Combination with an Antisense Oligonucleotide Synergistically Improves the Antileukemic Efficacy of Erucylphospho-N,N,N-trimethylpropylammonium in Chronic Myeloid Leukemia Cell Lines

Spiro M. Konstantinov,1 Miika C. Georgieva,2 Margarita Topashka-Ancheva, Hansjörg Eibl, and Martin R. Berger3

Institute of Zoology, Bulgarian Academy of Sciences, Sofia 1000, Bulgaria [M. T.-A.; Max Planck Institute of Biophysical Chemistry, 37077 Göttingen, Germany [H. E.]; and Unit of Toxicology and Chemotherapy, German Cancer Research Center, 69120 Heidelberg, Germany

Abstract

The aim of this study was to enhance the antileukemic efficacy of the alkylphosphocholine erucylphospho-N,N,N-trimethylpropylammonium (ErPC3) in chronic myeloid leukemia (CML)-derived cell lines by a bcr-directed antisense oligonucleotide (ASO-bcr). The mechanism was substantiated by Western blotting of the BCR-ABL expression level of CML cells, and the efficacy was substantiated by inhibition of colony formation compared with normal hematopoietic cells. The clonogenicity of K-562 cells expressing high levels of p210BCR-ABL was inhibited significantly by the ASO-bcr (T/C%: 30; P < 0.05) but not by ErPC3 (T/C%, 70). Combined sequential exposure to ErPC3 and the ASO-bcr, however, inhibited synergistically colony growth (T/C%, 3; P < 0.01). The colony growth of BV-173 cells expressing lower levels of p210BCR-ABL than K562 cells was inhibited to a greater extent by the ASO-bcr (T/C%, 15; P < 0.01). AR-230 cells that express high levels of p230BCR-ABL showed an intermediate decrease in colony formation in response to the ASO-bcr (T/C%, 20; P < 0.05). BCR-ABL levels of BV-173, CML-T1, and LAMA-84 cells were reduced in response to the ASO-bcr, as evidenced by Western blot. However, K-562 and AR-230 cells showed reduced BCR-ABL expression only after repeated treatment. ErPC3 and the ASO-bcr did not reduce colony formation (CFU-GM) of normal mouse bone marrow cells from long-term bone marrow cell cultures; instead, ErPC3 stimulated colony formation (P < 0.05) and did not induce chromosomal aberrations in mouse bone marrow. In conclusion, the combination of ErPC3 with a suitable antisense oligonucleotide inhibited synergistically colony formation of CML cell lines without damaging normal cells and thus might have a bearing on the purging of autologous hematopoietic transplants in CML patients.

Introduction

CML is a malignant disease of the human hematopoietic stem cell, which is characterized by the reciprocal translocation t(9,22)(q34;q11) and in most cases is associated with the Philadelphia chromosome. This genetic rearrangement leads to the expression of a BCR-ABL fusion protein with elevated and dysregulated tyrosine kinase activity (1). The oncoprotein is necessary and sufficient for transformation and growth factor-independent growth of hematopoietic and nonhematopoietic cell lines (2, 3). Hydroxyurea and other cytotoxic agents can control the clinical symptoms of CML such as myeloid hyperplasia, leukocytosis, and organomegaly but have little or no effect on progression into blast crisis or myelofibrosis with subsequent bone marrow failure. An IFN-based therapy is currently the standard regimen for CML, but the tyrosinase kinase inhibitor imatinib (Glivec, STI-571) has been introduced recently and shows promising therapeutic results (4–6). At the molecular level, CML is well characterized (1) and, because of its distinct genetic alteration, is suited for an antisense therapy by which short spans of nucleic acids are used to disrupt the expression of disease-related genes (7).

APCs are a new group of antineoplastic agents (8–10) that selectively damage leukemia cells without affecting the normal bone marrow (11). Recently, erucylphosphocholine and its congener ErPC3 were described as the first i.v.-injectable APCs that lack hemolytic properties and show activity in vitro as well as increased therapeutic ratio in vivo (12). In contrast with most antitumor agents, this class can even stimulate the production of hematopoietic progenitor cells (13, 14). An exception to the sensitivity of leukemia cells toward APCs is the erythroleukemic cell line K-562 (11), which expresses high levels of BCR-ABL (15). BCR-ABL can inhibit or at least delay apoptosis in leukemia cells, and its expression level influences the antileukemic efficacy of APCs (14). Therefore, we speculated that a reduced expression of BCR-ABL would render CML cells sensitive toward APCs.

Received 3/4/02; revised 5/24/02; accepted 6/6/02.

1 Present address: Department of Pharmacology and Toxicology, Medical University, Faculty of Pharmacy, 2 Dunav Street, Sofia 1000, Bulgaria.
2 Present address: Laboratory of Oncogenesis, National Oncology Centre, 6 Plovdivsko Pole Street, Sofia 1756, Bulgaria.
3 To whom requests for reprints should be addressed, at Unit of Toxicology and Chemotherapy, German Cancer Research Center, Im Neuenheimer Feld, 280 69120 Heidelberg, Germany, Phone: 49-6221-423310; Fax: 49-6221-423313; E-mail: M.Berger@DKfz-Heidelberg.de.

The abbreviations used are: CML, chronic myeloid leukemia; APC, alkylphosphocholine; ErPC3, erucylphospho-N,N,N-trimethylpropylammonium; ASO, antisense oligonucleotide; NSO, nonsense oligonucleotide; Comb1, combination index; LTBMCC, long-term bone marrow cell culture; CFU-GM, colony-forming unit-granulocyte/macrophage.
In the present study, we investigated the mono- and combination effects of ErPC3 and an ASO directed against the start codon of bcr (ASO-bcr) that was to reduce the expression of the BCR-ABL protein. From a panel of five leukemia cell lines with the bcr-abl rearrangement that was used in this study, the mono-effect and combination effects on colony formation were determined in the most resistant CML cell line (K-562) and in normal murine long-term bone marrow cell culture. The latter results were correlated with the induction of chromosomal aberrations of ErPC3 on murine bone marrow in vivo.

**Materials and Methods**

**Compounds.** ErPC3 was synthesized as published elsewhere (16). Phosphorothioate oligonucleotides were synthesized by the Central Group for Oligonucleotide Synthesis and Sequencing of the Deutsches Krebsforschungszentrum according to the sequences reported by McGahan et al. (17). The structures are as follows: antisense (ASO-bcr), 5'-GCC CAC CGG GTG CAC CAT-3', nonsense (NSO), 5'-GCC GCC TCG TCC CAA GCA-3'; junction site antisense (ASO-b2a2), 5'-CGCTGAAAGGGCTTCTCCATTGAT-3'; junction site antisense (ASO-b3a2), 5'-CGCTGAAAGGGCTTGAACCTCTGCC-3'; and TAT (mismatch), 5'-CTCTTCCCTT-3'.

**Cells and Culture Conditions.** The following BCR-ABL-expressing cell lines were used: K-562, LAMA-84, AR-230, CML-T1, and BV-173. In addition, HL-60, HL-60fox and SKW-3 cells were used as leukemia cell lines without BCR-ABL expression. All cells were grown as suspension cultures (RPMI 1640 supplemented with 10% FCS and 2 mM L-glutamine) in a humidified atmosphere of 5% CO2 at 37°C in an incubator with humidified atmosphere and 5% CO2 and were passaged two or three times/week to keep them in log phase.

**Electrotransfection and Clonogenicity Assay.** The efficiency of electrotransfection was determined by a fluorescein-labeled randomized sequence phosphorothioate oligonucleotide of 20-bp length (FTC-control). Single or double impulses of 2 ms duration ranging from 300 to 700 V/cm were used to transfect this oligonucleotide into K-562 cells. After cytosin centrifugation onto a slide, the treated cells were fixed in methanol, and the ratio of fluorescing cells was determined by a fluorescence microscope. For therapy, cells were transfected with specific phosphorothioate ASOs or NSOs by electroporation (two rectangle pulses at 200 V for 2 ms in 0.4-cm gap electroporation chambers, Eppendorf, Hamburg, Germany; corresponding to 500 V/cm) using an electrotransfection impulse generator (EPI2500; Dr. L. Fischer, Heidelberg, Germany) with the capacitance set at 1200 μF) in RPMI 1640 supplemented with 10% FCS without phenol red as indicated below. After incubation for 24 or more hours in the presence of the oligonucleotides or ErPC3, respectively, 2,500 (K-562) or 10,000 (AR-230, BV-173) cells were transferred into 5 ml of semisolid medium containing 0.8% RPMI-methylcellulose and 30% FCS. Cells were plated into 3.5-cm Petri dishes (1 ml/dish). Usual cell culture conditions (37°C, 5% CO2 in humidified air) were applied. Colony formation (clusters of 20 or more cells) was scored by an inverted microscope after 7 days. At least three dishes/treatment protocol were used.

**Statistics and Evaluation of Combination Effects.** Colony counts were presented as mean with corresponding SD. For comparison between treatment groups, the Kruskal-Wallis test was applied (18). P ≤ 0.05 was considered significant. Predicted theoretical values were calculated according to the equation: C = a × b/100, where a and b are colony counts in percentage of untreated control obtained in response to single agents. For each concentration, applied theoretical values were calculated and compared with the real value of the combination. Two-factorial ANOVA was applied independently to test for synergistic interaction between the two agents (19). In addition, isobologram and multiple drug effect analysis was used to examine the nature of the interaction observed (20, 21). To that purpose, the dose-effect curve for each drug alone was determined based on the experimental observations using the median-effect principle; the CombI for each combination was then calculated according to the following equation:

\[
\text{CombI} = \left( \frac{D_1}{I_{D_1}} + \frac{D_2}{I_{D_2}} + \frac{D_1}{I_{D_1}} \times \frac{D_2}{I_{D_2}} \right) \times \frac{1}{D_1 \times D_2}
\]

where \(D_1\) and \(D_2\) are the doses of drug 1 and drug 2 that have X effect when used in combination, and \(I_{D_1}\) and \(I_{D_2}\) are the doses of drug 1 and drug 2 that have the same X effect when used alone. When CombI = 1, this equation represents the conservation isobologram and indicates additive effects. CombI values < 1.0 indicate a more than expected additive effect (synergism).

**Western Blot.** Transfected cells were incubated, harvested, and washed in PBS. Thereafter, cell pellets (2 × 10^6 cells) were lysed in buffer [0.1 mM NaCl, 25 mM Tris-Cl (pH 8.0), 0.001 M EDTA (pH 8), 25% glycerol, and 10% β-mercaptoethanol] containing 1 mM phenylmethylsulfonyl fluoride (Sigma), 50 μg/ml aprotinin (Sigma), 1 mM Na3VO4, and 2 mg/ml leupeptin (Sigma). After vigorous vortexing, 6 x SDS loading buffer was added. Lysates were boiled for 5 min in a water bath at 100°C and centrifuged at 13,000 rpm for 10 min at room temperature.

Cell lysates corresponding to 300,000 cells were loaded on a 6% SDS polyacrylamide gel, separated by electrophoresis, and transferred to a polyvinylidene difluoride membrane, blocked with 5% skimmed milk in PBS, and incubated with a mouse monoclonal antibody against ABL or a rabbit polyclonal antibody against BCR (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h in 1 × PBS, 0.5% milk, and 0.5% Triton X-100. After washing in PBS containing TWEEN 20 (0.05%), an horseradish peroxidase-conjugated antimonoo or antirabbit IgG secondary antibody (Santa Cruz Biotechnology) and ECL (Santa Cruz Biotechnology) were used to detect both BCR-ABL and c-ABL or BCR constitutive proteins.

**LTBMCC and CFU-GM.** LTBMCCs were obtained as described previously (11). Briefly, female C57BL6 mice (Charles River, Wiga, Germany) were used as donors. After euthanizing the animals by cervical dislocation, femurs were removed and the marrow was flushed aseptically with a 21-gauge needle fitted to a syringe into LTBMCC medium (10% FCS, 10% horse serum, 10^-6 m hydrocortisone, 0.017 mg/ml transferrin, 0.006 μl/ml β-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B.
in MEM-a). A single cell suspension was prepared by repeated gentle aspiration of the marrow plug. Bone marrow cells (1 × 10^7) were plated in 25-cm² culture flasks and incubated at 37°C in 5% CO₂ in humidified air. Weekly, half of the medium in each flask was removed, and an equal volume of fresh medium was added. After an adherent layer had been established, the cultures were refed with 1 × 10^7 fresh bone marrow cells/flask. Two weeks later, ErPC3 was added, and electrotransfection of the nonadherent cells was performed. After incubation, cells were collected by trypsinization, and 250,000 cells were plated in 5 ml of semi-solid medium (0.8% RPMI-methylcellulose, 30% horse serum, and 0.1 ng/ml recombinant mouse granulocyte/macrophage-colony stimulating factor). Aliquots of 1 ml/dish (three dishes/treatment group) were seeded in 3.5-cm Petri dishes and cultivated for 10 days. Colonies (clusters of 20 or more cells) were counted using an inverted microscope.

**Cytogenetic Investigation.** The cytogenetic investigation of bone marrow cells was performed as described previously with some modifications (11). Briefly, male and female C57BL6 mice were treated i.p. with ErPC3 as indicated in Table 1. At the end of the treatment schedule, animals were injected with colchicine at a dose of 0.4 mg/kg body weight. Thereafter, mice were euthanized, and bone marrow cells were flushed from their femurs and hypotonized in 0.075 M KCl solution at 37°C for 20 min. After fixation in methanol:acetic acid (3:1), slides were made, air dried, and stained with 5% of Giemsa solution. To score chromosomal aberrations, at least 50 well-spread metaphases/animal were analyzed. In addition, the mitotic index was calculated by counting the number of dividing cells.

**Results**

**Efficiency of Electrotransfection.** Transfection with impulses of 2-ms duration ranging from 300 to 700 V/cm was associated with a voltage-dependent increase in fluorescing cells (Fig. 1). The ratio of strongly fluorescing cells increased from 20 to nearly 40% (1 × 10^7 V/cm) and that of weakly fluorescing cells from 40 to 60% (2 × 10^7 V/cm), thus adding up to 60–90% of total cells. A pulse strength over 500 V/cm did not contribute to a further increase in the ratio of fluorescing cells. Except for 500 V/cm, there was no significant difference between using one or two impulses with regard to oligomer uptake. In the absence of electrotransfection, there was no discernible uptake of the oligonucleotide (≤ 5% fluorescing cells after 1 h of incubation).

**CFU Growth after ASO-bcr Treatment.** A dose-response study in K-562 cells with the bcr-directed ASO and a posttreatment period of 72 h after electrotransfection showed a clear concentration dependency in colony growth inhibition ranging from 20% (2.5 μM) to 40% (10 μM). The control oligonucleotides ASO-b2a2 and NSO-bcr showed no significant inhibition of colony formation (Fig. 2A). Repeated electrotransfection with two posttreatment periods of 72 h each did not cause a further decrease in the clonal growth of K-562 cells (Fig. 2B).

To broaden the spectrum of cell lines, the inhibitory effect of the ASO-bcr was also investigated in BV-173 cells. In
accord with their low BCR-ABL level (see below), BV-173 cells showed a >80% inhibition of colony formation after a single round of electrotransfection (Fig. 2C). The inappropriate junction site ASO-b3a2, however, was completely devoid of an inhibitory effect.

Subsequent studies focused on the combination effect of the ASO-bcr with the APC ErPC3 (Figs. 3 and 4). To that purpose, cells of the more resistant cell line K-562 were treated with the ASO-bcr, NSO, ErPC3, or their combinations (Fig. 3). After 24 h of posttreatment incubation, the colony number was decreased by 45% in response to the ASO-bcr, or ASO-bcr plus ErPC3 \( (P < 0.05; \text{Fig. 3A}) \). Neither ErPC3 alone nor the NSO effected a significant decrease in colony numbers. After 48 h, the latter two agents caused a colony count reduction of 30%, whereas the ASO-bcr alone induced a 70% reduction in clonal growth \( (P < 0.05) \). Most strikingly, the combination of the ASO-bcr with ErPC3 was synergistically active, resulting in 97% reduction of colonies as opposed to 80% that would have been expected from additive interaction (Fig. 3B).

This synergism prompted us to perform a dose-response study with the ASO-bcr and ErPC3. On the basis of the optimal posttreatment period of 48 h, ErPC3 reduced the clonal growth of K-562 cells in a concentration-dependent manner; no effect was observed at 10 \( \mu \text{M} \), but 35\% colony growth inhibition was found at 20 \( \mu \text{M} \) and 65\% \( (P < 0.05) \) at 30 \( \mu \text{M} \). The ASO-bcr caused a growth inhibition of 30 and 35\% at 5 and 10 \( \mu \text{M} \) concentrations (Fig. 4A). The sequential treatment with ErPC3 and the ASO-bcr resulted in synergistic growth inhibition at each dose level tested (Fig. 4B). The combination of 10 \( \mu \text{M} \) ErPC3 and 5 and 10 \( \mu \text{M} \) ASO-bcr resulted in 57 and 33\% inhibition instead of 29 and 33\% \( (\text{open columns}) \) that would have been expected from additive interaction. Isobologram analysis demonstrated CombI values of 0.40 and 0.36, respectively. Likewise, combination of 20 \( \mu \text{M} \) ErPC3 with 5 and 10 \( \mu \text{M} \) ASO-bcr led to 72 and 75\% inhibition as opposed to 55 and 58\% that would have been expected, and isobologram analysis gave CombI values of 0.69 and 0.67, respectively. Finally, the combination of 30 \( \mu \text{M} \) ErPC3 with 5 and 10 \( \mu \text{M} \) ASO-bcr caused 85 and 92\% clonal growth inhibition instead of 77 and 79\%, and isobologram analysis yielded CombI values of 0.89 and 0.78, respectively. All observed colony...
formation in response to the sequential combination was >30% below the respective expected (additive) effects and can therefore be considered significantly reduced. This was confirmed by the presence of a significant ($P < 0.0001$) interaction between the two agents in a two-factorial ANOVA as well as by Combi values, which were <1 and thus indicate synergism.

**Comparative Expression of the BCR-ABL Fusion Protein.** Using an anti-ABL antibody, the expression levels of the 210-kDa oncoprotein were compared in five CML human cell lines (K-562, LAMA-84, CML-T1, BV-173, and AR-230) and in AML-derived HL-60 cells that were used as a negative control for BCR-ABL (Fig. 5). K-562 cells were found to have the highest amount of the 210-kDa fusion protein, followed by LAMA-84 cells; CML-T1 and BV-173 cells showed a comparable, lower expression of this BCR-ABL protein than the other two cell lines. AR-230 cells express a large amount of the 230-kDa BCR-ABL protein (Fig. 5). The level of this protein is comparable or even greater than that of K-562 cells. There was no band corresponding to the normal c-ABL protein in the 230-kDa BCR-ABL protein (Fig. 5). The level of this protein is comparable or even greater than that of K-562 cells. There was no band corresponding to the normal c-ABL protein in the 230-kDa BCR-ABL protein (Fig. 5).

**Influence of ASO-bcr on p210BCR-ABL Expression.** To investigate whether electroporation with the bcr-directed ASO would result in inhibition of BCR-ABL expression, the CML cell lines K-562, LAMA-84, CML-T1, and BV-173 were treated either with ASO-bcr, NSO, or ErPC3. The respective BCR-ABL levels were assessed by Western blot.

K-562 cells showed no difference in BCR-ABL expression after a 48-h incubation period with the ASO-bcr or NSO, regardless of whether the monoclonal anti-ABL (Fig. 6a) or the polyclonal anti-BCR antibodies were used (Fig. 6b). The constitutive proteins c-ABL or BCR served as internal controls. To overcome this resistance, the electroporation treatment was repeated once (total treatment period, 144 h), and by this the corresponding BCR-ABL level was distinctly reduced in response to the ASO-bcr but not the NSO (Fig. 6c, Lanes 2 and 3). Densitometry of the BCR-ABL bands indicated 100 and 27% of the respective control for NSO and ASO treatments. For compensating any differences in loading, these percentages were normalized to the amount of ABL detected concomitantly that was used as internal control. Thus, the relative level of BCR-ABL was 83% (NSO) and 31% (ASO) of the control (Fig. 6c). In contrast to the K-562 cells, two rounds of this treatment reduced the BCR-ABL level significantly, as evidenced by Western blot (Fig. 7A). Densitometry analyses revealed 50% (TAT), 35% (ASO-b3a2), 46% (NSO), and 23% (ASO-bcr) of control BCR-ABL. After normalization to actin, the figures were 52% (TAT), 59% (ASO-b3a2), 96% (NSO), and 27% (ASO-bcr) of control BCR-ABL. The same treatment schedule resulted in 80% reduction of AR-230 colony growth (Fig. 7B). This effect contrasts with the distinctly lower inhibition of BCR-ABL level and clonogenicity of AR-230 cells after electroporation with the oligonucleotides NSO, TAT (mismatch), and the junction site-directed ASO-b3a2.

**LTBMCCs and CFU-GM after ErPC3 and ASO-bcr Treatment.** Treatment of murine LTBMCCs with different concentrations of ErPC3 (5–40 μM) did not inhibit the growth of CFU-GM. In contrast, 40 μM ErPC3 induced a significant stimulation of colony growth (Fig. 8A). To control for a possible influence of the antisense therapy on normal hematopoietic cells, nonadherent LTBMCC cells were exposed to 10 μM ASO-bcr administered by electroporation. Remarkably, at 24 h after the electroporation, there was no reduction in CFU-GM colony formation (Fig. 8B).

**Cytogenetic Investigation of the Clastogenic Activity of ErPC3.** Previous investigations have shown that alkylphosphocholines have very low if any clastogenic activity in vivo.

**Fig. 5.** Western blot with a monoclonal mouse anti-cAbl antibody (A) and polyclonal rabbit anti-BCR antibody (B). Comparison of expression levels: A, ABL and BCR-ABL proteins in K-562 (Lane 1), HL-60 (Lane 2), LAMA-84 (Lane 3), CML-T1 (Lane 4), BV-173 (Lane 5), K-562 (Lane 6), and AR230 (Lane 7) cells; B, expression of BCR and BCR-ABL proteins in BV-173 (Lane 1), SKW-3 (Lane 2), CML-T1 (Lane 3), K-562 (Lane 4), LAMA-84 (Lane 5), and AR-230 (Lane 6) cells.

**Fig. 6.** Western blot with monoclonal mouse anti-abl (a and c) and polyclonal rabbit anti-bcr (b) antibodies in K-562 cells. a and b, HL-60 control cells (Lane 1), untreated K-562 cells (Lane 2), K-562 cells treated with ASO-bcr (Lane 3), or NSO (Lane 4), c, untreated K-562 cells (Lane 1), K-562 cells treated with NSO (Lane 2), or ASO-bcr (Lane 3), HL-60 (Lane 4), and HL-60 control cells (Lane 5).
To verify this expectation for ErPC 3, we performed cytogenetic analyses on bone marrow metaphase plates of ErPC3-treated female and male mice. The results are summarized in Table 1.

The dose of 124 mg/kg (corresponding to 100 mg/kg of hexadecylphosphocholine) was well tolerated within the observation period of 48 h as compared with 24 h, chosen for the positive control, mitomycin C. Even after this prolonged observation period, there was no significant increase in the percentage of bone marrow cells with chromosomal aberrations nor in the mitotic index. In contrast, mitomycin C increased the number of cells with aberrations 11-fold and decreased the mitotic index by a factor of almost four.

**Discussion**

APCs are candidates for antileukemic therapy because they combine properties such as cytotoxic activity, induction of apoptosis, and stimulation of normal hematopoietic cells (11, 13, 14, 22, 23). In the present study, we focused on ErPC 3, a derivative closely related to erucylphosphocholine, which is the prototype of i.v.-injectable APCs (12). This subgroup of APCs is appropriate for systemic i.v. therapy because it lacks significant hemolytic activity. Comparing the two agents, ErPC 3 forms micellar and water-clear solutions, whereas erucylphosphocholine tends to arrange in gel-like lamellar structures at temperatures \( \approx 37^\circ\text{C} \). Both compounds are equally efficient against human leukemia cells and combine cytotoxic properties as well as those restoring cellular adherence (11, 14). However, we have selected ErPC 3 for a more detailed analysis for its better solubility and increased bone marrow stimulatory activity. The experimental data obtained indicate that ErPC 3 can significantly reduce the growth of K-562 cells that have been described as relatively resistant to ether lipids and APCs (11, 22). However, the inhibitory effect of ErPC 3 is not sufficient to completely inhibit the colony growth of this BCR-ABL-expressing cell line. Our assumption was that specific inhibition of the BCR-ABL protein would help to further reduce the K-562 colony growth. Our panel of human CML-derived cell lines consisted of three cell lines with myeloid (K-562, LAMA-84, and AR-230) and two cell lines of lymphoid phenotype (CML-T1 and BV-173). They express the typical CML oncoprotein, although they differ by the type of fusion site: CML-T1 and BV-173 cells contain a b2a2 fusion, K-562 and LAMA-84 are characterized by a b3a2 fusion (15). AR-230 cells express a fusion protein with a molecular mass of 230 kDa because of the insertion of 180 amino acids encoded by 3' exons of bcr (B4 to C3; Ref. 24). Their resistance against APCs (14) correlates with the respective level of the BCR-ABL expression as evidenced by Western blot (Fig. 5). K-562 and AR-230 cells showed the highest expression levels among the five cell lines.

**Fig. 7.** Inhibition of BCR-ABL expression (A) in AR-230 cells after two rounds of electrotransfection: Lane 1, control; Lane 2, TAT (mismatch); Lane 3, ASO-b3a2; Lane 4, NSO; and Lane 5, ASO-bcr. Inhibition of clonogenicity (B) of AR-230 cells after two rounds of electrotransfection. Oligonucleotides were applied at 10 \( \mu\text{M} \). An asterisk denotes a significant difference versus the respective control (Kruskal-Wallis test; \( P < 0.05 \)).

**Fig. 8.** CFU-GM colony formation from murine LTBMCC after exposure to ErPC 3 (upper panel) or ASO-bcr (lower panel). Bars, SD of the mean of three Petri dishes, respectively. An asterisk indicates a significant difference versus the respective control (Kruskal-Wallis test; \( P < 0.05 \)).
The mechanistic basis of this synergy is largely unknown to date. We speculate that both agents increase the capability of the cell to undergo either apoptosis or differentiation. Experiments along these lines are planned to be performed in the near future. A similarly strong inhibitory effect was observed in CML blasts after combination treatment with mafosfamide and an ASO directed against the b2a2 junction site (28). CML blasts, however, generally contain lower amounts of BCR-ABL than K-562 and AR-230 cells, and therefore, the present combination appears to be more powerful and is expected to be more effective in eradicating CML blasts. Interestingly, the inhibitory activity depended on the time after electrotransfection because an incubation period of 48 h (24 h for ErPC3 and the ASO, respectively; Fig. 3A) was much less effective than 96 h, i.e., 48 h, respectively (Figs. 3B and 4B). It was optimal to perform the antisense therapy after the APC treatment. Furthermore, the electrophoresis was of importance, because the bcr-directed ASO that has been known since 1994 (17) gained its remarkable efficacy because of the rectangular impulses with controlled duration used. The conditions of electrophoresis used in this study were in the same range as described as optimal for K-562 cells (29). The advantages of electrophoresis over other transfection techniques have been described (30, 31). This corresponds to our preliminary experiments with transfecting agents such as DOTAP and Lipofectamine that resulted in unsatisfactory transfection using DOTAP and in unspecific inhibition of colony formation after Lipofectamine (data not shown). It is conceivable that the simple phosphorothioate backbone of the ASO-bcr used can be chemically optimized. Such a development seems promising because its target, i.e., the transcription start of bcr, makes it universally active against BCR-ABL expression, even in unusual fusion types such as that of AR-230 cells (Fig. 7).

To check whether this therapeutic schedule can affect normal hematopoietic cells, ErPC3 and ASO-bcr effects were studied in normal LTBMCCs as well. As expected, ErPC3 did not reduce the number of CFU-GM-forming cells and even stimulated the colony number significantly at a concentration of 40 μM (Fig. 8A). Similar to other APCs investigated previously (11), ErPC3 did not show any significant clastogenic or antimitotic effect on mouse bone marrow cells in vivo. The other combination therapy partner, the anti-bcr ASO administered by electrophoresis, also did not show any toxicity on hematopoietic progenitor cells (nonadherent murine LTBMCCs; Fig. 8B). Because transfection with the anti-bcr ASO did not significantly inhibit CPU-GM colony formation, it is derived that inhibition of BCR synthesis does not impair cellular functions of normal hematopoietic progenitor cells that are required for their proliferation capacity as assessed by a CFU assay.

The combination of an optimized anti-bcr ASO with ErPC3 appears to be a promising treatment for purging of bone marrow from contaminating CML blasts, because the ex vivo situation allows us to apply the necessary electrophoresis. The treatment could also be useful for patients who have developed resistance against the tyrosine kinase inhibitor imatinib (Glivec, STI571), as has been described recently (32–34).

Taken together, our results indicate that the combination treatment of ErPC3 and the ASO-bcr is synergistically active in CML cells and well tolerated by normal hematopoietic cells. The BCR-ABL expression level is responsible at least in part for the therapeutic resistance of CML-derived leukemic blasts. The combined application of the agents might be a useful tool for purging the bone marrow from contaminating leukemic cells before an autologous bone marrow transplantation.

Acknowledgments

We are indebted to Dr. Robert Owen for stylistic improvements of the manuscript.
References
Combination with an Antisense Oligonucleotide Synergistically Improves the Antileukemic Efficacy of Erucylphospho-\(N_3\)trimethylpropylammonium in Chronic Myeloid Leukemia Cell Lines

Spiro M. Konstantinov, Milka C. Georgieva, Margarita Topashka-Ancheva, et al.