Highly Efficient Elimination of Philadelphia[1] Leukemic Cells by Exposure to bcr/abl Antisense Oligodeoxynucleotides Combined with Mafosfamide

Tomasz Skorski,* Margaret Nieborowska-Skorska,* Cosimo Barletta,* Lucia Malaguarnera,* Cezary Szczylik,* Sing-Tsung Chen,‡ Beverly Lange,§ and Bruno Calabretta*

**Jefferson Cancer Institute and Departments of *Microbiology and ‡Pathology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107; and §Division of Oncology, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104*

Abstract

Synthetic oligodeoxynucleotides complementary to the breakpoint junction of bcr-abl transcripts selectively inhibit the proliferation of Philadelphia[1]-positive leukemic cells, but residual leukemic cells persist in antisense oligodeoxynucleotides-treated cultures. Cyclophosphamide derivatives such as mafosfamide and 4-hydroperoxycyclophosphamide are used at high doses for purging of Philadelphia[1] leukemic cells from marrows but such treatment can be associated with delayed engraftment and prolonged cytopenias. To develop a more effective procedure that might optimize the killing of leukemia cells and the sparing of normal hematopoietic progenitor cells, a 1:1 mixture of Philadelphia[1] leukemic cells and normal bone marrow cells was exposed to a combination of a low dose of mafosfamide and bcr-abl antisense oligodeoxynucleotides and assayed for growth ability in clonogenic assays and in immunodeficient mice. Bcr-abl transcripts were not detected in residual colonies, and cytogenetic analysis of individual colonies revealed a normal karyotype. Normal but not leukemic hematopoietic colonies of human origin were also detected in marrows of immunodeficient mice 1 mo after injection of the treated cells. Our results indicate that a combination of a conventional chemotherapeutic agent and a tumor-specific antisense oligodeoxynucleotide is highly effective in killing leukemic cells and in sparing a much higher number of normal progenitor cells as compared with high-dose mafosfamide treatment. This offers the prospect of a novel and more selective ex vivo treatment of chronic myelogenous leukemia. (*J. Clin. Invest.* 1993. 92:194–202.) Key words: chemotherapy • DNA • leukemia • oncogenes

Introduction

Bone marrow transplantation (BMT)[1] has been introduced in the therapy of chronic myelogenous leukemia (CML) to increase the probability of long-term remission or even cure of these patients (1). Allogeneic or syngeneic BMT has improved the clinical prognosis, but this therapy is applicable only to a minority of patients because histocompatible marrow donors are scarce. In the absence of appropriate donors, autologous BMT may be performed, although the results of such treatment are usually worse compared with allogeneic marrow transplantation (2, 3), owing to residual clonogenic leukemia cells within the autograft and lack of graft-vs.-leukemia effect (4). However, a beneficial effect of autologous BMT in CML patients has been reported, but remission (when achieved) was usually brief (5, 6). Thus, improvement in CML treatment depends on the establishment of a better method of ex vivo and in vivo treatment of leukemic cells.

The presence of the bcr-abl break point in CML cells (7, 8) and the demonstration that bcr-abl transcripts play an essential role in the induction and maintenance of the leukemic phenotype (9, 10) offers the unique opportunity to utilize these antisense oligodeoxynucleotides, alone or in combination with conventional chemotherapeutic agents, as tumor-specific in vitro purging agents. Among the candidate chemotherapeutic agents mafosfamide has been used with promising results as a purging agent in acute leukemia in a nonrandomized trial (11). A theoretical objection to the purging of bone marrow cells (BMC) from CML patients rests in the observation that the vast majority of hematopoietic cells in bone marrow or peripheral blood of CML patients are neoplastic so that repopulation with normal hematopoietic cells upon reintroduction of purged cells is questionable. However, nonclonal, presumably nonneoplastic hematopoiesis can be temporarily restored in vivo after chemotherapy (12), interferon treatment (13), or autologous BMT using unpurged or purged autografts (14–16). In in vitro long-term bone marrow cultures, the dominant Philadelphia[1] (Ph^1)-positive cell population disappeared, and karyotypically normal progenitors remained detectable for a long period of time (17). Accordingly, the development of an effective strategy for the ex vivo treatment of CML cells might have therapeutic utility.

Using clonogenic assays, we have examined the in vitro sensitivity of CML-blast crisis (BC), and normal BMC to high-dose mafosfamide (ASTA Z 7654), or to a combination of low-dose mafosfamide and bcr-abl antisense oligodeoxynucleotides. This combination purge is highly effective in eliminating Ph^1 leukemic cells, and in sparing a larger number of normal hematopoietic progenitors as compared with high-dose

Dr. Skorski is on leave from the Department of Cytophysiology, Medical Center of Postgraduate Education, Warsaw; Dr. Nieborowska-Skorska is on leave from the Medical Center of Industrial Medicine (OMPB), Warsaw; and Dr. Szczylik is on leave from the Postgraduate Medical Center CSK WAM, Warsaw.

Address reprint requests to Dr. Calabretta, Room 469, Jefferson Alumni Hall, Jefferson Cancer Institute, Thomas Jefferson University, 1020 Locust Avenue, Philadelphia, PA 19107-6799.

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1. *Abbreviations used in this paper:* A^-T^-, adherent cells and T lymphocytes removed; BC, blast crisis; BMC, bone marrow cells; BMT, bone marrow transplantation; BNX, bg/nu/xid (mice); CALLA, common acute lymphoblastic leukemia antigen; CML, chronic myelogenous leukemia; IMDM, Iscove's modified Dulbecco's medium; MNC, mononuclear cells; Ph^1, Philadelphia[1] (leukemic cell); SCF, stem cell growth factor; SCID mice, severe combined immunodeficient mice.

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mafosfamide alone, suggesting that this approach might prove clinically useful, if the findings in small cell samples can be extended to conditions more closely mimicking those used in the ex vivo treatment for BMT.

Methods

Cells. Marrows were obtained as iliac crest aspirates from healthy volunteers after informed consent. Light-density mononuclear cells (MNC), separated on Histopaque-1077 (Sigma Chemical Co., St. Louis, MO) density gradient were enriched for hematopoietic progenitors by removal of adherent cells and T lymphocytes (A^-T^-MNC) as described (18). MNC from untreated, CML-BC patients (> 50,000/μl of white blood cells, > 95% Ph^1 metaphases) were similarly isolated from bone marrow. The Ph^1 leukemia cell line BV173 was established from a patient in lymphoid blast crisis (19) and is characterized by the presence of the b2/a2 break point and common acute lymphoblastic leukemia antigen (CALLA), and the absence of CD45 antigen (19, 20 and unpublished observations).

Mice. Triple-immunodeficient bg/nu/xid (BNX) female mice and immunodeficient severe combined immunodeficient (SCID) male mice were obtained from Taconic (Germantown, NY). Mice were maintained under pathogen-free conditions and were 6–8 wk old when used in the experiments. BNX and SCID mice received 600 and 300 cGy of total body irradiation, respectively, 1 d before injection of cells. After irradiation and injection of cells, SCID mice were supported with stem cell growth factor (SCF, 20 μg), a fusion protein of human IL-3 and GM-CSF (PIXY321, 8 μg) and erythropoietin (20 U). Mice were injected intraperitoneally with the growth factors every 2 d for a total of 15 times. SCF and PIXY321 were obtained from Immunex Corp. (Seattle, WA) and erythropoietin from Amgen, Inc. (Thousand Oaks, CA).

Oligodeoxynucleotides. These were synthesized on a DNA synthesizer (model 380B, Applied Biosystems, Inc., Foster City, CA) by means of β-cyanoethylphosphorymidite chemistry. The sequences of b2/a2 and b3/a2 antisense oligodeoxynucleotides, the 5′ primer of bcr exon 2, the 3′ primer of abl exon 2, and the abl probe recognizing the amplified 257-bp bcr-abl transcript have been described (10). The sequences of 5′ and 3′ primers of human α-satellite DNA are as described (21) and the 32-base probe corresponds to nucleotides 77–108 of the published sequence of human α-satellite (22). The β_2-microglobulin (β_2) 5′ and 3′ primers correspond to nucleotides 280–301 and 510–531, respectively, and the 50-base probe corresponds to nucleotides 351–400 (23). β-Actin oligomers were made based on the published sequence of mouse β-actin (24). Primers synthesized for murine β-actin cross-reacted with human β-actin (not shown), and the probe corresponds to nucleotides 258–296 (25).

Mafosfamide treatment. Mafosfamide (ASTA Z 7654) was kindly provided by Drs. H. Sindermann and M. Peukert (Asta-Werke AG, Frankfurt am Main, FRG). The drug was dissolved in Iscove's modified Dulbecco's medium (IMDM) and sterilized by filtration through a 0.22-μm Acrodisc filter (Gelman Sciences, Inc., Ann Arbor, MI) before use. 10^6 to 10^7 cells/ml in IMDM supplemented with 10% heat-inactivated human AB serum were incubated in the presence of different concentrations of mafosfamide for 30 min at 37°C, washed, and resuspended in IMDM medium.

Oligomer treatment. For in vitro studies, 1×10^5 cells/ml were seeded into 24-well cell culture plates (Costar Corp., Cambridge, MA) in 0.4 ml of IMDM supplemented with 2% heat-inactivated human AB serum, Hepes buffer, and recombinant human IL-3 (50 U/ml) and GM-CSF (12.5 ng/ml) (Genetics Institute, Cambridge, MA). For in vivo studies, 1×10^6 cells/ml were seeded into 75-cm^2 tissue culture flasks (Corning Glass Works, Corning, NY) in IMDM supplemented with 10% heat-inactivated human AB serum, Hepes buffer, IL-3, GM-CSF, and SCF (100 ng/ml). Sense and antisense b2/a2 oligodeoxynucleotides were added at 80 μg/ml on time 0. The second and third dose (40 μg/ml) were added 18 and 40 h later. 65 h after starting the treatment, cells were plated without washing or injected into mice after washing in IMDM. Control groups were plated/injected without prior oligodeoxynucleotide exposure.

Colony assays. CFU-GM colonies were grown and counted as described (18). 5×10^4 of A^-T^-MNC or CML cells were plated in HCC-4230 medium (Terry Fox Laboratories, Vancouver, BC) supplemented with IL-3 (20 U/ml), GM-CSF (5 ng/ml), and 0.125 mM L-glutamine in duplicate 35-mm Petri dishes (Nunc Inc., Naperville, IL) at 1 ml per dish. Growth of human colonies derived from 10^5 mouse marrow cells plate was, in addition, supported with recombinant human SCF (100 ng/ml) and recombinant human erythropoietin (2 U/ml). Colonies and clusters were scored after 9–12 d of culture in a humidified 5% CO_2 incubator.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Cells were collected separately from each experimental group, centrifuged on Histopaque-1077 density gradient, and washed before total RNA extraction in the presence of 20 μg of *Escherichia coli* ribosomal RNA as described (26). RNA from each group was divided into two aliquots. One sample was reverse-transcribed using 400 U of Moloney murine leukemia virus RT (BRL, Gaithersburg, MD) and 0.1 μg of 3′ primer of abl exon 2 for 1 h at 37°C. The second sample was reverse-transcribed using the β_2-microglobulin 3′ primer. The resulting cDNA fragments were amplified with 5 U of *Thermus aquaticus* (Taq) polymerase (Perkin Elmer Cetus, Norwalk, CT) in the presence of the 5′ primer of bcr exon 3 or β_2-microglobulin 5′ primer, generating a 257-bp fragment corresponding to the bcr/abl break-point region, and a 252-bp fragment of β_2-microglobulin during 56 cycles of PCR (27). Reaction products were electrophoresed in 2% agarose gel, transferred to Zeta-probe blotting membranes (Bio-Rad Laboratories, Richmond, CA) and hybridized overnight at 50°C using the abl exon 2 or β_2-microglobulin probes end-labeled with [γ-^{32}P]ATP and polynucleotide kinase as described (28). Filters were washed with 2× SSC + 0.1% SDS at 49°C for 30 min. Using this RT-PCR protocol we were able to detect 10^2 BV173 or 10^3 CML-BC cultured primary cells among 10^6 cells.

Single-colony PCR analysis. Single colonies were picked separately and divided into two portions; RNA was prepared as described (18) from one portion, and DNA was extracted by repeated freezing and thawing from the other. RNA was reverse-transcribed using the abl exon 2 or β-actin 3′ primers. The resulting cDNA was amplified using the bcr exon 2 and the β-actin 5′ primers, respectively, and Taq polymerase as described above. DNA was amplified with human α-satellite 3′ and 5′ primers and Taq polymerase, and PCR amplification products were electrophoresed, transferred, and hybridized as described for RT-PCR.

Immunofluorescence studies. For flow cytometry analysis, single-cell suspensions were prepared from bone marrow of mice injected with human cells. Cells (10^5) were stained with FITC-conjugated mouse anti-HLe-1 (anti-CD45) or anti-CALLA (anti-CD10) monoclonal antibody (Becton-Dickinson Immunocytometry Systems, San Jose, CA), washed, and analyzed by flow cytometry using the Epics Profile Analyzer (Coulter Corp., Hialeah, FL). For each cell type, two negative controls were used: the same cell population stained with FITC-conjugated anti-human CD3 monoclonal antibody, and BMC stained with FITC-conjugated anti-HLe-1 or anti-CALLA from non-injected mice. Human peripheral blood mononuclear cells (99% positivity) and BV173 cells (95% positivity) served as positive control for anti-CD45 and anti-CD-10 monoclonal antibodies, respectively. The level of sensitivity for each antibody was $\sim 10^{-2}$. Negative and positive controls were utilized in each set of experiments.

Cytogenetic analysis of single hematopoietic colonies. A 1:1 mix of normal marrow progenitors and leukemic cells was cultured in methylcellulose in the presence of bcr-abl antisense oligodeoxynucleotides, alone or in combination with mafosfamide. After 7–9 d, plates were exposed to a colcemid solution (0.3 μg/ml) for 1 h. Single colonies were plucked from the methylcellulose, diluted with physiological saline, and centrifuged to remove residual methylcellulose. After 25 min of hypotonic treatment in 0.075 M KCl, cells were fixed by washing several times with methanol/glacial acetic acid (3:1). Slides were air-dried and G-banded as described (29).

Results

In vitro sensitivity of CML and normal marrow cells to mafosfamide. The sensitivity of CML-BC cells from 11 patients, and normal bone marrow hematopoietic progenitor cells from 8 healthy volunteers to mafosfamide was examined. Leukemic cells were more sensitive than normal hematopoietic cells to mafosfamide at concentrations of 25, 50, and 100 μg/ml (Fig. 1). At the highest concentration of mafosfamide (100 μg/ml), formation of colonies derived from CML-BC cells was completely inhibited in samples from 9 of 11 patients and almost completely in two other cases (~ 99.8% inhibition). At the same time, 3.15% (0.6–10.3%) of normal hematopoietic colonies were spared in 8 of 8 samples from healthy volunteers (Fig. 1). The $CD34^+$ subpopulation of CML cells was also more sensitive than that of normal hematopoietic progenitors to mafosfamide (not shown). Secondary colony formation assays to demonstrate further that mafosfamide treatment (100 μg/ml) permanently inhibited the growth ability of leukemic cells but not of normal progenitors indicated no visible secondary colonies derived from CML primary cells but several CFU-GM colonies from normal marrow MNC (A^-T^-MNC) incubated with the same concentrations of the drug. Untreated CML-BC cells formed numerous secondary colonies (not shown).

Effect of different concentrations of mafosfamide on a mixture of CML-BC and normal marrow cells. To determine whether mafosfamide preferentially eliminates leukemic cells as compared to normal progenitor cells, normal A^-T^-MNC and CML-BC cells mixed at a 1:1 ratio were incubated with increasing concentrations of mafosfamide and plated in methylcellulose and the resulting colonies were counted (Fig. 2, *top*). Cells were then harvested and centrifuged on Histopaque-1077 to eliminate dead cells before RNA extraction, and RT-PCR was performed to identify leukemic cells expressing the bcr/abl transcript among normal BMC; no bcr-abl mRNA was detected after treatment with mafosfamide at 100 μg/ml, and the bcr-abl transcript was barely detectable after exposure to mafosfamide at 50 μg/ml (Fig. 2, *bottom*). By contrast, control β_2-microglobulin mRNA, was clearly detectable in all samples (Fig. 2, *bottom*). Similar results were obtained with cells from two other CML-BC patients indicating that cells carrying the Ph^1 translocation and generating the bcr/abl transcript were below the level of detection, whereas normal progenitor cells survived exposure to mafosfamide at 100 μg/ml.

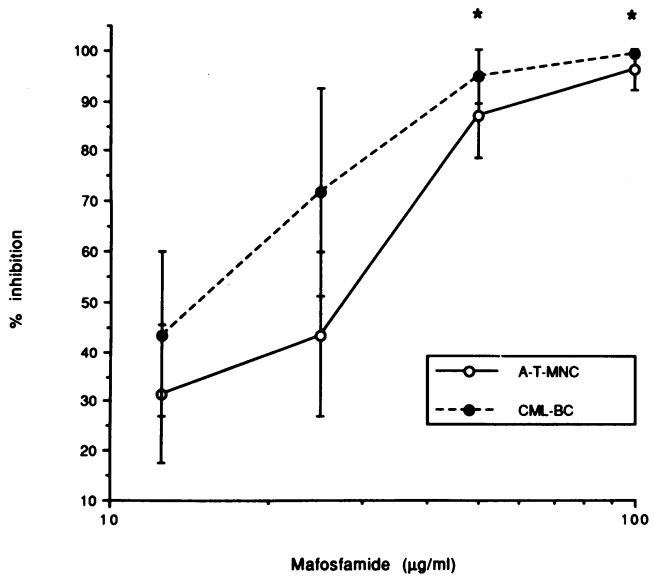

Figure 1. Effect of mafosfamide on in vitro colony-forming ability of A^-T^-MNC (○), or CML-BC cells (●). Cells were incubated with the indicated concentrations of mafosfamide and plated in methylcellulose, and colonies were counted. Results are expressed as mean±SD (*$P < 0.05$).

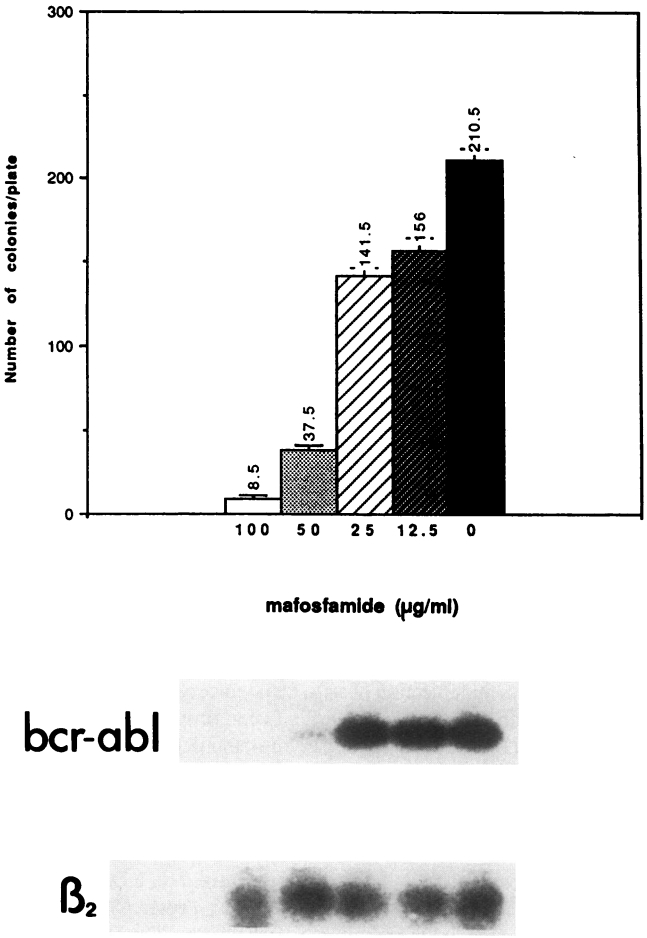

Figure 2. Effect of mafosfamide at various concentrations on colony formation (*top*) and bcr/abl or β_2-microglobulin mRNA expression (*bottom*) in a 1:1 mix of CML-BC primary cells and normal marrow cells. Duplicate cultures for each experimental group were counted 12 d after plating in methylcellulose. Bars represent mean±SD. Cells were pooled from each group, and total RNA was extracted and divided in two equal aliquots for analysis of bcr-abl and β_2-microglobulin mRNA levels by RT-PCR.

Effect of mafosfamide plus bcr-abl antisense oligodeoxynucleotides on a 1:1 mixture of Ph^1-positive BV173 cells and normal marrow cells. The Ph^1 leukemia cell line BV173 is characterized by the presence of the b2/a2 break point in which the bcr exon 2 is fused to the abl exon 2 (20). BV173 cells form colonies with high efficiency when plated in semisolid medium, and proliferation of these cells was inhibited by exposure to a b2/a2 antisense oligomer (18-mer) complementary to the break-point junction, but not by the sense oligomer b2/a2 or the antisense oligomers b1/a2 or b3/a2 (not shown). BV173 cell proliferation was also inhibited by exposure to mafosfa-

mide (not shown). Colony formation assays of a 1:1 mixture of BV173 cells (cloning efficiency ~ 30%) and normal marrow cells (cloning efficiency ~ 0.3–0.6%) exposed to mafosfamide, b2/a2 antisense oligodeoxynucleotides or both revealed residual colonies in the presence of 2.5 μg/ml of mafosfamide or b2/a2 antisense oligodeoxynucleotides (80 μg/ml followed by 40 μg/ml 18 and 40 h later), and also when the drug (2.5 μg/ml) was combined with the b2/a2 antisense oligomer (80 + 40 + 40 μg/ml) (Fig. 3, *top*). However, a bcr-abl transcript was clearly present in colonies derived from cells treated with mafosfamide or with the antisense oligomer alone, but was not detectable after exposure to a combination of the two reagents suggesting that mafosfamide and b2/a2 antisense oligodeoxynucleotides have a synergistic effect against leukemic cells (Fig. 3, *bottom*). In contrast, control β_2-microglobulin mRNA, was clearly detectable in each sample (Fig. 3, *bottom*), suggesting that normal clonogenic cells were spared. In fact, cytogenetic analysis of individual colonies derived from the 1:1 mixture of normal marrow MNC and BV173 cells, untreated or exposed to a combination of bcr-abl antisense oligodeoxynucleotides and mafosfamide revealed that 15 of 20 untreated colonies were Ph^1-positive, whereas all 18 colonies examined after treatment with bcr-abl antisense oligodeoxynucleotides and mafosfamide, were karyotypically normal (Table I). Bcr-abl sense oligodeoxynucleotides did not exert any additional effect against leukemic cells when combined with mafosfamide (not shown).

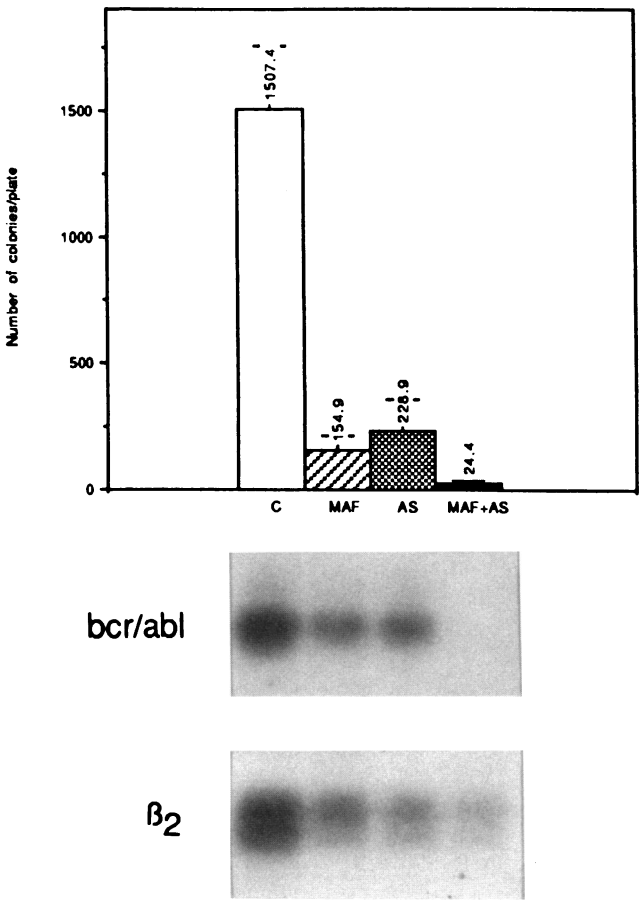

Figure 3. Effect of mafosfamide (*MAF*), bcr-abl antisense oligodeoxynucleotides (*AS*), or both (*MAF + AS*) on colony formation (*top*) and bcr/abl or β_2-microglobulin mRNA expression (*bottom*) in a 1:1 mix of BV173 cells and normal marrow cells. Cell culture conditions, RNA extraction, and RNA phenotyping by RT-PCR technique were as described in Methods. Bars represent mean±SD.

Effect of mafosfamide plus bcr-abl antisense oligodeoxynucleotides on CML-BC or A$^-$T$^-$ MNC colony formation. Clonogenic assays of CML-BC cells from five patients and A$^-$T$^-$MNC cells from four healthy volunteers were utilized to determine the sensitivity of these cells to a combination of mafosfamide (25 μg/ml) and bcr-abl antisense oligodeoxynucleotides (80 + 40 + 40 μg/ml) (Table II). Compared with untreated cultures, CML-BC colony formation was completely (100% inhibition in two cases) or almost completely (99.8% inhibition in three cases) suppressed by exposure to mafosfamide plus antisense oligodeoxynucleotides. Under identical culture conditions 20.8–43.8% of normal hematopoietic progenitors were spared (Table II). At those concentrations mafosfamide or antisense oligodeoxynucleotides alone were not able to inhibit so efficiently the growth of CML-BC cells. It should be also noticed that antisense oligodeoxynucleotides exerted a moderate nonspecific toxic effect against normal bone marrow progenitors which varied between donors and batches of oligomers.

Effect of mafosfamide plus bcr-abl antisense oligodeoxynucleotides on a 1:1 mixture of CML-BC and normal marrow cells. Primary cells from CML-BC patients (cloning efficiency ~ 0.6–3%) in whom cytogenetic analysis indicated that 100% metaphases were Ph^1-positive and molecular analysis revealed the expression of bcr-abl transcripts containing the b2/a2 break-point junction were mixed with an equal number of A$^-$T$^-$MNC (cloning efficiency ~ 0.3–0.6%) and incubated with mafosfamide (25 μg/ml) and a bcr-abl antisense oligomer complementary to the break-point junction (b2/a2, 80 + 40 + 40 μg/ml) before plating in methylcellulose. Colonies were counted after 9 d of culture (Fig. 4, *top*), and then total RNA was isolated from the cells. A bcr-abl transcript was clearly present in untreated cells and in cells treated with mafosfamide or with the antisense oligomer alone, but, in all three cases, was not detectable after exposure to a combination of mafosfamide and bcr/abl antisense oligodeoxynucleotides. (Fig. 4, *bottom*). In contrast, control β_2-microglobulin mRNA, was clearly detectable in each sample. Fig. 4 shows results from a representative case. Bcr-abl sense oligodeoxynucleotides in combination with mafosfamide had no additional effect over that of mafosfamide alone (not shown). Cytogenetic analysis of individual colonies derived from a 1:1 mix of normal marrow MNC and

Table I. Cytogenetic Analysis of the Presence of Ph^1 Chromosome in Colony-forming Cells In Vitro before or after Purging

Cells*	No. of Ph^1 colonies per no. of colonies analyzed‡	
	Untreated	Treated
BV173+A$^-$T$^-$MNC	15/20	0/18
CML-BC+A$^-$T$^-$MNC	12/18	0/23

* 1:1 mixture of leukemic and normal cells.
‡ Metaphases of BV173 and CML-BC patient cells were 100% positive for Ph^1 before the studies. Data represent results from two independent experiments.

Table II. Effect of Mafosfamide + bcr-abl Antisense Oligodeoxynucleotides on the Number of Colonies Derived from CMI-BC and A^-T^-MNC

Cells	Mafosfamide					
	0 μg/ml			25 μg/ml		
Oligodeoxy nucleotide:	C	S	AS	C	S	AS
CML-BC						
UPN 1 (b2/a2)	363.5±36.1	321.5±26.1	146.5±21.9	37.0±1.4	30.5±2.8	1.5±1.4
UPN 2 (b2/a2)	295.8±72.0	279.8±24.2	84.0±17.6	68.5±13.4	60.2±2.5	0
UPN 3 (b2/a2)	412.5±95.5	ND	181.5±3.5	75.5±13.4	ND	1.0±0.0
UPN 4 (b2/a2)	410.0±76.4	ND	130.5±24.7	41.5±20.5	ND	0.3±0.1
UPN 5 (b2/a2)	1527.5±128.4	1480.2±151.7	285.8±57.2	212.5±78.2	198.8±51.0	0
A^-T^-MNC						
A	143.4±2.1	ND	124.0±22.6	80.5±17.1	ND	47.5±1.4
B	165.5±13.4	ND	155.5±7.8	82.0±10.8	ND	34.5±6.4
C	256.0±28.3	ND	162.5±17.7	172.0±1.4	ND	112.0±9.9
D	286.2±37.8	256.2±27.8	268.5±44.0	158.5±10.2	ND	102.5±13.2

Cells were incubated with indicated concentrations of mafosfamide and left untreated (C) or treated with break-point-specific sense (S) or antisense (AS) oligodeoxynucleotides as described in Methods. The results (mean ± SD are from duplicate cultures. ND, not determined.

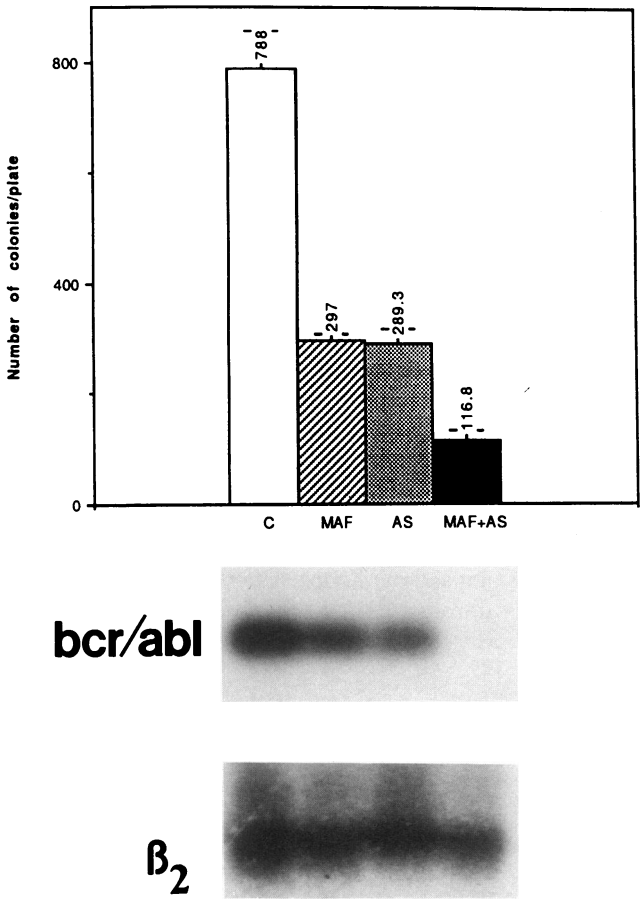

Figure 4. Effect of mafosfamide (*MAF*), bcr-abl antisense oligodeoxynucleotides (*AS*), or both (*MAF + AS*) on colony formation (*top*) and bcr/abl or β_2-microglobulin mRNA expression (*bottom*) in a 1:1 mix of CML-BC primary cells and normal marrow cells. Cell culture conditions, RNA extraction, and RNA phenotyping by RT-PCR technique were as described in Methods. Bars represent mean±SD.

leukemic cells exposed to a combination of bcr-abl antisense oligodeoxynucleotides and mafosfamide revealed a normal karyotype in all 23 colonies examined, whereas only 6 of 18 untreated colonies were normal and the remaining 12 were Ph^1-positive (Table I). Together, these results indicate that only the combination of mafosfamide and bcr-abl antisense oligodeoxynucleotides reduced below the levels of detection cells carrying the Ph^1 translocation and spared a high number of normal progenitor cells.

Detection of normal hematopoietic colony-forming cells but not leukemic cells in SCID mice injected with a 1:1 mixture of marrow MNC and BV173 cells treated with mafosfamide and bcr-abl antisense oligodeoxynucleotides. A 1:1 mixture of A^-T^-MNC and BV173 cells, untreated or treated with a combination of mafosfamide (2.5 μg/ml) and b2/a2 antisense oligodeoxynucleotides (80 + 40 + 40 μg/ml), was injected intravenously into sublethally irradiated SCID mice systemically treated with human hematopoietic growth factors as described in Methods. This model allows detection of normal human hematopoietic cells and BV173 cells after injection of a least 10^6 and 10^4 cells, respectively. Flow cytometric analysis of bone marrow cells isolated from these mice killed 1 mo after injection revealed $CD45^+$ cells in SCID mice injected with purged and nonpurged human bone marrow (Table III), indicating the presence of normal human hematopoietic cells, (BV173 leukemia cells do not express this antigen); $CALLA^+$ cells (mainly BV173 cells) were detected only in mice injected with nonpurged bone marrow. Under conditions which presumably allow only the growth of human cells (bone marrow progenitors and BV173 cells), several colonies (34.5±21.9/10^5 cells plated) were detected in bone marrow of two mice injected with the 1:1 mixture of normal marrow cells from different donors and BV173 cells which was exposed to mafosfamide (2.5 μg/ml) and b2/a2 antisense oligodeoxynucleotides (80 + 40 + 40 μg/ml) (Table III). These colonies (five colonies tested per mouse) were of human origin, as indicated by the detection of human-specific α-satellite DNA sequences,

Table III. Effect of Mafosfamide + bcr-abl Antisense Oligodeoxynucleotides on the Number of Leukemic and Normal Hematopoietic Colony-forming Cells in Bone Marrow of SCID Mice

Cells	Treatment*	$CD45^+$ cells	$CALLA^+$ cells	Colonies/ 10^5 cells
		%	%	
A-T-MNC+	None	7.0 + 0.3	15.3 + 7.3	3121.0 + 210.7
BV173	MAF + AS	3.2 + 1.2	0	34.5 + 21.9
A-T-MNC+	None	24.0 + 3.6	ND	149.8 + 18.3
CML-BC	MAF + AS	1.5 + 0.2	ND	9.8 + 4.0

1:1 mixture at A^-T^-MNC + BV173 cells (45×10^6) or A^-T^-MNC + CML-BC cells (60×10^6) were divided onto two portions and were untreated (none) or treated with mafosfamide + antisense oligodeoxynucleotides as described. Then the cells were injected i.v. into the SCID mice which then were supported with human recombinant growth factors. 1 mo later mice were killed and BMC were analyzed. Each experiment was performed twice using A-T-MNC from different donors.

* Two mice per group were analyzed. The results represent mean±SD from two independent experiments (six determinations).

and not leukemic because of the lack of bcr/abl mRNA expression (Fig. 5 *A*). Colonies showed a characteristic myeloid or erythroid phenotype. Numerous colonies (3,121.0±210.7/10^5 cells plated) arose from bone marrow of two SCID mice injected with nonpurged cells. The human α-satellite DNA sequence and bcr-abl mRNA were easily detectable in single colonies (five colonies tested per mouse) (Fig. 5 *A*), indicating the leukemic origin of these colonies. The phenotype of these colonies was typical for colonies derived from BV173 cells. Analysis of single colonies derived from experiments conducted in two additional mice gave similar results.

Engraftment of human normal but not patient leukemic hematopoietic cells in SCID mice injected with a 1:1 mixture of marrow MNC and CML-BC primary cells after mafosfamide and bcr/abl antisense oligodeoxynucleotides treatment. A 1:1 mixture of A^-T^-MNC and CML-BC patient cells (UPN 5, b2/a2 break point) was untreated or treated with mafosfamide (25 μg/ml) plus b2/a2 antisense oligodeoxynucleotides (80 + 40 + 40 mg/ml) and then injected i.v. into sublethally irradiated SCID mice, which were then supported with human recombinant hematopoietic growth factors as described in Methods. This murine model allowed detection of normal and primary CML-BC cells after injection of at least 10^6 cells.

Flow cytometric analysis of bone marrow cells isolated from the mice 1 mo after cells injection revealed the presence of $CD45^+$ cells in both groups of mice (Table III). Under conditions that favor the growth of human hematopoietic cells numerous colonies were grown from bone marrow of mice injected with nontreated cells, whereas only few colonies grew after injection of purged cells (Table III). All colonies (five colonies tested per mouse) contained human-specific α-satellite DNA sequences (Fig. 5 *B*). Bcr/abl mRNA was only detected in four colonies which arose from bone marrow of the mouse injected with nontreated cells (five colonies tested per mouse) (Fig. 5 *B*). The analysis of single colonies from the second group of mice gave similar results. As expected, bcr/abl expression was detected in marrow cells of mice injected with untreated cells but not after in vitro treatment with bcr/abl

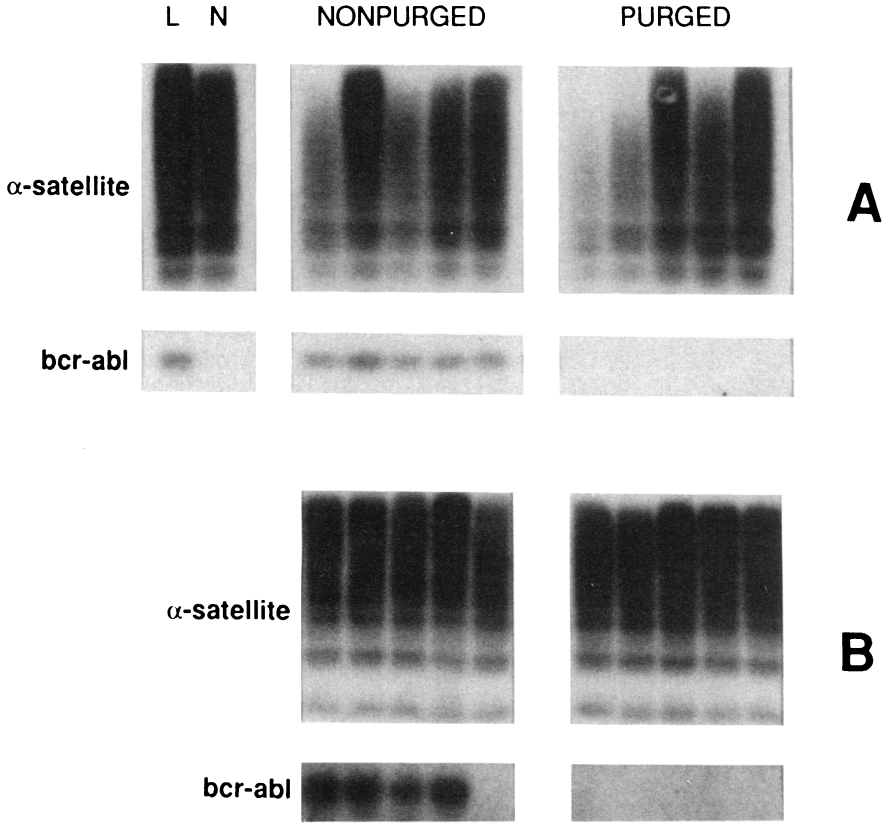

Figure 5. Identification of human α-satellite DNA and bcr-abl mRNA in colonies of BMC of SCID mice injected with mafosfamide + antisense-treated human BMC mixed 1:1 with (*A*) BV173 leukemia cells or (*B*) CML-BC cells. BMC were obtained from sublethally irradiated SCID mice 1 mo after injection of a 1:1 mixture of BV173 or CML-BC cells and human BMC (A^-T^-MNC) pretreated with mafosfamide plus bcr-abl antisense oligodeoxynucleotides. PCR and RT-PCR were used to detect nucleic acids. Colonies of BV173 leukemia (*L*) or normal bone marrow (*N*) cells served as positive and negative control for bcr-abl mRNA detection, respectively.

Table IV. Engraftment of Human Hematopoietic Colony-forming Cells in BNX Mice

Mice*	A^-T^-MNC injected (10^6/mouse)‡	$CD45^+$ cells/10^5 cells	Colonies/10^5 cells§
Control			
A	3.3 1.5	349±70	5, 12, 4, 2
B	2.3 2.5	140±8	4, 4, 8, 6
C	4.0 2.8	355±177	3, 0, 1, 4
Mafosfamide (100 μg/ml)			
A	3.3 0.7	0	0
B	2.3 1.1	0	0
C	4.0 1.6	0	0
Mafosfamide (25 μg/ml) + antisense			
A	3.3 1.0	398±98	7, 11, 3, 1
B	2.3 1.9	60±11	8, 4, 4, 10
C	4.0 2.2	335±35	1, 1, 0, 3

* Mice were analyzed 1 (experiments A and B) or 2 mo (C) after implantation of human BMC. Each experiment was performed independently.
‡ Left column, numbers of human A^-T^-MNC in each experiment (A, B, C) before treatment; right column, numbers of the cells injected into the mouse after treatment.
§ Number of colonies per plate.

antisense oligodeoxynucleotides and mafosfamide (not shown).

Engraftment of human hematopoietic colony-forming cells treated with mafosfamide and bcr/abl antisense oligodeoxynucleotides in BNX mice. To compare the toxicity of two effective regimens of CML cells elimination for the hematopoietic cells responsible for in vivo engraftment, A^-T^-MNC from donors were treated ex vivo with mafosfamide alone (100 μg/ml) or mafosfamide (25 μg/ml) and b2/a2 antisense oligodeoxynucleotides (80 + 40 + 40 μg/ml) as described above, and assayed for colony-forming ability in vitro in the presence of recombinant human IL-3 and recombinant human GM-CSF before injection into sublethally irradiated BNX mice. Untreated cells formed numerous colonies (707±80 colonies per 10^5 cells), whereas colony formation was diminished (183±50 colonies per 10^5 cells), after mafosfamide plus bcr/abl antisense oligodeoxynucleotides treatment, and almost totally inhibited (3±3 colonies per 10^5 cells), after treatment with mafosfamide (100 μg/ml) alone.

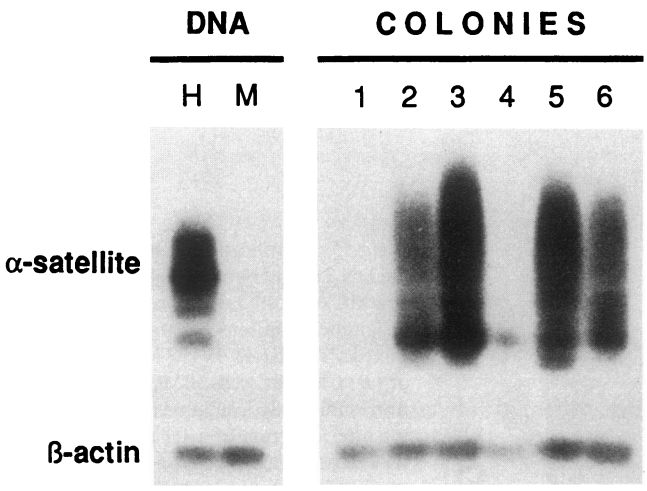

Figure 6. PCR-based detection of human α-satellite DNA in individual colonies from methylcellulose cultures of BMC obtained from sublethally irradiated BNX mice injected with mafosfamide (25 μg/ml) + antisense-treated human BMC. DNA from human (*H*) and murine (*M*) peripheral blood mononuclear cells served as positive and negative controls, respectively.

1 or 2 mo after cell injection, the mice were killed and BMC were isolated from sacrificed mice and examined for the presence of human hematopoietic colony-forming cells by monoclonal antibody staining and colony-forming ability. $CD45^+$ cells were detected among bone marrow cells obtained from mice injected with untreated or mafosfamide (25 μg/ml) plus b2/a2 antisense oligodeoxynucleotide-treated human BMC, but not from mice injected with mafosfamide (100 μg/ml)-treated cells (Table IV). Under conditions that allow only growth of human bone marrow progenitor cells, several myeloid and erythroid colonies were detected in bone marrow of mice injected with untreated or mafosfamide (25 μg/ml) plus b2/a2 antisense oligodeoxynucleotides-treated human BMC (2–12 or 1–11 per 10^5 cells, respectively), but not in bone marrow of mice injected with mafosfamide (100 μg/ml)-treated cells (Table IV). The human origin of most of these colonies (5/6) was confirmed by detection of human α-satellite DNA sequence in PCR reaction products obtained from single colonies (Fig. 6). In this murine model human colony-forming cells were detected after injection of BNX with at least 10^6 normal BMC.

Discussion

Intensive chemoradiotherapy followed by allogeneic or syngeneic BMT has become an effective and potentially curative therapy for patients with CML (30). If appropriate donors are not available, autologous BMT can be utilized, but the almost certain reinfusion of large numbers of clonogenic Ph^1-positive leukemic cells may eventually minimize the benefit of this treatment. Gene transfer techniques to mark BMC before reinfusion into the patient would allow determination of the origin of the relapsed CML cells (31), thereby addressing the contro-

versial issues on the ability of reinfused CML cells to engraft in vivo (14, 32).

CML is a disease of the hematopoietic stem cells, which generate an expanded pool of unipotent progenitors that retain the ability to differentiate during the chronic phase, but undergo severe differentiation arrest during blast crisis. There is evidence that residual normal hematopoiesis persists during both disease stages (33, 34), which would support autologous BMT as an alternative therapy for CML. The maintenance of normal hematopoiesis and the effective elimination of Ph^1 leukemic cells from the autograft would significantly improve the results of such treatment. However, a real impact on the treatment of the disease, can be expected only if we improve also the efficacy of patient's in vivo treatment.

Mafosfamide, a cyclophosphamide derivative, is utilized to purge acute myeloid leukemia cells from autologous bone marrow grafts. A decreased probability of relapse is associated with the use of such drugs at a concentration sparing as few as 5% CFU-GM (11). Lowest relapse rates appear to be achieved using a drug concentration that spares < 1% of CFU-GM (35). For this reason, effective purging is often accompanied by delayed engraftment and persistent thrombocytopenia (36).

In light of conflicting observations on the efficacy of cyclophosphamide derivatives for bone marrow purging in CML patients (15, 37), we utilized colony formation assays (38) and the PCR technique (39) to determine whether Ph^1 leukemic cells are killed while normal progenitor cells are spared.

Our data from colony-forming assays (primary and secondary) and RT-PCR detection of bcr-abl transcripts indicate that the colonies arising from mafosfamide (100 μg/ml) purged bone marrow were nonneoplastic, and that the number of leukemic cells was diminished below the level of detection. In light of the unaltered expression of P210 bcr/abl tyrosine kinase in the presence of cytotoxic agents that inhibit cell growth (40), it was likely that the disappearance of the bcr/abl signal after mafosfamide treatment reflects the elimination of leukemic cells rather than the effect of the drug on bcr-abl gene expression. Although the findings reported here cannot be extrapolated to actual transplants, they nevertheless suggest that mafosfamide might be useful in some cases for purging of contaminating CML cells from autologous bone marrow, consistent with previous observations of other investigators (41, 42). However, the combination of different purging techniques might be more efficient in the elimination of leukemic cells and less toxic for normal hematopoietic stem cells.

We have recently described the selective elimination of CML-BC cells from the mixture of CML patients cells and normal BMC using antisense oligodeoxynucleotides targeted against the bcr/abl transcript (10), but the procedure did not eliminate all leukemic cells. In an effort to improve the efficiency of CML treatment, we tested the combination of mafosfamide and bcr/abl antisense oligodeoxynucleotides at concentrations which, when used separately, did not eliminate totally the leukemic cells but spared 47% and 85% of normal hematopoietic clonogenic cells, respectively. In combination, mafosfamide and antisense oligodeoxynucleotides caused complete disappearance of the bcr/abl transcripts which suggests a very efficient elimination of detectable leukemic cells from bone marrow. Cytogenetic analysis of the cells that grew after purging revealed no Ph^1-positive metaphases, which excludes the possibility of growth of "silent" leukemic cells that possess the Ph^1 chromosome but do not express bcr/abl transcripts (43), and confirms the efficacy of this treatment procedure. The in vitro results were confirmed in vivo when human BMC mixed with BV173 leukemic cells or CML-BC primary cells were treated with mafosfamide and bcr/abl antisense oligodeoxynucleotides before injection into immunodeficient SCID mice (44) subsequently supported with recombinant human hematopoietic growth factors. Normal human hematopoiesis after implantation of purged bone marrow, and primarily leukemic hematopoiesis after injection of nonpurged bone marrow were detected in bone marrow of the mice. In addition, the in vitro results (clonogenic assays) and the in vivo data (detection of human hematopoiesis in BNX mice) provide strong evidence that purging with mafosfamide plus antisense oligodeoxynucleotides spares a higher number of normal hematopoietic progenitors compared with purging with high concentrations of mafosfamide alone.

Another described procedure of bone marrow purging from CML cells is that based on the differential sensitivity of normal and Ph^1-positive hematopoietic cells to in vitro culture conditions (16). However, the effectiveness of this strategy is still questionable because the Ph^1-positive clonogenic progenitors usually disappear within 4–6 wk of long-term cultures (16), and there is variation among patients in the number of Ph^1-negative cells with repopulating potential after in vitro culture (34). A combination of mafosfamide treatment followed by in vitro culture purging and bcr-abl antisense oligodeoxynucleotide treatment might prove to be more therapeutically useful in some patients.

In summary, these data suggest that in our experimental model the combination of low-dose mafosfamide, with bcr/abl antisense oligodeoxynucleotides offers an efficient approach to eliminate CML-BC cells from BMC while significantly improving the sparing of normal hematopoietic stem cells, as compared with that seen using mafosfamide. Preliminary evidence that antisense oligodeoxynucleotides (bcr-abl or c-myb) in combination with other chemotherapeutic agents (adriamycin, etoposide, or *cis*-platinum) enhance the elimination of hematopoietic and nonhematopoietic neoplastic cells suggests the possibility of using this therapeutic strategy in the treatment of human malignancies.

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References

1. Bortin, M. M., M. M. Horowitz, and R. P. Gale. 1988. Current status of bone marrow transplantation in humans: report from the International Bone Marrow Transplant Registry. *Nat. Immun. Cell Growth Regul.* 7:334–350.

2. Butturini, A., and R. P. Gale. 1989. How we can cure leukemia. *Br. J. Haematol.* 72:479–485.

3. Bron, D., and P. Styckmans. 1989. Removal of tumour cells from bone marrows: overview. *Eur. J. Cancer Clin. Oncol.* 25:163–166.

4. Apperley, J. F., F. R. Mauro, J. M. Goldman, W. Gregory, C. K. Arthur, J. Hows, W. Arcese, W. Papa, F. Mandelli, D. Wardle, et al. 1988. Bone marrow transplantation for chronic myeloid leukemia in first chronic phase: importance of a graft-versus-leukemia effect. *Br. J. Haematol.* 69:239–245.

5. Goldman, J. M., and D. P. Lu. 1982. New approaches in chronic granulocytic leukemia: origin, prognosis and treatment. *Semin. Hematol.* 19:241–256.

6. Reiffers, J., G. Vezow, P. M. Bernard, B. David, J. Chevaleyere, P. Richard, M. R. Boisseau, J. Moulimer, and A. Broustet. 1983. Haemopoietic stem cell autografting for granulocytic leukemia in transformation. *Exp. Hematol.* 11:148.

7. Groffen, J., J. R. Stephenson, N. Heisterkemp, A. de Klein, C. R. Bartram, and G. Grosveld. 1984. Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. *Cell.* 36:93–99.

8. Shtivelman, E., B. Lifshitz, R. P. Gale, and E. Canaani. 1985. Fused transcript of abl and bcr genes in chronic myelogenous leukemia. *Nature (Lond.).* 315:550–554.

9. Daley, G. A., R. A. Van Etten, and D. Baltimore. 1990. Induction of chronic myelogenous leukemia in mice by the $p210^{bcr/abl}$ gene of the Philadelphia chromosome. *Science (Wash. DC).* 247:824–830.

10. Szczylik, C., T. Skorski, N. C. Nicolaides, L. Manzella, L. Malaguarnera, D. Venturelli, A. M. Gewirtz, and B. Calabretta. 1991. Selective inhibition of leukemia cell proliferation by BCR-ABL antisense oligodeoxynucleotides. *Science (Wash. DC).* 253:562–565.

11. Gorin, N. C., P. Aegertner, B. Auvert, G. Meloni, A. H. Goldstone, A. Burnett, A. Carella, M. Korbling, P. Herve, D. Maraninchi, et al. 1990. Autologous bone marrow transplantation for acute myelocytic leukemia in first remission: a European survey of the role of marrow purging. *Blood.* 75:1606–1614.

12. Singer, J. M., Z. Arlin, V. Najfeld, J. W. Adamson, S. J. Kempin, B. D. Clarkson, and P. J. Fialkow. 1980. Restoration of non-clonal hematopoiesis in chronic myelogenous leukemia (CML) following a chemotherapy-induced loss of the Ph^1 chromosome. *Blood.* 56:356–360.

13. Talpaz, M., K. B. McCredie, G. M. Mavligit, and J. U. Gutterman. 1983. Leukocyte interferon-induced myeloid cytoreduction in chronic myelogenous leukemia. *Blood.* 62:689–692.

14. Hughes, T. P., F. Brito-Babapulle, D. J. Tollit, P. Mariat, S. Bowcock, K. H. Th'ng, C. Dowding, and J. M. Goldman. 1991. Induction of Philadelphia-negative hemopoiesis and prolongation of chronic phase in patients with chronic myeloid leukemia treated with high dose chemotherapy and transfusion of peripheral blood stem cells. *In* Autologous Bone Marrow Transplantation: Proceedings of the Fifth International Symposium. K. Dicke, J. O. Armitage, and M. J. Dicke-Evinger, editors. University of Nebraska Medical Center, Omaha, NE. 219. (Abstr.)

15. Degliantoni, G., L. Mangoni, and V. Rizzoli. 1985. In vitro restoration of polyclonal hematopoiesis in a chronic myelogenous leukemia after in vitro treatment with 4-hydroperoxycyclophosphamide. *Blood.* 65:753–757.

16. Barnett, M. J., C. J. Eaves, G. L. Phillips, D. E. Hogge, R. K. Humphries, H. G. Klingermann, P. M. Lansdorg, D. E. Reece, J. D. Shepherd, and A. C. Eaves. 1991. Autografting with curative intent for patients with chronic myeloid leukemia. *In* Autologous Bone Marrow Transplantation: Proceedings of the Fifth International Symposium. K. Dicke, J. O. Armitage, and M. J. Dicke-Evinger, editors. University of Nebraska Medical Center, Omaha, NE. 237. (Abstr.)

17. Coulombel, L., D. K. Kolousek, C. H. Evans, C. M. Gupta, and A. C. Eaves. 1983. Long term marrow culture reveals chromosomally normal hematopoietic progenitor cells in patients with Philadelphia chromosome-positive chronic myelogenous leukemia. *N. Engl. J. Med.* 306:1493–1498.

18. Skorski, T., C. Szczylik, M. Z. Ratajczak, L. Malaguarnera, A. M. Gewirtz, and B. Calabretta. 1992. Growth factor-dependent inhibition of normal hematopoiesis by N-ras antisense oligodeoxynucleotides. *J. Exp. Med.* 175:743–750.

19. Pegoraro, L., L. Matera, J. Ritz, A. Levis, A. Palumbo, and G. Biagini. 1983. Establishment of a Ph^1 positive human cell line (BV 173). *JNCI (J. Natl. Canc. Inst.).* 70:447–450.

20. van Denderen, J., D. van der Plas, T. Meeuwsen, N. Zegers, W. Boersma, G. Grosveld, and W. van Ewijk. 1990. Immunologic characterization of the tumor-specific bcr-abl junction in Philadelphia chromosome-positive acute lymphoblastic leukemia. *Blood.* 76:136–141.

21. Kamel-Reid, S., and J. E. Dick. 1988. Engraftment of immune deficient mice with human hematopoietic stem cells. *Science (Wash. DC).* 242:1706–1709.

22. Waye, J. S., and H. F. Willard. 1987. Nucleotide sequence heterogeneity of alpha satellite repetitive DNA: a survey of alphoid sequences from different human chromosomes. *Nucleic Acids Res.* 18:7549–7569.

23. Suggs, S. V., R. B. Wallace, T. Hirose, E. H. Kawashima, and K. Itakura. 1981. Use of synthetic oligonucleotides as hybridization probes: isolation of cloned cDNA sequences for human β_2-microglobulin. *Proc. Natl. Acad. Sci. USA.* 78:6613–6617.

24. Tokunaga, K., H. Taniguchi, M. Shimizu, and S. Sakiyama. 1986. Nucleotide sequence of a full length cDNA for mouse cytoskeletal β-actin mRNA. *Nucleic Acids Res.* 14:2829–2838.

25. Nicolaides, N. C., R. Gualdi, C. Casadevall, L. Manzella, and B. Calabretta. 1991. Positive autoregulation of c-myb expression via myb binding sites in the 5′ flanking region of the human c-myb gene. *Mol. Cell. Biol.* 11:6166–6176.

26. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

27. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scherf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1984. Primer-directed enzymatic amplification of DNA: a thermostable DNA polymerase. *Science (Wash. DC).* 239:487–491.

28. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

29. Seabright, M. 1971. A rapid banding technique for human chromosomes. *Lancet.* 2:971–972.

30. Thomas, E. D., R. A. Clift, A. Fefer, F. R. Appelbaum, P. Beatty, W. I. Bensinger, C. D. Buckner, M. A. Cheever, H. J. Deeg, K. Doney, et al. 1986. Marrow transplantation for the treatment of chronic myelogenous leukemia. *Ann. Intern. Med.* 104:155–157.

31. Claxton, D., S.-P. Sah, M. Filaccio, D. Ellerson, E. Gaozza, B. Anderson, M. Brenner, C. Reading, A. Feinberg, R. Moen, et al. 1991. Molecular analysis of retroviral transduction in chronic myelogenous leukemia. *Hum. Gene Ther.* 2:317–321.

32. Shiffer, C. A., J. Aisner, J. P. Dutcher, and P. H. Wiernik. 1983. Sustained post-transfusion granulocyte count increments following transfusion of leukocytes obtained from donors with chronic myelogenous leukemia. *Am. J. Hematol.* 15:65–74.

33. Dube, I. D., Z. A. Arlin, D. K. Kalousek, C. J. Eaves, and A. C. Eaves. 1984. Non-clonal hemopoietic progenitor cells detected in long-term marrow cultures from a Turner syndrome mosaic with chronic myeloid leukemia. *Blood.* 64:1284–1287.

34. Turhan, A. G., R. K. Humphries, C. J. Eaves, M. J. Barnett, G. L. Phillips, D. K. Kalousek, H. G. Klingemann, P. L. Lansdorg, D. E. Reece, J. D. Shepherd, et al. 1990. Detection of breakpoint cluster region-negative and nonclonal hematopoiesis in vitro and in vivo after transplantation of cells selected in cultures of chronic myeloid leukemia marrow. *Blood.* 76:2404–2410.

35. Rowley, S., R. Jones, S. Piandatosi, H. G., Braine, O. M. Colvin, J. Davis, R. Saral, A. Yeader, and G. Santos. 1988. CFU-GM recovery is a measure of effective ex vivo purging for autologous bone marrow transplantation in the treatment of acute nonlymphoblastic leukemia. *Exp. Hematol.* 7:543.

36. Donay, L., J. P. Laporte, and J. P. Mary. 1987. Differences in kinetics of hematopoietic reconstitution between ALL and ANLL after autologous bone marrow transplantation with marrow treated in vitro with mafosfamide (ASTA Z7557). *Bone Marrow Transplant.* 1:33–41.

37. Mortensen, B. T., P. Ernst, and P. Philip. 1988. Mafosfamide (ASTA-Z-7654) in vitro treatment of bone marrow does not eradicate Philadelphia-positive cells in chronic myeloid leukemia. *Eur. J. Haematol.* 41:218–222.

38. Wiley, J. M., and A. M. Yeager. 1991. Predictive value of colony-forming unit assays for engraftments and leukemia-free survival after transplantation of chemopurged syngeneic bone marrow cells. *Exp. Hematol.* 19:179–184.

39. Negrin, R. S., H-P. Kiem, I. G. H., Schmidt-Wolf, K. G. Blume, and M. L. Cleary. 1991. Use of polymerase chain reaction to monitor the effectiveness of ex vivo tumor cell purging. *Blood.* 77:654–660.

40. Takahira, H., J. Nishimura, K. Shibata, J. Hirata, T. Umemura, and H. Nawata. 1990. Lineage non-specific downregulation of P210bcr/abl in the CML cell line, KU-812-F, during differentiation. *Leuk. Res.* 14:801–808.

41. Carlo-Stella, C., L. Mangoni, G. Piovani, C. Amici, M. Savi, A. M. Carella, and V. Rizzoli. 1991. In vitro marrow purging in chronic myelogenous leukemia: effect of mafosfamide and recombinant granulocyte-macrophage colony-stimulating factor. *Bone Marrow Transplant.* 8:265–272.

42. Fouillard, L., J. Van Den Akker, J. P. Laporte, A. Najman, A. Perot, M. Lopez, L. Douay, F. Isnard, J. L. Taillemite, and N. C. Gorin. 1989. Bone marrow autograft treated with mafosfamide in the acceleration phase in chronic myeloid leukemia. Inversion of the clinical development. *Presse Med.* (Paris). 18:1785–1788.

43. Keating, A., and X.-H. Wang. 1991. Variable expression of bcr-abl and bcr gene mRNA in hematopoietic colonies from PH(+) chronic myeloid leukemia. *Exp. Hematol.* 19:539.

44. Lapidot, T., F. Pflumio, M. Doedons, B. Murdoch, D. E. Williams, and J. E. Dick. 1992. Cytokine stimulation of multilineage hematopoiesis from immature human cells engrafted in SCID mice. *Science (Wash. DC).* 255:1137–1141.