Pharmacokinetics, Toxicity, and Efficacy of Ends-modified raf Antisense Oligodeoxyribonucleotide Encapsulated in a Novel Cationic Liposome

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ABSTRACT

Raf-1 protein serine threonine kinase plays an important role in cell survival and proliferation. Antisense inhibition of Raf-1 expression has been shown to enhance the cytotoxic effects of radiation and anticancer drugs. Here we have evaluated the toxicity, pharmacokinetics, and antitumor efficacy of a novel formulation of liposome-entrapped raf antisense oligodeoxyribonucleotide (LErafAON). The LErafAON preparation showed high liposome entrapment efficiency of rafAON (>85%) and stability at room temperature. In CD2F1 mice, administration of LErafAON produced no morbidity/mortality (5–35 mg/kg/dose, i.v., ×12). Dose-related elevations in liver enzymes (alanine aminotransferase and aspartate aminotransferase) and histopathological changes in liver were noted in LErafAON and blank liposome groups. No morbidity/mortality and changes in clinical chemistry or histopathology were observed in New Zealand white rabbits (3.75 mg/kg/dose, i.v., ×8; 6.5 mg/kg/dose, i.v., ×6) or in cynomolgous monkeys (3.75 or 6.25 mg/kg/dose, i.v., ×9). Transient decrease in total hemolytic complement activity (~62–74%) and increases in C3a (~35-fold) and Bb levels (~5–12-fold) were observed in LErafAON and blank liposome groups of monkeys. A 30 mg/kg i.v. dose of LErafAON in human prostate tumor (PC-3)-bearing BALB/c athymic mice gave a terminal plasma half-life of 27 h, and intact rafAON could be detected in plasma and in normal and tumor tissues for up to at least 48 h. In monkeys, the terminal plasma half-life of 30.36 ± 23.87 h was observed at an i.v. dose of 6.25 mg/kg. LErafAON (25 mg/kg/dose, i.v., ×10) or ionizing radiation (3.8 Gy/day, ×5) treatment of PC-3 tumor-bearing athymic mice led to tumor growth arrest, whereas a combination of LErafAON and ionizing radiation treatments resulted in tumor regression. LErafAON treatment caused inhibition of Raf-1 protein expression in normal and tumor tissues in these mice (>50%, versus controls). These data have formed a basis of the clinical Phase I studies of LErafAON for cancer treatment.

INTRODUCTION

Raf-1, a cytoplasmic serine and threonine kinase, plays an important role in mitogen and stress-induced signaling response, cell survival, and proliferation (1–3). Depending on the cell type and stimulus, a number of growth factors, growth factor receptors, cytokines, and antiapoptotic molecules have been identified as components of the Raf-1 signaling pathway(s) (4, 5). Although the classical Ras/Raf/MEK/ERK pathway is generally associated with cell proliferation and survival via engagement of downstream transcription factors (4–9), growing evidence suggests that the survival effects of Raf-1 may also be a result of its mitochondrial translocation and interaction with the members of the Bcl family (10, 11) or the phosphatidylinositol 3-kinase/akt survival module (12). Reports using genetic knockouts of Raf-1, mouse c-raf-1 gene imply an MEK-independent role of Raf-1 in differentiation and survival (13, 14). Raf-1 has been linked with cell cycle regulation via its interaction with the retinoblastoma protein (15) and with cytoskeletal reorganization via its association with vimentin, vimentin kinases, and myosin phosphatase (16, 17). Raf-1 also regulates the transcription of a number of diverse molecules (9, 18, 19). The importance of Raf-1 activity has been demonstrated both in solid tumors and hematological malignancies (2, 6, 20). These and other data showing an association of the expression of exogenous Raf-1 with increased survival against radiation or chemotherapeutic agents (2, 7, 21, 22) imply that a relatively complex balance between the anti- and proapoptotic molecules may be a determinant of the Raf-1-mediated survival response. Antisense therapeutics is an attractive target-specific strategy for cancer drug development (23–28). We and others have demonstrated that human tumor cells transfected with antisense c-raf-1 cDNA show inhibition of Raf-1 expression, delayed tumor growth, and enhanced sensitivity to anticancer agents (2, 22). Down-regulation of Raf-1 expression via antisense raf oligonucleotides is associated with enhanced radiosensitivity, tumor growth arrest, activation of a proapoptotic molecule caspase-3, poly(ADP-ribose) polymerase cleavage, and apoptosis (20, 29–31). These observations underscore the importance of Raf-1 as a critical target in cancer therapy.

Liposomal delivery systems for anticancer drugs have

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demonstrated significant clinical benefits including extended circulation time, reduced drug-related toxicity, and improved efficacy (32–36). More recently, a variety of lipid formulations have been used for intracellular delivery of nucleic-acid-resistant phosphorothioate, P-ethoxy, or methylphosphonate oligonucleotides (37–40). Cationic liposomes have been shown to enhance cellular uptake and alter intracellular trafficking of phosphorothioate oligonucleotides. While free phosphorothioate oligonucleotides are localized within endosomes or lysosome-like structures, liposome-complexed phosphorothioates are present in cytosolic and nuclear compartments, thereby reducing the efflux of oligonucleotides (41).

Limited information is available on the in vivo liposomal delivery of nucleic sensitive phosphodiester oligonucleotides. Although anionic liposomes have been shown to protect unmodified oligonucleotides against serum nuclease degradation, understandably these carriers exhibit relatively low oligonucleotide entrapment efficiency (42). To date, preclinical evaluation of nuclease-sensitive, liposome-entrapped antisense oligonucleotides has not been conducted. We have designed novel cationic liposomes for in vivo delivery of a 15-mer antisense oligonucleotide complementary to the translation initiation region of c-raf-1 mRNA, rafAON4 (43). The phosphorothioate modification in rafAON is limited to only the terminal base at the 5' and 3' ends. Using fluorescence-labeled rafAON, we have demonstrated that rafAON is entrapped in the lipid bilayer of the liposome, facilitating endocytosis via association of the cationic lipids with negatively charged plasma membrane. Plasma pharmacokinetic and tissue distribution studies indicate that although free rafAON undergoes degradation within minutes after i.v. administration in athymic mice, LERafAON is stable in plasma and tissues for up to at least 24–48 h (Ref. 43 and unpublished data). The cationic liposome formulation was also shown to improve the antitumor effects of a fully phosphorothioate antisense raf oligonucleotide complementary to a 3'-untranslated region of c-raf-1 mRNA (44). The goal here was to perform the preclinical studies of ends-modified and nuclease-sensitive rafAON entrapped in liposomes using rodent and primate model systems. The pharmacokinetics, biodistribution, safety, and efficacy data presented here demonstrate that LERafAON is a promising cancer therapeutic agent.

MATERIALS AND METHODS

Cell Culture. Human prostate carcinoma cells (PC-3) were grown as a monolayer in Improved Minimum Essential Medium (Biofluids Inc., Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum, 100 µg/ml streptomycin, and 100 units/ml penicillin.

The abbreviations used are: rafAON, raf antisense oligodeoxyribonucleotide; LERafAON, liposome-entrapped raf antisense oligodeoxyribonucleotide; rafSON, raf sense oligodeoxyribonucleotide; BL, blank liposome; LE-MMAON, liposome-entrapped mismatch antisense oligodeoxyribonucleotide; NBF, neutral-buffered formalin; CBC, complete blood cell; IR, ionizing radiation; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH50, total hemolytic complement activity; UT, untreated.

Oligodeoxyribonucleotides and Chemicals. Antisense oligodeoxyribonucleotide sequence (5'-GTGCTCATTGATGC-3') directed toward the translation initiation site of human c-raf-1 mRNA (rafAON), the complementary sense strand sequence (5'-GCATCAATGGCC-3'; rafSON), and a mismatch sequence (5'-GTGTTCACCTAGTGC-3'; MMAON) were custom synthesized at Hydron Speciality Products (Milford, MA). The terminal base linkage at the 5' and 3' ends of these sequences was modified to a phosphorothioate group. The sequence of the GMP grade product (97% pure) was confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry by the manufacturer.

Preparation of LERafAON. LERafAON formulation was prepared by one of the following procedures. In method 1, lipids (dimethyldioctadecyl ammonium bromide, egg phosphatidylcholine, and cholesterol) were dissolved in chloroform and evaporated to dryness in a round-bottomed flask using a rotary vacuum evaporator in a 37°C water bath for ~20 min. LERafAON was prepared by hydrating the dried lipid film overnight at 4°C with rafAON solution in normal saline using rafAON to lipid ratio of 1:15 (w/w). The film was dispersed by vigorous vortexing, and the liposome suspension was sonicated at room temperature for 2 h, and sonication for 10 min in a bath-type sonicator (Model XL 2020; Misonix, Inc., Farmingdale, NY). In method 2, the lipids were dissolved in r-butanol and lyophilized. Lyophilized lipids were reconstituted at room temperature with rafAON solution as above. The mixture was vortexed vigorously for 2 min, followed by hydration at room temperature for 2 h, and sonication for 10 min. Alternatively, lyophilized lipids supplied by NeoPharm, Inc., were reconstituted with rafAON solution as above (method 3). LERafAON was stored at 4°C and used within 3 days (mice and rabbit studies) or 8 h after preparation (monkey studies). Control LE-MMAON was prepared as above, and the BLs were prepared in the absence of rafAON.

LERafAON Entrapment Efficiency and Stability Assays. The amount of rafAON entrapped in liposomes was determined by adding traces of 32P-end-labeled rafAON to the excess of unlabeled rafAON, and the entrapment efficiency was calculated by scintillation counting as reported earlier (43). Alternatively, the entrapment efficiency and stability of intact rafAON were determined by the denaturing gel electrophoresis method. An aliquot of the LERafAON preparation was designated as the prewash sample. The unentrapped rafAON was removed from the remainder of the preparation by ultracentrifugation at 100,000 × g for 20 min, followed by washing the liposomes twice with normal saline (postwash sample). RafAON was extracted from pre- and postwash samples with phenol-chloroform, and the extracts were electrophoresed on a 20% polyacrylamide/8 M urea gel, followed by electroblotting on to a nylon membrane in 0.5× Tris-borate EDTA buffer at 20 V for 1 h. The blot was probed with 32P-end-labeled complementary rafSON probe in ExpressHyb solution (Clontech, Palo Alto, CA). The radiolabeled rafSON was generated by 5'-end labeling with γ32P-ATP using T4 polynucleotide kinase and purification over Chroma Spin-10 columns (Clontech). The
i.v. injections of 5.0, 15.0, 25.0, or 35.0 mg/kg LErafAON (specimens for complement activity (CH50) were obtained within 30 min after injection on day 12 in cohort I and before euthanasia in cohort II. Various tissues were collected in 10% NBF for histopathology. Gross and histopathological findings were graded as minimal, slight, moderate, or severe based on the degree of involvement relative to the normal saline group.

**Animals.** Male CD2F1 mice, 20–22 g, and male BALB/c nu/nu mice, 5–6 weeks old, were purchased from the National Cancer Institute (Frederick, MD). Male and female New Zealand white rabbits, 2.3–3.0 kg, were purchased from Covance Research Products, Inc. (Denver, PA). Mice and rabbits were maintained at the AAALAC-accredited Research Resource Facility of the Division of Comparative Medicine, Georgetown University Medical Center. Cynomolgus monkeys (Macaca fascicularis), 1.7–2.2 kg, were purchased from Covance Research Products, Inc., and maintained according to the Food and Drug Administration’s GLP regulations at Covance Laboratory, Inc. (Vienna, VA).

**Mouse Toxicology.** Male CD2F1 mice received multiple i.v. injections of 5.0, 15.0, 25.0, or 35.0 mg/kg LErafAON (n = 5). LErafAON was administered via tail vein on days 1, 2, 4, 5, 7, 8, 10, 11, 13, 14, 16, and 17. Control groups received BLs containing LErafAON equivalent lipid doses or were left untreated. Mice in each group were observed daily and group-weighted at least twice a week. Two mice/group were sacrificed on day 19, 48 h after the last dose and representing the short-term response. The remaining three mice/group were sacrificed on day 32, 15 days after the last dose and representing the long-term response. Blood was collected via intracardiac stick under anesthesia, and tissue samples from euthanized animals were collected in 10% NBF. Specimens were processed for clinical pathology, including CBC count and serum chemistry and histopathology evaluations. Gross and histopathological findings were graded as minimal, slight, moderate, or severe based on the degree of involvement relative to the UT control.

**Rabbit Toxicology.** The two i.v. doses of 3.75 and 6.5 mg/kg in rabbits were selected based on allometric scaling of 15.0 and 25.0 mg/kg doses in mice, respectively. However, the total number of injections in rabbits had to be reduced because of technical limitations. Two male and two female rabbits received eight injections of 3.75 mg/kg LErafAON via marginal ear vein over 12 days (days 1, 2, 4, 5, 8, 9, 11, and 12; cohort I), and two male and two female rabbits received six i.v. injections of 6.5 mg/kg LErafAON over 8 days (days 1, 2, 4, 5, 7, and 8; cohort II). Control groups, one male and one female rabbit, received BLs containing LErafAON equivalent lipid doses, and one male and female rabbit received normal saline on treatment days. The study was terminated 24–72 h after the last dose. Blood samples for CBC and chemistry panel were collected on day 1 from the auricular artery (predose) and before euthanasia under sedation through cardiac stick. Blood samples for coagulation profile (prothrombin time, partial thromboplastin time, and fibrinogen) were collected within 30 min after injection on day 12 in cohort I and within 30 min after injection on days 5 and 8 and before euthanasia in cohort II. Blood specimens for complement activity (CH50) were obtained within 30 min after injection on day 12 in cohort I and before euthanasia in cohort II. Various tissues were collected in 10% NBF for histopathology. Gross and histopathological findings were graded as minimal, slight, moderate, or severe based on the degree of involvement relative to the normal saline group.

**Monkey Toxicology.** Monkeys received nine slow bolus injections of 3.75 mg/kg (three males) or 6.25 mg/kg LErafAON (three males and three females) administered via saphenous vein over 15 days (days 1, 2, 4, 5, 8, 9, 11, 12, and 15). Control group received BLs containing a lipid dose equivalent to 6.25 mg/kg LErafAON on treatment days (three males). Blood samples for CBC and chemistry panel were obtained from femoral vein before initiation of dosing and before study termination. Blood specimens were also collected on day −7 and at 10–15 min after dose on days 5, 12, and 15 for coagulation tests (prothrombin time and activated partial thromboplastin time) and complement marker evaluations (CH50, C3a, and C4b). Animals were anesthetized and exsanguinated 24 h after the last dose, and various tissues were collected in 10% NBF for histopathology. Pathological findings were graded relative to the vehicle control.

**Mouse Pharmacokinetics and Biodistribution.** Plasma pharmacokinetic analysis was performed in male CD2F1 mice and in male BALB/c nu/nu mice bearing a s.c. PC-3 tumor. Mice received injections of 30.0 mg/kg single i.v. bolus dose of LErafAON via tail vein. Blood samples were obtained via cardiac puncture under anesthesia in microtainers containing sodium heparin as an anticoagulant at predose and at 5, 15, and 30 min and 1, 2, 4, 8, 24, and 48 h after LErafAON administration. Athymic mice were euthanized, and liver, spleen, kidney, lung, heart, and PC-3 tumor tissue were rapidly excised, rinsed in ice-cold normal saline, and snap frozen on dry ice. Blood samples were centrifuged at 3000 rpm for 10 min at 4°C to separate the plasma. The plasma and tissue samples were stored at −80°C until additional analysis. rafAON concentrations in plasma, and normal and tumor tissue samples were determined as described earlier (43). Briefly, rafAON was isolated from plasma samples using the phenol-chloroform extraction method and from tissue samples using a DNA extraction kit (Stratagene, La Jolla, CA). The rafAON concentration standards were prepared by adding known amounts of rafAON in blank plasma or blank tissue samples, followed by extraction. The extracts were analyzed by gel electrophoresis, blotting, and hybridization as detailed above for entrapment efficiency. A 10–50-fold excess of the probe was used to ensure saturation of all bands. The autoradiographs were scanned and quantified using ImageQuant software (Molecular Dynamics), and the amounts of rafAON in various samples were calculated by comparison to standards.

**RafAON concentrations in plasma, and normal and tumor tissue samples were determined as described earlier (43).** Briefly, RafAON was isolated from plasma samples using the phenol-chloroform extraction method and from tissue samples using a DNA extraction kit (Stratagene, La Jolla, CA). The RafAON concentration standards were prepared by adding known amounts of RafAON in blank plasma or blank tissue samples, followed by extraction. The extracts were analyzed by gel electrophoresis, blotting, and hybridization as detailed above for entrapment efficiency. A 10–50-fold excess of the probe was used to ensure saturation of all bands. The autoradiographs were scanned and quantified using ImageQuant software (Molecular Dynamics), and the amounts of RafAON in various samples were calculated by comparison to standards.

**Monkey Pharmacokinetics.** Monkeys received a slow bolus injection of LErafAON via saphenous vein (3 males, 3.75
**RESULTS**

**LErafAON Formulation Shows High Entrapment Efficiency and Is Stable at Room Temperature.** The liposome entrainment efficiency of LErafAON at 0 h after preparation was found to be >85.0% (four independent experiments, cumulative n = 14). A reduction in the hydration time from 2 h to 30 min had no effect on the entrapment of rasAON (percentage of entrapment efficiency, 89.0 ± 6.9 n = 3). Stability of entrapment was determined by comparing the entrainment efficiency of the LErafAON formulation stored at room temperature for 8 h with its freshly prepared counterpart. This formulation was stable at room temperature for up to at least 8 h after preparation (percentage of stability: hydration time 2 h, 93.0 ± 7.2, n = 5; hydration time 30 min, 94.1 ± 4.2, n = 2). In other studies, particle size analysis of LErafAON was carried out using Nicosmp particle sizer (Particle Sizing Systems, Inc., Santa Barbara, CA). The vesicle size was found to be 467.2 ± 72.0 nm (n = 2).

**LErafAON Is Nontoxic in Vivo.** The toxicity of LErafAON was evaluated after i.v. administration in CD2F1 mice, NZW rabbits, and cynomolgous monkeys as described in “Materials and Methods.” Two animals from each group were intended sacrifices 48 h after the last dose (short-term response), and the remaining three animals were intended sacrifices 15 days after the last dose (long-term response). BL, blank liposome dose was equivalent to the lipid dose in 35.0 mg/kg LErafAON group. The values shown are mean ± SD.

**Statistical Analysis.** Student’s t test was performed to determine the significance of changes in clinical pathology values obtained in CD2F1 mice treated with four different doses of LErafAON or BLs versus untreated control mice. Analysis included both short-term and long-term treatment groups of these mice. ANOVA (one-way ANOVA) was used to compare the clinical pathology values observed in male monkeys treated with two different doses of LErafAON versus BLs. One-way ANOVA was also performed to determine the statistical significance of a treatment-related change in mean tumor volume in BALB/c nu/nu mice.

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**Table 1 Effects of LErafAON on liver enzymes and histology in CD2F1 mice**

<table>
<thead>
<tr>
<th>LErafAON dose (mg/kg)</th>
<th>AST (units/liter)</th>
<th>ALT (units/liter)</th>
<th>Liver histology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Short-term</td>
<td>Long-term</td>
<td>Short-term</td>
</tr>
<tr>
<td></td>
<td>response (n = 2)</td>
<td>response (n = 3)</td>
<td>response (n = 2)</td>
</tr>
<tr>
<td>5.0</td>
<td>99 ± 22</td>
<td>130 ± 32</td>
<td>37 ± 21</td>
</tr>
<tr>
<td>15.0</td>
<td>267 ± 145</td>
<td>225 ± 72</td>
<td>56 ± 14</td>
</tr>
<tr>
<td>25.0</td>
<td>396*</td>
<td>237 ± 114*</td>
<td>111*</td>
</tr>
<tr>
<td>35.0</td>
<td>564 ± 209</td>
<td>473 ± 78*</td>
<td>161 ± 57</td>
</tr>
<tr>
<td>BL</td>
<td>291 ± 26</td>
<td>267 ± 114*</td>
<td>78 ± 80</td>
</tr>
<tr>
<td>UT</td>
<td>125 ± 120</td>
<td>77 ± 20</td>
<td>30 ± 17</td>
</tr>
</tbody>
</table>

*LErafAON was injected i.v. at indicated doses (n = 5) and clinical and histopathological evaluations were performed as described in “Materials and Methods.” Two animals from each group were intended sacrifices 48 h after the last dose (short-term response), and the remaining three animals were intended sacrifices 15 days after the last dose (long-term response). BL, blank liposome dose was equivalent to the lipid dose in 35.0 mg/kg LErafAON group. The values shown are mean ± SD.

1 P < 0.03 compared with UT controls.

2 P < 0.001 compared with UT controls.

3 P < 0.05 compared with UT controls.

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mg/kg; 3 males and 3 females, 6.25 mg/kg). Heparinized blood samples were obtained from each monkey at following time points: predose and at 5, 10, and 30 min and 1, 2, 4, and 24 h after LErafAON administration. Blood tubes were centrifuged at 3000 rpm, and plasma was separated and stored at −80°C until additional analysis as described above. Predose specimens spiked with known amounts of rafAON were used as concentration standards.

**Antitumor Efficacy Studies: Experimental Design.** Logarithmically growing PC-3 cells were injected s.c. (5 × 10⁶ cells) in the left flank region of male BALB/c nu/nu mice. Tumors were allowed to grow to a mean tumor volume of ~60 mm³ before initiation of treatment. Tumor volumes were determined from caliper measurements of the three major axes (a,b,c) and calculated using abc/2, an approximation for the volume of an ellipse (π abc/6). Tumor-bearing mice were randomly divided into various treatment groups (n = 6–8). LErafAON and LErafAON + IR treatment groups received 10 i.v. injections of 25.0 mg/kg/dose LErafAON over 14 days (day 0, 1, 3, 4, 5, 7, 9, 10, 12, and 13). Radiation was delivered to the tumors of mice in the IR alone and LErafAON + IR treatment groups once daily from day 7 through day 11 using a [137Cs] irradiator (J. L. Shepard Mark I; 2.37 Gy/min, 3.8 Gy/day) as described earlier (44). In the combination group, the two treatments were given 8 h apart. Control groups received i.v. doses of BLs or liposome-entrapped mismatch oligo (LE-MMAON) on the same dosing schedule as LErafAON or were left untreated.

Tumor sizes were monitored once or twice weekly. Individual tumor volume was calculated as the percentage of pretreatment tumor volume (day 0, the first day of dosing; 100%) and the mean tumor volume (percentage of initial) ± SE for each treatment group was plotted.

**Raf-1 Expression Assay.** Normal and tumor tissues of PC-3 tumor-bearing BALB/c nu/nu mice were excised within 6–12 h after the last LErafAON treatment given as above. Raf-1 protein expression was examined in whole cell extracts from tissues by immunoprecipitation with polyclonal anti-Raf-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by immunoblotting with monoclonal anti-Raf-1 antibody (Transduction Labs, Lexington, KY) using standard procedures as detailed earlier (3, 17, 43–45). Raf-1 protein levels were quantified using ImageQuant software (Molecular Dynamics).
over 17 days. The cumulative dose of LErafAON administered in these groups was 60.0, 180.0, 300.0, and 420.0 mg/kg, respectively \((n = 5)\). Control groups received BLs at LErafAON equivalent lipid doses (BL) or were left untreated \((n = 5)\). Mice in untreated control group gained weight throughout the duration of study with a maximum group weight gain of 18.5% at the time of sacrifice (day 32, 15 days after last dose). At 5.0 mg/kg, BL and LErafAON groups exhibited group weight loss of 0.5 and 0.2%, respectively, by day 7, with return to weight gain by day 10. At 15.0 mg/kg, BL group had a moderate group weight gain, whereas LErafAON group showed a mild weight loss of 3.3% on day 12, with weight gain thereafter. Mice receiving 25.0 and 35.0 mg/kg BL had a maximum weight loss of 6.0 and 10.5%, respectively, by day 18, followed by weight gain by day 25. Mice treated with 25.0 and 35.0 mg/kg LErafAON had a maximum weight loss of 11.0 and 13.4%, respectively, by day 18 and weight gain by day 21. Weight loss did not exceed 14% in any of the groups, and all groups demonstrated positive growth curves within 5 days after treatment cessation.

No hematological abnormalities were noted in any of the groups of mice except for a dose-dependent increase in total neutrophil count with the total WBC count remaining in the normal range. This effect was seen in BL and LErafAON groups as compared with UT controls and was attributed to liposome administration. Within 48 h after the last dose, serum chemistries revealed dose-related elevations in liver enzymes (Table 1, ALT, short-term response). However, these elevations were not statistically significant as compared with control untreated mice. Additionally, the total bilirubin or alkaline phosphatase remained unchanged as compared with control untreated mice (data not shown). Fifteen days after the last dose, AST was significantly elevated in BL and LErafAON groups, and ALT was significantly elevated in only the highest dose LErafAON group (Table 1, AST/ALT, long-term response). The total bilirubin or alkaline phosphatase level remained unchanged as compared with control untreated mice (data not shown).

Changes in liver enzymes were manifested histologically. Mild to moderate diffuse hepatitis with minimal hepatocyte necrosis was observed in LErafAON and BL groups of mice as compared with untreated mice. These effects appeared to be dose related and relatively more pronounced in the LErafAON groups (Table 1). No clear difference was observed between the short-term and long-term responses of BL or LErafAON animals. In addition, mild to moderate splenic enlargements were seen in mice receiving mid to high doses of BL and LErafAON as compared with UT controls. However, no clinical chemistry or histological correlation was found. No other gross or microscopic pathology was observed in any of the groups.

The cumulative i.v. doses of LErafAON administered in rab-

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**Fig. 1** LErafAON or BLs induce transient changes in complement profile in cynomolgus monkeys. Indicated dose of LErafAON or BLs was injected i.v. on various days \((arrows)\), and blood specimens were collected as described in “Materials and Methods.” The CH50 in serum \((A)\) and plasma concentrations of complement split products, C3a \((B)\) and Bb \((C)\), were determined 10–15 min after treatment on days 5, 12, and 15 \((n = 3)\).
Liposome-entrapped Antisense raf Oligonucleotide

respective baseline values (day treatment groups on day 5 and/or day 12 compared with their transient changes in complement profile in monkeys treated /H11011 products C3a (baseline values (day 7–12) in all treatment groups compared with their respective (Fig. 1). A 62% decrease in the CH50 was observed on days 5 and 12 in all treatment groups versus BLs were identical and appear to be related to the liposome treatment per se. Histomorphological changes characterized by the presence of vacuolated macrophages were observed in spleen, liver, and kidneys of monkeys in LErAfAON and BL groups and are likely to be associated with liposomes. In addition, mild chronic inflammation was recorded in liver and kidney tissues in all treatment groups.

LErafAON Exhibits a Favorable Pharmacokinetic Profile. After i.v. administration of 30 mg/kg LErafAON, the peak plasma concentration of 10.17 ± 2.62 μg/ml was achieved in BALB/c nu/nu mice bearing PC-3 tumor, and intact rafAON (15-mer) could be detected in plasma for up to 48 h (Fig. 2). The decrease in plasma concentration of LErafAON followed a biexponential pattern with initial distribution half-life ($t_{1/2\alpha}$) of 1.09 ± 0.53 h and a terminal half-life ($t_{1/2β}$) of 26.95 ± 0.99 h, and area under the plasma concentration-time curve (AUC) was 10.12 ± 5.37 μg.h/ml (Table 2). Comparable pharmacokinetic parameters were observed in CD2F1 mice treated with a similar dose of LErafAON (Table 2).

The tissue distribution profile of LErafAON in PC-3 tumor-bearing BALB/c athymic mice is presented in Fig. 3. Liver, spleen, and kidneys were the preferential sites of rafAON accumulation with intact rafAON detectable up to at least 48 h (Fig. 3, bottom panel). Heart and lungs also showed substantial uptake of rafAON up to 48 h. Remarkably, intact rafAON was

Table 2 Plasma pharmacokinetic parameters of LErafAON in mice

<table>
<thead>
<tr>
<th>Species</th>
<th>$C_{max}^{a}$ (μg/ml)</th>
<th>$t_{1/2\alpha}$ (h)</th>
<th>$t_{1/2β}$ (h)</th>
<th>$AUC_{0-infty}$ (μg.h/ml)</th>
<th>Cl (liters/h/kg)</th>
<th>Vd (liters/kg)</th>
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<tr>
<td>Balb/c nu/nu</td>
<td>10.17 ± 2.6</td>
<td>1.09 ± 0.53</td>
<td>26.9 ± 0.99</td>
<td>10.12 ± 5.37</td>
<td>3.45 ± 1.83</td>
<td>132.94 ± 66.37</td>
</tr>
<tr>
<td>CD2F1</td>
<td>5.22</td>
<td>0.48</td>
<td>22.50</td>
<td>9.02</td>
<td>3.32</td>
<td>107.80</td>
</tr>
</tbody>
</table>

$^{a} C_{max}$, peak plasma concentration; Vd, volume of distribution.
$^{b}$ Values shown are mean ± SD (n = 2).
Fig. 3 The tissue concentration-time profile of LErafAON in PC-3 tumor-bearing male BALB/c nu/nu mice. Mice received i.v. injections of a single dose of 30.0 mg/kg LErafAON. The raAON concentration in various tissue samples was determined by denaturing gel electrophoresis as described in “Materials and Methods.”

Top, representative autoradiograph showing intact raAON in PC-3 tumor tissues excised at various time points after dose. S1, S2, and S3 are the standards representing 2.5, 5.0, and 10.0 ng of raAON prepared using predose tumor tissue homogenate. Bottom, tissue concentration-time curve of LErafAON (n = 1, except PC-3 tumor, n = 2). Quantification data were calculated based on comparison with known concentrations of the standard samples and then normalization against the weights of organs collected.

also detected in PC-3 tumor tissue for up to at least 48 h (Fig. 3, top and bottom panels). A peak raAON concentration of 5.56 ± 3.47 μg/g tumor tissue was obtained at 30 min to 1 h after dose (n = 2). The tissue AUC(0→48 h) (μg·h/g) values were: liver, 2248.26; spleen, 2031.00; kidneys, 796.00; lungs, 423.30; heart, 111.63; and PC-3 tumor, 25.26 ± 6.38.

The plasma pharmacokinetic parameters of LErafAON after a single bolus i.v. dose of 3.75 or 6.25 mg/kg in cynomolgus monkeys are shown in Table 3. The peak plasma concentrations achieved were 0.85 ± 0.63 μg/ml (n = 3) and 1.25 ± 0.53 μg/ml (n = 6) at 3.75 mg/kg dose and 6.25 mg/kg, respectively. Intact raAON could be detected up to 24 h (data not shown). The decrease in plasma concentration of LErafAON followed a biexponential pattern with an initial half-life (t1/2α) of 9.0 min seen at both dose levels. The terminal half-lives (t1/2β) were 36.39 and 30.36 h at low and high dose, respectively. The AUC increased from 2.35 ± 0.76 μg·h/ml at 3.75 mg/kg dose to 5.73 ± 6.30 μg·h/ml at 6.25 mg/kg dose.

**LErafAON Treatment Inhibits Raf-1 Expression in Normal Tissues.** The effect of multiple i.v. injections of LErafAON on Raf-1 protein expression was examined in limited sets of normal tissues from athymic mice and monkeys. Within 24 h after the last dose of LErafAON (25 mg/kg, 10×), Raf-1 protein levels in liver, kidney, and splenic tissues of mice treated with LErafAON were 7.2 ± 6.1, 17.0 ± 3.7, and 4.8 ± 2.3%, respectively, compared with BL-treated group (100%; n = 2; Fig. 4, top panel). BL treatment did not alter Raf-1 expression in these tissues compared with UT controls (data not shown). In monkeys, within 24 h after the last dose of LErafAON (6.25 mg/kg, 9×), Raf-1 protein expression in liver specimens from male and female monkeys was found to be 60.9 ± 14.1 and 56.6 ± 26.6%, respectively, as compared with BL-treated group (n = 3; Fig. 4, bottom panel). A lower dose of LErafAON (3.75 mg/kg, 9×) was found to be ineffective.

### Table 3 Plasma pharmacokinetic parameters of LErafAON in cynomolgus monkeys

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Cmax (μg/ml)</th>
<th>t1/2α (h)</th>
<th>t1/2β (h)</th>
<th>AUC0→∞ (μg·h/ml)</th>
<th>CI (L/h/kg)</th>
<th>Vd (L/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.75 3</td>
<td>0.85 ± 0.63</td>
<td>0.15 ± 0.04</td>
<td>36.39 ± 12.77</td>
<td>2.35 ± 0.76</td>
<td>1.74 ± 0.69</td>
<td>12.77 2</td>
</tr>
<tr>
<td>6.25 c</td>
<td>1.25 ± 0.53</td>
<td>0.15 ± 0.08</td>
<td>30.36 ± 23.87</td>
<td>5.73 ± 6.30</td>
<td>1.99 ± 1.29</td>
<td>64.72 ± 36.79</td>
</tr>
</tbody>
</table>

a Cmax peak plasma concentration; Vd, volume of distribution.

b Values shown are mean ± SD (n = 3, males).

c Values shown are mean ± SD (n = 6, three males and three females).
The mismatch antisense group LE-MMAON continued to show significant tumor growth compared with LErafAON group (mean ± SE percentage of initial tumor volume, day 52: LE-MMAON, 1209 ± 577%; LErafAON, 390 ± 106%). The data demonstrates that a combination of LErafAON and IR treatments is highly efficacious in a hormone-refractory prostate cancer.

Within 6–12 h after the last LErafAON treatment, Raf-1 expression in tumor tissue was 23.4 ± 15.4% of the UT control group. In PC-3 tumors from mice treated with a combination of LErafAON and IR, Raf-1 expression was 13.2 ± 2.7% of the UT control group. Raf-1 expression in PC-3 tumors of mice treated with IR, LE-MMAON (MM), or BL was comparable with UT control PC-3 tumors (Fig. 5C). These data indicate that rafAON selectively inhibits Raf-1 protein expression in vivo.

**DISCUSSION**

Raf-1 protein kinase is an established molecular target for cancer drug discovery (2, 20, 46). Although recent drug discovery efforts are directed at the structure-based design of suitable peptides or chemicals that may interfere with Raf-1 signaling (47, 48), the antisense oligonucleotide approach is at the forefront of clinical endeavors to block Raf-1 expression and activity (26, 49). With the advent of high-throughput technologies, the number of clinically relevant biological molecules is fast growing; however, delivery systems for safe and effective targeting of such agents to advanced lesions are relatively limited (50–53). This study offers a novel systemic liposomal oligonucleotide delivery system. Our data demonstrate favorable pharmacokinetic and biodistribution profiles of liposomal formulation of raAON as compared with raAON reported earlier (43), tumor tissue uptake of intact raAON, sequence-specific inhibition of Raf-1 protein in normal and tumor tissues, and raAON-associated enhanced radiation response in a human hormone-refractory prostate tumor model.

The antisense oligonucleotide chemistry, validity of the antisense sequence-specific inhibition of gene expression, and pharmaceutical application of the antisense technology have generated considerable interest in the past decade (23–28). Earlier, we have established the sequence specificity of the 15-mer raAON oligonucleotide used in this study (29, 43). In vitro and in vivo data using control sense oligonucleotide (29, 43) or a mismatch antisense oligonucleotide (present study) have established the raAON sequence as an antisense oligonucleotide capable of inhibiting Raf-1 protein expression in cells (29, 43) in normal and tumor tissues of athymic mice (Ref. 43 and present study), and in liver specimens of monkeys. The specimens obtained from BL or untreated animals served as additional controls. The LErafAON formulation developed here shows high entrapment efficiency of raAON (>85%), and LErafAON is stable at room temperature for at least up to 8 h. Toxicology studies indicated that cumulative doses of 420.0, 156.0, and 56.0 mg/kg LErafAON are well tolerated in mice, rabbits, and monkeys, respectively. A transient increase in complement activity observed in monkeys is restored to the baseline value during the course of treatment with BLs or LErafAON (Fig. 1). Unlike phosphothioate oligonucleotides where complement activity has been related to plasma concentrations of the oligonucleotide (54), raAON per se does not appear to contribute to changes in complement activity observed in monkeys. These changes are likely to be attributed to liposomes rather than raAON. Additional investigations should address the mechanism of liposome-mediated changes in complement activity. All other toxicology parameters observed in monkeys are clinically insignificant. Pharmacokinetic studies in mice and monkeys indicated that intact raAON is detectable in plasma for up to at least 24 h. The intact raAON could be detected in all normal and tumor tissues examined in mice for up to at least 48 h. These data demonstrate that liposomes entrapment protects circulating raAON and clearly enhances the biodistribution profile of these oligonucleotides. On the basis of our observations of normal clinical chemistry and histopathology in monkeys and a concomitant inhibition of Raf-1 expression observed in liver specimens from the same monkeys, it appears that inhibition of Raf-1 per se does not cause any toxicity in normal tissues. Furthermore, antitumor efficacy studies in athymic mice indicated that 50% inhibition of Raf-1 expression in prostate tumor
tissues is sufficient to cause tumor regression in conjunction with radiation treatment (Fig. 5).

The knowledge of the mechanisms of radiation-induced signal transduction and biological response has increased enormously during the past decade (3, 7, 45, 55–60); however, a limited number of radiation/chemosensitizers are currently approved for clinical use by the United States Food and Drug Administration (61, 62). Systemic administration of LErafAON treatment also results in enhanced radiation sensitivity in human head and neck squamous cell carcinoma (SQ-20B) and pancreatic carcinoma models (AsPC-1; unpublished data). In addition, LErafAON i.v. treatment causes tumor growth inhibition and chemosensitization of human lung (A549), breast (MDA-MB 231), prostate (PC-3), and pancreatic tumor xenografts grown in athymic mice (AsPC-1 and COLO-357; Refs. 63–65). Recently, the clinical Phase I studies of LErafAON as a monotherapy and LErafAON in combination with radiation therapy have been initiated (66–69). The present report has a broad application in the area of evolving antisense technology. Our data demonstrate that cationic liposome entrapment is a safe and effective mode of in vivo delivery of intact antisense oligonucleotides. This delivery system eliminates the need for a significant modification of the oligonucleotide chemistry and preserves the two salient features of the antisense drug: potency and target selectivity. The cationic liposomes developed here may be used to deliver more than one type of antisense oligonucleotide or decoy oligonucleotides (70) to validate components of a signaling pathway and associated biological response.

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Liposome-entrapped Antisense raf Oligonucleotide


Pharmacokinetics, Toxicity, and Efficacy of Ends-modified \textit{raf} Antisense Oligodeoxyribonucleotide Encapsulated in a Novel Cationic Liposome

Prafulla C. Gokhale, Chuanbo Zhang, Joseph T. Newsome, et al.


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