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COMPARISON OF THE EFFECTS OF TREATMENT WITH THE POLYAMINE ANALOGUE N^1,N^8BIS(ETHYL)SPERMIDINE (BESpd) OR DIFLUOROMETHYLORNITHINE (DFMO) ON THE TOPOISOMERASE II MEDIATED FORMATION OF 4'-(9-ACRIDINYLAMINO) METHANESULFON-M-ANISIDIDE (m-AMSA) INDUCED CLEAVABLE COMPLEX IN THE HUMAN LUNG CARCINOMA LINE NCI H157

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Summary: The positively charged polyamines putrescine, spermidine, and spermine are thought to be important in the maintenance of chromosomal structure. Polyamine depletion by the ornithine decarboxylase inhibitor, 2-difluoromethyl-ornithine (DFMO) is known to alter the effect of several DNA active agents, presumably resulting from the altered conformation of the polyamine depleted DNA. Here we compare the polyamine depletion effects of DFMO and the spermidine analogue N^1,N^8 bis(ethyl)spermidine (BESpd) on the formation of Topoisomerase II mediated, 4'-(9-acridinylamino) methane-sulfon-m-anisidide (m-AMSA) induced cleavable complex formation in human large cell undifferentiated lung carcinoma NCI H157 cells. This human cell line responds in the normal cytostatic manner to DFMO, whereas it responds in an unusual cytotoxic manner to treatment with BESpd. Here we report that neither DFMO nor BESpd alone affects the formation of cleavable complex. However, both compounds significantly enhance the m-AMSA induced formation of cleavable complex, each by approximately 1.6 fold. These results indicate that both DFMO and BESpd lead to a similar depletion of nuclear polyamines. Additionally, although BESpd closely resembles the natural polyamine spermidine, it appears that it cannot substitute for Spd at the level of DNA.

INTRODUCTION: The polyamines Put , Spd, and Spm have long been known to be important in cell proliferation; however, the precise mechanisms by which these polycationic alkylamines function are unknown (1-4). Of the many functions postulated for polyamines, good evidence exists that one of the important roles played by the polyamines is their influence on DNA conformation. The activity of several DNA-active antiproliferative agents are altered when cells are first depleted of Put and Spd by pretreatment with the ODC inhibitor DFMO (5-12). The activity of the alkylating agent BCNU is markedly enhanced by polyamine

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Abbreviations used: BESpd, N^1,N^8 bis(ethyl) spermidine; DFMO, 2-difluoromethylornithine; DMSO, dimethylsulfoxide; m-AMSA, 4'-(9-acridinylamino) methanesulfon-m-anisidide; ODC, ornithine decarboxylase; Put, putrescine; SDS, sodium dodecylsulfate; Spd, spermidine; Spm, spermine; Topo II, Topoisomerase II.

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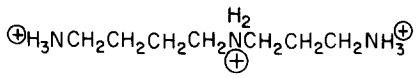
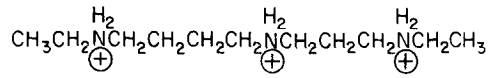
depletion by DFMO, whereas the activity of cis-platinum is greatly reduced (8, 9, 11). Marton and co-workers have proposed that these results indicate polyamine depletion can change DNA conformation to more or less favorable configurations, respectively, for drug activity (13). Similarly, DNA conformational changes by polyamine depletion have been implicated in the enhancement of strand scission activity associated with the drug, Etoposide (VP-16) (14).

DFMO has been the most studied inhibitor of the polyamine biosynthetic pathway and its use in several tumor models has validated the strategy of polyamine inhibition as a potential chemotherapeutic tool in the treatment of human cancers. However, the clinical results with DFMO thus far have not been encouraging. As an alternate approach to the direct inhibition of ODC, we and others have studied the effects of several polyamine analogues on cell growth (4,15-20). These analogues are similar to DFMO in their effects on the polyamine pools, but do not present some of the problems which may be responsible for the clinical inefficacy of DFMO, particular uptake and cellular compensatory mechanisms (20). Similar to DFMO, however, most of the analogues studied thus far result in a cytostatic response rather than overt cytotoxicity (16, 19, 20).

Recently, we have reported an unusual cytotoxic response to the polyamine analogue BESpd (Figure 1) by the relatively DFMO resistant human lung cancer cell line NCI H157 large cell undifferentiated carcinoma (15). BESpd treatment results in near complete inhibition of ODC and causes a rapid reduction in all intracellular polyamines, including Spm. Since BESpd causes a profound toxic response in H157 cells, yet closely resembles the natural polyamine Spd (Figure 1), we were interested to determine potential effects of BESpd in combination with a DNA active agent. Polyamine depletion by DFMO has been shown to increase the amount of strand scission produced by the DNA intercalator m-AMSA (7). This scission appears to be a result of Topo II activity, resulting in the formation of a covalent complex of Topo II and DNA in the presence of m-ASMA (21). Therefore, we chose to compare the effects of BESpd treatment on



SPERMIDINE



N^1,N^8-BIS(ETHYL) SPERMIDINE

Figure 1. Structures of spermidine and N^1,N^8 bis(ethyl)spermidine.

the formation of the intermediate protein Topo II/DNA covalent complex in the presence of m-ASMA to the effects of DFMO. The current studies were designed to determine whether the polyamine depletion caused by BESpd treatment in NCI H157 caused a similar increase in cleavable complex formation as reported in other cell types pretreated with DFMO. However, BESpd closely resembles the natural polyamines, both in structure and charge (Figure 1). Thus, we sought to investigate the possibility that BESpd may maintain DNA in a conformation refractory to increased cleavable complex formation in response to m-AMSA exposure.

METHODS AND MATERIALS

Chemicals: BESpd as the trihydrochloride salt was synthesized and graciously provided by the laboratory of Professor R.J. Bergeron, University of Florida, Gainesville (22). DFMO was kindly provided by Merrell-Dow Research Institute (Cincinnati, OH). BESpd was prepared as a 10 mM stock solution in 0.1 M HCL. DFMO was prepared as a 1.0 M stock solution in 0.1 M NaOH, pH 7.0. m-AMSA was obtained from the Drug Synthesis and Chemistry Branch, DCT, NCI and prepared fresh as a 1 mM stock in DMSO.

Cell Culture: The NCI H157 human large cell undifferentiated lung carcinoma cell line was maintained as previously described (16) in RPMI 1640 with 9% fetal calf serum, penicillin (100 u/ml) and streptomycin (100 ug/ml). Cultures were treated as indicated in Results. After treatments, cells were harvested and assayed for cell growth, polyamine content, and cleavable complex formation as described below.

Cleavable complex assay: To estimate the amount of TOPO II/DNA complex formed, the previously published method of Rowe et al. (23) based on the method of Nelson et al. (24) was used. Treated and control cells were incubated for the entire drug treatment time in the presence of [^{3}H]-TdR (1 uCi/ml of 50 Ci/mmol, Amersham, Arlington Heights, IL) to label their DNA. This labeled medium was then removed, and cells were washed and exposed to 2 uM m-AMSA for 1 hr. in serum free medium. m-AMSA containing medium was then removed and cells were lysed in 1 ml of 65°C lysis buffer (1.25% SDS, 5mM EDTA [pH 8.0] and 0.4 mg/ml carrier DNA). Each sample was then passed 4 times through a 22 gauge needle, transferred to a 1.5 ml Eppendorf tube and incubated at 65°C for 10 minutes. Protein linked DNA was precipitated by adding 250 ul of 325 mM KCL to each sample. Samples were vortexed for 10 seconds and then cooled in ice for 10 minutes. Precipitates were pelleted for 15 minutes at 12,000 x g at 4°C. Supernatants were aspirated, and 50 ul of the supernatant was added to 5 ml of

Hydrofluor scintillation fluid (National Diagnostics, Somerville, New Jersey) and counted. Pellets were resuspended in 1 ml of wash buffer (10 mM Tris [pH 8.0], 100 mM KCL, 1 mM EDTA and 0.1 mg/ml carrier DNA) and incubated at 65°C for ten minutes. Samples were again cooled to 4°C, pelleted, and washed twice. After the last wash the samples were pelleted, and dissolved in 200 ul of H_2O at 65°C for 10 minutes, then added to 5 ml of scintillation fluid and counted. The counts in the pellet and the first supernatant were used to determine the percentage of DNA precipitated.

Polyamine Analysis and Ornithine decarboxylase activity. Intracellular polyamine and BESpd concentrations were estimated by the methods of Kabra et al. (25). However, the 10 mM KH_2PO_4 suggested was replaced with distilled H_2O. Perchloric acid extracts of whole cells were dansylated and chromatograms were resolved by reverse-phase high performance liquid chromatography with an increasing acetonitrile/H_2O gradient.

ODC activity was measured by the previously published method of Pegg and Seely (26). Proteins were quantitated by the method of Lowry et al. (27).

RESULTS: We were interested in examining those events which precede the overt cytotoxic effects of BESpd; therefore, a 3 day treatment of 60 uM BESpd was chosen. Based on previous studies (15), concentrations of DFMO and BESpd that produced similar growth inhibition by day 3 were chosen.

Each of the compounds effected approximately a 50% reduction in growth by day 3, with BESpd being somewhat more effective (Figure 2). It is important to note that consistent with previous studies (15,19), BESpd is considerably more potent than DFMO. Both DFMO and BESpd decreased intracellular Put and Spd (Table 1) to below detectable levels. Significantly, BESpd decreased the level of intracellular Spm by greater than 50%, whereas in the DFMO treated cells, Spm was relatively unaffected. BESpd and DFMO were approximately equally

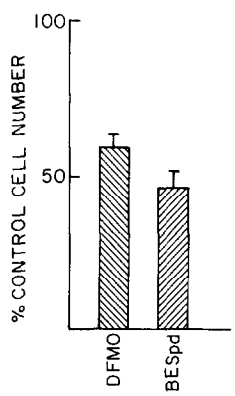


Figure 2. Effect of 5 mM DFMO or 60 uM BESpd on growth of NCI H157 cells after 3 day treatment. The initial seeding density was 5 x 10^6 cells/75 cm^2 flask. The total number of control cells on day 3 was 1.58 + .16 x 10^7 cells/75 cm^2 flask. The error bars indicate the standard deviation of 3 determinations.

Table 1. Effects of DFMO or BESpd treatment on polyamine levels and ornithine decarboxylase activity in NCI H157 human small cell lung carcinoma cells.

Treatment	Polyamines (nMol/10^6 cells)[1]				% Control ODC activity[2]
	Put	Spd	Spm	BESpd	
None	0.74+.03	1.40+.01	1.07+.02	---	100%
5 mM DFMO	<0.005	<0.005	1.04+.14	---	3.7
60 uM BESpd	<0.005	<0.005	0.51+.06	0.90+.17	ND[3]

[1] Means of three determinations + standard deviation.

[2] Means of at least 5 determinations each with a standard deviation less than 10%. The value for control cells was 280 pmol CO_2 released/10^6 cells/hr.

[3] ND, not detectable.

effective in reducing ODC activity to nearly undetectable levels. Viability of each treatment group was determined to be greater than 90% (by trypan blue exclusion).

To determine the effects of polyamine depletion by each agent on the association of Topo II and DNA, the formation of m-AMSA induced cleavable complexes were assayed using the K+-SDS precipitation assay described above (Figure 3). Typical for this assay (28), less than 1% of the cellular DNA was precipitable in control cells not exposed to m-AMSA. No appreciable change in the amount of precipitable complex occurred in cells treated with only DFMO, or BESpd (no m-AMSA exposure). In cells exposed to m-AMSA (no pretreatment) there was a 14-fold increase in the amount of TOPO II/DNA complex precipitated.

When the human lung cancer cells were depleted of polyamines with either DFMO or BESpd, then exposed to m-AMSA, there was a resultant significant increase (22.8 and 22.5%, respectively) in the amount of cleavable complexes precipitated as compared to those cells only exposed to m-AMSA. It is important to note that there was no significant difference between the DFMO induced increase in cleavable complex formation and that induced by BESpd.

DISCUSSION: Only recently, with the availability of specific inhibitors of polyamine biosynthesis such as DFMO, has it been possible to investigate *in situ* the earlier hypotheses that polyamines have functionally important

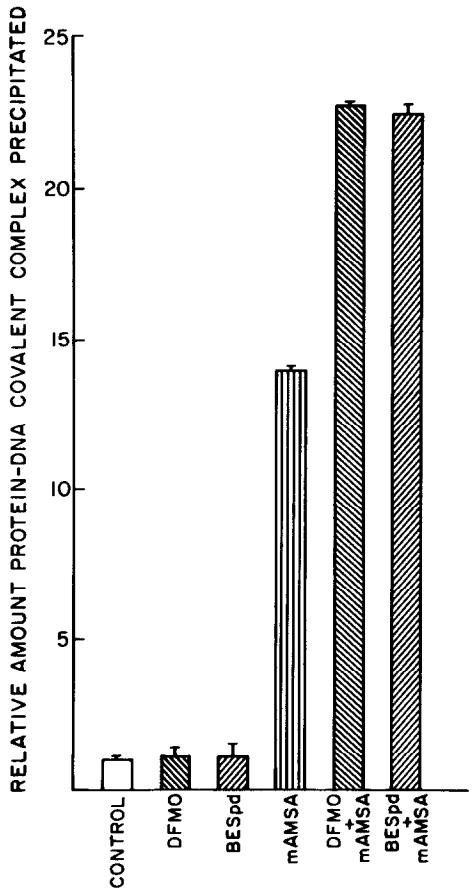


Figure 3. Effect of DFMO or BESpd 3 day pretreatment on m-AMSA induced cleavable complex formation in NCI H157 human large cell undifferentiated lung carcinoma cells. Results are expressed as a mean of at least 3 determinations with the standard deviation indicated by error bars. The actual value of the control cells was 5610 cpm precipitated from a total of 401,000 cpm labeled DNA.

associations with DNA (29). In vitro and in situ experiments suggest that the polyamines Put, Spd, and Spm alter DNA conformation, and their depletion can significantly affect the activity of DNA active cytotoxic agents (5-13,30). Most of the previous studies were performed with DFMO which irreversibly inhibits ODC and depletes cells of Put and Spd (generally not Spm) and typically leads to cytostasis (2,4). Our current study compares the effects of DFMO and the polyamine analogue BESpd, another inhibitor of polyamine biosynthesis, on the m-AMSA induced formation of Topo II/DNA cleavable complex.

BESpd treatment effectively depletes the human lung cancer line NCI H157 not only of Put and Spd, but in contrast to DFMO, also significantly reduces

the intracellular concentration of Spm (Table 1). This activity is thought to result from feedback mechanisms similar to those which occur when the natural polyamines are added to cultures, and presumably occurs because of the structural similarities between Spd and BESpd (15,18-20, Figure 1). These apparent structural similarities are sufficient to allow BESpd to act as a regulator of ODC (15,18-20); however, there are no indications that BESpd can substitute in function for the depleted polyamines (18).

Our current results indicate that polyamine depletion by either DFMO or BESpd leads to nearly equivalent enhancement of Topo II mediated DNA strand-scission. Previous reports have shown that pretreatment of murine L1210 leukemia cells with DFMO increases both Etoposide (VP-16) and m-AMSA induced DNA-scission (7,14) both of which are presumably mediated by Topo II activity (21). DFMO pretreatment was also found to synergistically enhance the cytoxic effects of Etoposide (VP-16) in both murine L1210 leukemia and 8226 human myeloma (14).

Previous reports have shown that in L1210 cells, DFMO treatment increases the number of single strand breaks produced by either m-AMSA or Etoposide by a factor of 1.4 - 1.9 (7,14). The 1.6-fold increase in m-AMSA induced cleavable complex formation with DFMO pretreatment described here, is in good agreement with these reports. Additionally, this agreement suggests that the mechanism of Topo II/DNA mediated reaction is qualitatively very similar between mammalian species. BESpd pretreatment results in essentially the same 1.6-fold enhancement of cleavable complex formation, further supporting the hypothesis that polyamine depletion produced by either DFMO or BESpd is specifically associated with the observed enhancement.

Our data are consistent with previous findings that BESpd does not substitute in general for the natural polyamines, and implies it specifically does not substitute at the chromatin level. It is however, important to note that BESpd treatment did not result in greater enhancement of m-AMSA activity than DFMO even though: 1) BESpd leads to a greater depletion of total polyamines than DFMO (including Spm) (15,18), and 2) BESpd treatment is

ultimately cytotoxic to NCI H157 human lung cancer cells (15), whereas DFMO is only cytostatic. These data suggest that the mechanism responsible for the observed cytotoxicity with BESpd may not be directly related to BESpd treatment effects on chromatin structure.

Although considerably more experimentation is necessary, the combination of agents which deplete polyamines and Topo II poisons such as m-AMSA provide intriguing clinical possibilities. DFMO alone has provided encouraging results in human small cell lung cancer models (31-33), actually resulting in significant cytotoxicity. However, as a single agent against the clinical disease, the results have not been encouraging (34). Human large cell lung cancers are not susceptible to the cytotoxic effects of DFMO (35,36). Currently, treatment modalities for nonresectable large cell lung cancers are severly lacking. However, we have recently shown that the large cell undifferentiated lung carcinoma NCI H157 is rapidly killed by the spermidine analogue, BESpd (15). Therefore, the potential enhancement of BESpd's cytotoxic activity by DNA active agents represent an avenue deserving further investigation.

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