Antitumor Efficacy of N1,N11-Diethlnorspermine on a Human Bladder Tumor Xenograft in Nude Athymic Mice

Abstract
The spermine analogue N1,N11-diethlnorspermine (DENSPM) has been shown to induce the polyamine-acetylating enzyme spermidine/spermine N1-acetyltransferase, disrupt polyamine pool homeostasis, and inhibit tumor growth. DENSPM is currently being developed as an antineoplastic agent and is about to enter Phase II clinical trials. In this report, the antitumor efficacy of DENSPM was evaluated against a human transitional cell bladder BL13 carcinoma xenograft implanted orthotopically and s.c. in nude athymic mice. DENSPM was administered via continuous s.c. infusion at 93 mg/kg/day for 5 days. Treatment with DENSPM was well tolerated and produced tumor regressions in all mice with a significant proportion (up to 50%) of apparent cures. On the basis of low toxicity and good therapeutic efficacy, there is a strong rationale for evaluation of the therapeutic efficacy of DENSPM against bladder carcinomas in Phase II clinical trials.

Introduction
Bladder cancer is the fourth and ninth most common cancer in men and women, respectively, in the United States. In 1996, an estimated 52,900 new cases were reported, with a male:female ratio of about 3:1, and an estimated 11,700 patients died of bladder cancer (1). In the majority of cases (50–65%), although the lesions are superficial at time of diagnosis, the recurrence rate is 55–70% despite aggressive treatment, including transurethral resection of the tumor, radiation, and/or chemotherapy (2). Conventional chemotherapeutic regimens in all mice with a significant proportion (up to 50%) of apparent cures. On the basis of low toxicity and good therapeutic efficacy, there is a strong rationale for evaluation of the therapeutic efficacy of DENSPM against bladder carcinomas in Phase II clinical trials.

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Corporation (Palo Alto, CA). Five- to 6-week-old female athymic nude mice were purchased from Taconic (Germantown, NY) and were maintained on a 5% fat, sterilized chow (C485-HSD) in a barrier facility for 1–2 weeks before use.

Human Bladder Tumor Lines. Human bladder transitional cell carcinoma lines BL13 and BL17 were originally developed from pretreatment biopsies of an invasive stage T3, grade II bladder tumor (13) and a stage T4b, grade III bladder tumor (14), respectively. The BL17 cell line expresses morphological features of squamous and glandular differentiation within the same cell, and it may represent a stem cell of transitional cell carcinoma of the bladder. Human bladder line BL28 was established from a patient with stage T4, grade II transitional cell carcinoma with focal glandular differentiation who had received cisplatin and radiotherapy treatments some years before (15). These tumor cell lines were maintained as monolayer cultures in RPMI 1640 supplemented with L-glutamine, 0.21% sodium bicarbonate, and 10% FCS. Cell lines were free from Mycoplasma contamination, as indicated by the lack of hybridization with a [3H]DNA probe homologous to Mycoplasma and Acholeplasma rRNA (Gen-Probe, San Diego, CA).

Cell Growth Inhibition Experiments. The antiproliferative potency of DENSPM was determined by the sulforhodamine B assay, which is based on the colorimetric determination of total cell protein remaining after drug treatment (16, 17). Briefly, human bladder tumor cells were plated at a density of 400 cells/well in 96-well plates and were allowed to attach overnight. DENSPM was dissolved in PBS and further diluted with 0.1% acetic acid and air dried. The dye was then solubilized with water to remove trichloroacetic acid and serum proteins, followed by a 5-mm incubation, plates were rinsed five times with ice-cold 50% trichloroacetic acid were added to each well and incubated under the skin of the right flank (the side opposite to the s.c. tumor). A PBS- or drug-filled pump, delivering at a rate of 1 µl/h, was inserted into the pocket. The wound was closed with sterilized clips, and the pumps were removed after 5 days of continuous drug delivery. Treatment and control groups consisted of 10 mice each, and experiments were repeated twice.

Antitumor Activity. Human bladder tumor cell line BL13, established originally from a biopsy of an invasive stage T3, grade II transitional cell carcinoma before chemotherapy (13), was selected for xenograft implantation and the evaluation of the antitumor activity of DENSPM. BL13 xenograft was selected for this study because it retains the phenotype of the original patient tumor (13). Furthermore, BL13 is a doxorubicin- and cisplatin-resistant human bladder cancer xenograft model (18, 19).

Xenografts were initiated by injections of 5 × 10⁶ BL13 cells in 0.2 ml saline on the left flank. For o.i. implantation, an incision was made in the skin of anesthetized mice, the bladder was externalized, and 1 × 10⁶ BL13 cells suspended in 10 µl of growth medium were injected through a 30-gauge needle into the bladder wall. Chemotherapy was started 7 and 11 days after o.i. and s.c. tumor implantation, respectively. Therapy consisted of s.c. infusion of DENSPM using miniosmotic Alzet pumps at 93 µg/kg/day for 5 days to administer a cumulative dose of 465 mg/kg. PBS-filled pumps were used in the control treatments. For implantation of Alzet pumps, mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), and a pocket was made under the skin of the right flank (the side opposite to the s.c. tumor). A PBS- or drug-filled pump, delivering at a rate of 1 µl/h, was inserted into the pocket. The wound was closed with sterilized clips, and the pumps were removed after 5 days of continuous drug delivery. Treatment and control groups consisted of 10 mice each, and experiments were repeated twice.

For experiments in which bladder tumors were implanted o.t., the percentage increase in lifespan of DENSPM-treated mice compared to controls was determined. Survival was defined as the time at which mice became moribund due to the growth of tumor in their bladder. For humane reasons, moribund animals were sacrificed. The survival data were analyzed for statistical significance by the Cox-Mantel test using the program Solid Tumor (20).

For s.c. tumors, orthogonal diameters were measured twice weekly, and tumor volumes were calculated as 0.4π(a² × b), where a is the smaller of two perpendicular tumor diameters. Mice were euthanized when tumor volumes reached 1000 mm³. Body weights were recorded at the time of tumor volume measurements as an indicator of drug toxicity. Efficacy of the drug treatment was assessed by the delay in tumor growth compared to the control groups. Partial tumor regression is defined as a decrease in tumor to ≤50% of initial volume. Complete tumor regression is defined as a tumor that is not palpable for a period of time, and cure is defined as an established tumor that is not palpable for a period of ≥10 tumor.
Fig. 2 Activity of DENSPM against BL13 tumor xenograft in nude athymic mice. s.c. BL13 tumors were implanted as described in “Materials and Methods.” On day 11, drug treatment was initiated via a s.c.-implanted Alzet pump delivering 93 mg/kg/day continuously for 5 consecutive days (Rx and bar along the abscissa). Controls received continuous s.c. infusion of PBS via an Alzet pump. Individual tumor sizes for the treatment and control groups are shown. Treatment and control groups consisted of 10 mice each. For humane reasons, animals were sacrificed when tumor volumes exceeded 1000 mm³.

Table 1 Activity of DENSPM on human bladder tumor implanted s.c. in nude athymic mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dosageab (mg/kg/day)</th>
<th>Total dose (mg/kg)</th>
<th>Timeb (days)</th>
<th>Tumor regression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cure</td>
</tr>
<tr>
<td>Experiment 1</td>
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<td>0</td>
</tr>
<tr>
<td>PBS</td>
<td>0</td>
<td>0</td>
<td>17</td>
<td>3</td>
</tr>
<tr>
<td>DENSPM</td>
<td>93</td>
<td>465</td>
<td>36</td>
<td>5</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>PBS</td>
<td>0</td>
<td>0</td>
<td>17</td>
<td>5</td>
</tr>
<tr>
<td>DENSPM</td>
<td>93</td>
<td>465</td>
<td>31</td>
<td></td>
</tr>
</tbody>
</table>

a Administered via continuous s.c. infusion for five days starting on day 11 after s.c. tumor implantation.

b Time required for median tumor to reach a 500 mm³ volume.

c Cure is defined as an established tumor not being palpable for a period of time ≥10 tumor volume-doubling days; CR, complete tumor regression (a tumor that for a period of time is not palpable); PR, partial tumor regression (a decrease in tumor size to ≤50% of initial); NR, no response.

volume-doubling times. Doubling time is the time (in days) that it takes for control tumor to double in volume. Tumor volume data were analyzed for statistical significance by the Cox-Mantel test using the program Solid Tumor (20). Therapeutic studies were performed in accordance with Institutional Animal Care and Use Committee guidelines.

Tissue Processing. Five representative control and DENSPM-treated mice with both s.c. and o.t. tumors were sacrificed immediately after the end of drug treatment. Nonnecrotic areas of tumor (~100 mg) were excised, weighed, and sonicated to homogeneity in 10 mM Tris-HCl buffer containing 0.5 mM EDTA, 5 mM DTT, and 50 mM pyridoxal phosphate. The homogenate was centrifuged at 10,000 × g for 20 min, and the supernatant extract was frozen and later used for polyamine pool-size analyses and enzyme assays. Duplicate measurements were performed on each tissue sample.

Enzyme Assays. SSAT activity was determined according to the method described by Libby et al. (21). The frozen tumor extract was thawed and centrifuged for 1 h at 35,000 rpm using a Spinco 40 rotor. This assay also measures enzyme activities other than SSAT that are capable of acetylating spermidine, which may account for a significant portion of the basal enzyme activity.

Polyamine Pools. Tissue extracts (obtained as described in “Tissue Processing”) were treated with an equal volume of 1.2 M perchloric acid and were centrifuged, and the supernatant
Antitumor Efficacy of DENSPM was dose dependent and resulted in similar drug-sensitivity profiles for the three human bladder tumor cell lines (Fig. 1). In contrast, BL13 was the most sensitive to DENSPM compared with the other tumor lines tested.

**Antitumor Activity**

Previously, we determined that continuous infusion of DENSPM was the most effective dosing regimen against human MALME-3M melanoma xenografts implanted s.c. in nude athymic mice (11). Therefore, we selected continuous-infusion delivery of DENSPM for evaluating its antitumor efficacy against s.c. and/or o.t.-implanted BL13 xenografts.

**s.c. BL13 Tumor.** The therapeutic effect on s.c. BL13 tumor growth in nude athymic mice of DENSPM was evaluated at a cumulative dose of 465 mg/kg that was administered as a continuous 5-day infusion. Drug was administered by continuous s.c. infusion using an Alzet pump. Mice in the control group received an infusion of PBS. The treatment and control groups consisted of 10 mice each, and experiments were repeated twice.

In both experiments, treatment of BL13 s.c. tumors with DENSPM showed a significant delay in the tumor growth compared to PBS controls (P < 0.001). Treatment with DENSPM produced tumor regression in all mice, followed by a sustained tumor growth inhibition (Fig. 2). Furthermore, 10–30% of mice were cured in the drug-treated groups (Table 1). The times required for the median tumor volume to reach 500 mm$^3$ were 31 and 36 days in the DENSPM-treated groups in the two experiments (Table 1) and 17 days for the control group.

**o.t. BL13 Tumor.** The antitumor activity of DENSPM was also evaluated against BL13 tumor implanted in the bladder wall of nude athymic mice. DENSPM was administered continuously for 5 days at a cumulative dose of 465 mg/kg using a s.c.-implanted Alzet pump. The treatment and control groups consisted of 10 mice each, and experiments were repeated twice.

In both experiments, treatment with DENSPM resulted in a significant increase (78 and 100%) in life span compared to that of controls (Fig. 3; Table 2). In two experiments, the median survival times were 30 and 38 days in the DENSPM groups, and 15 and 18 days for the control groups (Table 2). Treatment with DENSPM resulted in long-term cures (>60-day survival) in 20–50% of mice (Table 2).

**s.c. and o.t. BL13 Tumors.** To compare the sensitivity of s.c.- and o.t.-implanted bladder tumors to DENSPM, BL13 tumors were implanted s.c. on day 0 and o.t. on day 4 in the same nude athymic mouse. Treatment was started on day 11 via a s.c.-implanted Alzet pump for 5 days at a cumulative DENSPM dose of 465 mg/kg. At the end of the treatment on day 16, animals were euthanized, and tumor sizes were measured (Fig. 4).

Treatment with DENSPM produced a significant (P < 0.001) growth inhibition of both s.c. and o.t. tumors. Furthermore, the therapeutic effect of DENSPM was similar (P > 0.05) on both s.c. and o.t. tumor xenografts in nude athymic mice (Fig. 4). The similar antitumor activity of DENSPM against the s.c. and o.t. tumors may be partly due to the continuous infusion schedule used in this study because the pharmacokinetic advantage in the distribution of DENSPM in o.t. tumors would not be apparent when the system is at steady state. However, due to the rapid excretion of DENSPM, the exposure of o.t. tumors to drug may be significantly higher than that of s.c. tumors if a clinical...
### Table 2 Activity of DENSPM on human bladder tumor implanted o.t. in nude athymic mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dosage† (mg/kg/day)</th>
<th>Total dose (mg/kg)</th>
<th>Median survival time (day)</th>
<th>% increase in lifespan</th>
<th>Long-term survivors b</th>
<th>Mice/group</th>
</tr>
</thead>
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<td>PBS</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>0</td>
<td>1</td>
<td>10</td>
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<td>93</td>
<td>465</td>
<td>38</td>
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<td>465</td>
<td>30</td>
<td>100</td>
<td>2</td>
<td>10</td>
</tr>
</tbody>
</table>

† Administered via continuous s.c. infusion for 5 days starting on day 7 after o.t. tumor implantation.

b Animal alive on day 60 after o.t. tumor implantation.

### Table 3 Polyamine metabolism in human bladder BL-13 tumor xenograft after treatment with DENSPM

<table>
<thead>
<tr>
<th>Tumor implantation</th>
<th>DENSPM treatment</th>
<th>SSAT (pmol/implantation)</th>
<th>Putrescine</th>
<th>Spermidine</th>
<th>Spermine</th>
<th>DENSPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>s.c.</td>
<td>PBS</td>
<td>50 ± 11†</td>
<td>225 ± 75</td>
<td>1145 ± 165</td>
<td>882 ± 66</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>465 mg/kg</td>
<td>12,070 ± 4,850</td>
<td>390 ± 185</td>
<td>130 ± 90</td>
<td>132 ± 75</td>
<td>3425 ± 820</td>
</tr>
<tr>
<td>Bladder wall</td>
<td>PBS</td>
<td>25 ± 5</td>
<td>185 ± 70</td>
<td>1165 ± 255</td>
<td>760 ± 160</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>465 mg/kg</td>
<td>16,310 ± 2595</td>
<td>200 ± 25</td>
<td>120 ± 30</td>
<td>110 ± 30</td>
<td>2480 ± 580</td>
</tr>
</tbody>
</table>

† Tumors were implanted s.c. (on the left flank) and o.t. (in the bladder wall) on days 0 and 4, respectively.

b Administered via continuous s.c. infusion for 5 days starting on day 11 after s.c. tumor implantation.

Values are mean ± SD for tumors excised from five mice at the end of drug infusion.

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**Fig. 4** Activity of DENSPM against BL13 tumor xenograft in nude athymic mice. In the same mouse, BL13 tumors were implanted s.c. on day 0 and o.t. on day 4 as described in “Materials and Methods.” On day 11, drug treatment was initiated via a s.c.-implanted Alzet pump delivering 93 mg/kg/day continuously for 5 consecutive days. At the end of treatment on day 16, animals were sacrificed, bladder tumors were excised and calibrated, and mean tumor size was plotted with SD. Treatment and control groups consisted of 10 mice each.

**Site of Tumor Implantation**

- **Control - PBS**
- **DENSPM - 465 mg/kg**

**Biochemical Correlates**

The effect of DENSPM on polyamine pools and related enzymes of s.c. and o.t. bladder tumors was examined (Table 3). The activity of polyamine-acetylating enzyme SSAT in DENSPM-treated animals was enormously induced and was 250–650-fold higher compared to PBS controls (Table 3). Significant depletion of tumor spermidine and spermine levels were observed following treatment with DENSPM (Table 3). In contrast, there was some increase in putrescine pools, which may be due to back-conversion of spermine and spermidine by massively induced SSAT. There was little variation in the amount of DENSPM accumulated in the s.c. and o.t. tumors, perhaps due to the continuous infusion schedule used, as described earlier.

**Discussion**

Previously, we showed that the continuous infusion delivery of DENSPM at a cumulative dose of 600 mg/kg was well tolerated and the most efficacious regimen for the treatment of human MALME-3M melanoma xenograft in nude athymic mice (11). The present work revealed high antitumor efficacy of DENSPM against human bladder tumor xenografts. Treatment with DENSPM at 465 mg/kg cumulative dose resulted in complete bladder tumor regression, sustained tumor growth inhibition, and long-term survival in a large
number of animals. The biochemical results were consistent with previous findings of SSAT induction and polyamine pool decrease with various other human tumor xenografts (9–11). DENSPM has demonstrated a good antiproliferative activity against human bladder carcinoma cell lines (8). However, this is the first report describing antitumor efficacy for DENSPM against a clinically relevant o.t. human bladder tumor model. Furthermore, DENSPM exhibited significant antitumor activity against this relatively fast-growth human bladder tumor, which was previously shown to be refractory to drugs such as doxorubicin and cisplatin (18, 19).

Thus, there is a strong rationale for the evaluation of DENSPM against bladder cancer in upcoming Phase II clinical trials. Moreover, it may also be feasible to exploit the pharmacokinetic behavior of DENSPM for the treatment of bladder cancer; DENSPM is rapidly excreted in the urine after systemic administration (7) and may be taken up selectively to high levels by the bladder tumor. Although our studies found similar excellent activities for DENSPM against both o.t. and s.c. tumors, it must be remembered that these findings occurred under steady-state conditions (i.e., long-term constant infusions). The current clinical drug administration schedule (daily short-term i.v. infusion) may favor higher differential exposures to bladder tumors. In any case, due to its novel mechanism of action and potent preclinical activity against human lung, melanoma, and bladder tumor xenografts, DENSPM is being considered for further clinical development as an antitumor agent.

Acknowledgments

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References


Antitumor efficacy of N1,N11-diethylnorspermine on a human bladder tumor xenograft in nude athymic mice.

A Sharma, D Glaves, C W Porter, et al.


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