9-Nitrocamptothecin Liposome Aerosol Treatment of Melanoma and Osteosarcoma Lung Metastases in Mice

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ABSTRACT

The response rates of relapsed osteosarcoma and melanoma pulmonary metastases to traditional i.v. chemotherapy regimens have been disappointing. Direct delivery of chemotherapy to the lungs could increase the drug concentration in the tumor area and may offer a new therapeutic approach for these patients. Previous studies demonstrated that drugs delivered to the respiratory tract in liposomal formulation resulted in high pulmonary drug concentration, reduced systemic toxicity, and reduced dosage requirements compared with parenteral and oral administration. To determine whether this approach has utility against pulmonary metastases, the efficacy of aerosol therapy with liposome-encapsulated 9-nitrocamptothecin (L-9NC) was determined using two different experimental lung metastasis models. C57BL/6 mice were treated the day after the i.v. injection of B16 melanoma cells with aerosol L-9NC for 1 h (153 μg 9-nitrocamptothecin/kg) for 5 days per week for up to 3 weeks. Aerosol L-9NC treatment resulted in a reduction in lung weights (P = 0.005) and number of tumor foci (P < 0.001). Visible tumor nodules were fewer and smaller in the 9-nitrocamptothecin-treated group than in untreated control mice (P < 0.001). Using a newly developed human osteosarcoma experimental metastasis model in nude mice, we demonstrated that aerosol L-9NC was also effective against established lung metastases. Aerosol therapy initiated on the ninth week after i.v. tumor injection and continued for 8 or 10 weeks produced highly significant reductions in the number of animals with both visible and microscopic disease (P < 0.02), the total number of tumor foci in the lungs (P < 0.005), and the size of the individual tumor nodules (P < 0.02). These data suggest that L-9NC aerosol therapy may offer significant advantage over existing methods in the treatment of melanoma and osteosarcoma pulmonary metastases.

INTRODUCTION

The treatment of osteosarcoma and melanoma lung metastases continues to present a challenge to the oncologist. The majority of these tumors are unresponsive to standard chemotherapy regimens. In osteosarcoma, the success of high-dose ifosfamide in generating a response in patients where no response was seen initially using standard dosage suggests that increasing both the drug concentration and exposure may provide therapeutic benefit. Direct delivery of chemotherapy to the lungs could offer a new therapeutic approach for patients with pulmonary metastases.

Inhalation of small-particle aqueous aerosols generated by jet nebulizers containing medications incorporated in liposomes is a developing technology that may prove useful in the treatment of asthma, respiratory virus infections, and some other diseases (1, 2) and may be an effective method of treating pulmonary cancer in man.

The validity of that approach was suggested by the observation that 9NC administered in a liposome formulation (L-9NC) by small-particle aerosol is remarkably effective in the treatment of s.c. xenografts of human cancer in nude mice (3). The dosage of drug found to be effective when administered in this manner was severalfold lower than that required by other routes of administration. Pharmacokinetic studies with camptotheclin, the parent molecule of 9NC, showed that when inhaled by mice, it promptly reached high concentrations in the lungs followed by immediate distribution to the liver and other viscera (4).

In the present study, aerosol L-9NC was found to be highly active against B16 murine melanoma and SAOS-LM6 human osteosarcoma lung metastases in mice. These studies suggest that L-9NC aerosol might be useful in the treatment of osteosarcoma and melanoma and other tumors that metastasize to the lungs.

MATERIALS AND METHODS

Chemicals. 9NC was purchased from ChemWerth (Woodbridge, CN). DLPC was purchased from Avanti Polar Lipids (Alabaster, AL). Tertiary butanol was obtained from Fisher Scientific, DMSO was obtained from Sigma, and pyrogen-free, sterile water for irrigation was obtained from Baxter Healthcare Corp. (Deerfield, IL).

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3 The abbreviations used are: 9NC, 9-nitrocamptothecin; DLPC, dilauroylphosphatidylcholine; L-9NC, liposomal formulation of 9NC.
Cells and Pulmonary Tumor Metastases in Animal Models. B16F10 murine melanoma cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in tissue culture using DMEM supplemented with 10% FCS. Medium supplements were obtained from Life Technologies, Inc. (Grand Island, NY). To induce pulmonary metastases, B16F10 cells (1 × 10³) were injected i.v. in 0.2 ml of media via tail vein in female C57BL/6 mice (Harlan Sprague Dawley). The lung metastases could be detected visually within a week after cell inoculation. The aerosol treatment of mice started the day after injection.

The SAOS-LM6 cell line was derived from SAOS-2 human osteosarcoma cells by repeating cycling of tumor cells in nude mice (5). SAOS-LM6 cells were maintained in vitro using EMEM media supplemented with 10% FCS. SAOS-LM6 cells (1 × 10⁸) in 0.2 ml of media were injected into the tail vein of male nu/nu mice. After i.v. injection, visible macroscopic pulmonary metastases were present by 8 weeks. The aerosol treatment of mice bearing osteosarcoma lung metastases started on the ninth week after cell injection.

Liposome Preparation and Aerosol Dosage of 9NC in Liposome Aerosol. The procedure for L-9NC preparation has been described elsewhere (3). The DLPC:9NC ratio was 50:1. The lyophilized powder was reconstituted with water for irrigation (Baxter Laboratories) to a concentration of 0.5 mg 9NC/ml DLPC. Empty liposomes were prepared as described previously (3) without adding 9NC to the formulation, and the concentration of lipid after adding water for aerosol administration was 25 mg/ml.

Dosages in mice were based on a 30-g mouse with a minute volume of 30 ml [1 liter/min/kg body weight (6)] and an average of 30% deposition of inhaled aerosol (3). The deposited doses in this study were calculated as shown by the following example: 8.5 μg 9NC/liter in the aerosol × 0.03 liter/min (mouse minute volume) × 0.3 (fraction of inhaled aerosol deposited) × 30 min (treatment time)/0.03 kg (weight of mouse) = 76.7 μg/kg. Total dose equaled the daily dose times treatment days. The deposited dose of DLPC in the lungs was about 50-fold higher (3).

Administration of Aerosol. A clear plastic box [7 × 11 × 5 (depth) inches] with a sealed top and a wire netting floor was used for treatment. Aerosol was introduced via an 1-cm accordion tube a short distance from the nebulizer at one end and discharged at the other end. The apparatus was housed in a safety hood. The animals were exposed to aerosol for 30 min. The 10-ml volume of liquid in the reservoir of the nebulizer was nearly consumed in 30 min. The concentration of 9NC in the aerosol was measured using the Andersen/ACFM nonviable ambient particle sizing sampler (3).

Treatment with Aerosol L-9NC. In the melanoma model, aerosol treatment was started the day after B16 cells were injected into C57BL/6 mice. Mice were divided into groups and treated 5 days per week over a span of 16–21 days. Aerosol was administered to groups of mice in sealed cages (3). Ten ml of drug suspension containing 0.5 mg of 9NC and 25 mg of DLPC per milliliter were added to an AERO-MIST nebulizer (CIS-USA, Bedford, MA). It was operated at a flow rate of 10 liters air/min and produced aqueous aerosol droplets with a mass median aerodynamic diameter particle size of 1.2 μm and a geometric SD of 2.1. If the mice were treated for longer periods per day, they received half of the treatment in the morning and the other half in the afternoon.

Experiments with B16 melanoma were stopped after the first mouse in the control group died. Tumors were measured in B16 melanoma mice on lung surfaces with a dissecting microscope with a micrometer eyepiece.

In the osteosarcoma model, at week 9 after injection of SAOS-LM6 cells, when visible metastatic tumor nodules on the surface of the lungs (macrophagic metastases) were expected to be present (5), mice were divided into groups, and aerosol treatment was given for 30 min for 5 days per week for 8 or 10 weeks, as described above for the B16 model. Mice were sacrificed with the Isoflourane USP (Vedco, St. Joseph, MO) at the end of treatment. Tumors in the lungs of osteosarcoma mice were measured with microcalipers on the surfaces of the separated five lobes of the lungs. The calipers measured distances as small as 0.5 mm. Tumors were classified according to size at 1-mm intervals. Lungs without visible tumors were sectioned later, and microscopic metastases were counted.

Statistics. Comparisons of lung weights and various tumor indices were made using the two-tailed Student’s t test, two-by-two tables of probability (Epistat v 5.30; Epistat Services, Richardson, TX), or the two-tailed Mann-Whitney rank-sum test. For statistical analysis, tumor foci “too numerous to count” (i.e., >200) was assigned a value of 200, and tumor foci diameters less than 0.5 mm were assigned a value of 0.25 mm. For experiment 1 in Table 2, a mean tumor diameter of 4 mm (range, 0–8 mm) was used in the calculation of tumor areas for the untreated group.

RESULTS

Effect of L-9NC Aerosol on Experimental B16 Murine Melanoma Metastases. The lungs of mice treated for 5 weeks with aerosol L-9NC had markedly fewer B16 melanoma lung metastases than untreated control mice. The summarized data of the lung weights from three experiments performed with this animal model are presented in Fig. 1.

In experiment 1 (Table 1), 10 mice received no treatment,
and 12 mice received 153 µg 9NC/kg during a 1-h aerosol exposure for 5 days a week over a 21-day period. The total dosage of 9NC received during the treatment was 2.3 mg/kg. Statistical analysis gave the following P values (two-tailed Student’s t test) for treated animals versus controls: (a) lung weight, P = 0.0005; (b) number of tumor foci, P = 0.001; and (c) number of mice with foci larger than 1 mm, P = 0.001. The lungs shown in Fig. 2 were representative of the groups in the experiment 1. The number of tumor foci was greatest in the control mice (top row). There were many large pigmented and nonpigmented tumors visible on the surface of the lungs. Mice treated with L-9NC aerosol showed fewer and smaller tumors on lung surfaces.

In a second experiment, (Fig. 1), 12 mice were treated with L-9NC aerosol, and 10 mice served as untreated controls. An-
Pulmonary Metastases.

**Effect of L-9NC Aerosol on SAOS-LM6 Osteosarcoma Pulmonary Metastases.** L-9NC aerosol treatment was also effective against SAOS-LM6 osteosarcoma metastases in the lungs. As shown in Table 2, experiment 1, 10 of 11 control mice had large visible tumors in the lungs 16 weeks after tumor injection. Lung sections also revealed multiple microscopic foci in the 10 animals with tumors. The remaining animal had no detectable visible or microscopic disease. In contrast, none of the 11 animals treated for 8 weeks with L-9NC aerosol (treatment received on weeks 9–16; total deposited dose of 9NC throughout the respiratory tract. Pharmacokinetic studies done with the parent compound, camptothecin, showed a rapid increase in drug concentration in the lungs during aerosol treatment, with smaller but substantial amounts deposited in the liver, kidney, and spleen (4). The drug was shown to disappear from the lungs soon after treatment was stopped, with a somewhat slower disappearance from other sites. It may be that this methodology preserves more of the active lactone form of 9NC on the lung surfaces, where there is little albumin (10) to bind and inactivate the drug. The speed of the first-pass transport of 9NC to s.c. xenograft tumors after aerosol liposomal therapy has been reported previously (4) and may also be important in producing the antitumor effects seen in the present aerosol studies.

**DISCUSSION**

Here we report the use of liposome aerosol therapy consisting of the anticancer drug 9NC and the neutral lipid DLPC formulated into liposomes in the treatment of B16 murine melanoma and human osteosarcoma lung metastases in mice. L-9NC aerosol therapy was effective in inhibiting the growth of both tumors and, in some cases, resulted in near elimination of tumor cells from the lungs. Aerosol treatment of B16 melanoma pulmonary metastasis demonstrated a strong inhibition of tumor growth after 16–21 days of treatment. Treatment for 8–10 weeks was required to achieve a significant reduction of the SAOS-LM6 osteosarcoma tumor nodules. The dosages used in this study were 3–20 times lower than the dosages of 9NC previously found to be effective when given by the i.m., i.v., or intragastric routes in mice (7–9). A possible explanation for the apparent greater therapeutic efficacy of aerosol treatment may stem from the immediate deposition of high concentrations of 9NC throughout the respiratory tract. Pharmacokinetic studies done with the parent compound, camptothecin, showed a rapid increase in drug concentration in the lungs during aerosol treatment, with smaller but substantial amounts deposited in the liver, kidney, and spleen (4). The drug was shown to disappear from the lungs soon after treatment was stopped, with a somewhat slower disappearance from other sites. It may be that this methodology preserves more of the active lactone form of 9NC on the lung surfaces, where there is little albumin (10) to bind and inactivate the drug. The speed of the first-pass transport of 9NC to s.c. xenograft tumors after aerosol liposomal therapy has been reported previously (4) and may also be important in producing the antitumor effects seen in the present aerosol studies.

**Table 2 9-L-9NC aerosol treatment of human osteosarcoma lung metastases in a nude mouse model**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mice with visible lung metastases/total&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Microscopic lung metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median no. (range)</td>
<td>Mean no. ± SD</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated (n = 11)</td>
<td>10/11</td>
<td>16 (0–39)</td>
</tr>
<tr>
<td>9-NC (n = 11)</td>
<td>0/11</td>
<td>0 (0–4)</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated (n = 11)</td>
<td>9/11</td>
<td>15 (0–200)</td>
</tr>
<tr>
<td>DLPC only (n = 11)</td>
<td>7/11</td>
<td>2 (0–15)</td>
</tr>
<tr>
<td>9-NC (n = 11)</td>
<td>1/11</td>
<td>0 (0–1)</td>
</tr>
</tbody>
</table>

<sup>a</sup> SAOS-LM6 human osteosarcoma cells (1 × 10⁶ cells per 0.2-ml injection) were given i.v. and allowed to grow for 8 weeks. Beginning on week 9, mice were treated five times weekly for 8 weeks (experiment 1) or 10 weeks (experiment 2) with L-9NC aerosol (76.7 μg/kg/30-min treatment). At the end of 16 weeks (experiment 1) or 18 weeks (experiment 2), mice were sacrificed, lungs were removed, and tumor nodules were counted and measured. n = number of mice/group.

<sup>b</sup> Number of nude mice with lung metastases/number of mice injected with cells.

**Linear regression analysis of lung weights in experiment 1 demonstrated a significant correlation with the number of tumors (P = 0.76 and P < 0.0001, two-tailed Pearson correlation). Lung weights were more than four times greater in the untreated animals than in the L-9NC-treated group [Fig. 1, P < 0.001 (two-tailed Student’s t test)]. In experiment 3 (Fig. 1), lung weights of both untreated and mice given empty liposomes showed similar increases.**
It is of interest to note the differences in the handling of inhaled aerosols by mice and men (6). The minute ventilation per unit of body weight in mice is 1.0 ml/g, compared with 0.1 ml/g in man, a 10-fold difference. Mice deposit approximately 30% of inhaled particles in the respiratory tract, which includes the nose, trachea, and lungs (11), whereas humans deposit about 70% of the inhaled particles throughout the respiratory tract with nose breathing. Pulmonary deposition in mice accounts for only about 7% of the inhaled volume (12), whereas humans with mouth breathing will deposit about 20% of inhaled particles in the lung parenchyma (17–23 generations of the Weibel model of human lung branches).  

The net effect of these differences is that, overall, mice will deposit approximately 3-fold more particles in the lungs per unit of body weight than man. Because the maximum tolerated oral dose of 9NC in man is 1.0 – 1.5 mg/m² per day (26–39 µg/kg in an adult; Ref. 13), it should be easily possible to administer therapeutic doses of 9NC to humans by liposome aerosol. Whereas our studies indicated that 9NC is rapidly cleared from the pulmonary tissue after aerosol treatment in mice (4), DLPC remains in the lungs for many hours in humans (14, 15) and rats (16) studied with 99mTc-labeled DLPC.

In previous studies with s.c. xenografts (3), we found that the oral dosage of L-9NC in amounts greater than those given by aerosol had no detectable anticancer effect. This suggests that direct delivery of the drug to the tumor in the lungs by aerosol administration may be the main basis for the activity of this agent. Aerosol L-9NC therapy may also be effective against metastatic disease outside the lungs. Aerosol liposomal camptothecin treatment resulted in appreciable concentrations of the drug in tumors and in brains of treated animals (4). We anticipate a similar pharmacokinetic pattern for L-9NC.

It is current practice to administer aerosol to patients through a mouth-only breathing mask, which substantially avoids nasopharyngeal deposition of drug. This provides a deposition of the particles from approximately 20% of the inhaled volume in the lung parenchyma; moreover, less than 5% of inhaled particles will deposit elsewhere in the respiratory tract. Therefore, mouth breathing can target particles to the lungs with great efficiency and is an efficient way to administer liposomal aerosol chemotherapy for the treatment of pulmonary metastases. Mouth-only breathing masks avoid particle deposition in the nasopharynx; particles deposited in the nasopharynx are promptly swallowed, and therefore deposition in the nasopharynx constitutes oral medication rather than aerosol medication.

In summary, these studies have demonstrated that L-9NC aerosol therapy is effective in the treatment of melanoma and osteosarcoma pulmonary metastases in mice. Because drugs in the camptothecin family are presently being used in the treatment of primary lung cancer, aerosol L-9NC therapy may offer an additional therapeutic approach for this disease. A number of other anticancer drugs can also be delivered in the liposomal formulation by aerosol. Thus, the aerosol route could provide a broad base for this type of cancer chemotherapy. Previous studies showing the activity of aerosol L-9NC against various s.c. tumor xenografts in nude mice (3) indicate that this approach may be useful in the treatment of metastatic disease outside the lungs as well. Inhalation therapy has the advantage of being a noninvasive route for administration of medications. It can also be given at home under supervision, thus reducing clinic visits and the cost of therapy and yielding greater patient freedom. A Phase I clinical trial using aerosol L-9NC in patients with advanced pulmonary malignancies is now underway (17).

REFERENCES

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