Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells

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The *bcr-abl* oncogene, present in 95% of patients with chronic myelogenous leukemia (CML), has been implicated as the cause of this disease. A compound, designed to inhibit the Abl protein tyrosine kinase, was evaluated for its effects on cells containing the Bcr-Abl fusion protein. Cellular proliferation and tumor formation by Bcr-Abl-expressing cells were specifically inhibited by this compound. In colony-forming assays of peripheral blood or bone marrow from patients with CML, there was a 92–98% decrease in the number of *bcr-abl* colonies formed but no inhibition of normal colony formation. This compound may be useful in the treatment of *bcr-abl*-positive leukemias.

Chronic myelogenous leukemia (CML) is a malignancy of a pluripotent hematopoietic stem cell. The disease is characterized by a stable phase in which there is a massive expansion of myeloid lineage cells with full differentiative capabilities. Blast crisis ensues following an average of approximately three years as myeloid lineage cells lose the capacity for terminal differentiation, resulting in an acute leukemia.

Currently, allogeneic bone marrow transplantation is the only known curative therapy for CML. However, only 20–25% of patients with CML are eligible for allogeneic bone marrow transplantation because of age or lack of a suitable donor. α-Interferon has been reported to significantly prolong survival in CML (ref. 1–4), however, this is not a curative therapy. A major current focus in the treatment of CML is the use of autologous bone marrow transplantation. Although no adequate prospective randomized trials have been performed, there is a suggestion that autografting may prolong survival[5,6]. Attempts to improve the success of autologous transplantation are currently under investigation and include selection of benign hematopoietic precursors from CML patient's bone marrow or stem cells[7].

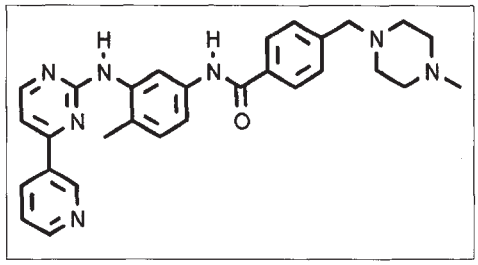
Chronic myelogenous leukemia is associated with a specific chromosomal translocation known as the Philadelphia (Ph) chromosome that is identifiable throughout the course of the disease[8,9]. The Ph chromosome is a somatic mutation that results from a reciprocal translocation between the long arms of chromosomes 9 and 22 and fuses genetic sequences on chromosome 22 (*bcr*) with c-*abl* sequences translocated from chromosome 9 (ref. 9–11). This t(9;22) translocation fuses *bcr* sequences upstream of the second exon of c-*abl* and generates one of two Bcr-Abl fusion proteins, p185 and p210. The p210 form of Bcr-Abl is seen in 95% of patients with chronic myelogenous leukemia and up to 20% of adult patients with *de novo* acute lymphocytic leukemia, whereas the p185 form is seen in approximately 10% of patients with acute lymphocytic leukemia[12,13]. Both of these chimeric proteins are capable of inducing leukemias in mice, implicating these proteins as the cause of these diseases[14–16].

As the tyrosine kinase activity of the Bcr-Abl proteins is known to be essential to their transforming abilities[17,18], a specific inhibitor of the Abl protein tyrosine kinase might be useful as a therapy for CML and other *bcr-abl*-positive leukemias. One of the first steps toward producing a useful drug was reported by Yaish *et al.*, who synthesized compounds, referred to as tyrphostins, that display specificity for individual tyrosine kinases[19]. These and other similar compounds and their potential uses in cancer and other diseases that involve tyrosine kinase-induced cellular proliferation have recently been reviewed[20]. One of the tyrphostins, capable of inhibiting the Abl protein tyrosine kinase, was reported to kill K562 cells[21,22]. We have produced a novel compound of the 2-phenylaminopyrimidine class that was selected for specificity against the Abl tyrosine kinase. This compound is more active against the Abl tyrosine kinase than other tyrosine kinase inhibitors previously reported, and we have extended the studies by Anafi *et al.*[22], by showing that not only are K562 cells killed by this compound, but cellular proliferation of Bcr-Abl-expressing cells was specifically inhibited by this compound with no effects on normal cells. Activity was also demonstrated *in vivo* against Bcr-Abl-induced tumors. Further, in colony-forming assays of peripheral blood or bone marrow from patients with CML, there was a 92–98% decrease in the number of *bcr-abl* colonies formed with no inhibition of normal colony formation. Thus, this compound may be useful in the treatment of *bcr-abl*-positive leukemias.

Design of CGP 57148

Using the known structure of the ATP binding site of protein kinases, a series of compounds of the 2-phenylaminopyrimidine class were synthesized and screened for the ability to inhibit a panel of protein kinases. CGP 57148 (Fig. 1) was found to be a potent inhibitor of the Abl protein tyrosine kinase that was identified from this screen. Inhibitors of this class act as competitive inhibitors of protein kinases with respect to ATP (ref. 23).

Fig. 1 Structure of CGP 57148.



In vitro profiling of CGP 57148

The ability of CGP 57148 to inhibit the *in vitro* kinase activity of a panel of purified protein tyrosine and serine/threonine kinases was assayed. Additional studies were performed by analyzing the effects of CGP 57148 on tyrosine phosphorylation induced by ligand binding to a transmembrane tyrosine kinase receptor (epidermal growth factor receptor, Her-2/neu, insulin receptor, insulin-like growth factor-1 receptor, and platelet-derived growth factor receptor) or by cellular expression of a tyrosine kinase oncoprotein (Bcr-Abl, v-Src, v-Fms). The concentration of compound resulting in a 50% reduction of kinase activity or a 50% reduction in tyrosine phosphorylation of the specific tyrosine kinase (IC_{50}) is reported. Results presented in Table 1 show that CGP 57148 is a potent inhibitor of the Abl protein tyrosine kinases with an IC_{50} of 0.038 μM *in vitro* for substrate phosphorylation by v-Abl, 0.025 μM for Bcr-Abl and c-Abl, and 0.25 μM in cell-based assays of autophosphorylation of v-Abl or Bcr-Abl (Table 1). There was no significant inhibition of other protein kinases tested with the exception of the platelet-derived growth factor (PDGF) receptor tyrosine kinase. There was no inhibition of Bcr-Abl protein expression by this compound (data not shown).

Table 1 Profile of inhibition of protein kinases by CGP 57148

Protein kinase	Substrate phosphorylation IC_{50} value (μM)	Cellular tyrosine phosphorylation IC_{50} value (μM)
v-Abl	0.038	0.25
Bcr-Abl	0.025	0.25
c-Abl	0.025	
EGFR-R-ICD	>100	>100
Her-2/neu		>100
Insulin receptor		>100
IGF-1R		>100
PDGF-R		0.3
c-Src	>100	
v-Src		>100
c-Fgr	>100	
c-Lyn	>100	
v-Fms		>100
TPK-IIB	>100	
PKA	>500	
PPK	>100	
PKC α, β1, β2, γ, ε, σ, η, ζ	>100	
Casein kinases - 1 and 2	>100	
cdc2/cyclin	>100	

Abbreviations: EGF-R-ICD, epidermal growth factor receptor–intracellular domain; IGF-1R, insulin like growth factor-1 receptor; TPK, tyrosine protein kinase; PKA, protein kinase A; PPK, phosphorylase kinase; PKC, protein kinase C.

Inhibition of cellular proliferation

Cellular expression of *bcr-abl* is known to render myeloid cell lines growth factor independent for proliferation[24–26]. The growth properties of factor-independent cell lines expressing $p210^{Bcr\text{-}Abl}$ (MO7p210 and 32Dp210) derived from a human factor-dependent megakaryocytic cell line (MO7e) and a murine factor-dependent cell line (32Dcl3) were assayed in the presence or absence of growth factor and in the presence or absence of CGP 57148. Incubation with up to 10 μM CGP 57148 resulted in no inhibition of growth of the parental cells in the presence of exogenous growth factor (Fig. 2, *a* and *b*). This is consistent with our observation that we see no inhibition of tyrosine phosphorylation induced by granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin-3 (IL-3) in these cell lines (data not shown). However, incubation of the MO7p210 cells with a concentration of CGP 57148 of 1 or 10 μM resulted in cell death in the presence or absence of exogenous growth factor (Fig. 2*a*). Although 32Dp210 cells were killed in the absence of growth factor, addition of IL-3 partially rescued these cells from the effects of CGP 57148. However, there remained a significant inhibition of proliferation of the 32Dp210 cells even in the presence of IL-3, and increased numbers of cells were observed to be killed under these conditions, as compared to controls, as assessed by trypan blue viability and assays for apoptosis (data not shown). CGP 57148 also induces erythroid differentiation and cell death of K562 cells, a human *bcr-abl*-positive cell line (Fig. 3). The proliferation of a derivative of 32Dcl3 cells that express v-Src was not affected by incubation with either 1 or 10 μM CGP 57148 (Fig. 2*d*). Thus, the compound appears to be selectively toxic to cells expressing the constitutively active Bcr-Abl protein tyrosine kinase.

Inhibition of tumor formation

32Dcl3 cells expressing Bcr-Abl or v-Src are capable of forming tumors in syngeneic C3H/HEJ mice. The ability of CGP 57148 to inhibit tumor formation was tested following inoculation of C3H/HEJ mice with 32Dp210 or 32Dv-Src cells. Tumors typically developed within one week of inoculation of 5×10^6 cells subcutaneously. Figure 4 is representative of three separate experiments that demonstrate a dose-dependent inhibition of tumor growth of Bcr-Abl-inoculated animals treated with CGP 57148, with no effects on animals inoculated with v-Src-expressing cells.

Inhibition of colony formation

To characterize these inhibitors further, their effects were analyzed in assays of *in vitro* hematopoietic colony formation of peripheral blood and bone marrow. Colony-forming assays allow an assessment of committed progenitors in the sample that are capable of forming erythroid colonies in the presence of granulocyte colony-stimulating factor (G-CSF) and erythropoietin (BFU-E) or granulocyte-macrophage colonies in the presence of IL-3 and G-CSF (CFU-GM). Bone marrow samples from patients undergoing bone marrow transplantation for a variety of nonleukemic disorders were incubated with 1 μM of CGP 57148. There was no inhibition of colony formation at this dose (Fig. 5). At a dose of 10 μM, colony formation of normal bone marrow was inhibited by approximately 15–20% (data not shown). In contrast, when peripheral blood or bone marrow was obtained from CML patients known to have the t(9;22) chromosomal translocation, a 60–80% inhibition of colony formation of com-

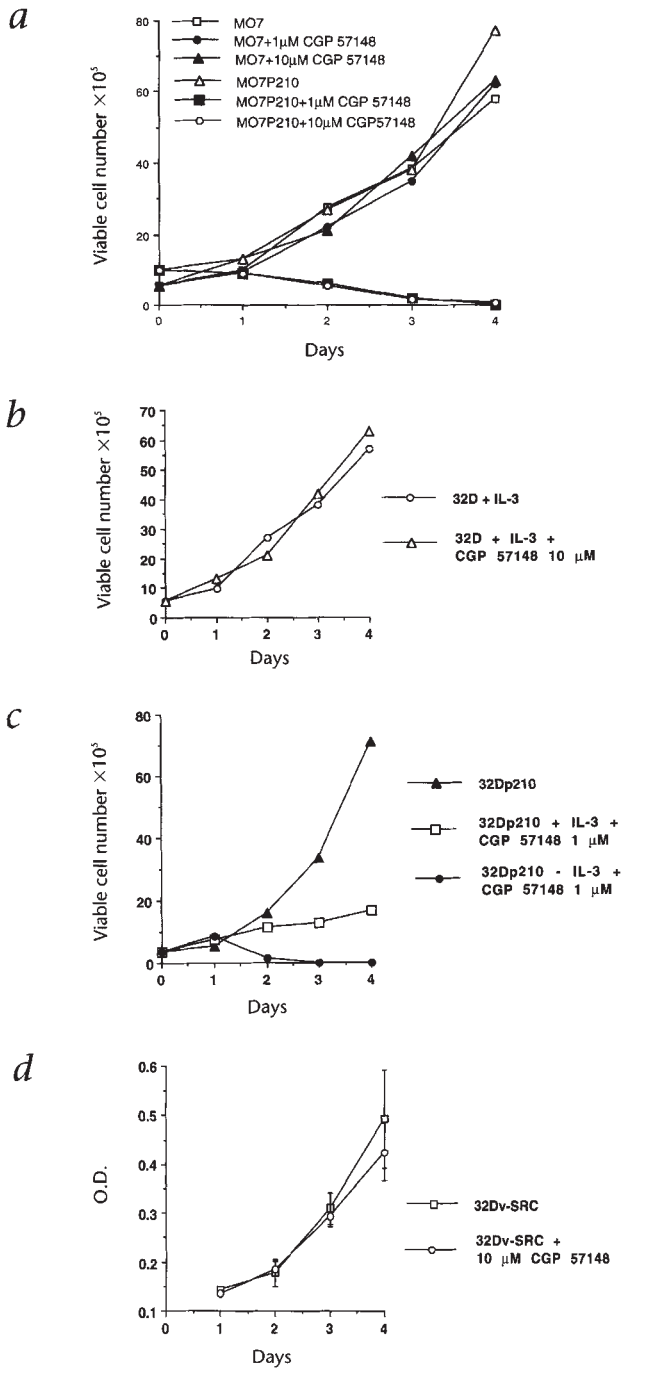


Fig. 2 Cell count and MTT assays. Cells were plated in the presence of regular growth medium with or without exogenous growth factor or the indicated amount of inhibitor. Each day, viable cells were counted as assessed by exclusion of trypan blue. *a*, MO7e and MO7p210 cells. GM-CSF and IL-3 were included in all cultures presented in this portion of the figure. Similar data have been obtained using MO7p210 cells incubated in the absence of growth factor (data not shown). *b*, 32Dcl3 cells. *c*, 32Dp210 cells in the presence or absence of 15% WEHI-3B-conditioned medium with the indicated amount of inhibitor. *d*, 32Dv-src cells were plated in quadruplicate in the presence of regular growth medium without exogenous growth factor and the indicated amount of inhibitor. Wells were assayed for uptake of MTT at daily intervals, and results are expressed as the mean *A* plus or minus the standard deviation.

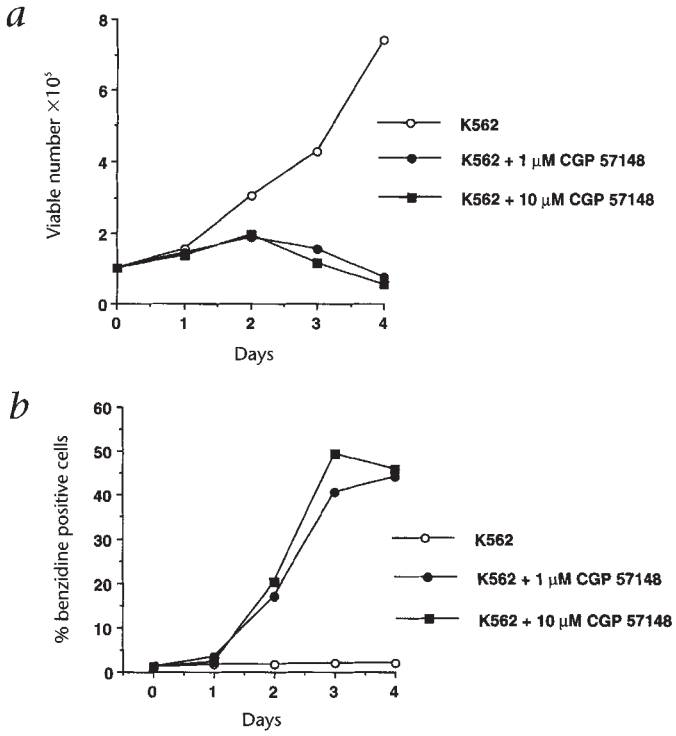


Fig. 3 Effects of CGP 57148 on K562 cells. *a*, Cell count assays were performed as described in the Methods section and the legend to Fig. 2. *b*, An aliquot of the cell culture was assessed daily by staining with 0.2% benzidine and the percentage of benzidine-positive cells are reported.

mitted progenitor cells was seen by incubation with CGP 57148 at a concentration of 1 μM (Fig. 5). When colonies that formed were assayed for the presence of *bcr-abl* transcripts by reverse-transcriptase polymerase chain reaction (RT-PCR), 92–96% of the colonies formed in the absence of inhibitor contained *bcr-abl*. However, of the colonies that formed in the presence of 1 μM CGP 57148, less than 20% of the colonies contained *bcr-abl* transcripts. This is represented in Fig. 5 as the solid portion of the graph of CML colonies. Combining this data with the 60–80% inhibition of colony formation, there was an overall decrease of *bcr-abl*-positive colonies of 92–98%. Interestingly, one Ph chromosome-negative CML patient, who is also negative for *Bcr-abl* transcripts by PCR, had no inhibition of colony formation by this compound.

Discussion

In this study, we have outlined the preclinical studies of a compound designed specifically for the treatment of *bcr-abl*-positive leukemias. A compound of the 2-phenylaminopyrimidine class was synthesized that was based on the structure of the ATP binding site of protein kinases, and a series of structurally modified congeners were produced. CGP 57148 was found to be a potent inhibitor of the Abl protein tyrosine kinase identified from this screen. Although several tyrosine kinase inhibitors have been demonstrated to inhibit Bcr-Abl-induced proliferation[22,27], little information regarding their specificity for Bcr-Abl-expressing cells has been presented. In this study, we have demonstrated that CGP 57148 selectively inhibits the proliferation of Bcr-Abl-expressing cells *in vitro* and *in vivo*.

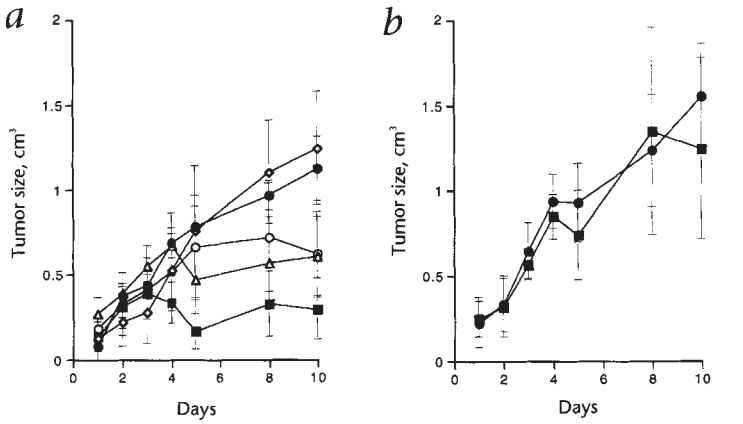


Fig. 4 *In vivo* antitumor activity of CGP 57148. *a*, 32Dp210 tumors. *b*, 32Dv-src tumors. Results are presented as mean tumor volume (± s.d.) in cubic centimeters. A Kruskal-Wallis test[52] demonstrated a significant difference at $P < 0.05$ beginning on day 5 and persisting through day 10. A Dunn's post test[53] on the day 10 data demonstrated a significant difference, $P < 0.05$, comparing placebo-treated control animals to treatment with 10 or 25 mg/ml of CGP 57148 and a $P < 0.01$ comparing animals treated with 50 mg/ml to controls.

In vitro profiling of this compound demonstrated specificity for the Abl protein kinase with an IC_{50} of 0.025–0.038 μM for substrate phosphorylation by v-Abl, Bcr-Abl, and c-Abl. In cell-based assays of autophosphorylation the IC_{50} was 0.25 μM. Although the *in vitro* profiling of CGP 57148 demonstrated a high degree of specificity, the cellular proliferation assays demonstrated that other kinases that might be required for cellular growth and proliferation are not inhibited by this compound. Although *abl* knock-out mice have decreased neonatal viability and lymphopenia[28], there is no clear evidence that c-*abl* is required for cellular proliferation and overexpression of c-*abl* has suggested that it may negatively regulate cellular proliferation[29]. As we see no inhibition of normal cellular proliferation at doses of this compound that inhibit c-Abl protein tyrosine kinase activity, our data are consistent with the lack of a requirement for c-Abl tyrosine kinase activity for cellular proliferation. The lack of inhibition of IL-3-dependent proliferation of the parental cells suggests that this compound does not inhibit tyrosine kinases, such as Jak-2, that are known to be required for IL-3-induced mitogenesis[30]. Although we have not tested CGP 57148 against Jak kinases specifically, we have seen no inhibition of tyrosine phosphorylation induced by either IL-3 or GM-CSF (data not shown).

CGP 57148 was capable of selecting for the growth of benign hematopoietic progenitor cells from CML patients, with this compound having little or no effect on normal hematopoiesis. Previous studies have demonstrated that antisense oligonucleotides to c-*abl* inhibit colony formation by committed progenitor cells[31]. The difference between our findings and the report using an antisense oligonucleotide strategy to target c-*abl* could reflect nonspecific toxicity of the antisense constructs, a kinase-independent function of c-Abl in supporting hematopoietic colony formation, or could represent an effect of the compound on endogenous c-Abl protein kinase. In any case, our data demonstrate that CGP 57148 has little effect on the ability of normal marrow to form colonies at a concentration of up to 10 μM, while selecting against the growth of Bcr-Abl-expressing hematopoietic colonies.

Inhibition of the Bcr-Abl protein tyrosine kinase did not simply cause cells to revert to factor-dependent growth. However, this effect was more pronounced in MO7p210 cells than 32Dp210 cells. Previous studies have shown that expression of antisense oligonucleotides to Bcr-Abl in cells rendered factor independent by Bcr-Abl, cause these cells to revert to factor dependence[32]. One possible explanation for these data is that with the tyrosine kinase inhibitor, Bcr-Abl protein is still present, but kinase inactive, whereas in the antisense experiment, Bcr-Abl protein levels decline. As kinase inactive Bcr-Abl is known to bind to cellular proteins such as CRKL and others[33], this suggests that the discrepancy could be due to kinase-inactive Bcr-Abl complexing with a protein or proteins that are required for IL-3-induced mitogenesis, thereby not allowing the protein or proteins to be available for the IL-3 signaling pathway. In this scenario, the difference between the two cell lines could either be differences in the level of expression of Bcr-Abl or in the expression of a protein required for IL-3 signaling. As several subclones of each line with overlapping levels of Bcr-Abl expression yielded similar results, the latter explanation seems more likely.

Regardless, in MO7p210 cells, exposure to CGP 57148 resulted in cell death that could not be rescued by IL-3. This suggests that Bcr-Abl may be responsible for more than simply growth factor independence in these cells. Another possible explanation for our inability to rescue cells with growth factor include a reported role for Bcr-Abl in the prevention of programmed cell death[34,35]. In this case, inhibition of Bcr-Abl tyrosine kinase activity would allow programmed cell death to proceed. Consistent with this notion, treatment of Bcr-Abl-expressing cells with CGP 57148 results in these cells undergoing programmed cell death (data not shown). However, as IL-3 is known to prevent apoptosis, we would have to postulate that Bcr-Abl exerts a dominant negative effect on the IL-3 antiapoptotic pathway, such that IL-3 is unable to rescue these cells.

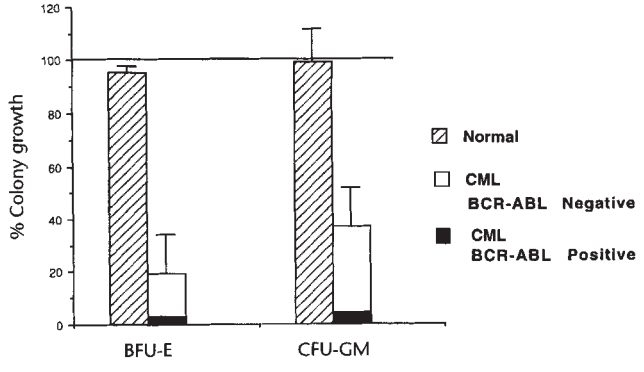


Fig. 5 Colony-forming assays. Results are presented as the number of colonies formed with the line at 100% representing the average number of colonies formed without inhibitor and the columns represented the average numbers of colonies formed in the presence of inhibitor. Error bars represent standard deviations. The solid boxes represent the average number of *bcr-abl* positive colonies formed in the presence of inhibitor as assessed by RT-PCR.

The only other kinase that we have found thus far to be inhibited by CGP 57148 is the PDGF receptor tyrosine kinase. PDGF has been suggested to be a mediator of pathological cellular growth in tumors such as glioblastoma and nonmalignant proliferative diseases such as atherosclerosis and fibrosis[20,36]. Thus, this compound could also be useful in diseases that involve abnormal PDGF receptor activation. It is also possible that inhibition of the PDGF receptor could prove detrimental to processes such as wound healing.

The intended use of this compound would be for the *in vivo* therapy of CML and other *bcr-abl*-positive leukemias. In support of this intended use, preliminary data have shown little, if any, toxicity in animals. However, if this compound proves too toxic *in vivo*, another possible use would be *in vitro* purging of Bcr-Abl-expressing cells for use in autologous bone marrow transplantation. A variety of techniques have been proposed and are being tested to eliminate Bcr-Abl-expressing cells from patients with CML for use in this procedure[7]. Additional studies are in progress to determine whether this compound can successfully purge bone marrow or peripheral stem cells of Bcr-Abl-expressing cells in preparation for clinical trials using this compound in combination with autologous bone marrow transplantation.

This compound serves as an example of a drug that was rationally designed to inhibit the function of a specific protein when the protein's function was known to be involved in the pathogenesis of a specific disease state. It is hoped that by directing therapy toward the underlying disease mechanism, this will result in more effective and less toxic therapies. Ultimately, clinical trials will be required to determine the potential therapeutic benefit of this compound.

Methods

Antibodies and reagents. Highly purified recombinant GM-CSF and IL-3 were gifts of G. Segal, Oregon Health Sciences University. The antiphosphotyrosine antibody 4G10 was generated using phosphotyramine as the immunogen and was used as described[37]. The anti-Abl monoclonal antibody 24-21 was a gift from N. Rosenberg, New England Medical Center, Boston, Massachusetts. Other reagents were obtained from Sigma Chemical Company unless otherwise indicated. A stock solution of CGP 57148 at a concentration of 10 mM was prepared by dissolving the compound in sterile PBS and was diluted in tissue culture medium before use.

Cells and cell lines. The 32Dcl3 cell line is a murine myeloid cell line that is dependent on IL-3 for proliferation and was obtained from J. Greenberger, University of Massachusetts Medical Center, Worcester, Massachusetts[38]. Cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS (UBI, Lake Placid, New York) and 15% WEHI-3B-conditioned medium as a source of IL-3. MO7e cells are from a human megakaryocytic leukemia cell line that requires GM-CSF, IL-3 or steel factor for proliferation[39] and were obtained from J. Griffin, Dana-Farber Cancer Institute, Boston, Massachusetts. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 10 ng/ml of GM-CSF and 10 ng/ml of IL-3.

Derivatives of these cell lines expressing $p210^{Bcr\text{-}Abl}$ were created by electroporation with a retrovirus, pGD containing a full-length *bcr-abl* cDNA as described[25,40]. 32Dp210 and MO7p210 cells are factor independent for proliferation and were grown in RPMI 1640 medium supplemented with 10% FBS. 32Dcl3 cells expressing v-Src were obtained courtesy of S. Anderson, University of Colorado[41].

Proliferation and differentiation assays. *Cell count assays.* Cells were plated at a density of 2×10^5 cells per milliliter in RPMI 1640 medium with 10% FBS with or without growth factors. Inhibitor was added at various concentrations; controls using an identical dilution of buffer in which the inhibitor had been dissolved were also performed. Viable cells were counted, as assessed by exclusion of trypan blue, at 24-h intervals.

MTT assays. Cells (5×10^3 to 2×10^4) were plated per well in quadruplicate in RPMI 1640 medium with 10% FBS with or without growth factors and various concentrations of the inhibitor. Controls were performed using an identical dilution of buffer in which the inhibitor had been dissolved. Wells were assayed for uptake of MTT at daily intervals as described[42]. One plate per day contained serial dilutions of cells to ensure that the assay remained linear with respect to the cell concentration used. Results are expressed as the mean of the absorbance (A) at 570 nm, plus or minus the standard deviation.

Differentiation of K562 cells. The differentiation of K562 cells was assessed by staining with 0.2% benzidine as previously described[43].

***In vitro* assays of protein kinase inhibition.** Purification of protein kinases and *in vitro* enzyme assays were performed as previously described[44–46]. The concentration of compound resulting in a 50% reduction of kinase activity (IC_{50}) is reported. c-Abl kinase assays were performed on immunoprecipitates of c-Abl from lysates of Cos-7 cells transfected with a full-length c-*abl* or in vitro translated c-*abl* (gifts of C. Sawyers). *In vitro* kinase assays were performed as described without exogenous substrate[47] or in the presence of GST-CRK II as described[48]. Cell-based assays were performed as described[44,45] or cells expressing activated tyrosine kinases were incubated in the presence of various concentrations of inhibitor. Following a 1.5-hour incubation, cells were lysed and equal amounts of cellular lysates were analyzed by immunoblotting with a monoclonal anti-phosphotyrosine antibody, 4G10, as described[37]. Gels were scanned using a laser densitometer to quantify the signal intensity. Serial dilutions of a cellular lysate were performed to ensure that densitometric measurements were in a linear range. The concentration resulting in a 50% reduction in tyrosine phosphorylation of the specific tyrosine kinase is reported. IC_{50} values are the average of at least three separate determinations.

***In vivo* antitumor activity.** Syngeneic C3H/HEJ mice, four per group, were injected in the left flank with 5×10^6 32Dp210 or 32Dv-Src cells. Beginning on day 8 and continuing for 10 days, mice were injected intraperitoneally daily with various amounts of CGP 57148 dissolved in sterile saline or an equal volume of sterile saline. Tumor growth was monitored by daily measurements of perpendicular tumor diameters. Tumor volumes were calculated as described[49] using the formula $(\pi \times L \times D^2/6)$.

Colony-forming assays. Normal bone marrow or CML bone marrow or peripheral blood samples were assayed for colony formation as described[50], either without inhibitor or in the presence of 1 μM CGP 57148. Assays were performed on six normal bone marrow samples and seven CML patients, of which five patients were in the stable phase of the disease and two patients in accelerated phase or blast crisis. Assays of each patient were performed in triplicate. Individual colonies from CML patients arising in the CFU-GM and BFU-E assays were picked with a pipette. RT-PCR was performed as described[51] with half of the RNA incubated with *bcr-abl*-specific primers and the other half with *abl* primers, including a 5' primer from *abl* exon 1a, as a control for the quality of the RNA preparation. Only two samples were negative for c-*abl* and were excluded from this analysis.

Acknowledgments

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