Chapter 8

Growth modulatory effects and altered cell cycle phase distribution in cultured human breast adenocarcinoma cells depleted of polyamines by inhibitors of polyamine biosynthesis
Introduction

Polyamines putrescine, spermidine and spermine are essential for the maintenance of eukaryotic cell proliferation (96). Several inhibitors of polyamine biosynthesis have been developed as antiproliferative reagents (98,343). These inhibitors deplete the intracellular polyamines.

S-adenosylmethionine decarboxylase (AdometDC) is a critical enzyme in the polyamine metabolic pathway leading to the synthesis of spermidine and spermine (344). These distal polyamines are known to play a major role in cell proliferation (158,345) therefore AdometDC has been considered as a potential target for antitumor therapy. The first identified inhibitor of AdometDC (346) which had an antiproliferative effect in vitro (347) and to a lesser effect in vivo (348) was methylglyoxal bis (guanyl hydrazone) (MGBG). This is not a specific inhibitor of AdometDC and has effects unrelated to polyamine depletion such as antimitochondrial effect (349). New cyclic analogs of MGBG, CGP48664 [4-amidinoindano-1-(2’amidino)hydrazone dihydride chloride] has been synthesized in order to improve the specific action, such as antitumor effect and reduce the nonspecific action (165).

Several analogs of polyamines such as bis(ethyl)polyamines have also been developed as antiproliferative agents (350,351). These inhibitors negatively regulates ornithine decarboxylase (ODC) and AdometDC activity (352). They are known to induce spermidine/spermine N’-acetyltransferase (SSAT) activity (353,354). These analogs are known to inhibit cell growth through the depletion of intracellular
polyamines completely (355). It has been reported earlier that N¹,N¹²-bis(ethyl)spermine can substitute for the function of spermine and accumulated in the cells at a concentration of up to 5 fold that of spermine in control cells (168,356). It was therefore suggested that it is not the depletion of polyamines but also the accumulation of N¹,N¹²-bis(ethyl)spermine may be involved in the inhibition of cell growth (357). These accumulated polyamines and bis(ethyl)polyamine analogs caused the inhibition of protein synthesis especially mitochondrial protein synthesis leading to decrease in ATP (171,358). These analogs of spermine such as bis(ethyl)pentamines and bis(ethyl)tetraamines have been synthesized and tried against various human tumors xeno transplanted into nude mice, and it was observed that the analogs have strong antitumor activities (359).

Besides the inhibitors of polyamine biosynthetic pathway, adriamycin, an antineoplastic antibiotic is known to intercalate into cellular DNA (360). It is an antibiotic of anthracyclin group isolated from *Streptomyces* var. *caesius* (361). It has been reported to be an effective growth inhibitor of several human tumors as well as leukemic cells. It remains unclear if the primary antitumor effects of adriamycin result from its intercalation or from other action of the drug (362-365). A previous report shows that combination of adriamycin with DFMO results in greater than additive suppression of *in vivo* tumor growth in rodents (366).

In the present study the antiproliferative effects of CGP48664, bis(ethyl)-pentamines (BE3333, BE4444) and adriamycin, an antineoplastic agent, on MCF-7 human breast adenocarcinoma cell line has been worked out. It still remains a matter of some controversy, however, if cells deficient in polyamine content are capable of
moving through various phases of the cell cycle or are blocked at one or more specific points. The present work clearly demonstrates an apparent inhibition of cell cycle traverse in human breast adenocarcinoma cells treated with inhibitors of polyamine biosynthesis.

**Materials and Methods**

**Chemicals:** All tissue culture chemicals viz. RPMI-1640, antibiotics (Streptomycin and penicillin), sodium bicarbonate were purchased from Sigma Chemical Co. (St. Louis MO, USA). Dansyl chloride, propidium iodide, RNase A, putrescine, spermidine and spermine were also obtained from Sigma Chemical Co. CGP48664 [4-amidinoindanon-1-(2'amidino)hydrazone dihydrochloride monohydrate] was a generous gift from Ciba Geigy Ltd. Switzerland. BE3333.5HCl [1,15-bis(ethylamino)-4,8,12-triazapentadecane], BE4444.5HCl [1,19-bis(ethylamino)-5,10,15-triazanonadecane] were kindly provided by Akira Shirahata, Josai University, Sakado Japan. Adriamycin (Doxorubicin) was purchased from Sigma Chemical Co. Fetal calf serum (FCS) was obtained from Biological Industries (Kibbutz Beit Haemek, Israel). All other chemicals used were of analytical grade.

**Cell culture:** MCF-7, a human breast adenocarcinoma cell line, was obtained from the National Facility for Animal Tissue and Cell Culture (NFATCC), Pune, India. The cell line was regularly maintained in RPMI-1640 medium supplemented with 0.2% sodium bicarbonate, 10% FCS and antibiotics (50 μg/ml of penicillin and 100 μg/ml of streptomycin). The cells were routinely grown in 25 cm² flasks under humidified atmosphere of 95% air and 5% carbon dioxide at 37°C and
were subcultured until growth reached near confluency. Cell growth was monitored by counting viable cells after trypan blue dye exclusion test using hemocytometer.

**Drug study:** Cells were seeded \((10^5 \text{ cells/ml})\) in 200 \(\mu\text{l}\) of medium in 96-well culture plates 48h before drug treatment. Following drug addition to medium, 2.5 \(\mu\text{Ci/ml}\) of \(^3\text{H}\)thymidine was added per well and cultures were kept for 48h. Cells were washed with PBS, harvested by trypsinization and radioactivity, incorporated was measured by scintillation spectrometer (Beckman LS1800, Beckman Instrument Inc. CA, USA). Cell number was also monitored by counting viable cells after trypan blue dye exclusion test using a hemocytometer. The pattern of cell growth obtained by thymidine incorporation versus trypan blue exclusion was similar. Each experiment was repeated at least 3-4 times and each treatment was in triplicate.

**Polyamine estimation:** For polyamine estimation \(5 \times 10^5\) cells were inoculated in 90mm culture plates and 48h after seeding, indicated concentration of drugs were added. Cells were incubated for another 24h, harvested in 2\% perchloric acid and stored at 4\(^\circ\)C. Cell debris was pelleted from perchloric acid and supernatant was used for polyamine estimation. Dansyl derivatives were prepared (196) and were separated by thin layer chromatography (TLC) on 0.2mm thick silica gel plates, using ethylacetate:cyclohexane (2:3, v/v) as a solvent. Quantification of polyamines was accomplished using Camag TLC scanner with the TLC II software program Cats3 (Camag, Sonnenmattsr, Switzerland). The concentration of unknown samples was determined against standard polyamines.
Cell cycle analysis: Cells were grown and treated with drug(s) for 48h, harvested by trypsinization, washed with cold PBS and pelleted by centrifugation at 2000 x g for 10 min at 4°C. Cells were kept in 70% ethanol for 24h for fixation. Cells were centrifuged again at 2000 x g for 10 min at 4°C. Ethanol was removed without disturbing the pellet and 1.0 ml of propidium iodide staining solution (50 mg/ml of propidium iodide, 100 μg/ml of RNase A and 1.0 mg/ml of glucose in PBS) was added to the cells with continuous vortexing. The cells were incubated for 30 min at room temperature. Samples were examined using EPIC® XL software (Coulter Corporation, Miami, Florida, USA) and then analyzed using MULTICYCLE® software (Phoenix Flow System Inc. San Diego CA, USA) for cell cycle analysis.

Each experiments was repeated at least three to four times and each treatment was in triplicate. The percentage of trypan blue excluded cells (live cells) were more than 95% in untreated group. SD < 5% of average are not shown.

Results

The structure and abbreviation of various inhibitors used is shown in Fig.1. Fig.2 and Table 1 summarize the results of dose response studies testing the antiproliferative effects of CGP48664, BE333, BE4444 and Adriamycin on hormone sensitive MCF-7 human breast adenocarcinoma cell line after 48h of incubation. These findings show that CGP48664 (Fig.1A) exerted a potent antiproliferative effect with IC_{50} of approximately 0.2 μM. The effect of BE3333 (Fig.1B) and BE4444 (Fig.1C) was examined at concentration of 0.1-1000 μM. The inhibition of cell growth by BE3333 and BE4444 was dose dependent with IC_{50} of 0.4 μM and 250 μM.
respectively. Adriamycin (Fig.1D) also resulted in inhibition of cell growth in a dose dependent manner with an $IC_{50}$ of approximately 0.01 $\mu\text{M}$.

The effects of CGP48664, BE3333, BE4444 and adriamycin on cellular polyamines was assessed in hormone sensitive MCF-7 cell line. The inhibitors were tested at concentration of 0.5 $\mu\text{M}$ CGP48664, 10 $\mu\text{M}$ BE3333, 100 $\mu\text{M}$ BE4444 and 1.0 $\mu\text{M}$ adriamycin. As can be seen from the Fig.3, 48h after treatment CGP48664 resulted in increase in putrescine levels (Fig.3A) and decrease in spermidine and spermine levels (Fig.3B, 3C). At 0.5 $\mu\text{M}$ concentration of CGP48664, putrescine concentration increased by 65% whereas spermidine and spermine levels decreased by 62% and 60% respectively over the control values.

Polyamine content of cells treated with bis(ethyl)polyamine analogs were also measured (Fig.3A,B,C). When cells were treated with 10 $\mu\text{M}$ BE3333 or 100 $\mu\text{M}$ BE4444 for 48h a very significant decrease in putrescine, spermidine and spermine content was observed. Only a marginal effect of adriamycin on polyamine levels was observed (Fig.3A,B,C).

Fig.4 show representative DNA histograms obtained with MCF-7 cell lines for untreated controls and culture treated with 0.5 $\mu\text{M}$ CGP48664, 10 $\mu\text{M}$ BE3334, 100 $\mu\text{M}$ BE4444 or 1.0 $\mu\text{M}$ adriamycin, 48h after seeding and harvesting after an additional 48h incubation. The histograms show that 48h in the presence of 0.5 $\mu\text{M}$ CGP48664, a decrease in S phase population and no change in G2/M fraction of MCF-7 cells was observed. However corresponding increase in G1 phase fraction was observed.
Bis(ethyl)polyamine analogs BE3333 and BE4444 at concentrations of 10 μM and 100 μM respectively, resulted in decrease in S phase fraction and increase in G1 phase fraction. Both these analogs did not however alter the G2/M fraction. BE4444 was more effective in inhibiting S phase cells than BE3333.

Adriamycin (0.5 μM) resulted in inhibition of S phase cells and a sharp increase in G2/M fraction. However only a smaller decline in the G1 phase fraction of cells was observed.

To quantitate the alteration in cell cycle phase distribution resulting from treatments with these drug, phase fractions were estimated by computer analysis using MULTICYCLE® software program. The results are shown in Table 2. From the quantitative analysis of the data it is clear that BE4444 was more effective in inhibiting S phase fraction of cells then BE3333. All the inhibitors of polyamine biosynthesis resulted in significant increase in the percentage of cells in G1 phase and did not alter G2/M phase of cells. Adriamycin on the other hand resulted in decrease in percentage of cells in G1 phase and increase in G2/M fraction.

Discussion

Polyamines are known to play a critical role in breast cancer cells proliferation (177,179,180,367,368). Hormone responsive breast cancer cells are known to have polyamine biosynthesis under estrogen control (369). Polyamines are known to influence estrogen action at multiple levels. These include a) the association kinetics of the estrogen receptor to specific DNA sequences (174), b) The synthesis of estradiol regulated cell cycle-specific gene (175) c) the synthesis and/or action of
estradiol modulated growth factors (176,177,370). Even in hormone independent cell lines, polyamines have also been shown to be involved in their proliferation (371). All this clearly shows that polyamine biosynthetic pathway is a logical target for anti-tumor therapy in breast cancer. Inhibitors of the enzyme ornithine decarboxylase, like difluoromethylornithine have been shown to exert a potent antiproliferative effect in several experimental breast cancer system in vitro (367,368,371) and to a lesser extent in vivo (183,372). The other enzyme responsible for synthesis of spermidine and spermine is AdometDC, is also an important target for the development of improved therapeutics modalities in breast cancer and other malignancies. Similarly bis(ethyl)polyamine analogs have also been developed as antiproliferative reagents (350,351). These analogs can deplete intracellular polyamines almost completely and they are thought to inhibit cell growth through this process (355).

Development of new AdometDC inhibitors which are less toxic and are more specific, has led to testing of these inhibitors in breast cancer. Among the inhibitors of AdometDC available, CGP48664 has the best therapeutic profile (165). The data reported here clearly indicates the antiproliferative action of CGP48664 with an IC$_{50}$ of 0.2 $\mu$M. Also, CGP48664 induced the expected increase in putrescine and also the levels of two polyamines spermidine and spermine were reduced to less than 35% and 40% respectively of control.

The effect of two bis(ethyl)polyamine analogs on cell growth was studied to determine their antiproliferative potential in breast cancer cells and also to look into the mechanism of inhibition of cell growth by these analogs. The results indicate that both BE3333 and BE4444 inhibit the cell growth with IC$_{50}$ of 0.4 $\mu$M and 250 $\mu$M
respectively. Both these analogs resulted in inhibition of polyamines. BE3333 was found to be more potent in antiproliferative activity than BE4444. Earlier reports have shown that accumulation of bis(ethyl)polyamine analogs is important for the inhibition of cell growth by the analogs (357). It was not clear whether polyamine deficiency is required for the growth inhibition which was not restored by provision of polyamines. The accumulated bis(ethyl)polyamine analogs are known to cause the inhibition of protein synthesis, especially of mitochondrial protein synthesis (358). Thus protein involved in the ATP production were markedly decreased, followed by the decrease of ATP content and swelling of mitochondria and depletion of mitochondrial DNA was also observed (171).

In order to address the question of role of polyamines in the regulation of cell cycle traverse, I used specific inhibitors of polyamine biosynthesis. These inhibitors were capable of partially depleting the cells of their polyamine content. These polyamine depleted cells were used to investigate the consequences of such treatment on cell cycle phase distribution and rates of cell cycle traverse. Inhibitor of AdometDC, CGP48664 and polyamine analogs BE3333 and BE4444 which were found to results in partial polyamine depletion in MCF-7 breast cancer cells resulted in decrease in S phase fraction and accumulation of cells in G1 phase. The overall picture emerging from these studies indicate a requirement for polyamines to maintain optimal rates of cell cycle traverse.

The results presented here suggest that the extreme caution is needed before using inhibitors of polyamine biosynthesis to enhance the antitumor specificity of S phase specific cytotoxic agents.
Adriamycin, an antibiotic of the anthracyclin group, has been reported to be an effective growth inhibitor of several human tumors as well as leukemic cells. In the present study, the effect of adriamycin on human breast cancer cell line was studied. Cells were found to be more sensitive to adriamycin induced cell kill during S-phase and arrests the cells in G2/M phase. The mechanism by which adriamycin exerts its antitumor cytocidal effects remains controversial (360,362-365). Among factors thought to contribute are inhibition of DNA and/or RNA synthesis arising from intercalation of the drug (360,362). DNA strand breakage resulting from peroxide and/or radicals generated from the adriamycin semiquinone radical (363) and DNA strand breaks resulting from effects of adriamycin on mammalian DNA topoisomerase II (364).

In conclusion, this data demonstrates that AdometDC inhibitor, CGP48664 and polyamine analogs BE3333 and BE4444 exert a potent anti-tumor activity on breast cancer cells in culture. The mechanism of antitumor action is partially mediated through the polyamine pathway. Furthermore polyamine depletion by these inhibitors results in accumulation of cells in G1 and inhibition of percentage of cells in S phase. These results suggest that extreme caution is needed before using these inhibitors to enhance the antitumor specificity of S phase specific cytotoxic agents.
CGP48664
4-aminooindanon-1-(2-amidino)hydrazone
dihidrochloride monohydrate

BE3333
1,15-bis(ethylamino)-4,8,12-triaza pentadecane

BE4444
1,19-bis(ethylamino)-5,10,15-triazanonadecane

Adriamycin
(Doxorubicin)

Fig. 1 Structure of an AdometDc inhibitor, CGP48664; polyamine analogs, BE3333 and BE4444; and an antibiotic anthracycline, adriamycin.
Fig. 2  Sensitivity of a human breast adenocarcinoma cell line to different concentrations of A: CGP48664, B: BE3333, C: BE4444 and D: adriamycin. 10^5 cells/ml were plated in 96-well flat bottom microtitre plate and were allowed to grow for 24h. At the end of 24h varying concentration of different drugs along with [^3]H]thymidine (0.25 mCi/well) were added and cells were harvested 48h later. The results were expressed as percentage of cells present at each drug concentration. Each point is mean ± SD of three determinations. SD < 5% of average is not shown.
A \[ \text{% of Control} \]

- CGP 48664 (uM)

B \[ \text{% of Control} \]

- BE3333 (uM)

C \[ \text{% of Control} \]

- BE4444 (uM)

D \[ \text{% of Control} \]

- Adriamycin (uM)
Fig. 3  Effect of an AdometDC inhibitor (CGP48664), polyamine analogs (BE3333 and BE4444) and an anthracycline (adriamycin) on the polyamines after 48h of treatment (A: putrescine, B: spermidine, C: spermine).
Lane 1: Control, Lane 2: BE3333 (10 μM), Lane 3: BE4444 (100 μM), Lane 4: CGP48664 (0.5 μM), Lane 5: Adriamycin (1.0 μM).
Bars represent the SD values of triplicate values.
DNA histograms as analyzed by flowcytometry of MCF-7 cells. Cells (5 x 10$^5$) were treated for 48h with different drugs, harvested and fixed in 70% ethanol followed by staining with propidium iodide. Samples were examined using EPIC$^\text{®}$ XL-software. A: Control; B: CGP48664 (0.5 $\mu$M), C: BE3333 (10 $\mu$M), D: BE4444 (100 $\mu$M), E: Adriamycin (1.0 $\mu$M).
Table 1  IC$_{50}$ values of CGP48664, BE3333, BE4444 and adriamycin on MCF-7 cells

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>IC$_{50}$ Values$^a$</th>
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<tbody>
<tr>
<td>CGP48664</td>
<td>0.2 μM</td>
</tr>
<tr>
<td>BE3333</td>
<td>0.4 μM</td>
</tr>
<tr>
<td>BE4444</td>
<td>250 μM</td>
</tr>
<tr>
<td>Adriamycin</td>
<td>0.01 μM</td>
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$^a$ IC$_{50}$ represent the effective concentration of the drug that causes inhibition of growth by 50% when compared to untreated control.
Table 2  Flow cytometric analysis of MCF-7 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Distribution of cells in</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1</td>
</tr>
<tr>
<td>None</td>
<td>76</td>
</tr>
<tr>
<td>CGP48664 (0.5 μM)</td>
<td>79</td>
</tr>
<tr>
<td>BE3333 (10 μM)</td>
<td>79</td>
</tr>
<tr>
<td>BE4444 (100 μM)</td>
<td>83</td>
</tr>
<tr>
<td>Adriamycin (1.0 μM)</td>
<td>70</td>
</tr>
</tbody>
</table>

Cell cycle phase distribution of human breast adenocarcinoma cell line MCF-7 after 48h treatment with different drugs at indicated concentrations. Drugs were added to cultures after seeding 10⁵ cells/90mm culture plates for 48h. Cells were harvested and fixed in 70% ethanol followed by staining with propidium iodide. Phase distribution were estimated by computer analysis of DNA histograms by MULTICYCLE® software. Each value represent the mean ± SD of triplicates.