Polyamine analogues: potent inducers of nucleosomal array oligomerization and inhibitors of yeast cell growth

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Polyamines are naturally occurring intracellular polycations that are essential for viability and growth of eukaryotes. Dysregulation of polyamine metabolism is a hallmark of cancer and the carcinogenic process, and consequently development of polyamine analogues has emerged as a viable strategy for therapeutic intervention. Previously, we showed that the naturally occurring polyamines spermidine and spermine were quite effective at inducing the oligomerization of nucleosomal arrays in vitro, suggesting that polyamines may play a key role in regulating higher order chromatin structures in vivo. Here, we analyse the ability of a number of synthetic polyamine analogues to potentiate formation of higher order chromatin structures in vitro. We find that a class of long-chain polyamines called oligoamines are potent inducers of nucleosomal array oligomerization in vitro and that these same polyamine analogues rapidly block yeast cell growth.

Key words: chromatin, nucleosomal array, oligoamine, polyamine analogue, spermine, yeast.

INTRODUCTION

The polyamines, putrescine, spermidine and spermine, are naturally occurring intracellular polycations that are essential for viability and growth of eukaryotes [1–3]. Polyamines are synthesized by an exquisitely regulated biosynthetic pathway, and regulation of their catabolism and transport is likewise finely tuned [1,4,5]. Dysregulation of these pathways is the hallmark of cancer and the carcinogenic process. Elevation of polyamine levels and of the polyamine biosynthetic enzyme ODC (ornithine decarboxylase) are observed in neoplastic tissues and are induced by the application of tumour promoters [4]. ODC transcription is a target for the proto-oncogene c-myc [6,7], and activation of c-jun and c-fos pathways involves polyamines [8]. Overexpression of ODC has been shown to transform cells in culture, and high levels of ODC production in transgenic mice cause increased susceptibility to tumour formation [4]. Thus the polyamine pathway has been identified as a target for therapeutic intervention for cancer, as well as for other hyperproliferative diseases.

While pharmacological inhibition of polyamine biosynthetic enzymes has found some clinical utility, inherent problems related to the availability of polyamines in food and from normal bacterial flora in the gastrointestinal tract have necessitated the evolution of new approaches to the creation of potentially useful compounds. The development of a variety of polyamine analogues that gain entry into cells through active polyamine transport, that may modulate normal polyamine biosynthesis through natural feedback mechanisms, that may up-regulate catabolism and, importantly, that may compete with natural polyamines for intracellular binding sites, but with altered function, is an emerging approach that is now being tested clinically [9–14].

While the biochemistry and molecular biology of the polyamines are being elucidated rapidly, knowledge of their specific molecular functions is more embryonic. Emerging functions include association with nucleic acids [15], maintenance and modulation of structure and function of chromatin [16,17], regulation of specific gene expression, ion channel regulation [18], maintenance of membrane stability [19], essential incorporation into active eIF5A (eukaryotic translation initiation factor 5A) [20] and free radical scavenging. Of particular interest are the effects of polyamines on chromatin structure. Previously we found that depletion of cellular polyamines bypassed the transcriptional requirements for the yeast histone acetyltransferase Gcn5p [17]. Furthermore, we found that polyamines facilitated nucleosomal array condensation in vitro and that this activity was antagonized by histone hyperacetylation [17]. These genetic and biochemical data indicated that cellular polyamines function in opposition to chromatin-modifying enzymes to facilitate formation of chromatin higher order structures, leading to transcriptional repression. Likewise, Gilmour and co-workers [21–23] have found that increased levels of polyamines in mammalian cells also lead to changes in chromatin structure and alterations in the activities of histone-modifying activities. Thus it seems likely that chromatin is a key cellular target for polyamines and that dysregulation of this chromatin function may play a key role in development or progression of cancers.

Given the therapeutic promise of several polyamine analogues, we have investigated the potency of a variety of polyamine analogues in facilitating condensation of chromatin in vitro. All polyamine analogues tested retained the ability to induce oligomerization of nucleosomal arrays in vitro, and our results lead to a classification of polyamine analogues into the following two groups: (i) group I analogues are similar in potency to the natural polyamine, spermine, in chromatin condensation assays, and (ii) group II analogues are 3–4-fold more potent than spermine, and thus induce chromatin condensation at low micromolar concentrations. These novel group II polyamine analogues are composed mostly of oligoamines [24], and each member of this class is a potent inhibitor of yeast cell growth. Previous studies have indicated that members of the group II class of polyamine analogues show promise as therapeutics for treatment of breast cancer and other cancers [25], and our results suggest that chromatin structure may play a role as a key molecular target.
**EXPERIMENTAL**

**Polyamine analogues**
The polyamine analogues were obtained from Cellgate and each was resuspended in sterile water and stored at $-20^\circ\text{C}$. These compounds are highly stable in aqueous solutions even at elevated temperature.

**Nucleosomal array reconstitution**
Array DNA template was isolated by digestion of plasmid pCL7c with NotI, HindIII and HhaI (New England Biolabs) followed by FPLC purification on Sephacryl-500 (Amersham Biosciences) essentially as described in [26,27]. Chicken erythrocyte histone octamers were purified from chicken whole blood (Pel-Freez Biologicals) as described previously [28]. Arrays were reconstituted on the 208-11S DNA template in a Slide-a-Lyzer dialysis cassette (Pierce) by using the salt dialysis protocol of Hansen et al. [29]. Octamer concentrations were determined by measuring $A_{260}$ (absorbance) [30].

**Array oligomerization assay**
Arrays (50 nM) were incubated with polyamines or polyamine analogues in TE buffer (10 mM Tris, pH 7.4, and 0.1 mM EDTA). Samples were incubated for 15 min at room temperature ($24^\circ\text{C}$), and then samples were centrifuged for 10 min in a microfuge. $A_{260}$ of the resulting supernatant was then determined and compared with the initial reading prior to polyamine addition to yield the percentage array remaining in supernatant.

**Analysis of polyamine levels within yeast cells**
Yeast cultures (50 ml) were grown at $30^\circ\text{C}$ in rich YPD medium [1% (w/v) yeast extract, 2% (w/v) bactopeptone and 2% (w/v) glucose] to an $A_{600}$ of 1.0. Polyamine analogues were added to 100 $\mu$M final concentration and cultures were grown for an additional 2 h at $30^\circ\text{C}$. An identical cell culture was grown in the absence of polyamine analogue. Cells were harvested by centrifugation (4000 $g$ for 5 min at $4^\circ\text{C}$) and washed three times with distilled water. Cell pellets (0.25 g) were resuspended in ice-cold 5% HClO$_4$ and a cell pellet volume equivalent of zirconium beads (Biospec Products) was added. Cells were broken by vigorous vortex-mixing. Extracts were clarified by centrifugation (14000 $g$ for 10 min at $4^\circ\text{C}$), and extracts were stored at $-20^\circ\text{C}$. Polyamine extracts were subjected to dansylation reactions and analysed by HPLC. Since different preparations vary due to different efficiencies of cell breakage, polyamine analogue levels were normalized to spermine, since spermine levels are relatively constant within cells.

**RESULTS AND DISCUSSION**
In order to investigate the potential for various polyamine analogues to induce chromatin condensation, model nucleosomal arrays were reconstituted with purified histones. Nucleosomal arrays were assembled by salt dialysis using purified chicken erythrocyte histone octamers and a DNA template composed of 11 head to tail repeats of a 208 bp 5S rRNA gene from *Lytechinus variegatus* (208-12S template). Each 5S repeat can rotationally and translationally position a nucleosome after in vitro salt dialysis reconstitution, yielding a positioned array of nucleosomes. These model nucleosomal arrays undergo complex hierarchical structural changes in vitro in the presence of bivalent cations [31]. Low concentrations of Mg$^{2+}$ ions ($<2$ mM) induce intramolecular compaction of individual nucleosomal arrays through association of neighbouring nucleosomes (‘folding’), while progressively higher concentrations of Mg$^{2+}$ ($>2$ mM Mg$^{2+}$) or low concentrations of polyamines ($\sim200$ $\mu$M) can induce nucleosomal arrays to reversibly oligomerize. The intramolecular folding of model arrays at low Mg$^{2+}$ concentrations is believed to mimic the formation of 30 nm-like chromatin fibres, while intermolecular oligomerization generates relatively defined, soluble structures that sediment in the thousands of S, and are believed to mimic the fibre–fibre interactions that stabilize higher order chromosomal domains such as chromonema fibres [16].

Figure 1 illustrates the polyamine analogues used in the present study. Several of the analogues are conformationally restricted spermine-like compounds (CGC-11047, CGC-11093, CGC-11102, CGC-11123, CGC-11217, CGC-11158, CGC-11157, CGC-11144, CGC-11150).
Chromatin functions of polyamine analogues

Figure 2 Representative nucleosomal array oligomerization profiles for four different polyamine analogues

Nucleosomal arrays (50 nM) were incubated with increasing concentrations of spermine, spermidine or the polyamine analogues CGC-11047 or CGC-11217 for 15 min at room temperature. Samples were centrifuged for 10 min and the concentration of nucleosomal array remaining in the supernatant was determined by measuring A260.

Table 1 Summary of chromatin condensation by polyamine analogues

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration inducing 50% chromatin condensation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
</tr>
<tr>
<td>Spermidine</td>
<td>140 µM</td>
</tr>
<tr>
<td>Spermine</td>
<td>18 µM</td>
</tr>
<tr>
<td>Group I analogues</td>
<td></td>
</tr>
<tr>
<td>CGC-11047</td>
<td>36 µM</td>
</tr>
<tr>
<td>CGC-11102</td>
<td>20 µM</td>
</tr>
<tr>
<td>CGC-11093</td>
<td>16 µM</td>
</tr>
<tr>
<td>CGC-11098</td>
<td>18 µM</td>
</tr>
<tr>
<td>CGC-11237</td>
<td>60 µM</td>
</tr>
<tr>
<td>Group II analogues</td>
<td></td>
</tr>
<tr>
<td>CGC-11157</td>
<td>5 µM</td>
</tr>
<tr>
<td>CGC-11158</td>
<td>5 µM</td>
</tr>
<tr>
<td>CGC-11150</td>
<td>5 µM</td>
</tr>
<tr>
<td>CGC-11144</td>
<td>4 µM</td>
</tr>
<tr>
<td>CGC-11217</td>
<td>7 µM</td>
</tr>
</tbody>
</table>

CGC-11098 and CGC-11102 [32], several analogues are longer chain oligoamines (CGC-11157, CGC-11158, CGC-11144 and CGC-11150), while CGC-11217 is mesoporphyrin IX covalently bound to two molecules of CGC-11093, and CGC-11237 is mesoporphyrin IX covalently bound to two molecules of a cyclic polyamine. All of these compounds are active against a variety of human tumour cells both in vitro and in vivo [9,12,24,25,32]. CGC-11047 and CGC-11093 are in human clinical trials.

Polyamine analogues induce chromatin condensation

To investigate the ability of polyamine analogues to induce oligomerization of nucleosomal arrays, increasing concentrations of each polyamine analogue were incubated with nucleosomal arrays (50 nM) for 10 min at room temperature, and oligomerized arrays were pelleted by centrifugation. The amount of array remaining in the supernatant was then quantified and plotted as a function of analogue concentration. Similar to our previous study [17], spermidine was quite effective at oligomerizing nucleosomal arrays, as a concentration of 140 µM was sufficient to oligomerize 50% of the arrays (Figure 2 and Table 1). Spermine was approx. 10-fold more potent than spermidine, as 50% oligomerization occurred at 18 µM. When each of the analogues was analysed in parallel, two groups emerged from the data (Figure 2 and Table 1). Group I analogues were similar in effectiveness to spermine, with 50% oligomerization occurring between 16 and 60 µM concentrations (CGC-11047, CGC-11093, CGC-11098, CGC-11102 and CGC-11237). On the other hand, group II analogues were clearly more effective than spermine, with oligomerization requiring only 4–7 µM of each compound (CGC-11157, CGC-11158, CGC-11144, CGC-11150 and CGC-11217). Interestingly, with only one exception, CGC-11217, all of the group II polyamine analogues are oligoamines, indicating that potent nucleosomal array oligomerization is a common feature of this class of compounds.

In a therapeutic setting, polyamine analogues must compete with cellular polyamine pools for interactions with cellular targets such as chromatin. One possibility is that chromatin, which is already bound by cellular polyamines, will be insensitive to the effects of polyamine analogues added subsequently. To test this idea, nucleosomal arrays were pre-incubated with 80 µM spermidine, a concentration in which ∼95% of the arrays are soluble, and then increasing concentrations of the polyamine analogues were added and array oligomerization was assayed by centrifugation. As shown in Figure 3, prebinding spermidine to nucleosomal arrays did not inhibit the ability of the polyamine analogues to mediate array oligomerization. For instance, analogue CGC-11102 which induces 50% oligomerization at ∼20 µM by itself, only requires ∼10 µM when the arrays were prebound by low concentrations of spermidine. Likewise, each of the group II polyamine analogues (e.g. CGC-11144) were potent in the presence of spermidine, with greater than 50% oligomerization occurring at 2.5 µM. Thus the results suggest that endogenous polyamines do not grossly antagonize polyamine analogue-mediated nucleosomal array oligomerization.

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Figure 4  Group II polyamine analogues inhibit yeast cell growth

Yeast cells (CY1019) were grown at 30°C in rich YPD medium until mid-exponential phase and then diluted to an A 600 of 0.1 prior to addition of spermine (A), spermidine (A), CGC-11047 (B), CGC-11144 (C), CGC-11150 (D), CGC-11157 (E) or CGC-11158 (F) at the indicated concentrations. Cultures were grown at 30°C with shaking and A 600 was monitored at the indicated times. Results shown are representative of at least two independent experiments. spm, spermine; spd, spermidine.

Group II polyamine analogues inhibit yeast cell growth

The potent chromatin condensation activity of the group II polyamine analogues may have a detrimental effect on cell growth and/or nuclear function. As an initial investigation, increasing concentrations of each polyamine analogue were added to cultures of yeast cells and growth rates were monitored over time. A representative set of results for a subset of the polyamine analogues is shown in Figure 4. As was the case for the chromatin condensation assays, these yeast cell studies were consistent with the classification of the polyamine analogues into the same two groups. The group I polyamine analogues were identical with spermine and spermidine, as no effects were observed on yeast cell growth at the concentrations used (10–100 µM). In contrast, the group II polyamine analogues, which were more potent in chromatin condensation, dramatically inhibited yeast cell growth within the first cell cycle following addition of the polyamine analogue. For instance, the group II analogue, CGC-11144, blocked yeast cell growth at a concentration of 40 µM, while addition of 100 µM of any of the group I analogues had no detrimental effect. Previous studies have shown that polyamine synthesis is required for yeast cell growth and that depletion of cellular polyamines leads to cell cycle arrest in the G1-phase of the cell cycle [33,34]. Likewise, FACS analysis indicates that yeast cells treated with the group II analogue, CGC-11144, rapidly accumulate in G1 (results not shown). Thus the ability of polyamine analogues to inhibit yeast cell growth correlates quite well with their ability to induce chromatin condensation.

One possibility is that the toxicity of group II polyamines may be due to enhanced uptake or stability within yeast cells compared with group I polyamines. To test this idea, yeast cultures were treated for 2 h with 100 µM CGC-11144 or CGC-11093, cells were harvested, and 5 % HClO4 extracts were analysed for polyamine content by HPLC analysis. Results from two independent extracts indicate that both of the polyamine analogues accumulate to similar levels within yeast cells. The class I analogue CGC-11093 was found at 183 ± 9 nmol/g of yeast cells (normalized to endogenous spermine levels), while the potent class II analogue CGC-11144 was found at 150 ± 22 nmol/g of yeast cells (normalized to endogenous spermine). In both cases polyamine analogues were ~ 10 % the level of cellular spermidine. Thus these results indicate that the increased toxicity of the class II polyamine analogues is not due to greatly enhanced uptake, but that this phenotype may be due to their effects on chromatin structure.

As select polyamine analogues are being advanced in preclinical and clinical studies for cancer and other hyperproliferative diseases, such as macular degeneration, further understanding of the mechanisms of action of these compounds is essential.
REFERENCES


