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ORIGINAL ARTICLE

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Favorable therapeutic index of a p210$^{BCR-ABL}$-specific tyrosine kinase inhibitor; activity on lineage-committed and primitive chronic myelogenous leukemia progenitors

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Abstract *Purpose*: In order to assess the effect of the tyrosine kinase inhibitor CGP57148B on lineage-committed and primitive chronic myelogenous leukemia (CML) progenitor cells, peripheral blood progenitor cells (PBPC) mobilized in chronic phase CML were exposed to this compound in vitro. *Methods*: Both short-term (≤1 week) and long-term exposure (≥2 weeks) to CGP57148B were investigated using suspension culture, semisolid (methylcellulose) assay or stroma-dependent long-term culture (LTC). The proportion of bcr/abl-positive progenitors was determined after direct plating [2 weeks in colony-forming cell (CFC) assay] as well as after 2 or 6 weeks LTC (LTC always followed by CFC replates). *Results*: Incubation of CML PBPC over 48 h in suspension culture with 100 μ*M* CGP57148B reduced the proportion of bcr/abl-positive colonies to 4.4 ± 4.3% ($n=5$) after direct plating, 6.6 ± 4.2% ($n=5$) after 2 weeks LTC and 5 ± 5.6% ($n=2$) after 6 weeks LTC. At this dose, survival of drug-exposed normal PBPC was 53 ± 4.2%, 51 ± 2.8% and 54.5 ± 4.9% ($n=2$), respectively. Incubation with CGP57148B at a concentration of 10 μ*M* over 1 week under LTC conditions reduced the number of bcr/abl-positive colonies to 11.8 ± 6.1% ($n=5$) after direct plating, 12 ± 6.4% ($n=4$) after 2 weeks LTC and 14.3 ± 11.4% ($n=3$) after 6 weeks LTC; survival of normal PBPC was 84.5 ± 2.1%, 93 ± 4.2% and 86 ± 1.4% ($n=2$), respectively. Following long-term exposure to CGP57148B at a concentration of 1 μ*M*, the proportion of remaining bcr/abl-positive colonies was 35%, 9% and 25% of untreated CML samples after direct plating as well as after 2 and 6 weeks LTC, respectively. The respective values for 10 μ*M* CGP57148B were 10%, 11% and 19%. Long-term exposure of normal progenitors to CGP57148B yielded a survival of 98%, 100% and 93% (1 μ*M*) or 77%, 86% and 80% (10 μ*M*), respectively. *Conclusion*: The results support the use of CGP57148B either for short-term exposure in vitro (e.g. purging) or for continuous treatment of CML in vivo.

Key words CML treatment · Tyrosine kinase inhibitors · Stroma-dependent long-term cultures · Mobilized peripheral blood progenitor cells · Purging

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Introduction

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disorder originating from neoplastic transformation of a primitive hematopoietic stem cell [5]. The disease is characterized by the reciprocal translocation (9;22)(q34;q11) with fusion of the bcr and abl genes [20] resulting in an abnormally short chromosome 22, the so-called Philadelphia (Ph) chromosome. The bcr/abl fusion gene encodes for a fusion protein of 210 kDa that has enhanced tyrosine kinase activity [22], increases proliferation and reduces apoptosis [6, 13, 14].

Yaish et al. first reported the therapeutic use of a specific inhibitor of tyrosine kinases [27]. Inhibition of the abl tyrosine kinase activity has been demonstrated for the benzopyranones, the benzothiopyranones [15] and the tyrphostine class of substances [1]. However, these compounds show either limited selectivity on bcr/abl-expressing cells in vitro or low potency. Recently, a number of tyrosine kinase inhibitors of the 2-phenylaminopyrimidine class have been synthesized, among them CGP57148 [3], which is more active against the abl tyrosine kinase than other inhibitors previously reported [21]. Druker et al. have demonstrated that this

compound selectively inhibits the tyrosine kinase activity of abl as well as the platelet-derived growth factor receptor (PDGFr) protein tyrosine kinases [11]. These findings have been confirmed and extended by Carroll et al. who have shown an inhibition of growth of cells expressing BCR-ABL, TEL-ABL and TEL-PDGFr fusion proteins [4]. Druker et al. have reported that proliferation of lineage-committed CML cells is specifically inhibited by CGP57148 with no effects on normal cells [11]. These findings were confirmed and supplemented in the present study by assessing the effect of CGP57148B on proliferation of primitive progenitors using stroma-dependent long-term cultures (LTC).

In contrast to treatment with DNA-directed agents like busulfan, hydroxyurea and cytarabine or to treatment with interferon alpha (IFN-α) [19], inhibition of the abl tyrosine kinase may be a more specific strategy for CML treatment [9]. Either ex vivo purging prior to autografting or systemic administration of the compound are possible approaches; accordingly the effects of short-term exposure (≤1 week) and of long-term exposure (≥2 weeks) to CGP57148B were investigated in the present study.

Materials and methods

Drugs

CGP57148 and its methane sulfonate salt (CGP57148B) were kindly provided by Novartis Pharma AG, Basel, Switzerland. A stock concentration of 10 m*M* CGP57148B was prepared in Me_2SO (DMSO) and stored at −20 °C. All experiments were performed with the methane sulfonate salt (CGP57148B) at different concentrations.

Cells

Cryopreserved human normal and malignant cells from CML patients were obtained from the Department of Internal Medicine V, University of Heidelberg. Five different leukapheresis products from two chronic phase CML patients were collected. The study was approved by the Ethics Committee of the Medical Faculty of the University of Heidelberg and informed consent was obtained from all patients. Cells were rapidly thawed at 37 °C, washed and resuspended in a medium containing Iscove's modified Dulbecco's medium (IMDM; Gibco, Eggenstein), 10 m*M* HEPES (Gibco) and 0.01% DNAse (Boehringer Mannheim, Mannheim). After purification and separation of mononuclear cells using Ficoll separation solution (Biochrom KG Seromed, Berlin), unselected cells were counted, resuspended in IMDM and kept on ice until the culture assays. As untreated controls, normal human leukapheresis cells from three nonleukemic patients were used.

Experimental design

Short-term exposure to CGP57148B was performed either over 48 h in suspension culture (experiment I) or over 1 week under LTC conditions (experiment II) using unselected peripheral blood (PB) cells from CML patients in chronic phase. In experiment I, after exposure to CGP57148B over 48 h in suspension culture , the cells were taken out of the Petri dishes, and the drug was removed by washing. The cells were then either transferred to the colony-forming cell (CFC) read-out assay comprising 2 weeks further observation in HMC before evaluation (experiment Ia) or to the LTC assays, which comprised either 2 weeks LTC plus 2 weeks CFC assay before evaluation (experiment Ib) or 6 weeks LTC plus 2 weeks CFC assay before evaluation (experiment Ic). In experiment II, after exposure over 1 week under LTC conditions, cells were taken out of the flasks, cells and the stromal feeder were washed and the drug was removed. An aliquot of these hematopoietic cells was transferred directly to a CFC read-out assay (experiment IIa; compare experiment Ia). The remainder of these cells were transferred back into the same flasks that contained the stromal feeder cells. The cells were kept under LTC conditions as described above for experiments Ib and Ic. To summarize, for both short-term exposure setups, following the period of drug exposure, evaluation was performed (a) after 2 weeks in HMC (CFC assay), (b) after 4 weeks (2 weeks LTC + 2 weeks CFC) or (c) after 8 weeks (6 weeks LTC + 2 weeks CFC). These different periods allowed hematopoietic progenitor cells at different stages of primitivity as initially described by Dexter et al. [10] to be assessed.

For long-term exposure to CGP57148B (experiment III), the drug was present at all times and during all read-out assays. Accordingly, continuous exposure was performed (a) over 2 weeks in the CFC assay, (b) over a total of 4 weeks (2 weeks LTC + 2 weeks CFC) or (c) over a total of 8 weeks (6 weeks LTC + 2 weeks CFC).

In vitro assays

Suspension culture

For short-term exposure in suspension culture [23] cells were exposed to CGP57148B for 48 h. Unselected CML cells (1.5×10^6) were resuspended in 5 ml myeloid LTC medium (MyeloCult^{TM}) H5100 (Stem Cell Technologies, Vancouver, Canada; referred to as LTC-medium) containing 12.5% horse serum (HS), 12.5% fetal bovine serum (FBS), 0.2 m*M* i-inositol, 20 m*M* folic acid, 10^{-4} *M* 2-mercaptoethanol and 2 m*M* L-glutamine. Incubation was performed in the presence of the human growth factors interleukin-3 (IL-3, 10 ng/ml; Novartis Pharma, Nürnberg) and Flt-3 (100 ng/ml; R&D Systems, Wiesbaden) at 37 °C in a humidified atmosphere containing 5% CO_2.

CFC assay

Colony formation of human normal and malignant CML cells was studied in a CFC assay. Usually, 1×10^4 or 1×10^5 cells were plated in duplicate in MethoCult GF H4434 methylcellulose (MC; Stem Cell Technologies) supplemented with IL-3 (10 ng/ml) plus G-CSF (20 ng/ml) and incubated for 14 days at 37 °C in an atmosphere containing 5% CO_2. Individual colonies, burst-forming units-erythroid (BFU-E) and colony-forming units granulocyte/macrophage (CFU-GM) were counted.

Stroma-dependent LTC

To assess the repopulation characteristics of human normal and malignant stem cells from CML patients and to study the long-term effect of CGP57148B on primitive progenitors [24], the stroma-dependent Dexter-type LTC assay was used as described by Breems et al. [2]. Confluent stromal layers of FBMD-1 cells, a stromal cell line derived from female adult C57BL/6 mouse bone marrow, were overlaid with $0.5–2 \times 10^6$ mononuclear cells, obtained as primary material from patients. Cells were incubated at 33 °C in LTC-medium supplemented with IL-3 (10 ng/ml) plus G-CSF (20 ng/ml) (LTC conditions) over a period of 2 or 6 weeks. CFC assay replates of adherent and nonadherent cells were performed after 2 or 6 weeks LTC.

Polymerase chain reaction

To study the presence of leukemic progenitors in CFC assays, individual colonies were plated and a nested reverse transcriptase-

polymerase chain reaction (RT-PCR) for single colonies, based on the detection of the CML-specific bcr/abl translocation, was perfomed. Using an RNeasy Mini Kit (Qiagen, Hilden), RNA was extracted according to the protocol provided. cDNA synthesis was performed as described by Cross et al. [8]. Half of the final volume of the reverse transcription (20 µl) was used for the first PCR amplification in a total volume of 50 µl, and reamplification was performed under the same conditions using 2.5 µl of the first amplificate. Reagents, primer sequences and PCR conditions were as described by Schulze et al. [25]. Primer sequences used for the first PCR round were 5′CATGGCCTTCAGGGTGCACAG3′ (bcr exon 3) and 5′CCATTTTTGGTTTGGGCTTCACACCATTCC3′ (abl exon 3), and for the second PCR round were 5′GGAGCTGCAGATGCTGACCAA3′ (bcr exon 3) and 5′TCAGACCCTGAGGCTCAAAGTC3′ (abl exon 2). As an internal control, β-actin PCR was performed using the human β-actin control amplimer set by Clontech (Clontech Laboratories, Palo Alto, Calif.). Primer sequences used for this β-actin PCR were 5′ATCTGGCACCACACCTTCTACAATGAGCTGCG3′ (5′ primer) and 5′CGTCATACTCCTGCTTGCTGATCCACATCTGC3′ (3′ primer). The PCR reaction products (10 µl) were analysed by electrophoresis in a 1.5% agarose gel (Qualex Gold Agarose; Angewandte Gentechnologie Systeme, Heidelberg), stained with 0.01% ethidium bromide and visualized under UV light. As a positive control, cDNA from the bcr/abl-expressing cell line K562 was used.

Determination of bcr/abl-positive and normal colonies

Colony formation and bcr/abl expression were determined by CFC assays and RT-PCR, respectively, at different time-points after the start of exposure. The number of CFU-GM colonies (Table 1) was determined and is given with reference to the number of cells seeded. The proportion (percentage) of bcr/abl-positive colonies was determined by RT-PCR analysis using an average 25 colonies. Numbers (n) of bcr/abl-positive and normal colonies (Table 1) were calculated as follows. If, for example, in the control RT-PCR sample 21/25 colonies (84%) were bcr/abl-positive (Fig. 3), for a total of 80 CFU-GM colonies the corresponding value of bcr/abl-positive colonies would be 67 (84%; Table 1). The number of normal colonies is the difference between total colony count and number of bcr/abl-positive colonies.

Statistics

For all data mean values ± standard deviation ($SD = [(1/n-1)\sum(x_i - x)^2]^{1/2}$) were calculated and are given.

Results

Short-term exposure (≤1 week) to CGP57148B

In a pilot experiment, exposure of cells to 10 µ*M* CGP57148B over 48 h in suspension culture resulted in a borderline activity with a proportion of remaining bcr/abl-positive colonies of 71%, 49% and 75% of untreated CML samples after direct plating, after 2 weeks and 6 weeks LTC, respectively (data not shown). Therefore, the tenfold dose (100 µ*M*) was chosen for the 48-h exposure.

For the 1-week exposure under LTC conditions a concentration of 10 µ*M* CGP57148B was chosen. The results of both experiments are shown in Figs. 1 and 2.

Figure 1 shows the effect of CGP57148B on colony formation. When PB CML cells were incubated over

Table 1 Long-term exposure of mobilized CML PBPC to CGP57148B. Colonies (CFU-GM) were evaluated after 2 weeks in CFC assay, after a total of 4 weeks (2 weeks LTC + 2 weeks CFC assay) and after 8 weeks (6 weeks LTC + 2 weeks CFC assay) (*CFU-GM* colony-forming unit-granulocyte/macrophage, *CFC* colony-forming cell, *LTC* long-term culture)

	CFC assay					CFC replate after 2 weeks LTC			CFC replate after 6 weeks LTC		
	Total CFU-GM colony count[a]	bcr/abl-positive colony count	Normal colony count[b]	bcr/abl-positive colonies (% of control)	Normal colonies (% of control)	Total CFU-GM colony count[a]	bcr/abl-positive colony count	bcr/abl-positive colonies (% of control)	Total CFU-GM colony count[a]	bcr/abl-positive colony count	bcr/abl-positive colonies (% of control)
Control	80	67	13	100	100	240	230	100	295	177	100
1 µ*M* CGP57148B	35	23	12	35	92	86	21	9	53	45	25
10 µ*M* CGP57148B	14	7	7	10	54	57	25	11	36	33	19

[a] CFU-GM colonies counted 2 weeks after (re)plating of CML PBPC (related to 1×10^5 cells per dish)
[b] Numbers of normal colonies are given only for the CFC assay to enable the therapeutic index of CGP57148B to be estimated (see text)

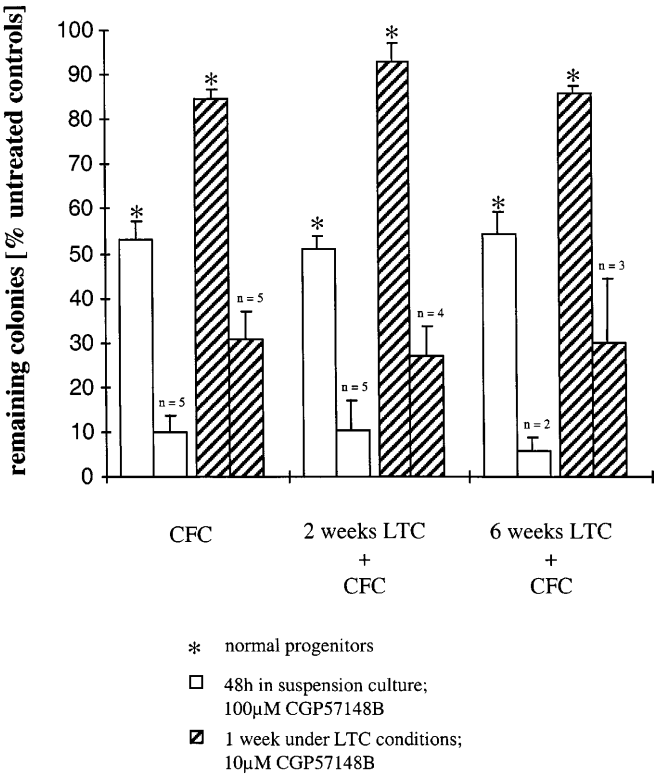

Fig. 1 Short-term exposure of CML progenitors to CGP57148B was either performed for 48 h in suspension culture containing 100 μ*M* CGP57148B (□) or for 1 week under LTC conditions with 10 μ*M* CGP57148B (▨). The inhibition of colony formation of malignant cells is compared to that of normal progenitors (*). Values are means ± SD

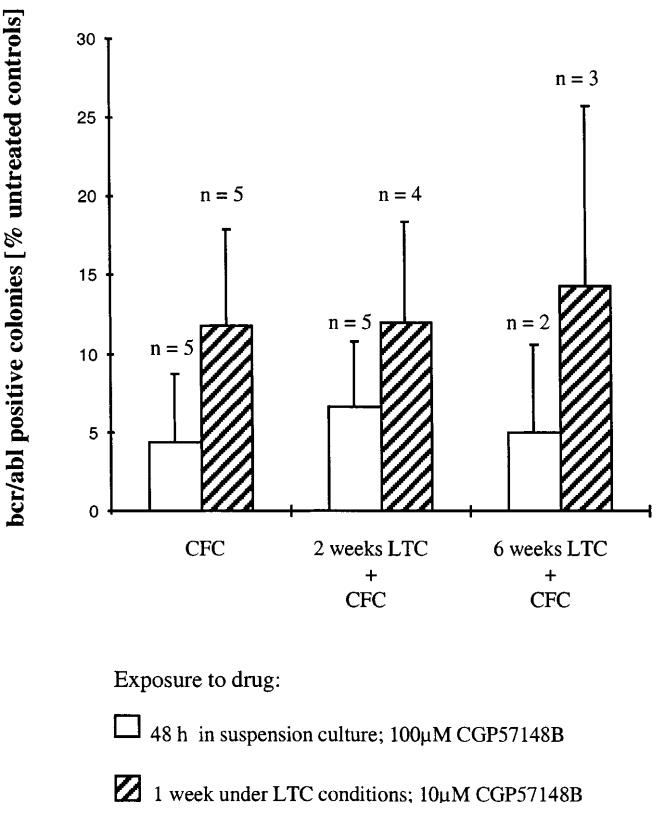

Fig. 2 Colonies positive for bcr/abl following short-term exposure (48 h or 1 week) of CML progenitors compared to untreated CML samples (=100%). Values are means ± SD

48 h with 100 μ*M* CGP57148B, the remaining colonies were 10 ± 3.8% ($n=5$) after direct plating (2 weeks in CFC assay), 10.2 ± 6.8% ($n=5$) after 2 weeks in LTC and 6 ± 2.8% ($n=2$) after 6 weeks in LTC, respectively (LTC always followed by 2-week CFC replates). The corresponding values for normal drug-exposed progenitors were 53 ± 4.2%, 51 ± 2.8% and 54.5 ± 4.9% ($n=2$), respectively. Incubation over 1 week with 10 μ*M* CGP57148B reduced the colony counts to 30.6 ± 6.3% (n=5) after direct plating, to 27 ± 6.5% ($n=4$) after 2 weeks in LTC and to 30 ± 15.6% ($n=3$) after 6 weeks in LTC, respectively. The corresponding values for normal drug-exposed progenitors were 84.5 ± 2.1%, 93 ± 4.2% and 86 ± 1.4% ($n=2$), respectively.

Comparing the proportion of remaining bcr/abl-positive colonies showed (Fig. 2), that 48 h incubation with 100 μ*M* CGP57148B led to percentages of bcr/abl-positive colonies of 4.4 ± 4.3% ($n=5$) after direct plating, 6.6 ± 4.2% ($n=5$) after 2 weeks in LTC and 5.0 ± 5.6% ($n=2$) after 6 weeks in LTC, respectively. Incubation over 1 week under LTC conditions with 10 μ*M* CGP57148B reduced the proportion of remaining bcr/abl-positive colonies to 11.8 ± 6.1% ($n=5$) after direct plating, to 12.0 ± 6.4% ($n=4$) after 2 weeks in LTC and to 14.3 ± 11.4% ($n=3$) after 6 weeks in LTC, respectively.

Long-term exposure (≥2 weeks) to CGP57148B

When PB cells from CML patients were incubated over 2 weeks (CFC assay only), 4 weeks (LTC plus CFC assay) or 8 weeks (LTC plus CFC assay) with 1 μ*M* CGP57148B, 35%, 9% and 25% of bcr/abl-positive colonies were found. Exposure to 10 μ*M* CGP57148B resulted in a residual CML-CFC growth of 10%, 11% and 19%, respectively. Comparison of these values with the respective control values indicated a tenfold inhibition of bcr/abl-positive colonies (100% vs 10%) as compared to a twofold inhibition of normal CFU-GM growth (100% vs 54%) at 10 μ*M* CGP57148B indicating a therapeutic index of CGP57148B of about 5 (Table 1). A representative RT-PCR of bcr/abl-positive colonies is given in Fig. 3.

In this experimental setting, leukapheresis cells from patients with nonleukemic disorders were used as negative control. Continuous incubation with 1 μ*M* CGP57148B yielded 98%, 100% and 93% survival and with 10 μ*M* CGP57148B 77%, 86% and 80% survival of these normal PBPC colonies (data not shown).

Discussion

CGP57148B, a compound of the 2-phenylaminopyrimidine class, has been found to be a potent inhibitor of the

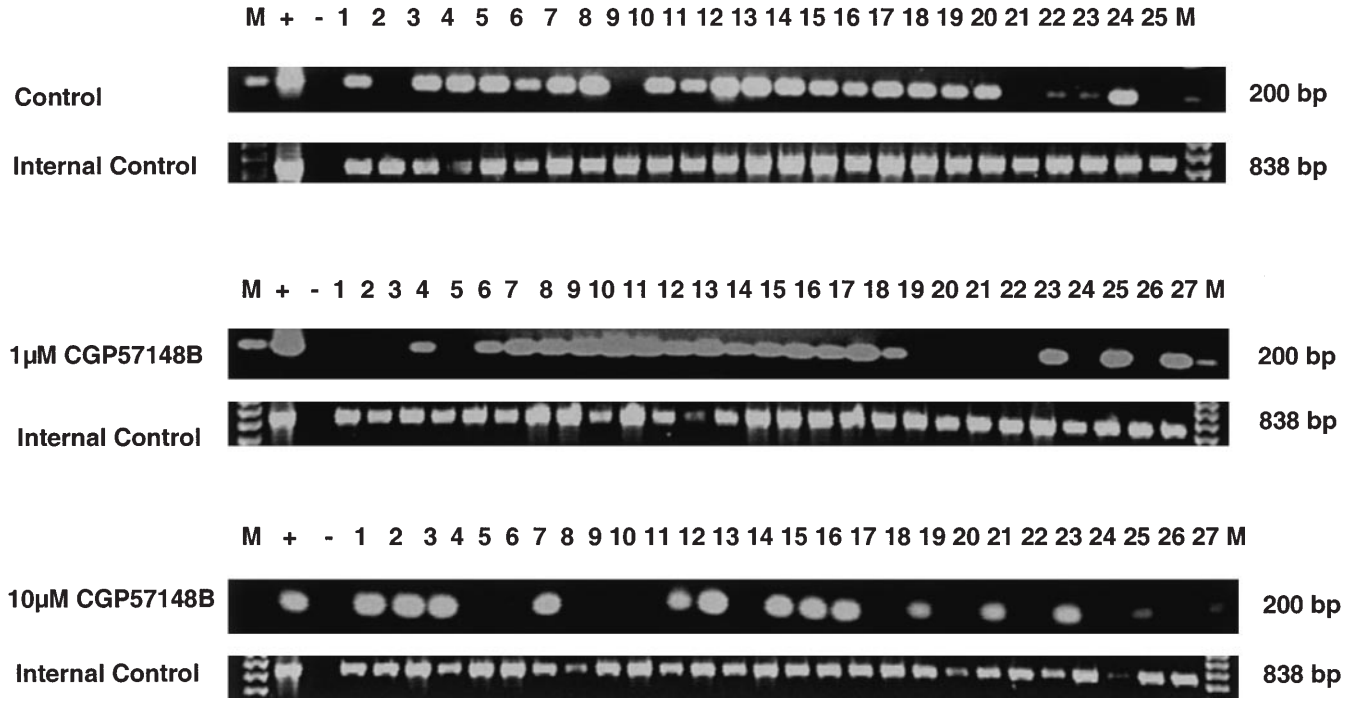

Fig. 3 Molecular monitoring by a nested RT-PCR technique of bcr/abl-positive CFU-GM colonies following continuous exposure to CGP57148B. In the control sample (no drug) 21/25 colonies (84%) were bcr/abl-positive, with 1 μ*M* CGP57148B 18/27 colonies (67%) were bcr/abl-positive and with 10 μ*M* CGP57148B 13/27 colonies (48%) were bcr/abl-positive, respectively. As positive control (+), cDNA from the bcr/abl-expressing cell line K562 was used. H_2O was used as a negative control template (−). As an internal control, RT-PCR was performed with β-actin

abl tyrosine kinase [11]. In the present study, the effects of short- and long-term exposure of bcr/abl-positive cells from chronic phase CML patients to this drug were investigated. CFC and LTC assays demonstrated drug activity towards *lineage-committed progenitors* and towards *primitive progenitors* that give rise to long-term hematopoiesis.

The primary material used in our studies carried the bcr/abl translocation before drug exposure; this was confirmed by RT-PCR. Owing to the clonal origin of CML [5], cells that did not show the translocation after treatment with CGP57148B were considered to be Ph-negative normal cells. We have no reason to suppose that cells proving fusion-negative by RT-PCR might nevertheless have been Ph-positive, as reported for isolated cases [7, 26].

Incubation over 48 h with 100 μ*M* CGP57148B resulted in an effective reduction of human malignant CML progenitors. The percentage of remaining bcr/abl-positive colonies was reduced to about 5% for as long as 8 weeks postincubation. However, at this concentration, CGP57148B showed relatively high toxicity to normal PBPC which showed a survival of approximately 50%. In contrast, a tenfold lower dose, has been shown to result in no [11] or only marginal toxicity as presented here.

Short-term incubation over 1 week under LTC conditions with CGP57148B at a concentration of 10 μ*M* resulted in a *sustained* inhibition of bcr/abl-positive progenitors of 80–90%. Continuous exposure to the compound over 2 to 8 weeks led to a sustained inhibition of primitive bcr/abl-positive progenitors of up to 90% with marginal or no toxicity to normal cells.

For the treatment of CML, DNA-directed chemotherapeutic agents such as hydroxyurea, busulfan or cytarabine are used either alone or in combinations including IFN-α. The median survival of patients treated with hydroxyurea, that proved to be superior to busulfan, was 48–58 months [17]. IFN-α produces complete cytogenetic remissions and prolongs the median survival to 55–89 months [18]. At present, however, the only curative treatment for CML is allogeneic bone marrow transplantation. High-dose chemotherapy combined with autologous transplantation of PB or BM cells after ex vivo purging is an attractive alternative treatment modality for patients without a HLA-matched donor or those too old (> 50 years) for allogeneic transplantation [16]. Supplementation of the present treatment armamentarium with more specific approaches such as abl tyrosine kinase inhibitors appears to be promising.

The presented findings support the view that CGP57148B has significant effects on CML progenitor cells. Inhibition of bcr/abl-positive CML progenitors by up to 95% at a dosage and with a treatment schedule showing only marginal toxicity towards normal progenitors suggests that CGP57148B might be an interesting new candidate for CML treatment [12].

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