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# Regulatory and antiproliferative effects of *N*-alkylated polyamine analogues in human and hamster pancreatic adenocarcinoma cell lines*

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**Summary.** *N*-Alkylated polyamine analogues have been shown to exert antiproliferative effects in several tumor models, with the bis-ethyl derivatives exerting the greatest suppression of polyamines by virtue of down-regulation of the polyamine biosynthetic enzymes. Pancreatic adenocarcinoma presents a challenge both clinically and experimentally due to its inherent resistance to conventional therapy, which results in its having the worst 5-year survival rate of all cancers. We have previously shown that $N^1,N^{12}$-bis(ethyl)spermine (BESPM) is much more potent than the polyamine enzyme inhibitor α-difluoromethylornithine (DFMO) against pancreatic adenocarcinoma cell lines. In the present study, we compared the biochemical and antiproliferative effects of two *N*-alkylated polyamine analogues, $N^1,N^{14}$-bis(ethyl)homospermine (BEHSPM) and $N^1,N^{11}$-bis(ethyl)norspermine (BENSPM) in two human pancreatic ductal adenocarcinoma cell lines, PANC-1 (poorly differentiated) and BxPC-3 (moderately well-differentiated), and in the WD PaCa (well-differentiated ductal) hamster cell line. BENSPM displayed greater antiproliferative activity in the human pancreatic cancer cell lines, whereas BEHSPM was more potent in the hamster cell line. Both BEHSPM and BENSPM suppress the activity of the major biosynthetic enzymes ornithine decarboxylase and *S*-adenosylmethionine decarboxylase. However, the induction of polyamine depletion in the human cell lines was only modest for BENSPM and minimal for BEHSPM, which suggests that the substantial antiproliferative activity of these analogues may result from mechanisms other than polyamine depletion. The somewhat greater polyamine depletion seen following treatment with BENSPM is thought to result from its striking induction of spermidine/spermine $N^1$-acetyltransferase. The biochemical and antiproliferative activity of BENSPM makes it an attractive agent for further preclinical and clinical development, especially in pancreatic cancer.

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## Introduction

Polyamines are essential for cell growth, proliferation, and DNA synthesis [30]. Hence, polyamine inhibitors have been developed as antitumor agents, which exploit the polyamine depletion that they induce and take advantage of the aberrant polyamine biosynthesis and metabolism in malignantly transformed cells [28, 42, 45]. Recently, polyamine analogues have been designed to take advantage of the cellular transport systems for natural polyamines and to induce polyamine depletion by negatively regulating the biosynthesis of polyamines while failing to support their essential functions in cell proliferation [35, 39]. We have shown in pancreatic adenocarcinoma cell lines that this regulatory approach to influencing the polyamine pathway is far more effective than the classic approach involving the inhibition of biosynthetic enzymes [15].

Until recently, screening and initial characterization of the metabolic effects of the polyamine analogues have mainly been conducted in murine leukemia cell lines (especially L1210) [36–38]. Published data are now available regarding the antitumor activity or metabolic effects of these polyamine analogues in human lung cancer [7], colon cancer [33], brain tumor [1], and malignant melanoma cell lines [41]. In the present study, we compared the biochemical and antiproliferative effects of two *N*-alkylated polyamine analogues, $N^1,N^{14}$-bis(ethyl)homospermine (BEHSPM) and $N^1,N^{11}$-bis(ethyl)norspermine (BENSPM) in two human pancreatic ductal adenocarcinoma cell lines, PANC-1 (poorly differentiated) and BxPC-3 (moderately well-differentiated), and in the WD PaCa (well-differentiated) hamster cell line. The structures of the analogues are depicted in Table 1. α-Difluoromethy-

**Table 1.** Structural representations of the spermine analogues BENSPM, BESPM, and BEHSPM

| $CH_3CH_2NH(CH_2)_nNH(CH_2)_nNH(CH_2)_nNHCH_2CH_3$ | | | |
|---|---|---|---|
| | ⇓ | ⇓ | ⇓ |
| BENSPM | 3 | 3 | 3 |
| BESPM | 3 | 4 | 3 |
| BEHSPM | 4 | 4 | 4 |

lornithine (DFMO), the irreversible inhibitor of ornithine decarboxylase (ODC, a rate-limiting enzyme in polyamine biosynthesis), was included for comparison. We have previously reported that $N^1,N^{12}$-bis(ethyl)spermine (BESPM) is much more potent than DFMO against pancreatic adenocarcinoma cell lines due to the more complete polyamine depletion induced by BESPM [15]. Data on L1210 murine leukemia show that BEHSPM is more active than either BENSPM or BESPM [3]. We have found that BEHSPM is indeed more potent in the rodent cell line WD PaCa. However, BENSPM is most potent in the human pancreatic cancer cell lines, which has implications for its clinical development and has been confirmed in melanoma cell lines [41].

In our efforts to explain the relative differences in antiproliferative response to the two *N*-alkylated spermine analogues, we examined their uptake and effects on polyamine biosynthetic and interconversion enzymes in the pancreatic cancer cell lines. In contrast to the limited effect of DFMO, the polyamine analogues affected the polyamine pathways at multiple levels. BENSPM proved to be the most active agent tested in the human pancreatic cancer cell lines, with BEHSPM being most active in the hamster cell line. The major differences in the cellular responses to the analogues were that BENSPM caused the greatest depletion of polyamines and massively induced the interconversion enzyme spermidine/spermine $N^1$-acetyltransferase (SSAT) in all of the cell lines tested. Nevertheless, both BENSPM and (especially) BEHSPM caused less polyamine depletion than that reported for BESPM [15] in two of the three cell lines used in the present study, which raises the question as to whether mechanisms of action other than polyamine depletion might be involved in the growth inhibition induced by BENSPM and BEHSPM (as contrasted with BESPM).

## Materials and methods

*Materials.* DFMO was supplied courtesy of Marion Merrell Research Center (Merrell Dow Pharmaceuticals, Cincinnati, Ohio). BENSPM and BEHSPM were synthesized as hydrochloride salts as previously described [2].

*Cell lines.* The characteristics of the cell lines used in this study have been described elsewhere [12, 26, 27]. PANC-1 and BxPC-3 were obtained from the American Type Culture Collection (Rockville, Md.). WD PaCa was developed from a carcinogen-induced, transplantable, well-differentiated pancreatic ductal adenocarcinoma in the Syrian golden hamster.

**Table 2.** Characterization and responses of human and hamster pancreatic-cancer cell lines to polyamine inhibitors

| Cell line (origin) | Degree of differentiation | DT (h) | $IC_{50}$ at 96 h (μM)[a] | | |
|---|---|---|---|---|---|
| | | | BENSPM | BEHSPM | DFMO |
| PANC-1 (human) | Poorly differentiated | 24–36 | 3.2 | 14 | 1,945 |
| BxPC-3 (human) | Moderately well-differentiated | 48–58 | 6.4 | 15 | 2,815 |
| WD PaCa (hamster) | Moderately well-differentiated | 24–36 | 30 | 4.2 | 3,670 |

[a] Mean values for 3–5 experiments, each involving quadruplicate controls and duplicate drug-exposed cultures
DT, Doubling time

*Culture conditions.* All cell lines were grown as adherent monolayers in culture and were passaged or grown for assays in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, Utah), glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml), and gentamicin (50 μg/ml) (all nonserum additives obtained from Grand Island Biological Co., Grand Island, N.Y.). Cells were harvested by short exposure to 0.25% trypsin. Cell counts were semiautomatically performed using a Cell-Dyn 900 counter (Sequoia-Turner Corp., Mountain View, Calif.). Cell counts determined electronically agreed well with manual counts using trypan blue staining.

*Inhibition determinations.* Polyamine inhibitors were added at 24 h after plating of cells in T-25 flasks; 100 μM aminoguanidine, an inhibitor of serum oxidase, was added to all cultures as a standard laboratory practice, although its addition was not necessary for the experiments reported herein since the ethylated polyamine analogues are not substrates for polyamine oxidase (unlike natural polyamines). For growth-inhibition assays, cultures were terminated after 4 days' incubation at 37° C in an atmosphere containing 5% $CO_2$. Results were expressed as T/C values using the equation,

$$\frac{\text{Recovery}_{\text{treated}}}{\text{Recovery}_{\text{control}}} \text{ or } \frac{(n/n_0)_{\text{treated}}}{(n/n_0)_{\text{control}}},$$

where $n_0$ represents the numbers of cell seeded on day zero and $n$ represents the average number of cells counted on the day of harvest. Concentrations inhibiting the growth of 50% of the cell population ($IC_{50}$ values) were determined using the median-effect equation for each dose-effect curve [16, 17].

*ODC and S-adenosylmethionine decarboxylase activity determinations.* ODC and *S*-adenosylmethionine decarboxylase (AdoMetDC) activities were determined on cell sonicates using slightly modified standard methods based on the enzymatic liberation of $[^{14}C]$-$O_2$ from carboxyl-labeled substrate [31, 32].

*Measurement of polyamine and analogue levels.* Cells were extracted with 0.6 M $HClO_4$. After dansyl chloride derivatization, polyamines were assayed by high-performance liquid chromatography (HPLC) according to a modification of the method of Bergeron et al. [3], on a gradient of 45% aqueous acetonitrile:methanol (5%:95%, v/v over 30 min) using fluorescence detection and external standards.

*SSAT activity determinations.* SSAT activity was measured according to the conversion of the labeled substrate $[^{14}C]$-acetyl-concanavalin A to $[^{14}C]$-acetylspermidine as previously described [24]. The results were expressed in picomoles per minute per milligram of protein.

**Table 3.** Effect of BENSPM, BEHSPM, and DFMO on polyamine enzymes in log-phase and 72-h pancreatic-adenocarcinoma cell lines

| Cell line | Treatment | Decarboxylase activity (nmol mg protein$^{-1}$ h$^{-1}$)[a] | | | |
|---|---|---|---|---|---|
| | | ODC (%control) | | AdoMetDC (%control) | |
| | | Log phase[b] | 72 h | Log phase[b] | 72 h |
| PANC-1 | None | 1.2 | 1.3 | 0.44 | 0.25 |
| | 10 μM BENSPM | 0.05 (4.1%) | 0.06 (4.7%) | 0.02 (3.4%) | 0.11 (45%) |
| | 10 μM BEHSPM | 0.12 (9.9%) | 0.06 (4.7%) | 0.10 (23%) | 0.06 (25%) |
| | 5 mM DFMO | 0.23 (19%) | 0.38 (29%) | 1.9 (441%) | 2.9 (1196%) |
| BxPC-3 | None | 0.59 | 0.17 | 0.34 | 0.10 |
| | 10 μM BENSPM | 0.03 (5.1%) | ND (0) | ND (0) | ND (0) |
| | 10 μM BEHSPM | 0.03 (5.1%) | ND (0) | 0.05 (15%) | 0.01 (11%) |
| | 5 mM DFMO | 0.07 (12%) | 0.04 (24%) | 0.87 (256%) | 2.4 (2548%) |
| WDPaCa | None | 25 | 8.7 | 0.52 | 0.23 |
| | 10 μM BENSPM | 0.41 (1.6%) | 0.07 (0.8%) | 0.14 (26.0%) | ND (0) |
| | 10 μM BEHSPM | 0.39 (1.6%) | 0.06 (0.7%) | ND (0) | ND (0) |
| | 5 mM DFMO | 3.4 (14%) | 0.56 (6.5%) | 2.4 (460%) | 1.9 (849%) |

[a] Mean values for 2–3 experiments, each involving duplicate samples
[b] 24 h
ND, Not detectable

**Table 4.** Effect of BENSPM, BEHSPM, and DFMO on polyamine pools in log-phase and 72 h cultures of pancreatic-adenocarcinoma cell lines

| Cell line | Treatment | Polyamine pools (nmol/10$^7$ cells)[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Log phase (%control)[b] | | | | 72 h (%control) | | | |
| | | PUT | SPD | SPM | BEN-/BEH-SPM | PUT | SPD | SPM | BEN-/BEH-SPM |
| PANC-1 | None | 2.1 | 9.9 | 9.6 | | 1.6 | 5.2 | 6.4 | |
| | 10 μM BENSPM | 2.0 (95%) | 2.3 (32%) | 3.5 (37%) | 26 | 1.5 (86%) | 1.8 (34%) | 3.0 (47%) | 20 |
| | 10 μM BEHSPM | 1.76 (82%) | 5.0 (53%) | 8.1 (85%) | 21 | 1.4 (82%) | 1.9 (36%) | 6.1 (95%) | 28 |
| | 5 mM DFMO | 1.4 (67%) | 5.6 (49%) | 14 (141%) | | 1.3 (82%) | 1.0 (19%) | 9.3 (146%) | |
| BxPC-3 | None | 1.6 | 16 | 10 | | 0.94 | 11 | 11 | |
| | 10 μM BENSPM | 0.90 (58%) | 2.8 (34%) | 2.5 (24%) | 13 | 0.78 (83%) | ND (0) | 1.6 (15%) | 29 |
| | 10 μM BEHSPM | 0.70 (42%) | 5.8 (35%) | 7.9 (77%) | 16 | 0.82 (88%) | 4.1 (38%) | 9.0 (82%) | 29 |
| | 5 mM DFMO | 0.64 (41%) | 5.6 (34%) | 8.9 (87%) | | 0.85 (91%) | 1.5 (13%) | 11 (101%) | |
| WDPaCa | None | 0.45 | 4.2 | 15 | | 0.38 | 5.5 | 8.7 | |
| | 10 μM BENSPM | 0.30 (67%) | 0.71 (17%) | 2.5 (17%) | 19 | 0.24 (63%) | ND (0) | 1.9 (21%) | 18 |
| | 10 μM BEHSPM | 0.31 (70%) | ND (0) | 14 (90%) | 28 | 0.28 (73%) | ND (0) | 3.8 (49%) | 20 |
| | 5 mM DFMO | 0.44 (99%) | 0.96 (23%) | 19 (128%) | | 0.28 (73%) | 0.12 (2.2%) | 8.2 (94%) | |

[a] Mean values for 2–3 experiments, each involving duplicate samples
[b] 24 h
ND, Not detectable; PUT, putrescine

## Results

The $IC_{50}$ values obtained for the polyamine analogues and DFMO in the three pancreatic adenocarcinoma cell lines are summarized in Table 2. Considerable in vitro activity against human pancreatic cancer cell lines was demonstrated for BENSPM and BEHSPM, with BENSPM being slightly more active. In contrast to the human pancreatic cancer cell lines but similar to murine L1210 [3], WD PaCa, a hamster cell line, showed a greater antiprolifera-

**Table 5.** Effect of BENSPM and BEHSPM on SSAT in log-phase and 72-h cultures of pancreatic-adenocarcinoma cell lines

| Cell line | Treatment | SSAT activity (pmol $min^{-1}$ mg $protein^{-1}$)[a] | |
|---|---|---|---|
| | | Log phase[b] | 72 h |
| PANC-1 | None | 31 | 34 |
| | 10 μM BENSPM | 8,029 (256) | 23,304 (694) |
| | 10 μM BEHSPM | 89 (2.9) | 184 (5.5) |
| BxPC-3 | None | 21 | 27 |
| | 10 μM BENSPM | 3,456 (165) | 15,399 (580) |
| | 10 μM BEHSPM | 32 (1.5) | 67 (2.5) |
| WDPaCa | None | 32 | 33 |
| | 10 μM BENSPM | 22,716 (718) | 23,394 (714) |
| | 10 μM BEHSPM | 1,532 (48) | 1,390 (42) |

[a] Data represent the mean orders of magnitude of SSAT activity for 2 (24-h) and 3 (72-h) experiments, each involving duplicate samples
[b] 24 h

tive response to BEHSPM than to BENSPM. For reference, the $IC_{50}$ values for BESPM in the PANC-1 and BxPC-3 cell lines were 6.1 and 48.3 μM, respectively [15].

As shown in Table 3, BENSPM and BEHSPM suppressed both major biosynthetic enzymes of the polyamine pathway, ODC (to $\leq 10\%$ of the control value) and AdoMetDC (to 0–45% of the control level), in all cell lines. In contrast to the analogues and in confirmation of our previous findings for BESPM [15], DFMO inhibited only ODC and stimulated AdoMetDC. Table 4 shows the results of polyamine pool determinations and the cellular uptake of the analogues. No major difference was found in the cellular accumulation of the two polyamine analogues in any of the cell lines. In all cell lines, BENSPM caused greater polyamine depletion than did BEHSPM. Indeed, BEHSPM produced only minimal polyamine depletion in the two human pancreatic-cancer cell lines despite its substantial antiproliferative activity in comparison with that of DFMO. Although BENSPM caused greater depletion of spermidine (SPD) and spermine (SPM) than did BEHSPM, it should be noted that the observed depletion of these polyamines was much lower than that previously reported for BESPM, which caused the depletion of $\geq 89\%$ of the SPD and SPM in confluent cultures of PANC-1 and BxPC-3 [15]. These results raise questions about both the relationship of polyamine pool depletion to antiproliferative activity and the mechanism by which polyamine pools remain higher following incubation with certain analogues than after treatment with others despite the similar ability of the analogues to down-regulate the activity of the polyamine biosynthetic enzymes.

One mechanism by which the greater SPD and SPM depletion caused by BENSPM as compared with BEHSPM might have resulted involves the interconversion pathway. Our finding of a striking increase in the SSAT activity induced by BENSPM (see Table 5) may support this hypothesis. In terms of SSAT induction, the hamster cell line showed a pattern of response similar to that displayed by the human cell lines; however, WDPaCa exhibited even greater induction of SSAT by both analogues.

## Discussion

Our previous studies in experimental models of pancreatic adenocarcinoma have demonstrated that these cell lines display relative *intrinsic* resistance to a variety of anticancer agents [10–12]. The resistance has been characterized as "atypical" multidrug resistance, since these cell lines do not overexpress P-glycoprotein [13]. Because of the clinical [19, 23] and experimental chemoresistance of pancreatic cancer to conventional anticancer agents, we have pursued novel approaches to its therapy. The approach taken has been called "regulatory," in that the polyamine analogues investigated have been shown to substitute for the natural polyamines and to act as enzyme regulators, suppressing both ODC and AdoMetDC by posttranscriptional control of enzyme synthesis [3, 38–40]. The greater efficacy of the analogues as compared with DFMO has been presumed to be based on the profound polyamine depletion they induce. The activity of the analogues makes them attractive agents for further study in pancreatic cancer, for which no effective therapy is available.

SSAT is the first enzyme in the so-called interconversion pathway, whereby the $N^1$-acetyl-SPM and $N^1$-acetyl-SPD formed by SSAT can be back-converted to SPD and putrescine, (PUT), respectively, by elimination of the acetamidopropyl moiety as catalyzed by polyamine oxidase [5, 18, 20, 21]. The induction of SSAT by the *N*-alkylated analogues is presumably a greatly exaggerated form of the normal induction of SSAT in response to increased intracellular polyamines, with $N^1$-acetyl-SPD (but not $N^1$-acetyl-SPM) being considered a normal excretory product [44]. SSAT protein and mRNA have been shown to increase in cells exposed to BENSPM, which may cause a change in the transcription or stability of the mRNA [9, 25, 34]. In addition, recent data support the possibility that SSAT protein is stabilized by competitive analogue inhibition, which may contribute to its massive intracellular accumulation [41]. The extent to which the striking increase in SSAT induced by BENSPM (as compared with the other bis-ethyl analogues) can be attributed to differences in binding properties remains unknown at present.

Our present finding of the relative lack of depletion of PUT in BENSPM-treated cells suggests that the source of the PUT is the interconversion pathway. Since about 30% of PUT is usually purported to originate from the interconversion of SPD and the other 70%, from the decarboxylation of ornithine [43], the only possible source of PUT in the presence of massive suppression of ODC would involve the interconversion pathway. Although work with NIH 3T3 cells has suggested that malignant cells may preferentially excrete rather than reutilize acetylpolyamines [29], our data suggest that pancreatic adenocarcinoma cell lines can back-convert a considerable proportion

of the acetylated derivatives to PUT. Our findings differ from those of Casero et al. [8], who found that the massive induction of SSAT in lung cancer cell lines was associated with nonmeasurable PUT levels. The reason for this discrepancy is unclear at present; however, enhanced polyamine transport is *unlikely* to play a role, since the *N*-alkylated polyamine analogues have been shown to turn off polyamine transport effectively (unlike DFMO, which stimulates polyamine transport) [6, 22].

The present study affirms the activity of the *N*-alkylated polyamine analogues and suggests that BENSPM is the most promising analogue to pursue in human models. In addition, BENSPM is the most interesting agent from the standpoint of its regulatory effects on polyamine biosynthetic enzymes and SSAT induction. Nevertheless, a number of questions remain unanswered. We cannot yet provide a good explanation for the relative differences in the responses observed among the various cell lines, especially the difference in antiproliferative effects noted between the human cell lines and the hamster WD PaCa line (which showed the same hierarchy of response to the analogues as previously found for murine L1210 cells). WD PaCa showed similar, if not exaggerated, patterns of enzyme responses to BENSPM and BEHSPM as compared with the responses seen in the two human pancreatic cancer cell lines. Indeed, WD PaCa exhibited SSAT-induction levels that were among the highest found in any rodent line tested to date [3, 14, 34; Bergeron et al., submitted for publication]. However, the role played by the induction of SSAT in the antiproliferative effects of BENSPM remains unclear, especially since the striking SSAT induction by this analogue in the present study appeared to be inconsistently related to its relative antiproliferative activity in various cell lines. The present findings argue against the hypothesis that the antiproliferative effects of BENSPM and BEHSPM would be attributable to net effects resulting in polyamine depletion, despite the attractiveness of this conclusion for comparisons of the polyamine analogues with an enzyme inhibitor such as DFMO. Recent preliminary data suggest that differential binding to or interaction of the analogues with other intracellular sites such as DNA may account for some of the dissociation of effects on polyamine pools and antiproliferative activity (unpublished observations). Our present data demonstrating only minimal polyamine depletion for BEHSPM and moderate polyamine depletion for BENSPM (in comparison with the nearly 90% depletion of pools induced by BESPM) in association with substantial antiproliferative activity support this supposition.

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