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Antileukemia effect of c-*myc* N3′→P5′ phosphoramidate antisense oligonucleotides *in vivo*

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ABSTRACT ***In vitro*, uniformly modified oligonucleotide N3′→P5′ phosphoramidates are apparently more potent antisense agents than phosphorothioate derivatives. To determine whether such compounds are also effective *in vivo*, severe combined immunodeficiency mice injected with HL-60 myeloid leukemia cells were treated systemically with equal doses of either phosphoramidate or phosphorothioate c-*myc* antisense or mismatched oligonucleotides. Compared with mice treated with mismatched oligodeoxynucleotides, the peripheral blood leukemic load of mice treated with the antisense sequences was markedly reduced, and such effects were associated with significantly prolonged survival of the antisense-treated mice. Moreover, with each of three different treatment schedules (100, 300, or 900 μg/day for 6 consecutive days), survival of the phosphoramidate-treated mice was significantly longer than that of the phosphorothioate-treated mice. Both phosphoramidate and phosphorothioate oligonucleotides were efficiently taken up by leukemic cells *in vivo* and were capable of specifically down-regulating c-Myc expression. Moreover, tissue distribution of the phosphoramidate derivatives was undistinguishable from that of the phosphorothioate derivatives. Collectively, these studies suggest that phosphoramidate oligonucleotides can serve as potent and specific antisense agents in the treatment of human leukemia and probably of other malignancies.**

Synthetic oligonucleotides targeting activated oncogenes have attracted considerable interest for their potential to serve as rationally designed therapeutic agents in the treatment of neoplastic disorders (1–3). Several of the *in vivo* studies designed to assess the therapeutic potential of these compounds have been conducted using phosphorothioate oligonucleotide derivatives in severe combined immunodeficient (SCID) mice inoculated with human leukemia cells (4–7). In SCID mice, inoculation of acute leukemia cells is reproducibly associated with the induction of a disease process highly reminiscent of that occurring in humans (8).

In such a model, phosphorothioate oligonucleotides targeting the c-*myb*, *bcr-abl*, and c-*myc* oncogenes have been effective in suppressing the disease process (4–7). However, the effect was only temporary. This might have reflected an intrinsic limitation in the choice of the target gene, or it might have been due to less than ideal characteristics of the antisense phosphorothioate derivatives.

Phosphorothioate analogues have a relatively low (compared with natural phosphodiesters) binding affinity to single-stranded RNA (9) and sequence-independent binding to a variety of intra- and extracellular proteins in monomeric or multimeric forms (10–12). Thus, it would be important to identify new classes of compounds with physicochemical and pharmacokinetic properties more suitable for systemic use as anticancer agents.

Recently, a new type of oligonucleotide analogue, uniformly modified N3′→P5′ phosphoramidates, where a 3′-amino group was substituted for the 3′-hydroxyl group of the 2′-deoxyribose ring, was synthesized (13, 14). This analogue is characterized by high binding affinity to single-stranded RNA and by nuclease resistance (14, 15). *In vitro*, sequence-specific antisense effects were demonstrated by treatment of leukemic cells with c-*myc*, *bcr-abl*, or c-*myb* antisense phosphoramidate oligodeoxynucleotides (ODNs) (16). The effects were 8- to 10-fold more potent of those obtained with corresponding doses of isosequential thioate derivatives (16). On the basis of these promising results, a study was undertaken to assess whether the phosphoramidate derivatives are also effective *in vivo* and whether the potency of such compounds compares favorably to that of the phosphorothioate oligonucleotide derivatives.

MATERIALS AND METHODS

Mice and Leukemia Cells. Outbred ICR-SCID male mice (7–9 weeks old from Taconic Farms) and HL-60 leukemic cells were used as previously described (6). According to testing performed by the vendor, the frequency of "leaky" SCID mice in the population is less than 1%.

ODNs. Oligonucleotide N3′→P5′ phosphoramidates were prepared on an Applied Biosystems model 390Z or 394 automated synthesizers as reported (15). The c-*myc* (15-mer from the initiation codon) antisense (AS) and mismatched (MM) phosphoramidate [A] and phosphorothioate [S] ODNs are as follows: AS, AACGTTGAGGGGCAT; MM, AACGAGTTGGGGCAT (mismatched bases are underlined). The mismatched sequence includes the "G-quartet" motif shown to exert nonspecific antiproliferative effects on some cells growing in monolayer (17, 18). For *in vivo* uptake studies, the oligonucleotide phosphoromidate containing a terminal 3′-amino group was prepared as described (19) and labeled at the amino group with fluorescein isothiocyanate as described (20). Oligonucleotide phosphorothioates (21) were labeled with 5′-fluorescein by using fluorescein phosphoramidite.

ODN Treatment of HL-60 cells *in Vivo*. SCID mice inoculated intravenously with 10^6 HL-60 cells 7 days prior to oligonucleotide treatment were treated intravenously with the indicated dose of ODNs [in 0.2 ml of phosphate-buffered saline (PBS), bolus injection] for 6 consecutive days.

Detection of Leukemic Cells by Reverse Transcription (RT)-PCR. On days 21 or 35 after injection of HL-60 cells, blood (0.1 ml) was withdrawn from the tail vein and mononuclear cells

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Abbreviations: SCID mice, severe combined immunodeficient mice; ODNs, oligodeoxynucleotides; AS, antisense; MM, mismatched; [A], phosphoramidate; [S], phosphorothioate; RT, reverse transcription.

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were isolated on Lympholyte-M (Cedarlane Laboratories). The presence of leukemic cells (expressing human-specific c-*myb* transcripts) was analyzed in total RNA extracted from 5×10^5 cells, by RT-PCR amplification using 3′ and 5′ human-specific primers (22), followed by Southern blotting detection with a ^{32}P-labeled complementary oligoprobe, as previously described (22).

β-Actin transcripts were also measured to control quality and quantity of the amplification products recovered from each sample, as described (7). Samples obtained 21 or 35 days after injection of leukemic cells were analyzed at the same time.

Uptake of ODNs *in Vivo*. Leukemic SCID mice (6 weeks after inoculation of 10^6 HL-60 cells) were injected with ODNs (900 μg per mouse per day) on 3 consecutive days. The injected AS-ODNs consisted of a mix of unlabeled ODNs and fluorescein-labeled ODNs (9:1 ratio). Twelve hours after the last injection, DNA was extracted from splenocytes, fractionated by denaturing PAGE, and electrotransferred onto Nytran-Plus membranes as described (5). ODNs were detected by specific hybridization with complementary oligoprobes as previously described (5). For fluorescence-activated cell sorting analysis, splenocytes and bone marrow cells were harvested and labeled with phycoerythrin-conjugated anti-human CD45 antibody. The $CD45^+$ population represents infiltrating HL-60 cells, while the $CD45^-$ population corresponds to normal murine cells. Then, number and fluorescence intensity of fluorescein-positive cells was determined in both the $CD45^+$ (HL-60 cells) and the $CD45^-$ (normal murine cells) cell fractions.

Tissue Distribution of Systemically Injected c-*myc* AS-ODNs. For analysis of the tissue distribution of phosphoramidate and phosphorothioate ODNs, SCID mice were injected with 300 μg/day of phosphoramidate or phosphorothioate AS-ODNs for 6 consecutive days. Cell suspensions from several tissues were harvested 1, 3, or 7 days after completion of the injections and ODN distribution was determined by Southern blot hybridization, as described (5, 6).

Down-Regulation of c-*myc* Expression *in Vivo*. Splenocytes (10^6; 75–85% $CD45^+$ cells) were solubilized in RIPA lysis buffer [10% deoxycholate/2% Nonidet P-40/0.2% SDS/10% (vol/vol) glycerol in Tris-buffered saline, pH 7.2 (TBS)]. Proteins from each sample were separated by SDS/7.5% PAGE and transferred onto nitrocellulose membranes (Schleieher & Schuell). The membranes were blocked with 5% low-fat milk in TBS and then incubated with polyclonal anti-c-MYC antibody (Upstate Biotechnology, Lake Placid, NY) or monoclonal anti-heat shock protein (HSP 72/73) antibody (Oncogene Science, Uniondale, NJ). The membranes were washed five times with 0.25% Tween 20/0.25% Nonidet P-40 in TBS and blotted with an appropriate secondary antibody linked to horseradish peroxidase (Amersham). Immunocomplexes were detected using a chemiluminescent Western blotting detection system (ECL, Amersham).

RESULTS

Inhibition of Leukemia Growth *in Vivo*. The antileukemia effects of c-*myc* antisense phosphoramidate and phosphorothioate ODNs were assessed in SCID mice (males, 7–9 weeks old, 20–22 g) injected intravenously with 10^6 human HL-60 acute myeloid leukemia cells. In this model, injection of 10^6 cells reproducibly induces a disease process reminiscent of that in humans, with an early infiltration of hematopoietic tissues followed by spreading to nonhematopoietic organs. Seven days after injection of HL-60 cells, mice (five per group) were systemically treated for 6 consecutive days with three different doses (100, 300, or 900 μg/day) of phosphoramidate or phosphorothioate AS- or MM-ODNs. Control mice received diluent only. Three and five weeks after injection of leukemic cells, the leukemic load was examined in the peripheral blood of three mice from each group by measuring levels of human c-*myb* transcripts (as a marker of human leukemia cells in the peripheral blood of SCID mice) by RT-PCR amplification followed by Southern blotting.

Twenty-one days after inoculation of leukemic cells, RT-PCR revealed the presence of leukemic cells in all control mice and in those treated with MM-ODNs (Fig. 1 *Upper*). Levels of c-*myb* transcripts were almost undetectable in mice treated with 900 or 300 μg/day phosphoramidate AS-ODNs (Fig. 1 *Upper*). Low levels of c-*myb* transcripts were detected in samples from mice injected with 100 μg/day AS-ODNs. The levels of human c-*myb* transcripts detected in the mice injected with equivalent doses of phosphorothioate AS-ODNs were much higher, especially in mice injected with 300 or 100 μg/day (Fig. 1 *Upper*). Thirty-five days after injection of HL-60 cells, human c-*myb* transcripts were readily detected in mice treated with MM-ODNs and in controls (Fig. 1 *Lower*). Once again, the lowest levels of human c-*myb* transcripts were detected in mice treated with phosphoramidate AS-ODNs at the doses of 900 or 300 μg/day. Treatment with 100 μg/day phosphoramidate AS-ODNs reduced the c-*myb* transcripts to levels similar to those detected after treatment with the highest dose of phosphorothioate AS-ODNs (900 μg/day). In mice treated with lower doses (100 or 300 μg/day) of phosphorothioate AS-ODNs, there was no apparent reduction in the levels of human c-myb transcripts (Fig. 1 *Lower*), consistent with regrowth of leukemic cells after temporary suppression of the disease process.

The detection of low levels of c-*myb* transcripts in the peripheral blood of leukemic mice treated with AS-ODNs suggests that the leukemia load in these mice was markedly reduced by the antisense treatment. As expected, survival of

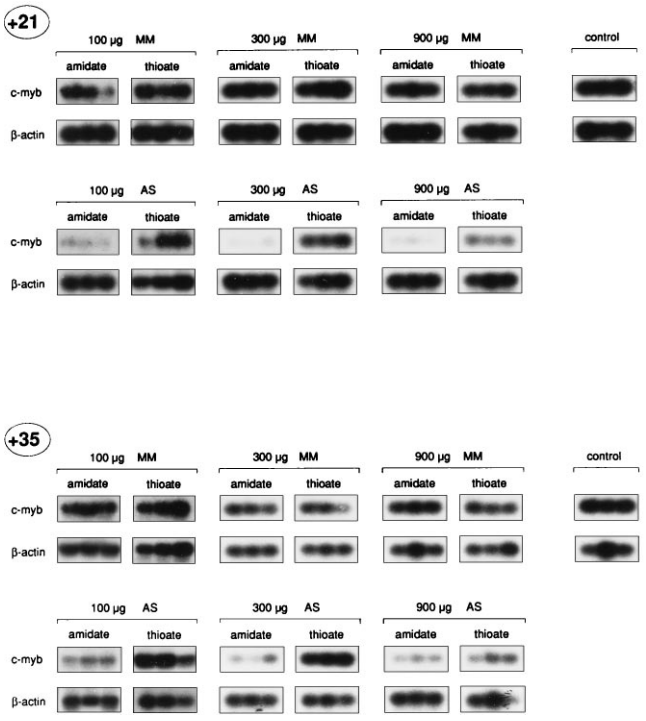

FIG. 1. Detection of HL-60 cells in peripheral blood of leukemic SCID mice by RT-PCR amplification of human c-*myb* transcripts. c-*myb* and β-actin transcripts were separately detected by RT-PCR using leukocyte total RNA from 0.1 ml of peripheral blood from leukemic mice (three mice per group), 21 or 35 days after injection of HL-60 cells and systemic treatment with MM or AS c-*myc* ODNs. Blots were hybridized with a γ-^{32}P-end-labeled c-*myb* or β-actin oligomer complementary to a region of the amplified 230-bp c-*myb* or 210-bp β-actin segment, respectively. Blots were exposed for 24 (c-*myb*) and 2 (β-actin) hr and are representative of two different experiments.

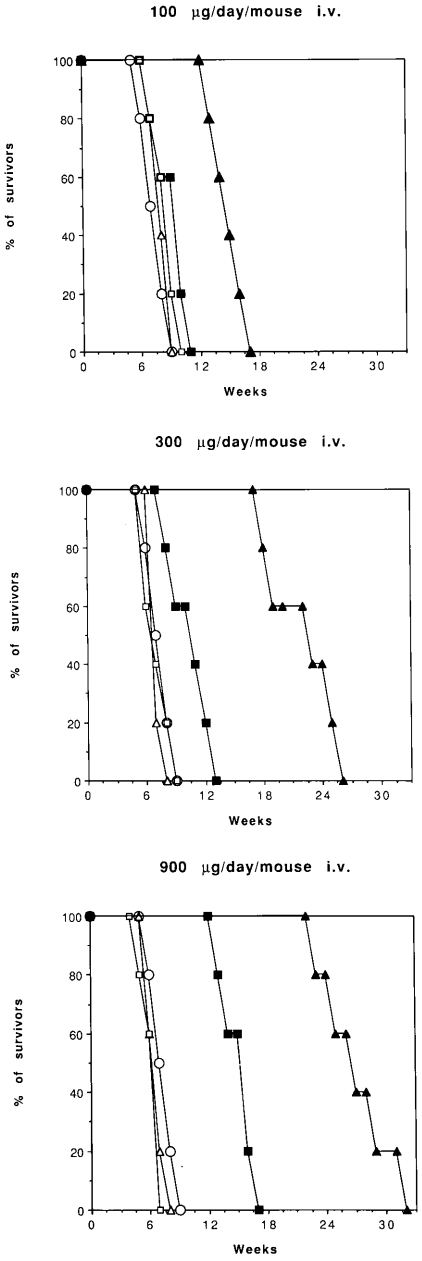

FIG. 2. Survival of leukemic SCID mice treated with different doses of thioate or amidate c-*myc* AS-ODNs. Mice injected with 10^6 HL-60 cells were treated intravenously 7 days later with 900, 300, or 100 μg/day for 6 consecutive days with □, c-*myc* phosphorothioate MM-ODN; △, c-*myc* phosphoramidate MM-ODN; ■, c-*myc* phosphorothioate AS-ODN; or ▲, c-*myc* phosphoramidate AS-ODN. Control mice (○) received diluent only. Five mice were included in each group.

the mice treated with the AS-ODNs was markedly prolonged, in correlation with the effects of the antisense compounds on the levels of c-*myb* transcripts. Median survival time (±SD) of mice treated with 900, 300, or 100 μg/day of phosphoramidate AS-ODNs was 25.8 ± 2.4, 20.8 ± 2.2, or 14.0 ± 1.0 weeks, respectively; in marked contrast, the survival of mice treated with the MM-ODNs was 6–7 weeks (Fig. 2). Mice injected with the corresponding doses of phosphorothioate AS-ODNs survived 14.1 ± 1.0, 9.5 ± 1.3, or 8.0 ± 1.1 weeks, respectively. Survival of phosphorothioate MM-treated mice was 5–7 weeks. Control mice survived 6.6 ± 0.7 weeks. Compared with the phosphorothioate c-*myc* AS-ODN-treated mice, the longer survival of the phosphoramidate AS-ODN-treated mice was statistically significant ($P < 0.001$, unpaired Student's *t* test). Thus, the phosphoramidate AS-ODN derivatives exert a selective antileukemia effect *in vivo*, and they are significantly more potent than the isosequential thioate derivatives.

Down-Regulation of c-Myc Expression by AS-ODNs *in Vivo*. To determine whether the antileukemia effect exerted by c-*myc* AS-ODNs is dependent on an antisense mechanism, SCID mice with hematopoietic organs massively infiltrated by leukemic cells (6 weeks after inoculation of 10^6 HL-60 cells) were treated for 3 consecutive days with phosphorothioate or phosphoramidate c-*myc* AS-ODNs (900 μg/day) and then assessed for c-Myc protein levels. Splenocytes (75–85% leukemic cells, as indicated by percent of the $CD45^+$ cell fraction) were harvested 12 hr after the last dose of ODNs, and the expression of c-Myc protein was analyzed in total cell lysates from 10^6 cells. The levels of c-Myc protein were significantly reduced in the splenocyte lysates obtained from mice treated with AS-ODNs, in comparison to those found in the lysate from mice injected with MM-ODNs (Fig. 3). Detection of similar amounts of heat shock protein 72/73 (HSP 72/73) indicated equal protein loading in each lane.

Uptake of Phosphoramidate ODNs by Leukemic Cells *in Vivo*. To correlate *in vivo* down-regulation of c-Myc expression with ODN uptake by leukemic cells and to compare ODN uptake in normal and leukemic cells, leukemia-bearing SCID mice (two mice per group) were injected for 3 consecutive days with a 10:1 mix of unlabeled and fluorescein-conjugated ODNs (900 μg/day). Twelve hours later, splenocytes and bone marrow cells harvested from leukemic mice were labeled with the phycoerythrin-conjugated anti-human CD45 monoclonal antibody to distinguish between HL-60 leukemia cells ($CD45^+$) and normal murine splenocytes ($CD45^-$). Then, ODN uptake was measured in leukemic and in normal cells by biparametric flow cytometry. A significant proportion (40–70%) of normal splenocytes and bone marrow cells had detectable levels of fluorescence (Fig. 4). However, fluorescence intensity of such normal populations was heterogenous, with the majority of cells characterized by modest levels of fluorescence. In marked contrast, more than 90% of leukemic cells in spleen and bone marrow were fluorescently labeled. Furthermore, fluorescence intensity of leukemic cells was very homogenous and was approximately 4–5 times higher than that detected in normal cells. The fluorescence intensity in leukemic cells harvested from mice injected with phosphoramidate AS-ODNs was 2- to 3-fold higher than that detected after injection of the phosphorothioate analogues, consistent with a more efficient phos-

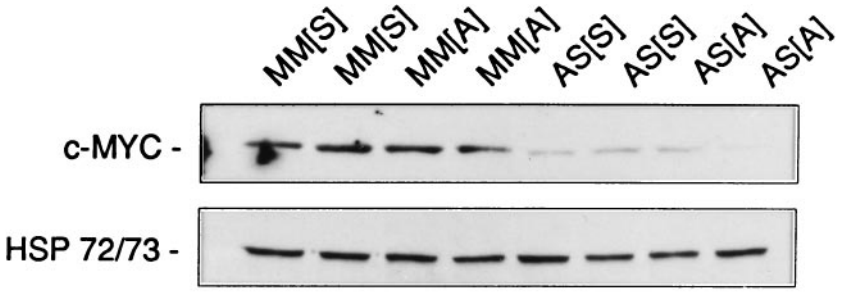

FIG. 3. *In vivo* down-regulation of c-Myc levels by c-*myc* AS-ODNs. Total cellular lysates were isolated from 10^6 splenocytes obtained from leukemic SCID mice systemically treated with 2.7 mg (900 μg/day for 3 consecutive days) of MM or AS phosphorothioate [S] or phosphoramidate [A] c-*myc* ODNs. Proteins were separated by SDS/PAGE, transferred onto nitrocellulose membranes, and sequentially incubated with an anti-Myc and an anti-heat shock protein (HSP) 72/73 monoclonal antibody. Immunocomplexes were detected using a chemiluminescent Western blotting detection system (ECL; Amersham).

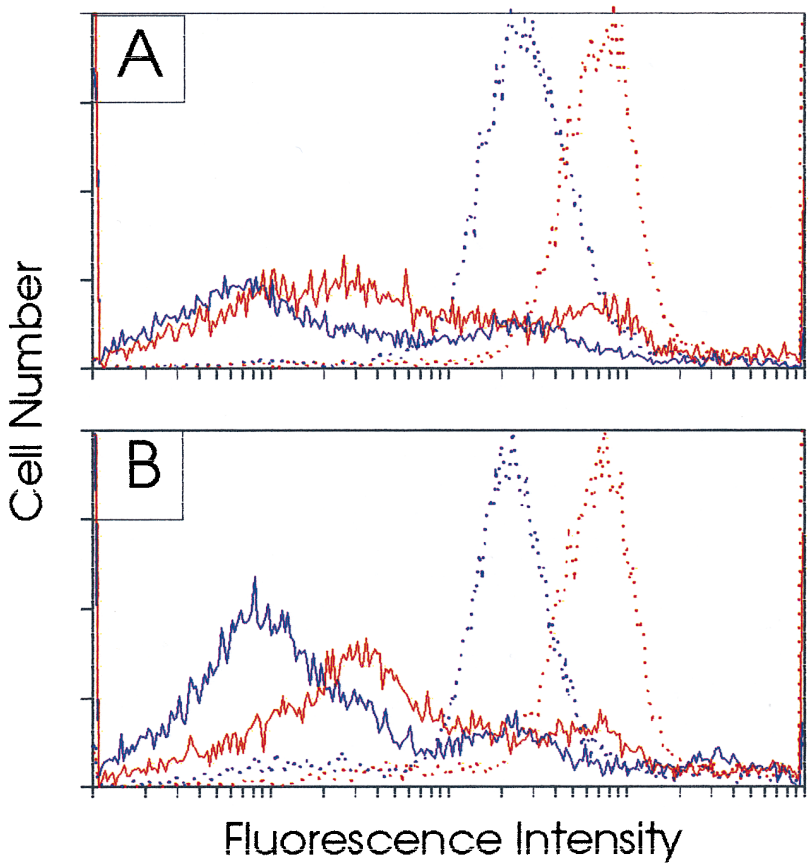

FIG. 4. Detection of fluorescein-labeled thioate and amidate c-*myc* AS-ODNs in normal splenocytes ($CD45^-$ cells) and in HL-60 cells ($CD45^+$ fraction) *in vivo*. Spleen (*A*) and bone marrow (*B*) cell suspensions obtained 12 hr after the last injection of a 10:1 mix of unlabeled and fluorescein-labeled ODNs were incubated with a phycoerythrin-conjugated anti-CD45 monoclonal antibody. The fluorescence of the $CD45^+$ and the $CD45^-$ fractions were determined by fluorescence-activated cell sorting analysis. Solid red line indicates fluorescent phosphoramide ODN in $CD45^-$ cells; dotted red line indicates fluorescent phosphoramide ODN in $CD45^+$ cells. Solid purple line indicates fluorescent phosphorothioate ODN in $CD45^-$ cells; dotted purple line indicates fluorescent phosphorothioate ODN in $CD45^+$ cells.

phoramidate ODN uptake by leukemic cells *in vivo*. Cell-associated ODN was independently assessed by Southern blot hybridization of splenocyte DNA with a ^{32}P-labeled c-*myc* complementary oligoprobe. Levels of cell-associated phosphoramidate c-*myc* AS-ODN were at least twice that of the phosphorothioate derivative (Fig. 5), consistent with the results of flow cytometry analysis.

Tissue Distribution of Phosphoramidate c-*myc* AS-ODNs. To examine the tissue distribution of phosphoramidate c-*myc* AS-ODNs, SCID mice were injected with 300 μg/day of AS-ODNs for 6 consecutive days. Southern blotting analysis of ODN content in various organs 1, 3, and 7 days after the last injection revealed that ODNs were distributed, in intact form, throughout the body (Fig. 6). No major difference was observed in the tissue distribution of phosphorothioate and phosphoramidate ODNs.

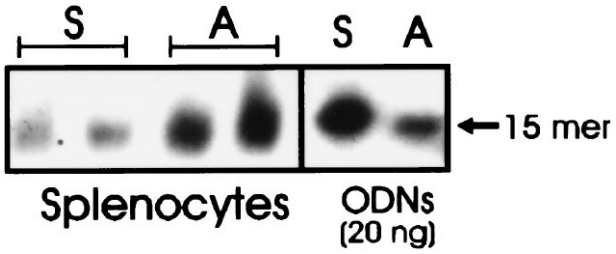

FIG. 5. Detection of intact c-*myc* AS-ODN in splenocytes of leukemic SCID mice. c-*myc* ODN was detected in splenocytes (75–80% leukemia cells, 15–25% normal cells) from two different mice after ODN systemic injection (for details, see the text). Standard 15-mer phosphorothioate and phosphoramidate c-*myc* AS-ODNs were run as controls.

Of note, the hybridization signal of the control phosphoramidate oligonucleotide was weaker than that of the phosphorothioate sequence, despite the loading of identical oligonucleotide amounts. This might reflect a reduced affinity of the phosphoramidate oligonucleotide to nylon membranes, as compared with the phosphorothioate counterpart.

DISCUSSION

In this study, we assessed the therapeutic effects of phosphoramidate ODNs directed to the c-*myc* oncogene transcripts and compared such effects with those achieved upon administration of identical doses of isosequential phosphorothioate derivatives. Using SCID mice harboring HL-60 myeloid leukemia cells, whose proliferation is inhibited by c-*myc* AS-ODNs both *in vitro* and *in vivo* (6, 23, 24), we found that treatment with c-*myc* antisense phosphoramidate ODNs markedly reduced the leukemic cell load, as indicated by the detection of low levels of c-*myb* transcripts in peripheral blood leukocytes of leukemic mice.

This effect was associated with a marked survival prolongation of the phosphoramidate-treated mice, in comparison to those treated with equal doses of either the mismatched compounds or the antisense phosphorothioate sequences.

The antileukemia effects of the phosphoramidate derivatives are consistent with an antisense mechanism, since a marked down-regulation of c-Myc expression was achieved *in*

vivo after treatment with three doses of c-*myc* phosphoramidate ODN, but not with the mismatched compound. Down-regulation of c-Myc expression correlated with homogenous uptake of a fluorescently labeled phosphoramidate c-*myc* ODN by leukemic cells *in vivo*.

Collectively, these findings demonstrate that c-*myc* phosphoramidate ODNs (and perhaps other oncogene-targeted ODNs) can act as potent antileukemia agents *in vivo*. The therapeutic effect of such derivatives was significantly more potent than that observed upon systemic injection of equal doses of isosequential phosphorothioate ODNs. However, the reasons underlying such a difference in the potency of these two compounds are not completely clear yet.

Phosphorothioate and phosphoramidate derivatives have very similar tissue distribution, are effectively taken up by leukemic cells *in vivo*, and are both capable of efficiently down-regulating c-Myc expression in leukemic cells infiltrating the spleen. ODN uptake by leukemia cells *in vivo* was 4- to 5-fold higher than that in normal splenocytes and marrow cells. To our knowledge, this is the first evidence for preferential *in vivo* uptake by leukemic cells, and it is consistent with recent *in vitro* findings demonstrating increased ODN uptake by leukemic cells in comparison to normal hematopoietic cells (25). Such increase in ODN uptake, if a general feature of leukemic cells, might be important in ODN-based therapies, especially when target sequences to which ODNs are directed are identical in normal and neoplastic cells. The leukemic cell uptake of c-*myc* phosphoramidate ODNs (as detected by flow cytometry) was approximately 2-fold of that of the thioate derivative. The observed variation in fluorescence intensity between thioate and amidate derivatives could conceivably reflect differences in oligonucleotide compartmentalization, rather than true intracellular concentrations, because the extinction coefficient of fluorescein varies with pH (26). However, such interpretation might not explain the findings reported here, because the higher phosphoramidate oligonucleotide uptake was independently observed by use of a Southern blot method of detection (Fig. 5). Phosphorothioate oligonucleotide uptake occurs primarily through concentration-dependent endocytosis mediated by a receptor-like mechanism at low extracellular concentration (<1 μM) while fluid-phase endocytosis is apparently more important at higher concentrations (27). Phosphoramidate oligonucleotide uptake might be different from that of the phosphorothioates because of differences in the physicochemical properties of these compounds (16, 18), and it could conceivably explain the higher intracellular concentration of phosphoramidate ODN in leukemic cells. At the time of measurement of c-Myc level (12 hr after the last injection) and at the dose of ODNs used (900 μg/day per 3 days), there was no significant difference in the level of c-Myc down-regulation by phosphoramidate and phosphorothioate antisense compounds. However, it is possible that down-regulation of c-Myc may last longer after treatment with the phosphoramidate derivative, since the amount of full-length phosphoramidate in mouse tissues and in leukemic cells was considerably higher than that of the cognate phosphorothioate. Moreover, a difference in binding affinity to the target RNA may be also a major factor in explaining the

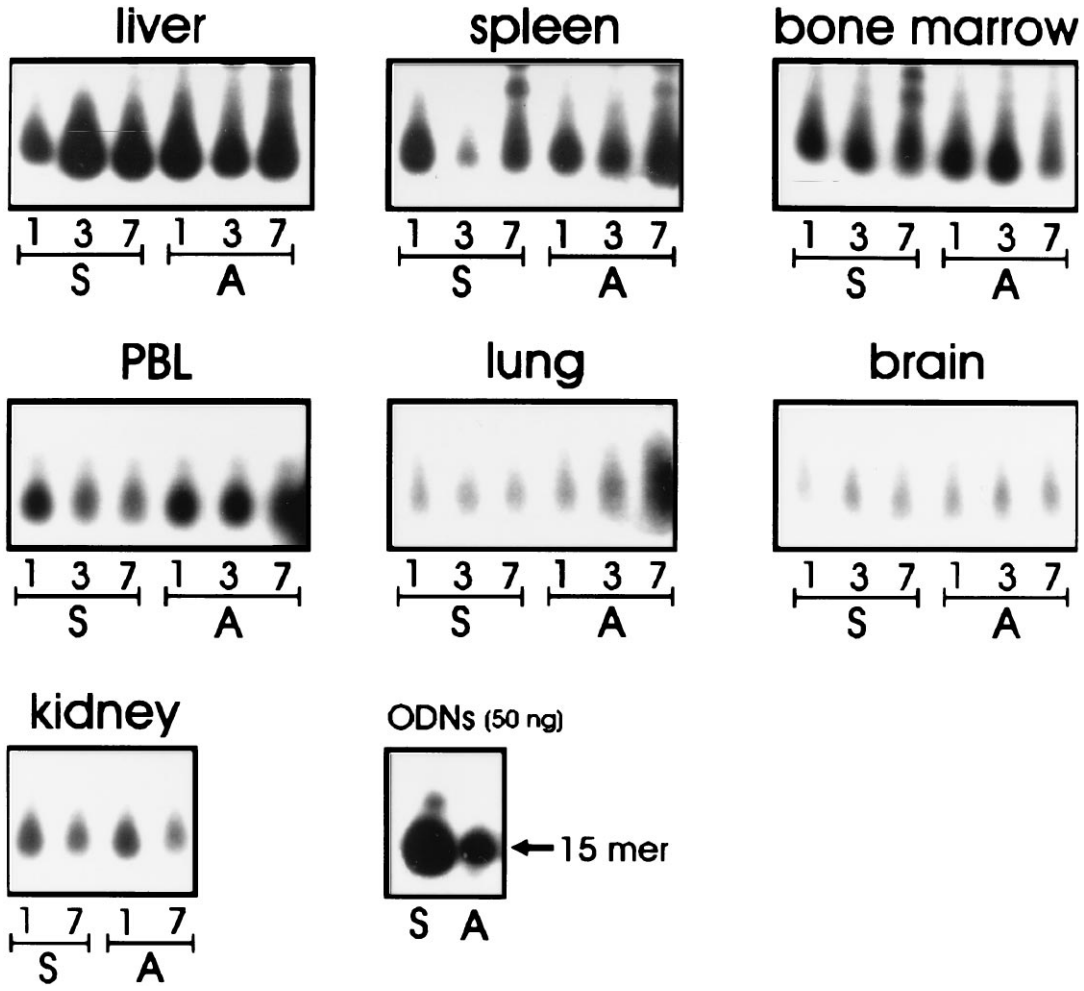

FIG. 6. Tissue distribution of systemically injected phosphorothioate and phosphoramidate c-*myc* AS-ODNs. Intact ODNs were detected in the indicated mouse tissues by specific hybridization with complementary oligoprobes as described (5, 6).

more potent therapeutic effects of the phosphoramidate derivatives. The melting temperature of the duplexes formed by the anti-c-*myc* phosphoramidate and phosphorothioate oligomers with the RNA target was 90.0°C and 54.8°C, respectively, under close to physiological conditions (150 mM NaCl, pH 7.0). Accordingly, down-regulation of c-Myc expression after treatment with the phosphoramidate derivatives might be longer lasting than that induced by the thioate derivative, or it might be associated with more rapid kinetics of leukemia cell death *in vivo*.

The activity of the phosphoramidate oligomers might also be target mRNA site-specific. In this regard, another c-*myc* antisense phosphoramidate oligonucleotide (TTTCATTGTTTTCCA) directed against nucleotides 1709–1724 of c-*myc* mRNA had only marginal activity in the HL-60/SCID mouse model as indicated by a 4- to 5-week longer survival of phosphoramidate-treated mice (900 μg/day for 6 consecutive days), relative to untreated or mismatched phosphoramidate-treated mice (data not shown).

In these studies, treatment with the isosequential antisense phosphorothioate oligonucleotide did not prolong survival of leukemic SCID mice, compared with controls. Thus, the results of both studies are consistent with a more potent activity of the phosphoramidate derivatives. The sequence specificity in reduction of leukemia load, survival prolongation, and *in vivo* suppression of c-Myc protein levels, all support an antisense mechanism of action for the phosphoramidate derivatives. However, sequence-specific nonantisense effects cannot be excluded at this time.

In summary, a c-*myc* antisense phosphoramidate ODN exerts potent, sequence-specific therapeutic effects when injected systemically in leukemic SCID mice. Such effects compare favorably with those of an isosequential phosphorothioate ODN and portend use in humans.

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