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ORIGINAL ARTICLE

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Polyamine analog bis(ethylamino)-5,10,15-triazanonadecane (BE-4-4-4-4) enhances simian virus 40 late gene expression

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Abstract *Purpose*: The polyamine analog bis(ethylamino)-5,10,15-triazanonadecane (BE-4-4-4-4) depletes cellular polyamines and inhibits malignant cell growth. We have previously shown that BE-4-4-4-4 inhibits nucleosome condensation on supercoiled DNA in a cell-free system. Here we sought to determine whether BE-4-4-4-4 inhibits nucleosome condensation in cells, and whether that effect alters the expression of specific genes. *Methods*: We used the simian virus 40 (SV-40) minichromosome as a model system and studied the expression of the viral late genes. It is known that the SV-40 late genes are regulated by the steroid receptor elements that, in turn, control gene expression by altering nucleosomal organization. *Results*: We observed a more than six fold increase in SV-40 late gene expression in cells pretreated with BE-4-4-4-4 for 18 h. The polyamine analog bisethyl norspermine (BE-3-3-3), that does not affect nucleosomal condensation in cell free systems and has little effect on chromatin structure in cultured human tumor cells, had a negligible effect on SV-40 late gene expression under treatment conditions identical to those used with BE-4-4-4-4. *Conclusion*: Similar to the findings in the cell-free system, the polyamine analog BE-4-4-4-4 inhibited nucleosome formation and, thereby, altered the expression of specific genes in a cellular system.

Key words Polyamine analog · Chromatin structure · SV-40 · Late gene expression

Abbreviations *BE-3-3-3* bis(ethyl) norspermine · *BE-4-4-4-4* bis(ethylamino)-5,10,15-triazanonadecane · *DMEM* Dulbecco's minimal essential medium · *IBP* initiator binding protein · *ori* origin of replication · *PBS* phosphate-buffered saline · *SRE* steroid receptor element · *SSAT* spermidine/spermine acetyl transferase · *SV-40* simian virus 40 · *T-antigen* tumor antigen

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Introduction

The polyamines spermidine and spermine, and their precursor diamine putrescine, are organic cations found in all mammalian cells. They are absolutely required for cell growth and differentiation [22]. Cellular polyamines interact with negatively charged nucleic acids and alter the secondary structure of specific DNA sequences in cell-free systems (reviewed in reference 9). We have previously demonstrated that some polyamine analogs interact with DNA in a cell-free system in a different manner than do the naturally occurring polyamines. Most of these analogs inhibit cell growth and may even kill cells [9, 17]. One such analog, bisethyl norspermine (BE-3-3-3), is now undergoing phase I clinical trials as an anticancer agent and another analog, bis(ethylamino)-5,10,15-triazanonadecane (BE-4-4-4-4), is in the final stages of preclinical evaluation.

It has been shown that spermine facilitates chromatin condensation in cell-free systems (reviewed in reference 2). We have shown that BE-4-4-4-4 but not BE-3-3-3 inhibits nucleosome formation on covalently closed circular plasmid DNA in a cell-free system [4]. Although there is some indirect evidence that natural polyamines are involved in chromatin condensation in cells as well (reviewed in reference 2), it has not been clearly demonstrated so far. The use of polyamine analogs has only now enabled us to more directly address this issue. We used a genetically engineered simian virus 40 (SV-40) minichromosome as a model system to determine how polyamine analogs may specifically affect chromatin structure and gene expression in a cellular system.

The SV-40 genome consists of two genetic regions, early and late, oriented in opposite directions [8, 13, 14,

18, 23, 24]. Two divergent promoters and the viral origin of replication (*ori*) reside in close proximity in a genomic control region in the SV-40 DNA [18]. The early genes are transcribed soon after infection to produce two viral proteins, large and small tumor antigens (T-antigens). The large T-antigen binds to the 65 bp *ori* site and disrupts the chromatin structure at the *ori* site before the start of DNA replication [21]. In vitro studies have shown that the chromatin structure and the nucleosomal organization of the viral genomic control region can inhibit T-antigen functions [11, 12, 18, 25]. Subsequent to the start of DNA replication, the transcription of the viral late genes that code for three viral capsid proteins VP1-3 and an agnoprotein LP1 begins [8, 24]. During the initial phase of the viral lytic cycle, the late gene promoters remain associated with a group of cellular proteins called the initiator-binding proteins (IBPs). Mertz and coworkers [26, 27] have shown that the IBPs are members of the steroid/thyroid receptor hormone superfamily. The binding of IBPs either directly to the promoter sequence or to histones and/or nonhistone chromosomal protein(s) bound at the promoter sequence regulates the transcription of the late genes. It is well established that steroid hormone receptor elements (SREs) that respond to glucocorticoids (reviewed in reference 5) and estrogen [6] are regulated by specifically positioned nucleosomes [23]. Any changes in DNA structure that may remove the nucleosomes from the SREs may enhance the transcription from the promoters containing the SREs.

We pretreated host CV-1 African green monkey kidney cells for 18 h with BE-4-4-4-4 and BE-3–3-3. BE-4-4-4-4 markedly inhibits nucleosome formation on circular plasmid DNA in a cell free system while BE-3-3-3 has no effect on nucleosome formation in the same system [4]. We used a reporter gene to study the expression of the SV-40 late genes in the analog-treated cells. We observed a more than sixfold increase in reporter gene expression in BE-4-4-4-4-treated cells. In contrast, BE-3-3-3 treatment induced less than a twofold increase in reporter gene expression.

Materials and methods

Materials

BE-3-3-3 was a kind gift from Warner-Lambert-Parke-Davis (Rochester, N.Y.) and BE-4-4-4-4 was obtained from the drug synthesis and development section of the National Cancer Institute (Bethesda, Md.). The host CV-1PD African green monkey kidney cells and the *E. coli* plasmid pluc-WT containing a genetically engineered SV-40 genome in which the viral capsid protein VP-1 is truncated and a luciferase reporter gene is inserted [26, 27] were kind gifts from Dr. Janet Mertz's laboratory at the McArdle Laboratory for Cancer Research, University of Wisconsin, Madison.

Methods

Plasmid

The plasmid pluc-WT containing an ampicillin-resistant marker was transfected into a competent *E. coli* strain DH5a. Ampicillin-resistant clones were selected from an ampicillin-containing agar plate and were grown for 18 h at 37 °C in 500 ml LB broth supplemented with 60 μg/ml ampicillin with constant shaking. The plasmid was isolated using the Qiagen Plasmid Maxi Kit (Qiagen, Chatsworth, Calif.) following a previously reported procedure [16].

Cell culture

CV1-PD cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Gaithersberg, Md.), supplemented with 5% fetal bovine serum (Gemini Bio-Products, Calabasas, Calif.), 40 U/ml penicillin and 40 μg/ml streptomycin (Gibco-BRL, Gaithersberg, Md.) at 37 °C supplemented with a mixture of 5% CO_2 in air.

Transfection of CV1-PD cells with pluc-WT

Approximately 1×10^6 CV-1 cells were incubated in 100-mm dishes in 10 ml growth medium about 2 days before transfection. Approximately 24 h after seeding, appropriate concentrations of polyamine analog were added to the plates. About 18 h after the drug treatment, the cells were transfected with the plasmid pluc-WT following a previously reported procedure [10, 26].

Determination of luciferase activity

At the end of the drug treatment, the cells were washed twice with 5 ml ice-cold PBS (137 m*M* NaCl, 2 m*M* KCl, 8 m*M* $Na_2HPO_4 \cdot 7H_20$, 1.5 m*M* KH_2PO_4). Then 417 μl 1 × lysis reagent (25 m*M* Tris-phosphate, pH 7.8, 2 m*M* dithiothreitol, 2 m*M* 1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid, 10% glycerol, 1% Triton X-100) was added to each plate and distributed evenly over the plate. The lysate was scraped into a microcentrifuge tube, spun at 12 000 *g* for 5 s and the supernatant was transferred to a fresh tube. The supernatants were kept at −70 °C until use. The luciferase activity in the supernatants was determined using the Luciferase Assay System (Promega, Madison, Wis.) following the manufacturer supplied protocols [20].

Quantitation of SV-40 DNA from the transfected cells

After drug treatment, cells were washed twice each time with 5 ml ice-cold trisphosphate buffer (137 m*M* NaCl, 5 m*M* KCl, 0.7 m*M* Na_2HPO_4, 25 m*M* Tris-HCl; pH 7.4). To each dish 0.5 ml lysis buffer (0.2 *M* Tris-HCl, pH 7.5, 25 m*M* EDTA, 0.3 *M* NaCl, 2% SDS) was added. The lysis solution was distributed evenly over the plate by rocking gently and 25 μl proteinase K (20 mg/ml) was added to each plate and distributed by further rocking. The lysate was scraped into a microcentrifuge tube and SV-40 DNA from the transfected cells was isolated following a previously reported procedure [1]. The DNA was electrophoresed in a 0.8% agarose gel [15] and subjected to Southern hybridization and fluorescence detection following established procedures [1, 16]. The SV-40 construct pluc-WT was fluorescence labelled by random priming [16] and was used as the probe for Southern analysis. The blots were quantitated using a photomediplier tube (PMT) Fluoroimager SI (Molecular Dynamics, Sunnyvale, Calif.), using a PMT voltage of 650 mV and a 570 nm DF -30 band filter. The image was analyzed using Image Quant software (Molecular Dynamics).

Results and discussion

Effects of polyamine analog treatment on SV-40 DNA copy numbers

We first sought to determine whether polyamine analog treatment affected the amount of SV-40 DNA in the

host CV-1 cells. Near-confluent cultures of the host cells were treated with either BE-3-3-3 or BE-4-4-4-4 for 18 h. As reported previously, the treatment of near-confluent cell cultures did not appreciably affect cell growth even though the cells [17, 22] took up large amounts of analog. The analog-treated CV-1 cells were then transfected with the plasmid pluc-WT and incubated at 37 °C for 48 h before harvesting and lysis. One aliquot of the cell lysate was used to determine the amount of plasmid DNA in the transfected cells using Southern blot analysis. DNA isolated from the same number of transfected host cells was used in each lane and the blots were fluoroimaged and quantitated. The Southern blots before and after restriction enzyme Dpn I treatment are shown in Fig. 1a and Fig. 1b, respectively. The bands were compared with untreated and topoisomerase I-treated plasmid DNA to identify the relaxed and supercoiled plasmid DNA (Fig. 1a). Both the relaxed and the supercoiled DNA bands were taken together to estimate the amount of viral DNA content.

While BE-3-3-3 had little effect on the SV-40 DNA content, BE-4-4-4-4 markedly reduced the amount of SV-40 DNA both in the relaxed and in the supercoiled form (Fig. 1a). These findings suggest that BE-4-4-4-4 treatment possibly inhibited viral DNA replication. Plasmid DNA that is grown in prokaryotic systems gets methylated by bacterial methylases. Dpn I specifically digests methylated DNA sequences. Thus, Dpn I treatment removed the original plasmid DNA that was used for transfection leaving only the SV-40 DNA that had been replicated in the host CV-1 cells. No appreciable difference in the SV-40 band intensities before and after the Dpn I treatment was observed (compare lanes 2, 3 and 4 in Fig. 1a and b). This suggests that most of the intracellular SV-40 DNA was from the virus that had replicated in the host cells and, therefore, must have been existing in the minichromosomal state [8, 13, 24].

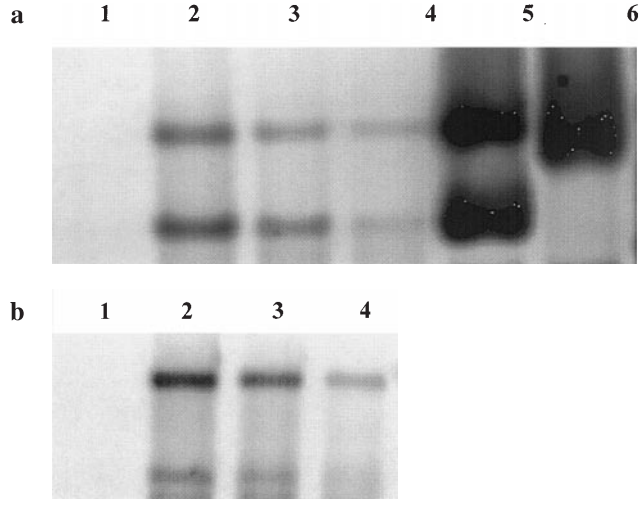

Fig. 1 **a** Digitized fluoroimage of the Southern blot of SV-40 DNA before Dpn I digestion: *lane 1* control (−pluc-WT), *lane 2* control (+ pluc-WT), *lane 3* 100 μ*M* BE-3-3-3 (+pluc-WT), *lane 4* 100 μ*M* BE-4-4-4-4 (+pluc-WT), *lane 5* plasmid (pluc-WT) DNA, *lane 6* plasmid DNA + topoisomerase I (100 U for 10 min). **b** Digitized fluoroimage of the Southern blot of SV-40 DNA after Dpn I digestion: *lane 1* control (− pluc-WT), *lane 2* control (+pluc-WT), *lane 3* 100 μ*M* BE-3-3-3 (+pluc-WT), *lane 4* 100 μ*M* BE-4-4-4-4 (+pluc-WT)

Table 1 Effect of an 18-h treatment with 100 μ*M* BE-3-3-3 and BE-4-4-4-4 on the expression of the luciferase reporter gene from the SV-40 late promoter

Sample	Luciferase activity (RLU)	Protein content (μg/assay)	Luciferase activity (RLU/μg protein)
Control (−luc)	20.7	5.04	4.10
Control (+luc)	1.2×10^6	4.60	2.6×10^5
BE-3-3-3 (+luc)	1.2×10^6	4.20	2.9×10^5
BE-4-4-4-4 (+luc)	1.9×10^6	3.70	5.1×10^5

luc luciferose; *RLU* relative light units

Effects of polyamine analogs on SV-40 late gene expression

We used the pluc-WT plasmid to study the effects of the polyamine analogs on viral late gene expression. This plasmid can transfect, transcribe and replicate in the host cells but cannot continue its lytic cycle due to truncation of the viral coat protein VP-1 [26, 27]. The transcription of the late genes in the replicated plasmid DNA was monitored by following the expression of the reporter luciferase gene in the cell lysate. Because the virus cannot complete its lytic cycle, the observed transcription of the late genes is without interference from any transcription due to a secondary transfection and replication cycle.

The effects of analog treatment on the luciferase activity are shown in Table 1. The data shown are representative of three separate sets of experiments each with two replicates with approximately 20% variations between separate sets of experiments. While BE-3-3-3 treatment had no effect on luciferase activity, slightly less than a twofold increase in luciferase activity was observed in the BE-4-4-4-4-treated cells. The data shown in Fig. 1, however, suggest that BE-4-4-4-4 treatment caused a marked reduction in the amount of viral DNA. Therefore, the luciferase activity in analog-treated cells was normalized for the viral DNA content. A representative plot of the data from three separate sets of experiments on the relative changes in the luciferase activity normalized for the amounts of viral DNA content is shown in Fig. 2. After normalization, BE-3-3-3 treated cell lysates showed only a slight (less than twofold) increase in luciferase activity. In contrast, in all three repeats, about a six- to sevenfold increase in luciferase activity was consistently observed in BE-4-4-4-4-treated cells. Most of the SV-40 DNA was from the viral genome that replicated in the host cells (as determined from the Dpn I treatment data). Therefore, the increase in the reporter gene activity was most likely due to a change in

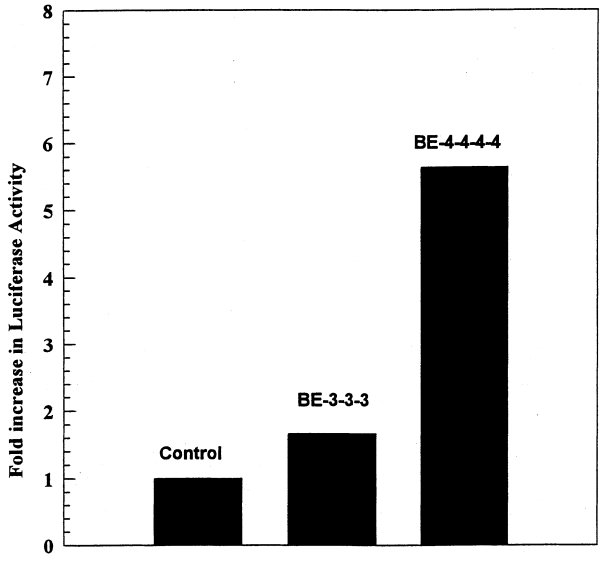

Fig. 2 Relative increase in luciferase activity in host CV-1 cells transfected with pluc-WT and treated with 100 μ*M* analog for 18 h

the minichromosomal structure of the viral genome that was synthesized in the BE-4-4-4-4-treated cells.

A representative plot of the data from three separate sets of experiments on the relative changes in the luciferase activity at various analog concentrations normalized for the amounts of viral DNA content is shown in Fig. 3. The luciferase activity increased sharply up to a BE-4-4-4-4 concentration of 10 μ*M* and then increased gradually with further increase in the analog concentration. We have previously reported that the dose dependence of BE-4-4-4-4 cytotoxicity in numerous human

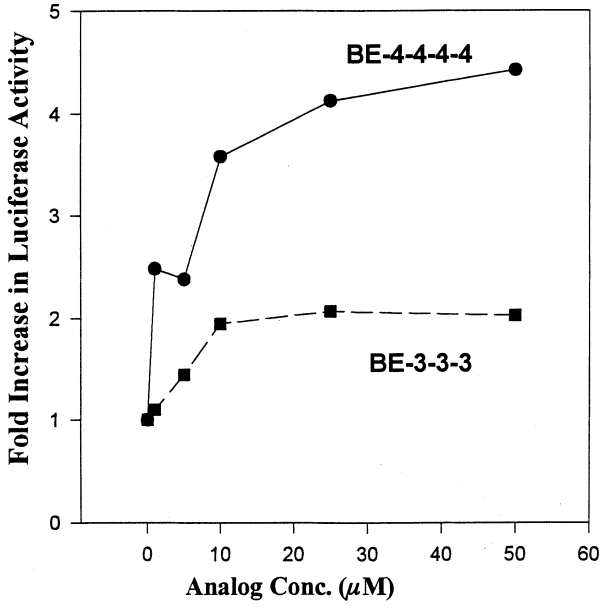

Fig. 3 Relative increase in luciferase activity in host CV-1 cells treated with increasing concentrations of BE-3-3-3 (■) and BE-4-4-4-4 (●)

tumor cells also increases sharply up to a concentration of 10 μ*M* before starting to level off [3]. Such a correlation between the dose dependence of the cytotoxicity and the dose dependence of SV-40 late gene expression strongly indicates that BE-4-4-4-4 cytotoxicity may be induced by changes in the chromatin structure and resultant changes in cellular metabolism. Although a similar concentration-dependent cell kill was observed in BE-3-3-3-treated cells [7, 19]. This analog caused a negligible increase in luciferase activity. This suggests that BE-3-3-3 differs from BE-4-4-4-4 in its mechanism of cytotoxicity. A superinduction of the polyamine catabolic enzyme spermidine/spermine acetyl transferase (SSAT) was observed for BE-3-3-3 but not for BE-4-4-4-4 [7]. Effects of SSAT on cellular oxidative stress and the resultant increase in apoptosis have been suggested as a possible mechanism of BE-3-3-3-induced cell kill [19].

The observed increase in luciferase activity in BE-4-4-4-4-treated cells may also have been due to an analog-induced increase in the cellular translational efficiency. Although it has been demonstrated that some polyamine analogs increase the translational efficiency of SSAT mRNA [19], this effect has been shown to be due to an analog-induced stabilization of a specific mRNA [19] and the increase in the SSAT activity was minimal in the case of BE-4-4-4-4-treated cells [7]. Moreover, none of the polyamine analogs tested so far had any effect on general cellular translation processes and in the CV-1 cells, we found no increase in the amount of cellular protein content in any of the analog-treated cells (Table 1). Thus, the increase in luciferase activity was most likely not due to an increase in the translational efficiency of the enzyme mRNA. We are now using Northern analysis to confirm this conclusion. To the best of our knowledge, this is the first report that the effect of the cytotoxic polyamine analog BE-4-4-4-4 on the secondary and higher order chromatin structure may have a specific biological function in regulating DNA replication and the expression of specific gene(s) in a cellular system.

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