The structure of polyamine analogues determines haemoglobin production and cytotoxicity in murine erythroleukaemia cells

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The naturally occurring polyamine spermine induces haemoglobin synthesis in murine erythroleukaemia (MEL) cells. We have studied the ability of various polyamine analogues to inhibit cell growth and induce haemoglobin production. Polyamine analogues with free terminal amino groups were good inducers of haemoglobin production in MEL cells. Haemoglobin levels correlated with the number of positive charges: pentamines

(five positive charges) were stronger inducers than tetramines (four positive charges). Compounds ethylated at their terminal amines were poor inducers of haemoglobin production but good inhibitors of MEL cell growth. These results provide evidence that polyamine analogues support specific biological functions of polyamines in MEL cells and suggest relationships between polyamine structure and function.

INTRODUCTION

The polyamines putrescine, spermidine and spermine are biological polycations that are essential for the growth and differentiation of mammalian cells [1,2]. Depletion of polyamines in cultured cells or addition of polyamines to isolated nuclei alters the expression of genes participating in cellular growth, and differentiation is altered [3–6]. We have found that spermine induces haemoglobin production in murine erythroleukaemia (MEL) cells without affecting the expression of actin [6a]. The demonstration that polyamines directly affect DNA and chromatin both *in vitro* and in tissue culture [7–11] suggests that polyamines regulate transcription of specific genes by interacting directly with chromatin elements.

Polyamine analogues designed to mimic the structure of naturally occurring polyamines have been useful in probing the role of polyamines in cellular proliferation [12,13] and differentiation [14,15]. These analogues deplete cells of naturally occurring polyamines and stop cell growth [13]. The growth-inhibitory effect is accompanied by repression of ornithine decarboxylase activity [16] and induction of spermidine/spermine acetyltransferase activity [17]. However, some analogues do not cause growth arrest even when they deplete natural polyamines [18], which suggests that they are able to mimic the growth functions of naturally occurring polyamines, perhaps by interacting directly at sites of polyamine function. Several polyamine analogues are in clinical trial for human cancer.

MEL cells are a model for the regulation of erythroid differentiation [17]. Chemicals such as dimethyl sulphoxide or hexamethylenebisacetamide (HMBA) stimulate MEL cells to differentiate along the erythroid lineage [19]. These agents induce a co-ordinated programme of molecular events, including expression of globin RNA, haem synthesis, growth inhibition, decrease in cell volume, chromatin condensation and expression of erythroid membrane antigens. We have found that spermine stimulates haemoglobin synthesis in MEL cells, but does not induce the entire differentiation programme [6a]. Spermine

increased levels of globin mRNA and haemoglobin but did not induce the cell-cycle block or the decrease in cell size seen with HMBA-induced differentiation. This effect is reminiscent of the effects of compounds such as haemin or tiazofurin [20,21]. In the present study we have examined the ability of several polyamine analogues to induce haemoglobin production. The goal of the study was to evaluate relationships between polyamine structure and function in the MEL cell model.

EXPERIMENTAL

The polyamine analogues (Table 1) were kindly provided by Dr. R. J. Bergeron (University of Florida, Gainesville, FL, U.S.A.) and Dr. K. Samejima (Josai University, Saitama, Japan). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Mycoplasma-free MEL DS-19 cells, taken from exponentially growing cultures in minimal essential medium supplemented with 10 mM Hepes, 10% fetal calf serum, non-essential amino acids, penicillin and streptomycin, were seeded at 5×10^4 cells/ml and incubated at 37 °C in 5% CO₂. HMBA (5 mM) or polyamine analogue (100 µM) was added along with aminoguanidine (2 mM) to prevent the formation of toxic end-products by serum diamine oxidase [22].

Cells were counted daily using a haemocytometer. Cell viability was measured by standard Trypan Blue exclusion. Cell volume was determined using a particle counter (Coulter Counter S-Plus VI; Coulter Electronics, Hialeah, FL, U.S.A.).

Haemoglobin synthesis was assessed by a cytochemical staining procedure using 3,3',5,5'-tetramethylbenzidine [23]. Cellular haem content was determined fluorimetrically after boiling the cells in the presence of oxalic acid [24].

Intracellular levels of polyamines and analogues were measured after dansylation by reverse-phase HPLC and quantified fluorimetrically [25].

Abbreviations used: MEL, murine erythroleukaemia; HMBA, hexamethylenebisacetamide.

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Table 1 Names and chemical structures of polyamine analogues used in this study

Abbreviation	Trivial name	Systematic (IUPAC) name	Chemical formula
3-3-3	Norspermine	1,11-Diamino-4,8-diazaundecane	$\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_3\text{NH}_2$
BE-3-3-3	Bis(ethyl)norspermine	1,11-Bisethylamino-4,8-diazaundecane	$\text{C}_2\text{H}_5\text{NH}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_3\text{NH}\text{C}_2\text{H}_5$
BE-3-4-3	Bis(ethyl)spermine	1,12-Bisethylamino-4,9-diazadodecane	$\text{C}_2\text{H}_5\text{NH}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NH}\text{C}_2\text{H}_5$
BE-4-4-4	Bis(ethyl)homospermine	1,14-Bisethylamino-5,10-diazatetradecane	$\text{C}_2\text{H}_5\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_4\text{NH}\text{C}_2\text{H}_5$
3-3-3-3	Caldopentamine	1,15-Diamino-4,8,12-triazapentadecane	$\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_3\text{NH}_2$
4-4-4-4		1,19-Diamino-5,10,15-triazanonadecane	$\text{NH}_2(\text{CH}_2)_4\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_4\text{NH}_2$
BE-4-4-4-4		1,19-Bisethylamino-5,10,15-triazanonadecane	$\text{C}_2\text{H}_5\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_4\text{NH}\text{C}_2\text{H}_5$

Figure 1 Induction of benzidine-positive cells by HMBA (5 mM) or polyamine analogues (100 μM)

MEL cells were treated with (a) 3-3-3 ($\square$), BE-3-4-3 ($\circ$), BE-3-3-3 ($\blacksquare$), BE-4-4-4 ($\blacklozenge$), (b) 3-3-3-3 ($\bullet$), 4-4-4-4 ($\triangle$), BE-4-4-4-4 ($\blacktriangle$) or HMBA ($\circ$). $\diamond$, Control (no drug). At the times indicated, samples were withdrawn and benzidine-positive cells were counted. Values represent the means $\pm$ S.E.M. of three different experiments.

To determine the relative levels of transcription of actin and β -globin, cytoplasmic RNA was analysed by dot-blot analysis as previously described [6a].

RESULTS

Haemoglobin synthesis

Each of the polyamine analogues and HMBA induced haemoglobin synthesis in MEL cells (Figure 1), and the cellular

Table 2 Haem content and proportion of benzidine-positive MEL cells after 4 days of treatment with polyamine analogue (100 μM) or HMBA (5 mM)

Note that the two values move in the same direction. Results are means $\pm$ S.D.

Treatment	Benzidine-positive cells (%)	Haem content (pmol/ 10^6 cells)
None	1 $\pm$ 1	18 $\pm$ 1
HMBA	98 $\pm$ 1	383 $\pm$ 1
3-3-3	30 $\pm$ 7	94 $\pm$ 12
BE-3-3-3	8 $\pm$ 3	51 $\pm$ 3
BE-3-4-3	9 $\pm$ 1	41 $\pm$ 6
BE-4-4-4	6 $\pm$ 3	49 $\pm$ 11
3-3-3-3	54 $\pm$ 7	281 $\pm$ 42
4-4-4-4	72 $\pm$ 19	267 $\pm$ 39
BE-4-4-4-4	22 $\pm$ 1	95 $\pm$ 5

Figure 2 Dot-blot analyses of cytoplasmic β -globin (a) and actin (b) mRNAs in MEL cells treated with 100 μM 3-3-3-3 or 5 mM HMBA

mRNAs prepared from MEL cells were dotted on to nitrocellulose and hybridized to ^{32}P -labelled actin and β -globin DNA probes.

haem content paralleled benzidine-positivity (Table 2). Of the polyamine analogues, the pentamines with free terminal amino groups (3-3-3-3 and 4-4-4-4; see Table 1) were the most efficient inducers of haemoglobin synthesis (Figure 1b). Benzidine-positive cells were detected 2 days after addition of either drug, and

Figure 3 Effects of HMBA (5 mM) and polyamine analogues (100 μ M) on the growth of MEL cells

Cells were treated with (a) 3-3-3 (□), BE-3-4-3 (○), BE-3-3-3 (■), BE-4-4-4 (◆), (b) 3-3-3-3 (●), 4-4-4-4 (△), BE-4-4-4-4 (▲) or HMBA (○). ◇, Control (no drug). Values are the means $\pm$ S.D. of at least three separated determinations. Error bars, where not visible, are smaller than the symbol size.

75% were positive after 5 days. The tetramine with free terminal amino groups (3-3-3) was less potent, inducing benzidine-positivity in only 35% of cells after 5 days of treatment (Figure 1a).

The bis-ethylated analogues were less effective inducers of haemoglobin synthesis. Of these compounds, the bis-ethylated pentamine BE-4-4-4-4 was the best inducer (Figure 1b), inducing benzidine-positivity in 25% of cells after 5 days. The bis-ethylated tetramines (BE-3-3-3, BE-3-4-3 and BE-4-4-4) induced benzidine-positivity in less than 10% of cells (Figure 1a). After 2 days of treatment with HMBA, 90% of cells were benzidine-positive (Figure 1b).

A dot-blot analysis of mRNA from analogue- or HMBA-treated MEL cells showed that haemoglobin production was preceded by an accumulation of β -globin mRNA; the level of actin mRNA was unaffected (Figure 2).

Cell growth, viability and volume

Analogues with free terminal amines (3-3-3, 3-3-3-3 and 4-4-4-4) had limited but significant effects on cell growth; growth in-

Figure 4 Effects of polyamine analogues (100 μ M) on the viability of MEL cells

MEL cells were treated with 3-3-3 (□), BE-3-4-3 (○), BE-3-3-3 (■), BE-4-4-4 (◆), 3-3-3-3 (●), 4-4-4-4 (△), or BE-4-4-4-4 (▲). Cell viability was assessed by Trypan Blue exclusion. Values are the means $\pm$ S.D. of at least three separate determinations. Error bars, where not visible, are smaller than symbol size.

hibition ranged from 35% to 50% (Figure 3). These analogues had little effect on cell viability: more than 90% of cells were viable after 5 days (Figure 4). Bis-ethylated analogues had a greater effect, each inhibiting growth by more than 80% at day 3 (Figure 3); they also decreased cell viability after 3 days (Figure 4). The toxicity of the bis-ethylated analogues increased in the order BE-3-3-3 < BE-3-4-3 < BE-4-4-4-4 < BE-4-4-4. After 5 days of treatment with BE-4-4-4, only 20% of cells were viable. MEL cells treated with HMBA did not grow after 2 days (Figure 1b) and their viability after 3 days was similar to that of controls (nearly 100%; results not shown). 3-3-3, 3-3-3-3 and 4-4-4-4 had no significant effect on MEL cell volume (mode approx. 200 fL; results not shown), while HMBA induced a decrease in cell volume (mode approx. 100 fL; results not shown). Because bis-ethylated compounds induced little haemoglobin synthesis, their effect on cell size was not assessed.

Polyamine levels

Figure 5 gives intracellular levels of natural polyamines and polyamine analogues in cells treated with 100 μ M 3-3-3-3 (representative of other analogues) or 5 mM HMBA. The pentamine 3-3-3-3 (Figure 5a) and the other analogues (Table 3) accumulated rapidly, although absolute levels differed. In general, intracellular levels were maximal within 24 h (levels at earlier times were not measured), remained stable until day 2, and then fell at day 4.

In cells treated with analogues, intracellular putrescine and spermidine levels decreased to low or undetectable levels after 1 day, while spermine dropped to 30–60% of control levels after 1 day. After 4 days, spermine was undetectable except in cells treated with BE-4-4-4, where the level was 17% of control.

HMBA induced a striking decrease in putrescine (Figure 5b) and also decreased spermidine and spermine, but polyamine levels remained generally higher than in MEL cells treated with polyamine analogues (Figure 5).

Figure 5 Effects of 3-3-3-3 and HMBA on (a) 3-3-3-3, (b) putrescine, (c) spermidine and (d) spermine levels in MEL cells

MEL cells were treated with 100 μ M 3-3-3-3 (●) or 5 mM HMBA (○), or were untreated (◇).

Table 3 Levels of polyamine analogues in MEL cells after 1 day of treatment with the analogue

Results are means $\pm$ S.D.

Polyamine analogue (100 μ M)	Analogue content (nmol/10 ⁶ cells)
3-3-3	2.52 $\pm$ 0.51
BE-3-3-3	6.39 $\pm$ 0.44
BE-3-4-3	4.66 $\pm$ 0.13
BE-4-4-4	5.93 $\pm$ 0.20
3-3-3-3	1.54 $\pm$ 0.09
4-4-4-4	3.60 $\pm$ 1.04
BE-4-4-4-4	2.38 $\pm$ 0.28

DISCUSSION

This study shows that polyamine analogues induce haemoglobin synthesis in MEL cells. The analogues increased the haem content and the amount of mRNA coding for β -globin, and induced benzidine-positivity. The analogues also accumulated in MEL cells, depleted natural polyamines within 24 h and inhibited cell growth. These findings are similar to those observed for spermine, which also induces haemoglobin production in MEL cells,

depletes intracellular spermidine and putrescine and inhibits cell growth slightly [6a], suggesting that polyamine analogues can support some of the functions of naturally occurring polyamines. The cellular responses were uniquely determined by the chemical structure of the polyamine analogue, demonstrating a relationship between polyamine structure and function.

Analogues with free terminal amino groups were good inducers of haemoglobin synthesis in MEL cells. The tetramine 3-3-3, which differs from spermine (3-4-3) by one methylene group in the central aliphatic backbone, was slightly less active (35% benzidine-positive cells; Figure 1a) than spermine (45% benzidine-positive cells) [6a]. The pentamines 3-3-3-3 and 4-4-4-4 induced benzidine-positivity in 75% of cells (Figure 1a). Thus the number of charges in unethylated polyamine analogues correlates with the ability to induce haemoglobin production in MEL cells: pentamines, with five positive charges, were much more efficient than tetramines, with four positive charges. In addition, spermine (3-4-3) was better at inducing haemoglobin production than 3-3-3, and 4-4-4-4 was better than 3-3-3-3; these findings suggest that carbon chains with four methylene carbons promote haemoglobin production better than do carbon chains with three methylene carbons. Since the pK_a values of secondary amines progressively decrease when the number of methylene carbons falls below five [27], alterations in the protonation, average charge density and hydrophobicity of these molecules may affect the ability of unethylated polyamine analogues to

induce haemoglobin synthesis. The differences in uptake between 3-3-3-3 and 4-4-4-4 may also influence the differences we see in haemoglobin production.

Bis-ethylated analogues were poorer inducers of haemoglobin production than their unethylated cousins. BE-3-3-3 induced a maximum of 15% benzidine-positivity, while 3-3-3 induced over 30%; and BE-4-4-4-4 induced only 25% benzidine-positivity, while 4-4-4-4 induced 75%. Bis-ethylation transforms the terminal primary amines into secondary amines, with a consequent drop in pK_a [27], and increases the hydrophobicity of the analogue. This again suggests that pK_a and hydrophobicity are related to the ability of the analogue to induce haemoglobin synthesis. Alteration of the number of methylene carbons between the amino groups of bis-ethylated tetramines had little effect on their ability to induce haemoglobin synthesis, probably because very little haemoglobin production was induced. However, the ability of BE-4-4-4-4 to induce more benzidine-positivity than bis-ethylated tetramines confirms that induction of haemoglobin synthesis is correlated with the number of charges.

Other reasons for the decreased ability of bis-ethylated analogues to induce haemoglobin synthesis are possible. Our data confirm that ethylation of the terminal amines is associated with cytotoxicity and growth inhibition [13]. Terminal ethyl groups could hinder interaction between polyamine analogues and a cellular polyamine binding site(s) such as chromatin [8,13], or they could increase affinity at a competing site. The impaired ability of bis-ethylated analogues to induce haemoglobin production may also result from a general deficiency of macromolecule synthesis caused by the cytotoxic bis-ethylated analogues, as reported in cells treated with BE-3-4-3 [28,29].

Other polyamine-like compounds, such as the diacetylated diamines, HMBA, diacetylputrescine and acetylspermidine, are potent inducers of differentiation of MEL cells and other transformed cell lines *in vitro* [30–33]. HMBA was a better inducer of haemoglobin production in MEL cells than the polyamine analogues studied here (90% versus 75%; Figure 1a), although it is active at more than 100-fold higher doses. In addition to inducing haemoglobin synthesis, treatment with HMBA terminated cell growth and reduced the modal cell volume. Unethylated polyamine analogues induced haemoglobin synthesis and had a limited effect on cell growth, but did not affect cell volume. Thus unethylated polyamine analogues and HMBA both induced haemoglobin production in MEL cells, but polyamine analogues, like spermine [6a], did not induce the entire differentiation programme seen in MEL cells treated with HMBA.

In conclusion, our experiments with polyamine analogues suggest two activities associated with their actions in MEL cells. One activity, associated with bis-ethylation, determines cytotoxicity; the other, associated with free terminal amines and the distance between amino groups, determines the production of haemoglobin. Our experiments also confirm that this class of differentiating agents operates differently from HMBA.

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