Selective Inhibition of Cell Proliferation and BCR-ABL Phosphorylation in Acute Lymphoblastic Leukemia Cells Expressing Mr 190,000 BCR-ABL Protein by a Tyrosine Kinase Inhibitor (CGP-57148)

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ABSTRACT

The excessive proliferation of the myeloid marrow compartment in Philadelphia chromosome (Ph)-positive acute and chronic leukemias has been largely attributed to a hyperactive and autonomously acting hybrid tyrosine kinase BCR-ABL, a product of the fusion between the second exon of the c-ABL proto-oncogene and 5’ portions of the BCR gene on chromosome 22. This specific molecular event, amenable to attack with specifically designed inhibitors, has recently been successfully influenced by the drug CGP-57148 in mammalian cells transfected with full-length BCR-ABL gene and expressing full-length p210BCR-ABL protein, as well as in primary human leukemic cells expressing p210BCR-ABL fusion protein. In view of the heterogeneity of BCR-ABL transcripts associated with various phenotypes, we investigated the effect of CGP-57148 on p190BCR-ABL and p210BCR-ABL-expressing, patient-derived cell lines and primary intact blast cells. In particular, we were interested in whether the variations in molecular events and/or the phenotype of Ph-positive cells would affect their susceptibility to the specific tyrosine kinase inhibitor CGP-57148. We have demonstrated that the sensitivity of human cells with lymphoblastic immunophenotype expressing p190BCR-ABL protein is comparable to that for leukemic myeloid cells expressing p210BCR-ABL protein. After documenting profound and phenotype-independent suppression of both autophosphorylation and cell growth, we explored the importance of time and dose of exposure on the manifestation and stability of the induced events. Although there were variations between target cells, in vitro exposure for 24–48 h induced extensive and apparently irreversible apoptosis in BCR-ABL-expressing but not other normal or BCR-ABL-negative leukemic cells. These findings support the potential use of CGP-57148 to purge Ph-positive cells from autologous bone marrow in vitro. Another important finding was the comparable suppressive effect of temporary CGP-57148 exposure on both clonogenic KBM-5 cells and the whole cell population. Exposure time and dose appeared to be important variables among various cell types. Moreover, effective doses appeared uniformly harmless to cells lacking BCR-ABL protein functioning as tyrosine kinase. Thus, the continuous exposure of target cells, at least during the initial period of 24–48 h, may prove to be an important variable in the design of in vitro and in vivo therapy using tyrosine kinase inhibitors.

INTRODUCTION

The cytogenetic abnormality termed Ph,2 a product of a reciprocal translocation between chromosomes 9 and 22, results in the fusion BCR-ABL gene (1, 2). This gene encodes a fusion protein with tyrosine kinase activity (p210BCR-ABL or p190BCR-ABL), which plays a pathogenetic role in CML (5–7). The t(9;22) translocation is also found in 10–20% cases of adult ALL (8–10) and in rare cases of AML (11). It has been speculated that the location of the breakpoint in BCR, which determines the composition of the fusion BCR-ABL protein, may also affect the disease phenotype (12, 13).

The development of novel therapies directed toward molecular mechanisms of malignant disease depends on the detailed knowledge of such mechanisms. Recently, a series of 2-phenylaminopyrimidine derivatives capable of inhibiting protein tyrosine kinases with varying degrees of specificity have been synthesized (14–16). One compound, CGP-57148, selectively inhibits the c-ABL tyrosine kinase (15, 17) and was recently shown to specifically inhibit proliferation of p210BCR-ABL-expressing cells with little or no effect on cells lacking p210BCR-ABL (15, 17). This was correlated with the potent inhibition of isolated ABL kinase in vitro, as well as v-ABL kinase in intact tissue culture cells (17). Moreover, CGP-57148 was shown to specifically inhibit BCR-ABL-associated prolif-

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2 The abbreviations used are: Ph, Philadelphia chromosome; CML, chronic myelogenous leukemia; ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; TKI, tyrosine kinase inhibitor; IMDM, Iscove’s modified Dulbecco’s medium; RT, room temperature; CFC, colony-forming cell; GM-CFC, granulocyte-macrophage CFC.
eration, not only in BCR-ABL-transfected cell lines (14, 16) but also in primary cells derived from patients with chronic-phase CML (17).

The diversity of BCR-ABL fusion proteins is well known. However, the enzymatic activity of the protein product of this fusion gene and the effects of its inhibition in leukemias with a lymphoblastic phenotype is not. We, therefore, sought to investigate whether CGP-57148 would be equally effective in modulating the p190Bcr-Abl tyrosine kinase activity and proliferation of p190Bcr-Abl-expressing cells of the lymphoid phenotype as it is at inhibiting CML cells expressing p210Bcr-Abl.

As a result, we found that the TK1 CGP-57148 inhibited proliferation of Ph-positive ALL cell lines, as well as primary fresh Ph-positive ALL blast cells containing p190Bcr-Abl fusion protein, but not cell lines and cells derived from ALL patients lacking this protein. We also found the inhibition of cell growth to be associated with decreased phosphorylation of p190Bcr-Abl protein. Finally, we showed that the dose-response relationship documented an inhibition comparable in magnitude to that seen in myeloid leukemia cell lines.

Donated the extremely poor outcome of adult patients with Ph-positive ALL (9), our data on TKIs may provide a basis for an alternative therapeutic strategy in vivo in this disease and for use of such inhibitors in vitro to purge cells in the setting of autologous bone marrow transplantation. For this purpose, we sought to define activity and the optimal dose and exposure time needed to achieve the effect of the drug on the target cells.

MATERIALS AND METHODS

Leukemia Cell Lines

Myeloid Leukemia Cell Lines. With the exception of K-562 cells (American Type Culture Collection, Rockville, MD) and M3.16 and Mo7e cells (provided by R. B. A.; Ref. 18), all cell lines were established in our laboratory. The KBM-3 cell line was originally established from the peripheral blood of a patient with relapsed acute myelomonocytic leukemia; these cells have been described previously (19). The KBM-5 cell line was derived from a patient with CML in myeloid blast crisis. This line contains Ph, expresses p210Bcr-Abl (20) and is remarkable for the absence of normal c-ABL (21). KBM-7, a near-haploid cell line, was derived from a patient with CML in myeloid blast crisis; this line contains the Ph chromosome and expresses p210Bcr-Abl protein (22).

Lymphoblastic Leukemia Cell Lines. Lymphoblastic cell lines were established in the laboratory of Z. E. (23, 24). Three cell lines designated Z-119, Z-181, and Z-33 were derived from three Ph-positive ALL patients; they all retained typical B-cell characteristics and phenotypes of the original tumors. Karyotypic analysis revealed t(9;22), and kinase assay detected 190Bcr-Abl in all three lines. Z-138, a Ph-negative cell line, was derived from a patient with chronic lymphocytic leukemia and supervening ALL.

Primary Leukemic Cells. Primary leukemic cells were obtained from the peripheral blood of two patients with Ph-positive ALL, expressing 190Bcr-Abl. The first patient had not been treated, whereas the second patient was in a chemotherapy-refractory terminal stage of the disease. Leukemic cells were also isolated from the blood of two other patients with relapsed, therapy-resistant AML using Ficoll-Hypaque gradient (1.077 g/cm³).

Cell Cultures. The primary leukemic cells and myeloid leukemia cell lines were cultured in IMDM supplemented with 15% FCS. The lymphoblastic leukemia cell lines were cultured in RPMI 1640 with 10% FCS.

Drugs. The 2-phenylaminopyrimidine derivative CGP-57148 was synthesized by CIBA-Geigy Pharmaceuticals Division. Its structure has been published (17). A 10 mM CGP-57148 stock concentration in MeSO was stored at −20°C until dilution in medium on the day of experiments.

Monoclonal Antibodies. Mouse monoclonal antibodies P6D and 8E9 directed against two different regions of the ABL gene were prepared in the laboratory of R. B. A. and have been described previously (25, 26). Monoclonal antiphosphotyrosine antibody PY20 coupled to horseradish peroxidase was obtained from ICN Radiochemicals (Irvine, CA).

Proliferation Assays. Cells were plated in quadruplicate into 96-well plates at 5 × 10⁴/ml/well in their respective media, supplemented with 15% FCS without growth factors. BCR-ABL TK1 CGP-57148 was then added in various concentrations. After 72–96 h of exposure, the viable cells in each well were assayed for their ability to transform 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide into purple formazan as described previously (27). Alternatively, viable cells were counted in a counting chamber after staining with trypan blue.

Cytotoxicity Assay in Primary Cells. Mononuclear, light-density cells (≥ 90% blasts), separated from the blood of patients with acute leukemia as described above, were plated in triplicate into 24-well plates at 3 × 10⁴/ml/well and then exposed to different concentrations of the CGP-57148 for 24, 48, and 72 h. At each time point, cells were monitored for viability using trypan blue staining and counted in a counting chamber. The number of viable cells per ml was then calculated.

Tyrosine Kinase Inhibition in Intact Cells. Exponentially growing cells (1 × 10⁶/ml for cell lines and 1.3 × 10⁷/ml for patient-derived leukemic samples) were incubated in IMDM supplemented with 15% FCS (except for Z-119 cells, which were grown in RPMI) for various periods of time (90 min to 3 h). This was done in the presence of various concentrations of CGP-57148 (final concentration, 2.5, 5.0, or 10 μM). Concentrations were selected based on the results of the cell survival experiments in vitro. At the end of the incubation, the cells were washed twice in 10 ml of ice-cold PBS and split into halves for further studies: one half for BCR-ABL Western blotting, and the other half for immunoprecipitation and kinase assay.

Western Blot Analysis. Cells (5 × 10⁶ for cell lines or 2 × 10⁷ for primary leukemic cells) were suspended in 60 μl of hot 2X Laemmli buffer and then boiled in a water bath for 7 min. After spinning at 40,000 rpm for 30 min, the supernatant was collected and loaded onto a 6.5% acrylamide gel (29:1) for SDS-PAGE. Each gel was then electroblotted onto an Immobilon (Millipore) membrane (800 mA for 3 h in transfer buffer.

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48 mM Tris, 39 mM glycine, and 0.001% SDS (pH 9.2). Each membrane was incubated in washing buffer containing primary antibody at RT for 120 min. After washing, the membrane was then incubated in washing buffer containing 0.01% of second antibody (antimouse immunoglobulin, hors eradish peroxidase-linked whole antibody; Amersham) at RT for 120 min. After washing, the chemoluminescence of the blots was enhanced using an ECL kit (A mersham).

**Immunoprecipitation.** Cells (5 × 10⁶ for cell lines and 2 × 10⁷ for primary leukemic cells) were lysed in 0.1 ml of lysing buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 100 mM NaCl, 10 mM phosphate buffer, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 100 kallikrein inhibitor units/ml aprotinin) on ice for 30 min. Then, after spinning the lysate in a microfuge at 14,000 rpm for 40 min at 4°C, the supernatant was removed and mixed with 30 µl of P6D antibody, incubated for 120 min at 4°C, and then spun at 14,000 rpm for 1 min. The protein complex in the sediment was then washed three times: once with 0.5 ml of RIPA buffer [0.1% Triton X-100, 5.0 mM EDTA, 100 mM NaCl, and 100 kallikrein inhibitor units of aprotinin in 10 mM sodium phosphate buffer (pH 7.2)], once with washing buffer [0.1% Triton X-100, 0.05% SDS, 0.5% deoxycholate, and 100 mM NaCl in 10 ml of sodium phosphate buffer (pH 7.2)], and, finally, once with 50 mM Tris-HCl buffer (pH 7.5). The material was then subjected to kinase assay.

**Kinase Assay.** Each immunoprecipitate was mixed with 40 µl of kinase buffer [20 mM HEPES (pH 7.2), 100 mM NaCl, 0.1% Triton X-100, 10 mM MnCl₂, and 10 µCi of [γ-32P]ATP] and incubated on ice for 10 min (26). Then, after washing three times with 0.5 ml of 50 mM Tris buffer (pH 7.5), each immunoprecipitate was mixed with 50 µl of sample buffer [125 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.02% bromphenol blue, and 10% β-mercaptoethanol] and boiled for 10 min. Supernatant obtained after spinning at 14,000 rpm for 10 min at RT was then loaded on a 6.5% acrylamide gel (29:1) and subjected to SDS-PAGE (26). The resulting blots were transferred to Immobilon P membranes under the conditions described above. The membranes were dried in air and exposed to X-ray film (Kodak) at RT for 60 min. In some experiments, the membranes were also scanned using Image Quanta equipment.

**Growth Inhibition as a Function of Drug Exposure Time.** The kinetics of growth inhibition as a function of drug exposure time and the reversibility of CGP-57148’s effect on cell growth were studied in vitro using intact cells expressing p190 (Z-119) and p210 (KBM-5), as well as cells that were negative for BCR-ABL (KBM-3). Cells were grown in the presence of CGP-57148 (2.5 or 10 µM) or in DMSO only (controls). After exposure for various times, both controls and CGP-57148-containing cultures were evaluated for total number of viable cells, washed twice with PBS to remove drug, and after resuspension in fresh, drug-free medium, cultured for another 72 h. At several intervals after drug removal, duplicate cultures were terminated, and the total number of viable cells was determined as described. In selected cultures, the percentage of apoptotic cells was determined using flow cytometry. Growth curves were constructed to evaluate the impact of drug exposure on subsequent cell growth.

**Apoptosis Assays**

**Immunocytochemistry.** Cells were fixed in 4% neutral-buffered formalin for 10 min at RT. Then, 50–100 µl of each cell suspension were dried on a microscopic slide and washed twice for 5 min with PBS. Then, cells were deproteinated with a 1:100 solution of proteinase K (20 mg/ml) for 15 min at 37°C, followed by washing with distilled water four times for 2 min each, and cell smears were incubated in TDT buffer for 15 min at RT. Next, a TDT (0.3 unit/ml, 1:40) and biotinylated dUTP (20 mM, 1:100) in 1 X TDT buffer were than added, and incubation continued for 1 h at 37°C. Slides were then washed for 5
Fig. 2. *In vitro* survival of p190Bcr-Abl-expressing primary blasts derived from Ph-positive ALL (A) but not of Ph-negative primary blasts from a patient with AML (B) was impaired by addition of CGP-57148. Cells were grown in suspension cultures for up to 3 days. Data points, mean numbers of viable blast cells (trypan blue exclusion test) from triplicate cultures.

![Graph A](image1)

**A.** CGP57148

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**B.** CGP57148

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![Graph B](image2)

**Fig. 3** Tyrosine kinase assay. Effect of CGP-57148 on autophosphorylation of BCR-ABL proteins in leukemic blasts. A, inhibition of autophosphorylation by 2.5 μM CGP-57148 in myeloid and lymphoid BCR-ABL-expressing cell lines. Lanes 1 and 2, p210Bcr-Abl (KBM-5); Lanes 3 and 4, p190Bcr-Abl (Z-119); Lanes 5 and 6, p190Bcr-Abl (Z-181); and Lanes 7 and 8, p190Bcr-Abl (Z-33). B, effect of CGP-57148 on autophosphorylation of p190Bcr-Abl in intact blast cells derived from two patients with Ph-positive ALL, patients SS (Lanes 9 and 10) and BA (Lanes 11–14). Lanes 9 and 11, samples without drug. Lane 10, sample exposed to 2.5 μM for 180 min; Lanes 12–14, samples exposed for the same time to 0.5, 2.5, and 10 μM, respectively.

Flow Cytometry. After exposure to CGP-57148, 1 × 10⁶ viable KBM-5 or Z-119 cells were washed with ice-cold PBS and fixed in ethanol. DNA content was then measured after propidium iodide staining and RNase treatment using a Coulter EPICS Profile Analyzer (Hialeah, FL). Cell cycle distribution and the percentage of apoptotic cells were determined using Software MultiCycle AV (Phoenix Flow System, Inc., San Diego, CA). Apoptotic cells having less than diploid DNA content were identified on DNA distribution histograms as a population of cells to the left of G₁ diploid peak (28).

Immunoprecipitation and Western Blotting with PY 20 Antibodies. Cells were incubated for 90 min in the presence of 2.5 μM CGP-57148, after which BCR-ABL was immunopre-
Cell line M3.16 derived from Mo7e and engineered to express phosphorylation using PY 20 antiphosphotyrosine antibodies. 

Coprecipitates were then assayed by Western blotting for tyrosine coprecipitated from cell lysates by P6D antibody. The immunoprecipitation generated using an ECL kit (Amersham).

... derived from a patient with lymphoma and supervening ALL...

... selected for their expression of two molecular variants of BCR-ABL fusion sequences, and BCR-ABL protein. CGP-57148 showed strong and comparable antiproliferative activity only against the Ph-positive cell lines (IC50 ~0.08–0.6 μM; Fig. 1A). In contrast, it did not inhibit the growth of Ph-negative cell line Z-138, even up to concentrations of 1.0 mM. Both 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and trypan blue exclusion tests yielded comparable results in repeated experiments (data not shown). In Fig. 1B, three myeloid leukemic cell lines had single or multiple copies of Ph present and expressed p210Bcr-Ab1 protein. The human myelogenous leukemia cell line KBM-3, which lacks both Ph and the BCR-ABL fusion protein, was used as a control along with growth factor-dependent megakaryocytic cell line Mo7e (Fig. 1B). A factor-independent cell line M3.16 derived from Mo7e and engineered to express p210Bcr-Ab1 was studied for comparison as well.

...KBMB3 (Ph-negative AML; Lanes 1 and 2), KBM-5 (p210Bcr-Ab1; Lanes 3 and 4), Z-138 (Ph-negative ALL cell line; Lanes 5 and 6), Z-119 (Lanes 7 and 8), Z-33 (Lanes 9 and 10), and Z-181 (Lanes 11 and 12), all p190Bcr-Ab1-expressing ALL cell lines. Note differences in the electrophoretic motility but not the intensity of bands in samples exposed to the drug, suggesting effect on the phosphorylation but not amount of the proteins (Lanes 4, 8, 10, and 12).

Soft Agar Colony Growth. Cells were plated in duplicate in 35-mm Petri dishes in 1 ml of IMDM, supplemented with 0.35% agar and 15% FCS. Plates were then incubated for 7–10 days, after which colonies of ≥50 cells were counted directly under phase contrast.

RESULTS

Inhibition of Cellular Proliferation in Human Leukemic Cell Lines. The activity of CGP-57148 was studied in a panel of human leukemic cell lines, both lymphoid and myeloid, selected for their expression of two molecular variants of BCR-ABL protein. The selectivity of CGP-57148-mediated inhibition of cell growth was first studied in a series of cell lines derived from patients with ALL. Of major interest to us were the antiproliferative actions of the drug in three Ph-positive ALL cell lines (Z-119, Z-181, and Z-33) independently derived from three patients with Ph-positive ALL. All three cell lines expressed hybrid p190Bcr-Ab1. The fourth cell line (Z-138) was derived from a patient with lymphoma and supervening ALL and is characterized by the absence of Ph, BCR-ABL hybrid sequences, and BCR-ABL protein. CGP-57148 showed strong and comparable antiproliferative activity only against the Ph-positive cell lines (IC50 ~0.08–0.6 μM; Fig. 1A). In contrast, it did not inhibit the growth of Ph-negative cell line Z-138, even up to concentrations of 1.0 mM. Both 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and trypan blue exclusion tests yielded comparable results in repeated experiments (data not shown). In Fig. 1B, three myeloid leukemic cell lines had single or multiple copies of Ph present and expressed p210Bcr-Ab1 protein. The human myelogenous leukemia cell line KBM-3, which lacks both Ph and the BCR-ABL fusion protein, was used as a control along with growth factor-dependent megakaryocytic cell line Mo7e (Fig. 1B). A factor-independent cell line M3.16 derived from Mo7e and engineered to express p210Bcr-Ab1 was studied for comparison as well.

Incubation of cells in the presence of increasing concentrations of CGP-57148 (0.01–10 μM) revealed a remarkable and dose-dependent inhibition of cellular proliferation in all myeloid cell lines containing either native or vector-transfected BCR-ABL fusion sequences and expressing p210Bcr-Ab1 fusion protein. The IC50 varied between 0.25 μM for KBM-7 cells and 0.5 μM for KBM-5 cells. Similarly, the proliferation of M3.16 cells (a p210Bcr-Ab1-expressing derivative of Mo7e cells) was effectively inhibited by the drug concentrations shown to inhibit the leukemic cells containing the native p210Bcr-Ab1. In contrast, the proliferation of Mo7e and KBM-3 cells was not affected at all by concentrations of up to 0.5 μM and was only marginally affected in some experiments by concentrations of 10 μM CGP-57148 (Fig. 1B).

Effect of CGP-57148 on the Survival of Ph-positive Leukemic Blasts Freshly Derived from Patients with ALL. Leukemic blasts were isolated from the blood of two patients with Ph-positive ALL on a density gradient. The blasts were then incubated at high density (1 × 106 cells/ml) in suspension cultures in the presence of various concentrations of CGP-57148. The survival of viable cells was assessed daily by the means of the trypan blue exclusion test. As shown in Fig. 2A, the survival of cells from one patient was impaired by the drug in dose-and-time dependent fashion; similar results were obtained with cells from a second patient, whose disease was terminal and drug resistant (data not shown). Concentrations of CGP-57148 as low as 0.3 μM decreased survival on days 1 and 2 of culture (Fig. 2A). No such differences were noted in primary, BCR-ABL-negative myeloid leukemia blasts exposed to similar CGP-57148 concentrations (Fig. 2B).

Selective CGP-57148 Inhibition of BCR-ABL Protein Kinase in Intact Cells. The effect of CGP-57148 on BCR-ABL and ABL tyrosine kinases in intact cells was studied in a variety of ALL and AML cells expressing p190 or p210Bcr-Ab1 (Fig. 3). Inhibition of cell proliferation was associated with reduced autophosphorylation of the BCR-ABL gene product. Tyrosine kinase assay (26) revealed decrease in autophosphorylation in cells expressing p190Bcr-Ab1 as well as p210Bcr-Ab1 proteins. Importantly, the inhibition of autophosphorylation revealed by kinase assay was seen not only in cell
lines but also in fresh blast cells derived from patients expressing p190Bcr-Abl (Fig. 3). Under these conditions, the extent of inhibition of autophosphorylation varied with cell line: p190Bcr-Abl-expressing Z-119 and Z-33 cells were most sensitive, whereas p210Bcr-ABL-myeloid cell line KBM-5 was least sensitive (Fig. 3). Inhibition of autophosphorylation was also documented in fresh intact cells from two patients with Ph-positive ALL (Fig. 3). The inhibition of p210Bcr-ABL and p190Bcr-Abl phosphorylation was even more clearly demonstrated by immunoprecipitation with P6D antibody followed by Western blotting of the immunoprecipitates using antiphosphotyrosine antibodies (Fig. 4). There was no effect on the level of expression of BCR-ABL proteins, as evidenced by the intensity of BCR-ABL bands on Western blotting analysis (Fig. 5). The consistent shift in electrophoretic mobility of both p210Bcr-ABL and p190Bcr-Abl proteins clearly indicated the effect on the extent of autophosphorylation in treated samples (Fig. 5).

**Effect of CGP-57148 on the Growth of Leukemic Cells.** The results of a series of experiments on the effect of CGP-57148 are summarized in Figs. 6 and 7. First, the fact that exposure of up to 72 h in the presence of 2.5 and 10 μM of CGP-57148 did not inhibit the BCR-ABL-negative KBM-3 AML cells (data not shown) again confirmed high degree of specificity of the drug’s action. In both p210Bcr-ABL- and p190Bcr-Abl-positive cell lines respectively, cell proliferation was clearly inhibited (Figs. 6A and 7A) in the continuous presence of 2.5 and 10 μM of the drug, respectively. At these concentrations, the exposure time affected the proliferative potential of the target population subsequent to drug removal. In
Fig. 7 The CGP-57148 concentration-dependent growth inhibition of p190Bcr-Ab1-expressing Z-119 ALL cells. Cells were grown in triplicate suspension cultures in the presence of the drug. After various periods of time (horizontal bar), the drug was removed by washing, and the cells were cultured in drug-free medium. The total number of viable cells was assessed at indicated times. Data points, means of triplicate cultures at each experimental point. Presence of a 10 μM concentration of the drug irreversibly abolished the cell growth (A), with cells failing to regrow in the drug-free medium after exposure as short as 24 h (B–D). At 2.5 μM concentration, Z-119 cells resumed growth after removal of the drug (E and F).

particular, the p190Bcr-Ab1-expressing Z-119 cells required a longer exposure to impair the subsequent recovery of proliferation after drug removal. In fact, the inhibition was not complete, and delayed recovery was noted after exposures to 2.5 μM concentrations (data not shown and Fig. 7, E and F). In contrast, exposure of KBM-5 cells to the drug for 48 h completely prevented subsequent regrowth of the cells in drug-free medium, suggesting a permanent inactivation of cell proliferation (Fig. 6). This “sterilizing” effect was evidently drug dose dependent because increasing the concentration of CGP-57148 to 10 μM resulted in the complete sterilization of Z-119 cells after 24–72 h of exposure, with no indication of cell regrowth within 72 h after drug removal (Fig. 7, B–D).

Induction of Apoptosis in p210Bcr-Ab1- and p190Bcr-Ab1-expressing Cells. After exposure of KBM-5 cells (p210Bcr-Ab1) for 2 days and Z-119 (p190Bcr-Ab1) for 3 days, the cells were analyzed by immunocytochemical staining for the induction of apoptosis. At a concentration of 2.5 μM, CGP-57148 induced apoptosis in both cell lines at very high frequency, thus documenting the irreversible induction of death rather than a reversible inhibition of proliferation (Fig. 8). Incubation for at least 24 h was necessary to document the apoptosis. The frequency of apoptotic cells was further analyzed as a function of exposure time by flow cytometric analysis of the cells with subdiploid DNA in two cell lines expressing BCR-ABL (p210Bcr-Ab1) expressing KBM-5 and p190Bcr-Ab1-expressing Z-119) and one BCR-ABL-negative cell line (KBM-3). The results are summarized in Fig. 9. In the presence of 2.5 μM CGP-57148, myeloid (KBM-5) Ph-positive cells showed an increase in apoptotic cell number, starting after 24 h of exposure. The increase persisted through 72 h of exposure, with a maximum of 40% apoptotic cells at 48 h (Fig. 9A). No increase in apoptotic cells above
background was seen in BCR-ABL-negative KBM-3 cells (Fig. 9B). Because of the slightly lower sensitivity of Z-119 cells (Fig. 7), the induction of apoptosis was studied in the presence of 10 μM concentration of the drug. As shown in Fig. 9C, 24-h exposure induced apoptotic changes in very few cells, but this proportion increased to 60% at 48 and 72 h.

Removal of the drug after 24 h exposure of Z-119 cells was followed by increase of apoptotic cells in subsequent 24 h in drug-free medium. When drug was removed after 48 or 72 h and the cells were again grown in drug-free medium, the percentage of apoptotic cells tended to decrease, paralleling the decrease in the total number of cells.

**Lack of Recovery of the Clonogenic Potential of Leukemic Cells after Temporary Exposure to CGP-57148.** As shown in previous experiments in whole leukemic cell populations, the presence of CGP-57148 suppressed cellular growth, apparently by inducing apoptosis. Moreover, removing the drug after a minimal exposure of 24–32 h failed to reinitiate cell growth. This suggests a sterilizing effect on the cells that are essential for the perpetuation of the population, i.e., clonogenic cells. It poses a major question regarding the potential use of CGP-57148 to purge autologous marrow of BCR-ABL-expressing cells.

To study this question, we exposed leukemic cells to CGP-57148 in liquid cultures and studied the frequency of clonogenic growth after removal of the drug. The results, summarized in Fig. 10, demonstrate that exposure of p210Bcr-Abl-expressing KBM-5 clonogenic cells to CGP-57148 resulted in a sustained damage, from which the cells failed to recover during subsequent growth for 72 h in drug-free medium. In contrast, the KBM-3 clonogenic population was not affected.

**DISCUSSION**

The fusion protein p210Bcr-Abl has been shown to possess tyrosine kinase activity and, thus, to stimulate cellular proliferation. In agreement with such function, attempts to selectively inhibit the activity of this protein have been made using two classes of drugs. Yaish et al. (29) were first to exploit series of TKIs called tyrphostines (30, 31), initially modeled on erbstatin structure. Although tyrphostines later proved to inhibit both tyrosine kinase activity and growth in p210Bcr-Abl-expressing K562 leukemic cells (32, 33), their specificity and a lack of effect on Ph-negative cells remains to be convincingly documented (34). The second class of TKI was developed from 2-phenylaminopyrimidine, and one, CGP-57148, showed promising specificity and activity in BCR-ABL-expressing cells (15, 17). These results led us to investigate whether CGP-57148, which inhibits p210Bcr-Abl, may also work against Ph-positive ALL, an aggressive disease with an extremely poor prognosis.

First, we investigated the association of the CGP-57148-induced proliferation arrest and decreased survival *in vitro* with the inhibition of autophosphorylation of BCR-ABL tyrosine kinase. We found that a detectable decrease in the autophosphorylation, as documented by kinase assay, Western blotting, and immunoprecipitation with antiphosphotyrosine antibodies, was not accompanied by a decrease in the amount of the BCR-ABL protein. The extent of the inhibition of autophosphorylation, however, did not strictly correlate with the *in vitro* cellular sensitivity to CGP-57148. The extent of the inhibition of autophosphorylation, however, did not strictly correlate with the *in vitro* cellular sensitivity to CGP-57148. This was shown by comparing the extent of autophosphorylation in two cell lines that were comparably sensitive to the antiproliferative action of CGP-57148, i.e., p210Bcr-Abl-expressing KBM-5 and p190Bcr-Abl-expressing Z-33. The extensive inhi-
bition of autophosphorylation in the Z-33 cells contrasted with the barely detectable inhibition in KBM-5 cells. This may, however, be related to the method used because no differences were observed using antiphosphotyrosine antibodies. The specificity of the antiproliferative action of this drug remained remarkable and limited to BCR-ABL protein-expressing cells, with comparable activity in p190- and p210-expressing populations.

Our results, thus, confirm the pioneering data of Buchdunger et al. (15) and Druker et al. (17), which documented the specificity of CGP-57148 in cells engineered to express p210<sup>p210<sup>Bcr-AbI</sup></sup> (15), as well as “native” p210<sup>p210<sup>Bcr-AbI</sup></sup>-expressing cells, derived from patients with Ph-positive CML (17). In addition, our data further extend these results to p210<sup>p210<sup>Bcr-AbI</sup></sup>-expressing blast cell lines derived from patients with CML in myeloid blast crisis and, more importantly, to ALL patient-derived cell lines, as well as primary ALL blasts expressing p190<sup>p190<sup>Bcr-AbI</sup></sup>. Similar results in BCR-ABL expressing cell lines were recently reported by others (35). In comparing our results in cell lines derived from blast-crisis CML-expressing p210<sup>p210<sup>Bcr-AbI</sup></sup> and cell lines derived from Ph-positive ALL-expressing p190<sup>p190<sup>Bcr-AbI</sup></sup>, we noted comparable sensitivity. This, in
turn, argues against the notion of phenotype-associated differences in the tyrosine kinase function of various classes of BCR-ABL fusion proteins.

Finding the correlation of CGP-57148 exposure time with inhibition of proliferation and cell death is new and may have important implications for the use of TKIs both in vitro (i.e., cell purging) and in vivo (therapy). Irreversible apoptosis requires continuous exposure of cells for at least 24 h; removal of the drug after <24 h of exposure results in recovery of cell growth. The reversibility of CGP-57148’s action must be taken in account in the design of both purging protocols and clinical trials. Interestingly, Druker et al. (17), using growth factor-dependent cell lines rendered factor independent by BCR-ABL transfection, have shown that cells inhibited by the continuous presence of CGP-57148 cannot be rescued by the presence of growth factors. Although experiments with, e.g., primary CML cells demonstrated loss of colony-forming potential in the presence of CGP-57148, the time dependency of the de facto killing of Ph-positive progenitor CFCs was not known. It could be speculated that, although the colony-forming ability of CFCs is blocked by the compound, they could survive a limited exposure to it, restoring their clonogenic potential in the drug’s absence with resulting regrowth of leukemia. Such questions could conceivably be studied further in suspension cultures, with removal of the drug after defined periods of time and assay of the whole population for cell regrowth and for CFCs during various subsequent, drug-free periods. Indeed, we have shown previously that, although time-limited exposure of bone marrow mononuclear cells to selected doses of various chemotherapeutic agents eliminates all GM-CFC growth, subsequent short-term culture in drug-free liquid medium leads to restoration of the GM-CFC-forming capacity. In those studies, the kinetic of the recovery process was dependent on the drug used and suggestive of either differences in the degree of the damage by a drug to the ancestors of GM-CFC cells or the ability of GM-CFC-forming cells to repair drug-induced damage, with kinetics dependent on the mechanism of action of respective drugs. Our finding with KBM-5 clonogenic cells supports the notion of a permanent loss of colony-forming ability of CFCs exposed to CGP-57148.

The mechanism by which CGP-57148 influences the proliferative potential of leukemic cells and the kinetics as well as reversibility of the process is of major importance for both the selective killing of leukemic cells in vitro in the setting of marrow purging and the design of dosing schedules in clinical trials.

The results we obtained with Ph-positive ALL cell lines have been confirmed with primary, ALL patient-derived blast cells. CGP-57148 inhibited both cell survival and autophosphorylation in Ph-positive ALL blasts but had no effect on Ph-negative ALL and AML blast cells. This extends the previous

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Fig. 10 The comparison of the effect of CGP-57148 on total cell numbers and the numbers of leukemic clonogenic cells (CFCs). The total number of cells and the number of CFCs in suspension cultures was determined at the beginning of culture (0 h), during exposure to drug (24 and 48 h), and after washing off the drug and replacement with fresh, drug-free medium (72, 96, and 120 h). CGP-57148 effectively inhibited survival of clonogenic leukemic cells during continuous exposure for 48 h in short-term liquid suspension cultures of KBM-5BCR-ABL cells, while leaving the population of BCR-ABL-negative KBM-3 clonogenic cells virtually intact. Removal of the drugs from medium after 24 h of exposure was not associated with recovery of BCR-ABL-containing KBM-5 clonogenic cells. Data points, means of triplicate cultures.

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4 M. Beran, unpublished observations.
observations in primary human CML-derived cells (17, 35) to Ph-positive ALL. Ph-positive ALL is a disease that needs new therapeutic approaches. It comprises 10–20% of all adult ALL cases, most of which, in turn, express p190Bcr-Abl, while a minority express p210Bcr-Abl. Although 70–80% of adult Ph-positive ALL patients can be induced into a complete remission with, for example, the HYPER-CVAD regimen (36), virtually all patients relapse and die of the disease regardless of various maintenance regimens, even after allogeneic bone marrow transplantation. In cases of disease with minimal tumor burden, innovative approaches are presently the only hope for long-term, disease-free survival. Therefore, tyrosine kinase inhibition may be an important therapeutic modality during complete remission. Indeed, our present results would support clinical trials involving the in vitro purging of bone marrow by TKIs prior to autologous transplantation and in vivo use of TKI as part of total maintenance chemotherapy. A similar logic would apply to patients with lymphoid blast-crisis CML, in whom currently available treatments are unable to sustain remission and prolong disease-free survival. Finally, our results suggest that prolonged, continuous exposure to a TKIs such as CP-571485 in vitro, as well as in vivo, will likely be necessary for optimal therapeutic results.

REFERENCES


Selective inhibition of cell proliferation and BCR-ABL phosphorylation in acute lymphoblastic leukemia cells expressing Mr 190,000 BCR-ABL protein by a tyrosine kinase inhibitor (CGP-57148).

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