Chapter-2

Materials and methods

Materials and Methods

2.1. Materials

2.1.1. Commonly used Buffers/Reagents

SL. No.	Buffers/ reagents	Composition
1	Phosphate Buffer Saline (PBS)	140 mM NaCl; 2.7 mM KCl; 10 mM KH_2PO_4 /K_2HPO_4 (pH 7.5)
2	Tris Buffer Saline (TBS)	25 mM Tris; 0.15M NaCl pH 7.5
3	TBS-T	TBS; 0.05% (V/V) Tween 20
4	Cell lysis buffer	50 mM Tris-HCl (pH7.4); 150 mM NaCl; 5 mM EDTA; 50 mM NaF; 1mM $NaOV_4$; 0.1% (v/v) NP-40; 1 mM PMSF; 1X Protease inhibitor cocktail; pH 7.5
5	30% Acrylamide solution	29.2 g acrylamide; 0.8 g bis acrylamide; Volume make up to 100 ml by water.
6	Stacking gel buffer	0.5 mM Tris HCl; pH 6.8
7	Resolving gel buffer	1.5 mM Tris HCl; pH 8.8
8	4X Sample buffer (SDS PAGE)	6.5 ml 1M Tris (pH 6.8); 10 ml 20% SDS; 10 ml glycerol; pinch of bromophenol blue; 10 µl β Mercaptoethanol
9	Running buffer (SDS-PAGE)	14.4 g/l glycine; 3.0 g/l Tris-Cl; 20% methanol; 0.1% (w/v) SDS. Volume 1 L
10	Western transfer buffer	14.4 g/l glycine; 3.0 g/l Tris-Cl; 20%

		Methanol. Volume make up with water to 1 L.
11	Ponceau S staining solution	2% (w/v) Ponceau S in 5% (v/v) acetic acid.
12	Blocking buffer	5% BSA in TBST
13	BCIP	1.5 mg in 100µl water if used as Na salt.
14	NBT	3.75 mg NBT/250µl of 70% DMF
15	BCIP / NBT buffer	0.1 M Tris base; 0.05 M $MgCl_2$; 0.01M NaCl (pH~9) 100 µl NBT and 100 µl BCIP 10 ml BCIP/NBT buffer.
16	Coomassie Brilliant Blue solution (gel staining solution).	2.5 mg Coomassie Brilliant Blue R; 500 ml Methanol; 100 ml Acetic acid. Volume make up with water to 1 L
17	Gel destaining solution	250 ml Methanol, 100 ml Acetic acid; volume make up to 1 L with water.
18	Bradford reagent	25 mg Serva Blue-G in 25 ml 85% H_3PO_4; 12.5 ml Ethanol and 12.5 ml 1N NaOH, 200 ml H_2O_2

2.1.2. Mammalian cell lines

The human breast cancer cell lines, MCF-7, MDA-MB-231, T47D, MDA-MB-468 were procured from National Cell Science Centre (Pune, India) with proper licensing.

2.1.3. Bacterial Strain

XL1Blue (*E. coli* strains) were used for general plasmid amplification and cloning purposes.

2.1.4. Plasmid

The pDest-mCherry-EGFP-LC3B plasmid was kindly provided by Prof. Terje Johansen, University of Tromso (1).

2.1.5. Instruments

Fluorescence measurement for ROS detection was done using Fluorescence Spectrophotometer (Hitachi F-7000, USA). Cell viability, Caspase-3 activity assay and ELISA based apoptosis assay were done using Epoch Microplate Spectrophotometer (Biotek, USA). Cell cycle analysis and mitochondrial membrane potential measurement were done using Becton Dickinson flow cytometer (FACS Calibur). Folin Lowry protein estimation was done using Bio Photometer plus (Eppendorf, Germany). DNA quantification was done using Nanodrop 2000 (Thermo Scientific, USA). SDS-PAGE was done using Hoeifer SE260 Mini-vertical gel electrophoresis unit (GE Healthcare Bio-Science AB, USA), Western blot transfer was done by Hoeifer Minive Blotter (GE Healthacare Bio-Science AB, USA). Blots were scanned using HP Scanjet. Cell imaging was done using LSM 710 Confocal Microscope (Zeiss, USA) and Apotome Fluorescence Microscope (Zeiss, USA).

2.1.6. Antibodies

Primary antibody	Molecular Weight (kDa)	Dilution	Manufacturer
Caspase-3 (cleaved)	17,19	1:1000	Cell Signalling Technologies, USA
PARP (cleaved)	89	1:1000	Cell Signalling Technologies, USA
PhosphoAkt(Thr 308)	60	1:1000	Cell Signalling Technologies, USA
PhosphoAkt (Ser 473)	57	1:1000	Abcam, UK
Pan Akt	60	1:1000	Cell Signalling Technologies, USA
Beclin-1	60	1:1000	Cell Signalling Technologies, USA
LC-3B	17,19	1:2000	Novus Biologicals, USA
β-Actin	42	1:10000	Sigma, USA

Secondary antibody	Dilution	Manufacturer
Anti Rabbit ALP	1:1000	Sigma, USA
Anti Mouse ALP	1:1000	Sigma, USA
Anti Rabbit HRP	1:5000	Cell Signalling Technologies, USA
Anti Mouse HRP	1:5000	Cell Signalling Technologies, USA

2.1.7. Chemicals/inhibitors

Inhibitors/ drugs	Concentration used	Manufacturer
Piroxicam	10-100 μM	Sigma, USA
Resveratrol	10, 20 and 30 μM	Cayman Chemicals, USA
Resveratrol analog, C1	1, 5 and 10 μM	NA
Hydrogen peroxide	300 μM	Merck Chemicals, India

N-acetyl cysteine	5 and 10 mM	Sigma, USA
Akt Inhibitor V, triciribine	20 μM	Calbiochem, USA
Wortmannin	1 μM	Cayman Chemicals, USA
Akt1/2/3 Kinases ShortCut siRNA mix	7.5, 15 and 22.5 nM	New England Biolabs, USA
3-Methyladenine	10 mM	Sigma, USA
Ac-DEVD-CHO	1 μM	Cayman Chemicals, USA

2.2. Methods

2.2.1 Cell culture and drug treatment

The human breast cancer cell lines were maintained at 37^{o}C and 5 % CO_2 in a humidified incubator in media composed of DMEM high glucose (Invitrogen, USA) supplemented with 10 % Fetal Bovine Serum (Invitrogen, USA), 1 mM non-essential amino acids (Hi-Media laboratories, India), 1 mM sodium pyruvate (Invitrogen, USA), and 100 units/ml penicillin and 100 μg/ml streptomycin (Hi-Media laboratories, India). For drug exposure, cells were plated in 60 mm sterile petriplates (BD Falcon, USA) and incubated overnight. Following day media was replaced with fresh media. Piroxicam (Px) dissolved in DMSO (Merck Chemicals, India) was added at 30 μM either alone or in combination with inhibitors N-acetyl cysteine (NAC) and Akt Inhibitor V, Triciribine (T), Wortmannin (Wrt). Resveratrol (Res) and analog C1 were dissolved in DMSO and added at 10, 20, 30 and 1, 5, 10 μM respectively. The cells were pre-treated with the inhibitors 3-Methyladenine (3-MA) or Ac-DEVD-CHO for 2-3 h prior to Res and C1 exposure. The final DMSO concentration was maintained at 0.1 % for all the exposure studies.

2.2.2. Transfection of short interfering RNA (siRNA) and plasmid.

Cells ($2X10^6$) were seeded in 60 mm sterile petriplates to achieve 50 % confluence next day. Cells were transfected with three concentrations: 7.5, 15 and 22.5 nM of Akt 1/2/3 and also Polylinker RNA using the Akt1/2/3 Kinases ShortCut siRNA mix. Polylinker-transfected cells served as the control for siRNA experiments. The concentration 22.5 nM of siRNA was then selected for subsequent experiments. After 24 hrs post transfection cells were treated with Px (30 μM) or DMSO for 24 hr and samples were collected for western blot analysis. For plasmid transfection, 300 ng of GFP-LC3 plasmid was transfected in cells and incubated for 24h, followed by Res/ C1 exposure. All the transfection was done using Lipofectamine 2000 (Invitrogen, USA) as per the manufacturer's instructions.

2.2.3. Cell Viability and IC_{50} determination by MTT assay.

Cells ($5 X 10^3$) were seeded in 96 well plates (Thermo Scientific, Denmark) and incubated overnight. Next day cells were treated with Px either alone or in combination with NAC/ Triciribine/ DMSO for 72 h (in triplicates) and incubated at 37^oC in 5 % CO_2. For the time dependent effect on viability, incubations were carried out for various time points: 6, 12, 24 and 48 h. For IC_{50} determination, cells treated with Res and C1 at different concentrations (starting from 200 μM, 2 fold dilutions) for 72 h. Following incubation, media was replaced by 200 μl of fresh DMEM media and 10 μl of MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] reagent (Sigma, USA) was added in each well and incubated at 37^oC for 3 h. Media was then carefully discarded and 200 μl/well of DMSO was added and kept at orbital shaker for 15 min in dark to dissolve the formazan crystal. Absorbance was measured at 570 nm. IC_{50} values were calculated using GraphPad Prism 5 software (GraphPad, USA).

2.2.4. Analysis of ROS.

Cells (5 X 10^5) were seeded in 60 mm sterile petriplates and incubated overnight at 37^{o}C in 5 % CO_2. Next day cells were pre treated with NAC/DMSO for 2 h followed by Px (30 μM) exposure for 3, 6, 12, 24 and 48 h. Following exposure, cells were incubated with 25 μM of 2′,7′-Dichlorofluorescin diacetate (H_2DCFDA; Sigma, USA) dye at 37^{o}C for 15 min in dark. The cells were then washed with Phosphate Buffer Saline (PBS, pH-7.4) and harvested. Fluorescent intensity was measured at excitation/ emission wavelength of 506/ 526 nm. The fluorescent intensity of Px treated samples were normalized with their corresponding untreated control for each of the time points. The fold change was calculated w.r.t control. Cells were also stained for visualization at 63X magnification in confocal microscope. For this cells were seeded in confocal dish (SPL Life Sciences, Korea) and incubated overnight. Next day cells were treated with Px/ DMSO for 3, 6, 24 and 48 h and processed for imaging in fluorescence microscope, following the protocol mentioned above.

2.2.5. Cell Cycle Analysis.

Cells (2X10^6) were seeded in 90 mm sterile petriplates (BD Falcon, USA) and incubated overnight. Following day cells were treated with Px (30 μM) for 6, 12, 24 and 48 h. Cells were harvested and fixed with 70 % chilled ethanol (Merck Chemicals, India) and kept at 4 ºC for 24 h. The cells were washed with PBS twice and suspended in PBS containing 200 μg RNase (Sigma, USA) and incubated at 37^{o}C for 2 h. Cells were then treated with 100 μg/mL propidium iodide (Sigma, USA) and kept in the dark for 15 min. The cell cycle was analyzed using FlowJo software (Tree Star Inc., USA). Time-dependent change in the percentage of cells in the sub G0/ G1 population were plotted.

2.2.6. Annexin-V FITC/ PI staining

Cells were treated with Res and C1 at different concentration for 24 h. Following incubation 5 X 10^5 cells were harvested and re-suspended in 500 µl of binding buffer. 5 µl of each Annexin V- FITC and propidium iodide (PI) were then added and incubated for 15 mins in dark. The assay was done as per the manufacturer's instruction using the Annexin V- FITC Apoptosis Detection kit (Abcam, USA). The samples were then quantitated for fluorescent intensity in a flow cytometer.

2.2.7. Protein Extraction for Western Blot

Cells were harvested using trypsin and washed with PBS buffer. The PBS-washed pellets from cell lines were then lysed on ice in lysis buffer for 30 min with mild vortexing and centrifuged at 13,000 rpm for 15 mins. The supernatant was collected and protein concentration was determined by Folin Lowry method (2).

2.2.8. Western Blot

The control and drug treated cell samples were lysed and 20–50 µg protein extracts were separated by 10 or 12.5 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS– PAGE) and were transferred onto PVDF membrane (Millipore, India). The membrane was blocked with 5 % BSA (Hi-Media laboratories, India) in TBST buffer for 1-2 h at room temperature. The membrane was then probed with primary antibodies against specific proteins, washed with TBST and again incubated with horseradish peroxidase tagged or alkaline phosphatase tagged secondary antibody for 2 h at room temperature. The membrane was then developed by chemiluminescence using SuperSignal West Pico (Thermo Scientific, USA) or by colorometric detection using NBT/BCIP solution. Bands were quantified by densitometric analysis using Image J software

(National Institute of Health, USA). Quantified bands were normalized with their respective β-Actin levels and the fold change in expression was calculated w.r.t control.

2.2.9. Preparation of polyacrylamide gel

Components	Resolving Gel			Stacking Gel
(Conc)	5%	10%	12.5%	4.5%
30% acrylamide	1.5	3	3.75	.45
1.5 mM Tris HCl, pH 8.8	2.25	2.25	2.25	-
0.5 mM Tris HCl, pH 6.8	-	-	-	0.75
10% APS	0.04	0.04	0.04	0.02
TEMED	0.01	0.01	0.01	0.01
H_2O	5.25	3.75	3.00	1.80

2.2.10. Caspase- 3 activity assay.

Cells ($2X10^6$) were seeded in 60 mm sterile petriplates and incubated overnight at 37^oC in 5 % CO_2. Next day cells were pre treated with Px (30 µM) for 6, 12, 24 and 48 h. The cells were then harvested and lysed. The protein concentrations were determined by the Folin-Lowry method. For enzyme assay, 100 µg of protein sample diluted in 50 µl of lysis buffer/well (in triplicates) of 96 well plate. Then 50 µl of 2X reaction buffer (containing 10 mM DTT) was added in each well, followed by addition of 5 µl of 4 mM of DEVD-p-NA substrate (final concentration- 200 µM) in each well. The plates were incubated for 2 h at 37ºC after mixing well. The absorbance was measured at 405 nm. The protocol was followed using the colorometric Caspase 3 Assay Kit (Abcam, UK).

2.2.11. Mitochondrial Membrane Potential.

The mitochondrial membrane potential (MMP) of intact cells was measured by flow cytometry and microscopy using JC-1 Mitochondrial Membrane Potential assay Kit, Cayman Chemicals, USA). 5 X 10^5 cells were seeded in 60 mm sterile petriplates. Following day the 70 % confluent MCF-7 cells were treated with Px (30 μM) for 6, 12 and 24 h either alone or in combination with inhibitor Triciribine (20 μM) at 37^{o}C in 5 % CO_2. JC-1 dye (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) was then added directly to the culture medium (3 μM final concentration) and incubated in dark for 30 min at 37^{o}C. The cells were then processed as per the manufacturer's instruction. Cells were quantitated for fluorescent intensity in a flow cytometer (FACS Calibur, Becton Dickinson, USA) to detect green fluorescence at excitation/emission wavelengths of 485/530 nm and red fluorescence at excitation/ emission wavelengths of 550/595 nm. JC-1 exhibits potential dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~ 525 nm) to red (~ 590 nm). The mitochondrial depolarization is indicated by the increase in the green fluorescent intensity (3). The ratios of green and red fluorescent cells were calculated for each time points and fold change in the ratio was calculated w.r.t control. Cells were also stained for visualization under Apotome Fluorescence Microscope (Zeiss, USA) at 20X magnification. For this cells were seeded in confocal dish and incubated overnight. Next day the cells were treated with Px (30 μM) for 24 h in combination with inhibitor Triciribine (20 μM)/ DMSO at 37^{o}C in 5 % CO_2 and processed for imaging as per manufacturer's instructions.

2.2.12. ELISA Apoptosis Detection assay.

Cells (7 X 10^3) were seeded in 96 well flat bottomed plates and incubated overnight. Cells were then treated with 30 μM Px or DMSO (in triplicates) for 24 h and processed using

the ApoStrand ELISA Apoptosis Detection Kit (Enzo Life Sciences, USA) following manufacturer's instruction. Absorbance was measured at 405 nm using Epoch Microplate Spectrophotometer (Biotek, USA). The absorbance was directly proportional to the ssDNA levels in the cell which is a prominent marker for apoptosis. The fold change in the ssDNA level was calculated w.r.t control.

2.2.13. Preparation of competent cell

E. coli competent cells were prepared using standard $CaCl_2$ method (4). *E. coli* XL1 blue cells were grown overnight in LB broth to saturation. Saturated bacterial culture (2 ml) was inoculated in 100 ml of LB broth (1:100) and the cells were grown at 37ºC to an OD 600 of 0.5-0.6. Cells were chilled on ice for 1 h and then pelleted at 5000 rpm for 10 min at 40ºC. The cell pellet was suspended in ice-cold 100 mM $CaCl_2$ solution ($1/4^{th}$ of the original volume) and chilled on ice for 15 min. It was then pelleted at 5000 rpm for 10 min at 40ºC to remove the residual medium. The cells were then resuspended in ice-cold $CaCl_2$ ($1/20^{th}$ of the original volume) and kept on ice for 2-16 h. Glycerol was added dropwise with gentle swirling to a final concentration of 15% (v/v). Cells were then stored in 0.5 ml aliquots at -80ºC.

2.2.14. Transformation of bacterial cell

For transforming the bacteria with the desired plasmid, 200 µl of the competent cell were taken from the fresh aliquots and mixed with plasmid DNA 100-300 ng (<5 µl) and incubated on ice for 30 min. cells were then subjected to heat shock at 42º C for 90 sec and immediately chilled on ice for 2 min. 1 ml of LB broth was added and incubated at 37 ºC with shaking for 1 h. Cells were then spread on LB agar supplemented with appropriate

antibiotic. The plate was incuabated at 37 ºC with constant shaking for 16-18 h. Following incubation the single colonies were selected and used for plasmid isolation.

2.2.15. Plasmid isolation

The single colonies from the transformed bacterial cells were used for plasmid isolation using the QIAprep Spin Miniprep Kit (Qiagen, USA) following the manufacturer's instructions. The DNA yield was measured and used for transfection studies.

2.2.16. GFP-LC3 dot assay

LC3-II after its conversion from LC3-I is recruited to autophagosomal membrane during induction of autophagy therefore, GFP-LC3 expressing cells have been used as an important tool for autophagy detection (5). MCF-7 and MDA-MB-468 cells were transiently transfected with the GFP-LC3 vector using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. After overnight culture, cells were treated with 30 μM of Res and 10 μM of C1 for 24 h, fixed with 4% paraformaldehyde and examined under a fluorescence microscope.

2.2.17. Statistical analysis.

Statistical significance were determined using a two-tailed non-paired Student's t test for two group comparison or one-way analysis of variance (ANOVA) followed by Dunnett post test for multiple group comparison, and differences were considered significant if the p values were <0.05 (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Statistical analyses were performed using GraphPad Prism 5 software (GraphPad, USA). The mean and S.D values were used from 2-3 independent experiments.

References

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