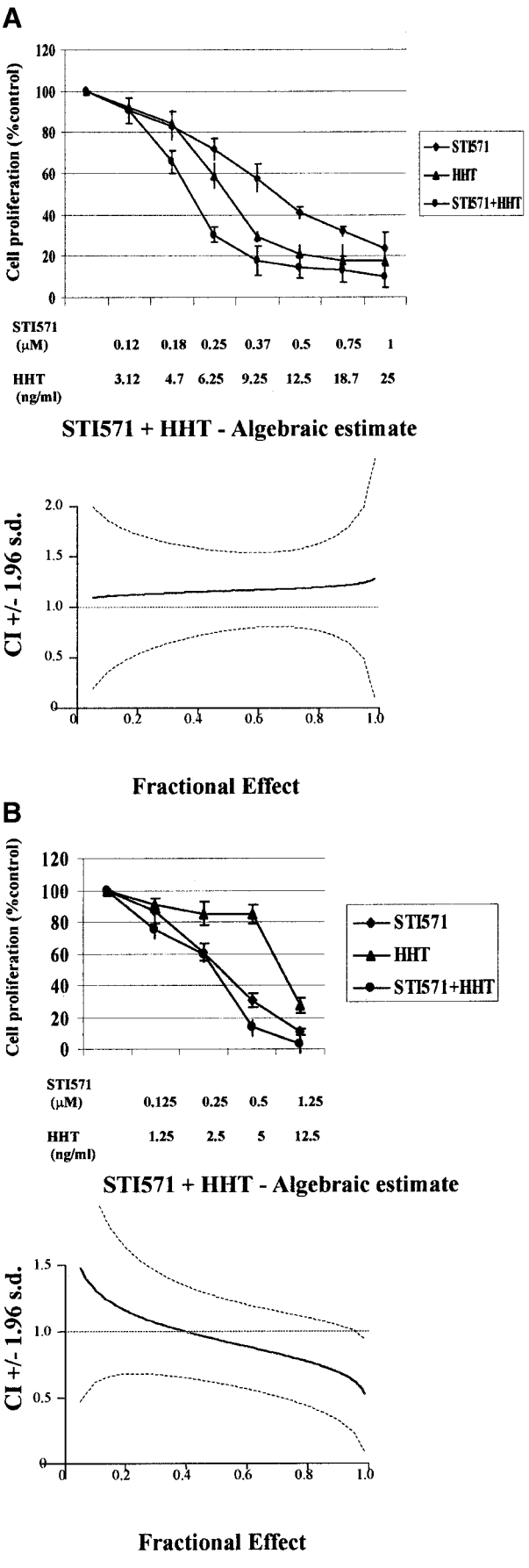


FIGURE 6.

STI 571 Plus HHT

In the KBM5 cell line, STI571 and HHT were additive at a ratio of 1:25 (Fig. 6A) and synergistic at a ratio of 1:10 (Fig. 6B). KBM7 cells were more sensitive to HHT than were KBM5 cells. STI571: HHT ratio of 1:25 (Fig. 7A) but not 1:10 (Fig. 7B) indicated synergistic effects. Thus, in both cell lines, STI571 and HHT produced additive or synergistic effects in a dose range that affected 80–90% of cells.

Among the patient samples, STI571 plus HHT at a ratio of 1:10, in the same concentration range of STI571 as that tested for STI571 plus cytarabine showed again a large inter-patient variability, although a mostly additive effect was observed in all cases. Figure 8 illustrates results of clonogenic assays in Cases 1 and 5.

DISCUSSION

The prognosis of patients with CML has improved in recent years with the use of IFN-α, alone or with cytarabine, and allogeneic transplantation.[19–21,35–37] However, even with interferon-based regimens, the CML usually evolves into the accelerated and blastic phases,[38] and bone marrow transplantation is limited to a minority of younger patients.[36,37] Inhibition of the Bcr-Abl tyrosine kinase with specific compounds, such as STI571, represents a new and unique strategy for treating CML. Results of clinical trials of STI571 in chronic phase CML have been encouraging. In accelerated or blastic phases of CML, the effects of STI571 are moderate and transient, and remissions are short.[14–16] Resistance to STI571 therapy has also been observed also in patients with chronic phase disease and may become more frequent with longer follow–ups of STI571-treated patients.

One possible strategy to improve the results is to combine STI571 with other agents with different mechanisms of action and proven efficacy against CML. The current study investigated the effects of STI571 plus cytarabine or HHT on the growth of CML cells. We hoped to provide experimental evidence to support such clinical trials of STI571 combinations, first by excluding the possibility of antagonism, and second by documenting additive or synergistic interactions between these agents.

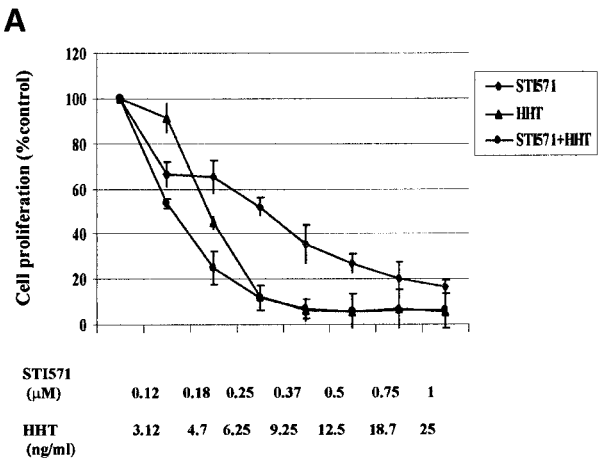
We found that STI571 inhibited cell growth in the BCR-ABL-positive KBM5 and KBM7 cell lines in a dose-dependent manner. KBM7 cells were more sensitive, probably because of their near-haploid karyotype. The combination of STI571 with cytarabine was more effective in reducing proliferation of both these cell lines than was either drug used as a single agent. These experiments confirmed a recent report of the favorable interaction between STI and cytarabine.[22] We also found that combinations of STI571 and HHT produced additive/synergistic antiproliferative effects in both cell lines. The synergistic effects of STI571 with cytarabine and HHT were observed at low doses in ranges that affected 80–90% of the cells.

As expected, the results of studies on specimens collected from patients with chronic phase CML were variable. Drugs were tested using mononuclear blood cells collected after Ficoll separation. Such populations presumably contained STI571-sensitive Ph-positive clonogenic cells but also STI571-insensitive Ph negative hematopoietic progenitors. Thus, a higher survival of clonogenic cells derived from some patients could reflect an increased frequency of circulating normal progenitors rather than a lower sensitivity to STI571. Polymerase chain reaction-assisted analysis of STI571-surviving clones would be necessary to identify whether they are BCR-ABL positive or normal. Using patient-derived cells and a clonogenic experimental system, we confirmed in part data observed in cell lines. Indeed, circulating progenitor cells from all five patients were sensitive to STI571, and the combination of STI571 with cytarabine or HHT had at least an additive effect. Differences in phenotype, biologic characteristics, or disease behavior commonly observed in chronic-phase CML, as well as in the experimental procedures (MTT assay versus clonogenic assay), might account for the variability in results.

We conclude that inhibition of CML cell growth can be improved by using STI571 in combination with cytarabine or HHT. These findings, together with a lack of evidence of antagonistic actions, lend support

FIGURE 5. Effect of STI571 and cytarabine on colony formation by mononuclear white blood cells from Cases 1 and 5. Cells were plated in 1 mL Iscove's modified Dulbecco's medium containing 0.8% methylcellulose, 30% fetal calf serum, and 5% phytohemoagglutinine-stimulated lymphocyte-conditioned medium . STI571 and cytarabine were added singly or in combination at a ratio of 1:100. Colony forming unit–granulocyte macrophage (aggregates composed of more than 50 cells) were counted after 8 days of culture. A) Inhibition of growth as a percentage of colony growth in the control (no drug) sample. B) Plot of the combination index (CI) versus cytotoxicity calculated from the data in A by CalcuSyn software.

FIGURE 6. Effect of combinations of STI571 and homoharringtonine (HHT) in KBM5 cells. A) Effect of combination STI571-HHT at a ratio of 1:25. B) Effect of combination STI571-HHT at a ratio of 1:10. Proliferation at 72 hours was measured by 3(4,5-dimethylthiazol-2yl)-2,5 diphenyl-tetrazolium bromide assay. Individual values for each condition are means of triplicate cultures. Upper figures: results are expressed as percentage of maximal proliferation (control without STI571). Lower figures: plot of the combination index (CI) versus cytotoxicity calculated by CalcuSyn software.



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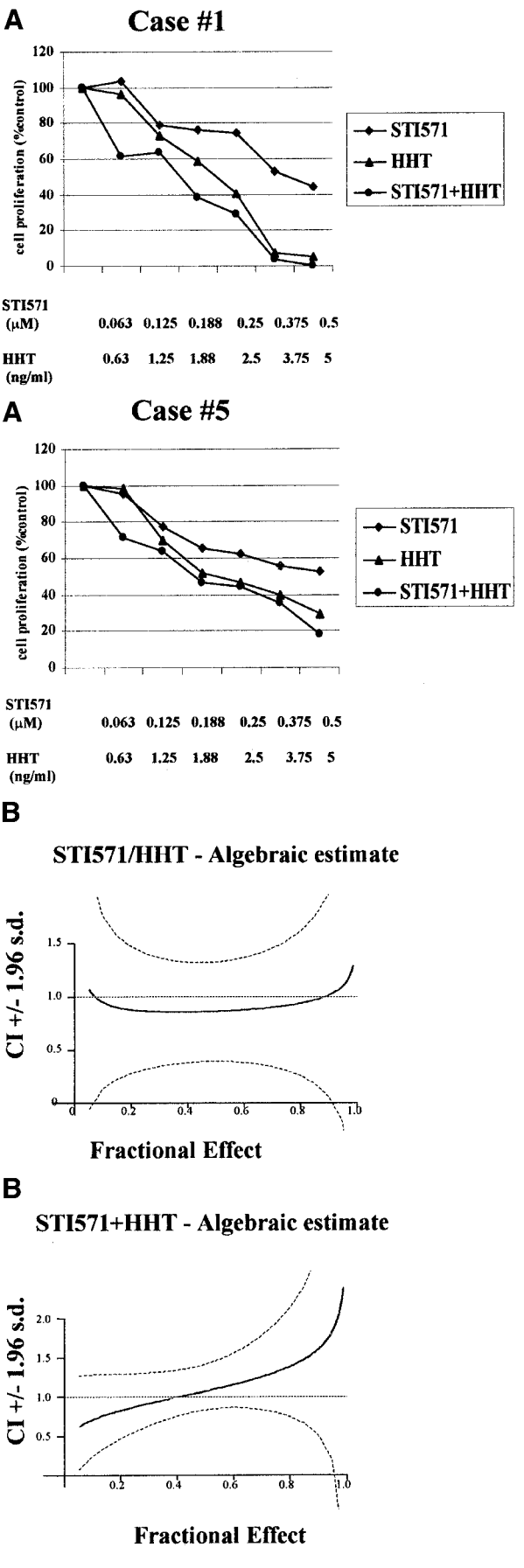

FIGURE 8.

FIGURE 7. Effect of combinations of STI571 and homoharringtonine (HHT) in KBM7 cells. A) Effect of combination STI571-HHT at a ratio of 1:25. B) Effect of combination STI571-HHT at a ratio of 1:10. Proliferation at 72 hours was measured by 3(4,5-dimethylthiazol-2yl)-2,5 diphenyl-tetrazolium bromide assay. Individual values for each condition are means of triplicate cultures. Upper figures: results are expressed as percentage of maximal proliferation (control without STI571). Lower figures: plot of the combination index (CI) versus cytotoxicity calculated by CalcuSyn software.

FIGURE 8. Effects of STI571 and homoharringtonine (HHT) on colony formation by mononuclear white blood cells from Cases 1 and 5. Cells were plated in 1 mL Iscove's modified Dulbecco's medium containing 0.8% methylcellulose, 30% fetal calf serum, and 5% phytohemoagglutinine-stimulated lymphocyte-conditioned medium. STI571 and HHT were added, singly or in combination, at a ratio of 1:10 as shown. Colony forming unit–granulocyte macrophage (aggregates of more than 50 cells) were counted after 8 days of culture. A) Inhibition of growth as a percentage of colony growth in the control (no drug) sample. B) Plot of the combination index (CI) versus cytotoxicity calculated from the data in A by CalcuSyn software.

to evaluations of these combinations in clinical trials, especially for the treatment of STI571–resistant disease.

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