Chapter 2

Materials and Methods
2.0 MATERIALS

Biological Materials

Cell line: MCF-7, a human breast adenocarcinoma cell line was obtained from the National Facility for Animal Tissue and Cell Culture (NFATCC) Pune, India and maintained at the School of Life Sciences, Jawaharlal Nehru University, New Delhi, India.

Serum: Fetal calf serum (FCS) was obtained from the Biological Industries, Kibbutz Beet Haemek, Israel.

Animals: Sprague-Dawley Wistar rats and Swiss mice were purchased from the National Institute of Nutrition (NIN) Hyderabad, India and maintained under standard conditions of light and humidity at the animal house facility of Jawaharlal Nehru University, New Delhi, India. The animals were fed with the standard animal food pellets provided by the Hindustan Lever Ltd. Delhi, India.

The studies were conducted according to the ethical guidance of the Indian National Science Academy (INSA) on the use of animals for scientific research.

Biochemical: Tissue culture chemicals viz. RPMI-1640, DMEM phenol red free growth media, estradiol, tamoxifen, antibiotics streptomycin and penicillin, sodium bicarbonate, activated charcoal, dithiothreitol (DTT), L-ornithine, putrescine, spermidine, spermine and pyridoxal phosphate were purchased from Sigma Chemical Co. St.Louis, MO, USA. All common chemicals were of analytical grade and were purchased locally.
Chemomodulators/drugs: Pacilitaxel (taxol), sodium selenite, retinoic acid, methylglyoxal bis(guanyl)hydrazone (MGBG), doxorubicin (adriamycin) were purchased from Sigma Chemical Co., St. Louis, MO, USA. DL-α-difluoromethylornithine (DFMO) was kindly provided by Marrion-Merrel Dow Research Center, Cincinnati, OH (USA). CGP48664 [4-amidinoindanon-1-(2’amidino)hydrazone dihydrochloride monohydrate] was a kind gift from Ciba-Geigy Ltd. (Switzerland), BE3333.5HCl [1,15-bis(ethylamino)-4,8,12-triazapentadecane] and BE4444 [1,19-bis(ethylamino-5,10,15-triazanona decane], the pentamines were generous gift provided by Akira Shirhata, Josai University, Sakado, Japan.

Radiochemicals: \([^{14}\text{C}]\text{ornithine (51.6 mCi/mmole)}\) was obtained from NEN Research Products, DuPont, Boston, USA. \([^{35}\text{S}]\text{methionine and }[^{3}\text{H}]\text{thymidine were from Bhabha Atomic Research Centre (BARC), Bombay, India.}\)

Immunochemicals: Goat anti-rabbit IgG-horse radish peroxidase conjugate and protein A sephrose beads were obtained from Sigma Chemical Co., St. Louis, MO, USA. Anti ODC antibody was kindly provided by Anthony E. Pegg, Milton S. Hershey Medical Centre, Pennsylvania State University PA, USA. ECL enhanced chemiluminescence western blotting reagents were purchased from Amersham, Arlington Height, IL, USA.

Other chemicals/materials: Dansyl chloride, propidium iodide (PI),7,12-dimethyl-1,2,-benz(a)anthracene (DMBA), N-nitrosomethylurea (NMU), L-butathione sulfoximine (L-BSO), RNAase A, 4-6,diamidino-2-phenyl-indole (DAPI), ethylene
diamine tetra acetic acid (EDTA), methylbenzothionium hydroxide, TritonX-100, sodium azide, Tris(hydromethyl)aminomethane (Tris), Nonidet P-40, sodium dodecylsulphate (SDS), 2-mercaptoethanol, glycerol, acrylamide, 5,5'-dithiobis(2-nitrobenzoic)acid (DTNB) were from Sigma Chemical Co., St. Louis, MO, USA. Diphenylamine (DPA) sulfuric acid, sodium dihydrogen-phosphate, disodium hydrogen phosphate, perchloric acid, D-glucose, glycine, sodium chloride, ethyl acetate, L-proline, picric acid, dimethyl sulfoxide (DMSO), formalin were all of analytical grade and were purchased from either Qualigen Fine Chemicals, Bombay, India or S.D fine chem. Ltd. Boisar, India. Scintillation fluid (toluene based) and Bray’s solution was obtained from Spectrochem Pvt. Ltd. Bombay, India. All other reagents used were of analytical grade.

Tissue culture wares like culture flasks, culture plates or petri dishes (60mm and 90mm), 24-well and 96-well flat bottom microtitre plates were supplied by Tarson Company Ltd. Precoated MERCK thin layer chromatography (TLC) plates (0.20 mm thickness and 20 X 20 cm size) were obtained from Anchrom Industries, Bombay, India. Silicated glass tubes (15 X 60 mm) were specially prepared for ODC assay by Mr Baljeet Singh, a senior technical assistant (Glass Blowing Unit), School of Life Sciences, Jawaharlal Nehru University, New Delhi, India. Propylene centre wells and rubber stoppers were procured from Kontes Vineland, New Jersey, USA.

2.1 METHODS

2.1.1 Cell culture: MCF-7, a human breast adeno-carcinoma cell line was 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 bicarbonate 10% fetal calf serum (FCS) and antibiotics 50.0 μg/ml of penicillin and 100 μg/ml of streptomycin. The cells were routinely grown in 25 cm² flask under humidified atmosphere of 5% carbon-dioxide and 95% air at 37°C and were subcultured when the growth reached near confluency.

For drug study and other biochemical assays, the cells were cultured in 60mm or 90mm petri-dishes as indicated in each experimental set up. Cells were harvested by trypsinization of cells from either flasks or plates by incubating with trypsin solution (0.25% w/v trypsin, 1 mM EDTA in PBS) for 1 min and then washed twice with PBS. Cell pellet was used for all the studies.

2.1.2 Selection of DFMO resistant cell line: Human breast adenocarcinoma cells 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. 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 the drug resistant study were performed with the cells adapted to grow in the presence of 1.0 mM DFMO.

2.1.3 Monitoring the cell growth: Cell growth was monitored by trypan blue dye exclusion test. Cell suspension in PBS (100 μl) was mixed with 100 μl of trypan blue solution (5 mg of trypan blue in 10 ml of PBS) was added and incubated for 1 min at 37°C. The trypan blue excluded cells (live cells) were counted and percentage of live cells were considered for monitoring the cell growth.
2.1.4 Drug studies: Cells were maintained in 60mm petri-dishes. A number of petri-dishes were plated with approximately $10^5$ cells in medium for 48h followed by drug treatment. The incubation period varied with each experimental set up as indicated. Following exposure to drugs, the cells were rinsed and harvested by trypsinization, cell growth was monitored by counting viable cells after trypan blue dye exclusion test using a hemocytometer. Each experiment was repeated at least 3-4 times and each treatment was in triplicate. The percentage of trypan blue excluded cells (live cells) were more than 95% in untreated groups (control). The growth was also measured by estimation of total DNA content (195) and by incorporation of $[^3\text{H}]$thymidine as discussed below.

2.1.5 Drug study by thymidine incorporation: Cells were seeded ($10^5$ cell/ml) in 200 $\mu$l of medium in 96-well culture plates 48h before drug treatment. Following drug addition to the medium, 2.5 $\mu$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 Instruments Inc. CA, USA). The results were expressed as percentage of cells present at each drug concentration.

2.1.6 DNA estimation: After harvesting of cell pellet, the cells were hydrolyzed in 10% TCA by heating at 95°C for 10 min. One part of the sample was diluted with two parts of water and five parts of diphenylamine (DPA) reagent (1.00 gm of DPA was dissolved in 100 ml of glacial acetic acid and 2.5 ml of concentrated sulfuric acid) and heated in boiling water bath for 10 min. The absorbance was measured at 595 nm (195) and DNA content was calculated after comparing with the standard DNA solution.
2.1.7 **Polyamine estimation:** Polyamines were estimated in this study by dansylation method described by Seiler, 1970 (2) with some modification.

2.1.8 **Principle:** Polyamines react with dansyl chloride to form dansylamide, which are then separated by thin layer chromatography. Dansylamides fluoresce and have an excitation wavelength of 366 nm and emission at 495 nm.

2.1.9 **Extraction and estimation:** About a million cells were sedimented by centrifugation at 5000 rpm for 15 min washed with phosphate buffered saline (pH 7.4) and resuspended in 250 μl of 2% perchloric acid. The suspension was kept overnight at 4°C to extract polyamines. The dansyl derivatives were prepared according to Seiler, 1970 (196). The debris was precipitated by spinning at 12000 rpm for 10 min. 200 μl of supernatant was first neutralized with 200 μl of super saturated sodium bicarbonate and then dansylated overnight by adding 400 μl of dansyl chloride (5 mg/ml in acetone) at room temperature in dark. After approximately 16h untreated dansyl chloride was removed by incubating the mixture with 100 μl of L-proline (150 mg/ml in ice cold water) for 30 min at room temperature. The dansyl chloride was extracted in 500 μl of toluene and separated by thin layer chromatography (TLC) on 0.22 mm thick silica gel precoated plates using ethyl acetate: cyclohexane (2:3 v/v) as the solvent. Quantification of plates was done in situ by TLC scanner (CAMAG/TLC-Scanner II) with Cats3/TLC II software program (Camag, Sonnenmattstr, Switzerland).

Tissue samples were processed for polyamine estimation by homogenization of tumor tissue or mammary epithelium using Polytron® PT-MR 3000 (Kinematica AG, Littau, Switzerland) for 5 min in 10 volumes of 2% perchloric acid. The
homogenate was kept overnight in order to extract the polyamines. The dansyl derivatives were prepared and separated by thin layer chromatography as described above. Data was expressed as nmole of polyamines/mg protein.

2.1.10 Assay of ornithine decarboxylase: The method of Seeley et al., 1982 (197) was followed for the measurement of ornithine decarboxylase activity.

Cell homogenate: A million cells were harvested by trypsinization and washed with ice-cold PBS and pelleted down. The pellet was suspended in 250 µl of harvest buffer (50mM Tris, 0.1mM EDTA and 2.5 mM DTT pH 7.5). The cells were homogenized by glass homogenizer at 4°C.

Tissue homogenate: The mammary epithelia and tumor tissue were homogenized in 10 volume of homogenizing buffer (50mM Tris, 0.1 mM EDTA and 2.5 mM DTT pH 7.5) after removing the blood by thoroughly washing with ice cold PBS followed by mincing of the tissue.

The homogenate was centrifuged at 12000 rpm for 10 min at 4°C. 200 µl of the supernatant was added to 50 µl of reaction mixture containing 200 µM of pyridoxal phosphate, 12.5 mM DTT, 250mM Tris (pH 7.5), 200 µM L-ornithine and 3 µCi of 14C-ornithine (Specific activity 51.6 mCi/mnmole). The mixture was incubated at 37°C for one hour in air tight tube and evolved CO₂ was trapped in 100 µl of methyl benzethonium hydroxide. Reaction was stopped by adding 5N sulfuric acid and further incubated for 30 min. The trapped radioactivity was counted in toluene based scintillation cocktail using Beckman scintillation counter (LS1800 model, Beckman Instruments Inc. Palo Alto, CA, USA).
2.1.11 GSH content: The acid soluble sulfahydryl (-SH) or GSH (glutathione) group binds with DTNB to form a yellow colored complex. This complex has an absorption maxima at 412 nm.

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\text{GSH} + \text{DTNB} \longrightarrow \text{2-thio-nitrobenzoate.}
\]

Procedure: The acid soluble sulfahydryl levels were estimated as described by Moron et al., 1979 (198). The homogenate (from a million cells or 10% w/v tissue) was prepared in 5M sodium chloride, 5 mM EDTA and 250 mM glacial acetic acid. The homogenate was centrifuged at 5000 rpm for 10 min. 100 μl of the supernatant was incubated in the presence of 0.06 M sodium phosphate buffer (pH 8.0) and 0.4 mM dithio (bis) nitrobenzoic acid (DTNB) and absorption was measured at 412 nm. The concentration of GSH in experimental groups was determined by running standards of known concentration of glutathione (reduced). The data was expressed as μM of thio-nitrobenzoate produced/mg of protein or per million cells as the case may be.

2.1.12 Cell cycle analysis: Cells were grown and treated for indicated time periods, harvested by trypsinization, washed with cold PBS and pelleted down by centrifugation at 2000 x g for 10 min at 4°C. The pelleted cells were fixed using ice cold 70% ethanol for 24h. 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 μg/ml of propidium iodide, 100 μg/ml of RNAase A and 1 mg/ml of glucose in PBS) was added to 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 than analyzed using
MULTICYCLE® software (Phoenix Flow System Inc. San Diego CA, USA) for cell cycle analysis.

2.1.13 Quantitative assay of DNA fragmentation: Assays were conducted in triplicate in flat bottom, 96-well microtitre plates in RPMI-1640 medium supplemented with 10% FCS in a total volume of 0.1 ml. Approximately 5 x 10^4 cells were seeded in each well. Wells for total count received 100μl of medium whereas experimental wells received appropriate concentration of drugs in the medium. DNA fragmentation was measured by release of [³H]thymidine labelled DNA fragments by adding 150 μl of 10 mM EDTA at 0.3% of TritonX-100 to each well. Incorporated [³H]thymidine was measured by washing cells on glass micro-fibre filters and counting in a Beckman scintillation spectrometer LS1800 (Beckman, Instrument Inc. CA, USA). The percentage of DNA released was calculated as follows:

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\% \text{ of DNA Fragmentation} = \frac{(\text{Total cpm} - \text{Experimental cpm})}{\text{Total cpm}}
\]

2.1.14 Cell nucleus morphology: Cells were exposed to indicated treatment for 24h. After the exposure period cells were washed with PBS fixed in 70% ethanol and stained by incubating with DAPI (10 μg/ml) for 10 min. Cells were washed with tap water for 30 min. Fixed and stained cells were observed under the fluorescent microscope (Leitz Wetzlar, Aristophot, Ernest Leitz GMBH Wetzlar, Germany) for cell nucleus morphology.

2.1.15 Tumor induction in animals: 56 day old female virgin Sprague Dawley rats were used for induction of mammary tumors by chemical carcinogens, [7,12-
dimethyl-1,2-benz(a)anthracene] (DMBA) or N-nitrosomethylurea (NMU). Animals were maintained in air-conditioned room of the animal house facility of Jawaharlal Nehru University, New Delhi, India with the standard diet and tap water ad-libitum.

Induction by DMBA: DMBA (20 mg/ml) solution was prepared in corn oil and administered intragastrically (65 mg/kg of body weight) in each animal for induction of mammary tumors in overnight starved 56 day old female Sprague Dawley rats (199).

Induction by NMU: NMU was dissolved in acidified 0.9% saline solution (0.05% acetic acid and 0.9% (w/v) sodium chloride). Mammary tumors were induced by single subcutaneous injection of NMU solution to 56 day old rats (200).

Control group animals were treated with vehicle corn oil or acidified saline solution in same amount and by same way as irrespective DMBA or NMU induced mammary carcinogenesis. The animals were palpated once a week for detection of tumors in their mammary glands for documentation of tumor incidence. Only continued adenocarcinoma were reported in the results. About 20-25 rats per group were used in the carcinogenesis experiments. All the rats were weighed every week during the first two months and alternate weeks for the remaining time. All the animals surviving after 25 weeks of carcinogenic treatment were sacrificed and an autopsy of mammary gland was exposed for detection of non-palpable tumors. Tumors or mammary epithelia were harvested and processed for studies.
2.1.16 Tumor incidence: The chemical carcinogen injected rats were weighed and observed every week after the carcinogen injection. The tumor induction was monitored by palpating the mammary glands. The tumor presence was recorded. The term used in the tables are: 1) Tumor incidence — it is percentage of tumors induced animals in a particular group. 2) Tumor load — average number of tumors induced per animals. 3) Latency period — the average time period between carcinogen injection and induction of tumors. 4) The total number of tumors induced in a group. 5) Observation period — the total time period in number of days the animals were kept for study after carcinogen injection.

The tumor incidence at the final point were compared by analysis and tumor yield compared by frequency distribution analysis (201). The statistical analysis of inter group differences in tumor incidence and number were corrected for multiple comparison.

2.1.17 Tumor isolation: The tumor induced rats were sacrificed by keeping them in anesthetic ether saturated chambers. The mammary epithelia and tumors were dissected out without opening the peritonieum. The tissue was minced and washed completely in ice cold PBS to remove blood and fat. The homogenate was prepared according to the respective assays and analyzed accordingly.

2.1.18 Histological studies: The mammary epithelium or tumor tissue were fixed by keeping them in Bouin's fixative (7.5 ml saturated picric acid solution, 25 ml formalin, 5 ml of glacial acetic acid) overnight and washed with tap water to remove excess fixative and transferred to 50%, 70%, 90% and 100% alcohol for 5 min in
each alcohol concentration and finally transferred to xylene for 2h. The tissues were transferred in molten paraffin wax (melting point 60-62°C) for 2h at 40-45°C to allow the wax to infiltrate inside the tissue. The wax blocks were prepared and 5 μm thin tissue sections were made using a senior rotary microtome (Model MT-1090A, Weswox Optix, India). The tissue sections were spread on albuminated glass slide and kept on hot plate (50°C-60°C) to remove wrinkles in the wax sections. The sections were stained with hematoxylin and eosin staining dye and mounted by using DPX mounting solution. The sections were photographed to study the histology of the tissue.

2.1.19 Estrous cycle in mice: Study of estrous cycle was done in 40 day old virgin female mice. The stage of estrous cycle was determined by observing the vaginal smears by inserting a flamed pipette (filled with 0.5cc of physiological saline) into vagina and sucking out some of the contents on microscopic slide and observing under dark field illumination in microscope (Nikon, Japan) for the estrous stage.

2.1.20 Determination of pregnancy period in mice: The time of pregnancy was determined by looking for vaginal plugs everyday in the morning between 6-8 A.M. after keeping four females and one male in one cage for mating. For the study, 8 day pregnant, 8 day lactating and 8 day weaned mice were selected. For control 40 day old virgin mice were used.

2.1.21 Mammary epithelium isolation: The mice were killed by cervical dislocation, the mammary epithelium was dissected out and kept on ice-cold PBS and washed thoroughly to remove the fat along with any blood stains. The tissue was
weighed and homogenized in indicated solution in the respective assays. The homogenate was processed further for respective analysis.

2.1.22 Ovariectomy: Virgin Sprague Dawley female rats were 50 day old when operated for ablation of ovaries (ovarectomization) under anesthesia and were kept for one week under normal conditions till they recovered and were then treated with the carcinogens for induction of mammary tumors. Control animals were kept under anesthetic condition for the same duration as overectomised rats. The animals were palpated once a week for detection and documentation of tumors in their mammary glands. After 25 weeks of carcinogenic treatment all the animals were sacrificed and tumor or mammary epithelia were harvested and processed for studies.

2.1.23 Estradiol study: The cells were transferred from normal RPMI-1640 supplemented 10% FCS growth media to DMEM without phenol red containing 10% dextran coated Charcoal (DCC) treated fetal calf serum. The DCC treated serum was prepared by incubating 100 ml of fetal calf serum with 5 ml of charcoal coated dextran solution (0.50% Norit A, 0.05% dextran T-70 in 0.14 M sodium chloride) at 55°C for 30 min (202,203). This procedure removed 98% of trace amounts of serum estradiol (202). However, such serum may still contain some estrogen sulfate. The charcoal was removed by centrifugation and filtration and filtered serum was stored at -20°C.

The cells were grown in the phenol red free DMEM medium supplemented with 10% DCC treated serum. Medium was changed after 48 h and growth monitored
by viable cell counting (trypan blue dye exclusion) or by estimation of total DNA
content.

2.1.24 Immuno-precipitation: The protein was labelled by addition of 15 μCi/ml
of [35S]methionine to the culture medium. It was incubated for 2h to label the newly
synthesized protein. The cells were lysed in lysis buffer (10mM Tris HCl pH 7.5, 2.0
mM EDTA 0.03% Triton X-100, 150 mM sodium azide, 100 mg/ml PMSF and 0.3% NP-40). The protein was precipitated by addition of 10 μl of antiserum to the lysate
and incubated for 1 h at room temperature. Protein A sephrose beads were added and
incubated for another 30 min by continuous shaking. The immuno-precipitated protein
was collected washed twice with lysis buffer and fractionated by electrophoresis on
10% polyacrylamide gel containing 0.1% SDS (204). After electrophoresis the gel
was fixed with DMSO and 22% diphenyloxazole and was exposed to X-ray film for
autoradiography.

2.1.25 Western blot analysis: Proteins in cell extracts (15 μg/lane) were separated
by SDS-PAGE on 10% acrylamide gel. The proteins were electrophoretically
transferred to a 0.45 μM nitrocellulose membrane. The membrane was incubated with
Tris-buffered saline (TBS), 0.1% Tween-80 (TBST) containing 3% skimmed milk and
then washed with TBST. The membrane was incubated with 20 ml TBST and 2 μl of
goat anti-rabbit IgG-horse radish peroxidase conjugate for one hour. The membrane
was washed four times with Tris-buffered saline, 0.3% Tween 80, and thrice with
TBST. The membrane was incubated for 10 min with ECL reagent and the emitted
light was detected by short exposure to Kodak X-ray film.
2.1.26 **Protein Estimation**: Protein content was estimated by the method of Lowry *et al*. 1951 (205). Different aliquots of unknown samples were taken and made up to 0.5 ml with PBS and to this 5.0 ml of alkaline copper reagent was added (1.0 ml of 1% sodium potassium tartarate and 1.0 ml of 0.5% copper sulfate mixed with 2% sodium carbonate in 0.1 N sodium hydroxide) and kept for incubation for 10 min at room temperature. After addition of 0.5 ml of 1N Folin-phenol reagents, it was vortexed and incubated for 30 min at room temperature. The color development was measured at 700 nm against the blank. The protein concentration of the sample was determined from standard curve using 10-250 μg of crystalline bovine serum albumin (fraction IV) as standard.

2.1.27 **Statistical analysis**: All the experiments were performed in triplicate and repeated at least 3-4 times. The concurrent observations were considered to find out the mean value and SD (standard deviation). Statistical analysis was done by student’s t-test. SD < 5% of the mean were not shown.