Liposomal Delivery of Methylphosphonate Antisense Oligodeoxynucleotides in Chronic Myelogenous Leukemia

By Ana M. Tari, Stanley D. Tucker, Albert Deisseroth, and Gabriel Lopez-Berestein

Chronic myelogenous leukemia (CML) is a hematologic malignancy characterized by the presence of the Philadelphia (Ph) chromosome. Bcr-abl, the fusion gene associated with the Ph chromosome, expresses a p210bcr-abl protein that promotes a selective expansion of mature myeloid progenitor cells. Methylphosphonate (MP) oligodeoxynucleotides complementary to specific regions of the bcr-abl mRNA were incorporated in liposomes. We studied the effects of liposomal MP (L-MP) on the growth inhibition of CML-like cell lines. L-MP targeted to the breakpoint junctions of the bcr-abl mRNA inhibited the growth of CML cells. Fifty percent inhibition was achieved at ~1 μmol/L of L-MP oligonucleotide concentrations. The inhibitory effect was selective because growth inhibition was observed only with CML but not with control cell lines. Moreover, CML cell growth inhibition was dependent on the sequence of the MP oligodeoxynucleotides incorporated in the liposomes. The growth inhibition of CML cells by L-MP resulted from selective inhibition of the expression of the p210bcr-abl protein.

MATERIALS AND METHODS

MP oligodeoxynucleotides were provided by Gesta, Inc (San Diego, CA). MP oligodeoxynucleotides no. 1, 2, and 4 (see below) were synthesized with a phosphodiester nucleotide at the 5' end. The length of the MP oligodeoxynucleotides ranges between 15 and 18 bases. Phospholipids were purchased from Avanti Polar Lipids (Birmingham, AL). Radioisotopes were obtained from Amer sham (Arlington Heights, IL).

Sequences of MP Oligodeoxynucleotides

The oligonucleotides with target and sequence from 5' to 3' end are as follows: (asterisks indicate the breakpoint junctions of the bcr-abl mRNA): (1) antisense to L6 breakpoint junction, CGGGCT T*CTTCC; (2) antisense to K28 breakpoint junction, GCGTA TAGTT CTCTC G*ITA; and (3) control, GCGTA TAGTT CTCTC G*ITA.

Cell Lines

K562 and BV173 (CML myeloid blast crisis) cells and HL60 myelomonocytic cells (as negative control) were used. All cell lines were grown in RPMI 1640 media (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum.

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Labeling of MP Oligonucleotides With Radioisotope

MP oligonucleotides were 5' end-labeled with [γ-32P]ATP using Escherichia coli T4 polynucleotide kinase (GIBCO) in 70 mmol/L Tris-HCl, pH 7.6, 0.1 mol/L KCl, 10 mmol/L MgCl2, 5 mmol/L diethylthreitol (DIT), 0.5 mg/mL bovine serum albumin (BSA), and 25% DMSO for 8 hours at 37°C. The oligonucleotides were precipitated with ethanol at −20°C overnight. After washing with 70% ethanol three times, the oligonucleotides were twice filtered with a Microcon-3 filter (Amicon, Beverly, MA) to separate the labeled oligonucleotide from free [γ-32P]ATP. For a typical reaction, MP was labeled with 104 to 106 cpm/μg. The [32P]-labeled MP was resuspended in DMSO.

Preparation of Inulin-Containing Liposomes

[14C]Inulin (ICN, Irvine, CA) was added to phospholipids in the presence of excess 50% vol/vol t-butanol. Hexadecyl [1H]cholestaney ether (New England Nuclear, Boston, MA) was included in the mixture as a lipid marker. The mixture was vortexed before being frozen in an acetonedry ice bath, lyophilized, and finally hydrated with HEPES buffered saline (1 mmol/L HEPES and 10 mmol/L NaCl) overnight at a final concentration of 10 mmol/L. Liposomes were twice sonicated for 10 minutes in a bath-type sonicator. The sonicated mixture was then dialyzed (molecular weight cutoff 12,000 to 14,000) against 2 L of HEPES-buffered saline overnight to remove unentrapped inulin.

Preparation of L-MP Oligonucleotides

MP oligonucleotides dissolved in DMSO were added to phospholipids in the presence of excess t-butanol. Trace amounts of hexadecyl [1H]cholestaney ether and [32P]-labeled oligonucleotides were also added to the mixture. The mixture was vortexed before being frozen in an acetonedry ice bath, lyophilized, and finally hydrated with HEPES-buffered saline (1 mmol/L HEPES and 10 mmol/L NaCl) overnight at a final concentration of 10 mmol/L. Liposomes were twice sonicated for 10 minutes in a bath-type sonicator.

Separation of MP Oligonucleotides Incorporated in Liposomes From Unincorporated MP

MP oligonucleotides incorporated in liposomes were separated from the unincorporated MP by a 10% wt/vol Ficoll solution. One milliliter of the sonicated liposomal mixture, which contained liposomal MP (L-MP) and unincorporated MP, was layered over 2 mL of a 10% wt/vol t-butanol. Hexadecyl [3H]cholestaney ether and [32P]-labeled oligonucleotides were taken before and after the Ficoll separation and then sent to liquid scintillation counting. The incorporation efficiency of MP in liposomes was calculated as (cpm of [32P]oligonucleotide per μmol of [H]lipids after Ficoll separation/cpm of [32P]oligonucleotide per μmol of [H]lipids before Ficoll separation) × 100%. Typically, MP was incorporated into liposomes with a 90% or greater efficiency.

Uptake of Liposomes by Leukemic Cells

Fifty thousand cells per well were seeded in a 24-well plate in 0.3 mL of RPMI 1640 medium containing 10% fetal bovine serum. After 2 hours of seeding, final concentrations of 30 to 500 μmol/L of [H]-labeled liposomes entrapping [14C]-labeled inulin were added to leukemia cells. After 24 hours of incubation at 37°C, the cells were resuspended in phosphate-buffered saline and layered over a 10% wt/vol Ficoll solution. The cells were centrifuged at 1,600 rpm for 30 minutes and collected at the interface between the phosphate-buffered saline and the Ficoll solution. The collected cells were then lyzed with 0.1 N NaOH and sent to liquid scintillation counting.

Delivery of L-MP to Leukemic Cells

Ten thousand cells per well were seeded in a 96-well plate in 0.2 mL of RPMI 1640 medium containing 10% fetal bovine serum. After 2 hours of seeding, final concentrations of 0.25 to 2.50 μmol/L of L-MP oligonucleotides were added to cells. Typically, the leukemic cells were incubated with L-MP oligonucleotides for 5 days. Each experiment was performed at least three times.

Viability of Leukemic Cells

The viability of leukemic cells was measured by the alamarBlue dye (Alamar, Sacramento, CA). After 5 days of L-MP oligonucleotide incubation, 25 μL of cells/well was aliquoted and added to 155 μL of medium. Twenty microliters of alamarBlue dye was added to each well. After incubation for 4 hours at 37°C, the plates were read directly on a microplate reader (Molecular Devices, Menlo Park, CA) at 570 and 595 nm. The difference in absorbance between 570 and 595 nm was taken as the overall absorbance value of the leukemic cells. All the experiments were analyzed by t-tests in which the viabilities of the L-MP-treated cells were compared with those of the untreated controls.

Western Blots for p210bcux Protein

Fifty thousand K562 cells or 1 × 104 BV173 cells were treated with protease inhibitors and then lysed in sodium dodecyl sulfate (SDS) sample buffer containing 1% SDS and 1% 2-mercaptoethanol and boiled for 5 minutes. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was run on 7.5% polyacrylamide gels, electrophoretically transferred to nitrocellulose membranes, blocked in 5% nonfat dry milk, and treated with 1 hour with 8E9 monoclonal antibody (Pharmingen, San Diego, CA), which is specific for the tyrosine kinase domain of the abl protein. This antibody reacts with both the normal p145SH2 protein and the p210bcux product. Detection was performed by enhanced chemiluminescence (ECL; Amersham) after reaction with horseradish peroxidase conjugated to rat antimouse secondary antibody. Densitometric scans were run on a Giford Response Gel Scanner (CIBA Corning, Medfield, MA). Area integration of absorbance peaks at 500 nm was used to determine the ratio of p210/p145.

RESULTS

Uptake of Liposomes by Leukemic Cells

Liposomes labeled with hexadecyl [1H]cholestaney ether entrapping [14C]-labeled inulin were prepared. The former and the latter serve as lipid and aqueous markers, respectively. Liposomes were added to K562, BV173, and HL60 leukemia cells. The uptake of lipids (Fig 1A) and inulin (Fig 1B) by leukemic cells increased as their input concentrations were increased. The uptakes of lipids and inulin were approximately 9% and 8% of their input, respectively. The uptake of lipids and inulin increased in a similar proportional manner, suggesting that the liposomes were taken up as intact liposomes by the leukemic cells.

Inhibition of CML Cell Growth by L-MP Antisense Oligonucleotides

Antisense oligonucleotides complementary to the L6 junction of the bcr-abl mRNA (oligonucleotide no. 1). L-MP
cells. The viability of K562 cells decreased as the concentration of L-MP antisense oligonucleotide no. 2 was increased (Fig 2B). When 0.25 to 2.50 μmol/L of L-MP was used, the viability of K562 cells decreased to 30% to 80% of that of untreated K562 cells. Thus, approximately 20% to 70% growth inhibition of K562 cells was observed. Under identical conditions, the viabilities of BV173 and HL60 cells were greater than 80% of the untreated cells, which were insignificant from that of the untreated cells (P = .053 and .064, respectively).

Control oligonucleotides that are not complementary to the bcr-ab1 mRNA. To ensure that the growth inhibition of BV173 cells by L-MP oligonucleotide no. 1 was sequence-dependent, we also used L-MP oligonucleotides no. 2 (Fig 2B) and 3 (Fig 3A) as control L-MP oligonucleotides. When L-MP antisense oligonucleotide no. 1 was added to BV173 cells, a dose-dependent growth inhibition was observed. However, under identical conditions, growth inhibition was not observed with control L-MP oligonucleotide no. 3.

K562 cells were treated with control L-MP oligonucleotides no. 1 (Fig 2A) and 4 (Fig 3B). Growth-inhibitory effects were observed with K562 cells in a dose-dependent manner on incubation with L-MP antisense oligonucleotide no. 2. When treated with L-MP oligonucleotide no. 4, the viability of K562 cells was greater than 80%, which was insignificant

Antisense oligonucleotides complementary to the K28 junction of the bcr-ab1 mRNA (oligonucleotide no. 2). L-MP antisense oligonucleotide no. 2, complementary to the K28 junction of the bcr-ab1 mRNA, was used to treat BV173, K562 (which bears the K28 breakpoint junction), and HL60 cells. The viability of K562 cells decreased as the concentration of L-MP antisense oligonucleotide no. 2 was increased (Fig 2B). When 0.25 to 2.50 μmol/L of L-MP was used, the viability of K562 cells decreased to 30% to 80% of that of untreated K562 cells. Thus, approximately 20% to 70% growth inhibition of K562 cells was observed. Under identical conditions, the viabilities of BV173 and HL60 cells were greater than 80% of the untreated cells, which were insignificant from that of the untreated cells (P = .053 and .064, respectively).

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Selective Inhibition

Our results indicate that L-MP oligonucleotides selectively inhibit the expression of p210<sup>bc-abl</sup> with a concomitant decrease in cell growth. The effect on growth inhibition was specific, because only CML cells expressing the p210<sup>bc-abl</sup> protein were inhibited and not HL60 cells, which are myelomonocytic and do not express the p210<sup>bc-abl</sup> protein. Moreover, when L-MP oligonucleotides targeted to the L6 junction of the bcr-abl mRNA were added to BV173 cells, which express the L6 junction, cell growth was inhibited, but not on K562 cells, which do not express the L6 junction. The converse is true when L-MP oligonucleotides targeted to the K28 junction of the bcr-abl mRNA were added to both BV173 and K562 cells; only the growth of K562, but not BV173 cells, was inhibited, therefore indicating that the effect was specific to each abnormality. No significant growth
Table 1. Inhibition of CML Cell Growth and p210bcR-abl Expression by L-MP

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Concentration (μmol/L)</th>
<th>% Cell Growth Inhibition*</th>
<th>p210:p145 Ratio†</th>
<th>% Inhibition‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. BV173 cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Untreated control</td>
<td>—</td>
<td>—</td>
<td>1.04</td>
<td></td>
</tr>
<tr>
<td>2. Oligonucleotide no. 1</td>
<td>1.0</td>
<td>59.4</td>
<td>0.27</td>
<td>73.0</td>
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<tr>
<td>3. Control oligonucleotide§</td>
<td>1.0</td>
<td>1.8</td>
<td>1.16</td>
<td>0</td>
</tr>
<tr>
<td>B. K562 cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Untreated control</td>
<td>—</td>
<td>—</td>
<td>1.69</td>
<td></td>
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<tr>
<td>5. Oligonucleotide no. 2</td>
<td>1.0</td>
<td>24.7</td>
<td>0.31</td>
<td>82.0</td>
</tr>
<tr>
<td>6. Oligonucleotide no. 2</td>
<td>1.5</td>
<td>51.8</td>
<td>0.77</td>
<td>54.5</td>
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<tr>
<td>7. Oligonucleotide no. 2</td>
<td>2.0</td>
<td>52.6</td>
<td>0.18</td>
<td>89.4</td>
</tr>
<tr>
<td>8. Control oligonucleotide§</td>
<td>1.0</td>
<td>10.4</td>
<td>2.03</td>
<td>0</td>
</tr>
</tbody>
</table>

The inhibition of CML cell growth and p210bcR-abl expression corresponded to the Western blots (Figs 4 and 5).
* Obtained by (1 - number of treated cells/number of untreated cells) x 100%.
† Obtained by densitometric scans on Western blots.
‡ Obtained by (1 - ratio of p210:p145 in treated cells/ratio of p210:p145 in untreated cells) x 100%.
§ L-MP oligonucleotide no. 2 was used as a random control for BV173 cells.
‖ L-MP oligonucleotide no. 1 was used as a random control for K562 cells.

Inhibition was observed in BV173 and K562 cells exposed to control L-MP oligonucleotides. At concentrations of and less than 2.5 μmol/L, L-MP antisense oligonucleotides induced sequence-specific growth inhibitory effects on the CML cell lines.

The growth inhibition of CML cells was not immediately reversed when L-MP oligonucleotides were removed. When CML-like cell lines were incubated with L-MP for 5 days and washed and the culture was regrown in medium free of L-MP, the inhibitory effect lasted for another 4 days, indicating that a single dose of L-MP could induce a cytotoxic effect in the CML-like cell lines that lasted for approximately 9 days. It is possible that the incomplete suppression of the growth of the CML cell lines by the L-MP antisense oligonucleotides is caused by the many somatic mutations that may have been acquired in these cell lines, which may make the cells relatively independent of the p210bcR-abl protein. Furthermore, many of the CML cell lines so far tested express the p160bcR protein, product of the unrearranged bcr allele, which has been shown to form a complex with the p210bcR-abl protein. This complex has been suggested to play a significant role in maintaining the leukemic state of CML cells, which suggests the unproven hypothesis that suppression of the expression of the p160bcR and p210bcR-abl proteins may be important for the suppression of CML cell growth. It is possible that, because the L-MP oligonucleotides used...
The major limitations of using phosphodiester antisense oligonucleotides in the inhibition of gene expression are their susceptibility to nuclease digestion and their low cellular uptake. The use of MP, a nonionic and nuclease-resistant phosphorothioate MP, is believed to involve passive diffusion, which is a slow and limiting process. Intracellular delivery of oligonucleotides is another limiting factor that may be overcome by liposomal carriers. The degree of liposomal lipids uptake achieved is comparable to that observed with human peripheral blood monocytes. Liposomal delivery of MP oligonucleotides offers two major advantages: intracellular delivery and a soluble carrier for the water-insoluble MP oligonucleotides. Such an efficient uptake of liposomes by leukemic cells may explain why growth inhibition was achieved at low oligonucleotide concentrations (~1 μmol/L).

**REFERENCES**


Liposomal delivery of methylphosphonate antisense oligodeoxynucleotides in chronic myelogenous leukemia [see comments]

AM Tari, SD Tucker, A Deisseroth and G Lopez-Berestein