

The Tyrosine Kinase Inhibitor CGP57148B Selectively Inhibits the Growth of BCR-ABL-Positive Cells

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The Philadelphia chromosome found in virtually all cases of chronic myeloid leukemia (CML) and in about one third of the cases of adult acute lymphoblastic leukemia is formed by a reciprocal translocation between chromosomes 9 and 22 that results in the fusion of BCR and ABL genetic sequences. This BCR-ABL hybrid gene codes for a fusion protein with deregulated tyrosine kinase activity that can apparently cause malignant transformation. CGP57148B, a 2-phenylaminopyrimidine derivative, has been shown to selectively inhibit the tyrosine kinase of ABL and BCR-ABL. We report here that this compound selectively suppresses the growth of colony-forming unit-granulocyte/macrophage (CFU-GM) and burst-forming unit-ery-

OVER NINETY PERCENT of cases of chronic myeloid leukemia (CML)¹ and 10% to 25% of cases of adult acute lymphoblastic leukemia (ALL)² are associated with a reciprocal translocation between chromosomes 9 and 22 that produces a BCR-ABL fusion gene. Depending on the breakpoint in BCR, one of three types of fusion proteins is generated: P210^{BCR-ABL} is characteristic of CML and also occurs in about one third of BCR-ABL-positive ALL; P190^{BCR-ABL} is found in two thirds of BCR-ABL-positive ALL; and P230^{BCR-ABL} is associated with the very rare Philadelphia chromosome (Ph)-positive chronic neutrophilic leukemia.^{3,4} All three BCR-ABL fusion proteins exhibit deregulated tyrosine kinase activity compared with the native ABL protein (P145^{ABL}).^{3,5}

During the chronic phase, CML is usually readily controlled by cytotoxic drugs, mainly hydroxyurea (HU) and busulfan. However, eventually, the disease transforms into an acute phase that is rapidly fatal. Treatment options in CML are still limited because only a minority of patients are eligible for allogeneic bone marrow transplantation, the only curative treatment known to date.⁶ Recently, treatment with interferon alfa has been shown to restore Ph-negative hematopoiesis in a subset of patients; whether it generally offers a survival advantage compared with HU is at present unclear.^{7,8}

Targeting the tyrosine kinase activity of BCR-ABL appears to be an attractive therapeutic strategy, since this activity is considered essential for malignant transformation.⁹ Selective inhibition of the BCR-ABL tyrosine kinase was obtained with AG1112, a member of the tyrphostin compounds,^{10,11} and herbimycin A, an antibiotic derived from *Streptomyces* species.¹² More recently, CGP57148B, a 2-phenylaminopyrimidine derivative, was reported to inhibit the tyrosine kinase of ABL and BCR-ABL with high selectivity¹³; only the tyrosine kinases of PDGF β ¹³ and *c-kit* are inhibited at similarly low concentrations (IC₅₀ = 0.25 μ mol/L for BCR-ABL autophosphorylation). The data indicated activity against BCR-ABL-positive cell lines and a 90% reduction of BCR-ABL-positive colonies formed by mononuclear cells (MNCs) derived from chronic-phase CML.¹³ However, only two concentrations of CGP57148B (1 and 10 μ mol/L) were used in the clonogenic assays, and apart from two cell lines transfected with P210^{BCR-ABL}, only one cell line (K562) was studied that naturally expresses BCR-

throid derived from CML over a 2-logarithmic dose range with a maximal differential effect at 1.0 μ mol/L. However, almost all CML colonies that grow in the presence of 1.0 μ mol/L CGP57148B are BCR-ABL-positive, which may reflect the fact that residual normal clonogenic myeloid precursors are infrequent in most patients with CML. We also studied the effects of CGP57148B on hematopoietic cell lines. Proliferation was suppressed in most of the BCR-ABL-positive lines; all five BCR-ABL-negative lines were unaffected. We conclude that this new agent may have significant therapeutic applications.

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ABL. In the present study, we analyzed in detail the differential effect of graded concentrations of CGP57148B on the clonogenic capacity of CML and normal hematopoietic progenitor cells to determine the optimal therapeutic window. We investigated whether the effect of CGP57148B on CML was mediated by the presence of regulatory cells in the plastic-adherent fraction of MNCs. We also studied the effect of CGP57148B, the JAK2 inhibitor AG490,¹⁴ and the HER2-Neu inhibitor AG879¹⁵ on the proliferation pattern of a number of BCR-ABL-positive and -negative cell lines.

SUBJECTS AND METHODS

Patients. Nine patients with CML in chronic phase (one at diagnosis and eight on HU, two in second chronic phase after relapse postallograft) and three normal individuals were studied in the first series of experiments (clonogenic assays at 1.0 μ mol/L). BCR-ABL status in individual colonies grown in the presence of 1.0 μ mol/L CGP57148B was studied in five of these patients and in three additional patients (one at diagnosis, one on HU, and one off HU at the time of study). Six CML patients in chronic phase (three at diagnosis, two on HU, and one off HU at the time of study) and six normal individuals were investigated in the clonogenic assays at graded concentrations of CGP57148B. All patients and normal individuals provided informed consent for these studies.

Tyrosine kinase inhibitors. CGP57148B¹⁶ was kindly provided by Novartis (Basel, Switzerland). AG490 and AG879 were purchased from Alexis Ltd (Bingham, UK). A 10-mmol/L stock solution of CGP57148B in distilled water was prepared, from which a series of dilutions were made. Stock solutions in dimethylsulfoxide were prepared from AG490 and AG879, from which fresh working solutions were prepared in RPMI medium before the experiments.

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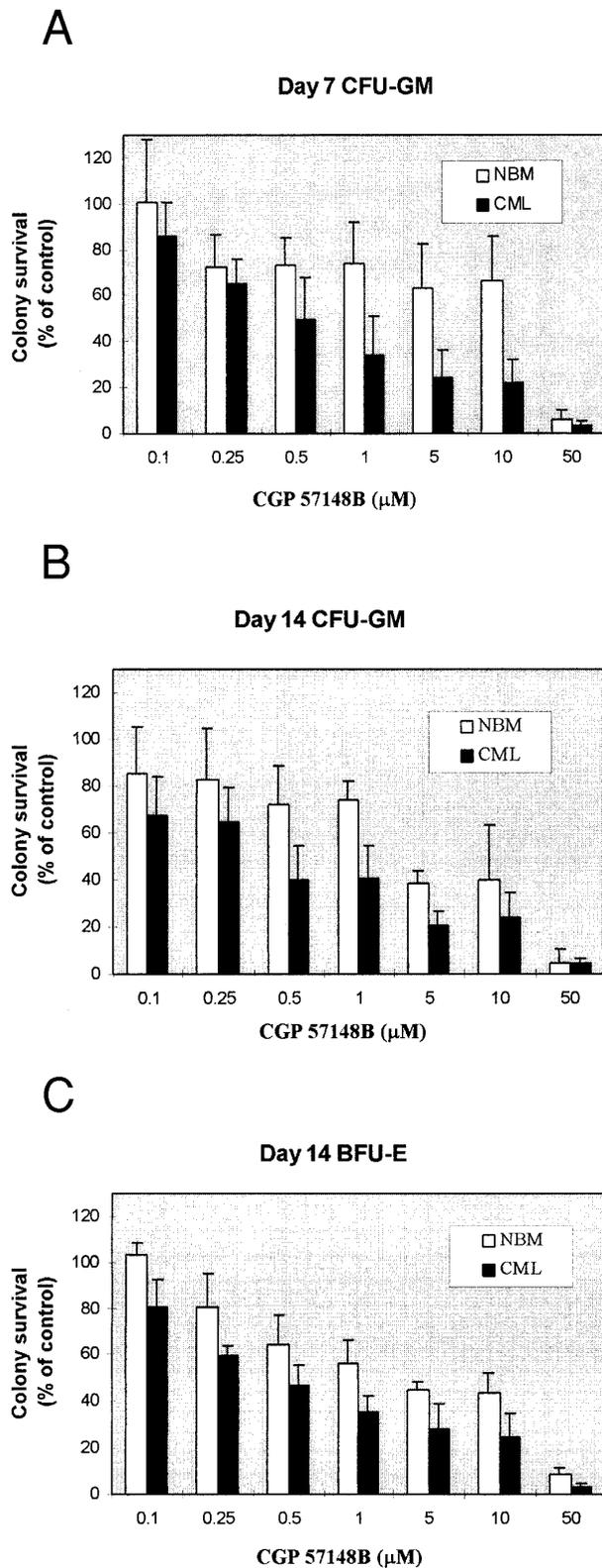


Fig 1. Effect of increasing concentrations of CGP57148B on formation of colonies derived from NBM (n = 6) and CML (n = 6). (A) Day 7 CFU-GM; (B) day 14 CFU-GM; (C) day 14 BFU-E. Vertical bars show 1 SD.

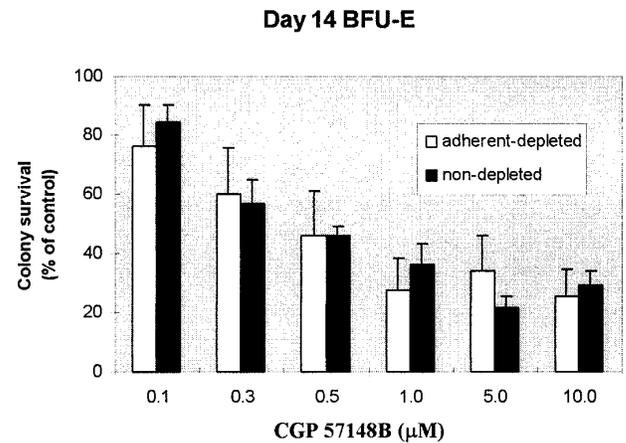


Fig 2. Effect of increasing concentrations of CGP57148B on formation of day 14 BFU-E derived from CML in adherent-depleted v nondepleted MNCs. Vertical bars show 1 SD.

Clonogenic assays. MNCs were separated on Lymphoprep (Nycomed, Oslo, Norway) from fresh or cryopreserved peripheral blood leukocytes or fresh normal bone marrow (NBM). For one experiment, CD34⁺ cells were separated from MNCs using a MiniMACS selection column, following the instructions of the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). For some of the experiments, MNCs were depleted of plastic-adherent cells by a 2-hour incubation at 37°C in α -MEM (GIBCO, Paisley, UK) supplemented with 15% fetal calf serum. Depending on the experiment, MNCs were then plated at 1×10^5 cells/mL in either (1) methylcellulose with 10% agar leukocyte-conditioned medium (LCM) and erythropoietin (Methocult H4431; Stemcell Technologies Inc, Vancouver, Canada) or (2) Iscoves' methylcellulose medium (Methocult H4430; Stemcell Technologies Inc) supplemented with 5 ng/ml recombinant human interleukin-3 (rhIL-3) (First Link UK Ltd, Wolverhampton) and 100 U/mL recombinant human granulocyte colony-stimulating factor (rhG-CSF) (Amgen, Thousand Oaks, CA) for growth of colony-forming unit-granulocyte/macrophage (CFU-GM) and 5 ng/mL rhIL-3 and 1.5 U/mL (rh)erythropoietin (R&D Systems, Abingdon, UK) for growth of burst-forming unit-erythroid (BFU-E). CGP57148B was added to the methylcellulose at the required concentration. All clonogenic assays were made in duplicate or triplicate. CFU-GM and BFU-E assays were scored under an inverted microscope. The scoring criteria for CFU-GM were at least 50 cells per colony on day 7, and at least 200 cells per colony on day 14. Counting was blinded for both doses of CGP57148B and type of sample (CML or normal). On day 14, individual and well-separated colonies were plucked with a micropipette fitted with a sterile plugged tip. A droplet of methylcellulose from an area without cells was plucked as a control. For reverse transcription-polymerase chain reaction (RT-PCR), the cells were transferred directly into 500 μ L guanidinium thiocyanate solution and stored at -20°C until processed further. To prepare cytopins for fluorescence in situ hybridization (FISH), the colonies were transferred into 100 μ L phosphate-buffered saline (PBS) plus 1% bovine serum albumin and cytocentrifuged at 400 rpm for 10 minutes. The slides were then stored at -20°C until analyzed by FISH. Colonies studied with both methods were first dispersed in 40 μ L PBS, half of which was used for FISH and half for RT-PCR.

FISH. After defrosting, the slides were incubated in methanol/glacial acetic acid (3:1) for 80 minutes at room temperature and, after a brief dip in 70% acetic acid, allowed to air-dry for at least 4 hours. This was followed by incubations in $2\times$ SSC (three times

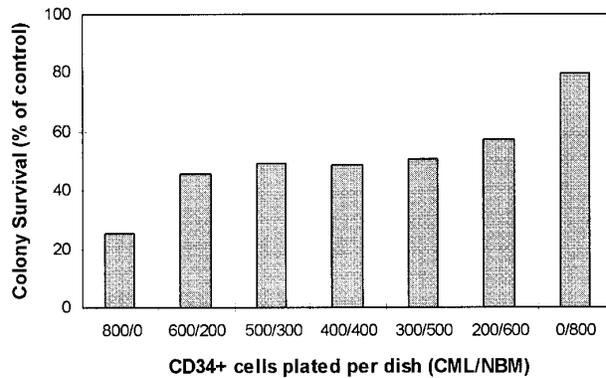


Fig 3. Formation of day 14 CFU-GM by CD34⁺ cells derived from CML and NBM mixed in various proportions in the presence and absence of 1.0 μ mol/L CGP57148B.

for 5 minutes at room temperature), 0.1 μ g/mL RNase (1 hour at 37°C in a moist chamber), 2 \times SSC (three times for 5 minutes at room temperature), 0.01 mol/L HCl (5 seconds), 0.1 mg Pepsin/mL 0.01-mol/L HCl (20 minutes at 37°C in a moist chamber), PBS (three times for 5 minutes at room temperature), 1% paraformaldehyde in PBS (10 minutes at room temperature), and PBS (three times for 5 minutes at room temperature). The cytopins were then dehydrated in ethanol (3 minutes' incubation in 70%, 90%, and 100%). The procedure for denaturation and hybridization followed the protocol suggested by the manufacturer of the specific probes (Vysis, Inc, Downers Grove, IL), with minor modifications. After counterstaining with DAPI (Sigma, Poole, UK), the slides were analyzed with a fluorescence microscope. Slides were scored as informative when at least four cells were analyzable (intact nucleus, no additional signals, and no signals lacking).

RT-PCR. RNA extraction and reverse transcription were performed as described previously.¹⁷ The reaction volume was either 40 μ L (44 colonies) or 20 μ L (160 colonies). Nested PCR amplifications for BCR-ABL and BCR were performed separately as described by Cross et al¹⁸ and Diamond et al.¹⁷ respectively. Five microliters of the second-step product were analyzed on an ethidium bromide-stained 2% agarose gel.

The cDNA was equally divided for replicate amplifications of BCR-ABL and BCR, comprising two or four independent PCR for each type of transcript depending on the reaction volume of the reverse transcription. Colonies with no amplification for either gene were scored as noninformative. Extensive precautions were taken to minimize the risk of false-positive results, as described elsewhere.¹⁹ All "blanks" included at the steps of colony plucking, RNA extraction, and cDNA synthesis were tested for BCR-ABL in the same way as true colonies, ie, two or four times depending on the volume of the reverse transcription reaction.

Table 2. Day 14 Colonies Grown at 1.0 μ mol/L CGP57148B Investigated by Both FISH and RT-PCR

Parameter	CGP57148B		Control	
	No.	%	No.	%
Colonies studied	81		49	
Informative by FISH	64	79.0	38	77.6
Informative by PCR	68	83.9	38	77.6
Informative by FISH and PCR	52	64.2	32	65.3
Pos for BCR-ABL (FISH and PCR)	49	94.2	29	90.6
PCR neg, FISH pos	2	3.8	3	9.4
PCR pos, FISH neg	1	1.9	0	

Abbreviations: Pos, positive; Neg, negative.

Cell line assays. Ten BCR-ABL-positive cell lines were used in this study: K562, KYO1, KU812, LAMA84, EM3, KCL22, BV173, AR230, TOM1, and SD1. The first eight cell lines were established from CML patients in blast crisis, whereas the latter two lines originated from Ph-positive ALL patients. Five Ph-negative hematopoietic cell lines were also analyzed: KG1 (acute myeloblastic leukemia), HL60 (promyelocytic leukemia), U937 (histiocytic lymphoma), Jurkat (acute T-lymphoblastic leukemia), and RPMI 8226 (multiple myeloma). The Ph status of all of these cell lines was confirmed by FISH. All cell lines were grown in RPMI 1640 medium supplemented with penicillin, streptomycin, L-glutamine, and 10% fetal calf serum. One day after subculturing, the cells were plated at 0.2 to 0.5 $\times 10^6$ /mL and the tyrosine kinase inhibitors were added to the medium at the required concentration. In one experiment, BV173 cells were preincubated with cycloheximide (Sigma) for 1 hour followed by addition of CGP57148B to the medium. Cell count and viability (by trypan blue dye exclusion) were determined at 24- or 48-hour intervals. All experiments were performed at least twice. When indicated by a change in the color of the supernatant, half of the cell suspension was replaced by fresh medium with or without inhibitor.

DNA fragmentation. An aliquot of 2 $\times 10^6$ cells from the cell line cultures was lysed in 0.4 mL 0.6% sodium dodecyl sulfate/10-mmol/L EDTA. Upon addition of 0.1 mL 5-mol/L NaCl, the lysate was mixed by gentle inversion and incubated at 4°C for at least 12 hours, followed by centrifugation at 13,000 rpm for 30 minutes at 4°C. The DNA was purified from the supernatant by standard phenol-chloroform extraction and ethanol precipitation, dissolved in 10 μ L TE buffer (10-mmol/L Tris, pH 8, and 1-mmol/L EDTA), and electrophoresed on a 2% agarose gel containing 0.5 μ g/mL ethidium bromide and 12.5 μ g/mL RNase A (Sigma).

Statistical analysis. Colony survival at a given concentration of CGP57148B was expressed as a percentage of the control (mean \pm

Table 1. Results of RT-PCR and FISH Performed on Day 14 Colonies Grown at 1.0 μ mol/L CGP57148B

Test	Tested	CGP57148B				Control				
		Informative		BCR-ABL-positive		Informative		BCR-ABL-positive		
		No.	%	No.	%	No.	%	No.	%	
PCR only	54	34	62.9	29	85.3	9	6	66.7	5	83.3
FISH only	11	8	72.7	8	100	—	—	—	—	—
PCR + FISH	81	76	93.8*	74	97.4*	49	45	91.8*	44	97.8*
Total	146	118	80.8	111	94.1	58	51	87.9	49	96.1

* Results informative by either FISH or RT-PCR or both.

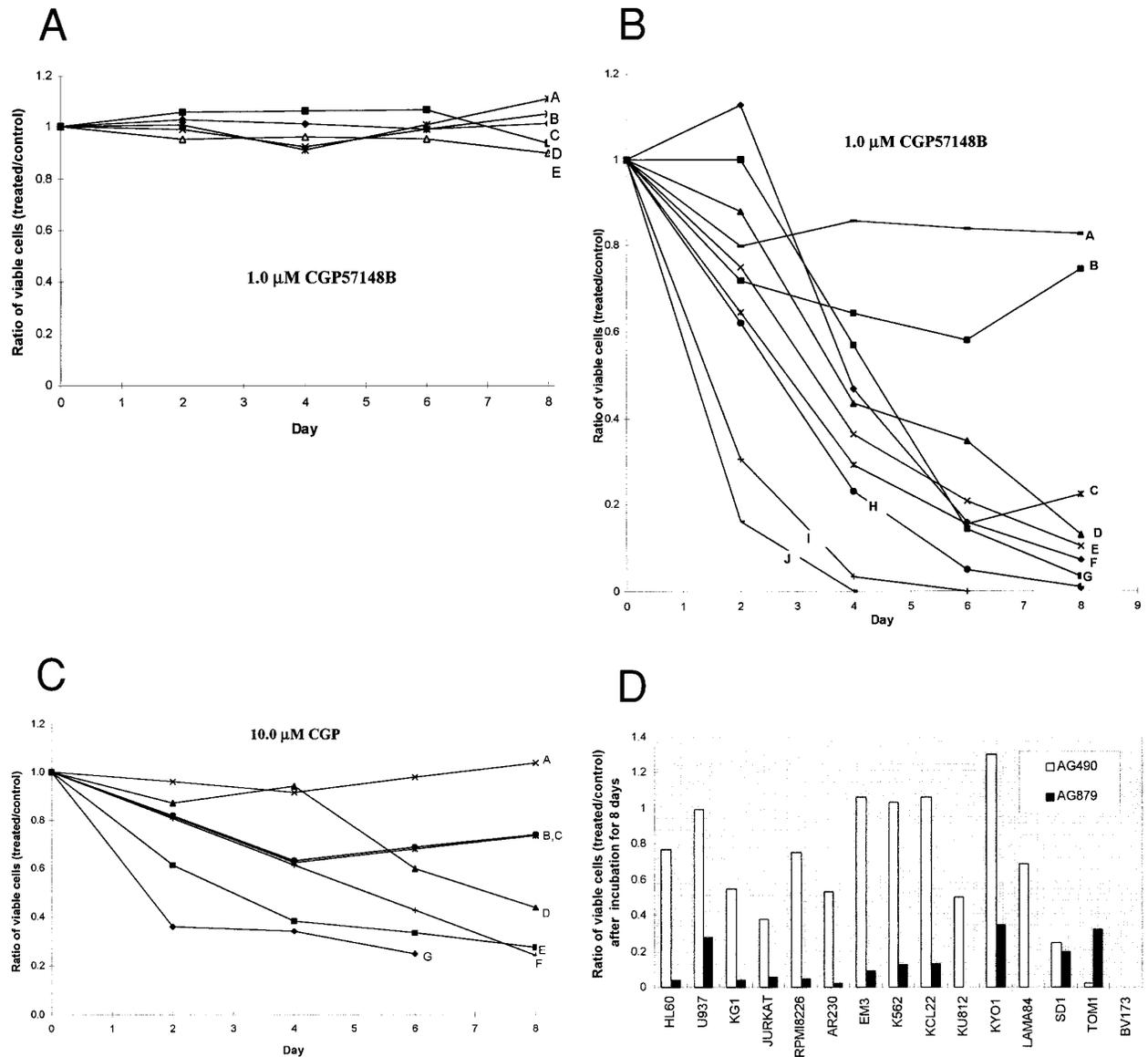


Fig 4. Leukemia cell lines exposed to tyrosine kinase inhibitors. (A) BCR-ABL-negative cell lines exposed to 1.0 $\mu\text{mol/L}$ CGP57148B (A, HL60; B, U937; C, KG1; D, RPMI 8226; E, Jurkat). (B) BCR-ABL-positive cell lines exposed to 1.0 $\mu\text{mol/L}$ CGP57148B (A, KCL22; B, SD1; C, KU812; D, LAMA84; E, KYO1; F, TOM1; G, K562; H, BV173; I, EM3; J, AR230). (C) HL60 (A), U937 (B), Jurkat (C), KG1 (D), SD1 (E), RPMI 8226 (F), and KCL22 (G) exposed to 10 $\mu\text{mol/L}$ CGP57148B. (D) Exposure to 10 $\mu\text{mol/L}$ AG490 and 10 $\mu\text{mol/L}$ AG879.

SD). Analysis of variance was used to compare colony survival by CML cells versus normal MNCs.

RESULTS

Clonogenic assays. In the first series of experiments, we studied the capacity of adherent-depleted MNCs from nine CML patients and three normal individuals to form day 14 CFU-GM and BFU-E colonies in the presence of 1.0 $\mu\text{mol/L}$ CGP57148B when plated in methylcellulose containing LCM and erythropoietin. In CML samples, CFU-GM were reduced to $40.9\% \pm 19.3\%$ and BFUs-E to $9.2\% \pm 13.4\%$ of control values. Individual day 14 colonies were plucked and analyzed for the presence of BCR-ABL. In the three

normal samples, CFU-GM colonies were largely unaffected, whereas BFU-E colonies were reduced to a variable degree.

To dissect this apparent differential effect of CGP57148B on granulocyte-macrophage and erythroid lineages, we analyzed the dose-response of each type of precursor over a wide dose range in separate methylcellulose assays optimized for the selective growth of CFU-GM (ie, supplemented with IL-3 and G-CSF) and BFU-E (supplemented with IL-3 and erythropoietin), respectively. Figure 1a to c shows the results for cells from six CML patients and six normal individuals studied for at least four (mean, 6.3) dose points. Analysis of variance confirmed a selective inhibition of colony formation by CML MNCs over the dose range of 0.1 to 10.0 $\mu\text{mol/L}$

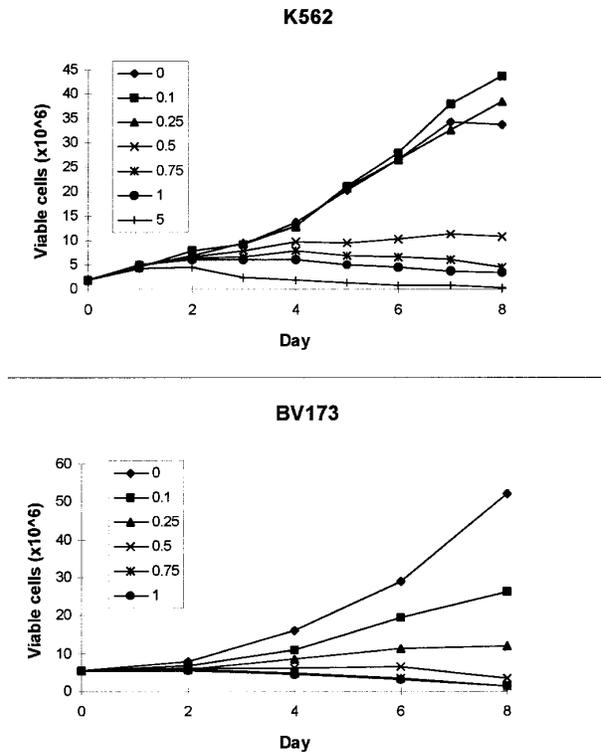


Fig 5. Survival of BV173 and K562 cells incubated with graded concentrations of CGP57148B by time.

CGP57148B ($P < .001$ for all three colony types) in a dose-dependent manner, with the maximal differential effect at $1.0 \mu\text{mol/L}$. There was a slight plateau-type reduction from 0.25 to $10.0 \mu\text{mol/L}$ in day 7 CFU-GM (Fig 1A) and from 0.1 to $1.0 \mu\text{mol/L}$ in day 14 CFU-GM (Fig 1B) derived from normal MNCs. Day 14 BFU-E formation by normal MNCs was also slightly reduced, without an apparent plateau (Fig 1C). When the dose of CGP57148B was increased to $50 \mu\text{mol/L}$, colony formation by both CML and normal MNCs was reduced to less than 10% of control. In four CML patients, MNCs depleted of the plastic-adherent fraction were plated in parallel, with similar results as for nondepleted MNCs (Fig 2, BFU-E).

In a separate experiment, $\text{CD}34^+$ cells derived from a patient in chronic-phase CML were plated together with increasing proportions of normal $\text{CD}34^+$ cells in the presence and absence of $1.0 \mu\text{mol/L}$ CGP57148B, and CFU-GM colonies were counted on day 14. Colony survival increased with the proportion of normal $\text{CD}34^+$ cells in the mixture (Fig 3). CFU-GM colonies grown in dishes that contained a 3:1 mixture of CML and normal $\text{CD}34^+$ cells were analyzed for BCR-ABL by FISH.

RT-PCR and FISH. A total of 146 day 14 colonies (128 CFU-GM and 18 BFU-E) grown in the presence of $1.0 \mu\text{mol/L}$ CGP57148B were investigated for BCR-ABL by RT-PCR, FISH, or both methods (Table 1). Fifty-eight day 14 colonies (51 CFU-GM and seven BFU-E) grown without CGP57148B served as controls. Eight patients were studied. The number of colonies analyzed per patient ranged from

eight to 30 (mean, 18.3) in the treated group and from two to 12 (mean, 8.3) in the controls. Results on colonies analyzed by both FISH and PCR were concordant in 92.9% of cases (Table 2). Five of six colonies in which discordant results were obtained were negative on RT-PCR but positive on FISH; the remaining colony was positive on RT-PCR but negative on FISH. Overall, informative results could be obtained for 118 colonies (80.9%) in the treated group and 51 colonies (87.9%) in the control group. When colonies with discordant results were scored according to FISH, 111 of 118 colonies (94.1%) in the treated group and 49 of 51 colonies (96.1%) in the control group were BCR-ABL-positive. A single colony was BCR-ABL-negative by FISH.

Day 14 CFU-GM colonies derived from a 3:1 mixture of CML and normal $\text{CD}34^+$ cells were analyzed for BCR-ABL by FISH (20 colonies grown in the presence and 20 grown in the absence of $1.0 \mu\text{mol/L}$ CGP57148B). Eleven of 18 colonies (58%) grown at $1.0 \mu\text{mol/L}$ CGP57148B were BCR-ABL-negative, compared with five of 19 (28%) in the control. The remaining colonies were not analyzable.

Cell lines. Seven CML cell lines expressing $\text{P}210^{\text{BCR-ABL}}$ (BV173, EM3, K562, KCL22, KYO1, KU812, and LAMA84), the CML cell line AR230 that expresses a $\text{P}230^{\text{BCR-ABL}}$, two ALL cell lines expressing $\text{P}190^{\text{BCR-ABL}}$ (TOM1 and SD1), and five Ph-negative hematopoietic cell lines (HL60, Jurkat, KG1, RPMI 8226, and U937) were exposed to $1.0 \mu\text{mol/L}$ CGP57148B. CGP57148B did not affect the growth and viability of Ph-negative cell lines (Fig 4A). In the majority of BCR-ABL-positive cell lines, there was growth inhibition within 48 to 72 hours, followed by a decline in viability over the following days (Fig 4B). In

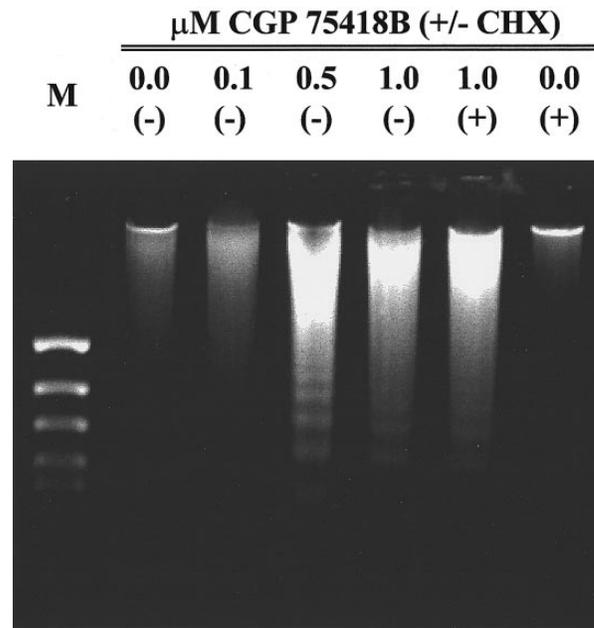


Fig 6. High-molecular weight DNA extracted from 2×10^6 BV173 cells exposed to graded concentrations of CGP57148B for 48 hours with (+) or without (-) preincubation with $0.5 \mu\text{g/mL}$ cycloheximide (CHX). M, molecular weight marker (pEMBL digested with *Taq* I).

contrast, KCL22 and SD1 were found to be resistant to CGP57148B at 1.0 $\mu\text{mol/L}$ (Fig 4B). Exposure to 10.0 $\mu\text{mol/L}$ CGP57148B inhibited the growth of KCL22 and SD1 but also of the BCR-ABL-negative cell lines KG1 and RPMI 8226, and to a lesser degree, Jurkat and U937 (Fig 4C). The JAK2 inhibitor AG490 and the HER2-Neu inhibitor AG879 did not affect the growth of any of the cell lines at a concentration of 1.0 $\mu\text{mol/L}$. Ten micromolar AG879 resulted in severe growth inhibition of all cell lines regardless of BCR-ABL status (Fig 4D). AG490 at 10 $\mu\text{mol/L}$ caused pronounced growth inhibition of BV173, TOM1, and SD1 (all Ph-positive cell lines with a lymphoid phenotype), as well as moderate inhibition in some other cell lines (Fig 4D).

Detailed studies of the dose-response characteristics of BV173 and K562 showed the former to be slightly more sensitive to the effect of CGP57148B than the latter. In BV173, there was growth inhibition at doses as low as 0.1 $\mu\text{mol/L}$, whereas inhibition of K562 required at least 0.5 $\mu\text{mol/L}$ (Fig 5). Furthermore, whereas in BV173 a clear dose-response relationship was observed, growth inhibition of K562 showed a threshold between 0.25 and 0.5 $\mu\text{mol/L}$ CGP57148B. Exposure of BV173 to CGP57148B resulted in DNA laddering typical of apoptosis (Fig 6). Similar results were obtained for K562 (not shown). Apoptosis could not be prevented by preincubation with 0.5 $\mu\text{g/mL}$ cycloheximide (Fig 6).

DISCUSSION

Our results show that CGP57148B selectively inhibits colony formation by CML progenitor cells over a 2-logarithmic dose range. A dose of 1.0 $\mu\text{mol/L}$ offers the maximal differential effect for all three types of colonies studied. At very high doses (50 $\mu\text{mol/L}$), colony formation by both normal and CML MNCs is reduced to less than 10% of control values. This loss of selectivity may be due to the inhibition of tyrosine kinases other than ABL and BCR-ABL that are essential for the proliferation of normal CFUs. Depletion of the plastic-adherent fraction of the MNCs did not affect colony survival. This indicates that the inhibitory effect of CGP57148B is not mediated by the inhibition of regulatory cells present in the plastic-adherent population.

Colony formation by normal MNCs was also moderately reduced, particularly for BFU-E. This is in contrast to the results from Druker et al,¹³ who reported no effect on normal MNCs at 1.0 $\mu\text{mol/L}$ CGP57148B. These discordant findings cannot be explained by a difference in the composition of the growth factors, which was identical in their experiments¹³ and in our dose-response study. However, low oxygen tension as used in their investigation is known to enhance colony formation by hematopoietic stem cells,²⁰ particularly CFU-E and BFU-E,²¹ and culture under these conditions may protect normal CFUs against the effect of CGP57148B. Furthermore, colony counting is highly subjective, and variations between different centers are large.²² Importantly, in our investigation the person counting the colonies was unaware of the dose of individual dishes, to avoid any possible bias.

We were unable to detect a significant proportion of BCR-ABL-negative day 14 colonies either in clonogenic assays

performed in the presence of 1.0 $\mu\text{mol/L}$ CGP57148B or in the controls. The results obtained by RT-PCR and FISH on the same colonies were concordant in over 90% of the cases. Only one colony scored as positive by RT-PCR was negative by FISH, but five colonies scored as negative by RT-PCR were positive by FISH. These results are in line with previous studies from our group¹⁷ and others^{23,24} indicating that Ph-negative CFUs are rare in chronic-phase CML. Therefore, a very large sample would be required to detect a significant number of Ph-negative colonies, even in the presence of a considerable growth advantage. To circumvent this problem, we assessed the formation of day 14 CFU-GM colonies grown from a mixture of normal and Ph-positive CD34⁺ progenitor cells. Colony survival increased in good correlation with the proportion of normal CD34⁺ cells. Moreover, the percentage of Ph-negative colonies derived from the 3:1 mixture of CML and NBM progenitor cells was increased from 28% to 58% by addition of 1.0 $\mu\text{mol/L}$ CGP57148B to the growth medium. This experiment demonstrates that incubation with CGP57148B results in a substantial enrichment for Ph-negative colonies, which may be of clinical relevance in the setting of autologous bone marrow transplantation for CML. The apparent discrepancy between our results and those of Druker et al,¹³ who found that most colonies grown in the presence of CGP57148B are Ph-negative, is likely due to technical reasons. The assessment of BCR-ABL expression in individual colonies by RT-PCR requires repeated testing to minimize the risk of false-negative results since, due to the small number of template molecules in the reaction, the assay is prone to sampling effects.²⁵ In the present study, colonies were tested at least twice for BCR-ABL, but nevertheless there were five false-negative RT-PCR results. Similar observations were made by Thijsen et al,²⁶ who found 15 of 107 CFU-GM colonies negative on RT-PCR but positive on FISH. Our results suggest that FISH is preferable to RT-PCR to assess the Ph status of individual colonies if the question is whether the BCR-ABL gene is present rather than its level of expression.

It is not clear what mechanisms allow some Ph-positive progenitor cells to escape the effect of CGP57148B. Possible explanations include the presence of additional mutations in a subset of the progenitor cells that allow proliferation independently of BCR-ABL. Alternatively, the level of BCR-ABL protein may vary from one progenitor cell to another. In this model, cells (and colonies) with low-level or no BCR-ABL protein at all would be unaffected by CGP57148B.

CGP57148B at 1.0 $\mu\text{mol/L}$ inhibited the growth of all BCR-ABL-positive cell lines tested, except for KCL22 and SD1. The proliferation of these two lines was inhibited at 10 $\mu\text{mol/L}$ CGP57148B. Since 10 $\mu\text{mol/L}$ also inhibited the growth of most Ph-negative cell lines tested, the effect of CGP57148B at such high doses is likely to be independent of BCR-ABL. The mechanism underlying the resistance of SD1 and KCL22 to 1.0 $\mu\text{mol/L}$ CGP57148B is presently unclear. However, it is interesting that normal ABL is expressed²⁷ (and J.V.M., M.W.N.D., unpublished observations, July 1996) in both Ph-positive cell lines resistant to 1.0 $\mu\text{mol/L}$ CGP57148B but in only two (K562 and TOM1) cell

lines sensitive to this concentration. Since ABL in the nucleus is known to be a negative regulator of cell growth,²⁸ its presence in BCR-ABL-expressing cells may have to be counteracted or modified by additional genetic changes to enable a Ph-positive cell to be immortalized in vitro. In contrast to CGP57148B, the two other tyrophostins tested did not show selectivity for BCR-ABL-positive cell lines. AG490, a JAK2 inhibitor, and AG879, a HER2-Neu inhibitor, did not affect the growth of any cell line at 1.0 $\mu\text{mol/L}$. Whereas AG879 at 10 $\mu\text{mol/L}$ nonselectively killed all cell lines tested, AG490 at 10 $\mu\text{mol/L}$ caused moderate inhibition in a subset of both BCR-ABL-negative and -positive lines. However, a pronounced effect was observed in the BCR-ABL-positive lymphoid cell lines BV173, TOM1, and SD1. This could indicate that JAK2 activity is required for the proliferation of these cell lines but not for Ph-positive cell lines with myeloid phenotypes.

Sensitive cell lines undergo apoptosis when exposed to CGP57148B. This observation lends some support to the view that BCR-ABL may act by preventing programmed cell death.^{29,30} Inhibition of protein synthesis with cycloheximide, which prevents apoptosis in certain circumstances,^{31,32} did not rescue BV173 from programmed cell death induced by CGP57148B. This finding suggests that in a sensitive cell, inhibition of the BCR-ABL tyrosine kinase activates a death program for which the components are already present. Studies on the mechanism of resistance to CGP57148B may help to clarify the manner in which programmed cell death is induced in sensitive cells.

In summary, CGP57148B selectively inhibits the in vitro growth of BCR-ABL-positive cells. Since preliminary data suggest minimal toxicity in animals,¹³ the compound represents a promising novel strategy for in vivo treatment of CML and BCR-ABL-positive ALL. Alternatively, it could prove useful for in vitro purging in the setting of autologous bone marrow transplantation.

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