Chapter 9

Isolation and Characterization of Human Breast Adenocarcinoma Cells Made Resistant to α-difluoromethylornithine
Introduction

Polyamines play an important role in the normal growth and development of mammalian cells (95, 96, 98). They appear to have essential role in carcinogenesis and neoplastic cell proliferation (98). A critical role of polyamines in breast cancer proliferation has been demonstrated by several laboratories (216). Polyamine biosynthesis in hormone responsive breast cancer cells has been shown to be regulated by estrogen (369). Majority of inhibitors have been directed against the activity of ODC and many studies have shown that inhibition of ODC activity has an antiproliferative effect. Difluoromethylornithine (DFMO), a mechanism based irreversible inhibitor of ODC activity (144), is known to inhibit estradiol stimulated breast cancer cell growth (176, 179, 180, 369). There have been several reports showing mouse cell lines having amplification of their ODC gene, on exposure to DFMO (373-376) for long periods of time. These ODC over producing cell lines can be produced from either chronic DFMO exposure (344) or gene transfection (185). Such cell lines have been useful tools for analyzing the biochemical and molecular biological properties of these enzymes. A transfected breast cancer cell line over producing ODC has been raised earlier to test the influence of increased polyamine biosynthetic activity on phenotypic features of breast cancer cells in culture (185).

In the present work, human breast cancer adenocarcinoma (MCF-7) cell line was exposed to increasing concentration of DFMO resulting in selection of cells resistant to 1.0 mM concentration of DFMO. Under the exposure to DFMO these MCF-7 cells appear to undergo a dose dependent increase in the ODC activity.
Polyamine metabolism and ODC synthesis has also been evaluated. The effect of potent inhibitors of S-adenosylmethionine viz. MGBG and CGP48664, certain polyamine analogs and adriamycin on these resistant cells has also been reported.

**Materials and Methods**

**Chemicals.** All of tissue culture chemicals, RPMI-1640, antibiotics (streptomycin and penicillin), sodium bicarbonate were purchased from Sigma Chemical Co. (St. Louis MO, USA). Dansyl chloride, dithiothreitol (DTT), L-ornithine, methyl benzethonium hydroxide, putrescine, spermidine, spermine, methylgloxal bis(guanyl) hydrazone (MGBG), doxorubicin (adriamycin) and all other reagents were also purchased from Sigma Chemical Co. (St. Louis MO, USA). Fetal calf serum (FCS) was obtained from Biological Industries (Kibbutz Beit Haemek, Israel). Antiserum to ODC protein was kindly provided by Anthony E. Pegg, Hershey Medical Centre, PA, USA. Radioactive $[^{14}C]$ornithine was obtained from NEN (DuPont, Boston USA). $[^{35}S]$methionine and $[^{3}H]$thymidine were purchased from Bhabha Atomic Research Centre (Bombay, India). Difluoromethylornithine (DFMO) was kindly provided by Marrion-Merrell Dow Research Center (Cincinnati, OH, USA). CGP48664 (4-aminoindanon-1-(2'-amidino) hydrazone dihydrochloride monohydrate) was a kind gift from Ciba-Geigy Ltd. (Switzerland), BE3333 (1,15-bis(ethylamino)-4,8,12-triaza pentadecane) and BE4444 (1,19-bis(ethylamino)-5,10,15-triazanonadecane) were generous gifts provided by Akira Shirahata, Josai University (Sakoda, Japan).
Cell line. Wild type MCF-7 cells were obtained from 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 carbonate, 10% fetal calf serum (FCS) and antibiotics (50 ug/ml of penicillin and 100 ug/ml of streptomycin). The cells were routinely grown at 37°C in humidified atmosphere of 95% air and 5% carbon dioxide and were subcultured till growth reached near confluency.

Selection of DFMO resistant cell line. Human breast adenocarcinoma cells MCF-7 were initially grown in the presence of 0.1 mM DFMO for about four weeks. The concentration of DFMO was increased stepwise (0.1 mM --> 0.2 mM --> 0.4 mM --> 0.6 mM --> 0.8 mM --> 1.0 mM) over a period of seven months. The cultures were kept at each concentration for several weeks till their growth pattern was comparable to that of the wild type. Most of the experiments described in this study were performed with cells adapted to grow in the presence of 1.0 mM DFMO.

Ornithine decarboxylase activity. In order to determine the ODC activity, the cells were washed with ice-cold phosphate buffered saline (PBS) and then suspended in lysis buffer containing 50 mM Tris, 0.1 mM EDTA, 2.5 mM DTT (pH 7.5) and stored at -70°C till use. The cells were lysed by repeated freezing and thawing before the assay. The cell lysate was centrifuged at 12000 x g for 30 min at 4°C and ODC activity was assayed (197).

Polyamine estimation. DFMO resistant cells (≈ 10^5) were plated in 90mm petri-dishes and grown for 48h in the absence of DFMO. Cells were harvested and
resuspended in 250 ul of 2% perchloric acid and kept at 4°C for 24h. The cells were centrifuged at 12000 x g for 15 min and supernatant was used for polyamine estimation. Dansyl derivatives were prepared according to Seiler (196) and polyamines were separated by thin layer chromatography (TLC) on 0.2 mm thick silica gel G plates using ethylacetate : cyclohexane (2:3 v/v) as a solvent. Quantification of polyamines was accomplished using a Camag TLC scanner (Camag, Sonnenmattstr, Switzerland) with the help of a TLC scanning software Cats3 (Camag, Sonnenmattstr, Switzerland). The concentration of unknown samples was determined against standard polyamines.

**Assay of ODC protein.** ODC protein was labelled by addition of 15 µCi/ml [35S]methionine to the culture medium. The synthesis of ODC protein was studied using a 2h labelling period. The cell extracts were prepared in lysis buffer (10 mM Tris HCl pH 7.5, 2.0 mM EDTA, 0.03% Triton X-100, 150 mM sodium azide, 100 g/ml PMSF and 0.3% Nonidet P-40). Immunoprecipitation of [35S]methionine labelled ODC molecule was performed by addition of 10 µl of antiserum to ODC protein. After 30 min at room temperature, 30 µl of 10% protein A sepharose was added and mixture was shaken for 30 min. The immuno-precipitated protein was collected and washed twice with lysis buffer, and solubilized by heating in 62.5 mM Tris-HCl pH 6.8, 0.1% sodium dodecylsulfate, 5% 2-mercaptoethanol and 10% glycerol and separated on 10% polyacrylamide gel containing 0.1% of SDS (204). After electrophoresis the gel was treated with DMSO followed by fluorography finally exposing the gel to Kodak X-ray film.

**Drug Studies.** Wild type and 1.0 mM DFMO resistant (DR 1.0) MCF-7 cells were plated in 96 well flat bottom microtitre plates. After 24h of incubation the medium
was changed and varying concentrations of the drugs were added and plates were incubated for another 48h. The cells were labelled with [$^3$H]thymidine (0.25 mCi per well) at the time of drug addition. The cells were harvested and counted in a scintillation counter (Beckman LS1800, Beckman Instrument Inc. Palo Alto CA, USA). The results are expressed as percentage of cells present at each drug concentration relative to the number of cells present in the absence of drug. Each point represents result of triplicate samples and is plotted as the mean ± standard deviation (SD).

All experiments were conducted in triplicate and repeated at least three to four times, and the average ± SD was calculated; SDs < 5% of the average are not shown.

Results

Resistance to DFMO was induced by growing cells in increasing concentration of DFMO. The cells were maintained at each concentration until a normal growth rate was obtained. The cells showed an increase in ODC activity as they acquired resistance to DFMO. The ODC enzyme activity of resistant cells when grown in the absence of DFMO for 48h was found to be 5 times more in case of 0.2 mM DFMO resistant cell and 7 times more in case of 0.6 mM DFMO resistant and 10 times more in 1.0 mM DFMO resistant cells as compared to wild type MCF-7 cells (Fig. 1).

Analysis of cellular polyamine profile in cells made resistant to different concentration of DFMO revealed gradual accumulation of putrescine, spermidine and spermine over wild type MCF-7 cells. (Table 1).
The differences in the synthetic rates as determined with biosynthetic labelling with \[^{35}\text{S}]\text{methionine} \text{ were greater in 1.0 mm DFMO resistant cells as compared with wild type. The molecular weight of immuno-reactive ODC in MCF-7 cells in both wild type and DFMO resistant cells was 52 kD. In the resistant cells, the ODC synthesis was enhanced 20 times over the wild type as measured by densitometric analysis (Fig. 2).}

Effect of inhibitors of S-adenosylmethionine decarboxylase (AdometDC), polyamine analogs and adriamycin on the growth of wild type MCF-7 cells and cells made resistant to 1.0 mM DFMO is shown in Fig. 3 (A-E) and Table 2. Effect of AdometDC inhibitors namely MGBG and CGP48664 on the resistant cell line is shown in Fig. 3A and 3B. As observed from the ED\(_{50}\) values the DR 1.0 strain is sensitive to CGP48664 but was resistant to MGBG (Table 2) The susceptibility of DFMO resistant strains to polyamine analogs BE3333 and BE4444 is shown in Fig.3C and 3D respectively. As observed from their ED\(_{50}\) values (Table 2) it was observed that DR 1.0 cells were almost equally sensitive to these drugs as their wild counterpart. Fig.3E shows the effect of adriamycin on DR 1.0 resistant MCF-7 cell line. The ED\(_{50}\) value shows that both the wild type and resistant cells were equally sensitive to adriamycin.

Fig.4 shows that cells grown in the presence of DFMO showed increase resistance towards the anti-proliferative action of DFMO. When cells were grown for 48h in the presence of DFMO, the ED\(_{50}\) was 0.4 mM for the wild type and it was > 10.0 mM for the cells made resistant to 1.0 mM concentration of DFMO.
Discussion

Polyamines and its enzymes have been postulated to play a critical role in cell proliferation and carcinogenesis (96,98,377). Ornithine decarboxylase is an important mammalian enzyme the gene of which easily undergoes amplification under appropriate selection pressure (375,376). ODC activity is known to increase transiently upon growth factor exposure, becomes constitutively active during cell transformation by variety of agents, such as chemical carcinogen (378), viruses (379,380) and oncogenes (381,382). A direct role of polyamine in mammary carcinogenesis is not known. Several findings however point to the fact that breast cancer specimens have considerably higher levels of polyamines then the surrounding normal tissue (383,384), suggesting the possible role of these compounds in the development of mammary tumors. Chronic administration of escalating doses of DFMO over a period of months has been shown to induce ODC over-expression in variety of biological systems, which in some cases persists despite discontinuation of the inhibitors (344,385,386). Transfection of hormone deficient MCF-7 breast cancer line with an ODC cDNA coding for a stable DNA showed that there was selective increase in cellular putrescine content while spermidine and spermine level were not altered (185). ODC over-expressing MCF-7 cells were resistant to the antiproliferative effect of low but not high concentration of α-DFMO.

In the present work we attempted to address the potential role of polyamines in breast cancer proliferation and progression. DFMO-induced overexpression of ODC has been found to be due predominately to gene amplification (344,385,386). In this case we studied the ODC protein in the DFMO-resistant strain versus wild
type strain. We found amplified product of the target gene and its enzyme activity. The resistant clones showed a selective increase in ODC activity and putrescine spermidine and spermine levels. Comparison of human breast cancer specimens to normal breast tissue reveals more profound alteration in the polyamine pathway than a selective increase in putrescine (383). These include the elevation of the cellular content of spermidine and spermine, acetylated polyamines and spermidine and spermine N-1-acetyl transferases (383). These results show that multiple alternation in the polyamine pathway as opposed to simple increase in ODC takes place during mammary carcinogenesis and possibly tumor progression.

Resistant cell line against DFMO seemed an excellent model to test the cytotoxicity of agents against this cell line which are potential inhibitors of the wild type. The results here clearly show that resistant MCF-7 cells were equally sensitive towards adriamycin, CGP48664 and polyamine analogs BE3333 and BE4444. DFMO resistant cells give an opportunity to exploit the multidirectional inhibitory approach in the polyamine biosynthetic pathway. It is interesting to note that if cells become resistant to one inhibitor of the pathway, other inhibitors of the same pathway like AdometDC inhibitor CGP48664 and polyamine analogs could be successfully employed in overcoming resistance. This indicates that it is possible to control breast cancer cell resistance to ODC inhibitors by interfering with other steps in the pathway. However MGBG was unsuccessful in controlling the growth of this cell line. MGBG is not a very specific inhibitor of AdometDC (155,156), it is possible that resistance to one ODC inhibitor can confer resistance to other inhibitor of ODC also.
The results confirm that the polyamine biosynthetic pathway can be exploited successfully not only against the resistant cell lines, but it also suggests that if breast cancer cells develop resistance to one of the inhibitors of the pathway, other targets in the pathway can be chosen for controlling the cancerous growth. The importance of the gene amplification to the emergence of resistance to cancer chemotherapy against anticancer agents and to the malignant process as well, is becoming increasingly acknowledged. Beside necessitating carefully planned combined chemotherapy to circumvent possible developing drug resistance it has been suggested that the gene amplification induced arrangements may play a role in the progression of the malignant process (387). Further studies could reveal changes that may be important not only to the metabolism of polyamines, but also to the very nature of human malignancy in general.
Fig. 1  Ornithine decarboxylase (ODC) activity in wild type (W) and DFMO-resistant MCF-7 cells. W, wild type; DR 0.2, resistant to 0.2 mM DFMO; DR 0.6, resistant to 0.6 mM DFMO; DR 1.0, resistant to 1.0 mM DFMO. Bar indicates ± SD of three determinations.
Fig. 2 Immunoprecipitated ODC (52 kDa) protein in wild type (W) and 1.0 mM DFMO-resistant (DR 1.0) cells. 5 x 10^6 cells were labelled by addition of 15 uCi/ml of \[^{35}\text{S} \]methionine to the culture medium. After a 2h labelling period the cells were harvested and extracts were prepared by using lysis buffer as mentioned in "Material and Methods" section. Immunoprecipitation of \[^{35}\text{S} \]methionine labelled ODC protein was performed by addition of antiserum to ODC protein. The immunoprecipitated protein was solubilized and separated on 10% SDS-PAGE as described in the "Material and Methods" section. Molecular weight markers (Pharmacia) are shown on the left.
Fig. 3  Sensitivity of wild type (W) and DFMO resistant MCF-7 cells (DR 1.0) to different concentration of A: MGBG, B: CGP48664, C: BE3333, D: BE4444, and E: adriamycin. Wild or DFMO resistant cells (1 x 10^5/ml) were plated in 96 well flat bottom microtitre plate and were allowed to grow for 24 h. At the end of 24h varying concentrations of different drugs along with [3H]thymidine [0.25 mCi per well] were added to the wells and cells were harvested 48h later. The results are expressed as percentage of cells present at each drug concentration relative to the number of cells present in absence of drug (control). (o) W (wild type MCF-7 cells), (●) DR 1.0 (resistant to 1.0 mM DFMO).

Each point is mean ± SD of three determinations. SD < 5% of average is not shown.
Fig. 4 Effect of different concentrations of DFMO on the growth of wild type (o) and 1.0 mM DFMO resistant (●) MCF-7 cells. Wild or DFMO resistant cells (1 x 10^5/ml) were plated in 96 well flat bottom microtitre plate and were allowed to grow for 24h. At the end of 24h varying concentrations of DFMO along with [3H]thymidine [0.25 mCi per well] were added to the wells and cells were harvested 48h later. The results are expressed as percentage of cells present at each drug concentration relative to the number of cells present in absence of drug (control) Bars indicate ± SD values of triplicate sample. SD < 5% of average is not shown.
Table 1: Polyamine levels in DFMO resistant MCF-7 cell line (DR) and wild type (W) MCF-7 cell line

<table>
<thead>
<tr>
<th>Groups</th>
<th>Putrescine</th>
<th>Spermidine</th>
<th>Spermine</th>
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<tr>
<td>Control</td>
<td>5.35 ± 0.2</td>
<td>11.83 ± 0.3</td>
<td>8.5 ± 0.2</td>
</tr>
<tr>
<td>DR 0.2</td>
<td>16.5 ± 0.47</td>
<td>20.9 ± 0.1</td>
<td>21.53 ± 0.44</td>
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<tr>
<td>DR 0.6</td>
<td>23.5 ± 0.5</td>
<td>38.27 ± 0.9</td>
<td>20.15 ± 0.5</td>
</tr>
<tr>
<td>DR 1.0</td>
<td>26.5 ± 0.4</td>
<td>45.2 ± 1.0</td>
<td>27.52 ± 0.9</td>
</tr>
</tbody>
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DR 0.2, resistant to 0.2 mM DFMO  
DR 0.6, resistant to 0.6 mM DFMO  
DR 1.0, resistant to 1.0 mM DFMO

a Mean ± SD of 3-4 replicates samples
Table 2  Comparative effects of growth inhibitory agents on wild type (W) and DFMO resistant (DR 1.0 mM) MCF-7 cells

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>ED$_{50}$ values$^a$</th>
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<tbody>
<tr>
<td></td>
<td>W</td>
<td>DR 1.0*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>μM</td>
<td>mM</td>
<td></td>
</tr>
<tr>
<td>MGBG</td>
<td>0.1 μM</td>
<td>&gt; 1.0 mM</td>
<td></td>
</tr>
<tr>
<td>CGP 48664</td>
<td>0.2 μM</td>
<td>0.25 μM</td>
<td></td>
</tr>
<tr>
<td>BE3333</td>
<td>0.4 μM</td>
<td>0.6 μM</td>
<td></td>
</tr>
<tr>
<td>BE4444</td>
<td>250 μM</td>
<td>0.6 mM</td>
<td></td>
</tr>
<tr>
<td>Adriamycin</td>
<td>0.01 μM</td>
<td>0.02 μM</td>
<td></td>
</tr>
<tr>
<td>DFMO</td>
<td>0.4 mM</td>
<td>&gt; 10.0 mM</td>
<td></td>
</tr>
</tbody>
</table>

* DR 1.0 represents DFMO resistant cells that were made resistant and adapted in presence of 1.0 mM DFMO.

$^a$ ED$_{50}$ represent the effective concentration of the drug that causes inhibition of growth by 50% when compared to untreated control.