Part-2
Materials and Methods
1. Source of Materials

1.1 Cell lines
MCF-7, MDA MB-231, DU-145, and B16F-10 cell lines were purchased from American Type Culture Collection (ATCC). All the cell lines were maintained in our in-house National Cell Repository, National Centre for Cell Science (NCCS), Pune, India.

1.2 Culture media, serum and antibiotics
Dulbecco’s minimum essential medium (DMEM), Leibovitz-15 (L-15) and RPMI were purchased from Invitrogen Corporation. Fetal bovine serum (FBS) was purchased from HyClone or Invitrogen Corporation. Penicillin and streptomycin were purchased from Invitrogen Corporation.

1.3 Drugs, inhibitors and SiRNAs
Carboplatin (Carb), Vinblastine (Vin), 5-fluorouracil (5-FU) were purchased from Sigma. Carb and 5-FU were dissolved in sterile water to make a stock of 25 mM and 50 mM respectively. Vin was dissolved in ethanol to make a stock of 5 mM. Radio labeled 5-flurouracil (C\textsuperscript{14}-5-FU) and etoposide (H\textsuperscript{3}-ETOP) were purchased from American Radio Labeled Chemicals (ARC). Methyl-β-cyclodextrin (MCD) was purchased from Sigma and dissolved in sterile water to prepare a stock of 500 mM. p53 selective inhibitor Pifithrin\textsubscript{α} (PFT\textsubscript{α}) was purchased from Sigma. ERK inhibitor U0126, Cdk-5 inhibitor Roscovitine and Cdk2/5; and p38 specific inhibitor SB203580 were purchased from Calbiochem. p53, Caveolin-1 and Control SiRNAs were purchased from Santa Cruz Biotechnology.
1.4 Antibodies

Antibodies against p53 (Mouse monoclonal;SC-126), Cdk5 (Rabbit Polyclonal;SC-173), p35 (Rabbit Polyclonal;SC-820), Caveolin-1(Rabbit Polyclonal;SC-894),p38 Rabbit Polyclonal;SC-535), Phospho-Akt (Ser-473)( Rabbit Polyclonal;SC-7985), Akt (Rabbit Polyclonal;SC-8312),Bcl-2 (Mouse monoclonal;SC-509), Bcl-2 (Rabbit Polyclonal;SC-492), ERK-(Tyr 204)(Mouse monoclonal;SC-7383), ERK-2 (Rabbit Polyclonal;SC-154) and β-Actin (Goat polyclonal;SC-1615) were purchased from Santa Cruz Biotechnology. Phospho-p38 (Rabbit Polyclonal) antibody was purchased from Cell-signaling. pCav-1 was purchased from BD Bioscience. HRP-linked secondary antibodies against mouse and rabbit were purchased from BioRad, HRP-linked secondary antibodies against goat IgG as well as FITC-linked secondary antibodies against rabbit and mouse IgG were purchased from Santa Cruz Biotechnology.

1.5 Plasmid constructs

PG13CAT which contains 13 repeats of p53 binding site inserted in the 5’ to polyomavirus basal promoter linked to CAT reporter gene and pWWPCAT which contains p53 binding sequence from p21 were kindly gifted by Dr. Bert Vogelstein, John Hopkins, Baltimore, MD USA. NF-κB reporter plasmid and pNF-κB-Luc was obtained from Dr. Gopal C. Kundu, NCCS, India. EGFP expression vector pEGFPN1 was purchased from Clontech.
1.6 General reagents

Reagents purchased from

[γ-32P] ATP (BRIT-India)
Acetyl CoA (Amersham)
Acrylamide (BioRad)
Agarose (Invitrogen Corporation)
APS (ICN)
Bis-acrylamide (BioRad)
[14C]-chloramphenicol (NEN)
DEPC (ICN)
dNTP (Gibco)
DTT (Sigma)
EDTA (ICN)
EGTA (Sigma)
Ethedium bromide (Sigma)
Glycine (ICN)
HEPES (ICN)
IGPAL (Sigma)
Iso-propanol (Merck)
KCI (Sigma)
KH2PO4 (ICN)
Lipofectamine 2000 (Invitrogen Corporation)
MgCl2 (Sigma)
M-MLV RT (Invitrogen Corporation)
MTT (Sigma)
Na2HPO4 (ICN)
Na3VO4 (Sigma)
NaCl (ICN)
NaF (ICN)
Oligo-dT12-18 Primer (Invitrogen Corporation)
Oligonucleotide Synthesized from (Geno-Mechanix)
2. Methods

Protocols described in this section are commonly used techniques and are well-established standard protocols of our laboratory.

2.1 Cell culture

All the cell lines were regularly cultured in their respective medium, supplemented with 10% heat inactivated (56°C treatment for 30 min) fetal bovine serum along with streptomycin (100 mg/ml) and penicillin (100 U/ml) at 37°C with 5% CO₂.

2.2 MTT (methylthiazole tetrazolium) cytotoxicity assay

The MTT assay, which is based on the conversion of the yellow tetrazolium salt-MTT to purple-formazan crystals by metabolically active cells, provides a quantitative determination of viable cells. Cells were seeded at the density of 7500 per well into 96 well plates and
allowed to adhere for 24 h at 37°C. Next day, cells were treated with drug or inhibitor as per experimental requirement. Cytotoxicity was assessed by MTT assay. Fifty microliter of MTT (1 mg/ml) was added to each well and incubated for 4 h at 37°C. Formazan crystals were solubilized in 50 µl of iso-propanol by incubating in shaking condition at room temperature for 10 min. Absorbance was taken at 570 nm using 630 nm as reference filter. Absorbance given by untreated cells was taken as 100% cell survival.

2.3 Western-blotting

2.3.1 Preparation of whole cell lysate

Following indicated treatments, cells were washed thrice with ice-cold phosphate buffered saline (PBS) and lysed in ice-cold lysis buffer (50 mM Tris.Cl, pH 7.5, with 120 mM NaCl, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1% NP-40 and protease inhibitor cocktail. Cellular-lysates were clarified by centrifugation at 15,000 rpm for 30 min. Supernatant was used as whole cell protein sample. Protein concentration was quantified using protein assay reagent (Pierce).

2.3.2 Cytosolic and nuclear extract preparation

The MCF-7 cells were plated at a density of 1x10^6 cells in 60 mm plates and allowed to grow for 24 h. Following desired experimental treatment, cells were harvested for preparation of cytoplasmic and nuclear fractions. The cells were washed thrice with ice cold PBS and the pellet was resuspended in hypotonic buffer [10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 1 mM PMSF, 1 mM DTT, and 5 mM Na₃VO₄ with protease inhibitor cocktail] for 15 min on ice. Nuclei were pelleted by centrifugation at 3300g for 15 min at 4°C. The supernatant was collected as the cytosolic fraction. Pellet was washed once by cold hypotonic buffer and again centrifuge by spinning at 3300g for 30 min.
The crude nuclear pellet was resuspended and lysed with nuclear extraction buffer [20mM HEPES (pH 7.9), 400 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 1 mM PMSF, 1 mM DTT, 0.1% Triton X-100, and 5 mM Na₃VO₄ with protease inhibitor cocktail] for 15 min on ice, and homogenized by passing through 25 G needle 10-15 times. Insoluble materials were pelleted at 15,000 rpm for 30 min. This supernatant was used as nuclear extract. Protein concentration was quantified using Coomassie Plus Protein Assay Reagent (Pierce). Equal amount of protein samples were used in experiment.

2.3.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Equal amount of protein samples (preferably equal volume, adjusted with lysis buffer) were heated with 1X SDS-loading buffer [50 mM Tris.Cl (pH 6.8), 2.5% β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol] at 95°C for 8-10 min. All the samples were chilled on ice for 5 min. Samples were centrifuged at 15,000 rpm for 5 minute and then loaded carefully in the well of polyacrylamide gel. The proteins were resolved on polyacrylamide gel on discontinuous buffer system using Bio-Rad mini-gel electrophoresis unit. Electrophoresis was carried out at constant voltage.

The components of polyacrylamide gel are as follows –

**10% resolving gel (component volumes are given per 5 ml of gel volume)**

- Deionized water..................................................1.9 ml
- 30% acrylamide mix
  (29.2% acrylamide and 0.8% bis-acrylamide)..............1.7 ml
- 1.5 M Tris.Cl (pH 8.8).............................................1.3 ml
- 10% SDS..............................................................50 µl
- 10% APS..............................................................50 µl
- TEMED..............................................................2 µl
12% resolving gel (component volumes are given per 5 ml of gel volume)

Deionized water.............................................................. 1.6 ml
30% acrylamide mix
(29.2% acrylamide and 0.8% bis-acrylamide) .......... 2.0 ml
1.5 M Tris.Cl (pH 8.8) .................................................... 1.3 ml
10% SDS..........................................................50 µl
10% APS..........................................................50 µl
TEMED..........................................................2 µl

One mm thick gels were prepared in between the gel plates of Mini PROTEAN 3 gel casting plates (BioRad). Samples were run in SDS-running buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS) using Mini PROTEAN 3 Bio-Rad mini-gel electrophoresis unit. SDS-PAGE was run at 60 volts till the dye was in stacking gel and then at 100 volts to separate proteins in resolving gel. To each gel one well was loaded with the standard molecular weight marker (BioRad).

2.3.4 Western blotting and detection of protein on membrane

For Western blot analysis, the proteins were electrotransferred to nitrocellulose membrane using a mini Trans-Blot electrophoretic transfer apparatus (Bio-Rad). Nitrocellulose membranes (Amersham) were placed over the gel to transfer the separated proteins from gel to membrane. Cassettes were fitted into the apparatus and transfer was done at constant current of 100 mA for 2-3 h (depending upon molecular weight of target protein) in chilled transfer buffer (25 mM Tris, 192 mM Glycine, 20% methanol). To detect the transferred proteins on the membrane, nitrocellulose membranes were washed once in deionized water and stained with 0.2% Ponceau-S stain (Sigma). With this visible temporary staining of Ponceau-S, positions of the marker proteins were marked on the membrane. Membranes were washed with TBS, pH 7.5 (10 mM Tris, 150 mM NaCl, pH was adjusted
with HCl) to remove Ponceau-S staining and further used for immunoprobing.

2.3.5 Immunoprobing and detection

Nitrocellulose membranes were blocked with 10% skimmed milk (in TBS) for 2 h at room temperature or overnight at 4°C. Subsequently, membranes were washed 4 times with TBS and TBST (TBS+ 0.1% Tween 20) alternatively. Membranes were incubated with 1:1000 dilutions of primary antibodies (dilution may vary for different antigen) for 3 h in TBS at room temperature. Next, membranes were washed 4 times with TBS as well as TBST alternatively. Thereafter, membranes were incubated with 1:10,000 dilutions of HRP-linked secondary antibodies in TBS for 2 h at room temperature. Membranes were washed 4 times with TBS as well as TBST alternatively. The activity of HRP was detected by chemiluminescent substrate (Pierce). Dilutions of HRP-linked secondary antibodies mainly depend upon nature of chemiluminescent substrate used. Blots developed with chemiluminescent substrate were covered by plastic wrap, exposed to Konica X-ray film (AX), and developed using developer (Kodak DA-163).

2.3.6 Stripping of the membrane

Stripping is used to re-probe the nitrocellulose membrane with another antibody. It is generally required to detect the structural proteins which ensure equal loading in experiment or basal expression of phosphoprotein. The blots were stripped by incubating the membrane at 50°C for 30 min in stripping buffer (62.5 mM Tris.Cl pH 6.7, 100 mM mercaptoethanol, 2% SDS) with intermittent shaking (every 8-10 minutes). Membrane is washed thoroughly with TBST and re-probed with required antibodies according to the given protocol.
2.4 Maxi-preparation of plasmid DNA

Glycerol stock of plasmid was used for maxi-preparation of plasmid DNA. LB broth (10 g/L trypton, 10 g/L NaCl, 5 g/L Yeast extract; pH was set to 7.2 with HCl), containing 100 µg/ml of ampicillin or 37 µg/ml of kanamycin as per the selectivity of the plasmid was prepared and loop full of glycerol stock culture was inoculated in 5 ml of LB to make starter culture. Cultures were incubated for 12-16 h at 37°C in shaker incubator. Next day, 5 ml grown culture was transferred to 500 ml of antibiotic containing LB broth for 12-16 h at 37°C in shaker incubator. Optical density of culture was taken at 600 nm to ensure exponential phase. Cells were harvested by centrifugation at 6000g for 15 min at 4°C. Cell pellet was resuspended properly in buffer-P1 (Qiagen) [50 mM Tris.Cl, (pH 8.0), 10 mM EDTA, 10 µg/ml RNase A]. Gently mixed the resuspended cells with buffer-P2 (Qiagen) [200 mM NaOH, 1% SDS (w/v)] and incubated for 5 min at room temperature. Next, 5 ml of chilled buffer-P3 (Qiagen) [3.0 M potassium acetate, pH 5.5] was added in the tube and mixed gently and further incubated for 10 min on ice. Next centrifugation at 20,000g was carried out for 30 min at 4°C. Supernatant was passed through a pre-wet filter paper and collected in a fresh eppendorf tube.

DNA-binding columns (Qiagen) were equilibrated by passing 5 ml of QBT-buffer (Qiagen) [750 mM NaCl, 50 mM MOPS; pH 7.0, 15% isopropanol (v/v), 0.15% Triton X-100 (v/v)]. Clear supernatant was then poured into the column and allowed to pass through the column by gravity flow. Next, column was washed twice with 5 ml of QC-buffer (Qiagen) [1.0 M NaCl, 50 mM MOPS; pH 7.0, 15% isopropanol (v/v)]. Seven ml of QF-buffer (Qiagen) [1.25 M NaCl, 50 mM Tris·Cl; pH 8.5, 15% isopropanol (v/v)] was passed through the column to elute DNA. Eluted DNA was precipitated by adding 0.7 volume of iso-propanol. The tube was centrifuged at 15,000g for 30 min at 4°C and DNA was pellet down. Pellet was washed once in 70% ethanol. Next, the DNA
was air dried and dissolved in appropriate volume of TE (10 mM Tris·Cl, pH 8.0, 1 mM EDTA).

2.5 Reverse transcription PCR (RT-PCR)

2.5.1 Isolation of total cellular RNA from tissue (tumor) sample

Homogenize the 50-100 mg of tissue sample in 1 ml of TRIZOL™ reagent. Sample volume should not be exceeded than 10% of the volume of TRIZOL™ regent (Invitrogen Corporation). Total cellular RNA from treated or untreated tumor sample was extracted using TRIZOL™ reagent. After homogenization, insoluble material was removed from homogenate by centrifugation at 12,000g for 10 min at 4°C. Supernatant of homogenized samples were incubated at room temperature for 5 min. Thereafter 200 µl chloroform was added to each sample. Tubes were vigorously mixed by hand and incubated at room temperature for 2-3 min. In next step, the tubes were centrifuged at 12,000g for 15 min at 4°C. After centrifugation, the upper colorless aqueous phase was taken in fresh tubes. RNA was precipitated from the aqueous phase by adding 500 µl isopropanol. Tubes were incubated for 10 min at room temperature and centrifuged at 12,000g for 10 min at 4°C. Supernatant was removed and RNA was pellet down. This was washed once with 1 ml of 70% ethanol (prepared in DEPC treated distilled water). RNA pellets were momentarily air dried. RNA was dissolved in DEPC treated water at 55°C for 10 min and stored at -70°C. The concentration of total cellular RNA was estimated by measuring the absorbance at 260 nm in spectrophotometer (BioRad). Total RNA quantity calculated as standard absorbance of 1 (optical density) is known to be equal of 40 µg/ml of RNA.

2.5.2 Preparation of cDNA

RNA (7.5 µg) in diethyl pyrocarbonate (DEPC)-treated water was used for preparation of cDNA. Synthesis was initiated using 200 units of moloney murine leukemia virus reverse transcriptase (M-MLV-RT),
under conditions recommended by manufacturer. Details of the procedure are as follows -

Reagents were mixed as given below -
1. Oligo dT (0.5 mg/ml) - 1 µl / sample
2. RNA - 7.5 µg / sample
3. 4dNTPs (10 mM each) - 1 µl / sample
4. Deionized water (DEPC treated) - to make the volume 19 µl

All the components were mixed thoroughly and heated at 65°C for 5 min. All the tubes were quickly chilled to open the secondary structures of RNA.

Reagents, given below were mixed together and used as master mix to be added in this tube:
1. 1st strand synthesis buffer (5X) - 6 µl / sample
2. DTT (100 mM) - 3 µl / sample
3. RNase inhibitor (100 U/ µl) - 1 µl / sample
4. MMLV-RT (200 U/ µl) - 1 µl / sample

Contents of the tubes were well mixed and incubated at 37°C for 90 min. Thereafter, reaction was stopped by inactivating the enzyme at 70°C for 15 min. cDNA was stored at -20°C.

2.5.3 Reverse transcription PCR

Each RT-PCR sample contained 10% of cDNA, 20 pM of each primer in 1X PCR-buffer [20 mM Tris (pH 8.4) and 50 mM KCl], 0.2 mM dNTP mix, and 1 unit of platinum Taq DNA polymerase in a final volume of 20 µl. Polymerase chain reaction (PCR) was carried out for 20-25 cycles.

**Gene Primer Sequence Annealing**

**p53**
(F) 5’-GTT GGC TCT GAC TGT ACC -3’
(R) 5’-GTT CCG TCC CAG TAG ATT ACC -3’
Annealing Temp 55°C; Product size 473 (bp)
Bax
(F) 5’-TTT ATG GAC GGG TCC GGG GA-3’
(R) 5’-TGT CCA GCC CAT GAT GGT TCT-3’
Annealing Temp 59°C; Product size 419 (bp)
p21
(F) 5’-GAC ACC ACT GGA GGG TGA CT-3’
(R) 5’-GGC GTT TGG AGT GGT AGA AA-3’
Annealing Temp 59°C; Product size 165 (bp)
β-Actin
(F) 5’- ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG-3’
(R) CGT CAT ACT CCT GCT TGC TGA TCCACA TCT GC-3’
Annealing Temp 55°C; Product size 838 (bp)

2.6 Cell transfection methods

For transient transfection experiment, cells were either transfected with calcium phosphate method or by Lipofectamine 2000 reagent (Invitrogen Corporation). Details about the procedure are given below -

2.6.1 Transfection with calcium phosphate method

Calcium Phosphate transfection method is a very efficient means of introducing DNA into cells in many cell systems. It is important that on the day of transfection the cells are thoroughly separated on the dish and should be 65-70% confluent. Cells in 60 mm culture plates were transfectected with calcium phosphate method. 10 µg of plasmid DNA (including 1 µg of pEGFPN1 plasmids) was used for transfection. pEGFPN1 plasmid is used as an internal control to evaluate the transfection efficiency. Vector plasmids were used as carrier DNA to makeup the final DNA concentration to 10 µg. DNA was taken in 120 µl of sterile distilled water. 120 µl of filter sterile buffer-A (0.5 M CaCl2, 0.1 M HEPES pH 7.0) was added drop wise to DNA solution with vortexing and incubated for 10 min at room temperature. To the DNA-buffer-A
mixture, 240 µl of filter sterile buffer-B (0.28 M NaCl, 0.05 M HEPES, 0.75 mM NaH₂PO₄ and 0.75 mM Na₂HPO₄ (pH 7.0) was added drop wise. Simultaneously tube was vortexed for proper mixing. To allow the formation of calcium phosphate-DNA complex, tube was incubated for 10 min at room temperature. This complex was then added drop wise evenly over the 60 mm plates containing cells and left for 18 h. Cells were washed thrice with serum-free medium and complete medium was added to the cells and incubated for further 24 h before the experiment.

2.6.2 Transfection with Lipofectamine 2000 (LF2000) reagent

Lipofectamine or LF2000 is a common transfection reagent which alters the cellular plasma membrane, allowing nucleic acids to cross into the cytoplasm. All cell transfection experiments were carried out as described. Semi-confluent (80-90%) cells were transfected with 6 µg of DNA including 1 µg of pEGFPN1 plasmids as an internal control to assess the transfection efficiency. Vector plasmids were used as carrier DNA to makeup the final DNA concentration to 6 µg. One hour before the transfection, 1 ml of fresh medium was added to each plate. For each plate (35 mm) to be transfected, 6 µg of DNA was diluted in 250 µl in Opti-MEM (Invitrogen Corporation) medium. For each plate, 4 µl of LF2000 reagent was diluted to 250 µl in Opti-MEM medium and incubated for 5 min at room temperature. These two solutions were combined with gentle mixing and incubated at room temperature for 45 min to allow LF2000-DNA complex formation. Five hundred µl of LF2000-DNA complex was added drop wise to the plate and mixed gently by rocking the plate. Cells were incubated at 37°C for 6 h. Thereafter, cells were washed and incubated at 37°C for 18 h in fresh medium. Cells were incubated for further 24 h before treatment.
2.6.3 SiRNA transfection

Almost 80% confluent cells in 60 mm culture plate were transfected with SiRNA reconstituted in SiRNA dilution buffer. Fluorescent labeled non-specific control SiRNA was utilized as an internal control to assess the transfection efficiency. The SiRNAs, transfection medium, transfection buffer and transfection reagent were obtained from Santa Cruz Biotechnology. In brief, all the SiRNA (p53, Caveolin-1 or Control) transfection experiments were carried out as described. For each plate, 18 µl (1 µl/well for 96 well plate) of SiRNA from the stock (10 µM) was diluted into 200 µl (25 µl/well for 96 well plate) of transfection medium and 12 µl (0.5 µl/well for 96 well plate) of transfection reagent was diluted into 200 µl (25 µl/well for 96 well plate) transfection medium in separate tubes. After incubating for 5 min at room temperature, the diluted siRNA was mixed with diluted transfection reagent and further incubated at room temperature for 20-25 min to allow complex formation. The complex was added drop wise to the plate containing cells with 1600 µl (50 µl/well for 96 well plate) transfection medium. Cells were incubated at 37°C for 7 h. Thereafter, cells were washed and incubated with medium containing 20% serum at 37°C for further 18 h before experiment.

2.7 Trypan blue staining

Trypan blue is one of several stains recommended for use in dye exclusion procedures for viable cell counting. This method is based on the principle that live (viable) cells do not take up certain dyes, but dead cells do so. Staining with dye also facilitates the visualization of cell morphology. Viability assay for transfected cells was carried out by trypan blue staining method. Cells were transfected with the indicated amount of DNA for 18 h. Cells were washed and incubated in fresh media for further 48 h. Cell death was checked by trypan blue
exclusion method, as trypan blue is a membrane impermeable dye, which is not taken up by viable cells with an intact plasma membrane. Both adherent and floating cells were collectively taken and washed once with PBS. Cells were incubated at room temperature with 0.1% trypan blue in PBS for 5 min. Samples were kept on ice, trypan blue positive as well as total cell population were counted and number of viable cells was calculated.

2.8 Reporter assays

Reporter gene assays are an important tool used for transcriptional regulation because of their simplicity and versatility. In present studies, the Chloramphenicol acetyltransferase (CAT) and Luciferase assays were performed. The activities of these reporters were normalized with EGFP expression in the cells. Procedures of these assays are given below.

2.8.1 CAT Assay

Chloramphenicol Acetyl Transferase (CAT) is a bacterial enzyme which inactivates chloramphenicol by acetylating it at one or both of its two hydroxyl groups and used as a reporter gene for promoter studies. This gene is not found in eukaryotes so these cells do not exhibit endogenous CAT activity. This property makes the CAT gene as one of the first reporter genes used for mammalian gene expression studied. To determine when a promoter is activated, the promoter of interest is placed upstream of the CAT coding sequence. Transfections of this vector allow the expression of CAT protein in the cells. Activity of CAT is determined by analyzing the amount of acetylated chloramphenicol after the completion of the reaction which is directly proportional to activity of CAT in the sample.

The detailed procedure is given below –

The transactivation activity of p53 or p21 was estimated in MCF-7 cells transiently transfected with pG13CAT or pWWPCAT reporter construct
(kind gift from Dr. Bert Vogelstein, John Hopkins, Baltimore, USA) which has p53 binding site from p53 or p21 gene promoter. pG13CAT contains 13 repeats of p53 binding site inserted in the 5’ to polyomavirus basal promoter linked to CAT reporter gene. To perform CAT assay, cells were cotransfected with pG13CAT and pEGFP expression vector using Lipofectamine 2000. After 18 h post transfection, drug or inhibitor treatment was given as per experimental requirement. Thereafter, cells were collected and resuspended in 0.25 M Tris.Cl buffer (pH 7.5). Cells were disrupted by five cycles of freeze–thaw. Equal amount of protein was used for the CAT reaction. The CAT reaction mixture, containing 1 µCi 14C-chloramphenicol (NEN) and 100 µg of acetyl CoA (Amersham), was incubated at 37°C for 6 h. Topped by adding 1 ml of ethyl acetate to each sample tubes. Ethyl acetate was mixed vigorously and organic phase was separated from aqueous phase by centrifugation at 15,000 rpm for 15 min. Total 850 µl of organic phase was taken to another tube and dried by speed-vac (Thermo Savant). Content of the tube were again resuspended in 25 µl of ethyl acetate and loaded on thin layer chromatographic silica gel plates (Merck). Thin layer chromatography was performed using mixture of chloroform and methanol as a running phase in the ratio of 19:1. TLC plates were exposed to X-ray film (Kodak) for 12-18 h. Spots were quantified with phosphorimager (Bio-Rad). CAT activity was normalized with GFP readings at 510 nm for ensure equal transfection efficiency.

2.8.2 Luciferase assay

Firefly luciferase protein catalyzes luciferin oxidation, using ATP and Mg²⁺ and light is generated in the due to conversion of chemical energy of luciferin oxidation through an electron transition with the formation of the product molecule oxyluciferin. The luciferase protein is frequently used as a reporter gene for measuring promoter activity or transfection efficiency. Cells, transfected with luciferase reporter
plasmids express firefly luciferase which is a 61 kDa protein. The intensity of light which is captured by illuminometer during the reaction is directly proportional to luciferase activity.

All cell transfection experiments were carried out by Lipofectamine 2000 reagent. Semi-confluent cells were transfected with 6 µg of DNA. Each transfection mixture contained 5 µg of pNF-κB-Luc and 1 µg of EGFP expression vector, pEGFPN1 as an internal control for transfection efficiency. Eighteen hours after transfection, cells were washed and fresh medium was added. As per experimental requirement, cells in indicated plates were treated with MCD for 4 h and washed. Subsequently, 10 µM of Carb or 5-FU treatment was given for 24 h. Subsequently, drug was washed and cells were further incubated in drugs free media for 48 h. The transfection efficiency was monitored by co-transfection of GFP-expressing vector pEGFPN1. Cells were harvested and luciferase assays were performed using an assay kit (Promega). The luciferase readings were taken in illuminometer (Fluoroskan Ascent FL, Labsystems) and further normalized with EGFP expression to calculate relative fold activation. All transfections were performed in duplicate and repeated for at least two times.

2.8.3 GFP expression assay

GFP, a gene that encodes jellyfish green fluorescent protein (GFP), which causes cells that express it to glow green under blue light. GFP intensity was directly measured from the cell lysates of pEGFPN1 transfected cells by fluorometer to check or correct for equal transfection efficiency as well to normalize the reporter activity. Equal amount (50 µg) of cell lysate from pEGFPN1 transfected cells were taken in the wells of 96-black-well plates. The fluorescence intensity of GFP was recorded on a fluorometer (Fluoroskan Ascent FL, Labsystems) with filter set at excitation 485 nm and emission at 510 nm.
2.9 Electrophoretic mobility shift assay (EMSA)

EMSA or gel shift assay is an in-vitro assay, which is used to explore and characterize protein-nucleic acid interactions. In the present study this technique is used to visualize the DNA-binding activity of p53 in MCF-7 cells as well as to monitor the effect of drug induced stress (in presence or absence of Cav-1 SiRNA) on the DNA-binding activity of p53 in nuclear extracts. The details of the procedure is given below –

2.9.1 Cytosolic and nuclear extract preparation

The MCF-7 cells were plated at a density of 1x10^6 cells in 60 mm plates and allowed to grow for 24 h. Following desired experimental treatment, cells were harvested for preparation of cytoplasmic and nuclear fractions. The cells were washed thrice with ice cold PBS and the pellet was resuspended in hypotonic buffer [10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 1 mM PMSF, 1 mM DTT, and 5 mM Na₃VO₄ with protease inhibitor cocktail] for 15 min on ice. Nuclei were pelleted by centrifugation at 3300g for 15 min at 4°C. The supernatant was collected as the cytosolic fraction. Pellet was washed once by cold hypotonic buffer and again centrifuge by spinning at 3300g for 30 min. The crude nuclear pellet was resuspended and lysed with nuclear extraction buffer [20mM HEPES (pH 7.9), 400 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 1 mM PMSF, 1 mM DTT, 0.1% Triton X-100, and 5 mM Na₃VO₄ with protease inhibitor cocktail] for 15 min on ice, and homogenized by passing through 25 G needle 10-15 times. Insoluble materials were pelleted at 15,000rpm for 30 min. This supernatant was used as nuclear extract. Protein concentration was quantified using Coomassie Plus Protein Assay Reagent (Pierce). An equal amount of protein samples was used in experiment.

2.9.2 Annealing of complementary oligonucleotides

Complementary oligonucleotides containing the sequences corresponding to putative p53 binding site (forward, 5’-GAC ATG TCT
AAG CAT GCT G-3'; reverse, 5'-CAG CAT TCT TAG ACA TGT TC-30). Equimolar amounts (25 pico molar of each oligonucleotide) were taken in a final volume of 20 µl of PBS, heated at 94°C for 2 min. Subsequently heating was stopped and oligonucleotides were allowed to gradually cool at room temperature in a water bath. Annealing was confirmed by running the probes on 12% polyacrylamide native gel containing 5 µg/ml of ethidium bromide. Electrophoresis was performed as per the conditions given below. Annealed probes were stored at -20°C.

2.9.3 Labeling of annealed probe
Annealed probe (5 pico molar) was 5'-end-labeled with 2 micro curie (mCi) [γ-32P] ATP using 10 U of T4 polynucleotide kinase (Invitrogen Corporation). 2 µl of 10X T4 polynucleotide kinase buffer (Invitrogen Corporation) was added to the tube for labeling reaction in a final volume of 20 µl and incubated at 37°C for 90 min. To stop the reaction, activity of T4 polynucleotide kinase was inhibited by keeping the tube at 68°C for 10 min. The reaction mixture was diluted to 50 µl and labeled probes were stored at -70°C.

2.9.4 Binding reaction
Binding reaction was carried out in a final volume of 20 µl consisting of 10 mM Tris.Cl (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 2.5% glycerol, 1 µg deoxyinosinic deoxyctydyl acid [poly(dI–dC)], 300 ng bovine serum albumin, 5 µg nuclear extract, and 0.3 nmol of [γ-32P]-labeled oligonucleotide probe and 2 µl of binding buffer [100 mM Tris.Cl (pH 7.5), 500 mM NaCl, 10 mM DTT, 10 mM EDTA, 25% glycerol] and binding reaction was carried out. Reaction mixtures were incubated for 10 min at room temperature. Samples were loaded on to a native polyacrylamide gel. Ten times more unlabeled probe (cold probe) was used to competitively inhibit the binding of [γ-32P] - labeled oligonucleotide probe to the protein.
2.9.5 Native gel electrophoresis and autoradiography

To separate free probes from DNA-protein complex, native polyacrylamide gels were carried out. Gels (14X16 cm) were casted (Amersham). The components of the native polyacrylamide gel are mentioned below –

**Component volumes are given per 10 ml of gel mold volume**

**Ingredients:** 8% Gel; 12 % Gel

- **40% Acrylamide:** 2 ml; 3 ml
- **2% Bis-acrylamide:** 320 µl; 500 µl
- **10 X TGE:** 500 µl; 500 µl
- **50% glycerol:** 1 ml; 1 ml
- **Nuclease free water:** 6.18 ml; 5 ml
- **10% APS:** 100 µl; 100 µl
- **TEMED:** 10 µl; 10 µl

Pre-electrophoresis was carried out for 60 min at 80 V at 4°C before loading the samples. After binding reactions, all the samples were loaded into the wells of native polyacrylamide gel. For p53 binding 8% gel was prepared. Samples were run in TGE (25 mM Tris, 190 mM glycine, 1 mM EDTA). To visualize the sample movement, one well was loaded with 1 µl of 6X DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol). The blue colour of bromophenol blue dye indicates the running front of the samples. After completion of electrophoresis, gel was carefully removed from the glass plates, transferred to Whatmann filter paper (Whatman) and dried under vacuum at 80°C for 90 min by using gel dryer (BioRad). Dried gel was exposed to X-ray film (Konica X-ray film) and DNA-protein complex were visualized by autoradiography. Films were developed with Kodak DA-163 developer.

2.10 Immunofluorescence confocal microscopy

Coverslips were layered with collagen (50 µg/ml in 0.02 N acetic acid) and air dried to form a layer of collagen over the coverslips. Cells were
grown on collagen-coated cover slips and treated with different inhibitor or drugs as per experimental requirement. Cells were washed with PBS and fixed with 3.7% Para formaldehyde for 10 min at room temperature. Cells were permeabilized in PBS containing 0.1% Triton X-100 on ice and subsequently blocked with 10% FBS for 1 h at 4°C. Primary antibodies against pCav-1, Cav-1 (dilution in the blocking solution 1:100) were added and incubated for 2 h at room temperature. Following incubation, cells were washed three times with PBS. Fluorescein isothiocyanate (FITC) conjugated secondary antibodies (1:100) were added in blocking solution and incubated for 1 h at room temperature. After three washes with cold PBS, samples were examined on a confocal microscope (LSM510, Carl Zeiss, Germany). Images were subsequently processed by Adobe Photoshop software.

2.11 Flow cytometry for cell cycle analysis

Cells were plated at a density of approximately 5x10^5 cells in 35 mm plates and allowed to grow for 24 h. Cells were treated with drug/MCD/inhibitor/SiRNA as per requirement of the experiment. Cells were harvested by trypsinization and processed for flow-cytometry. In brief, cells were washed with chilled PBS and fixed in 70% ethanol on ice. RNAase (200 µg/ml) treatment was given for 30 min at 37°C. Fifty µg/ml propidium iodide (PI) was added in RNAase treated cells and incubated in dark for 30 min on ice. The fluorescence of PI was collected through a 585 nm filter in flowcytometer (FACS Vantage, Becton Dickinson). The data were analyzed using cell quest software for 10,000 cells.

2.12 Immunoprecipitation and kinase assay

An anti-Cdk5 (1 µg) polyclonal antibody (SC-173) was incubated with cell-lysate (400 µg) overnight at 4°C in an orbital shaker. Fifty microliter Protein G plus / protein A agarose (Invitrogen Corporation) was then added in antibody-lysate complex with gentle shaking for 4-5 hours at
4°C. The immune complex was separated by centrifugation at 4000 rpm. Kinase assay was performed by washing immunoprecipitates three times with kinase reaction buffer (50 mM HEPES pH 7.0, 10 mM MgCl₂ and 1 mM DTT). Protein G plus/protein A agarose beads bound antibody with target proteins were incubated with kinase reaction buffer containing 2 μg of substrate (Histone H1, Millipore) and 10 μCi of ³²P-ATP in a final volume of 40 μl at 30°C for 30 minutes. Termination of the assay at the appropriate time was carried out by adding SDS-sample buffer and subsequently all samples were electrophoresed. Dried gel was exposed to X-ray film and developed.

2.13 Drug uptake assay

To check the difference in cellular uptake of radiolabeled drugs in presence or absence of MCD, drug uptake assay was performed. C¹⁴ labeled 5-fluorouracil (C¹⁴-5-FU) and H³ labeled etoposide (H³-ETOP) was used in this experiment. Cells (1x 10⁵ /well) were grown in 24 wells plate. Next day cells were treated with MCD for 4 h. Subsequently, cells were washed to remove MCD and radio labeled drugs (500 nCi) was added in controls cells as well as cells subjected to MCD pretreatment. These cells were incubated for different time points (6 to 24 h). Drug uptake assay was terminated by washing the cells with ice-cold PBS and lysed with 0.1% SDS solution (40 μl volume). The lysates were transferred on to a membrane (Whatman). Incorporation of radio labeled drugs in cells was quantified by using a Top Count Micro plate Scintillation Counter (Pacard). Relative radiolabeled drug uptake was calculated as ratio of radioactivity measured in the cells as counts per minutes (CPM), either in presence or absence of MCD pretreatment.
2.14 Animals, animal-keeping conditions, and tumor growth regression model

2.14.1 Xenograft models

Eight weeks old female homozygous (nu+/nu+) nude athymic NIH mice (NIV, Pune, India), weighing 18–22 g, were used as human cancer xenograft models. The animals were housed in specific pathogen-free isolators (Harlan Isotec) in an environmentally controlled Animal Care facility of our institute. They were caged (six per cage) in polypropylene boxes and had free access to sterilized pelleted laboratory rodent chow and water. All animal experiments have been performed following the requirement of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, and after permission of the Institute’s Animal Care and Use Committee (IACUC). Five consecutive subcutaneous (sc) estradiol valerate (30 µg/day) injections were administered in each mouse before sc injection of MCF-7 tumor cells in the exponential growth phase (2x10^7 cells in 0.1 ml PBS) at right flank of each mouse. Drug treatments were initiated 2-3 weeks after palpable tumors developed. The mice were randomized into four groups having six mice per group (n=6) to receive vehicle alone (control), MCD (1.6 mg/mice/day), 5-FU (0.6 mg/mice/day), or MCD plus 5-FU by intraperitoneal (ip) injection for four consecutive days. 5-FU and MCD were dissolved in sterile water. On fifth day following last injection, mice were euthanized and tumors were surgically excised. Tumor growth was determined by weight measurements and host toxicity was monitored by body weight measurements. Representative tumor tissues (200 mg from each animal of a group) were processed and samples were pooled from three animals per treatment group for reverse transcriptase polymerase chain reaction (RT-PCR) analysis as mentioned.
2.14.2 C-57 mice

Eight weeks old C-57 mice (18–22 g) were caged (six per cage) in our in-house animal house facility. Tumor were raised against mouse melanoma cell line B16F10 (1 × 10^6 cells/0.2 ml PBS) injected subcutaneously. Drug treatments were initiated 2 weeks after palpable tumors developed. The mice were randomized into four groups having six mice per group (n = 6) to receive vehicle alone (control), MCD (1.6 mg/mice/day), 5-FU (0.6 mg/mice/day), or MCD plus 5-FU by intraperitoneal (ip) as well as local (at the tumor site) injection for four consecutive days. 5-FU and MCD were dissolved in sterile water. Four days following last injection mice were euthanized and tumors were surgically excised. Tumor growth was determined by weight measurements and host toxicity was monitored by body weight measurements.

2.15 Statistical analysis

Data are expressed as the mean of three independent results. Statistical comparisons are made using student’s t test and P value < 0.05 was considered as significant.