5.1 Introduction:

Estrogen is a key regulatory hormone, which in addition to its role in reproduction, affects a number of physiological systems, including the skeletal and cardiovascular system. The important role of estrogen in various tissues is perhaps most evident in postmenopausal women. In addition to menopausal symptoms, experience increases in osteoporosis and coronary heart disease as their estrogen levels decline (Henry U et al., 1998). Estrogen replacement, while effective against osteoporosis and heart disease, produces a number of side effects associated with the breast and uterus which limits compliance. Selective estrogen receptor modulators (SERMs), may exhibit an agonistic or antagonistic biocharacter depending on the context in which their activity is examined (Martin Dutertre et al., 2000). Tamoxifen, Raloxifene is an ER (Estrogen receptor) antagonist in breast and an agonist in bone. However, it does not exert ER agonist properties in the uterus (Mitlak et al., 1997; Cosman et al., 1999). Both tamoxifen and raloxifene also have beneficial, estrogen-like effects in the liver with respect to lipid metabolism (Mitlak et al., 1997). Taken together, current SERMs are useful pharmacological agents for the prevention and/or treatment of breast cancer. SERM biocharacter may vary depending on the activity of intracellular signaling pathways that are induced by extracellular factors (growth factors) and can cross talk with ERs (Smith et al., 1998). Indeed, in the ER-positive human breast cancer cell line MCF-7, activation of the MAP kinase pathway increases the partial agonist activity of tamoxifen and decreases its antagonist activity (Fujimoto et al., 1994).

In the previous chapter the molecular docking results have shown better ketosterol – estrogen receptor binding interactions compared with native ligand, E2.
Ketosterol and SERMs showed better binding affinity than ligand and molecular dynamics simulation studies concluded that when ketosterol bound to the estrogen receptor structure it forms stabled structure than native structure. From these results we hypothesize that ketosterol with anti-estrogenic activity could be used in the treatment of ER +ve breast cancer.

This chapter presents the experiments pertaining to impact of ketosterol on viability and proliferation of MCF-7 cells.

5.2 MATERIALS AND METHODS

5.2.1 CHEMICALS

Dithiothreitol, Glycerol, Acrylamide, Bis-acrylamide, Sodium dodecyl sulphate (SDS), β-mercaptoethanol, Agarose, Ethidium Bromide, DMSO, Tris base, Triton X-100, Tween-20, DMEM, MEM, Sodium bicarbonate, EDTA, Glycine were all obtained by SIGMA. Ponceau S and Potassium chloride were from HIMEDIA. Sodium Chloride from Laboratory Rasyan. Magnesium Chloride, EDTA, Calcium chloride and Methanol from SRL. DEPC, Medium molecular weight marker proteins for SDS PAGE from HIMEDIA. FBS is obtained from LONZA. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Bovine Serum Albumin fraction V (BSA) were purchased from Sigma Co., St Louis, MO, USA. 0.5% Trypsin –EDTA solution was procured from Invitrogen (Grand Island, NY, USA).
5.2.2 CHEMICAL STOCKS:

12% Resolving gel: Resolving gel-12% (1.5 M Tris HCl pH 8.8, 30% polyacrylamide and 10% SDS) were polymerized using TEMED and 10% Ammonium per sulphate. The gels were run using buffer containing 0.025M Tris, 0.192M glycine and 0.1% SDS.

1x Running Buffer: 25Mm Tris-HCl, 200mM Glycine, 0.1% (w/v) Sodium dodecyl sulphate.

Lysis Buffer: 50 mM Tris pH 7.4, 2mM DTT, 5mM MgCl2, 2mMATP, 250mM sucrose.

Assay Buffer for caspase activity: 200mM HEPES, pH7.4 with 1% CHAPS, 50mM DTT, and 20mM EDTA

DEPC water: Water for RNA isolation was treated with 1% DEPC, left overnight and subsequently autoclaved to remove excess DEPC.

MEM (1X): 13.4 g of MEM and 3.6 g of NaHCO₃ was dissolved in milli-Q water to make 1 litre of the medium. The pH is adjusted to 7.4 using HCl and subsequently filter sterilized.

Ethidium Bromide: 10 mg/ml solution in water.

SDS-PAGE sample buffer (3X): 180 mM Tris-Cl pH 6.8, 6% SDS, 15% glycerol, 7.5% β-mercaptoethanol and 0.01% bromophenol blue in double-distilled water and stored at -20°C.

Tris EDTA pH 8.0 (TE): 10 mM Tris HCl (pH 8.0) and 1 mM EDTA (pH 8.0).

Trypsin EDTA: 0.125% trypsin (cell culture grade) and 0.1% EDTA was dissolved in 1X PBS, filter sterilized and stored at -20°C.
5.2.3 Maintenance of Cell culture MCF-7 cell line:

MCF-7 is the acronym for Michigan Cancer Foundation-7 (human breast adenocarcinoma cell line) was obtained from the cell repository of National Centre for Cell Sciences, Pune, India. The cell line has bio safety level-1. MCF-7 cells seeded at 1.0x10^6 cells were grown in uncoated 25 mm^2 tissue culture grade flasks with DMEM medium supplemented with 10 % FBS. After attaining around 70% confluence, the cells were used for experimental studies. Cells were expanded in 1:3 ratio after attaining 80% confluence.

**Plastic ware:**

Tissue culture grade flasks like 25 mm^2, 75 mm^2, 35 mm and 60 mm plates as well as 6, 12 and 96 well plates and 15, 50 mL centrifuge tubes were purchased from Falcon (Becton Dickinson Labware, NJ, USA). Micropipette tips, micro-centrifuge tubes, PCR tubes, RNA grade tubes and tips were purchased from Axygen (Axygen Inc, CA, USA). Cryo-cooler was purchased from Tarsons (Tarsons Inc, Kolkata, India).

**Antibodies:**

- ERK1/2 (4695), phospho-ERK1/2 (4370) raised in Rabbit were purchased from Cell Signaling Technology.
- Anti α-tubulin (T5168) antibody raised in mouse was procured from sigma Aldrich, India.
- Secondary antibodies of anti-mouse IgG HRP (NA9310), anti-rabbit IgG HRP (NA9340) were procured from Amersham Pharmacia.
Antibiotics:

Antibiotics Penicillin and Streptomycin, Amphotericin, were purchased from Himedia Laboratories, Mumbai, India.

Buffers

**Phosphate Buffered Saline:**

A 10x stock solution was prepared by dissolving NaCl (80.0 gm), KCl (20.0 gm), Na$_2$HPO$_4$ (11.5 gm) and KH$_2$PO$_4$ (2.0 gm) in 1 L of distilled water. pH was adjusted to 7.4. 10x stocks are diluted with distilled water to make working solution prior to use.

**Phosphate buffered Saline with Tween-20 (PBST):**

To the 1x PBS, 0.1% Tween 20 (v/v) was added to prepare PBST.

**Acid-Ethanol solution:**

The solution was prepared by mixing 75 mL absolute ethanol, 1.5 mL 10N HCl and 33.5 mL distilled water. The mixture is stored at -20°C.

**MTT solution:**

5 mg/mL stock solution of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) was prepared in PBS and stored at 4 ºC. A working concentration of 250 µg/mL was added to cells.

**5.2.4 PREPARATION OF MEDIA AND CULTURE PLATES**

Serum Containing Medium (SCM): The lyophilized media were re-constituted in autoclaved distilled water and a 5x stock is prepared according to the manufacturer’s
instructions. After adjusting the pH to 7.2, the media was sterile filtered through 0.22 μm PVDF syringe filters (Millipore, Cork, Ireland) and stored at -20°C until use. Prior to use the media was diluted to 1x with autoclaved water and antibiotics (100 units/mL penicillin, 100 μg/mL streptomycin) added. Filtered fetal bovine serum was added to the reconstituted media to make 10% serum medium. RPMI1640 medium was used for maintenance of MCF-7 cell line.

Assessment of viability of the cells:

The cells were assessed for their viability by MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. MTT, a pale yellow substrate of the dehydrogenase enzymes in mitochondria; is converted to formazone, a purplish blue compound by metabolically active or viable cells. 1 mg/mL MTT reagent was added to the cells from different treatment conditions and incubated for a period of two hrs at 37°C. Cells were washed twice with PBS after incubation and allowed to dry for 4 hr at room temperature. DMSO was added to cells in order to dissolve formazone crystals. The purplish blue colour of formazone was measured at 562 nm and the values are expressed as percentage of viability relative to the control cells.

Assessment of Receptors in MCF7 Cells:

The MCF7-cells were grown for 24 h and assessed for estrogen α receptors in the cells, Western blot analysis revealed the presence of estrogen receptor α in the MCF-7 cell lines.
Effect of Ketosterol, Estradiol, Tamoxifene, Raloxifene on proliferation of MCF-7 cells:

Cell proliferation was determined by MTT [3-(4, 5-Dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide] assay (Campling et al., 1988). MCF-7 cells (5 x 10^3 cells/well) were seeded to 96 well culture plates in the presence or absence of Ketosterol (10µM), Estradiol (10µM), Raloxifene (10µM), and Tamoxifene (10µM) for 12, 24 and 48 h in a final volume of 100 µl. After treatment, the medium was removed and 20 µl of MTT (5 mg/ml of PBS) was added to the fresh medium. After 2 h incubation at 37°C in 5% CO2 incubator, removed the media by suction pump, 100 µl of DMSO was added to each well and plates were agitated for 1 min. Absorbance was read at 562 nm on a multi well plate reader. Percent inhibition of proliferation was calculated as a fraction of control (control was without Ketosterol, Estradiol, Tamoxifene and Raloxifene).

5.3 Caspase-3 activity assay

Principle

Caspases (Cysteine-requiring Aspartate proteases) belong to a highly conserved family of cysteine proteases with specificity for aspartic acid residues of their substrates. Caspases play central role in apoptosis. Caspases are a family of proteases that mediate cell death and are important to the process of apoptosis. Caspase 3 (also referred to as CPP32, Yama, and apopain) is a member of the CED-3 subfamily of caspases and is one of the critical enzymes of apoptosis. Caspase 3, is an effector caspase and it can process caspase 2, 6, 7, and 9 proenzymes and specifically cleave
most caspase related substrates known to date, including many key proteins such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP), the inhibitor of caspase-activated deoxyribonuclease (ICAD), and gelsolin and fodrin, which are proteins involved in apoptosis regulation.

This cleavage is part of the mechanism leading to cell death. In addition, caspase 3 plays a central role in mediating nuclear apoptosis including chromatin condensation and DNA fragmentation as well as cell blebbing. Caspase 3 activity is tissue, cell type, or death stimulus specific. The caspase 3 fluorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVDAMC) by caspase 3, resulting in the release of the fluorescent 7-amino-4-methylcoumarin (AMC) moiety. The excitation and emission wavelengths of AMC are 360 nm and 460 nm, respectively.

**Materials**

(4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), 3-[(3-cholamidopropyl) dimethyl ammonio]-1-propanesulfonate (CHAPS), Dithiothreitol [(DTT), Ethylene diamine tetra acetic acid (EDTA), Ac-DEVD-AMC (caspase3 substrate), 96-well black well plates were purchased from Sigma Aldrich.

**Buffers**

5X Lysis Buffer: 250 mM HEPES, pH 7.4, with 25 mM CHAPS and 25 mM DTT 10X Assay Buffer: 200 mM HEPES, pH 7.4, with 1% CHAPS, 50 mM DTT, and 20 mM EDTA.

Caspase 3 Substrate (Ac-DEVD-AMC): Solution (5 mM in DMSO)
Method

- At the end of treatments, the cells were collected in media by gentle scraping and centrifuged at 3000 rpm for 5min. The pellet was washed with PBS and the pellets were stored at -80°C until use.
- The pellets were re-suspended in 100µL of 1X lysis buffer and incubated for 10-15min.
- Samples were centrifuged for 15min at 13000 rpm at 4°C.
- Supernatants were transferred to fresh tubes.
- 50µL of the lysate and 50µL of 2X assay buffer along with 2µL of the substrate stock solution were added to the 96-well black plate.
- For the blank readings, 50µL of 1X lysis buffer was added to 50µL of assay buffer along with 2µL of substrate.
- Increase in the fluorescence was monitored for 1hr at 5 min intervals at 360 nm/460nm excitation and emission respectively using perkin elmer multi plate reader.
- Protein estimation was done by Bradford’s method.
- Fluorescence units/mg protein value was calculated for each of the treatments after taking the blank value into consideration.
- Bar graphs were drawn with respect to the percentage control values obtained.
5.4 Caspase-8 Activity assay:

**Principle**

The process of caspase activation in extrinsic apoptotic pathway is initiated by binding of ligands to death receptors. Caspase-8, also known as Mch5, 1 MACH, 2 and FLICE3, is localized at the top of the hierarchy of the caspase cascade and is a member of the initiator family of caspases. Caspase-8 exists in the cell as an inactive proenzyme of 55 kDa. It is converted to the active form, consisting of 18 and 12 kDa subunits, upon its recruitment to the cytoplasmic domain of activated death receptors such as Fas, via the adaptor protein FADD. The activation of the proenzyme is triggered by the protein’s aggregation, which leads to auto catalytic processing. Caspase-8 activates downstream caspases (3, 6 and 7) that cleave key cellular substrates and lead to apoptotic death of the cells. The Caspase-8 fluorimetric assay is based on the hydrolysis of the peptide substrate Acetyl-Ile-Glu-Thr-Asp-7-amino-4-methyl coumarin (Ac-IETD-AMC) by Caspase-8 resulting in the release of a 7-amino-4- methyl coumarin (AMC) moiety. The excitation and emission wavelengths of AMC are 360 nm and 460 nm, respectively. The concentration of the AMC released can be calculated from a calibration curve performed with defined AMC solutions.

**Buffers and Method:**

The buffers used for caspase 8 activity were as same caspase 3 assays except the substrate and the readings were measured at 360 nm/460 nm excitation and emission respectively.
5.5 Cell Cycle Analysis:

MCF-7 Cells (1 × 10^7) were treated with Ketosterol (50 μM), Tamoxifen (30 μM), Raloxifene (40 μM), Estradiol (50 μM) and also a untreated (control) were used for cell cycle analysis. Cells were incubated for 15 – 30 minutes on ice at 0–4°C then washed using BD Pharmingen™ staining buffer (BD Pharmingen™ Stain Buffer (FBS) or 1× Dulbecco’s PBS, 3% FCS, 0.09% NaN3, pH 7.4). Then cells were washed twice with 100uL of 1× PBS to remove any residual media or buffers that contain FBS or BSA. 5 mL of ice-cold 70% EtOH was added by dropper while slowly mixing or vortexing. Cells were incubated with EtOH for at least 1 hr below 4°C. Cell suspension was filtered through a 60 μM pore nylon mesh to remove any aggregates are present. Cells were washed twice with 1× PBS. Cells were pelleted by centrifugation at 300 × g for 5 minutes under cold conditions. Cell pellet was suspended in 1 ml of DNA staining solution containing 0.002% of propidium iodide, 0.02% of DNase free RNase in PBS and incubated for 1 hr at room temperature in the dark. The DNA contents of 20,000 events were measured by flow cytometer (DAKO CYTOMATION, Beckman Coulter, Brea, CA). Histograms were analyzed using Summit Software.

5.6 Western Blotting:

Preparation of whole cell extracts and immunoblot analysis

MCF-7 cells with treatment (Ketosterol, Estradiol, Tamoxifen and Raloxifene) and without treatment (control) at a density of 5 × 10^6 were seeded in 90 mm culture dishes. They were incubated with Ketosterol (50 μM), Estradiol (50 μM), Tamoxifene (30 μM), Raloxifene (40 μM). Cells, harvested after 24 h were used for preparation of
whole cell extract. The harvested, control and treated, MCF-7 cells were washed with PBS and suspended in lysis buffer (20 mM Tris, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM α-glycerophosphate, 1mM sodium orthovanadate, 1mM PMSF, 10 µg/ml leupeptin, 20 µg/ml aprotinin and phosphatase inhibitor cocktail 1 and 2 with 100 fold dilution). After 30 min of shaking at 4°C, the mixtures were centrifuged (10,000 x g) for 10 min, and the supernatants were used as the whole-cell extracts. The protein content was determined according to the Bradford method (Bradford, 1976). 40 µg of protein from each treatment was resolved on 10% gels along with protein molecular weight standards, and then transferred onto nitrocellulose membranes. Membranes were stained with 0.5% Ponceau S in 1% acetic acid to check the transfer. The membranes were blocked with 5% w/v nonfat dry milk and then incubated with the primary antibodies [p42-44 and p-p42-44 (1:1000), in 10 ml of antibody-diluted buffer (1X Tris-buffered saline and 0.05% Tween-20 with 1% milk) with gentle shaking at 4°C for 8-12 h and then incubated with peroxidase conjugated secondary antibodies. Signals were detected by using peroxidase substrate, TMB/H2O2. Equal protein loading was confirmed by reprobing the nitrocellulose membranes with α-tubulin antibodies (1:3000).

5.7 Results:

The ER belongs to a large superfamily of ligand-inducible transcription factors that includes steroid, thyroid, and retinoid receptors as well as numerous receptors whose ligands have not been delineated (Mangelsdorf et al., 1995). The hormone estrogen brings about changes in cell function through regulation of target gene
Ex vivo analysis

expression. Estrogen’s actions are mediated through the estrogen receptor (Margaret A. Loven et al., 2001). The ER interacts with estrogen response elements (EREs) present in target genes to initiate changes in transcription.

The steroid hormone, estradiol, plays an important role in the progression of breast cancer and a majority of the human breast cancers start out as estrogen dependent and express the estrogen receptor. The biological effects of estrogen are mediated by its binding to one of the structurally and functionally distinct ERs (ERα and ERβ) (C.Thomas et al., 2011). Endocrine therapy using Tamoxifene, Raloxifene a selective estrogen receptor modulator (J.S.Lewis-Wambi et al., 2005 and V.C.Jordan et al., 2005), and aromatase inhibitors, which ablate peripheral estrogen synthesis, has been shown to substantially improve disease-free survival (A.Leary et al., 2006 and M.Dowsett et al., 2006). Endocrine therapy has also been shown to have a positive effect on the treatment of ER-positive breast cancer (T.Utsumi et al., 2007, N.Kobayashi et al., 2007 and H.Hanada et al., 2007).

MCF-7 cells were treated with different concentrations (1 µM, 10 µM, 100 µM) of ketosterol (NP), Raloxifene (R), Tamoxifene (T), Estradiol (E) for 48 hr. After termination of treatments MTT (5mg/ml) was added to cells and incubated for 2 hr followed by drying of the cells by removing medium. DMSO was added to solubilize formazin crystals and purple colour was measured at 565 nm. IC50 values were determined (shown in Figure. 5.1).
Selective estrogen receptor modulators (SERMs)

**Tamoxifen** — multiple studies have examined the effectiveness of tamoxifen for the prevention of breast cancer. Overall, these studies suggest that tamoxifen can prevent hormone-positive breast cancers from developing in women at risk for the disease. In general, tamoxifen, given daily for five years, reduces the risk of developing breast cancer by about 50%. Tamoxifen can be used in women before and after menopause.

Despite the evidence that it reduces the risk of developing breast cancer in high-risk women, tamoxifen has not been widely accepted for breast cancer prevention. That is largely because there is no evidence that tamoxifen improves survival when given as
a preventive treatment and because the medication has a small risk of serious adverse events, including uterine cancer and blood clots in the legs or lungs.

**Raloxifene** — Raloxifene is currently used for the prevention and treatment of osteoporosis (low bone density) in postmenopausal women. Several studies suggest that in postmenopausal women at high risk of developing breast cancer, raloxifene can reduce the risk of developing an invasive hormone-positive (estrogen receptor-positive) breast cancer.

In Breast Cancer Prevention Trial that directly compared tamoxifen and raloxifene, it was found that raloxifene was slightly less effective than tamoxifen at preventing breast cancer. On the other hand, raloxifene was associated with fewer of the most serious side effects associated with tamoxifen, including a lower risk of uterine cancer. Raloxifene has been tested only in postmenopausal women and its benefit in premenopausal women is unknown. Therefore it is important to find novel therapeutic agents with lower side effects for the treatment of breast cancer. Thus, in the present study we have evaluated Ketosterol with SERMs to understand its efficacy.

The existence of a quantitative correlation between inhibition of MCF7 human breast adenocarcinoma cell line proliferation (measured by IC50 values) and estrogen receptor binding (relative binding affinity by competition with 17-Beta estradiol) has been reported for a series of SERM derivatives structurally related to raloxifene (Kuiper *et al.*, 1998).

We have carried out MTT experiments for 48 hrs and found cytotoxicity against MCF-7 cells (IC50 values) was used to approximate the ability of a compound to act as
SERMs (Tamoxifene and Raloxifene) by binding to ER receptor. Found the inhibition of MCF-7 cells by ketosterol.

To check the cell death, we performed caspase-3 and caspase-8 activity. Caspase-3 a critical executioner of apoptosis responsible for proteolytic cleavage of proteins triggering to cell death. In our experiments caspase-3 and caspase-8 have been evaluated to check the effect of ketosterol on MCF-7 cells. Increase in caspase-3 and caspase-8 activities were observed in ketosterol along with raloxifene, tamoxifene treatments, where as no apparent changes were observed in Estradiol treated cells when compared to control cells. Maximal levels of caspase-3 and caspase-8 were observed in co-treatments of raloxifene, tamoxifene with ketosterol. Increase in caspase-3 activity was started from 24 hr followed by maximum at 36 hr in all treatments, where as co-treatments has shown high caspase-8 activity at 24 hr followed by no significant change after 36 hr. However treatments of ketosterol, raloxifene, tamoxifene have shown higher caspase-8 activity at 36 hr (shown in Figure. 5.2).

Taken together these results suggests that ketosterol induced cell death was mediated through both the extrinsic and intrinsic activation of apoptosis and co-treatments synergistically enhanced cell death.

Our experiments have shown that caspase-3 and caspase-8 to be elevated compared with in combination with other SERMs and control.
Figure. 5.2: Synergistic induction of Caspase activities by ketosterol and SERMs
MCF-7 cells were treated with Raloxifene (40 µM), Tamoxifen (30 µM), Estradiol (50 nM) in the presence or absence of Ketosterol (NP: 50 µM) for 24 hr. Cell lysates were collected after termination of experiments. 50 µg of supernatant was added to the buffer containing the substrates for caspase3, caspase 8 and further activities were measured at 360 nm excitation 460 nm emission. Values are normalized to protein concentrations and expressed as fold change in activity with respect to control cells (n=3). *P = <0.05 compared to control, # p = P = <0.05 compared to K.

Caspase3 and 8 like activities were done with the combinations of the ketosterol with estradiol, raloxifene, tamoxifene. It is also proven that from our observations ketosterol have shown the decrease in the levels of the caspase-8 in 24 hrs.

Data shown are the mean ±SD of three separate experiments. *P<0.05 compared to Control group. *P<0.05 compared to K.

MCF-7 cells were treated with Raloxifene (40 µM), Tamoxifen (30 µM), Estradiol (50 nM) in the presence or absence of ketosterol (50 µM) for 48 hr. Cell lysates were collected after termination of experiments. 50 µg of supernatant was added to the buffer containing the substrates for caspase3, caspase 8 and activities were measured at 360 nm excitation 460 nm emission. Values are normalized to protein concentrations and expressed as fold change in activity with respect to control cells. Data shown are the mean ±SD of three separate experiments. *P<0.05 compared to control; # P<0.05 compared to ketosterol (shown in Figure.5.3).
Figure 5.3: Synergistic induction of Caspase activities by ketosterol and SERMs
Flow cytometric analysis was conducted for ketosterol, Estradiol, Tamoxifene and Raloxifene compounds in MCF-7 breast cancer cells. The cells were treated for 24h. cell cycle arrest was observed in Sub G1 phase (i.e., cells accumulated in G1 phase).

MCF-7 cells were treated with Raloxifene (40 µM), Ketosterol (50 µM), Tamoxifen (30 µM), Estradiol (50 nM) for 24 hr. After termination of treatments Cell cycle analysis was performed using propidium iodide as described in Materials and Methods. The percentage of cells in each phase of cell cycle was quantified by flowcytometry (shown in Figure.5.4).
Figure 5.4: Effect of ketosterol and SERMs on cell cycle
Extra cellular-signal-regulated kinases (ERK1/2) are Serine/Threonine kinases and their activities are positively regulated by phosphorylation mediated by MEK1 and MEK2. It is well known that phosphorylated ERK (pERK) is a key downstream component of the RAF/MEK/ERK signaling pathway (Hiromichi et al., 2013). It can be translocated to the nucleus after phosphorylation, where it leads to changes in gene expression by phosphorylating and regulating various transcription factors such as Ets family transcription factors (Elk-1). The Ras/Raf/MEK/ERK signaling cascades play a critical role in the transmission of signals from growth factor receptors to regulate gene expression and prevent apoptosis. Phosphorylated forms of p42/p44 are shown to decrease the expression of angiogenic VEGF involved in proliferation of tumors by p42/p44 MAP Kinase pathway. Further p42/p44 MAP kinase activity is rapidly activated by estradiol (Han J. et al., 1994). We have evaluated the activity ketosterol on p42/p44 expression levels in MCF-7 cell lines as anti-proliferative activity was proved by our earlier experiments (Julie Milanini et al., 1998).

Our experiments on expression levels of p42/p44 shown that Phosphorylated levels of (p-p42/p44) were decreased when treated with Natural product on MCF-7 cell lines compared with other SERMs and control. However un-phosphorylated forms of p42/p44 were observed to be unaffected in ketosterol treated and control and SERMs. Further expression levels of tubulin remained same as control for loading equal amount of protein.
Nearly confluent MCF-7 cells were treated with Raloxifene (40 µM), Ketosterol (50 µM), Tamoxifen (30 µM), Estradiol (50 µM) for 24 hr. After termination of treatments, cells were harvested and lysates were collected. 40 µg of protein from each lysates were used for immunoblot analysis of p42-44, p-p42-44 and Tubulin. Densitometric ratio of p-p42-44/p42-44 was deduced after normalizing with tubulin using gene tools (n=3).

Taken together, these experiments indicate that the decrease in phosphorylation levels of the P-p42 and P- p44 were more in ketosterol as compared to SERMs. However no change was observed in the levels of P-p42 and P- p44 in estradiol treated MCF-7 cells which behave similar to control. Also, the Un-phosphorylated form p42 and p44 remain unaffected.
5.8 Discussion:

Cytotoxic activity of Ketosterol was assayed by MTT cell assay on MCF-7 cell lines which showed IC-50 value for Ketosterol is 50 µM. For all the further experiments Ketosterol concentration used was 50 µM concentration. Flow cytometric analysis (FACS) was performed to evaluate the distribution of the cells through the cell cycle phases and FACS experiments showed that cells were arrested at sub G1 phase of cell cycle with Ketosterol treatment. Further to understand the mediators of cell death, we assessed caspase-3 and caspase-8 activity. Caspase activity experiments showed that Caspase 3 and Caspase 8 were significantly activated Ketosterol was used in combination with other SERMs than alone in MCF-7 cells. Further, to understand the ketosterol affect on p42/p44 signaling pathway phosphorylated and un-phosphorylated form of p42/p44 were assessed by western blotting in Ketosterol treated MCF-7 cells. Based on expression levels of p42/p44, it is understood that Ketosterol acts on p42/p44 MAP kinases by inhibiting the phosphorylation compared to other SERMs without affecting the p42/p44 protein levels.

Future work:

In future experiments the efficacy of the ketosterol on cancerous cell lines of different tissue origins need to be performed. The molecular mechanisms involved in the action of ketosterol on different cancer cells have to be elucidated. Ketosterol can be subjected to structural modifications to generate analogs with more efficacies. Further, Invivo studies with Ketosterol and its analogs can be performed for assessing the potential of Ketosterol as an alternative therapy to SERM for breast cancer.