The Tyrosine Kinase Inhibitor CGP 57148 (ST1 571) Induces Apoptosis in BCR-ABL-positive Cells by Down-Regulating BCL-X

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ABSTRACT

CGP 57148 is a potent inhibitor of the ABL protein tyrosine kinase and a promising new compound for the treatment of a variety of BCR-ABL-positive leukemias. We used this enzyme inhibitor to characterize the biological effects of BCR-ABL in primary cells and two growth factor-dependent BCR-ABL-transfected cell lines.

The effect of CGP 57148 on primary cells is dependent on the stage of differentiation. The growth of maturing chronic myeloid leukemia cells is independent of BCR-ABL in the presence of growth factors. However, the proliferation of leukemic immature cobblestone-forming area cells is almost completely blocked after the inhibition of the BCR-ABL kinase. In the BCR-ABL-transfected cell lines, M07/p210 and Ba/F3/p185, CGP 57148 induces apoptosis by releasing cytochrome c, activating caspase 3, and cleavage of PARP. No alteration of the expression level of the apoptosis regulator BCL-2 was observed. In contrast, BCL-X was down-regulated after exposure to CGP 57148. Inhibitors of signal transduction proteins such as PI-3 kinase, mitogen-activated protein/extracellular signal-regulated kinase kinase, and Janus-activated kinase 2 pathways were not capable of a comparable down-regulation of BCL-X. The Fas/Fas ligand system was not involved either in the induction of apoptosis by CGP 57148. We conclude that the inhibition of the BCR-ABL kinase by CGP 57148 (a) preferentially inhibits the growth of immature leukemic precursor cells, (b) efficiently reverts the antiapoptotic effects of BCR-ABL by down-regulation of BCL-X, and (c) is more effective than the inhibition of the downstream signal transduction pathways of PI-3 kinase, mitogen-activated protein/extracellular signal-regulated kinase kinase, and Janus-activated kinase 2.

INTRODUCTION

CML is a clonal stem cell disorder. The hallmark of the disease is the Philadelphia chromosome caused by a balanced reciprocal translocation between chromosomes 9 and 22, leading to the expression of a chimeric protein BCR-ABL. Fusion of the bcr sequences to the abl gene results in constitutive activation of the ABL kinase. BCR-ABL phosphorylates numerous cellular targets, leading to the activation of intracellular signaling pathways. Three biological key processes are affected in cells expressing BCR-ABL. BCR-ABL-transformed cells exhibit cell cycle entry in the absence of growth factors, decreased susceptibility to induction of apoptosis after exposure to DNA damage and growth factor withdrawal, and impaired adhesion to extracellular matrix proteins (1, 2). Numerous substrates of the BCR-ABL kinase have been identified in various cell lines. The majority of the proteins phosphorylated by BCR-ABL are components of pathways activated in normal cells by growth factor signaling. This includes proteins involved in the RAS pathway (3–5), the JAK/STAT pathway (6), and the PI-3 kinase pathway (7). Other substrates are involved in the process of cell adhesion, such as the cytoskeletal proteins paxillin and actin (8). It is not clear which of these intracellular transduction pathways are responsible for the antiapoptotic effect of BCR-ABL. The BCL-2 family members BCL-2 and BCL-X have been implicated in the BCR-ABL-mediated resistance to apoptosis induced by cytokine withdrawal (3) and cytotoxic agents (9).

Because the tyrosine kinase activity is the crucial enzymatic activity driving all known functions of the BCR-ABL protein (10) and is required for the protection from apoptosis by BCR-ABL, targeting this kinase activity is a promising approach for therapeutics strategies. Using the known structure of the ATP-binding site of protein kinases, a series of compounds of the 2-phenylaminopyrimidine have been synthesized and screened for inhibition of various tyrosine kinases. CGP 57148 and CGP 57148 B, the methane sulfonate salt, have been found to be potent inhibitors of the ABL-kinase without affecting various other intracellular tyrosine kinases. The only other ki-
nase inhibited to a similar extent by CGP 57148 is the platelet-derived growth factor β receptor tyrosine kinase (11, 12). Further investigations demonstrated a BCR-ABL-specific inhibition of cellular proliferation and tumor formation by both primary CML cells and BCR-ABL-expressing cell lines (12). In the colony-forming assays of peripheral blood and bone marrow from a patient with CML, a decrease of 92–98% in the number of BCR-ABL colonies was observed, whereas normal colony formation was not affected (12). Over a 2-logarithmic dose range with a maximum differential effect at 1 μmol/liter, CGP 57148 selectively suppressed the growth of CFU-GM and burst-forming unit-erythroid (13). Analyses of cell lines showed growth inhibition followed by decline in viability of 10 BCR-ABL-positive cell lines and BCR-ABL-transfected cells (13). These experiments showed dephosphorylation of the BCR-ABL protein after exposure to CGP 57148 (14). However, the intracellular mechanisms by which CGP 57148 inhibits proliferation and/or causes decreased viability are not known.

We show here that CGP 57148-mediated growth inhibition of primary BCR-ABL-positive cells depends on the stage of differentiation of hematopoietic cells. In addition, CGP 57148 induces apoptosis in the BCR-ABL-transfected human M07/p210 and the murine Ba/F3/p185 by down-regulating the BCL-X protein.

MATERIALS AND METHODS

Patients. Cells were harvested from newly diagnosed CML patients after obtaining informed consent before the experiments. All patients studied were in the stable chronic phase of disease and were not treated previously. Cells from patients with various malignancies in complete remission undergoing blood stem cell harvest were used as controls. All experiments were repeated at least three times. To evaluate the proliferation of maturing blast cells, cells derived from three different patients were used. The CFU-GM assay was performed five times using cells from four different patients. Accordingly, CD34+ cells from four patients were investigated in the CAFC assay.

Reagents. CGP 57148 (now renamed STI 571) was kindly provided by E. Buchdunger Novartis Inc. (Basel, Switzerland). A stock solution (10 mg/ml) was prepared by dissolving the compound in DMSO, and it was kept at −20°C. CGP 57148 was used at concentrations ranging from 0.1 to 10 μM. The PI-3 kinase inhibitor, LY, the MEK-inhibitor, PD, and the JAK-2 Inhibitor, AG, were purchased from Calbiochem (San Diego, CA). Stock solution of LY (25 mM in DMSO), PD (25 mM in DMSO), and AG (25 mM in DMSO) were kept at −20°C, and the final dilution (LY, 5 μM; PD 50, μM; and AG, 20 μM) was made immediately before use. The caspase inhibitor, ZVAD-fluoromethylketone (stock solution at −20°C: 20 mM in methanol; final dilution, 100 μM), was purchased from Bachem (Heidelberg, Germany). The blocking anti-FAS IgG1 monoclonal antibody ZB4 (1 μg/ml) and the neutralizing anti-FAS IgG monoclonal antibody 4H9 (100 ng/ml) were purchased from Immunotech (Marseilles, France).

Cell Source. Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation (Seromed, Berlin, Germany). After lysis of residual RBCs, CD34+ cells were purified using a Miltenyi MACS column (Miltenyi Biotech, Bergisch Gladbach, Germany) according to manufacturer’s instructions. Cell purity was checked by FACSscan analysis. The fraction of CD34+ cells ranged from 96% to 99%.

Cell Culture Techniques. The human M07 cell line was derived from a patient with megakaryocytic leukemia and required GM-CSF, IL-3, or stem cell factor for proliferation. Cells were cultured in RPMI 1640 medium (Seromed Inc., Berlin, Germany) supplemented with 10% FCS and 100 ng/ml GM-CSF (Leukomax, Sandoz, Switzerland). The M07/p210 cell line was obtained from Dr. M. Hallek (München, Germany; Ref. 15). The M07/p210 cell line expressed p210 BCR-ABL and was factor-independent. M07/p210 cells were grown in RPMI 1640 medium supplemented with 10% FCS.

The murine pro-B lymphocyte cell line, Ba/F3, expressing temperature-sensitive p185 BCR-ABL, was kindly provided by J. Duyster (Munich, Germany). The construction of plasmids and generation of transfected cell lines have been described (16). Factor-independent Ba/F3/p185 cultivated at a temperature of 33°C were grown without IL-3 supplementation. Ba/F3/p185 cultivated at 39°C showing no BCR-ABL phosphorylation were used as controls. The growth of factor-independent Ba/F3 clone expressing wtBCR-ABL was transformed with pSLXBer-Abl and cultivated at 37°C.

Primary CD34+ cells were grown at a density of 0.5 × 10⁶ cells/ml in 24-well plates in RPMI (Seromed Inc., Berlin, Germany) supplemented with 10% FCS and 200 ng/ml recombinant human G-CSF (Amgen Inc., Thousand Oaks) and recombinant human steel factor (50 ng/ml; Amgen Inc.). The culture system has been described in detail (17). All experiments were performed on days 3–5 of the culture. The majority of these cells lost expression of the CD34 antigen, but remained cycling. These cells represent maturing myeloid blast cells at an intermediate stage of differentiation. The percentage of 5-phase cells and the expression of the cell surface markers CD34, CD33, and CD15 were identical in leukemic and normal hematopoietic cells (17). Because of other groups reporting down-regulation of the BCR-ABL protein during hematopoietic differentiation, we further checked BCR-ABL expression in our culture system (18). No significant changes in the expression of BCR-ABL RNA were found between days 3 and 5 of the culture (data not shown). Cells were washed on day 3 of the culture, and 0.5 × 10⁶ cells were cultured in medium with or without 1 μM CGP 57148 for up to 48 h.

Hematopoietic Progenitor Cell Assay. The assay for detecting CFU-GM was performed as previously described (19). CD34+ cells were used at a cell density of 5 × 10³ cells/well. These cells were suspended in culture medium containing 0.3% agar in a volume of 0.25 ml and plated in multiwell tissue culture plates (Nunc, Wiesbaden, Germany). After solidification of the agar layer, a liquid overlay of culture medium was added to the culture plates containing the hematopoietic growth factors. The final concentration of growth factors was as follows: G-CSF, 200 ng/ml, and IL-3, 50 ng/ml. At the end of the incubation period, the agar cultures were fixed with glutaraldehyde, 2.5%, washed with distilled water for 4 h, transferred onto microscopic slides, dried at 40°C, stained with May-Grünwald-Giemsa, and permanently mounted. Colony number was evaluated by light microscopy.
CAFC Assay. CAFC assays were performed on a confluent layer of FBMD-1 cells, which were kindly provided by R. Ploemaker (Rotterdam, Germany), in 24-well plates (Falcon; Ref. 20). Indicated numbers of CD34+ cells in 0.5 ml of culture media were plated on the FBMD-1 cells in 24-wells and allowed to adhere at 37°C for 6 h. Afterward, the medium was removed, and cells were overlaid with culture medium containing 0.3% agar in a volume of 0.5 ml containing the hematopoietic growth factors. The final concentration of growth factors was as follows: G-CSF, 200 ng/ml, and IL-3, 10 ng/ml. The cultures were maintained at 33°C and 5% CO2 for 2 weeks. After 1 week, cells were fed with culture medium containing 0.3% agar in a volume of 0.5 ml containing the hematopoietic growth factors in 2-fold access. The number of CAFCs with at least one phase-dark hematopoietic clone of at least five cells (i.e., cobblestone area) beneath the stromal layer was counted on day 14 of culture.

Survival Assay. Cells were plated into 96-well plates at 1 × 10^5/well in their respective media supplemented with 10% FCS. CGP 57148 was added in various concentrations. For measurement of cytotoxic effects, the MTT assay was used. After 16–24 h of exposure, the viable cells in each well were assayed to their ability to transform MTT into a purple formazan (21).

Protein Extract Preparation. Protein extracts were prepared as follows: Cells were collected by centrifugation, washed twice with PBS, and dissolved in lysis buffer [50 mM Tris-HCl (pH 7.6); 250 mM NaCl; 0.1% Triton X-100; 5 mM EDTA; 1 μg/ml leupeptin; 1 mM phenylmethylsulfonyl fluoride; 1 mM DTT; 1 μg/ml aprotinin]. The protein content of the extracts was quantified using the Bradford protein assay with BSA as a protein standard (22).

For the analysis of cytosolic cytochrome c, cells were harvested by centrifugation, washed twice, and resuspended in 100 μl/10^7 cells lysis buffer [25 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml leupeptin]. After centrifugation at 800 g for 10 min at 4°C, supernatants were collected and 5 mM EDTA were added. Subsequently, the extracts were centrifuged at 280,000 g for 60 min. Two mM DTT were added to the supernatants (23), and protein concentration was determined by Bradford.

Western Blots. Western blots were performed according to standard techniques. Briefly, proteins (50 μg/lane) were run on SDS-polyacrylamide gels with the Tris glycine buffer system (24). Proteins were then transferred to polyvinylidene difluoride membranes (Boehringer Mannheim, Germany) using a semidy transfer apparatus as recommended by the manufacturer (Bio-Rad). The membranes were blocked by incubating in 1% BS (Boehringer Mannheim) in TBS (pH 7.6) for 1 h. The blots were then incubated with anti-BCL-2 [4 μg/ml (Santa Cruz, Heidelberg, Germany) and 10 μl/ml (Dako, Glostrup, Denmark)], anti-BCL-X [1 μg/ml; Transduction, Lexington], anti-p27 (2 μg/ml; PharMingen), anti-PARP (1 μg/ml; Upstate, Lake Placid), and anticytochrome c [1 μg/ml; PharMingen] dissolved in TBS with 0.5% BS overnight. Blots were washed twice in TBS containing Tween-20 (0.1%) for 10 min and afterward blocked two times with 0.5% BS. Then blots were incubated with secondary antibody conjugated to horseradish peroxidase (diluted 1:5000) for 30 min, and proteins were detected by using a chemiluminescence kit (Boehringer Mannheim).

Caspase 3 Activity. Caspase 3 activity was measured using a commercially available assay as described (PharMingen). Briefly, 2 × 10^6 cells were lysed in cell lysis buffer [10 mM Tris-HCl (pH 7.5), 27 mM KCl, 100 mM KH2PO4/K2HPO4 (pH 7.5)] and spun in an Eppendorf centrifuge at 14,000 rpm for 10 min. Five μl of supernatant were diluted with 80 μl of HEPES buffer [20 mM HEPES (pH 7.5), 10% glycerol, 2 mM DTT], and 5 μl of Ac-DEVD-AMC (200 μM in DMSO) were

![Fig. 1](link) CGP 57148 selectively inhibits the growth of immature CML cells. The proliferation of maturing blast cells is not significantly blocked by CGP 57148 (A), whereas a significant CML-specific inhibition is observed of CML CFU-GM at concentrations ranging from 0.05 μg/ml to 1 μg/ml (B). The most pronounced antiproliferative effect is seen on the growth of CML CAFC (C). Bars, mean ± SE values of three to five independent experiments. Light bars, experiments studying CML cells; dark bars, normal controls.
added. As a control lysate with both 5 μl of Ac-DEVD-AMC (200 μM in DMSO) and 5 μl Ac-DEVD-CHO (200 μM in DMSO), an inhibitor of caspase 3 was used. Fluorescence was measured using an excitation wavelength of 380 nm and an emission wavelength of 450 nm.

Cell Cycle Analysis. Cell cycle analyses were performed by determining the DNA content with propidium iodide. Cells were washed in PBS and fixed in precooled (−20°C) 70% ethanol for at least 1 h at 4°C. Shortly before flow cytometry analysis, cells were rehydrated in cold PBS, treated with 50 μg/ml RNase A (Boehringer Mannheim) for at least 15 min at room temperature, and stained with 50 μg/ml propidium iodide. The stained cells were analyzed by FACScan (Becton Dickinson). The fraction of cells with a DNA content of <2N, 2N, 2–4N, and 4N was determined by means of the Modfit 2.0 software (Becton Dickinson). The number of viable cells was determined by counting the number cells excluding trypan blue.

Fig. 2 CGP 57148 selectively induces apoptosis in BCR-ABL-transfected M07 cells. A, the FACS analysis of propidium iodide-stained cells exposed to 5 μmol of CGP 57148. Whereas only a few cells with a DNA content <2N are detectable in M07 cells, the majority of M07/p210 cells undergo apoptosis after this treatment. B, in a MTT assay, the IC_{50} of CGP 57148 for M07/p210 cells is 0.069 μg/ml, whereas the parental line is largely resistant, with an IC_{50} of 32.08 μg/ml. Cell death is not inhibited by the addition of exogenous growth factor. C, M07/p210 are cultivated in the presence of indicated CGP 57148 concentrations with and without GM-CSF and IL-3. CGP 57148 selectively inhibits the growth of the BCR-ABL-positive Ba/F3/p185 cell line; however, this effect is antagonized by the simultaneous addition of IL-3.
TUNEL Assay. In situ cell death detection was performed using a commercially available assay as described (Boehringer Mannheim). Briefly, 1 × 10^6 cells were washed and fixed in 2% paraformaldehyde in PBS for 30 min at room temperature. They were then washed once, permeabilized by incubating (2 min; 4°C) with 0.1% Triton X-100, and 0.1% sodium citrate, and then washed twice. The TUNEL reaction was carried out by incubating cells (60 min; 37°C) with 50 μl of TUNEL mixture (FITC-12-dUTP, dATP, CoCl₂, terminal deoxynucleotidyltransferase, and TdT buffer; Boehringer Mannheim).

RESULTS

Inactivation of the BCR-ABL Kinase Selectively Inhibits the Proliferation of Immature CML Cells. Proliferation of primary normal and leukemic cells at different stages of differentiation was studied in the absence or presence of different concentrations of the BCR-ABL kinase inhibitor CGP 57148. Twenty-four-h incubation of proliferating normal or leukemic myeloid blast cells with 1 μM CGP 57148 exhibited no significant effect on viability of both leukemic and normal cells (Fig. 1A). Even prolonged incubation of 48 h lead to a decrease in neither proliferation nor viability of leukemic cells (data not shown). Moreover, cell cycle kinetics and apoptosis were also not influenced by this treatment (data not shown), and thus in the presence of exogenous growth factors, both growth and survival of maturing CML cells is not dependent on the BCR-ABL kinase activity.

In contrast, the proliferation of leukemic progenitor cells was significantly inhibited after inactivation of the BCR-ABL kinase despite the presence of excess amounts of exogenous growth factors. At a concentration of 1 μM CGP 57148, CFU-GM colony formation of leukemic cells was inhibited by 36.14 ± 14%, whereas normal colony formation was not significantly reduced (Fig. 1B). The most pronounced effect of CGP 57148, however, was seen on the formation of immature hematopoietic cells. Whereas leukemic CAFCs were efficiently inhibited by CGP 57148, normal CAFC formation was not

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**Fig. 3** Treatment of M07/p210 cells with CGP 57148 induces cytosolic accumulation of cytochrome c, caspase 3 activity, and PARP cleavage. A, a Western blot of cytochrome c in the cytoplasmic fraction of protein extracts before and 4, 16, and 24 h after exposure to CGP 57148. B, the increase in activity of caspase 3 by using a fluorogenic caspase 3 substrate Ac-DEVD-AMC; as a control, an inhibitor of caspase 3, Ac-DEVD-CHO, is used. C, a Western blot of the PARP protein before and 4, 8, and 16 h after treatment with CGP 57148 with the appearance of the characteristic 85-kDa cleavage fragment 16 h after exposure to CGP 57148.
affected. Moreover, at lower concentrations of CGP 57148 (0.5 and 1.0 μM), normal CAFC formation was even enhanced (Fig. 1C).

**Inhibition of the BCR-ABL Kinase Efficiently Kills BCR-ABL-expressing M07/p210 and Ba/F3/p185 Cells.** The growth and survival of wild-type M07 and Ba/F3 cells are strictly dependent on the presence of exogenous growth factors such as GM-CSF or IL-3. BCR-ABL expression renders these cells growth factor-independent (25). Forty-eight-h incubation with 1 μM CGP 57148 did not affect the viability of wild-type M07 (Fig. 2) or Ba/F3 cells (data not shown) maintained in the presence of growth/survival factors. In contrast, inhibition of the BCR-ABL kinase with CGP57148 specifically killed BCR-ABL-positive cells. Incubation of M07/p210 and Ba/F3/p185 cells with 1 μM CGP 57148 rapidly resulted in cell death. Both M07/p210 cells (Fig. 2A) and Ba/F3/p185 cells (data not shown) undergo apoptotic cell death after exposure to CGP57148 as indicated by a large proportion of cells with a DNA content <2N. The inhibition of growth of M07/p210 by CGP 57148 is dose-dependent with an IC of 0.069 μg/ml (Fig. 2B). The two cell lines, however, differ in the BCR-ABL dependency of their proliferation. The addition of excess amounts of GM-CSF or IL-3 was not capable of preventing cell death from M07/p210 cells exposed to CGP 57148 (Fig. 2C). Thus, in this cell line, irreversible BCR-ABL dependency of proliferation can be observed. In contrast, the addition of murine IL-3 entirely rescued Ba/F3-p185 from cell death after inhibition of the BCR-ABL kinase with CGP 57148 (Fig. 2D).

**Inhibition of the BCR-ABL Kinase Rapidly Leads to a Cytosolic Accumulation of Cytochrome c with Resultant Activation of Caspase-3 and Cleavage of PARP.** To further investigate the mechanism leading to the induction of apoptotic cell death after inhibition of the BCR-ABL kinase, we studied mitochondrial release of cytochrome c, caspase-3 activation, and degradation of PARP. Recent data indicate that apoptotic stimuli cause a release of cytochrome c from the mitochondria to trigger with APAF and caspase 9 the proteolytic activity of caspase 3 (26). Fig. 3A shows increased cytosolic accumulation of cytochrome c in M07/p210 after exposure to 1 μM CGP 57148. Furthermore, in M07/p210 after 6-h incubation with 1 μM CGP 57148, caspase-3 was already active, and the maximum activation was detected after 16 h (Fig. 3B). As a consequence of caspase 3 activation, the 85-kDa cleavage product of PARP could be shown by means of Western blotting. After 12-h incubation of M07/P210 cells with 1 μM CGP 57148, the 85-kDa cleavage product of the 110-kDa PARP was visible (Fig. 3C). To confirm the significance of caspase activation, we analyzed CGP 57148 (1 μM)-induced apoptosis in the presence of a caspase inhibitor ZVAD-fluoromethylketone. The proportion of apoptotic cells was determined by FACS analysis of propidium iodide-stained cells. In M07/p210 cells, the fraction of cells containing <2N DNA decreased from 14.3% to 7.1% in the presence of ZVAD after 16 h of incubation and in Ba/F3/p185 from 36.9% to 9.9%.

Taken together, these data demonstrate that inhibition of the BCR-ABL kinase by CGP57148 induces apoptotic cell...
death in M07/p210 cells by activation of a mitochondrion-dependent proapoptotic pathway.

CGP 57148-induced Apoptosis Is Not Dependent on Fas-Fas Ligand Interaction. It has been proposed that induction of apoptosis by cytotoxic agents may involve ligand/receptor-driven amplifier systems such as the Fas/CD95 system (27). Also, controversial data exist on the induction of Fas/CD95 ligand and up-regulation of Fas/CD95 after treatment with cytotoxic drugs. To test whether CGP 57148-induced apoptosis is Fas-CD95 ligand/Fas-CD95-dependent, we used a Fas neutralizing and a Fas agonistic antibody in addition to CGP 57148. Neither of these antibodies could block or enhance CGP 57148-induced apoptosis (Fig. 4). Therefore, we conclude that the FAS system is not involved in the apoptotic process induced by CGP 57148 in the human cell line M07/p210.

Inhibition of the BCR-ABL Kinase Down-Regulates BCL-X but Has No Effect on BCL-2 Expression. Members of the BCL-2 protein family crucially regulate programmed cell death by affecting the mitochondrial membrane. Recently it has been described that BCR-ABL-mediated resistance to apoptosis is in part attributable to an up-regulation of BCL-X (9). We therefore analyzed the expression of BCL-X and BCL-2 after exposure to CGP 57148 in M07/p210 and Ba/F3/p185 cells.

BCL-X protein levels substantially decreased within 4–8 h after inhibition of the BCR-ABL kinase with CGP 57148 both in M07/p210 (Fig. 5A) and Ba/F3/p185 (Fig. 6) cells. In contrast, no variations in the protein level of BCL-2 was observed in these cell lines (Fig. 5A; Fig. 6B). The induction of apoptosis is closely accompanied by the down-regulation of BCL-X. Whereas BCL-X down-regulation was not reversed by the addition of GM-CSF in the M07/p210 cells (data not shown), the addition of IL-3 completely blocked down-regulation of BCL-X and apoptosis in the Ba/F3/p185 cell line (Fig. 6B). CGP 57148 had no effect on the expression levels of BCL-X in the BCR-ABL-negative M07 and Ba/F3 (Fig. 5, B and C; Fig. 6). Moreover, down-regulation of BCL-X after growth factor withdrawal could not be enhanced by CGP 57148 (Fig. 5C).

Inhibition of Several Signal Transduction Pathways neither Down-Regulates BCL-X nor Induces Apoptotic Cell Death as Does CGP 57148. Because numerous substrates of the BCR-ABL kinase are components of signal pathways activated in normal cells by growth factor signaling, such as RAS (3–5), JAK/STAT (6), and PI-3 kinase (7), we compared the effect of inhibitors of these pathways with inhibition of BCR-ABL kinase activity by CGP 57148. We studied the effects of the P-I3 kinase inhibitor LY, the MEK inhibitor PD, and the JAK-2 inhibitor AG on induction of apoptosis and regulation of BCL-X in Ba/F3/p185 cells. All Inhibitors had a dose-dependent inhibitory effect on growth of Ba/F3/p185 and the maternal Ba/F3 by means of MTT assay (Fig. 7A). None of these inhibitors revealed a significant induction of apoptosis as demonstrated by means of TUNEL assay (Fig. 7B) nor down-regulated the expression of BCL-X (Fig. 7C). As a control for viability of Ba/F3/p185, we investigated the induction of p27 (Fig. 7C). Induction of p27 expression levels suggests cell cycle arrest after exposure to the pathway inhibitors and CGP57148. A combination of LY, PD, and AG with a suboptimal dose of CGP 57148 (0.1 μM) did not result in a major increase of CGP 57148-induced apoptosis (Fig. 8A) nor in major variations of protein levels of BCL-X compared to the effect of CGP 57148 (0.1 μM).

DISCUSSION

Protection from apoptosis has been postulated to be one of the crucial biological consequences of the BCR-ABL translocation (1). The antiairopoptotic properties of the BCR-ABL protein may be responsible for both the accumulation of malignant cells as well as the genetic instability leading to clonal evolution of the malignant clone. Protection from apoptosis after growth factor deprivation and exposure to DNA-damaging agents has been consistently demonstrated in a variety of BCR-ABL-positive murine and human cell lines. However, the data in primary cells are less clear. No resistance to apoptosis has been demonstrated in proliferating myeloid blast cells after γ-irradiation and growth factor withdrawal (28). Moreover, the results from studies on the resistance of CFU-GM against DNA damage are conflicting (29, 30). The data from our studies on the activity of CGP 57148 on primary CML cells may at least partially explain this discrepancy. In primary cells, the effect of CGP 57148 depends on the differentiation status of the cell type studied. Neither growth inhibition nor induction of cell death was observed in exponentially growing maturing blast cells when cultured with an excess of exogenous growth factors. The persistent proliferation after removal of growth factors of these cells, however, was efficiently blocked by CGP 57148 (31). A significant antiproliferative activity despite the presence of growth factors similar to that described by Druker et al. (12) was seen after exposure of CFU-GM to CGP 57148. The most pronounced differential effect on leukemic and normal cells, however, was detected when immature cobblestone forming cells (CAFCs) were studied.

Therefore, in mature CML cells, BCR-ABL causes a subtle
abnormality of the cell cycle regulation detectable only under suboptimal growth factor conditions. In contrast, the growth of immature CML cells entirely depends on an active BCR-ABL kinase. Whether this BCR-ABL effect on immature CML cells is caused by induction of apoptosis or inhibition of cell cycle progression cannot be answered thus far because the biochemical consequences of CGP 57148 on cobblestone forming cells cannot be studied directly. The addition of CGP 57148 at the beginning of the culture period without refeeding of the inhibitor is sufficient to almost completely block the outgrowth of CML CAFCs. This observation argues against an antiproliferative effect and supports the view that CGP 57148 may induce apoptosis in these immature cobblestone forming cells.

The effect of CGP 57148 on cobblestone forming cells is leukemia-specific. The growth of normal differentiated and immature myeloid cells is apparently not affected by inhibition of the c-ABL kinase. The proliferation of normal CAFCs was even enhanced by CGP 57148. Accordingly, experiments studying

**Fig. 7** In contrast to CGP57148 inhibitors of PI-3 kinase (LY), MEK (PD) and JAK-2 (AG) pathways are not capable of blocking the antiapoptotic BCR-ABL signal. **A,** viability of Ba/F3 and Ba/F3/p185 after exposure to variable concentrations of LY, AG, and PD in a MTT assay. **B,** induction of apoptosis in a TUNEL assay. **C,** Western blotting of BCL-X and p27 of Ba/F3/p185 cells after exposure to LY (5 μM), AG (20 μM), PD (50 μM), and CGP 57148 (B, 0.5 μM; C, 0.1 and 1 μM).
the inhibition of the c-ABL kinase with a dominant-negative mutant demonstrated a stimulatory effect on cell cycle progression of murine fibroblasts (32). This differential effect on the growth of immature normal and leukemic cells offers an intriguing mechanism that can be used for selecting BCR-ABL-negative precursors, e.g., for autologous transplantation. Experiments testing the capacity of CGP 57148 to purge bone marrow cells from CML precursors are presently underway at our institution.

Because the mechanism leading to growth inhibition of primary CML cells could not directly be studied, we used two growth factor-dependent BCR-ABL-transfected cell lines for further analysis. CGP 57148 reverses the transformed phenotype of both M07/p210 and Ba/F3/p185. Specific inhibition of the tyrosine kinase of BCR-ABL induces exactly the same processes to which BCR-ABL confers increased resistance (33). The human M07/p210 and the murine Ba/F3/p185 cells undergo apoptosis within 24 h after exposure to CGP 57148. Cytochrome c release in the cytoplasm with subsequent caspase 3 activation and PARP cleavage could be observed in M07/p210 subsequent to exposure to CGP 57148. Thus, CGP 57148 induces apoptosis via a pathway involving the disruption of the mitochondrial membrane.

The BCL-2 protein family has been suggested to function as pore formers and adapter molecules in the mitochondrial membrane regulating mitochondrial alterations preceding the activation of apoptogenic proteases and nucleases (26). The effect of the BCR-ABL oncogene on BCL-2 expression is controversial. CGP 57148 treatment of BCR-ABL-positive cells did not cause a down-regulation of BCL-2 in either the Ba/F3/ p185 or the M07/p210 cell line. Other investigators reported conflicting results of BCR-ABL transfection on BCL-2 expression of Ba/F3/p210 cells and HL-60/p 185 cells (34, 35). BCL-X has been implicated in the antiapoptotic phenotype of BCR-ABL-positive cells. Inhibition of the BCR-ABL kinase by CGP 57148 results in a decrease of BCL-X preceding apoptotic cell death both in the murine BA/F3/p185 cell line and the human M07/p210, whereas BCL-2 remains unchanged. The regulation of BCL-X closely parallels the induction of cell death. Rescue of Ba/F3/p185 after exposure to CGP 57148 with IL-3 abrogates both the down-regulation of BCL-X and the induction of apoptosis. In contrast, IL-3 is not capable of rescuing M07/p210 cells after inhibition of the BCR-ABL kinase, and it does not prevent down-regulation of BCL-X. We find a close correlation between the expression of BCL-X and the prevention of apoptosis by the BCR-ABL protein. These data confirm previous reports showing BCL-X regulation in cells overexpressing BCR-ABL. A recent report showed that expression of BCR-ABL in HL-60, Ba/f3, DAGM, and 3T3 cells induced up-regulation of BCL-X and conferred resistance to apoptosis in the HL-60 in a variety of agents tested (AR-C, Vp16, VM26, camptothecin, and actinomycin). Moreover, antisense oligonucleotides to BCL-X partially restored staurosporine-induced apoptosis to about 28%. However, this cytotoxic effect was less than that observed in the control HL-60 vector cells, suggesting that BCL-X is one of several targets mediating BCR-ABL-induced resistance to apoptosis. Accordingly, BCL-X overexpression caused a lower level of resistance to apoptosis com-

Fig. 8 Combinations of suboptimal concentrations of CGP 57148 (0.1 μM) with inhibitors of PI-3 kinase (LY), MEK (PD), and JAK-2 (AG) pathways do not have an additive effect on induction of apoptosis or BCL-X expression. A, induction of apoptosis in a TUNEL assay. B, Western blotting of BCL-X of Ba/F3/p185 after exposure to combinations of CGP 57148 (0.1 μM) with LY (5 μM), AG (20 μM), and PD (50 μM).
pared to BCR-ABL despite a higher level of expression of the BCL-X protein (9).

Thus far little is known about the regulation of BCL-X. Several intracellular pathways have been implicated. The IL-2-receptor has been shown to induce BCL-2 and BCL-X via the adapter molecule, Shc, promoting proliferation but not viability in a murine T-cell line (36). In Ba/F3 cells, R-Ras and insulin-like growth factor I showed synergistic inhibition of IL-3 withdrawal-induced apoptosis. Insulin-like growth factor I and R-Ras activated the ERK and the Akt kinase, respectively, and cooperatively induced BCL-X expression (37). In our experiments, the addition of a selective ERK kinase inhibitor or LY, a PI-3 kinase inhibitor, did not block BCR-ABL-mediated survival, and no decrease of BCL-X expression after exposure to CGP 57148 was observed.

Recently, the JAK kinase-dependent pathway has been shown to regulate BCL-X(L). 32D.3, FDC-P1.2, and primary fetal liver-derived myeloid cells were shown to down-regulate BCL-X(L) in protein and RNA levels after IL-3 deprivation. Furthermore, transfected cells with an erythropoietin receptor defective in activation of JAK2 (EpoR-PB) died when it was shifted to medium containing Epo, whereas transfectants harboring EGF receptor-JAK2 kinase domain chimeric receptor maintained viability in the absence of IL-3 but were activated with EGF. BCL-X levels decreased in the dying EpoR-PB cells and increased in EGF receptor-JAK2 transfectants activated with EGF, suggesting that the JAK kinase pathway plays a crucial role in the cytokine-regulated BCL-X regulation as cell death antagonist (38). In our experiments, a JAK kinase inhibitor did not induce cell death of Ba/F3/p185, and there was no decrease of BCL-X in response to CGP 57148.

Combined treatment with inhibitors of the RAS-ERK, the PI-3 kinase, the JAK, and a suboptimal dose of CGP 57148 also did not abolish the antiapoptotic effect of BCR-ABL. Moreover, the inhibitors were also not capable of down-regulating BCL-X in the Ba/F3/p185 cell lines. Therefore, direct inhibition of the BCR-ABL kinase is apparently the most effective approach to block the biological effects of the BCR-ABL protein. In addition, these experiments indirectly support the view that either unknown or redundant biochemical pathways are responsible for both the down-regulation of BCL-X and the antiapoptotic function of BCR-ABL.

The discrepant results of the experiments analyzing the effect of exogenous growth factors on BCR-ABL-positive cells are not clear. Whereas Ba/F3/p185 cells can be readily rescued by IL-3 after exposure to CGP 57148, neither IL-3 nor GM-CSF nor TPO rescued the M07/p210 cells. Both species differences of the biological activities of the BCR-ABL protein or CGP 57148 as well as differing clonal selection of the cell lines might be responsible for this phenomenon.

In conclusion, our experiments demonstrate that inhibition of the BCR-ABL kinase by CGP 57148 predominantly affects immature primary CML cells. In growth factor-dependent BCR-ABL-transfected cell lines, CGP 57148 induces apoptosis by activation of mitochondrial cytochrome c release, activation of caspase 3, and PARP cleavage. This proapoptotic effect is accompanied by down-regulation of the BCL-X protein. These experiments therefore further support the role of BCL-X as a target for the transforming function of BCR-ABL. However, a thorough understanding of both the mechanisms of the antiapoptotic function of BCR-ABL is essential for a further development of an effective strategy using CGP 57148 for treatment of CML.

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