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ORIGINAL ARTICLE

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Effects of polyamine analogues on prostatic adenocarcinoma cells in vitro and in vivo

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Abstract *Purpose*: The overall purpose of this study was to determine the potential usefulness of 1,19-di-(ethylamino)-5,10,15-triazononadecane (BE-4-4-4-4) in the treatment of prostate cancer using in vitro and in vivo models. More specifically the objectives were: (1) to determine the in vitro and in vivo sensitivity of human and rat prostate cancer cells to two polyamine analogues N^1,N^{11}-di(ethyl)norspermine (DENSPM) and BE-4-4-4-4; (2) to determine whether the mechanism of cell kill occurred through an apoptotic pathway; and (3) to determine the toxicity associated with therapeutic doses of BE-4-4-4-4 using an animal model. *Methods*: In order to determine the ability of these drugs to cause in vitro cytotoxicity, colony-forming assays were performed utilizing the well-characterized Dunning rat prostate cancer cell lines AT3.1, AT6.1 and AT6.3, and the androgen-insensitive human prostate cancer cell lines DU145, DuPro-1 and TSU-Pr1. Apoptotic cell death was determined using DNA laddering and DAPI staining of nuclei. The antitumor activity of BE-4-4-4-4 was evaluated by treatment of DuPro-1 and PC-3 xenograft tumors in nude mice. *Results*: BE-4-4-4-4 was shown to be approximately 4 to 86 times more cytotoxic in clonogenic assays than DENSPM in both rat and human prostate carcinoma cell lines. Cells treated with cytotoxic doses of DENSPM or BE-4-4-4-4 showed no signs of apoptosis using either DNA laddering or DAPI staining of nuclei. There was a significant inhibition of DuPro-1 tumors for animals treated with BE-4-4-4-4 compared with control animals. Equitoxic doses of BE-4-4-4-4 resulted in greater tumor inhibition than DENSPM, although the difference was not significant. After treatment with therapeutic doses of BE-4-4-4-4, histopathologic evaluation indicated minimal to mild necrosis and inflammation in the kidneys on days 15 and 22 following treatment. On day 35, there was no necrosis or regeneration present in the kidney, indicating that the toxicity was transient and that regeneration of epithelial cells was complete with apparent return to normalcy. *Conclusions*: These initial studies demonstrate that BE-4-4-4-4 is cytotoxic against rat and human prostate cancer cells in culture and effective against DuPro-1 xenografts in nude mice. Polyamine analogues, such as DENSPM or BE-4-4-4-4, should be considered for clinical use in the treatment of prostate adenocarcinomas.

Key words Prostate cancer · Polyamine analogues · Apoptosis

Abbreviations *AdoMetDC S*-adenosylmethionine decar- boxylase · *BE-4-4-4-4* 1,19-di-(ethylamino)-5,10,15-triazanonadecane · *CPENSpm* N^1-ethyl-N^{11}-((cyclopropyl) methyl)-4,8-diazaundecane · *DAPI* 4,6-diamidino-2-phenylindole · *DENSPM* N^1,N^{11}-di(ethyl)norspermine (also known as N^1,N^{11}-bisethylnorspermine, BEN- SPM) · *DFMO* difluoromethylornithine · *EDTA* ethylenediaminetetraacetic acid · *HBSS* Hanks' balanced salt solution · *ODC* ornithine decarboxylase · *PBS* phosphate-buffered saline · *SDS* sodium dodecyl sulfate · *SSAT* spermidine/spermine acetyltransferase

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Introduction

In 1941, Huggins and Hodges demonstrated that the proliferation of prostate adenocarcinoma could be inhibited by surgical castration or neutralization of androgens with estrogen injection [13]. Since this discovery, androgen ablation therapy has remained the primary treatment for disseminated prostate cancer. Although this treatment is initially effective, ultimately the androgen-independent cells emerge to proliferate and metastasize [33]. At this time, there are no chemotherapeutic agents available that can effectively control the growth and metastasis of human androgen-independent prostate cancer cells [30]. Agents that decrease the rate of proliferation either directly or by increasing the rate of programmed cell death allowing for improved treatment of androgen-independent disease are clearly needed. One group of agents that may find utility against prostate tumors are polyamine analogues or inhibitors. The prostate gland is unique in its high levels of polyamine biosynthetic activity and is rich source of polyamines [34, 36]. The primary function of these substances as related to the physiology of the prostate gland is as a regulatory mechanism for the production, by prostatic epithelial cells, of protein and other constituents of glandular secretions [37].

Polyamines are essential biological cations that are required for normal cellular growth [23]. As positively charged molecules at physiologic pH, polyamines interact ionically with cellular anions such as nucleic acids, membrane phospholipids, and phosphoproteins. As a result of these interactions, the function of the anion can be significantly altered [23]. Relatively constant intracellular levels of polyamines are tightly regulated by the dynamic interaction of biosynthesis, degradation and cellular uptake pathways [26]. Depletion of intracellular polyamines results in inhibition of cell growth [27].

Initial efforts to deplete cellular polyamines focused on the use of specific inhibitors of biosynthetic enzymes, namely ODC and AdoMetDC. Treatment of cells using DFMO, the best characterized of these compounds, usually produces a cytostatic, not cytotoxic, effect [19]. DFMO is relatively inactive in vivo in many tumor models and has shown limited efficacy in clinical trials [11, 16, 23]. The lack of effectiveness of DFMO therapy in vivo has been attributed, at least in part, to its upregulatory effect on polyamine transport, which augments the compensatory uptake of exogenous polyamines [12, 32]. More recent approaches to the use of DFMO offer greater hope for utility. Over the past several years, polyamine analogues have been designed which have been found to downregulate ODC and AdoMetDC, deplete polyamines and inhibit cell growth [24, 27, 28]. Several bis(ethyl)polyamines, N^1,N^{14}-diethylhomospermine, DENSPM, and BE-4-4-4-4 have been studied in vitro and in vivo as antiproliferative agents [3, 5, 7, 14, 27, 29]. While several of these agents have been shown to induce the catabolic enzyme spermine/spermidine N^1-acetyltransferase, an event that assists in causing the near total depletion of intracellular polyamines which is thought to be responsible for their growth inhibitory effects [25, 28], BE-4-4-4-4 is a poor inducer of this enzyme, yet still depletes polyamines almost completely and is a potent cytotoxic drug [3, 7].

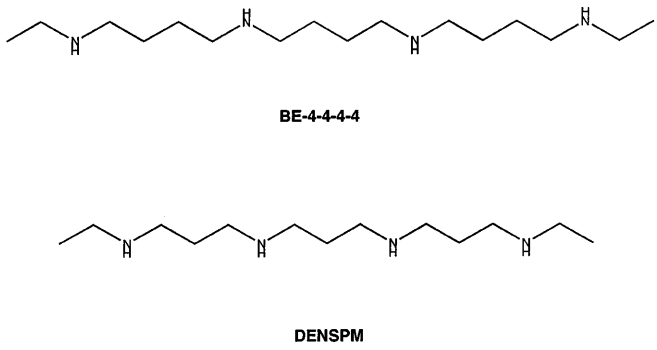

Fig. 1 Structures of polyamine analogues DENSPM and BE-4-4-4-4

On the basis of these findings and the high level of polyamines in the prostate, interference with polyamine biosynthesis and/or function is an attractive anticancer chemotherapeutic strategy for prostate cancer [23, 26]. Presently, there are very limited data on the usefulness of bis(ethyl)polyamines against prostate cancer. McCloskey et al. [21], have reported the sensitivity of prostate cancer cells to a symmetrical and unsymmetrical substituted polyamine analogue. They found that DENSPM inhibits the growth of DU145, PC-3 and LNCaP cells, but that it is only cytotoxic against LNCaP cells. In the current study, we evaluated the in vitro cytotoxic effects of DENSPM and BE-4-4-4-4 utilizing the well-characterized, highly tumorigenic and metastatic Dunning rat prostatic cell lines (AT3.1, AT6.1 and AT6.3) and the androgen-independent human prostate cancer cell lines DU145, DuPro-1 and TSU-Pr1. The structure of these analogues is shown in Fig. 1. We also investigated whether these two bis(ethyl)polyamine analogues produce their cytotoxic effect through the induction of apoptosis. Furthermore, both agents were evaluated for antitumor activity against human prostate tumor xenografts grown in nude mice. In efforts to ascertain the clinical potential of BE-4-4-4-4, histopathological studies were performed on mice following administration of therapeutic doses of this drug.

Materials and methods

Materials

DENSPM was generously supplied by the Drug Synthesis Branch of the National Cancer Institute. BE-4-4-4-4 was a generous gift from Drs. A. Shirahata and K. Samejima at Josai University (Keyakidai, Sakado, Saitama, Japan) [3]. Stock solutions of DENSPM and BE-4-4-4-4 were prepared in HBSS and filter-sterilized prior to use in cells or animals.

Cell culture

The Dunning rat prostate cell lines AT6.1, AT3.1 and AT6.3 were maintained as monolayer cultures in RPMI-1640 (Mediatech, Washington, D.C.) containing 100 units/ml penicillin G, 100 mg/ml streptomycin sulfate (Life Technologies, Grand Island, N.Y.), 8% fetal calf serum and 200 n*M* dexamethasone (Sigma, St. Louis, Mo.). The androgen-independent human prostate cancer cell lines DU145, DuPro-1 and TSU-Pr1 [10, 15] were maintained in RPMI-1640 containing 12% fetal calf serum. PC-3 cells used for animal studies were grown in Ham's F12 medium supplemented with 7% fetal bovine serum. All cells were grown in medium containing 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate (Life Technologies NY) in humidified air containing 5% CO_2 at 37 °C and passaged weekly.

Colony-forming assay

The cytotoxic effect of the polyamine analogues against each of the Dunning rat and human prostate cancer cell lines DU145, DuPro-1 and TSU-Pr1 was determined using a colony-forming assay. Briefly, 1×10^5 cells were plated on 25-cm^2 flasks 24 h prior to treatment. Following exposure of cells to increasing concentrations of drug for 96 h, cells were replated at a density of 100 to 10 000 cells per 60-mm^2 dish. Colonies (i.e. > 30 cells) were counted 7–14 days later after staining with 5% crystal violet/ethanol.

DNA fragmentation assays

Exponentially growing cells were plated at 4×10^4 cells/cm^2. After attachment, AT3.1 cells were treated with vehicle, 3.7 μ*M* DENSPM or 0.13 μ*M* BE-4-4-4-4, and AT6.1 cells were treated with vehicle, 25 μ*M* DENSPM or 0.55 μ*M* BE-4-4-4-4 for 72 h. At harvest, medium and cells were collected separately and pelleted by centrifugation (pellets were combined). For analysis of oligonucleosomal DNA fragmentation, genomic DNA was isolated, quantitated and equivalent amounts of DNA (20 μg) were loaded into wells of a 1.6% agarose gel that was electrophoresed in 1 × Tris borate EDTA (89 m*M* Tris base, 89 m*M* boric acid, 2 m*M* EDTA, pH 8.0) overnight at 20 V [1].

Cell morphology and nuclear visualization

AT3.1 and AT6.1 cells were treated with DENSPM or BE-4-4-4-4 at the ED_{50} or with vehicle for 72 h. At harvest, cells were collected and pelleted by centrifugation at 1000 *g*. Cells were resuspended in PBS containing 0.1% SDS and 0.1 mg/ml DAPI, dropped on microscope slides and examined by confocal and fluorescence microscopy. Images were obtained using a Zeiss Axiophot microscope coupled to a cooled charge device camera. As a control, cells were treated with thapsigargin, an apoptosis-inducing agent as previously described [8].

Animals

NIH Swiss nude mice and regular mice were obtained from the Frederick Cancer Research and Development Center (Frederick, Md.) Female nude mice were used for the PC-3 and DU145 study, male nude mice were used for the DuPro-1 study and NIH Swiss regular female mice were used for evaluation of drug toxicity. All mice were provided with food and water *ad libitum* and housed in the barrier facility at the University of Chicago, which is maintained under a controlled 12-h light/12-h dark cycle. Animal procedures followed guidelines set out by the Animal Care and Use Committee at the University of Chicago.[1]

[1]Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Resource Council, DHEW publication No. 8523, 1985

Tumor growth

Nude mice received subcutaneous injections of 7.5×10^6, 3.1×10^6 or 10×10^6 cells/animal of DuPro-1, PC-3 or DU145 cells, respectively. Body weight and tumor size were monitored weekly until the tumor reached a volume greater than 2000 mm^3, the criterion for euthanasia. Tumors were measured using calipers and the volumes were calculated from the following formula: length × $width^2 \times 0.52$ [35]. Treated groups were compared with control groups using a two-sided *t*-test using the Number Cruncher Statistical System developed by Dr. Jerry Hitze (Kaysville, Utah). Regressions were defined as a decrease in tumor volume over three consecutive measurements.

Animal treatment

BE-4-4-4-4 and DENSPM were administered intraperitoneally at a concentration of 0.5 mg/ml saline (0.9% NaCl, pH adjusted to 7.4 with 100 m*M* $NaHCO_3$). Treatment began 28, 53 or 38 days after injection with DuPro-1, PC-3 or DU145 cells when average tumor volumes had reached 59, 66 or 92 mm^3, respectively. Treatment animals received a cumulative dose of 80 mg/kg BE-4-4-4-4 per cycle on a schedule of 5 mg/kg twice per day for 4 days, followed by 3 days of no drug, then 5 mg/kg twice per day for 4 more days. A second cycle was administered in the PC-3 studies beginning from day 42. Control animals received the same number of injections of saline alone. Animals carrying the DU145 human tumor xenografts were treated with vehicle alone, BE-4-4-4-4 on the schedule described above, BE-4-4-4-4 at 4 mg/kg three times per day for 6 days or DENSPM at 40 mg/kg three times per day for 6 days. This dosing schedule was chosen since it was the optimal schedule for antitumor activity described previously [29].

Histopathological evaluation

Three animals that had received a total dose of 100 mg/kg BE-4-4-4-4 received complete necropsies to identify drug-related toxicity. From this study, it was determined that the kidney was the major site of toxicity and therefore, a more in-depth evaluation of the kidney was performed at various times following treatment. A group of 15 NIH Swiss mice were treated with 5 mg/kg BE-4-4-4-4 twice per day on the 4/3/4 schedule described above. A second group of five animals received the same number of injections with saline only. Four mice were sacrificed (three treated, one control) on days 4, 11, 15, 22 and 35. Following euthanasia of the animals, tissues were immediately removed and immersed in 10% neutral buffered formalin. After at least 24 h fixation, the tissues were trimmed and six to eight cross-sections of tissue were routinely processed through paraffin and stained with hematoxylin and eosin. Kidneys were bisected transversely, but not through the hilus. The mice were coded before histopathological evaluation to eliminate subjective interpretation of the microslide sections. Each slide was evaluated twice by a trained veterinary histopathologist. The microslide sections of kidneys were evaluated for necrosis, regeneration and inflammation on a scale of 0 (normal) to 5 (severe).

Results

In vitro studies

The in vitro cytotoxicity of DENSPM and BE-4-4-4-4 (Fig. 1) on human and rat prostate cancer cell lines was evaluated. Exponentially growing cells were treated with polyamine analogue for 96 h prior to replating for evaluation of clonogenic ability. Figure 2 illustrates the effect of increasing concentrations of DENSPM or BE-4-4-4-4 on the viability of AT3.1 rat prostate cancer

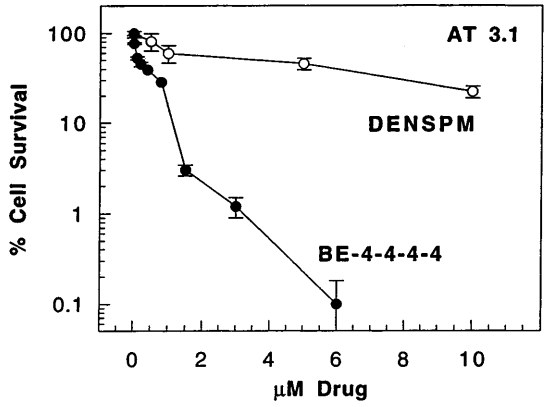

Fig. 2 Effect of DENSPM and BE-4-4-4-4 on the Dunning rat prostatic cell line AT3.1. The cells were treated with increasing concentrations of DENSPM or BE-4-4-4-4 for 96 h prior to replating at densities between 100 and 10 000 cells per 60-mm^2 dish. The data points are the mean percent cell survival. The *bars* represent the standard error

cells. These cells were dramatically more sensitive to the toxic effects of BE-4-4-4-4 than to the effects of DENSPM. For example, the ED_{50} of DENSPM was 6 μ*M*, but this same dose of BE-4-4-4-4 resulted in 0.1% cell survival. The ED_{50} for DENSPM or BE-4-4-4-4 against rat and human prostatic cell lines are shown in Table 1. In all three rat cell lines, BE-4-4-4-4 was 27- to 45-fold more cytotoxic than DENSPM.

The ability of androgen-independent human prostate cancer cell lines DU145, DuPro-1 and TSU-Pr1 to form colonies following polyamine analogue treatment was also evaluated. These studies showed that BE-4-4-4-4 was 4- to 85-fold more cytotoxic than DENSPM against the human prostate cells evaluated. Figure 3 illustrates the effect of these drugs on survival of TSU-Pr1, DuPro-1 and Du145 cells. Interestingly, even at high concentrations of DENSPM (up to 100 μ*M*) there was no increase in cell death over that observed with 5 μ*M* (data not shown). In contrast, there was a concentration-dependent cell kill with BE-4-4-4-4 with $<0.1\%$ survival at 6 μ*M* (Fig. 2).

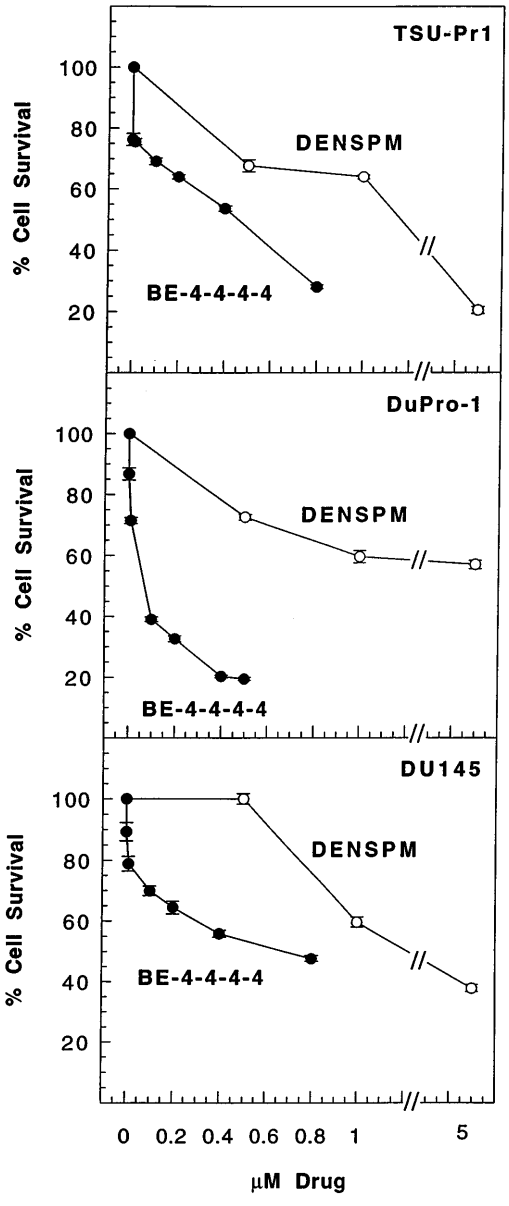

Fig. 3 Effect of DENSPM and BE 4-4-4-4 on human prostate cell lines. The cell lines TSU-Pr 1, DuPro-1 and DU145 were treated with increasing concentrations of DENSPM or BE 4-4-4-4 for 96 h prior to replating at densities between 100 and 10 000 cells per 60-mm^2 dish. The data points are the mean percent cell survival. Bars represent the standard error

Table 1 Cytotoxic activity of DENSPM and BE-4-4-4-4 on prostate tumor cell lines. Exponentially growing cells were exposed to increasing concentrations of polyamine analogue for 96 h. Cells were replated and colonies counted 7–14 days later

Cell line	Species	ED_{50} (μ*M*)		ED_{50} ratio (DENSPM/BE-4-4-4-4)
		DENSPM	BE-4-4-4-4	
AT3.1	Rat	3.7	0.13	28
AT6.1	Rat	25	0.55	45
AT6.3	Rat	8.7	0.32	27
DU145	Human	3.2	0.7	4
DuPro-1	Human	6	0.07	86
TSUPr1	Human	1.6	0.4	4

Apoptosis studies

To address the possibility that treatment with DENSPM and BE-4-4-4-4 induces apoptosis, AT3.1 and AT6.1 were treated with the ED_{50} for each drug or vehicle for 72 h, as discussed above. After treatment, cells were examined for evidence of apoptosis by two methods: nucleosomal laddering by DNA fragmentation analysis and nuclear condensation and fragmentation via fluorescence microscopic examination of DAPI-stained nuclei. As a control for apoptosis induction, these cell lines were also treated with 100 n*M* thapsigargin for 48 h. Cells treated with DENSPM or BE-4-4-4-4 showed no evidence of apoptotic nuclei or DNA degradation, while cells treated with thapsigargin, a known apoptosis-inducing agent, exhibited the hallmarks of having undergone apoptosis, namely nuclear condensation, cell shrinkage, and cytoplasmic blebbing (data not shown). These results indicate that the cytotoxic mechanism of DENSPM and BE-4-4-4-4 does not involve apoptosis.

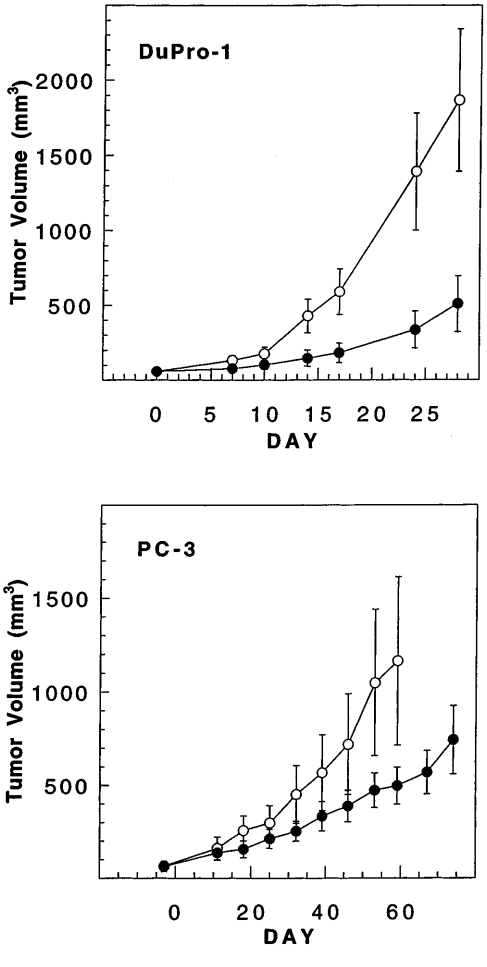

Fig. 4 Tumor growth of prostate tumor xenografts treated with BE-4-4-4-4. Nude mice carrying DuPro-1 or PC-3 xenograft tumors were given intraperitoneal injections of saline (*open circles*) or 5 mg/kg BE-4-4-4-4 twice daily (*closed circles*) on a 4/3/4 day schedule starting on day 0 (DuPro and PC-3) and day 42 (PC-3). The data points are the mean tumor volume for seven or eight mice per group. The *bars* represent the standard error

In vivo studies

There was a significant inhibition of DuPro-1 tumor growth observed for animals treated with BE-4-4-4-4 compared with control animals (Fig. 4). BE-4-4-4-4 resulted in one regression in eight animals treated compared with no regressions in eight animals in the control group (Table 2). The greatest weight loss (11%) was observed on day 11, but this was transient. There was no significant difference in weight between treated and control animals for the duration of the experiment. The tumor volume was significantly different ($P < 0.05$) on days 14, 17, 24 and 28. On day 28, the tumor volume was 1867 ± 476 mm^3 and 510 ± 189 mm^3 for control and treated animals, respectively. Although there was clearly inhibition of PC-3 tumor growth, the difference in tumor size between control and BE-4-4-4-4 treated animals was not significant.

The effect of BE-4-4-4-4 and DENSPM on the growth of human prostate tumor xenografts was evaluated. The dose and schedule for drug administration differed for the two drugs since BE-4-4-4-4 was more effective at lower doses. Drug scheduling was based on optimal conditions reported previously for DENSPM [4] and our own experience with BE-4-4-4-4 [7]. The in vivo studies with DU145 demonstrated a greater inhibition of tumor growth following treatment with BE-4-4-4-4 compared with DENSPM, although this was not significant (Table 2). Table 2 shows the number of tumor regressions, delay in tumor growth and animal weight loss resulting from each treatment.

Histopathological studies

Histopathology was performed on animals treated with BE-4-4-4-4. Complete necropsy was performed on three animals following treatment, and revealed necrosis of individual epithelial cells lining the lumens and submucosal glands in the larynx and trachea, and of the epithelial cells lining the convoluted tubules in the kidneys. Detailed histopathological studies of the kidneys showed necrosis of the epithelial cells of the proximal convoluted tubules that was reversed after cessation of drug treatment. Necrosis and regeneration of epithelial cells lining the convoluted tubules in the cortices of the kidneys were treatment-related (Table 3). They were found in three of three treated animals sacrificed on day 15, and in one of three treated animals sacrificed on day 22. Necrosis was always accompanied by regeneration, and in all of these animals, the degree of regeneration correlated well with the extent of necrosis. Regeneration consisted of the presence of cortical convoluted tubules of enlarged, often pleomorphic, epithelial cells with abundant basophilic cytoplasm and enlarged basophilic

Table 2 Effect of BE-4-4-4-4 or DENSPM on the mean tumor volume of human tumor xenografts. Animals were treated intraperitoneally with saline or 5 mg/kg BE-4-4-4-4 twice daily for 4 days on/3 days off/4 days on, starting on day 0. PC-3 animals were also treated on day 42 with a second cycle of the same schedule. DU145 animals: BE-4-4-4-4 (1) 5 mg/kg twice daily on the 4/3/4 schedule, BE-4-4-4-4 (2) 4 mg/kg three times daily for 6 days or DENSPM 40 mg/kg three times daily for 6 days

Tumor treatment	Regressions[a]	T−C[b]	% Mean weight loss at nadir (days 11–14)	Day	Tumor volume (mm^3) (mean ± SE)	*P*-value[c]
DuPro-1						
Saline	0/7		0	28	1867 ± 476	
BE-4-4-4-4	1/8	13	11	28	510 ± 189	0.029
PC-3						
Saline	0/7		0	59	1165 ± 449	
BE-4-4-4-4	1/8	13	9	59	498 ± 99	0.19
DU145						
Saline	4/9		0	52	305 ± 56	
BE-4-4-4-4 (1)	9/9	>9	11	52	223 ± 88	0.45
BE-4-4-4-4 (2)	6/9	2	9	52	392 ± 164	0.63
DENSPM	4/9	−8	1	52	383 ± 99	0.50

[a] Regressions were determined as three consecutive tumor measurements lower than the initial measurement
[b] T−C, growth delay in days defined as the difference between the median time for treated (T) and control (C) animals to reach five times the volume at time of treatment
[c] Two-sided *t*-test compared with control animals

nuclei with occasional mitotic figures. This change usually was present in most or all of the epithelial cells lining one or several adjacent cross or tangential sections of tubules, randomly scattered throughout the cortices of the kidneys. Inflammation consisted of the presence of small aggregates of lymphocytes and macrophages with a few plasma cells in the connective tissues surrounding large blood vessels at the corticomedullary junctions and in the connective tissues adjacent to the urothelium lining the calyces. The minimal inflammation observed in control or treated animals is probably incidental since this change is frequently seen in many strains of mice and is considered a background lesion. There was no necrosis or regeneration present in treated animals sacrificed on day 35, indicating that there was no persistent toxic effect of the test compound, and that the regeneration of replacement epithelial cells was completed with apparent return to normalcy.

Discussion

It has been reported that DENSPM has significant antitumor activity against several human melanoma, lung, ovarian and pancreatic tumor xenografts [4–6, 29]. We have previously demonstrated that BE-4-4-4-4 is effective against glioma, lung and colon tumor xenografts [7]. The purpose of this study was threefold: (1) to determine

Table 3 Histopathological condition of mouse kidney following administration of BE-4-4-4-4. Animals were treated intraperitoneally with saline or 5 mg/kg BE-4-4-4-4 twice daily for 4 days on/3 days off/4 days on, starting on day 0. On the days listed, animals were sacrificed, kidneys were removed, bisected transversely near the hilus and immediately placed in 10% formalin. Scoring is as follows: 0 = normal, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked, 5 = severe. Each number represents a separate animal

Day	Treatment	Number of animals	Necrosis[a]	Regeneration[b]	Inflammation[c]
4	Saline	1	0	0	1
	BE-4-4-4-4	3	0, 0, 0	0, 0, 0	0, 0, 0
11	Saline	1	0	0	1
	BE-4-4-4-4	3	0, 0, 0	0, 0, 0	0, 0, 0
15	Saline	1	0	0	0
	BE-4-4-4-4	3	1, 2, 1	1, 2, 1	0, 2, 0
22	Saline	1	0	0	0
	BE-4-4-4-4	3	0, 2, 0	0, 0.5, 0	0, 2, 0
35	Saline	1	0	0	0
	BE-4-4-4-4	3	0, 0, 0	0, 0, 0	0, 1, 2

[a] Necrosis of individual epithelial cells lining the proximal convoluted tubules of the kidneys
[b] Regeneration was the presence of enlarged, often pleomorphic, epithelial cells with abundant basophilic cytoplasm and enlarged basophilic nuclei with occasional mitotic figures. This was present in the same tubules as necrosis of individual cells
[c] Inflammation was the presence of small aggregates of lymphocytes and macrophages with a few plasma cells in the connective tissues surrounding large blood vessels at the corticomedullary junctions

whether either of these polyamine analogues is effective against prostatic cells or xenografts, (2) to determine whether these agents induce apoptotic cell death in prostatic cell lines, and (3) to determine the toxicity associated with BE-4-4-4-4 in animals. We showed that BE-4-4-4-4 and DENSPM are cytotoxic against Dunning rat prostatic cell lines, AT3.1, AT6.1 and AT6.3, and against the androgen-independent human prostatic carcinoma cell lines, DuPro-1, DU145 and TSU-Pr1. BE-4-4-4-4 was 4- to 8-fold more effective than DENSPM as measured by clonogenic assays. Furthermore, BE-4-4-4-4 exhibited significant antitumor activity against DuPro-1 human tumor xenografts. Although tumor growth was inhibited upon treatment of PC-3 and DU145 tumor xenografts with BE-4-4-4-4, the difference between control and treated animal tumors was not significant. DU145 cells grown in culture were at least two orders of magnitude more sensitive to BE-4-4-4-4 than to DENSPM, but this difference was not observed in vivo over the time frame we studied. A recent study of the in vivo effects of BE-4-4-4-4 in DU145 nude mouse xenografts conducted over a longer time has indeed shown a significant decrease in tumor size [17].

All of the rat and human prostatic cell lines evaluated were more sensitive to BE-4-4-4-4 than to DENSPM in vitro, suggesting that differences in analogue structure affect the compound's activity. This may be explained by differences in regulation of polyamine biosynthesis, differences in drug uptake, differential distribution of the drug in vivo or yet unknown factors. DENSPM has been shown to greatly induce the catabolic enzyme SSAT which may be, at least partially, responsible for polyamine depletion and inhibition of cell proliferation. BE-4-4-4-4, however, is not a potent inducer of SSAT [7]. Although there is evidence of a correlation between SSAT induction and antiproliferative activity [18], a direct relationship has not yet been demonstrated [9].

A second possible explanation for the differences in activity between the two analogues may involve their DNA binding properties, since direct interaction of polyamine analogues with DNA and consequent structural changes have been postulated as a mode of action [2]. DENSPM or BE-4-4-4-4 treatment may affect polyamine-controlled functions directly through analogue/DNA interactions as well as indirectly through decreasing the interaction between DNA and natural polyamines.

Recent reports have shown that the unsymmetrical alkylated polyamine analogue CPENSpm induces apoptosis, as measured by DNA fragmentation, following in vitro treatment of human breast carcinoma cells (MCF7 and MDA-MB-468) [20] and human lung tumor cells (NCI H157) [22]. The symmetrical substituted N^1,N^{12}-bis(ethyl)spermine (3-4-3) has been shown to induce programmed cell death in human lung tumor cells as well, but after much longer exposures (48 or 72 h), as determined by morphological changes characteristic of activation of programmed cell death. Interestingly, oligonucleosomal DNA fragmentation could not be demonstrated for the symmetrical analogue even at time-points up to 144 h. In this study DNA fragmentation in cells treated with DENSPM or BE-4-4-4-4, both symmetrical polyamines, was not observed. As a more sensitive measure of apoptotic cell death, the nuclei of cells treated with drug were examined by DAPI staining and analysis by fluorescence microscopy. These studies demonstrated that cells treated with DENSPM or BE-4-4-4-4 showed no signs of nuclear shrinkage, fragmentation, or cytoplasmic blebbing compared with cells treated with the known inducer of apoptosis, thapsigargin. These results indicate that the observed cytotoxicity of DENSPM and BE-4-4-4-4 may not be a result of apoptosis and that cell death may be occurring as a result of necrosis. However, since polyamine analogues themselves are capable of interacting with DNA, apoptotic end-points that relate to DNA modification may not be the best choice for assessing the involvement of apoptotic pathways. Additional mechanistic studies may lead to a better understanding of the mode of action of polyamine analogues as well as the design of new analogues with improved activity.

DENSPM is currently in phase I clinical trials. Animals require a lower dose of BE-4-4-4-4 than of DENSPM for antitumor activity since BE-4-4-4-4 is more potent. The toxicity associated with DENSPM has been alluded to by Bernacki et al. [5], although the species is not mentioned. Toxicities include reversible gastrointestinal mucositis and weight loss with no myelosuppression. Our results reveal that the toxicity associated with BE-4-4-4-4 was minimal to mild necrosis of epithelial cells lining the proximal tubules of the kidney. The effect was observed at 15 and 22 days posttreatment but was absent 35 days after treatment with return to normalcy. Regeneration and inflammation was associated with necrosis, indicating the reversible nature of the damage. In dogs, BE-4-4-4-4-related histopathological lesions occur in the kidney, lymph nodes/tissues, intestine and brain [31].

These studies indicate a role for DENSPM and BE-4-4-4-4 in the treatment of prostate cancer. BE-4-4-4-4 appears to be the better choice for prostate tumors, although this agent is not yet in clinical trials. Several bis(ethyl)polyamines might be pursued for clinical trials since there appear to be differences in the spectrum of tumors that respond to these unique therapeutic agents.

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