

Leukemia Treatment in Severe Combined Immunodeficiency Mice by Antisense Oligodeoxynucleotides Targeting Cooperating Oncogenes

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Summary

Transformation of hematopoietic cells by the p210^{bcr/abl} tyrosine kinase appears to require the expression of a functional MYC protein, suggesting that simultaneous targeting of BCR-ABL and *c-myc* might be a rational strategy for attempting treatment of Philadelphia¹ leukemia. To test this hypothesis, severe combined immunodeficiency mice injected with Philadelphia¹ leukemic cells were treated systemically with equal doses of *bcr-abl* or *c-myc* antisense oligodeoxynucleotides (ODNs) or with both ODNs in combination. Compared with the mice treated with individual agents, the disease process was much slower in the group treated with both ODNs, as revealed by flow cytometry, clonogenic assay, and reverse transcriptase-polymerase chain reaction analysis to detect leukemic cells in mouse tissue cell suspensions, and by enumeration of liver metastases. The retardation of the disease process was positively correlated with a markedly increased survival of leukemic mice treated with both ODNs. These data demonstrate the therapeutic potential of targeting multiple cooperating oncogenes.

Growing evidence suggests that cancer arises through a multistep process that involves activation of proto-oncogenes and loss of function of tumor suppressor genes (1, 2). Oncogene cooperation was originally demonstrated in vitro (3–5) and subsequently validated in vivo using transgenic mouse models (6). Chronic myelogenous leukemia (CML)¹ illustrates well the concept of a multistep process in human malignancies, because the clinical course consists of two well-defined stages, i.e., a relatively indolent and long-lasting chronic phase and a terminal, more aggressive blast crisis (7). At the genetic level, the predominant abnormality of the chronic phase is the Philadelphia chromosome (Ph¹) translocation, resulting in the formation of the BCR-ABL oncogene. BCR-ABL expression determines the growth advantage of Philadelphia¹ cells in part by protecting them from apoptosis (8–10). In blast crisis, several other abnormalities might be present, including inactivation of the p53 tumor suppressor gene, chromosomal trisomy, and additional translocations (11–13). Several lines of evidence also suggest that MYC cooperates with and is re-

quired for BCR-ABL or v-ABL transformation of hematopoietic and nonhematopoietic cells (14–18). Thus, to test whether simultaneous targeting of BCR-ABL (the initiating agent of the disease process) and *c-myc* (a transcriptional end point of BCR-ABL-mediated events) in CML cells represents a novel and effective modality of leukemia treatment, we compared the effects of phosphorothioate *bcr-abl* and *c-myc* antisense (AS) oligodeoxynucleotides (ODNs) alone and in combination in vitro and on leukemia development in immunodeficient (SCID) mice.

Materials and Methods

Mice and Leukemia Cells. Outbred ICR-SCID male mice (7–9 wk old from Taconic Farms, Inc., Germantown, NY) and BV173 leukemic cells were used as previously described (19). According to testing performed by the vendor, the frequency of “leaky” SCID mice in the population is <1%.

ODNs. ODNs were prepared on an automated synthesizer (model 390Z; Applied Biosystems, Foster City, CA). The *bcr-abl* (26-mer, b2/a2) and *c-myc* (26-mer from the initiation codon) sense (S) and AS phosphorothioate ODNs ([S]ODNs) were described previously (19, 20). Sequences of the 3′ primer of ABL exon 2 3′ primer of β-actin, 5′ primers of BCR exon 2 and 5′ primer of β-actin, and the ABL and β-actin probes recognizing amplified transcripts have been published (21, 22).

[S]ODNs Treatment of BV173 Cells In Vitro. BV173 cells (10⁴/

¹Abbreviations used in this paper: AS, antisense; BMC, bone marrow cell; CML, chronic myelogenous leukemia; ODN, oligodeoxynucleotide; PBL, peripheral blood leukocyte; Ph¹, Philadelphia chromosome; Qt/RT-PCR, quantitative RT-PCR; RT, reverse transcriptase; S, sense; [S]ODN, phosphorothioate ODN; SPL, spleen; TBS, Tris-buffered saline.

100 μ l per well) were placed in the 96-well culture plates in RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin. For protein studies and cell cycle analysis, 5×10^6 BV173 cells/20 ml of medium were placed in a 175-cm² tissue culture flask (LUX; Nunc, Inc., Naperville, IL). S or AS [S]ODNs were added at the beginning of culture and again (at 50% of the initial dose) 24 and 48 h later. Control groups were left untreated. Cells in 96-well plates were counted in trypan blue on days 4, 6, and 8. Cells in flasks were centrifuged on Histopaque-1077, washed, counted, and used for further studies. For protein analysis, 10^6 cells were solubilized in RIPA lysis buffer containing 10% deoxycholate, 2% NP-40, 0.2% SDS, and 10% glycerol, in Tris-buffered saline (TBS), pH 7.2. Proteins were separated on 7.5% SDS-PAGE and transferred to nitrocellulose (MCI, Westboro, MA). Filters were blocked in 0.5% gelatin in TBS and then incubated with murine monoclonal anti-ABL antibody (the kind gift of Dr. R. Arlinghaus, MD Anderson Medical Center, Houston, TX), murine monoclonal anti-c-MYC antibody (Oncogene Science Inc., Uniondale, NJ), and murine monoclonal anti-HSP 72/73 (Oncogene Science Inc.). Filters were washed five times with 0.25% Tween, 0.25% NP-40 in TBS buffer and blotted with anti-murine polyclonal antibody linked to horseradish peroxidase (Amersham Corp., Arlington Heights, IL). Proteins were detected using enhanced chemiluminescence Western blotting detection system (Amersham Corp.) For cell cycle analysis, cells (10^6) were fixed in 70% ethanol for 15 min at 4°C, washed, and incubated in 1 ml of PBS plus 0.1% NP-40 plus 1 μ g/ml of DNase-free RNase (Boehringer Mannheim Co., Indianapolis, IN) for 10 min at room temperature. Propidium iodide (5 μ g/ml) was added, and cells were analyzed (EPICS Profile analyzer; Coulter Corp., Hialeah, FL).

[S]ODNs Treatment of BV173 Cells or HL60 Cells In Vivo. SCID mice inoculated intravenously with 10^6 BV173 cells or 10^6 HL60 cells 7 d earlier were treated intravenously with [S]ODNs (0.2 ml of RPMI 1640 medium, bolus injection) at 1 mg/d for 12 consecutive days. 1 d after the treatment was stopped, cells were collected from various organs of the mice injected with BV173 cells, and the presence of leukemia was analyzed by flow cytometry (CD10⁺ cells), clonogenic assay (leukemia colony-forming cells), and reverse transcriptase (RT)-PCR (detection of bcr-abl transcripts) as previously described (19).

Quantitative RT-PCR (Q_t/RT-PCR). Total RNA from 5×10^6 bone marrow cells of b2/a2 AS- and b2/a2 plus c-myc AS-treated mice was extracted and adjusted to the same concentration, and equal amounts of β -actin were detected in 1 μ l of each sample by RT-PCR to confirm the same quality of isolated RNA. The material was divided into five equal samples, and an increasing amount (from 0 to 100 ng) of total RNA from K562 cells (b3/a2 junction) was added to each sample as a source of competitive RNA. RT-PCR performed using a 3' primer from ABL exon 2 and a 5' primer from BCR exon 2 generated two cDNA fragments, 257-bp b2/a2 and 332-bp b3/a2, during 25 cycles of PCR amplification. Fragments were detected by Southern blotting using as probe an internal oligomer corresponding to a segment of the c-ABL exon 2.

Tissue Distribution of Intact [S]ODNs. SCID mice were injected (1 mg/d for 12 consecutive days) with b2/a2 plus c-myc AS [S]ODNs. 24 h after the last injection, DNA obtained from 10^6 cells of various tissues was subjected to electrophoresis, and intracellular [S]ODNs were detected by specific hybridization with complementary oligoprobes as previously described (19). For detection of intact [S]ODNs in BV173 cells infiltrating mouse tissues, leukemic SCID mice were injected (1 mg/d for 12 consec-

utive days) with bcr-abl, c-myc, or b2/a2 plus c-myc AS [S]ODNs. 24 h after the last injection, CD10⁺ BV173 were isolated by immunosorting from bone marrow and spleen cell suspensions. After DNA isolation, intracellular [S]ODNs were detected as described (19).

Results

Effects of bcr-abl and/or c-myc AS [S]ODNs on BV173 Cells. In vitro, proliferation of Philadelphia¹-positive BV173 cells that carry the bcr exon 2-abl exon 2 (b2/a2) junction was completely inhibited in the presence of b2/a2 or c-myc AS ODNs at a concentration of 10 μ g/ml each (Fig. 1 A), whereas, in combination, the [S]ODNs inhibited proliferation at a two- and fourfold lower final concentration, i.e., concentrations at which the individual [S]ODNs were nearly or completely ineffective (Fig. 1 A). S [S]ODNs were non-inhibitory at any concentration tested. BV173 cell proliferation was equally inhibited by a 15-mer c-myc AS [S]ODN (5' AAC GTT GAG GGG CAT 3'). In contrast, a c-myc-scrambled sequence (5' AAG CAT ACG GGG TGT 3') including the G tetrad reported to exert nonspecific anti-proliferative effects on epithelial cells (23) was completely ineffective (not shown). Partial inhibition of BV173 cell proliferation using 5 μ g/ml of b2/a2 or c-myc AS [S]ODNs was accompanied by a partial down-regulation of BCR-ABL and c-MYC protein levels, respectively (Fig. 1 B). Incubation of the cells with higher concentrations of AS [S]ODNs, causing complete inhibition of cellular proliferation, induced much stronger down-regulation of BCR-ABL and c-MYC protein expression (19, 20). The combined treatment with low concentrations of b2/a2 plus c-myc AS [S]ODNs (2.5 μ g/ml each) down-regulated both BCR-ABL and c-MYC protein. Expression of MYC protein was also partially inhibited by b2/a2 AS [S]ODNs, which might rest in the functional linkage between BCR-ABL and c-myc, as suggested by studies indicating increased c-myc expression in BCR-ABL-transfected cells (17). Analysis of cellular DNA content (cell cycle distribution) by flow cytometry revealed that treatment with b2/a2 or c-myc AS [S]ODNs as well as with a combination of both AS [S]ODNs at concentrations affecting their proliferation led, after 48 and 72 h, to accumulation of cells in S phase of the cell cycle, concomitant with a decrease in the proportion of G₁ and G₂ cells and with the appearance of cells with fractional DNA content (Fig. 1 C). The cells with fractional DNA content are typical of cells dying by mode of apoptosis (24). The degraded, low-molecular weight DNA from apoptotic cells is generally extracted before and during the staining procedure; such cells, as well as the apoptotic bodies, stain with much lower intensity with the DNA fluorochromes, representing the "sub-G₁" cell population on the DNA frequency histograms. The apoptotic mode of cell death was confirmed by observation of cell morphology after differential staining of DNA and protein (Fig. 1 D). The changes characteristic of apoptosis, involving cell shrinkage, chromatin condensation, fragmentation of nuclei, hyperchromicity of chromatin, and shedding of apoptotic bodies,

were observed in all cultures treated with b2/a2, *c-myc*, or a combination of both AS [S]ODNs. After 48 and especially after 72 h, there were numerous very late apoptotic cells in these cultures, containing very little or almost no stainable DNA (Fig. 1 D). In all probability, these cells were represented on the DNA content frequency histograms by the distinct peaks near the origin of the DNA content coordinate, characteristic of cells with minimal DNA content.

Effects of bcr-abl, c-myc, or bcr-abl plus c-myc AS [S]ODNs on Leukemia Progression in SCID Mice. We assessed the antileukemic effects of *bcr-abl* and *c-myc* ODNs, alone and in combination, in immunodeficient SCID mice (males 8–10 wk old, 20–22 g) injected intravenously with 10^6 BV173 cells, a regimen that produces a disease process reminiscent of that in humans. 7 d later, mice were systemically injected for 12 consecutive days with 1 mg/d per mouse of b2/a2 S plus *c-myc* S (6 d each, every other day), b2/a2 AS, *c-myc* AS, or b2/a2 plus *c-myc* AS (6 d each, every other day). Control mice were injected with diluent only. 4 wk after leukemia implantation, peripheral blood, spleen (SPL), and bone marrow (BMC) from one mouse per group were analyzed to assess the disease process. Immunofluorescence assay (sensitivity 10^{-2}) did not detect CD10⁺ leukemic cells, whereas colony assay (sensitivity 10^{-3}) revealed several clonogenic leukemia cells in BMC suspensions of control and S [S]ODNs-treated mice, but none from cell suspensions of mice treated with AS [S]ODNs either individually or in combination (not shown). RT-PCR amplification of *bcr-abl* transcripts present in the total RNA isolated from BMC and SPL, followed by Southern blot hybridization, revealed a relatively strong signal from amplification products of RNA isolated from control and S [S]ODN-treated mice, but only a weak signal in RNA derived from tissue of mice treated with individual ODNs, and a nearly undetectable signal in RNA from the mouse treated with both b2/a2 and *c-myc* AS [S]ODNs (not shown). Equal amounts of β -actin transcript were detected in RNA samples from each tissue.

8 wk after leukemia cell injection, the disease process was examined again in three mice (A, B, and C) from each group by immunofluorescence, colony assay, and RT-PCR using RNA of various tissues. Immunofluorescence assay detected CD10⁺ cells in PBL (only one mouse was positive), SPL, and BMC of control and S [S]ODN-treated mice, but not in the corresponding tissues of the mice treated with AS ODNs (Table 1). The more sensitive clonogenic assay revealed several leukemic colonies in PBL and abundant colonies in SPL and BMC of control and S [S]ODN-treated mice; in contrast, cell suspensions of *c-myc* or b2/a2 AS-treated mice contained far fewer malignant clonogenic cells (Table 1). Only one of the mice treated with both b2/a2 and *c-myc* AS ODNs contained detectable clonogenic leukemic cells. Scoring of superficial liver metastases was consistent with immunofluorescence and clonogenic assays (Table 2). Numerous metastatic nodules were visible on the surface of livers from control and S-treated mice, several were visible on the liver of mice treated with single AS, and none were visible on the organs from mice treated with both AS [S]ODNs. RT-PCR amplification of *bcr-abl*

Table 1. Presence of CD10⁺ and Leukemia Clonogenic Cells in SCID Mice Injected with BV173 Cells and Treated with *bcr-abl* (b2/a2) or/and *c-myc* [S]ODNs

Treatment mice		% CD10 ⁺ cells			Leukemic colonies/ 10 ⁵ cells		
		PBL	SPL	BMC	PBL	SPL	BMC
Control	A	1.4	6.1	24.9	19	559	2,519
	B	0	4.9	11.6	7	252	1,579
	C	0	5.0	7.4	2	258	1,166
b2/a2 S + <i>c-myc</i> S	A	NT	6.3	38.5	NT	588	3,005
	B	0	6.5	10.4	4	239	1,389
	C	0	4.2	7.0	0	194	1,214
b2/a2 AS	A	0	0	0	0	5	4
	B	0	0	0	0	5	9
	C	0	0	0	0	9	19
<i>c-myc</i> AS	A	0	0	0	0	13	20
	B	0	0	0	0	8	37
	C	0	0	0	0	4	22
b2/a2 AS + <i>c-myc</i> AS	A	0	0	0	0	0	0
	B	0	0	0	0	0	1
	C	0	0	0	0	0	0

Mice were inoculated intravenously with 10^6 BV173 cells and, 7 d later, injected intravenously with S or AS [S]ODNs (1 mg per mouse per day) for 12 consecutive days; in the combination group (b2/a2 plus *c-myc*), [S]ODNs were injected every other day. Control mice were injected with diluent only. Leukemia growth in the mice was analyzed on day 56 by assessing PBL, SPL, and BMC for CD10⁺ cells by immunocytometry and for clonogenic growth in methylcellulose. Numbers show individual results obtained from three mice (A, B, and C). NT, not tested.

transcripts in RNA isolated from various tissues of control and S [S]ODN-treated animals (three mice per group) revealed *bcr-abl* transcripts in each of these tissues (Fig. 2 A). *bcr-abl* transcripts were also detected in all tissues except the brain of mice treated with single AS [S]ODNs, but the signal was much weaker than that observed with control and S [S]ODN-treated mouse tissues. Even weaker signals were detected in the RNA isolated from all the organs except the brain of mice injected with b2/a2 plus *c-myc* AS [S]ODNs, suggesting that the leukemic cell load in mice treated with [S]ODNs in combination was reduced as compared with that of mice treated with individual ODNs. The detection of equal amounts of β -actin in each group of organs indicated the integrity and equal loading of the amplified products.

To confirm that the differences in the intensity of the *bcr-abl* bands corresponding to tissues of single versus combined AS [S]ODN-treated mice reflected the difference in amounts of *bcr-abl* transcript in the tissues, we performed Q_t/RT-PCR using the same amount of RNA isolated from

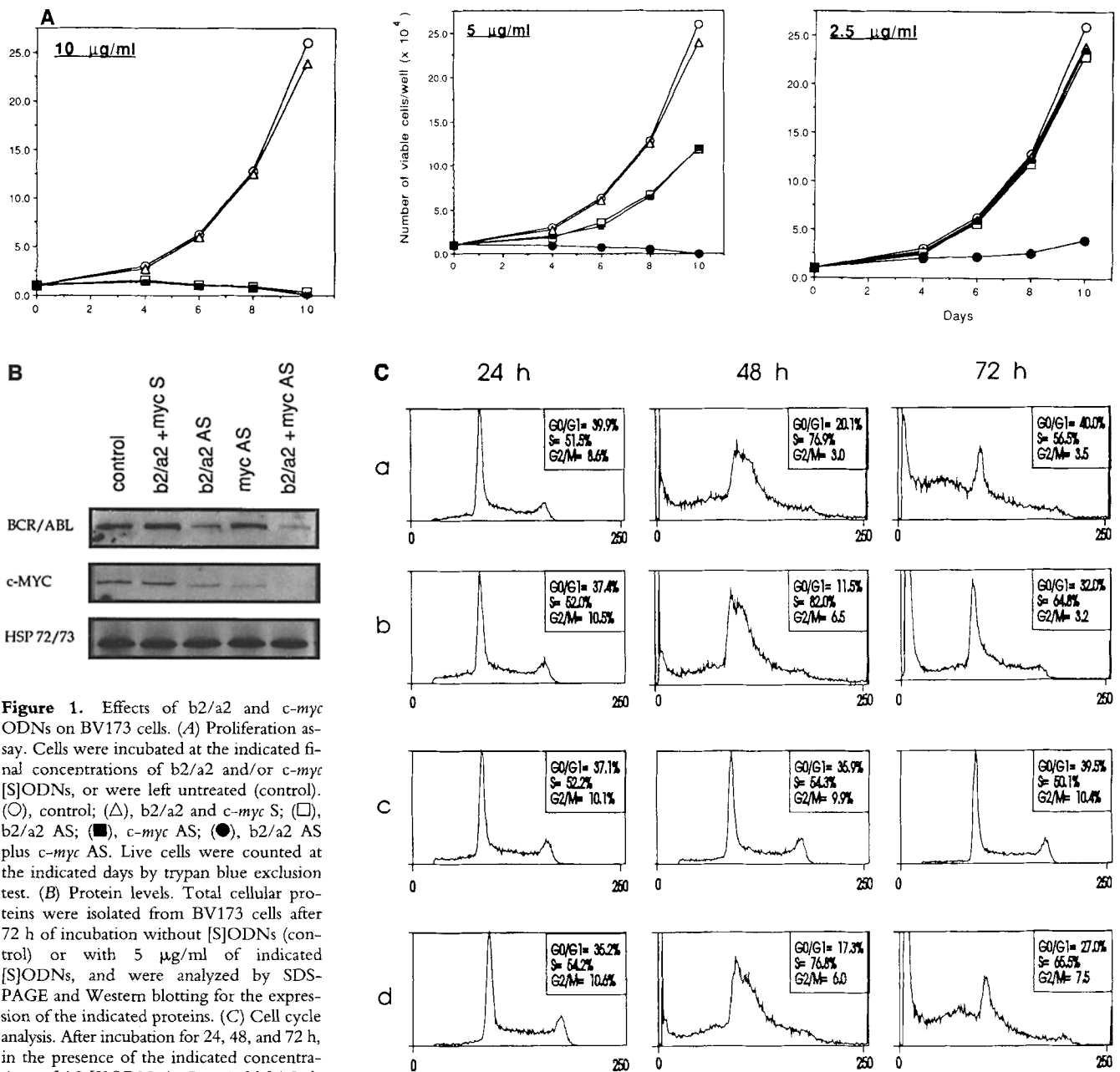


Figure 1. Effects of b2/a2 and *c-myc* ODNs on BV173 cells. (A) Proliferation assay. Cells were incubated at the indicated final concentrations of b2/a2 and/or *c-myc* [S]ODNs, or were left untreated (control). (○), control; (△), b2/a2 and *c-myc* S; (□), b2/a2 AS; (■), *c-myc* AS; (●), b2/a2 AS plus *c-myc* AS. Live cells were counted at the indicated days by trypan blue exclusion test. (B) Protein levels. Total cellular proteins were isolated from BV173 cells after 72 h of incubation without [S]ODNs (control) or with 5 µg/ml of indicated [S]ODNs, and were analyzed by SDS-PAGE and Western blotting for the expression of the indicated proteins. (C) Cell cycle analysis. After incubation for 24, 48, and 72 h, in the presence of the indicated concentrations of AS [S]ODNs (a, 5 µg/ml b2/a2; b, 5 µg/ml *c-myc*; c, 2.5 µg/ml b2/a2; d, 5 µg/ml b2/a2 plus *c-myc*), DNA content was determined by flow cytometry. (D) UV fluorescence photographs of untreated and [S]ODN-treated BV173 cells stained with the DNA fluorochrome 4',6' diamidino-2-phenylindole and protein fluorochrome sulforhodamine 101. (a) Control, untreated cells; (b) cells treated with b2/a2 AS [S]ODNs (5 µg/ml) for 48 h. Note the typical apoptotic cell with fragmented nucleus and highly condensed chromatin, uniformly and strongly stainable with 4',6' diamidino-2-phenylindole. (c) Cells treated with a combination of b2/a2 and *c-myc* AS [S]ODN (2.5 µg/ml each) for 48 h. Note one early apoptotic cell with typical chromatin condensation and nuclear fragmentation and four cells in very late stage of apoptosis, which shrunk and lost nearly all DNA. (d) Cells treated as in c, but for 72 h.

BMC of b2/a2 and b2/a2 plus *c-myc* AS [S]ODN-treated mice, in the presence of increasing amounts of RNA from K562 cells (b3/a2) as a source of competitive bcr-abl RNA, and using optimal concentrations of primers. Integrity of the isolated RNA was confirmed by RT-PCR, which detected similar amounts of β -actin transcript. Qt/RT-PCR revealed competitive blocking of the b2/a2 transcript (from BV173 cells present in the tissue) at lower K562 RNA concentrations when BMC were isolated from mice injected

with both AS ODNs as compared with those receiving only one antisense [S]ODN (Fig. 2 B), indicating the lower amounts of bcr-abl transcripts in BMC RNA from the combined versus single AS ODN-treated mice. These results are consistent with those obtained by nonquantitative RT-PCR, immunofluorescence, and clonogenic assays, and by assessment of liver metastases.

Analysis of two other b2/a2 plus *c-myc* AS [S]ODN-treated mice 20 wk after leukemia implantation, when all

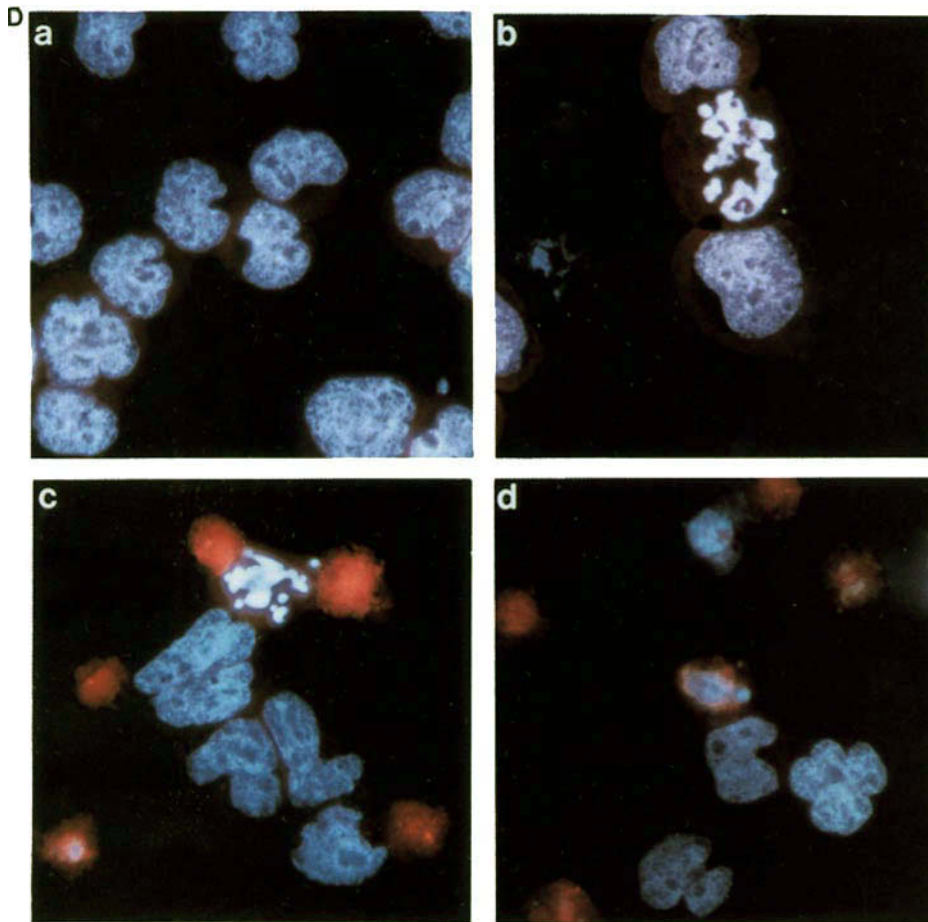


Figure 1. (Continued)

mice treated with individual [S]ODNs were dead, revealed different degrees of disease process as reflected by the tumor load of the two mice (Table 3). Analysis of leukemia progression in a separate set of experiments involving five mice per group confirmed the advantage of targeting cooperating versus individual oncogenes as a therapeutic strategy for tumor treatment (not shown).

Such differences among the groups of mice were reflected in their mortality rates (Fig. 3 A); all nine control and nine S [S]ODN-treated mice died with diffuse leukemia, as confirmed by necropsy, 7–10 wk after intravenous injection of 10^6 BV173 leukemia cells (mean survival time 7.7 ± 0.8 and 8.3 ± 0.5 wk, respectively). In contrast, the nine b2/a2 AS [S]ODN- and nine *c-myc* AS [S]ODN-treated mice died after 14–18 and 14–19 wk; of leukemia growth, respectively (mean survival time 14.7 ± 0.8 and 14.8 ± 0.9 wk, respectively; $P < 0.001$ compared with control or S-treated groups). Nine mice treated with both AS [S]ODNs survived significantly longer (mean survival time 30.8 ± 10.9 wk; $P < 0.001$ compared with mice treated with either AS ODNs).

In contrast, the combination of b2/a2 and *c-myc* AS ODNs did not exert synergistic antitumor effect in SCID mice bearing Ph^1 -negative HL60 leukemic cells (Fig. 3 B). All

eight control mice died with diffuse leukemia, as confirmed by necropsy, 7–8 wk after injection of 10^6 HL60 cells (mean survival time 7.0 ± 0.5 wk). The group treated with b2/a2 AS ODNs (1 mg/d for 12 consecutive days) died after 7–8 wk. The group treated with *c-myc* AS ODNs (1 mg/day for 12 consecutive days) died after 11–13 wk, and the group treated with b2/a2 plus *c-myc* AS ODNs (1 mg/d, 6 d each, every other day) died after 10–11 wk of leukemia. The

Table 2. Superficial Metastases in the Liver of SCID Mice Injected with BV173 Cells and Treated with b2/a2, *c-myc*, or b2/a2 + *c-myc* AS or S [S]ODNs

Treatment groups	Number of metastases
Control	89, 54, 88
b2/a2 + <i>c-myc</i> S	156, 107, 61
b2/a2 AS	12, 10, 8
<i>c-myc</i> AS	15, 15, 4
b2/a2 AS + <i>c-myc</i> AS	0, 0, 0

Mice were treated as described in the Table 1 legend. Numbers indicate visible liver metastases in three mice per group.

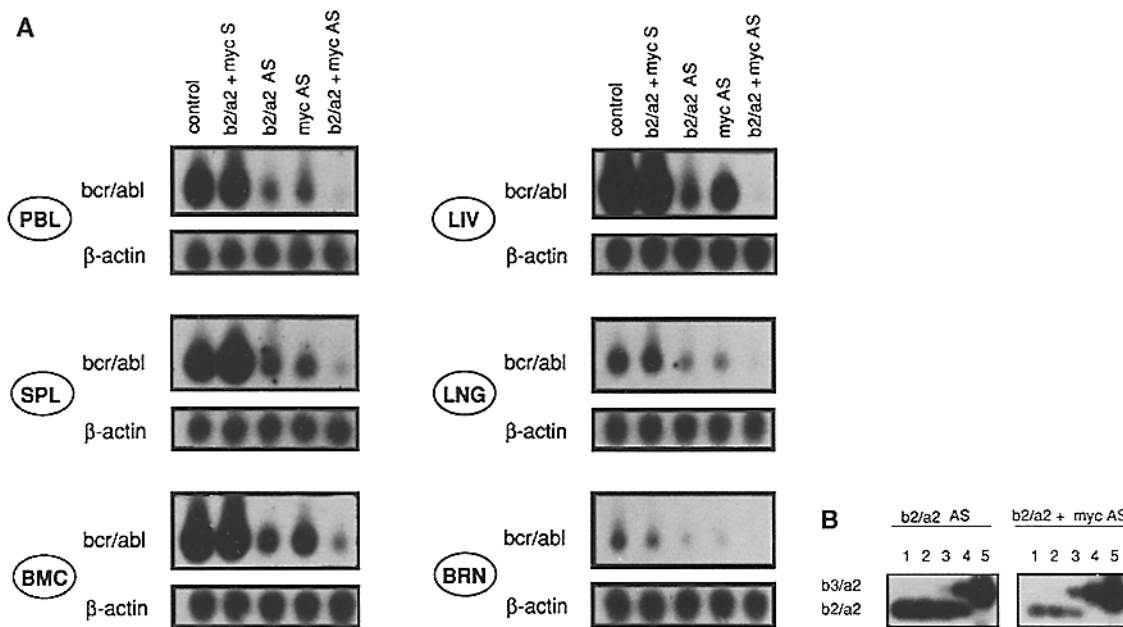


Figure 2. (A) Detection of *bcr-abl* transcripts by RT-PCR in RNA from tissues of [S]ODNs treated (b2/a2 plus *c-myc* S, b2/a2 AS, *c-myc* AS, or b2/a2 plus *c-myc* AS) or untreated (control) leukemic SCID mice. *bcr-abl* and β -actin transcripts were separately detected by RT-PCR (13) using total RNA from 5×10^5 cells for each tissue sample, 56 d after leukemia implantation. Blots were exposed after 24 h (*bcr-abl*) and 2 h (β -actin). The blot is representative of three different experiments using three mice per group. (B) Comparison of the amount of *bcr-abl* transcripts by Q_t/RT-PCR (16) in BMC of b2/a2 AS- and b2/a2 plus *c-myc* AS-treated mice. Various amounts of total RNA isolated from K562 (b3/a2 junction) cells (lane 1, no RNA; lane 2, 0.1 ng; lane 3, 1 ng; lane 4, 10 ng; lane 5, 100 ng) were added as a source of competitive *bcr-abl*-containing RNA to the same amount of total RNA isolated from 10^6 BMC obtained from b2/a2 AS- or b2/a2 AS plus *c-myc* AS-treated mice. Southern blot analysis of RT-PCR amplification products detects the b2/a2 fragment from BV173 RNA contaminating mouse BMC (lower band) RNA, and the b3/a2 fragment from the K562 RNA (upper band) added as competitor. The blot is representative of two different experiments.

mean survival times were 7.3 ± 0.8 , 11.6 ± 0.7 , and 10.4 ± 0.5 wk, respectively. Thus, the combination of *bcr-abl* (b2/a2) and *c-myc* AS ODNs exerted specific antitumor effects and was much more effective than individual AS ODNs in treating mice bearing Ph¹ leukemia.

Detection of *bcr-abl* and *c-myc* AS [S]ODNs in Mouse Tissues and Leukemic Cells. The leukemia-suppressive effects of AS [S]ODNs correlated well with their detection in all organs examined except the brain, although blot hybridization of tissue DNA isolated 1 d after the last injection showed the highest ODN concentrations in the liver and spleen (Fig. 4 A). [S]ODNs were still detectable in these organs 7 d after the last injection (not shown). Intact b2/a2 and *c-myc* AS [S]ODNs were simultaneously detected in vivo in leuke-

mic cells infiltrating BMC and SPL of SCID mice 1 d after completion of the injection protocol, by immunosorting of CD10⁺ cells and Southern blot hybridization of the isolated DNA with oligomer probes complementary to either *c-myc* or *bcr-abl* AS [S]ODNs (Fig. 4 B).

Discussion

The oncogene-targeted approach for the treatment of neoplastic disorders offers the possibility of selectively blocking the function of genes responsible for disease, as compared with the less selective antitumor effects of conventional chemotherapy and radiotherapy. Initial in vivo data in animal models suggest that targeting a single oncogene

Table 3. Leukemia Growth in SCID Mice 20 wk after Injection of 10^6 BV173 Cells and Treatment with b2/a2 Plus *c-myc* AS [S]ODNs

Mice	Leukemic colonies per 10^5 cells			bcr-abl messenger RNA levels						Liver metastases
	PBL	SPL	BMC	PBL	SPL	BMC	LIV	LNG	BRN	
D	0	2	236	-	+	++	+	+	-	0
E	53	283	2387	+	+++	+++	+++	+	+	23

Two mice (D and E) were analyzed. The abbreviations are the same as in Fig. 2. Leukemic colonies were counted after a 9-d culture in methylcellulose. Intensity of the RT-PCR band was evaluated after blotting with a junction-specific γ -³²P-labeled probe and exposing the filters for different times: (-), not detectable after 7-d exposure; (+), visible after 7-d exposure; (++) , visible after 24-h exposure; (+++) , visible after 1-h exposure.

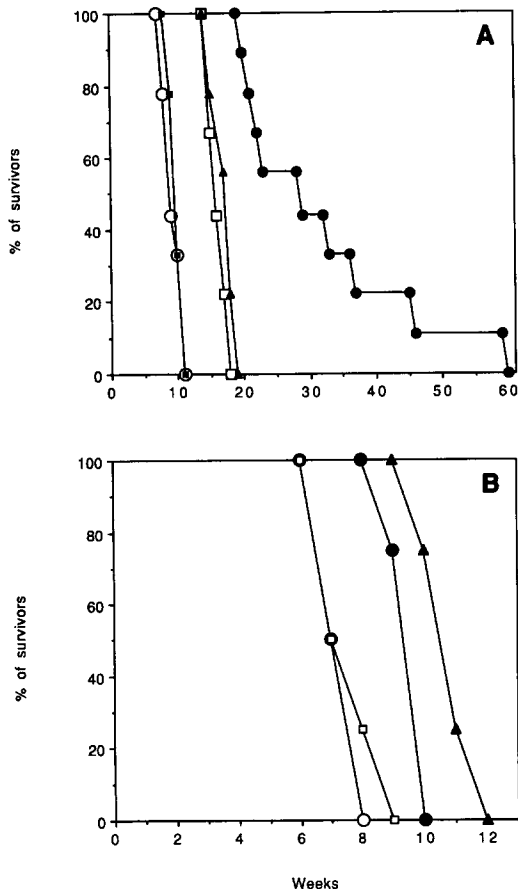


Figure 3. Survival of leukemic SCID mice treated with b2/a2 and/or *c-myc* [S]ODNs. Mice injected with 10^6 BV173 cells (A) or 10^6 HL60 cells (B) were treated intravenously 7 d later with 1 mg/mouse per day of b2/a2 S plus *c-myc* S (■); b2/A2 AS (□); *c-myc* AS (▲), or b2/a2 AS plus *c-myc* AS (●) [S]ODNs for 12 consecutive days. Control (○) mice received diluent only.

by AS [S]ODNs is associated with temporary suppression of tumor growth (19, 25–28). Because tumor formation is a multistep process involving altered function of several oncogenes, the success of oncogene-targeted therapy is likely to depend on the ability to target more than one gene critical for the maintenance of the tumor phenotype. In our studies involving multiple oncogenes targeting in CML cells, in addition to *bcr-abl*, we targeted *c-myc* because this gene appears to be necessary for the transforming ability of *bcr-abl* (14–18), perhaps as one of the nuclear end points of BCR-ABL activity. Both *bcr-abl* and *c-myc* AS [S]ODNs can inhibit in vitro proliferation of Philadelphia¹ cells while sparing, at the doses used, that of erythromyeloid hematopoietic progenitor cells (20–22, 29). In combination, these [S]ODNs exerted a specific synergistic antiproliferative effect on BV173 cells at concentrations at which individual AS [S]ODNs were only partially effective or were completely ineffective.

The flow cytometric data indicate that exposure of cells to b2/a2 or *c-myc* AS [S]ODNs or to a combination of these [S]ODNs, while not precluding cell entrance into S phase,

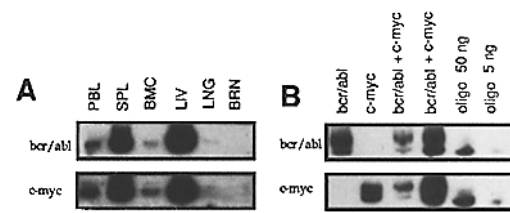


Figure 4. Detection of intact b2/a2 and *c-myc* [S]ODNs in mouse tissues (A) and in the CD10⁺ BV173 leukemia cells infiltrating bone marrow and spleen (B) by Southern blot hybridization. Intact [S]ODNs in mouse tissues were detected by specific hybridization with complementary oligoprobes as described (19). Intracellular detection of *bcr-abl* and *c-myc* [S]ODNs in BV173 cells infiltrating marrow and spleen was analyzed in two separate mice (lanes *bcr/abl* + *c-myc*). A single mouse was used for intracellular detection of either *bcr-abl* or *c-myc* [S]ODN. Standard 26-mer AS [S]ODNs were run as controls in Fig. 4 B.

does prevent cell progression through S. While the S phase function of *bcr-abl* has not been previously reported, the S phase arrest in *c-myc* AS [S]ODN-treated cells is in agreement with the reported ability of ectopically expressed *c-myc* gene to rescue S phase arrest of the hematopoietic IL-3-dependent BAF-B03 cell line stimulated via the epidermal growth factor receptor (30). The interference with S phase progression of BV173 cells treated with b2/a2 and/or *c-myc* AS [S]ODNs may provide a signal triggering apoptosis. This mechanism is analogous to the death of prostate epithelial cells after testosterone depletion, when the cells enter the “defective” cell cycle and die by apoptosis while progressing through S (31).

Leukemic SCID mice treated with both *bcr-abl* and *c-myc* AS [S]ODNs survived much longer than those injected with the same dose of single AS [S]ODNs. Although the reason for this effect remains unclear, it seems likely that AS [S]ODNs reach a plateau in their ability to down-regulate gene expression in vivo that is not sufficient to completely block cell proliferation. Targeting of a second oncogene involved in the disease process may arrest the growth of cells that escaped the inhibitory effect associated with individual gene targeting. Alternatively, the down-regulation of gene expression by single AS [S]ODNs at the relatively low concentrations reached in vivo (19) might be insufficient to inhibit cell proliferation, whereas “partial” inhibition of two cooperating oncogenes might induce a more permanent block in the ability to proliferate. Our in vitro experiments using different concentrations of AS [S]ODNs separately and in combination do not exclude either possibility. In additional experiments, we found that the simultaneous down-regulation of BCR-ABL and *c-myc*, but not BCR-ABL and DNA polymerase α , exerted synergistic antitumor effects (data not shown). This suggests that simultaneous inhibition of the expression of BCR/ABL and of other genes involved in proliferation of CML cells (i.e., DNA polymerase α) does not necessarily exert synergistic antitumor effects, and raises the possibility that a functional relationship between BCR-ABL and other genes is required for these effects. The enhanced antileukemic effect observed with ODNs in combination was not associated with nonspecific toxicity on normal cells. Our

previous studies showed that bcr-abl [S]ODNs used at doses similar to those used here were nontoxic to mice harboring leukemic cells (19). In similar experiments, control mice injected with murine *c-myc* AS [S]ODNs revealed no major toxicity-associated morphological changes in the organs examined and no decrement in the proliferative capacity of BMC as indicated by clonogenic assays (not shown), suggesting that bcr-abl and *c-myc* AS [S]ODNs have favorable pharmacological properties in vivo. Systemic treatment with [S]ODNs was associated, however, with moderate hepatosplenomegaly, a not uncommon observation after injection of [S]ODNs (19, 28, 32).

To exclude the possibility that the antileukemia effects were due to the nonspecific activity of [S]ODNs (33), several control experiments were performed. BV173 cells are not sensitive to b2/a2 or *c-myc* S [S]ODNs as well as to 6-bp mismatched AS [S]ODNs (19, 20). Because the *c-myc* AS [S]ODN contains the G tetrad, a sequence shown to exert antiproliferative effects independent of an AS mechanism on cells growing in monolayer (23), we have used a *c-myc*-scrambled [S]ODN preserving the G tetrad and found that it is nontoxic to BV173, HL60, and K562 cells (not shown). The recent observation that closely spaced repeats of the consensus sequence GGC are critical for inhibition of BCR-ABL tyrosine kinase autophosphorylation (34), which is crucial for its transformation ability (35), raises the possibility that AS [S]ODNs may act in an aptameric fashion. However, the [S]ODNs we had used do not contain closely spaced GGC sequences. Moreover, treatment of BV173 cells with b2/a2 AS [S]ODNs did not decrease the phosphorylation of p210 BCR-ABL and other proteins during the initial 48 h of incubation, and then the decrease in phosphorylation was proportional to the decrement in the p210^{BCR/ABL} protein levels (not shown). These two findings strongly argue against a primary aptameric mechanism as an explanation of the antileukemia effects seen with the

[S]ODNs used here. Induction of IFN production and stimulation of NK activity by [S]ODNs in mice (36) should also be considered as a possible cause of nonspecific inhibition of tumor growth in animals injected with [S]ODNs. Both *c-myc* S and AS [S]ODNs contain the hexamer palindromic sequence AACGTT, known to induce IFN and augment NK cell activity (36), but only the *c-myc* AS sequence was able to exert antitumor effect in SCID mice. Accordingly, it appears unlikely that this nonspecific mechanism of antitumor activity is an explanation for the AS effects we have observed in vivo. Moreover, b2/a2 AS [S]ODNs did not have antitumor effects against HL60 cells grown in SCID mice (19), and the combination of b2/a2 plus *c-myc* AS ODNs did not exert synergistic antitumor effect against HL60 cells in SCID mice. Finally, to strengthen our data obtained with a CML cell line, experiments performed with CML-blast crisis primary cells growing in vitro and in vivo in SCID mice clearly demonstrate that the combination of bcr-abl and *c-myc* AS [S]ODNs had synergistic antileukemia effects (Skorski, T., M. Nieborowska-Skorska, P. Wlodarski, G. Zon, R. V. Iozzo, and B. Calabretta, manuscript in preparation).

The synergistic antileukemic effect exerted by targeting two cooperating oncogenes is similar to that observed for other types of anticancer therapy such as combined chemotherapy or immunotherapy, in which different drugs are used to target tumor cells as they enter distinct cell cycle stages. The advantage of antioncogene therapy rests in its selectivity for disease-inducing agents, and, in turn, its sparing of normal cells. Improvement in therapeutic potential by selection of optimal combinations of agents able to inhibit gene expression at different stages of the disease process (for example, proliferation and metastasis) or the combined use of antioncogene and cytostatics agents (22) awaits more information on the functions and mechanism(s) of activation of oncogenes in tumor cells.

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