Polyamine drug inhibition of the proliferation of a small cell lung cancer cell line NIH-H82 with amplified myc oncogene

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Small cell lung carcinoma (SCLC) compromises rapidly proliferating, highly metastatic cancer cells leading to a negative prognosis for the patient. All such cells have elevated levels of polyamines and amplified myc oncogene expression. In the current study we evaluated the effects of two polyamine synthesis inhibitors (DFMO and methyl GAG) and two polyamine analogues (BE4-4-4-4 and BEPUT) on the cellular proliferation and polyamine levels in a human SCLC cell line (NCI-H82) which contained 25 fold elevated levels of both c-myc DNA and RNA. Individually, BE4-4-4-4 and BEPUT, showed 40% and 55% inhibition of cell proliferation, and gave 15% and 17% decrease in polyamine levels. However, when BE4-4-4-4 and BEPUT were simultaneously incubated with the SCLC cells, proliferation was inhibited 98%, while only a 20% decrease in polyamine levels occurred. BE4-4-4-4 and BEPUT have recently been reported to prevent/inhibit polyamine activation of casein kinase II, which activates both ornithine decarboxylase and myc oncoprotein via serine phosphorylations. It is possible that the above observed inhibition of SCLC cell proliferation may be related to specific, not general polyamine functioning, and probably involves more than one molecular target. [Turk J Cancer 2000;30(3):97-104]

Key words: Polyamines, polyamine drugs, SCLC, casein kinase II, myc

Approximately 25% of all lung cancers are small cell lung carcinomas (SCLCs) (1-2). SCLCs usually have a more rapid rate of cellular proliferation, are more aggressive, have higher metastatic rates and increased mortality when compared with non-SCLC (1-4). Even though the SCLC may be more sensitive to chemo- and radiotherapy, long term patient survival is poor. Much research has been performed to elucidate the molecular mechanisms involved
in the high SCLC cell proliferation rates and to improve novel treatment strategies (5-9).

The natural polyamines (putrescine, spermidine, spermine) are key regulators of mammalian cell proliferation and differentiation (7). They are elevated in fast growing normal and cancer cells and tissues, including SCLC. Several polyamine drugs, including polyamine synthesis inhibitors, polyamine metabolism stimulators, polyamine cellular uptake inhibitors, and polyamine analogues are currently being studied in attempts to control the growth of many types of cancer cells (10,11). Some of these polyamine drugs have already showed promise in vitro and in cancer chemotherapy clinical trials (5-7,9-16).

Even though polyamines were discovered over 50 years ago, the specific intracellular molecular mechanism(s) of the polyamines have been very elusive and are not yet understood. Current studies focus on polyamine binding to DNA, RNA, enzymes and other proteins (7,12,15,17-22). Over the past few years there have been reports on the specific binding of polyamines to CKII and their direct effects upon CKII activity (23,24). Recently we reported both activation and interference (inhibition/prevention) of activation by a series of natural and synthetic polyamines when purified lung CKII was studied with Myc as the phosphate acceptor protein substrate (18,25).

Various members of the family of myc oncogenes are amplified in many types of cancers (26). C-myc is amplified in most SCLCs (27,28). The intracellular levels of the biologically active Myc are controlled at the levels of transcription, post-transcriptor, and post-translation. Post-translational control occurs via the phosphorylation of specific serine/threonine residues in Myc by three protein kinases, including CKII (3,28). Such phosphorylations activate Myc in the cytoplasm of the cell, allow it to enter the nucleus and to dimerize with Max protein (also phosphorylated by CKII) (29). This heterophosphodimer controls the transcription of more that 50 genes involved in cellular proliferation, differentiation and apoptosis (27,28,30). One of the genes controlled by pMyc-pMax is ornithine decarboxylase (positive induction), the rate limiting enzyme for polyamine synthesis (31).

In the current study, we investigated the effects of several polyamine drugs on the growth of a human SCLC cell line NCI-H82 which has 25 fold elevated levels of c-myc DNA and RNA. By using different polyamine synthesis inhibitors and polyamine analogues, we evaluated the relationships between the inhibition of lung cancer cell proliferation and intracellular polyamine levels.

**Materials and Methods**

Most chemicals, biologicals, and tissue culture media were obtained from Sigma Chemical Co., St Louis, Mo. All polyamine drugs were a kind gift from the Drug Synthesis and Chemistry Branch of the National Cancer Institute, NIH, Bethesda, MD. Four polyamine drugs were used: 2 polyamine synthesis inhibitors, DFMO and methyl GAG; and 2 polyamine analogues, BE4-4-4-4 and BEPUT.

The NCI-H82 cells were obtained from the American Type Tissue Culture Collection, Rockville, MD. All experiments were carried out using RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum and 25mM Hepes. The SCLC H82 cells were grown in a 95% air, 5% CO2 mixture, at 37°C.
They grew as clumps of cells in suspension, showed a doubling time of 6-8 hrs, and were routinely passed 2-3 times weekly in T_{25} flasks. Cell proliferation experiments were performed as follows: The cells were grown to near confluency, then plated directly into 6 multi-well plates at a density of approximately 250,000 cells per well. For the inhibition studies, varying concentrations of the polyamine drugs (pre-prepared in the culture medium minus serum) were added to the cells. At the end of the incubation times, cell proliferation was evaluated by measuring the cellular DNA content using the fluorescent dye DAPI (32). All experiments were carried out in triplicate and repeated at 2-3 times.

Cellular polyamines were extracted with 3% perchloric acid, dansylated with dansyl chloride in acetone, separated on TCL silica chromatography plates using (2:3) ethyl acetate: cyclohexane, and quantified with a spectrofluorometer (excitation 360 nm; emission 500 nm (9,11).

All results are given as mean±standard deviation. The differences between the control and drug groups were analysed using the student’s t-test and p<0.05 was considered significant.

**Results**

Figure 1 shows the growth pattern of the NCI-H82 cells using our growth conditions. Continuous and steady proliferation continued for 24 hrs, but not for 48 hours (latter data not given). When each of the four drugs were present at final concentrations of 20 M at 20 hours, inhibition of cell proliferation ranged from 30 to 69%.

![Fig 1. The growth pattern of the NCI-H82 cells during a 24 hour culture period.](image)
Figure 2 and Table 1 give data on polyamine drug inhibition of H82 cell growth and polyamine levels. At 10 M concentration and after 24 hours of incubation, proliferation was inhibited and polyamines were decreased, respectively, as follows: DFMO, 21% and 34%; methyl GAG, 24% and 15%; BE4-4-4-4, 43% and 12%; BEPUT, 62% and 17%.

![Fig 2. The dose response effect of the polyamine drugs on the NCI-H82 cells during a 24 hour culture period. (□—□ DFMO; △—△ methyl GAG; ▼—▼ BE4-4-4-4; X—X BEPUT). The results are given as mean±standard deviation.](image)

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Putrescine</th>
<th>Spermidine</th>
<th>Spermine</th>
<th>Total</th>
<th>% decrease</th>
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<tbody>
<tr>
<td>None</td>
<td>0.36±0.05</td>
<td>0.32±0.04</td>
<td>0.34±0.05</td>
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<td>DFMO</td>
<td>0.23±0.04</td>
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<td>0.20±0.04</td>
<td>0.67</td>
<td>34</td>
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<tr>
<td>Methyl GAG</td>
<td>0.31±0.05</td>
<td>0.28±0.07</td>
<td>0.27±0.06</td>
<td>0.86</td>
<td>15</td>
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<tr>
<td>BE4-4-4-4</td>
<td>0.33±0.04</td>
<td>0.29±0.06</td>
<td>0.28±0.05</td>
<td>0.90</td>
<td>12</td>
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<tr>
<td>BEPUT</td>
<td>0.26±0.04</td>
<td>0.27±0.06</td>
<td>0.29±0.06</td>
<td>0.84</td>
<td>17</td>
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</table>

Polyamine drugs were 10 M each. Polyamine levels are calculated as nmol/g DNA. Results are given as mean±standard deviation and compared to the control group (p<0.05).

Combinations of the four drugs and their effects on cell proliferation and polyamine levels are given in Table 2. Combinations of the polyamine synthesis
inhibitors, the polyamine synthesis inhibitors plus polyamine analogues gave rates similar to the rates in Table 1 when each drug was used separately. However, the combination of the two polyamine analogues showed additivity of up to 98% inhibition of cell proliferation without additional changes in polyamine levels (decreased 20%).

### Table II

**Effects of combination of polyamine drugs on cell proliferation and polyamine levels**

<table>
<thead>
<tr>
<th>Drugs</th>
<th>% decrease in cell proliferation</th>
<th>% decrease in polyamine levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DFMO</td>
<td>18.5±3.3</td>
<td>36.3±4.5</td>
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<tr>
<td>Methyl GAG</td>
<td>22.3±3.0</td>
<td>20.5±3.2</td>
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<tr>
<td>BE4-4-4-4</td>
<td>40.4±4.8</td>
<td>15.4±3.7</td>
</tr>
<tr>
<td>BEPUT</td>
<td>55.3±6.1</td>
<td>16.6±3.9</td>
</tr>
<tr>
<td>DFMO + methyl GAG</td>
<td>23.2±3.8</td>
<td>34.4±5.1</td>
</tr>
<tr>
<td>DFMO + BE4-4-4-4</td>
<td>37.4±4.6</td>
<td>29.8±4.7</td>
</tr>
<tr>
<td>DFMO + BEPUT</td>
<td>51.4±6.7</td>
<td>29.2±4.1</td>
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<tr>
<td>Methyl GAG + BE4-4-4-4</td>
<td>36.1±5.9</td>
<td>25.8±4.0</td>
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<tr>
<td>Methyl GAG + BEPUT</td>
<td>54.3±7.5</td>
<td>21.7±5.9</td>
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<tr>
<td>BE4-4-4-4 + BEPUT</td>
<td>97.7±8.3</td>
<td>19.9±4.9</td>
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</table>

Single drug experiments were at 10 M; two drug experiments were at 5 M + 5 M to also equal 10 M final concentration. The percentage of changes in cell proliferation and polyamine levels was calculated from no drug controls and based upon g DNA at 24 hr incubation (cell proliferation), and nmol polyamine/g DNA, respectively. The results are given as mean±standard deviation (p 0.05)

### Discussion

Cancer cells have elevated levels of natural polyamines which help maintain rapid cell proliferations. Different types of polyamine drugs have been shown to deplete intracellular polyamines and inhibit the proliferation of many types of cancers (9,10,12-16). DFMO and methyl GAG are polyamine synthesis inhibitors which prevent the synthesis of polyamines by directly inhibiting ornithine carboxylase and propylamine transferases I and II, respectively (22,33). In the brief incubation period and under our culture conditions (24 hours), the effects of these 2 polyamine synthesis inhibitors upon the cell proliferation rates and polyamines levels were modest (20%-30%). Apparently inhibition of these cancer cells requires a longer time period if only polyamine synthesis inhibitors are employed. Also these growth inhibitory effects were not dose dependent. This may reflect the rate of cellular entry of these two drugs (not measured in the current study), or the rate of metabolism of the polyamines (not reported for the NCI-H82 cells). Or it may simply be related to the very high levels of polyamines (especially putrescine, Table 1) found in the SCLC cells.

Polyamine analogues are thought to inhibit cancer cell proliferation by multiple molecular mechanisms. Polyamine analogues, such as BE4-4-4-4, may induce enzymes involved in polyamine catabolism (e.g. spermidine/spermine
N1-acetyl-transferase) and thus deplete intracellular polyamine pools (12,13,22). However, this type of inhibition requires new protein synthesis, hence probably incubation times longer than 24 hours. As shown in Table 2, almost 100% inhibition of cell proliferation was observed within 24 hours when the two polyamine analogues (BE4-4-4-4 and BEPUT) were simultaneously added to the SCLC cultures. And during this time period, polyamine levels were only decreased near 20%. Therefore, the polyamine analogues which caused minimal depletion in polyamine levels showed maximal inhibition of cancer cell proliferation. This may be because these two polyamine analogs have several direct and specific intracellular molecular targets which, when combined, could allow for more immediate cell growth inhibition.

It is logical to assume that CKII may be one of these targets. Our recent studies with purified sheep lung CKII (purified using polyamine affinity chromatography) showed that spermidine, spermine, poly-lysine, and poly-ornithine, but not poly-arginine, stimulated CKII activity from five to 20 fold when casein or Myc was used as the protein substrate (18,25). Polyamine analogues, such as BE4-4-4-4, did not effect basal enzymatic activity, yet they inhibited/prevented the poly-lysine stimulated CKII phosphorylation of Myc up to 85%. In the SCLC cells employed in the current study, the c-myc oncogene was amplified 25 fold. Further, it has been shown that SCLC cells, which overexpress myc, have increased sensitivity to growth inhibition with polyamine analogues (5). Such data support the possible linkages between polyamines, CKII, myc/Myc, and ODC. Moreover, myc expression has recently been shown to be inhibited at the post transcriptional level by a polyamine analogue in a colon cancer cell line (34).

There are certainly many other intracellular molecular targets for these drugs. For example, a direct association between a nuclear factor and DNA has been reported (22). The DNA sequence (5'-TATGACTAA-3') was described as the polyamine responsive element and corresponded to the binding site of a specific transcription factor. Such data suggests that polyamine analogues may have diverse and specific molecular targets on DNA as well as on proteins/enzymes. These targets must be involved in cell proliferation, cellular differentiation, and apoptosis. Polyamine analogues have been shown to activate apoptosis and/or cause cytotoxic effects in many cancer cells (6,12,35). They have also been reported to render human cancer tissues more sensitive to other anti-neoplastic therapies (14-16).

Recent studies suggest that there are several molecular interactions (including signal transduction pathways, cell cycle controls, autocrine loop systems, etc.) in the proliferation of SCLCs (8,21,35). Many SCLCs have double minute chromosomes containing multiple copies of c-myc which may partially allow for amplified Myc in many SCLCs (1-4). And the normal control mechanisms of myc induction include growth factors, signal transduction pathways, and phosphorylation cascades (7,16,21). Amplified Myc could induce ODC which could increase polyamine levels in SCLCs. Future research efforts focusing on the identification of selected and specific intracellular targets of polyamine analogs, especially where they mask or interfere with the normal action of the natural polyamines, will provide oncologists with more rational approaches to cancer chemotherapy.
Acknowledgment

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References
