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Award Number: DAMD17-99-1-9231

TITLE: Therapeutic and Chemopreventive Actions of a Novel Polyamine Analog Against Breast Cancer

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REPORT DATE: September 2003

TYPE OF REPORT: Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20040226 035

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2003	Final Addendum (1 Sep 2002 - 31 Aug 2003)

4. TITLE AND SUBTITLE
Therapeutic and Chemopreventive Actions of a Novel Polyamine Analog Against Breast Cancer

5. FUNDING NUMBERS
DAMD17-99-1-9231

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The intracellular polyamines, spermidine, spermine, and putrescine, play an important role in the proliferation and death of normal and malignant cells. As a consequence, our work has focused on development of inhibitors of this metabolic pathway. A phase Ii trial of diethylnorspermine (DENSpm) for women with advanced breast cancer was completed. No major toxicity was observed but clinical activity was not sufficient to warrant further testing of DENSpm at the dose and schedule used. Analysis of DENSpm- and control-treated breast cancer tumors derived form discarded mastectomy tissue showed that DENSpm- can modulate expression of the SSAT enzyme on some specimens. This could potentially serve as a biomarker of effect the efficacy of several new polyamine ananogs as treatment for established breast cancer in a nude mouse model zenograft has been demonstrated. Future studies will focus on these agents.

14. SUBJECT TERMS
Polyamine, clinical trials, chemoprevention, breast cancer, bis(ethyI)polyamine

15. NUMBER OF PAGES
43

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

The intracellular polyamines, spermidine, spermine, and putrescine, play an important role in the proliferation and death of normal and malignant cells. As a consequence work focused on the preclinical and clinical development of one of these analogs for breast cancer. A final report for the grant period 1999-2002 was provided in 2002. The current addendum report to the final report covers only findings and accomplishments during a no-cost extension granted from 2002-2003.

BODY

Technical Objective 1: To test therapeutic efficacy of DENSpm against estrogen receptor-positive MCF-7 and estrogen-negative MDA-MB-468 human breast cancer cell lines growing in nude mice and assess tumors for possible biological or pharmacological end-points which predict subsequent tumor response.

During the addendum period, work focused on the SL11144 compound. In vitro studies suggested that it was a more potent inhibitor of human breast cancer cell proliferation than previously synthesized polyamine analogs including DENSpm. In vivo studies were undertaken using twice weekly injection of SL11144 in nude mice bearing MDA-MB-231 cell xenografts. These showed that the agent was well tolerated and inhibited tumor growth in a dose-dependent manner. These studies have now been published in Huang et al, Clinical Cancer Res 9:2769-2777, 2003.

Technical Objective 2: To use transient organ culture of normal and malignant human breast tissues to assess the effects of DENSpm on biological and pharmacological parameters relevant to antineoplastic activity as identified in Technical Objective 1.

Because of concents about the specificity of staining witth SSAT antibody, the immunohistochemistry technique was modified, and repeat staining and blinded interpretation of the DENSpm-and vehicle-treated explants were undertaken. These results are under analysis at the present time.

Technical Objective 3: To evaluate the efficacy of DENSpm in a phase II trial in women with metastatic breast cancer.

The results of the phase II trial are now in press in Wolff et al, Clinical Cancer Res, 2003.

Technical Objective 4: To evaluate the chemopreventive activity of DENSpm in the DMBA rat mammary tumor model.

These studies were not undertaken given results in Technical Objective 1 suggesting that SL11144 might be a more suitable compound for further study in the future.

ADDITIONAL KEY RESEARCH ACCOMPLISHMENTS

Anticipated publication of a phase II trial that assessed the efficacy of DENSpm in women with advanced breast cancer.

Analysis of the in vitro and in vivo effects of a novel polyamine analog, SL11144, in human breast cancer cells.

ADDITIONAL REPORTABLE OUTCOMES

Publications

Huang Y, Hager E, Phillips DL, Dunn VR, Hacker A, Frydman B, Kink JA, Valasinas AL, Reddy VK, Marton LJ, Casero Jr RA, and Davidson NE. A novel polyamine analog inhibits growth and induces apoptosis in human breast cancer cells. Clinical Cancer Res 9:2769-2777, 2003.

Wolff AC, Armstrong DK, Fetting JH, Carducci MK, Riley CD, Bender JF, Casero Jr RA, and Davidson NE. A phase II study of the polyamine analogue N1, N11-diethyinorspermine (DENSpm) daily for five days every 21 days in patients with previously treated breast cancer. Clinical Cancer Res, in press.

Grant applications awarded

Postdoctoral fellowship award to Yi Huang, M.D., Ph.D.
Antineoplastic efficacy of novel polyamine analogues in human breast cancer
DOD award DAMD17-03-1-0376
$171,000 June 1, 2003 – May 31, 2006

CONCLUSIONS

The in vitro and in vivo effects of a novel oligoamine polyamine analog, SL11144, against growth of human breast cancer cell lines have been reported. Final conclusions of a phase II trial of a second polyamine analog, DESNpm, are now accepted for publication.

APPENDICES

two reprints as listed above.

A Novel Polyamine Analog Inhibits Growth and Induces Apoptosis in Human Breast Cancer Cells[1]

Yi Huang, Erin R. Hager, Dawn L. Phillips, Valerie R. Dunn, Amy Hacker, Benjamin Frydman, John A. Kink, Aldonia L. Valasinas, Venodhar K. Reddy, Laurence J. Marton, Robert A. Casero, Jr., and Nancy E. Davidson[2]

The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, Maryland 21231 [Y. H., E. R. H., D. L. P., A. H., R. A. C., N. E. D.]; SLIL Biomedical Corp., Madison, Wisconsin 53711 [B. F., J. A. K., A. L. V., V. K. R., L. J. M.]; and Department of Surgery, Howard University, Washington, DC 20060 [V. R. D.]

ABSTRACT

Polyamine analogs have demonstrated considerable activity against many important solid tumor models including breast cancer. However, the precise mechanisms of antitumor activities of polyamine analogs are not entirely understood. The cytotoxicity of a newly developed polyamine analog compound, SL11144, against human breast cancer was assessed. Treatment of human breast cancer cell lines in culture with SL11144 decreased cell proliferation and induced programmed cell death in a time- and dose-dependent manner. SL11144 also profoundly inhibited the growth of MDA-MB-231 xenografts in host nude mice without overt toxic effects. Treatment of MDA-MB-435 cells with SL11144 led to the release of cytochrome *c* from mitochondria into cytosol, activation of caspase-3, and poly(ADP-ribose) polymerase cleavage. SL11144 decreased Bcl-2 and increased Bax protein levels in MDA-MB-231 cells. Furthermore, activator protein 1 transcriptional factor family member c-Jun was up-regulated by SL11144 in MDA-MB-435 and MDA-MB-231 cells, but not in MCF7 cells. In addition, significant inhibition of ornithine decarboxylase activity and a decrease in polyamine pools were demonstrated. These results demonstrate that the novel polyamine analog SL11144 has effective antineoplastic action against human breast cancer cells *in vitro* and *in vivo* and that multiple apoptotic mechanisms are associated with its cytotoxic effect in specific human breast cancer cell lines.

INTRODUCTION

The natural polyamines (Put,[3] Spd, and Spm) have been shown to be essential for cell growth. The critical role of polyamines in regulation of cell growth has led to the development of a number of inhibitors of key enzymes in the polyamine biosynthetic pathway as a therapeutic strategy (1–3). It has also been demonstrated that synthetic polyamine analogs can down-regulate polyamine biosynthesis by feedback mechanisms but are unable to act as substitutes for natural polyamines to promote cell growth. This approach has become an important means for the study of the physiological roles of natural polyamines and a potent application for creation of new antineoplastic agents (4–6). Indeed, several synthetic polyamine analogs have been reported to inhibit cell proliferation and induce PCD in a variety of tumor cell lines (7–11).

Apoptotic cell death is characterized by chromatin condensation, cytoplasmic blebbing, and internucleosomal DNA fragmentation and occurs in a variety of cellular systems in response to many different stimuli (12). We have demonstrated previously (9) that some polyamine analogs can induce PCD in hormone-responsive or -unresponsive human breast cancer cells. A highly regulated metabolic pathway finely controls intracellular polyamine concentrations. The rate-limiting enzymes ODC and S-Adenosylmethionine decarboxylase regulate biosynthesis, whereas catabolism is regulated by SSAT and human polyamine oxidase h1/spermine oxidase (13). Cell type-specific superinduction of SSAT and the subsequent depletion of natural polyamine pools have been reported in polyamine analog-induced growth inhibition and apoptosis in some tumor cell lines (9, 14). However, in other cell lines, polyamine analogs that do not highly induce SSAT can still inhibit tumor cell growth and produce apoptosis (15, 16). These divergent results suggest that polyamine analog-induced cell death may result from several agent-dependent mechanisms.

SL11144, a leading agent of a new generation of polyamine analogs designated as oligoamines, has shown significant activity against proliferating cells (17). In this study, we have evaluated the antineoplastic efficacy of SL11144 in human breast cancer cells *in vitro* and *in vivo*. The data presented in this study

Received 1/9/03; revised 3/5/03; accepted 3/12/03.
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[1] Supported by NIH Grants P50CA88843 (to N. E. D.) and CA51085 (to R. A. C.), Army DOD Grant DAMD 17-99-1-9242 (to N. E. D.), and The Breast Cancer Research Foundation (N. E. D.).
[2] To whom requests for reprints should be addressed, at CRB 409, Breast Cancer Research Program, The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, 1650 Orleans Street, Baltimore, MD 21231. Phone: (410) 955-8489; Fax: (410) 614-4073; E-mail: davidna@jhmi.edu.

[3] The abbreviations used are: Put, putrescine; Spd, spermidine; Spm, spermine; PCD, programmed cell death; ODC, ornithine decarboxylase; SSAT, spermidine/spermine N^1-acetyltransferase; BENSpm, N^1,N^{11}-bis(ethyl)norspermine; CPENSpm, N^1-ethyl-N^{11}-[(cyclopropyl)methyl]-4,8,-diazaundecane; CHENSpm, N^1-ethyl-N^{11}-[(cycloheptyl)methyl]-4,8,-diazaundecane; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; AP-1, activator protein 1; PVDF, polyvinylidene difluoride; FasL, Fas ligand.

suggest that SL11144 significantly inhibits growth and induces PCD in human breast cancer cells.

MATERIALS AND METHODS

Compound, Cell Lines, and Culture Condition. The polyamine analog SL11144 (Fig. 1) was provided by SLIL Biomedical Corp. (Madison, WI). Polyamine analog compounds BENSpm, CPENSpm, and CHENSpm were synthesized as described previously (18). Concentrated stock solutions (10 mM in double-distilled H_2O) of polyamine analogs were diluted with medium to the indicated concentrations. Human breast cancer MDA-MB-231 and MCF7 cells were maintained in DMEM supplemented with 5% fetal bovine serum, 2 mM glutamine, and 100 units/ml penicillin/streptomycin. MDA-MB-435 cells were maintained in improved modification of eagle's medium supplemented with 5% fetal bovine serum, 2 mM glutamine, and 100 units/ml penicillin/streptomycin. Cells were incubated at 37°C in a 5% CO_2 atmosphere.

MTT Survival Assays. MTT assays were performed using a method described previously (19). Briefly, 2000–5000 cells were plated in 96-well dishes and treated with the various concentrations of SL11144 for different lengths of time. At the end of each time point, 100 μl of a 1 mg/ml MTT solution (Sigma Chemical Co., St. Louis, MO), diluted in serum-free culture media, were added to each well. The plates were incubated at 37°C in 5% CO_2 atmosphere for 4 h, allowing viable cells to reduce the yellow tetrazolium salt into dark blue formazan crystals. At the end of the 4-h incubation, the MTT solution was removed, and 200 μl of 1:1 (v/v) solution of DMSO:ethanol were added to each well to dissolve the formazan crystals. The absorbance in individual wells was determined at $A_{540\ nm}$. All of the experiments were plated in quadruplicate, and the results of assays were presented as means ± SD.

Analysis of Intracellular Polyamine Pools, SSAT Activity, and ODC Activity. The intracellular polyamine content of treated and untreated cells was determined by precolumn dansylation and reversed phase high-performance liquid chromatography (20). SSAT and ODC activities were measured using cellular extracts as described previously (21, 22). Protein concentrations were determined according to the method of Bradford (23).

Hoechst Staining of Nuclear Chromatin. SL11144-treated cells were fixed with 4% formaldehyde in PBS at 37°C for 10 min and permeabilized with a 19:1 mixture of ethanol/acetic acid at –20°C for 15 min. Fixed cells were stained with 1 μg/ml Hoechst 33258 (Sigma Chemical Co.) in PBS at room temperature for 20 min. Hoechst staining of the cells was analyzed by fluorescence microscopy.

Determination of Internucleosomal DNA Cleavage. After tumor cells were treated with increasing concentrations of SL11144 for increasing times, cells were harvested, counted, and washed with PBS at 4°C. Cells were then suspended in lysis buffer (5 mM Tris-HCl, 20 mM EDTA, and 0.5% Triton X-100) and incubated for 20 min on ice. After incubation, samples were centrifuged at 14,000 × *g* for 20 min, and the supernatant was transferred to a reaction tube followed by phenol/chloroform/isoamyl (25:24:1) extraction. Two volumes of 100% ethanol were added to supernatant, followed by 5-min centrifugation at

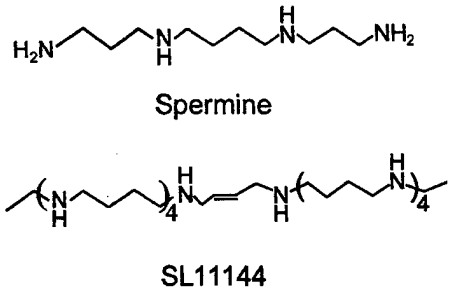

Fig. 1 Structures of Spm and polyamine analog SL11144.

14,000 × *g*. The pellet was resuspended in 0.1× SSC buffer and incubated with RNase for at least 30 min at 37°C. Then 50 μl of 5 M NaCl was added, followed by phenol/chloroform/isoamyl (25:24:1) extraction. After ethanol precipitation and centrifugation, the pellet was washed with 70% ethanol and dried. DNA samples were analyzed by electrophoresis in a 1.2% agarose slab gel containing 0.2 μg/ml ethidium bromide and visualized under UV illumination. This method isolates only DNA ladder fragments without genomic DNA.

Animal Studies. Female 4–6-week-old BALB c *nu/nu* athymic nude mice (Harlan Bioproducts for Science Inc., Indianapolis, IN) weighing between 16 and 18 g received injection in the right flank with 3.75 × 10^6 MDA-MB-231 cells. Cells were allowed to grow for 10 days to an average volume of 50–100 mm^3. Animals were then randomly assigned (eight mice for control group and seven mice for treatment groups) to receive vehicle control or SL11144 (2.5, 5, or 10 mg/kg) via i.p. injections twice weekly for 5 weeks. Tumor volumes were regularly assessed twice weekly by measuring 0.5 × length (mm) × width (mm) × width (mm). Mice were also weighed twice weekly.

Nuclear and Cytoplasmic Protein Extraction. The extractions of nuclear and cytoplasmic protein were performed using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce, Rockford, IL). MDA-MB-435 cells treated with 10 μM SL11144 for different times were harvested by trypsinization and washed with PBS. Two hundred μl of ice-cold Cytoplasmic Extraction Reagent I with protease inhibitors (0.5 mg/ml benzamidine, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 0.2 M phenylmethylsulfonyl fluoride) were added to the cell pellets. After a 10-min incubation on ice, 11 μl of ice-cold Cytoplasmic Extraction Reagent II without protease inhibitors were added, followed by a 5-min centrifugation at 14,000 × *g*. The supernatant containing the cytoplasmic extract was retained, and the insoluble pellet was resuspended in 100 μl of Nuclear Extraction Reagent and incubated on ice for 40 min. After a 10-min centrifugation, the supernatant that contained the nuclear extract was saved. Both cytoplasmic and nuclear extracts were analyzed by Western blot using anti-c-Jun and anti-c-Fos antibodies as described below.

Detection of Cytochrome *c* Release. To avoid artifacts due to mechanical breakage of the outer mitochondrial membrane, selective plasma membrane permeabilization with digitonin was used to examine the release of cytochrome *c* from mitochondria into cytosol (24). Briefly, cells treated with dif-

ferent concentrations of SL11144 for the desired exposure time were harvested by trypsinization, washed with PBS, and subsequently incubated in 100 μl of permeabilization buffer [210 mM D-mannitol, 70 mM sucrose, 10 mM HEPES, 5 mM succinate, 0.2 mM EGTA, and 100 μg/ml digitonin (pH 7.2)] for 5 min. After centrifugation for 10 min at 14,000 × *g*, the supernatant with protein content was saved, and protein concentrations were determined using the Pierce Micro Protein Assay Kit. Equal amounts of protein were fractionated using 12% SDS-PAGE and analyzed by Western blot as described below.

Western Blotting. Cells treated with different concentrations of SL11144 for the desired exposure times were harvested by trypsinization and washed with PBS. Cellular protein was isolated using the protein extraction buffer containing 150 mM NaCl, 10 mM Tris (pH 7.2), 5 mM EDTA, 0.1% Triton X-100, 5% glycerol, and 2% SDS. Protein concentrations were determined using the Pierce Micro Protein Assay Kit. Equal amounts of proteins (50 μg/lane) were fractionated using 12% SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with primary antibodies against caspase-3, PARP, Bcl-2, Bax, caspase-8, caspase-9, cytochrome *c*, c-jun, c-fos, or FasL (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA). After washing with PBS, the membranes were incubated with peroxidase-conjugated goat antimouse or antirabbit secondary antibody (1:3000; DAKO Corp., Carpinteria, CA) followed by enhanced chemiluminescence staining using the enhanced chemiluminescence system (Amersham Biosciences). Actin was used to normalize for protein loading.

RESULTS

Inhibition of Growth by SL11144. The sensitivity of three human breast cancer cell lines (MDA-MB-231, MDA-MB-435, and MCF7 cells) to the newly synthesized polyamine analog SL11144 (Fig. 1) was assessed by using a MTT cellular survival assay. These cells were chosen because they represent hormone-responsive (MCF7) and -unresponsive (MDA-MB-231 and MDA-MB-435) human breast cancer cell lines. All three cell lines exhibited time- and concentration-dependent growth inhibition by SL11144 (Fig. 2, *A*–*C*). The IC_{50} values for MDA-MB-231 and MCF7 cells are about 1–5 μM for a 72-h treatment and 0.5–0.75 μM for a 96-h treatment. MDA-MB-435 cells were more sensitive to SL11144 with IC_{50} values of 2.5 μM for 24-h drug exposure and 0.25–0.5 μM for 48-h drug exposure. In addition, the cytotoxicity of SL11144 against MDA-MB-231 cells was compared with the identified polyamine analogs BENSpm, CPENSpm, and CHENSpm. MTT studies demonstrated that SL11144 had a lower IC_{50} at 96-h treatment in MDA-MB-231 cells than BENSpm (1–2.5 μM), CPENSpm (1–2.5 μM), or CHENSpm [~2.5 μM (data not shown)]. These data indicate that SL11144 is a more potent inhibitor of human breast tumor cell proliferation than previously synthesized polyamine analogs.

Regulation of Intracellular Polyamine Pools and Metabolic Enzymes by SL11144. To address whether the observed growth-inhibitory effects of SL11144 in human breast cancer cells reflect its effects on the polyamine metabolic pathway, intracellular SL11144 accumulation, polyamine pools (Put, Spd, and Spm), and regulatory enzyme (SSAT and ODC) activities

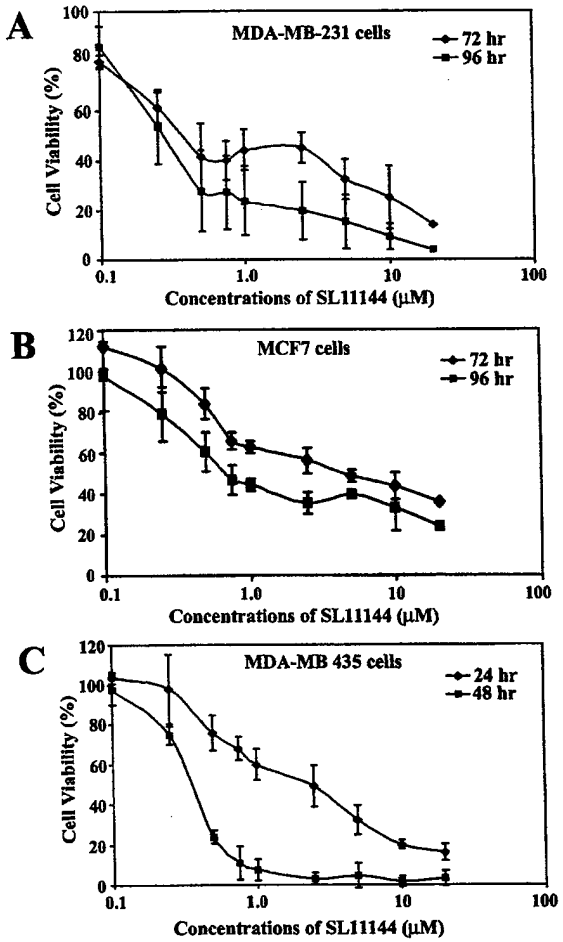

Fig. 2 SL11144 inhibits growth of human breast cancer cells in a time- and dose-dependent manner. MDA-MB-231 cells (*A*) and MCF7 cells (*B*) were treated with increasing concentrations of SL11144 for 72 or 96 h. MDA-MB-435 cells (*C*) were treated with increasing concentrations of SL11144 for 24 or 48 h. MTT assays were performed as described in "Materials and Methods." Shown are means ± SD of independent experiments performed in quadruplicate.

were assessed. As shown in Table 1, the accumulation rates of SL11144 in the three cell lines are similar after exposure of cells to 10 μM SL11144 for 24 h. SL11144 reduced all intracellular polyamines in MDA-MB-231 cells. Put and Spm were decreased, and Spd was slightly increased by SL11144 in MCF7 cells. In MDA-MB-435 cells, Spd level was down-regulated, and Spm was slightly up-regulated by SL11144. ODC activity was significantly inhibited, and SSAT activity was modestly increased in all three cell lines.

SL11144 Induces Apoptotic Cell Death. To determine whether observed SL11144-induced decrease in growth rate was a result of apoptosis, DNA fragmentation assays were performed. DNA ladders isolated from untreated and SL11144-treated cells were processed by agarose gel electrophoresis to detect the typical oligonucleosomal DNA fragmentation. The results (Fig. 3) indicate that SL11144 induces DNA fragmentation in all three human breast cancer cell lines, but the time and dose required for the induction of apoptosis varied by cell type.

Table 1 Effects of SL11144 on polyamine pools and SSAT and ODC activities in human breast cancer cells

Intracellular polyamine levels, SSAT activity, and ODC activity were determined as described in "Materials and Methods" after incubation of tumor cells for 24 h in the presence or absence of 10 μM SL11144. Values represent the means of duplicate determinations.

Cell lines	Treatment	SL11144 (nmol/mg protein)	Polyamines (nmol/mg protein)			SSAT activity (pmol/mg protein/min)	ODC activity (pmol CO_2/mg protein/h)
			Put	Spd	Spm		
MDA-MB-231	Control	ND[a]	3.65	52.04	21.17	2.22	2290.98
	SL11144	2.32	ND	15.27	8.14	6.02	ND
MCF7	Control	ND	6.22	49.38	28.52	1.39	2359.10
	SL11144	4.81	0.95	58.53	16.58	14.72	6.53
MDA-MB-435	Control	ND	ND	22.03	3.24	2.73	558.79
	SL11144	3.62	ND	8.84	4.35	23.39	4.58

[a] ND, not detected.

DNA fragmentation was clearly detected only after a 96-h exposure to SL11144 in MDA-MB-231 and MCF7 cells but was detectable in 12 h in MDA-MB-435 cells. A minimum concentration of 5 μM is required for SL11144 to induce DNA fragmentation in MDA-MB-231 and MDA-MB-435 cells, but MCF7 cells are more sensitive in that DNA fragmentation was observed with 0.1–0.25 μM SL11144 for 96 h.

The effect of SL11144 on cell morphology was also investigated. Both control and SL11144-treated cells were stained with the fluorescent dye Hoechst 33258 and visualized by fluorescence microscopy. Typical morphological changes of apoptosis including chromatin condensation and nuclear fragmentation were observed in all three treated cell lines (Fig. 4*B*), but not in the untreated control cells (Fig. 4*A*). Taken together, the DNA fragmentation and fluorescence results suggest that SL11144 induces apoptotic cell death in all three human breast cancer cell lines.

Therapeutic Effect of SL11144 against Human Breast Cancer MDA-MB-231 Xenografts. The *in vivo* therapeutic effect of SL11144 was evaluated using human breast cancer MDA-MB-231 xenografts in athymic nude mice. By 10 days after tumor cell inoculation, the average tumor sizes reached approximately 50–100 mm^3. Mice were then randomized into treatment ($n = 7$) and control ($n = 8$) groups. Different doses of SL11144 (2.5, 5, and 10 mg/kg) were administered via i.p. injections twice a week. SL11144 displayed antiproliferative effects against MDA-MB-231 xenografts in a dose-dependent manner (Fig. 5*A*). Whereas partial suppressions of tumor growth were observed at the doses of 2.5 and 5 mg/kg, increasing the dose to 10 mg/kg significantly inhibited the tumor growth. During the course of treatment, there was no obvious weight loss observed, except a slight decrease of weight (~5% of body weight) in the group treated with a dose of 10 mg/kg at 41 days (Fig. 5*B*). These results indicate that the treatment of SL11144 possesses significant *in vivo* growth suppression efficacy against MDA-MB-231 cells with no overt toxic effects.

Effects of SL11144 on Apoptosis-related Proteins. Several apoptosis-associated genes or proteins have been shown to play critical roles in regulating apoptosis. These include caspases, bcl-2 family members, FasL, cytochrome *c*, and PARP (25–29). To determine whether these proteins are involved in the mediation of SL11144-induced cell death in human breast cancer cells, we examined their expression by Western blotting. In MDA-MB-231 cells, as shown in Fig. 6*A*, treatment with 10 μM SL11144 decreased the amount of caspase-3 protein by 96 h of treatment, but no cleaved, active caspase-3 or its downstream target, PARP, was detected. We next examined whether two upstream proteases, caspase-9 and caspase-8, were affected by SL11144 in MDA-MB-231 cells. SL11144 treatment increased the cleavage of caspase-9 after 72 h, whereas caspase-8 was essentially undetectable under the conditions examined. Cytochrome *c* release from mitochondria was enhanced at 48 h and returned to its baseline level thereafter. Furthermore, Bcl-2 protein was down-regulated, and Bax was up-regulated by SL11144 beginning at 24 h. Finally, expression of FasL was increased by SL11144 after 48 h. The changes observed precede DNA fragmentation, which was not observed until 96 h of treatment.

Effects of SL11144 on apoptotic protein expression were further assessed in MCF7 cells. Our results (Fig. 6*B*) confirmed the previous finding (30) that caspase-3 is not expressed in MCF7 cells. The activities of caspase-8, caspase-9, and PARP were not affected by SL11144. SL11144 did not change Bcl-2 protein levels, whereas Bax expression was minimal. No release of cytochrome *c* was observed in SL11144-treated MCF7 cells. However, FasL level was increased by SL11144 after 48 h of treatment. These data suggest that SL11144 may induce apoptosis in MCF7 cells through caspase- and cytochrome *c* release-independent pathways. Another possibility is that the DNA fragmentation and apoptotic morphological changes noted in MCF7 cells are induced directly by SL11144. Many polyamine analogs bind strongly to DNA and are capable of inducing structural changes in chromatin (6).

In contrast to the above-mentioned results in MCF7 and MDA-MB-231 cells, SL11144 treatment of MDA-MB-435 cells led to caspase-3 activation and cleavage of PARP within 12 h of drug exposure (Fig. 6*C*). Also, caspase-8 was activated, and the proform of caspase-9 was completely cleaved by 48 h of exposure. Although Bcl-2 expression did not change, and Bax expression was essentially undetectable, cytochrome *c* was released into cytoplasm from mitochondria by 12 h. FasL expression was induced at 48 h. These results suggest that both caspase and mitochondrial pathways are activated by SL11144 in MDA-MB-435 cells. The time course of caspase activation and cytochrome *c* release parallels the course of DNA fragmentation, which was detected at 12 h of SL11144 treatment.

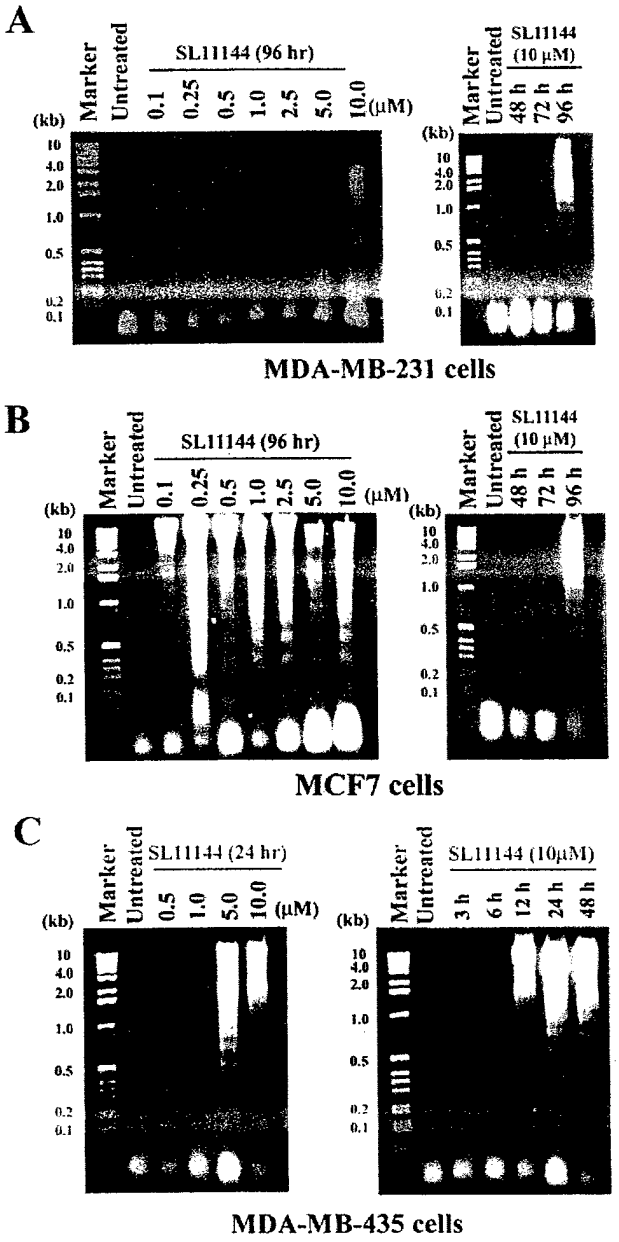

Fig. 3 SL11144 induces internucleosomal DNA fragmentation. MDA-MB-231 cells (*A*) and MCF7 cells (*B*) were treated with increasing concentrations of SL11144 (0.1–10 μM) for 96 h or treated with 10 μM SL11144 for 48, 72, and 96 h. MDA-MB-435 cells (*C*) were treated with increasing concentrations of SL11144 (0.1–10 μM) for 24 h or treated with 10 μM SL11144 for 3, 6, 12, 24, and 48 h. Cells were harvested, and fragmented DNA was extracted as described in "Materials and Methods." Fragmented DNA was analyzed by electrophoresis in a 1.2% agarose gel containing 0.1% ethidium bromide. Each experiment was done twice with similar results.

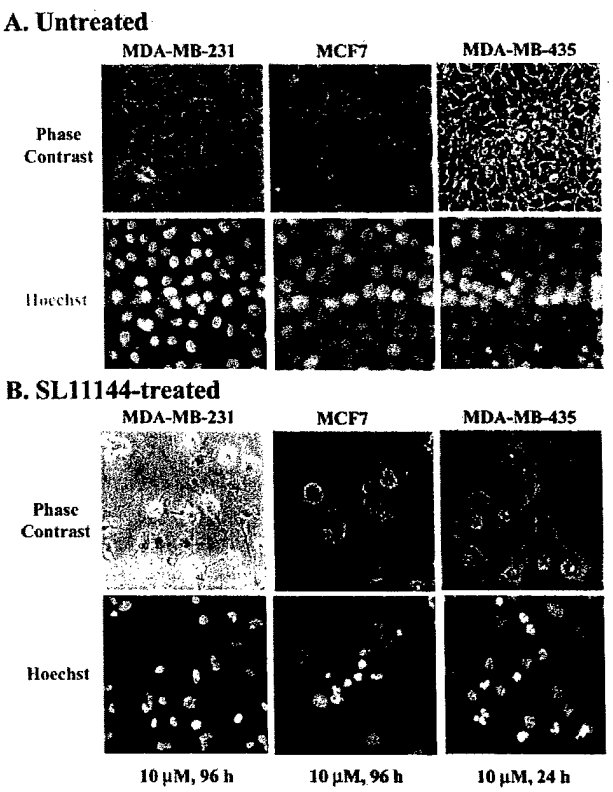

Fig. 4 Fluorescent micrographs of SL11144-treated cells. MDA-MB-231 cells, MDA-MCF7 cells, and MB-435 cells were exposed to 10 μM SL11144 for the indicated times. Then cells were fixed in formaldehyde and stained with Hoechst dye 33258. *A,* untreated cells; *B,* SL11144-treated cells.

SL11144 Up-Regulates c-Jun and c-Fos in MDA-MB-231 and MDA-MB-435 Cells. Because the effects of SL11144 on apoptotic pathways varied greatly between different human breast cancer cell lines, we examined the impact of SL11144 on other important apoptosis-related factors, particularly c-Jun and c-Fos. Both c-Jun and c-Fos are important members of the AP-1 transcription factor family, which plays a critical role in regulating transcription of a variety of genes involved in growth, differentiation, apoptosis, and so forth. In MDA-MB-231 cells, SL11144 treatment induced c-Jun phosphorylation after 48 h but did not alter the protein expression of either c-Jun or c-Fos (Fig. 7*A*). In MCF7 cells, no obvious changes in c-Jun and c-Fos were observed (Fig. 7*B*). In contrast, SL11144 significantly induced the phosphorylation of c-Jun and enhanced the protein level of c-Jun and c-Fos in MDA-MB-435 cells within 6–12 h (Fig. 7*C*). To study whether SL11144-enhanced c-Jun and c-Fos protein expression led to the increased nuclear localization of these proteins in MDA-MB-435 cells, the subcellular localization of c-Jun and c-Fos was examined. c-Jun was induced and expressed largely in the nucleus, whereas c-Fos was induced in both the cytoplasm and the nucleus (Fig. 7*D*). These results imply that up-regulations of AP-1 family proteins by SL11144 are cell type specific and may play an active role in the mediation of growth-inhibitory activities of SL11144.

DISCUSSION

Results of previous studies from our laboratory demonstrated that first and second generations of *N*-acetyl substituted polyamine analogs could inhibit growth and induce apoptosis in

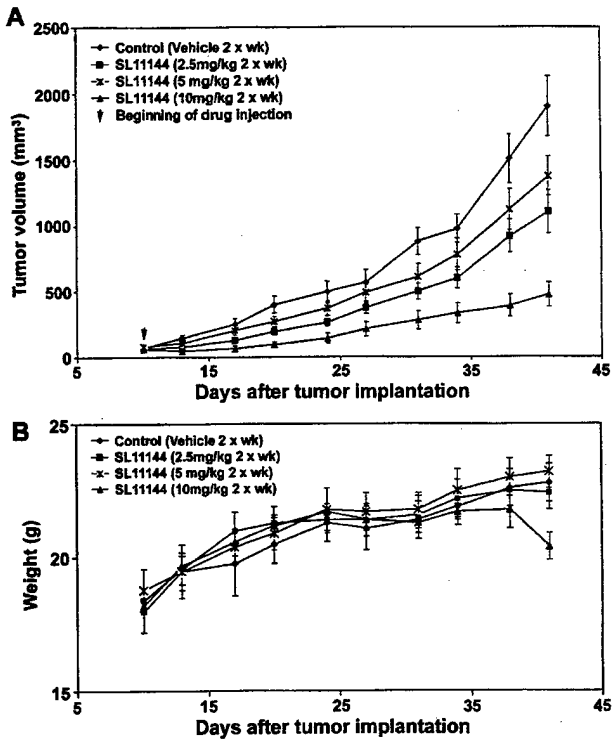

Fig. 5 Effects of SL11144 in nude mice bearing MDA-MB-231 xenografts. *A,* MDA-MB-231 cells were transplanted into the flank region of nude mice. Ten days after implantation, different doses of SL11144 (2.5, 5, or 10 mg/kg) or vehicle were given via i.p. injection twice weekly. Tumor volumes of mice were measured twice weekly. The *vertical bars* indicate mean tumor size (in mm^3) ± SE. *B,* weights of mice were measured twice weekly. The *vertical bars* indicate mean mouse weight (in g) ± SE.

MCF7 and other human breast cancer cell lines (3, 9, 31). However a Phase II clinical trial of one early polyamine analog, N^1,N^{11}-diethylnorspermine (DENSPM; also known as BENSpm), showed that it was not effective as a single agent in women with advanced breast cancer (32). Recently, a group of new polyamine analogs designated as oligoamines has been developed (17). Oligoamines were synthesized with longer chains than natural cellular polyamine molecules that occur in mammalian cells and are effective against a variety of proliferating cells (17). In this study, we demonstrate that one of the leading oligoamine compounds, SL11144, significantly inhibits the growth of and induces PCD in human breast cancer cells. It displays more potent antiproliferative activity against breast tumor line proliferation than the previously reported polyamine analogs, BENSpm, CPENSpm, and CHENSpm. SL11144 induced DNA fragmentation and typical apoptotic morphological changes in both hormone-responsive (MCF7) and hormone-unresponsive (MDA-MB-231 and MDA-MB-435) breast cancer cell lines. It appears that there is no relationship between hormone receptor status and cytotoxic effects of SL11144. SL11144 also inhibits the growth of MDA-MB-231 xenografts in nude mice in a dose-dependent fashion without apparent toxicity.

SL11144-induced apoptosis, based on morphological and DNA fragmentation criteria, was not detected until 96 h of treatment in MDA-MB-231 and in MCF7 cells, but 12 h of treatment with SL11144 resulted in apoptosis in MDA-MB-435 cells. Although the mechanisms of differential susceptibility among tumor cells to polyamine analog-induced cell death are unclear, this could reflect varied effects on apoptotic pathway members including caspases, bcl-2 family members, cytochrome *c*, and FasL, which have been demonstrated to play critical roles in regulating PCD (25–28). Caspases have been characterized as the effectors and executioners of apoptosis, and caspase-3 is a critical downstream apoptotic effector that cleaves specific substrates such as PARP. The observation that caspase-3 activation was followed by PARP cleavage in MDA-MB-435 cells indicates that caspase-3 may play a key role as an important executioner in SL11144-induced apoptosis in this cell line. However, the failure of SL11144 to activate caspase-3 in MDA-MB-231 cells and the absence of caspase-3 expression in MCF7 suggest that other factors or pathways can also function as apoptotic effectors in these two cell lines.

Mitochondria can be induced to release cytochrome *c* in response to many anticancer drugs and to other stresses by the opening of channels on the outer mitochondrial membrane (33). Release of cytochrome *c* activates the caspase adaptor, caspase-9, which then activates downstream caspases such as caspase-3 and caspase-8 (34). Our studies found that cytochrome *c* release was transiently enhanced by SL11144 with a 48-h drug exposure in MDA-MB-231 cells, whereas it was rapidly and consistently induced in MDA-MB-435 cells. In both cell lines, time-dependent activation of caspase-9 was observed, but caspase-8 activation was only seen in MDA-MB-435 cells. However, in MCF7 cells, SL11144 has no effect on cytochrome *c* release or on caspase-8 or -9 activation. The simultaneous activation of both caspase cascades and of the mitochondrial pathway in MDA-MB-435 cells by SL11144 might explain why cell death was more rapidly induced in these cells than in MDA-MB-231 or MCF7 cells. A recent study by Ellison *et al.* (35) reported that MDA-MB-435 cells might be of melanoma origin based on differential expressions of genes characteristic of breast cancer cells. If more compelling evidence ultimately confirmed this conclusion, the different sensitivity of MDA-MB-435 cells to SL11144 may possibly reflect its non-breast cancer identity.

Members of the Bcl-2 family play a central role in regulating the mitochondrial pathway of apoptosis. More than 20 Bcl-2 family members have been identified to date, including antiapoptosis members (Bcl-2, Bcl-X_L, Bcl-W, Bcl-G, Mcl-1, and so forth) and proapoptosis members [Bax, Bak, Bok, Bad, Bid, Bik, Bim, Bcl-Xs, and so forth (33, 34, 36–39)]. In response to various stimuli and stresses, Bcl-2 family proteins usually translocate to the outer mitochondrial membrane and modulate membrane permeabilization, leading to the release of cytochrome *c*. SL11144 decreased Bcl-2 and increased Bax expression in MDA-MB-231 cells but did not affect Bcl-2 and Bax in MDA-MB-435 and MCF7 cells, suggesting that the regulation of Bcl-2 family members by polyamine analog is cell type specific. Our data also demonstrate that SL11144 enhances FasL (the only protein to be uniformly affected) expression in all three cell lines. The Fas/FasL (CD95-CD95 ligand) system is another critical pathway that leads to the activation of apoptotic

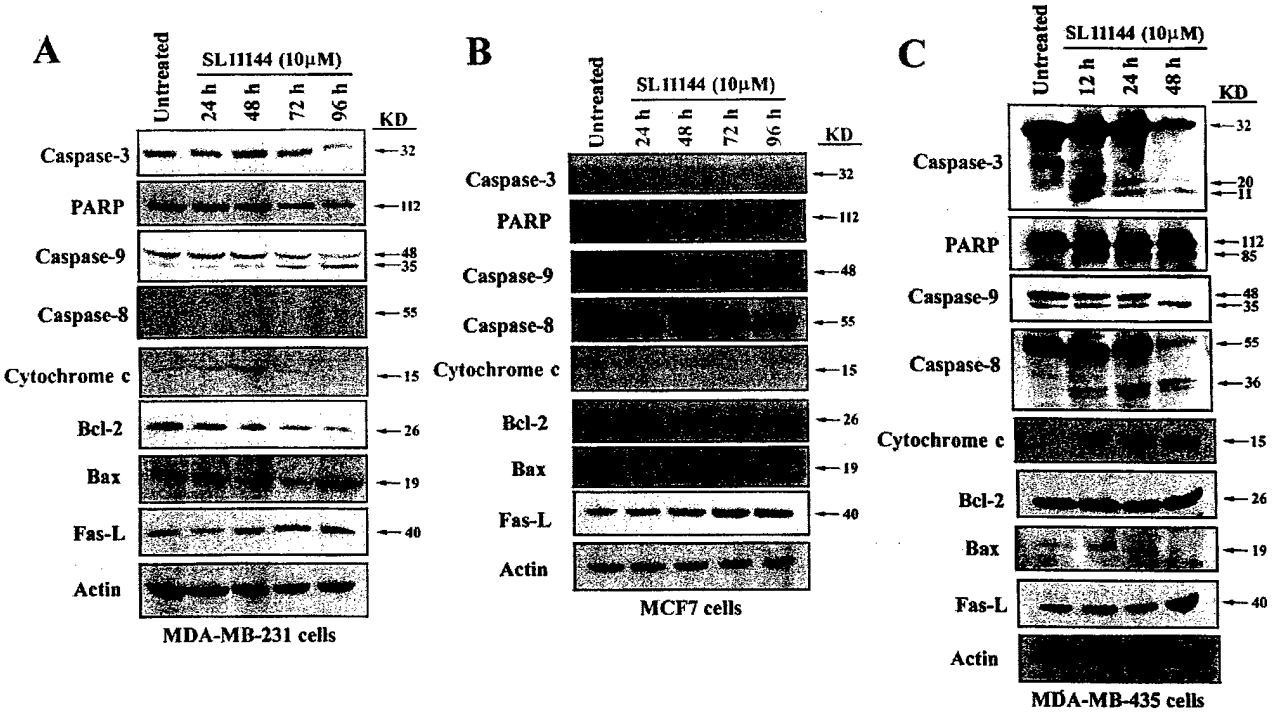

Fig. 6 Effects of SL11144 on apoptosis proteins. MDA-MB-231 cells (*A*) and MCF7 cells (*B*) were treated with 10 μM SL11144 for 24, 48, 72, and 96 h. MDA-MB-435 cells (*C*) were treated 10 μM SL11144 for 12, 24, and 48 h. Equal amounts (50 μg/lane) of cellular protein were fractionated on 12% SDS-PAGE gels and transferred to PVDF membranes followed by immunoblotting with anti-caspase-3, PARP, caspase-9, caspase-8, cytochrome *c*, Bcl-2, Bax, or FasL monoclonal or polyclonal antibodies and analyzed as described in "Materials and Methods." Actin protein was blotted as a control. Each experiment was repeated twice with similar results.

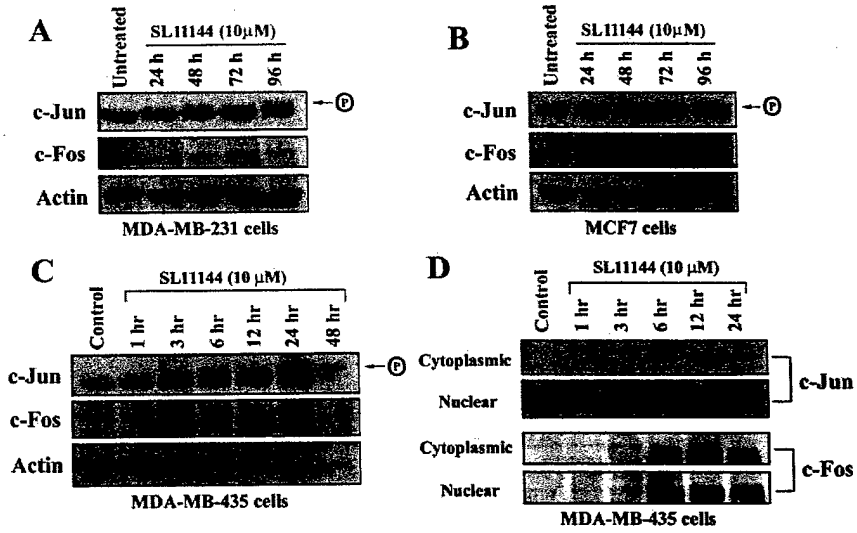

Fig. 7 Effects of SL11144 on c-Jun and c-Fos. Tumor cells were treated with 10 μM SL11144 for the times indicated. Equal amounts (50 μg/lane) of whole cell (*A*–*C*), cytoplasmic or nuclear protein (*D*) were fractionated on 12% SDS-PAGE gels and transferred to PVDF membranes followed by immunoblotting with anti-c-Jun and c-Fos polyclonal antibodies and analyzed as described in "Materials and Methods." Actin protein was blotted as a control.

machinery. Binding of FasL to Fas and to other death receptors results in receptor trimerization, recruitment of adaptor protein to the cytoplasmic death domain, and activation of a series of downstream apoptotic events (40, 41). Recent studies have shown that overexpression of FasL can lead to suicidal or fratricidal destruction in melanoma and leukemia cells via autocrine or fratricidal interactions between FasL and Fas (42, 43). Up-regulation of FasL level by SL11144 in all three human breast cancer cell lines implies that activation of Fas/FasL system might be a common mechanism for the cell death induced by SL11144.

We further investigated whether other important upstream

regulatory or signaling events were involved in the mediation of SL11144-induced growth inhibition and apoptosis. SL11144 induces expression and phosphorylation of c-Jun, an important member of the AP-1 family, in both MDA-MB-231 and MDA-MB-435 cells. It also significantly increased the protein expression of another important AP-1 family member, c-Fos, after 12 h in MDA-MB-435 cells. Nuclear extraction analysis showed that c-Jun protein was located largely in the nucleus, where it can potentially play an active role in mediation of a wide range of gene expressions. However, neither c-Jun or c-Fos levels nor phosphorylation status was significantly affected by SL11144 treatment in MCF7 cells. c-Jun-NH_2-terminal kinase signaling and AP-1 transcription factors have been implicated in the regulation of cell proliferation, differentiation, and apoptosis (44). The proapoptotic targets of c-jun include FasL, tumor necrosis factor α, c-Myc, p53, and members of the bcl-2 family (45–49). The activation of c-Jun in MDA-MB-231 and MDA-MB-435 cells, but not in MCF7 cells, suggests that c-Jun-NH_2-terminal kinase/AP-1 and the upstream regulator mitogen-activated protein kinase family might be a major polyamine analog response pathway in some but not all breast cancer cell lines.

The intracellular polyamines are highly regulated by several polyamine metabolic enzymes. ODC, the first and rate-limiting step of polyamine biosynthesis, increases levels of polyamines in cells during rapid proliferation or differentiation (6). High expression of ODC characterizes some cancers including breast cancer. As a result, there has been extensive effort to design compounds that can inhibit ODC activity in tumor cells. α-Difluoromethylornithine, an irreversible inhibitor of ODC, has proven to be effective in inhibiting growth in several *in vitro* and *in vivo* tumor models (6, 50). In this study, the effect of SL11144 on natural polyamine levels was variable in different cell lines. SL11144 treatment led to a decrease in all natural polyamines in MDA-MB-231 cells and had inconsistent effects in MCF7 and MDA-MB-435 cells. The increased level of Spd in MCF7 cells by SL11144 might explain why MCF7 cells are less sensitive to SL11144 than the other two cell lines. Although ODC activities were significantly suppressed by SL11144 in all these cells, it is not clear whether the attenuation of ODC activities contributes to SL11144 cytotoxicity. In addition, the activity of another critical polyamine metabolic enzyme, SSAT, was only modestly up-regulated by SL11144 exposure, indicating that SSAT activity is not responsible for the observed cytotoxic response. All these results imply that the effects of SL11144 may not be solely a function of its effect on polyamine pools.

In summary, a newly developed polyamine analog, SL11144, exhibits significant inhibitory actions against human breast cancer cell growth *in vitro* and *in vivo*. Apoptotic cell death was induced by SL11144 in a time- and dose-dependent manner. SL11144 modulated expression of apoptotic proteins in a cell type-specific manner, suggesting that multiple apoptotic pathways might be involved in SL1144-induced apoptosis in different human breast cancer cell lines.

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A Phase II Study of the Polyamine Analogue N^1, N^{11}-Diethylnorspermine (DENSpm) Daily for Five Days Every 21 Days in Patients with Previously Treated Metastatic Breast Cancer[1,2]

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RUNNING TITLE: Phase II Trial of DENSpm in Metastatic Breast Cancer

KEYWORDS: clinical trials

ORIGINAL SUBMISSION – May 9, 2003, REVISED SUBMISSION – August 5, 2003

ABSTRACT

Purpose: Polyamines are ubiquitous intracellular polycationic molecules essential for cell growth and differentiation. Polyamine analogues downregulate ornithine decarboxylase, induce spermidine/spermine N^1-acetyltransferase, deplete natural polyamine pools, inhibit growth, and induce programmed cell death in breast cancer models. This study evaluated the activity of the first generation analogue DENSpm in women with metastatic breast cancer.

Experimental Design: Overall accrual goal was 34 patients (30 evaluable) in a two-stage design. Second stage of accrual was to proceed if ≥two among first 15 evaluable patients were progression-free at four months. The primary objective was to determine if ≥20% of metastatic breast cancer patients treated with DENSpm as second or third-line therapy remained progression-free after four months.

Results: Sixteen patients (median age 52, range 34-65; median PS 1, range 0-1) enrolled in the first stage received 43 cycles (median 2, range 1-6) of DENSpm 100 mg/m^2 as 15-minute infusion IV days 1-5 every 21 days. All 16 patients were evaluable for toxicity; 15 were evaluable for response. All patients had disease progression by four months and the study closed after the first stage of accrual. Main toxicities included grade 1-2 abdominal pain, transient perioral numbness, nausea, and grade 1 thrombocytopenia. Two patients had grade 3 abdominal pain during cycle 2 infusion; one was hospitalized and another subsequently retreated at 80% dose without pain recurrence.

Conclusions: While this dose and administration schedule of DENSpm was quite tolerable, no evidence of clinical activity was detected. Encouraging preclinical activity of polyamine analogues alone and in combination with cytotoxic drugs support the continued evaluation of newer generation polyamine analogues for the treatment and prevention of breast cancer.

INTRODUCTION

Although many patients diagnosed with metastatic breast cancer benefit at first from systemic therapy with endocrine and chemotherapy strategies, most will develop progressive disease that is refractory to standard therapy. Thus, there is much interest in identifying novel therapeutic targets. Polyamines are ubiquitous intracellular positively charged aliphatic amines and their association with cancer was first reported in the early 1970's (1). Natural polyamines such as putrescine, spermidine, and spermine are essential for cell growth and differentiation of normal and malignant cells (2). Increased levels of polyamines have been observed in many cancers as compared to normal tissue; this observation has led to the exploration of the polyamine enzymatic pathway as a potential therapeutic target (Figure 1) (3).

The first such agent, α-difluoromethylornithine (DFMO or eflornithine), irreversibly inhibits ornithine decarboxylase (ODC), the first enzyme in the polyamine biosynthetic pathway, and leads to the depletion of putrescine and spermidine (3). It is used for the treatment of African sleeping sickness (4), and is also under investigation as a chemopreventive agent (5). A small study in women with breast cancer suggested a possible correlation between increased tumoral ODC activity and decreased survival (6). O'Shaughnessy *et al.* treated 21 patients with metastatic breast cancer with 4,800 mg of oral DFMO three times a day for 14 days of a 28-day cycle. While no patient had an objective response, one patient with heavily pretreated liver metastases achieved stable disease for 18 months, and urinary levels of putrescine, spermine, and spermidine levels were suppressed, suggesting biological activity (7). Levin *et al.* showed a possible survival advantage with the addition of DFMO to a standard chemotherapy regimen in patients with anaplastic gliomas (8). These preliminary studies with DFMO and other single enzyme inhibitors offer proof of principle

that the inhibition of the polyamine pathway could serve as a potential therapeutic target, but in all likelihood there will be a need to act on several levels of the pathway.

Polyamine analogues downregulate synthetic enzymes such as ODC and S-adenosylmethionine decarboxylase (AdoMetDC), and stimulate the catabolic enzyme spermidine/spermine N^1-acetyltransferase (SSAT) and polyamine oxidase h1 (PAOh1/SMO) (9, 10). This multistep interference with the polyamine pathway depletes the pools of natural polyamines and leads to the intracellular accumulation of analogues that do not support cell growth and differentiation. The first generation of polyamine analogues consisted of symmetrical terminally alkylated analogues of either spermine or spermidine, such as N^1, N^{11}-bis(ethyl)norspermine (DENSpm or BENSpm), bis(ethyl)spermine (DESpm or BESpm), and bis(ethyl)homospermine (DEHSpm or BEHSpm). These analogues demonstrated evidence of antitumor activity in several preclinical tumor models, such as melanoma and lung cancer (11-16). One analogue, DESpm, is a potent growth inhibitor of both estrogen receptor positive and negative human breast cancer cell lines, and its growth inhibitory effects were not diminished by the acquisition of resistance to doxorubicin or anti-estrogens (17). Similar growth inhibition against MDA-MB-468 and MCF-7 human breast cancer cell lines was seen with another analogue, DENSpm with IC_{50} achievable in serum in phase I human trials (1-10 μM after 120 hours of chronic exposure) (18). In addition, DENSpm is a particularly potent inducer of SSAT, the rate-limiting step in the two-step eukaryotic catabolism of polyamines. Thus it was selected for clinical development.

Preclinical toxicology studies identified hypotension with rapid infusions, gastrointestinal mucositis, and lethargy, but not myelosuppression, as potential toxicities of DENSpm. Based on promising pre-clinical activity, several human phase I trials were conducted (19, 20). A phase I trial

in our institution evaluated the effects of DENSpm given as a 15-minute daily intravenous infusion for 5 consecutive days and repeated every 21 days (one cycle) (21). Nine dose levels from 15.6 to 145 mg/m^2/day IV (doses reflecting the free base component of DENSpm; 1 mg free base = 1.59 mg salt) were explored in 29 patients, who received 83 complete courses of therapy. Dose-limiting toxicities included gastrointestinal bleeding, diarrhea, nausea, abdominal pain, asthenia, and CNS toxicity. The maximum tolerated dose was 116 mg/m^2/day IV days 1 – 5 (free base). A short half-life of up to 3.7 hours and a disproportional increase in maximum serum concentrations *versus* dose increase were observed. Transient, reversible elevations of creatinine occurred, but no patient developed neutropenia, thrombocytopenia, infectious complications, or immunosuppression at any of the dose levels. A phase 2 dose and schedule of 100 mg/m^2/day (free base) days 1-5 by IV infusion every 21 days was recommended.

Based on these data, we conducted an open-label, single center, phase 2 study of DENSpm in patients with measurable metastatic breast cancer who had received one or two prior chemotherapy regimens. The primary objective of this study was to estimate the proportion of patients who were free of disease progression at four months. Secondary objectives were to evaluate toxicities of DENSpm; and to determine the overall response rate, duration of response, survival, and clinical benefit.

PATIENTS AND METHODS

Patient Eligibility

Eligible patients were women age > 18 years with histologically confirmed adenocarcinoma of the breast, evidence of metastatic progression within three months before study registration and, at

least one site of measurable disease. Patients could have received a minimum of one but no more than two prior chemotherapy regimens for metastatic disease, including cytotoxic agents and/or trastuzumab. Continuation (but not initiation) of bisphosphonates was permitted. Prior hormonal therapy for metastatic disease, prior adjuvant systemic therapy of any type, and prior radiation therapy were allowed if completed ≥28 days before starting protocol therapy. Patients with a prior diagnosis of malignancy other than invasive breast cancer were allowed to enter only if disease-free for greater than five years, with the exception of curatively-treated basal or squamous cell carcinoma of the skin or carcinoma *in situ* of the cervix. Patients were required to have an Eastern Cooperative Oncology Group performance status (ECOG PS) of ≤2, and normal laboratory parameters (serum creatinine ≤ 1.5 mg/dL, absolute neutrophil count [ANC] ≥ 1,500/mm^3, platelet count ≥ 100,000/mm^3, SGOT ≤2x upper limit of normal and total bilirubin ≤1.5 mg/dL). Pregnant or lactating women were not eligible, and women of childbearing potential had to agree to use an effective method of contraception. Patients with known history of CNS metastases or any active serious medical illness were not eligible. The study was conducted in accordance with the Declaration of Helsinki. The protocol document and informed consent form were approved by the Institutional Review Board of the Johns Hopkins University and by the Surgeon General's Human Subjects Research Review Board as required by the United States Army Medical Research and Materiel Command (USAMRMC, Fort Detrick, MD). All patients were required to provide written informed consent.

Dosage and Drug Administration

DENSpm was provided free of charge for this study by Parke-Davis Pharmaceutical Research. Parke-Davis was then acquired by Pfizer, Inc., and the license for this compound was

transferred to GelTex Pharmaceuticals Inc. (now Genzyme Corporation). The drug was supplied as a lyophilized powder packaged in 10-mL clear glass vials containing 150 mg of free base. When reconstituted with 5 mL of Water for Injection, USP, the resulting solution contained 30 mg/ml of DENSpm (free base) and was chemically and physically stable for 96 hours at room temperature. The appropriate dose was withdrawn and further diluted in 50 mL of normal saline, and infused over 15 minutes into a peripheral vein once a day for 5 consecutive days every 21 days (one cycle). Toxicity was evaluated using the National Cancer Institute Common Toxicity Criteria version 2 (NCI CTC v.2).

Pretreatment and Follow-up Studies

At baseline, investigators identified one-three bidimensionally measurable lesion(s) within each patient. The same disease sites were reassessed during the treatment phase using the same methodology. Evaluations were conducted after every other cycle of treatment. These consisted at a minimum of a computerized tomography (CT) of the chest and abdomen every other cycle and a bone scintigraphy every 4^{th} cycle.

Treatment Plan

The initial dose of DENSpm was fixed for all patients at 100 mg/m^2/day (free base). Patients were premedicated with a 5-HT3 receptor antagonist prior to each daily infusion. If a course of treatment was interrupted for any reason, the remaining daily doses (maximum of five) were to be completed by day 7 or any remaining doses would be skipped. The next cycle of DENSpm would begin no sooner than 21 days after day 1 of the previous cycle, assuming that all associated non-hematologic toxicity had recovered to grade ≤ 1 (or to the patient's baseline) with normal neutrophil, platelets, and serum creatinine values. If these parameters not met, the start of the next course would

be delayed by weekly intervals.

The daily dose of DENSpm would be permanently reduced by 20% if the following occurred in a previous course: grade ≥2 treatment-related CNS adverse event, any grade ≥3 treatment-related adverse event (except grade 3 nausea and vomiting in the absence of antiemetics), < five doses given because of treatment-related adverse event, platelet count < 50,000/mm^3 or platelet transfusion, grade 4 ANC toxicity ≥5 days, or a microbiologically-documented infection while neutropenic treated with intravenous antibiotics.

Definitions and Statistical Design

Available data suggest that an increase in response rate is unlikely to impact on overall survival. Studies that allowed cross-over of patients from standard therapy to the investigational arm have not shown an improvement in overall survival, even if a higher response rate and longer time to treatment failure were observed following initial therapy with the investigational regimen (22). This phase II study used a design adopted by the Eastern Oncology Cooperative Group based on the hypothesis that an agent capable of stabilizing breast cancer for ≥four months in 20% of patients with progressive metastatic disease would be worthy of additional investigation in larger efficacy studies.

A Simon two-stage design was used (23) with early termination of the trial if a predetermined minimum level of activity was not observed after the first stage of accrual. Seventeen patients were to be enrolled in the first stage of this trial, expecting 15 to be eligible. A patient would have to complete one full course of treatment (21 days) with response assessment to be considered evaluable for response. If two or more patients were progression free at four months on study among 15 eligible patients, the second stage of accrual would enroll an additional 17 patients (expecting 15

eligible). At the end of the study, DENSpm would be considered worthy of further study if six or more patients (approximately 20%) were found to be free of disease progression after four months on study. The probability that this study would terminate at the end of the first stage of accrual (i.e., < two patients progression-free in the 15 eligible patients) was 8% if the true underlying proportion of patients that was progression free was 20%. However, the probability of terminating the study increased to 72% if the true underlying proportion of patients that was progression-free was only 7%. The compound and schedule of administration would be considered promising and worthy of further investigation if six or more among 30 eligible patients were progression free at four months. At the end of the study, the probability of concluding that the drug and schedule were effective would be 87% if the true rate is 25%, but only 5% if the true rate is 7%. All enrolled patients would be evaluable for the secondary endpoint of toxicity. If the true probability of rare toxicity among 34 patients was 3%, then the probability of observing one or more rare toxicities would be 64%. If the true probability was 5%, then this probability would increase to 83%. The 90% confidence interval for any grade 3 or higher toxicity would be $\leq$30%.

Time to progression is the interval (number of days) between the first day of study treatment and the first date of progression or development of new disease sites. Time to objective response was defined as the interval between the first study treatment and the start of the confirmed complete (CR) or partial response (PR). Duration of objective response among responders was defined as the interval between the start of the confirmed CR or PR and the first date of disease progression. Survival duration was defined as the interval between the date of first treatment and the date of death from any cause. CR was defined as disappearance of all measurable disease confirmed by a second evaluation done at least 3 weeks later with resolution of all sites of evaluable disease (e.g., ascites

and pleural effusion), no new sites of disease, and resolution of all symptoms including pain. PR was defined as a ≥50% decrease in the sum of the products of the sites of measurable disease identified at baseline and confirmed by a second evaluation done at least 3 weeks later, with no increase ≥25% in other areas of measurable disease, significant worsening of tumor-related evaluable disease, new lesions, or clinically significant worsening of tumor-related symptoms. Progressive disease (PD) was defined as appearance of a new lesion, increase in the sum of the products of any single lesion by ≥50% from baseline or best response or of multiple lesions by ≥ 25% each from baseline or best response, clinically significant increase in sites of evaluable disease, or clinically significant increase in tumor-related symptoms. Clinical benefit was defined as improvement in performance status and disease related symptoms, reduction in narcotic analgesic requirements, or mixed objective tumor response (reduction in size of some lesions without progression at any other site) that were considered to be related to the administration of DENSpm. Patients discontinued protocol treatment in case of disease progression, unmanageable adverse event, refusal to receive further therapy, or a decision by the investigator based on patient safety or any new information about the compound.

RESULTS

General

Sixteen patients were enrolled from April 2000 to April 2001. Patients' characteristics are listed in Table 1. Despite the overall good performance status, this was a heavily pretreated population. All patients were eligible and evaluable for toxicity and response. A total of 43 cycles of DENSpm were administered and the median number of cycles administered per patient was 2

(range, 1-6). Two patients received only one cycle of therapy: one died during cycle 1 from complications of her disease without evidence of disease progression and another had evidence of clinical and radiological progression prior to cycle 2. The additional 14 patients included eight patients who received two cycles of therapy, one who received three cycles, four who received four cycles, and one who received six cycles.

Antitumor Activity

Fifteen patients were evaluable for response and no responses were observed. One patient did not complete cycle 1 of therapy. This patient had developed ipsilateral supraclavicular recurrence while on adjuvant chemotherapy with doxorubicin and cyclophosphamide and did not respond to first-line therapy with paclitaxel. She received the first five days of cycle 1 without incident, was admitted on day 8 with acute onset of dyspnea at rest and severe hypoxia, and died on day 12. A CT scan on admission showed no evidence of disease progression, but an autopsy showed severe tumoral encasement and obstruction of the pulmonary vessels. Fourteen patients received a minimum of two cycles of therapy and six (40%) among the 15 evaluable patients had radiological evidence of stable disease after completing cycles 1 and 2. Six patients received a minimum of three cycles of therapy, but only one (6.6%) among 15 evaluable patients had radiological evidence of stable disease after completing cycles 3 and 4. This patient previously had a partial response with prolonged disease stabilization with paclitaxel 175 mg/m^2 IV every 21 days, and then another prolonged period of stable disease with letrozole. She was enrolled on this trial and had evidence of disease progression after receiving cycles 5 and 6 (four month evaluation) and went off study. Therefore, no patients among 15 patients evaluable for response received more than six cycles of therapy and none were progression-free at four months. As it would be impossible to observe two or

more patients free of disease progression at four months to fulfill the minimum requirement to proceed into the second stage of accrual, the study was closed in August 2001 after enrolling 16 patients and fulfilling its predetermined objectives.

Toxicities

All 16 patients were evaluable for toxicity (Table 2). No patients discontinued protocol therapy due to toxicity and there were no treatment-related deaths. One patient (described above) died from pulmonary vascular complications from the disease within 30 days of receiving study drug. No hematologic toxicity was observed aside from two patients with grade 1 thrombocytopenia. Two patients had grade 3 abdominal pain, but there were no other episodes of grade 3 or 4 toxicity.

The main toxicity observed was grade 3 abdominal pain (cramps) occurring after the third and fourth daily infusion during cycle 2 in two patients, respectively. The first patient was admitted to the hospital, and a CT showed small bowel wall thickening suggestive of jejunitis. Her symptoms of pain and constipation subsided with low doses of narcotics and IV hydration. She was not retreated as she then developed evidence of disease progression. A second patient developed abdominal cramps after cycle 2 day 4, and her day 5 infusion was held. Her symptoms required no intervention and subsided within the next 48-72 hours. She then received four additional cycles of therapy with a permanent 20% dose reduction without recurrence of abdominal symptoms or treatment delay.

Transient grade 1 or 2 perioral numbness occurred in eight of 16 patients during drug infusion around cycle 1 day 3 and in only two of 14 patients during cycle 2. No patients developed confusion, somnolence, or any other evidence of CNS toxicity. No patients reported tinnitus or symptoms suggestive of ototoxicity. Nine patients had grade 1/2 nausea and/or vomiting with cycle

1, but only one with cycle 2; all patients were premedicated with ondansetron 16 mg IV. Two patients each complained of grade 1 flushing and peripheral neuropathy during cycle 1. Previously described mid-cycle creatinine elevation or hypomagnesemia was not observed.

DISCUSSION

The primary endpoint of this phase II clinical trial was to determine if at least 20% of patients with metastatic breast cancer would be progression free after four months of treatment with DENSpm given as a 15-minute daily infusion repeated for five days every 21 days. This trial used a two-stage design to minimize the risks of exposing an excessive number of patients to a potentially ineffective therapy. The design requires a minimum predetermined level of activity during the first stage of accrual to allow expansion into the second stage. As none among 15 evaluable patients enrolled in the first stage of accrual were progression free at four months, it was considered unlikely that the predetermined minimum level of activity of interest would be observed at the end of the trial (i.e., 20% or six among 30 evaluable patients free of disease progression at four months). Therefore, the primary objective of the clinical trial was achieved and the study was closed after enrolling 16 patients.

Secondary objectives of this study were also fulfilled. This study confirmed the favorable toxicity profile of the administration schedule selected in the phase I study (21). Although two of 16 patients developed grade 3 abdominal pain, none withdrew from the trial due to complications from the study drug. Several patients presented with minor perioral numbness during the infusion, a toxicity also observed with other agents targeting the polyamine pathway (24). None had transient mid-cycle creatinine or electrolyte abnormalities. No patients developed confusion and somnolence,

symptoms commonly seen in the phase I trials that used a twice or three times daily schedule. Ototoxicity (e.g., tinnitus) was reported in prior studies of DFMO in glioma patients using an oral two-week administration schedule every eight hours followed by either one or two weeks off, (8, 25) despite a suggestion from a smaller phase II study of DFMO in breast cancer patients that ototoxicity could be prevented by a two-week drug holiday. (7) Preclinical data suggest that this neurosensory toxicity may result from polyamine depletion induced by DFMO in cochlear hair cells. (26) However, ototoxicity has not been described in any of the reported clinical trials of DENSpm. (19-21)

Several factors may explain the lack of observed clinical activity in this trial. They include the possibility that modulation of the polyamine pathway may not be a meaningful target for the treatment of breast cancer, patient selection, choice of DENSpm as the polyamine analogue for phase I/II testing, selection of the administration schedule, and the potential need for combination therapy with existing drugs.

First, although their primary mechanism of action is not fully understood, natural polyamines are essential for cell growth and differentiation, and levels are increased in malignant *versus* normal tissues. Polyamine analogues are incomplete substitutes that appear to displace natural polyamines from their DNA binding sites and induce enzymes in the catabolic pathway such as SSAT and PAOh1/SMO, resulting in the depletion of intracellular pools, cessation of cell growth, and cell death. Preclinical studies with the second generation compounds have offered additional insight on the role of polyamine analogues as anticancer drugs. Our group has shown that treatment with the unsymmetrically substituted analogue N^1-ethyl-N^{11}-[(cyclopropyl)methyl]-4,8-diazaundecane (CPENSpm) leads to accumulation of the analogue and depletion of natural polyamine pools, with

secondary growth inhibition and induction of programmed cell death in breast cancer models (27). Despite promising preclinical data, these data must be confirmed in clinical studies.

Second, the patients enrolled in this study may have been more heavily pretreated and less likely to respond to investigational therapies. Only a few drugs such as capecitabine (28) have been shown to be active in patients previously treated with anthracyclines and taxanes. Despite the original intent to recruit a less heavily pre-treated population, all patients had received two prior chemotherapy regimens, often including an anthracycline and a taxane. This pattern of late referral to studies with novel agents is commonly seen in tertiary centers. Breast cancer is a disease with a diverse biological behavior and patients with advanced disease often gain some clinical benefit from third or fourth-line cytotoxic regimens. Therefore, patients and their physicians are reluctant to consider clinical trials testing novel agents earlier in the course of their metastatic disease, despite evidence indicating that this approach does not negatively impact on their chances of benefiting from subsequent treatment with available standard regimens (29).

Third, our choice of DENSpm among the first generation of symmetrically substituted polyamines analogues for further testing was based on preclinical data. DENSpm is a homologue of DESpm, a prototypical spermine analogue initially evaluated in preclinical studies. While antitumor activity was seen with both compounds in several human tumor models (11-16), DENSpm showed greater antitumor activity and less associated toxicity in a human melanoma xenograft model (14).

Fourth, the administration schedule in this phase II trial was based on prior pre-clinical and phase I studies. DENSpm given as a single daily administration repeated for six days in a human melanoma xenograft model showed similar levels of drug accumulation, reduction of natural polyamine pools, and antitumor activity as compared to thrice-daily doses for six days (15).

Excessive CNS toxicity was detected in phase I trials that used administration schedules with dosing more frequent than once daily. Investigators at Roswell Park Cancer Institute evaluated DENSpm as a 1-hour twice daily infusion for five consecutive days repeated every 28 days in 15 patients treated over six dose levels (19). The highest dose level was 78 mg/m^2/day (free base) and the maximum tolerated dose (MTD) was limited to 52 mg/m^2/day (free base) due to neurological dose-limiting toxicities (DLT) such as headache, slurred speech, ataxia, dysphagia, dysarthria, and paresthesias. Another group of investigators examined DENSpm's administration as a 15-minute infusion given thrice daily for six consecutive days and repeated every 28 days in 28 patients treated over six dose levels (20). Once again the highest dose level given was 74 mg/m^2/day (free base) and the MTD was 52 mg/m^2/day (free base) due to similar neurological DLTs. Other toxicities observed in these two studies included asthenia, injection site reaction, and anemia, but not leukopenia or thrombocytopenia. No responses were observed with schedules using more than once a day dosing, and our daily single dose schedule was selected for further investigation (21).

Pharmacokinetic data from our previous phase I study showed a short half-life for DENSpm between 0.5 and 3.7 hours, which might suggest the need for a more frequent drug administration than five days every 21 days. However, a preclinical model by Porter *et al.* showed that DENSpm drug levels in tumor tissues were still retained (40%) two weeks after six single-daily doses of DENSpm, while almost gone in normal tissues (15). Therefore, serum pharmacokinetic observations noted in our phase I study may not truly reflect intratumoral pharmacodynamic exposure.

Finally, it is plausible that interference with the polyamine pathway may not be an effective therapeutic approach as a single-agent as in this trial. Our data show the ability of CPENSpm and N^1-ethyl-N^{11}-[(cycloheptyl)methyl]-4,8-diazaundecane (CHENSpm) to synergize with commonly

used cytotoxic agents in human breast cancer cell lines. (30) Therefore, the favorable human safety profile observed with this class of compounds as single agents supports the evaluation of polyamine analogues in combination regimens with standard therapies, and human trials will properly examine the impact of combinatorial strategies being developed in pre-clinical models. Prevention studies of polyamine analogues in women at high-risk for breast cancer and their evaluation in the pre-operative setting in patients with operable breast cancer will also permit tissue acquisition for assessment of the *in vivo* effect of these compounds on the polyamine pathway.

Newer analogues are under study and may be more effective. Indeed, a third generation of polyamine analogues has been synthesized (31, 32). Our group recently examined the preclinical *in vitro* and *in vivo* antineoplastic efficacy of SL-11144, a leading compound among the new generation of polyamine analogues designated as oligoamines. These compounds appear to have a more potent antiproliferative activity against breast cancer cells than previously observed with DENSpm, CPENSpm, and CHENSpm, with activation of multiple apoptotic pathways (33).

In summary, this phase II study of the first generation polyamine analogue, DENSpm, given once daily for five days and repeated every 21 days showed no evidence of clinical activity in women with previously treated metastatic breast cancer. Our trial highlights the difficulties of successfully recruiting a less heavily treated breast cancer patient population for studies with novel investigational compounds. These results confirm the tolerability and safety profile of polyamine analogues. Encouraging preclinical data with these compounds justify further clinical evaluation of other polyamine analogues, alone and in combination with standard cytotoxic regimens, for the treatment and possibly prevention of breast cancer.

ACKNOWLEDGEMENTS

We acknowledge the efforts of Dawn Young in data management and regulatory issues.

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FOOTNOTES

[1] Supported in part by U.S. Department of Defense award DAMD 17-99-1-9231, by the Breast Cancer Research Foundation, and by SPORE grant CA 88843 from the National Institutes of Health.

[2] Preliminary results presented in part at the Twenty-Fourth Annual San Antonio Breast Cancer Symposium, San Antonio, TX, December 10-13, 2001

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Table 1. Study Population

Characteristics	***Number***
No. of patients	16
Age, median (range)	52 (34 – 65) years
ECOG PS, median (0/1/2)	1 (6/10/0)
ER and/or PR positive disease	12
Sites of metastatic involvement	
Limited to soft tissue/soft tissue & bone	3/1
Limited to visceral disease	3
Both	9
Prior Therapy for Metastatic Disease	***Number***
Chemotherapy (one/two regimens)	1/15
Endocrine therapy	12
Both	12

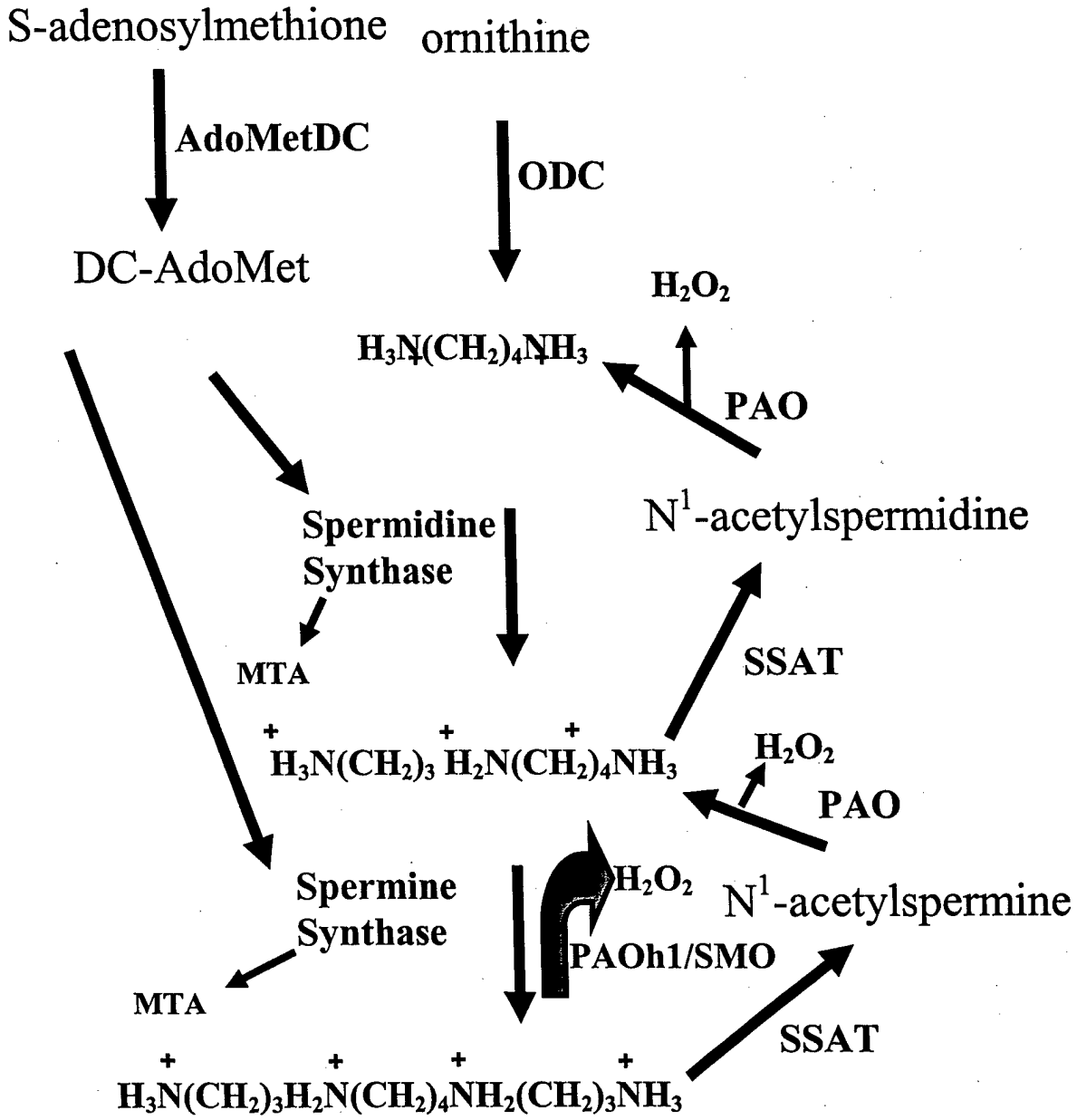
Table 2. Non-Hematologic Toxicities

Cycle No.	No. Patients/ Cycles	No. of Patients with Toxicity (CTC version 2)															
		CARDIOLOGY		DERMATOLOGY				GASTROINTESTINAL						NEUROLOGICAL			
		Edema		Flushing		Pruritus		Diarrhea		Nausea/Vomiting		Abdominal Pain		Perioral Numbness		Peripheral Neuropathy	
		Gr. 1	Gr. 2-3	Gr. 1	Gr. 2-3	Gr. 1-2	Gr. 3	Gr. 1-2	Gr. 3	Gr. 1-2	Gr. 3	Gr. 1-2	Gr. 3	Gr. 1-2	Gr. 3	Gr. 1	Gr. 2-3
Cycle 1	16/16	0	0	2	0	0	0	0	0	9	0	1	0	8	0	2	0
Cycle 2	14/14	0	0	0	0	2	0	1	0	1	0	1	2	2	0	0	0
Cycle ≥3	6/13	1	0	0	0	0	0	0	0	2	0	2	0	0	0	0	0

FIGURE LEGEND

Figure 1. The Polyamine Metabolic Pathway

AdoMetDC, s-adenosylmethionine decarboxylase; DC-AdoMet, decarboxylated AdoMet; ODC, ornithine decarboxylase; MTA, Methylthioadenosine; SSAT, spermidine/spermine N1-acetyltransferase; PAOh1/SMO, polyamine oxidase h1/spermine oxidase; PAO, N1-acetylpolyamine oxidase.


S-adenosylmethione
ornithine
AdoMetDC
ODC
DC-AdoMet
H_2O_2
$H_3N(CH_2)_4NH_3$
PAO
Spermidine Synthase
N^1-acetylspermidine
MTA
SSAT
$H_3N(CH_2)_3$ $H_2N(CH_2)_4NH_3$
H_2O_2
PAO
Spermine Synthase
H_2O_2
N^1-acetylspermine
PAOh1/SMO
MTA
SSAT
$H_3N(CH_2)_3H_2N(CH_2)_4NH_2(CH_2)_3NH_3$