MATERIALS AND METHODS:

Cell lines:

All cell lines were obtained directly from ATCC or procured via NCCS (National Centre for Cell Science, Pune, India).

1. **H460-** NCI-H460 cell line was derived by A.F. Gazdar and associates in 1982 from the pleural fluid of a patient with large cell cancer of the lung. It is an adherent epithelial cell line. The cells are tumorigenic and express easily detectable p53 mRNA at levels comparable to normal lung tissue. They exhibit no gross structural DNA abnormalities.

2. **A549-** This line was initiated in 1972 by D.J. Giard, *et al.* through explant culture of lung carcinomatous tissue from a 58-year-old Caucasian male. It is an adherent epithelial cell line.

3. **H1299-** This cell line was deposited at the ATCC by Dr. A. Gazdar and Dr. J. Minna and is provided for research purposes only. The cells have a homozygous partial deletion of the p53 protein, and lack expression of p53 protein. It is an adherent non-small cell lung cancer cell line carcinoma.

4. **HCT116-** It is an adherent colorectal carcinoma cell line. This line has a mutation in codon 13 of the ras protooncogene. HCT116 p53+/+ is a human colon carcinoma cell line, which contains a wild-type p53 gene, whereas the HCT116 p53-/- is a p53 knockout cell line derived from HCT116 p53+/+ by homologous recombination (Bunz, Dutriaux *et al.* 1998).

5. **Mouse primary fibroblasts-** Mouse primary fibroblast culture was established from skin explants from 2-3 day old pups.

6. **Buffalo primary cells-** Buffalo mammary primary culture was established from mammary tissue explants from Indian water buffalo (*Bubalus bubalis*).

All the above mentioned cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1X penicillin / streptomycin antibiotics (100u/ml penicillin and 100ug/ml streptomycin) under standard humid culture conditions of 37°C temperature 5% CO₂.
7. **RWPE-1** - It is an adherent normal prostate epithelial cell line. Epithelial cells derived from the peripheral zone of a histologically normal adult human prostate were transfected with a single copy of the human papilloma virus 18 (HPV-18) to establish the RWPE-1 cell line. These cells were maintained in Keratinocyte Serum Free Medium (K-SFM) with the following two additives:

- 0.05 mg/ml BPE (bovine pituitary extract)
- 5 ng/ml EGF (human recombinant epidermal growth factor)

**Cell culture and cryopreservation**

For subculturing or cryopreservation of cells, the culture medium was first removed and discarded. The cell layer was then briefly rinsed with Ca++/Mg++ free Dulbecco's phosphate-buffered saline (D-PBS). 1-2ml of 0.05% Trypsin - 0.53mM EDTA solution was added to a 90mm culture dish or T-25 flask and the flask was placed in a 37°C incubator for 2 to 3 minutes. The cells were observed under an inverted microscope until cell layer was dispersed (usually within 1 to 3 minutes). The trypsin-EDTA solution was then removed and cells were dislodged by shaking. For **passaging**, the cells were resuspended in complete growth medium (containing 10% FBS) and subcultured in 1:2 – 1:3 ratio. 6-7ml medium was used per 90mm dish and 2ml medium per 35mm dish.

For **cryopreservation**, following trypsinization, the cells were resuspended in FBS containing 5% DMSO. The cell suspension was transferred to cryovials and these cryovials were placed in an ethanol bath and kept at -80°C overnight. The following day the cryovials were stored in liquid nitrogen (-196°C).

**Chemicals and reagents**

Fenbendazole (FZ), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), colchicine, MG132, cycloheximide, JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimazolocarbocyanine iodide), Hoechst 33342, propidium iodide, N-acetyl cysteine, TNFa, Z-VAD-FMK, proteasomal and caspase substrates, anti β-actin, anti ubiquitin, anti GFP, anti p27Kip1, anti-mouse IgG-fluorescein isothiocyanate (FITC), horseradish peroxidase (HRP) conjugated anti-mouse, anti-
rabbit and anti-goat IgGs as well as all the cell culture reagents were purchased from Sigma. IKK inhibitor wedelolactone was obtained from Calbiochem. Anti p53 (Bp53-12 and DO1), anti-p21, anti MDM-2, anti cyclin B1, anti Bax, anti cytochrome c, anti caspase-3, anti caspase-9 and anti IκBα were purchased from SantaCruz Biotechnology. Keratinocyte Serum Free Medium (K-SFM) along with the additives (BPE and EGF) was obtained from Invitrogen (GIBCO). All the enzymes used in the study were obtained from either usb or Fermentas. Lipofectamine 2000 was obtained from Invitrogen. 2-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) and 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) were obtained from Molecular Probes (Invitrogen). Chemiluminiscent detection reagent as well as PVDF membrane for western blotting (Immobilon Western) was purchased from Millipore. All culture plasticware was purchased from Nunc. Regular plasticware like PCR tubes, microcentrifuge tubes, disposable pipette tips etc. were obtained from Eppendorf, BD or Axygen.

Expression vectors and constructs –

The pTet-On inducible system, pdlEGFP and pSEAP vectors were obtained from Clontech. pWWP-Luc construct was obtained from Dr. Bert Vogelstein, FLAG-Ub construct from Dr. Caixia Guo (Dept. of Pathology, The University of Texas Southwestern Medical Center, Dallas, Texas) and pNFκB-Luc was kindly provided by Prof. Macus T. Kuo (Dept. of Molecular Pathology, The University of Texas MD Anderson Cancer Center, Houston, Texas). The GFP-p53 construct was obtained from Prof. Antonis E. Koromilas (Lady Davis Institute for Medical Research, McGill University, Montréal, Québec H3T 1E2, Canada). The pCOC-Mdm2 construct was kindly provided by Prof. Moshe Oren (The Weizmann Institute, Israel).

Experimental Procedures -

Cell viability assays - Cell viability was measured using either MTT or cell count assays. Cells (5 x 10^3 cells/well) were seeded into 96-well plates, and 24 h after seeding, cells were treated with different doses of fenbendazole or other drugs as indicated in different experiments. For MTT assay, 20ul of 10mg/ml MTT stock solution (prepared in PBS) was added per well and cells were incubated at 37°C in
CO₂ incubator for 2h. Following incubation, the media was removed and the formazan crystals were resuspended in 100ul DMSO. Absorbance was then monitored at 570nm.

For cell count assays, cells were seeded at a density of 1 x 10⁴ cells/well in 24-well culture plates and then treated with FZ. Cells were harvested at the indicated time points and viable cells were counted using a haemocytometer by the trypan blue exclusion method. All experiments were done in triplicate.

**Immunofluorescence for p53** - Cells were grown on coverslips and treated with 1uM FZ or 0.05ug/ml colchicine for 24h. Following treatment, cells were washed with PBS and fixed using 4% paraformaldehyde fixative. Cells were again washed with PBS and incubated with anti-p53 (Bp53-12) antibody overnight at 4°C. Following day, after several washes with PBS the cells were incubated with FITC conjugated anti mouse secondary antibody for 1h at 37°C. Stained cells were mounted in DABCO mounting medium (Sigma) and observed using a Nikon fluorescence microscope (BA520, DM505, Ex 450-490).

**Immunofluorescence for tubulin** - For visualization of tubulin organization following treatment with microtubule interfering drugs, immunofluorescent staining was done. Briefly, A549 cells were grown on coverslips and treated with different drugs as indicated. Following treatment, cells were rinsed twice with PEM-PEG buffer (80mM PIPES, 1mM EGTA, 0.5mM MgCl₂, 4% PEG 8000) and permeabilized with PEM-PEG buffer containing 0.5% Triton X-100. The cells were then rinsed with PEM-PEG buffer and quickly fixed in 3% formaldehyde in PEM with 1% DMSO for 30 min at RT. After washing with PEM-PEG buffer, primary antibody (anti α-tubulin) incubation was carried out overnight at 4°C. After several washings with PBS, cells were incubated with fluorescein isothiocyanate-conjugated secondary antibody for 1 h at 37°C, washed several times, and visualized using a Nikon fluorescence microscope (BA520, DM505, Ex 450-490).

**Fractionation of soluble and polymerized tubulin** - Separation of soluble and polymerized tubulins from H460 cells was carried out as described by Legault et al (2000). Briefly, after drug exposure, about 5 X 10⁶ cells in 100mm petridishes were
washed with PBS at 37°C and harvested in 1ml of PBS containing 0.4ug/ml of paclitaxel using a rubber policeman. Cells were then centrifuged and lysed using 250ul of microtubule stabilizing buffer [20mM Tris-HCl (pH6.8), 140mM NaCl, 1mM MgCl2, 2mM EDTA, 0.5% NP40 and 0.4ug/ml paclitaxel] and centrifuged at 12,000Xg for 10 min at 4°C. The supernatants containing soluble tubulin were mixed with 2X Laemmli sample buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris HCl, pH 6.8.). Pellets containing the polymerized tubulin were resuspended in 250ul of water, followed by two freeze/thawing cycles and finally resuspended in Laemmli sample buffer. Samples were analyzed by western blot using anti α-tubulin antibody.

**Tubulin polymerization assay** - Polymerization of bovine tubulin was measured according to Beyer et al. (Beyer, Zhang et al. 2008). Briefly, bovine tubulin (1.8 mg/mL; Sigma) was added to ice-cold polymerization buffer (PEM: 80mM PIPES, 0.5mM EGTA, 2mM MgCl2, 10% glycerol, and 1mM GTP) and centrifuged at top speed in a microcentrifuge for 5 minutes at 4°C. Supernatant (100 uL/well) was immediately added to a 96-well plate, which contained 10uM FZ or dimethyl sulfoxide control in PEM buffer.

After addition of tubulin, the plate was immediately placed in the spectrophotometer, which was maintained at 37°C, and the absorbance measured every 5 minutes for 2.5 hours at 340 nm.

**Measurement of mitochondrial membrane potential and cytochrome c release** - H460 cells were plated onto cover slips in 35mm tissue culture plates at subconfluent density. Twenty four hours later, cells were exposed to the specified doses of FZ for 24 h or MG132 for 12 h following which they were incubated with 5 uM JC-1 dye for 30 min in the CO2 incubator. After several washes with prewarmed PBS, mitochondrial membrane potential was evaluated qualitatively under a fluorescence microscope using 568-nm filter.

The localization of cytochrome c was examined using immunofluorescent staining. Cells grown on cover slips were treated with FZ or MG132. Twenty four hrs after treatment, cells were washed twice with PBS, fixed with 4% paraformaldehyde in
PBS for 20 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min, washed extensively and then blocked with goat serum in PBS for 30 min. Primary antibody (anti-cytochrome c) incubation was carried out overnight at 4°C. After several washings with PBS, cells were incubated with FITC-conjugated anti mouse secondary antibody for 2 h at 37°C, washed several times, and visualized under a Nikon fluorescence microscope (BA520, DM505, Ex 450-490).

Cytochrome c release from mitochondria after FZ treatment was further confirmed by immunoblotting. H460 cells were grown on tissue culture dishes and treated with FZ for 24 h or MG132 for 12 h. The cells were then collected by scraping, washed in PBS followed by sucrose buffer [20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) containing 250 mM sucrose], and resuspended in the same buffer. After 1 h of incubation on ice, cells were lysed by passing through a 26 gauge needle several times. Homogenates were centrifuged at 750 x g for 10 min at 4°C, and the supernatants were re-centrifuged at 10,000 x g for 15 min at 4°C. The final supernatants were used for immunoblotting experiments of cytochrome c.

**Assay of proteasome and caspase-3-like protease activity** – After overnight culture in 35 mm culture dishes, cells were treated with varying doses of FZ or 10 μM MG132 for specified time periods. Cells were then isolated and processed for proteasome activity and caspase-3-like protease activity assays (Jana, Zemskov et al. 2001). After treatment, the cells were washed with PBS, pelleted and resuspended in 50 μl proteasome assay buffer [10 mM tris (pH7.4), 1 mM EDTA, 5 mM ATP, 5 mM DTT and 20% (v/v) glycerol]. The cells were lysed by passing through a 26 gauge needle several times and then centrifuged at 15,000 g for 15 min at 4°C. 10 μg of supernatant was incubated in proteasome activity assay buffer [50 mM Tris (pH7.4), 0.5 mM EDTA, 50 μM of each proteasome substrate] in 50 μl total reaction volume. For caspase-3 activity assay, the assay buffer used consisted of 10 mM HEPES (pH7.4), 2 mM EDTA, 0.1% CHAPS, 5 mM DTT and 1 mM PMSF. For measurement of caspase-3 like protease action, the activity assay buffer consisted of 20 mM HEPES (pH7.4), 2 mM DTT, 10% (v/v) glycerol and 50 μM of caspase-3 substrate. The fluorogenic substrates Succinyl-Leu-Leu-Val-Tyr-MCA, Z-Leu-Leu-Glu-7-amido-4-
MCA, Boc-Gln-Ala-Arg-7-amido-4-MCA and Ac-Asp-Glu-Val-Asp-MCA were used to determine chymotrypsin, peptidylglutamyl-peptide hydrolyzing activity, trypsin-like and caspase-3-like protease activity respectively.

For *in vitro* assay, control cell extracts were incubated with various doses of FZ for 4h or with 5uM FZ for different time points as indicated, and assayed for protease activity. To evaluate the direct effect of FZ on the protease activity of proteasome, pure 20S proteasome (100 ng/reaction) was used instead of cell supernatant in the protease activity assay buffer. Protease activities at a particular time point (30 min) within the linear range were used to calculate the data. The fluorescence intensity was measured at 380 nm excitation and 460 nm emissions using a PerkinElmer victor X3 fluorescence plate reader.

**RT-PCR** - Cells were treated with different drugs as indicated and total RNA was isolated using guanidium thiocyanate method (Chomczynski and Sacchi 2006) or using TRI reagent (Sigma) as per manufacturer’s instructions. Briefly, following treatment, cells were washed with PBS and lysed and collected in 400ul solution D (4M guanidium thiocyanate, 0.75M sodium citrate, 10% N-laurylsarcosine and 0.1M β-mercaptoethanol). This was followed by addition of 40ul 2M sodium acetate (pH4.0), 400ul water saturated phenol and 40ul 49:1 chloroform: isoamylalcohol. It was then mixed and incubated for 15 min on ice and centrifuged at 9000rpm for 10min at 4°C. RNA was precipitated with equal volume isopropanol. RNA was resuspended in DEPC treated water and quantified using spectrophotometer. RNA was reverse transcribed using oligo (dT)18 primers from RT-PCR kit obtained from Fermentas. RT-PCR analysis was performed for quantifications of different genes. Primers used for RT-PCR analysis of different genes are given in Table I. For real time PCR, RealMasterMix SYBR ROX kit from Eppendorf was used. The reactions were set up according to the manufacturer’s instructions and an Eppendorf Mastercycler realplex real-time PCR machine was used for analysis.
### Serial Gene

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Gene</th>
<th>Forward primer (5’-3’*)</th>
<th>Reverse primer (5’-3’*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>p53</td>
<td>GCCCCTCCTCGACGATCTTTAT</td>
<td>GGAGCTTCTCAGTGTAGATG</td>
</tr>
<tr>
<td>2.</td>
<td>p21/WAF1</td>
<td>GCACTGTCAAAACGGCGCT</td>
<td>ATTAGGCTTCTTTGG</td>
</tr>
<tr>
<td>3.</td>
<td>Bax</td>
<td>GACCGGTCCAGGCGACGCCC</td>
<td>TCAAGCCCCATTCCTCAGG</td>
</tr>
<tr>
<td>4.</td>
<td>Mdm2</td>
<td>TCAGATTCTAGTTCAGATA</td>
<td>CATTCTCAATAGTACGAGAGG</td>
</tr>
<tr>
<td>5.</td>
<td>PIG3</td>
<td>TGGCGTCCTCGCCGGAGGATA</td>
<td>GCTGGAGGCGCTGGAGCAG</td>
</tr>
<tr>
<td>6.</td>
<td>Survivin</td>
<td>GACGCCCTAAAGGGGCAGTC</td>
<td>ATGTTCTCTATGAGGCGTCATC</td>
</tr>
<tr>
<td>7.</td>
<td>Bak</td>
<td>GCTCTGAGGCGCCAGACGATT</td>
<td>TCAAGATTTCGAGAAATCCAGG</td>
</tr>
<tr>
<td>9.</td>
<td>HSP5A</td>
<td>GCTCTGCTCTTCCTGTTGA</td>
<td>GTAGCAGACTCTTGTTTCCAG</td>
</tr>
<tr>
<td>10.</td>
<td>IRE1α</td>
<td>GAGCAAGTCTGGAGCCACCTC</td>
<td>TCTGGAGGCCCTCCAGCAAA</td>
</tr>
<tr>
<td>11.</td>
<td>GADD153</td>
<td>CCTGAGAGAGATGTTCAAGAGG</td>
<td>TGACTGGAACAGCTTCCATG</td>
</tr>
<tr>
<td>12.</td>
<td>ATF3</td>
<td>CCTGAGAGCAAAATAGTGGCTCA</td>
<td>TCCGACTCTTCTGGACGACT</td>
</tr>
<tr>
<td>13.</td>
<td>Noxa</td>
<td>TCCAGCACGGCTGAGCTCGAGTGT</td>
<td>ATGATGCACCTCTACATCCT</td>
</tr>
<tr>
<td>14.</td>
<td>XBP1</td>
<td>TTACGAGAGAAACATCTCAGG</td>
<td>GGTTCAGAATGTTGTCAGAAAT</td>
</tr>
<tr>
<td>15.</td>
<td>ALDH4A</td>
<td>CCGTGGACGAGGCTGGGCCC</td>
<td>GACACTGGGCCCCAGCAGAGA</td>
</tr>
<tr>
<td>16.</td>
<td>GPX1</td>
<td>TTGGGATCATCGAGGAGGCAAGCCA</td>
<td>GCAAGAAGCGGCGGGAGGA</td>
</tr>
<tr>
<td>17.</td>
<td>Sestrin-2</td>
<td>TCTGGAGGCCGGAGGAGCAGC</td>
<td>AGTTCGAGGGCCGAAGGCTG</td>
</tr>
<tr>
<td>18.</td>
<td>Glutaminase-2</td>
<td>ACCCAAGGCGGCGGCACTTCT</td>
<td>CACGCGGTCGATCTCAGTCA</td>
</tr>
<tr>
<td>19.</td>
<td>Proline oxidase</td>
<td>GCTCTGGTGTCAGCGCAGCAT</td>
<td>CCTGCACCTTGTCAGCAGG</td>
</tr>
<tr>
<td>20.</td>
<td>Glut-1</td>
<td>ACGAGGGGCGTCGAGATCAG</td>
<td>TCTGCCACGGCCGAGATGA</td>
</tr>
<tr>
<td>21.</td>
<td>Glut-3</td>
<td>CCAAGAGGACAGCGAGATGCG</td>
<td>AGCAGGCTAGGGTGAGGC</td>
</tr>
<tr>
<td>22.</td>
<td>Glut-4</td>
<td>CAGAGATGCGGCCGCTCCAG</td>
<td>GGAAGAGGGCCGAGAGGTCG</td>
</tr>
<tr>
<td>23.</td>
<td>IKKα</td>
<td>GCCCTATGGGAGACGTCGAGGA</td>
<td>CTGCTGAGTGCGGCGGAGCG</td>
</tr>
<tr>
<td>24.</td>
<td>IKKβ</td>
<td>GAAACCAGAGGCGGTGTCGCG</td>
<td>GGCCAGCCTTCCGGCAGAGAC</td>
</tr>
<tr>
<td>25.</td>
<td>Hexokinase II</td>
<td>GGGCAGCAACCGCCTGCTACA</td>
<td>GGTCCAGGCCCCAAGGCAATC</td>
</tr>
<tr>
<td>26.</td>
<td>HIF1α</td>
<td>ACAAGCCAGACAGACTCAGTC</td>
<td>CCTGCAAGTGGTTTCTGCGCT</td>
</tr>
<tr>
<td>27.</td>
<td>TIGAR</td>
<td>CGGCTCCCAGGAAATCGCGCTC</td>
<td>GTGCTCCTCAGGGCGGTG</td>
</tr>
<tr>
<td>28.</td>
<td>SCO2</td>
<td>CCCGCAGGGAGGAGCAT</td>
<td>ACCGAGCTGGCACGCGGCAACA</td>
</tr>
<tr>
<td>29.</td>
<td>GADD45A</td>
<td>CCTGCACGTGGTCGTTGTA</td>
<td>TCCCGGCGGCCCGAACATC</td>
</tr>
<tr>
<td>30.</td>
<td>vWF</td>
<td>CGGCTTGTGACCCATGGCAGTA</td>
<td>TCCGAGAATGTTGATATCATAGGCAATC</td>
</tr>
<tr>
<td>31.</td>
<td>CD31</td>
<td>ATTGCAGTTGTTATCATCGGAGGT</td>
<td>CTCTGGTTGGAGTTCAGAATGG</td>
</tr>
<tr>
<td>32.</td>
<td>VEGF</td>
<td>CTACCTCCACACAGGCAAGT</td>
<td>GCAAGTCGCTGGCTGATAGA</td>
</tr>
<tr>
<td>33.</td>
<td>MMP-9</td>
<td>TTGCAGGCAGAAGAGGTG</td>
<td>GCCATTCACTGGCTCTTAT</td>
</tr>
<tr>
<td>34.</td>
<td>18sRNA</td>
<td>GTAACCCGAGGATCCCCATT</td>
<td>CCATCACATCGAATGAGC</td>
</tr>
<tr>
<td>35.</td>
<td>β-actin</td>
<td>TGCTATCGAGGCTGTCGAT</td>
<td>GATGGAGGTTGAAGGTTGTT</td>
</tr>
</tbody>
</table>

### Table I

**Two-dimensional gel electrophoresis** – Two dimensional (2-D) gel electrophoresis is used to separate complex mixtures of proteins into many more components than is possible with conventional one-dimensional electrophoresis. The first dimension is...
run in an isoelectric focusing (IEF) tube gel, which is then grafted horizontally onto the top of a polymerized regular slab gel. For making IEF tube gels, 4ml of IEF gel solution (2.19g urea, 0.42ml acrylamide solution (30 : 1.8g acrylamide : bisacrylamide/100ml), 0.41ml 20% NP-40, 0.20ml ampholytes (40%, pH 3-10), 2.5ul TEMED, 25ul 10% APS and 1.3ml milliQ H$_2$O) was poured into casting chamber with tubes. The gels were allowed to polymerize for 30 minutes. After 30 min, the bundle was carefully removed and any acrylamide mass sticking outside the tubes was carefully removed by cutting with a sharp blade. The gels were pre-run at 200V for 30 min after adding 5ul of sample overlay solution (0.481g urea, 0.05ml ampholytes (40%), H$_2$O to 1ml). The anolyte solution was 10mM phosphoric acid and the catholyte solution was 10mM NaOH. After pre-run, the samples were loaded using a Hamilton syringe and the samples were focused overnight at 300V. The lower ends of tubes were carefully secured using a muslin gauge to prevent the tube gels from slipping out.

For sample preparation, cells were pelleted by brief centrifugation and lysed in 240ul/2X 10$^6$ cells of Lysis Buffer 1 (0.3% SDS, 200mM DTT, 50mM Tris, pH7.5). Cells were further lysed by heating at 100°C for 5 min followed by chilling on ice. Nucleic acids were digested by adding DNase I (50ug) and RNase A (12ug) and incubating the lysates on ice for 30 min. Proteins were precipitated by adding ice cold acetone to 80% (v/v) and incubating on ice for 20 min. After the proteins were pelleted, they were air dried and solubilised in 50ul of Lysis Buffer 1. The proteins were further solubilised by adding 200ul of IEF sample buffer (9.9M urea, 4% NP-40, 2.2% pH 3-10 ampholytes, 100mM DTT).

After isoelectric focusing, the gels were carefully removed from tubes by squirting with equilibration buffer [0.0625 M Tris-Cl (pH 6.8), 2.3% w/v SDS, 5% β-mercaptoethanol, 10% glycerol and trace of bromophenol blue] using a syringe. The gels were equilibrated in buffer for 20 min at room temperature. They were then carefully overlaid on 10% SDS-PAGE gels, secured in place by 1% agarose in SDS equilibration buffer without β-mercaptoethanol, and run at 100 constant volts. The SDS-PAGE gels thus obtained were proceeded for silver staining or transferred on PVDF membranes for western blot analysis.
Co-immunoprecipitation and immunoblotting experiments - Twenty-four hours after transfection with pdlEGFP plasmid, cells were treated with different doses of FZ and MG132 for 8 h. Cells were then washed with cold PBS and lysed on ice for 30 min with Nonidet P-40 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, complete protease inhibitor mixture). Cell lysates were briefly vortexed, centrifuged for 10 min at 15000 x g at 4 °C, and the supernatants (total soluble extract) were used for immunoprecipitation. Protein concentration was measured according to the method of Bradford using bovine serum albumin as a standard (Bradford 1976). For each immunoprecipitation experiment, 200 ug protein in 0.2 ml of Nonidet P-40 lysis buffer was incubated with 5 ul (2.5 ug) of GFP antibody. After overnight incubation at 4°C with rotation, 20 ul of protein A/G-agarose beads were added, and incubation was continued at 4 °C for 5 h. The beads were washed six times with Nonidet P-40 lysis buffer. Bound proteins were eluted from the beads with SDS (1X) sample buffer, vortexed, boiled for 5 min, and analyzed by immunoblotting. The total cell lysates or the immunoprecipitated proteins were separated through 10% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were successively incubated in blocking buffer [5% skim milk in TBS-T (50 mM Tris, pH 7.5, 0.15 M NaCl, 0.05% Tween-20)] with primary antibody and then with secondary antibody conjugated with horseradish peroxidase. Detection was carried out with enhanced chemiluminiscense reagent from Millipore. All primary antibodies were used in 1:4000 dilutions for immunoblotting.

Histone H1 Kinase Assay - After treatment as indicated, cells were lysed on ice in H1 kinase lysis buffer [50mM Tris (pH7.4), 150mm NaCl, 0.5% Triton-X 100, 5mM EDTA, 1mM DTT and 1X protease inhibitor cocktail]. Cdk2 was then immunoprecipitated for 1h at 4°C using anti cdk2 antibody from SantaCruz. The immunoprecipitates were then washed with lysis buffer followed by two washes with kinase buffer [20mM HEPES (pH7.0), 5mm MgCl2 and 1mm DTT]. Commercially purified histone H1 (Sigma) at a concentration of 0.6 mg/ml was incubated with cdk2 immunoprecipitated from H460 whole-cell extracts equivalent to 100 µg of total protein in 50 µl of kinase reaction buffer (50 mM Tris, pH 7.2; 10 mM MgCl2; 1mM DTT; 5 µCi of [γ-32P]ATP and 5um cold ATP) for 10 min at 30°C. The reaction was
stopped by adding ice-cold 20mM EDTA (pH 8.0). Samples were analyzed by SDS-PAGE followed by autoradiography.

**SEAP assay for ER stress** - 293T cells were seeded in a 96-well plate and were transfected the next day with pSEAP-con plasmid which constitutively expresses secreted alkaline phosphatase (SEAP). 24h after transfection, the cells were either left unexposed or exposed to different concentrations of indicated drugs in fresh media containing 1% FBS. 24h after incubation, 10ul of conditioned culture media was removed and assessed for SEAP activity using 4-MUP substrate according to the protocol described by the manufacturer (Clontech, Palo Alto, CA). In brief, 10 ul medium was mixed with 15 ul 5X SEAP assay buffer (0.5 M Tris, pH 9.0 and 0.5% BSA) in a total volume of 50 ul in a 96-well plate and incubated at 65°C for 30 min to inactivate endogenous SEAP activity. The plate was chilled on ice for 2 min. Then 25 ul of 1 mM 4-methylumbelliferyl phosphate substrate was added to each well and incubated at 37°C for 2 h. The activity of SEAP was assayed using a 96-well fluorescence plate reader (PerkinElmer Multilabel reader Victor X 3) with excitation set at 355 nm and emission at 460 nm. Three independent experiments were performed in triplicates.

**Pulse-chase experiment for the analysis of protein stability** - Cells were plated onto 35mm tissue culture plates and on the following day, they were left untreated or treated with FZ and chased in the presence of cycloheximide for the indicated time points. Cells collected at each time point were then processed for immunoblotting using antibodies against p53, cyclin B1, IκBα or Bax. For studying GFP stabilization, H1299 cells were transfected with pdlEGFP plasmid using lipofectamine. On the following day, the cells were either left untreated or treated with 5μM FZ and chased in the presence of cycloheximide for the indicated time durations. They were observed under fluorescence microscope after indicated time points and then processed for immunoblotting using anti GFP antibody as described earlier.

**Hoechst / Propidium Iodide (PI) staining** - Following FZ treatment, Hoechst 33342 and PI were added into the medium at 100 ng/ml concentration for 15min and cells were then washed with pre-warmed PBS and examined under fluorescent microscope for apoptotic cells.
Electrophoretic Mobility Shift Assay (EMSA)- H460 cells were treated with different doses of FZ for 4h and nuclear extracts were then prepared according to Schreiber et al (Schreiber, Matthias et al. 1989). Briefly, $2 \times 10^6$ cells were washed with cold PBS and suspended in 0.4 ml of lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 2.0 μg/ml leupeptin, 2.0 μg/ml aprotinin, and 0.5 mg/ml benzamidine). The cells were allowed to swell on ice for 15 min, after which 12.5 μl of 10% Nonidet P-40 was added. The tube was then vigorously mixed on a vortex machine for 10 s, and the homogenate was centrifuged for 30 s. The nuclear pellet was resuspended in 25 μl of ice-cold nuclear extraction buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 2.0 μg/ml leupeptin, 2.0 μg/ml aprotinin, and 0.5 mg/ml benzamidine), and the tube was incubated on ice for 30 min with intermittent mixing. It was then centrifuged for 5 min at 4°C, and the supernatant (nuclear extract) was either used immediately or stored at −70°C for later use. The protein content was measured by the method of Bradford (Bradford 1976).

Electrophoretic mobility shift assays (EMSA) were performed by incubating 6 μg of nuclear extract (NE), with 16 fmol of $^{32}$P-end-labeled 45-mer double-stranded NF-kB oligonucleotide, 5'-TTGTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGGCGTG-3' (Singh and Aggarwal 1995), for 30 min at 37°C. The incubation mixture consisted of 2-3 μg of poly (dl-dC) in binding buffer (25 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM DTT, 1% Nonidet P-40, 5% glycerol, and 50 mM NaCl). The DNA-protein complex formed was separated from free oligonucleotide on 5% native polyacrylamide gel and then the gel was then dried. The specificity of binding was determined by using an excess of unlabeled oligonucleotide for competition.

ROS activity assay- A549 cells were grown on coverslips in 35mm culture dishes and treated with 5μM FZ or 10μM MG132 for 4h. NAC (10 mM) was added 2 h before the drug. To measure the production of reactive oxygen species, after treatment, cells were washed and incubated at 37°C with 1μM DCF-DA for 1h in serum free media without phenol red. After washing twice, samples were illuminated by a 100-W mercury lamp and viewed with an FITC filter on a Nikon fluorescent
microscope to view DCFDA fluorescence. For quantitative analysis, fluorescence intensity was measured at 530 nm after excitation at 485 nm in a Victor 3X fluorimeter (PerkinElmer).

**Reporter assays for p53 and NFκB gene transcription** - The effect of FZ on p53 and NFκB dependent reporter gene transcription was measured by luciferase assay using pWWP-Luc (el-Deiry, Tokino et al. 1993) or NFκB-Luc construct respectively. For p53 transcriptional activity, H1299 cells were co-transfected with pWWP-luc and either pCMV vector or pCMV-p53 (WT) in a 96 well plate. Transfections were done using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. 24 hrs post-transfection, cells were left untreated or treated with 1uM FZ for 24h. Similarly, for NFκB reporter assay, A549 cells were co-transfected with NFκB-Luc vector carrying NFκB binding sites and pRL-SV40. The following day, cells were treated with 5 μM FZ for 4h followed by 30ng/ml TNFα for 1h, where indicated. In both cases, the cell extracts were then prepared and luciferase activity monitored using a Dual Luciferase Assay kit from Promega as per manufacturer’s instructions. The luciferase activity was assayed using a 96-well fluorescence plate reader (PerkinElmer Multilabel reader Victor X 3). pRL-SV40 Renilla vector was used for co-transfection in all cases to normalize the data, and it was transfected at a lower concentration (5-fold lower than the reporter luciferase plasmid). The data were represented as relative luciferase activity (the ratio of firefly to Renilla values). The experiments were performed in triplicates.

**FACS analysis** - After treatment with FZ for the indicated time intervals, cells were harvested, washed with PBS and fixed in 70% ethanol overnight at 4°C. Next day, cells were centrifuged at 1000rpm for 5min, the supernatant was carefully aspirated and the pellet was resuspended in PBS containing 40ug/ml PI and 100ug/ml RNase A. FACS analysis was done on a BD FACS Array and the data was analyzed using FlowJo software.

**TdT assay** - H460 cells were plated onto cover slips in 35-mm tissue culture plates at subconfluent density. Twenty four hours after seeding, cells were either left untreated or exposed to 1uM FZ or 0.05ug/ml colchicine for 24h. In situ tailing reaction was performed as described by Gold et al. (Gold, Schmied et al. 1994). Following
treatment, cells were washed with PBS and fixed in 1:3 acetomethanol. After fixation, the cells were rinsed with PBS and incubated with 0.25u/ml λ exonuclease enzyme in a moist chamber at 37°C for 10min. Cells were again washed with PBS and incubated with 20u/ml TdT enzyme in the presence of 5ul of 50nmol biotin-dUTP in a humid chamber for 1h at 37°C. Endogenous peroxidase activity was then blocked by incubation in 3% hydrogen peroxide in methanol for 15min at RT. Detection was finally done using avidin-biotin complex from ABC kit (Vector) and DAB substrate (Sigma).

**DNA fragmentation assay** - DNA fragmentation assay was performed as described previously (Mukhopadhyay, Sasaki et al. 2002). Control and FZ-treated cells were washed with cold PBS. The cell pellets were lysed in lysis buffer [10 mM Tris (pH 7.4), 10 mM EDTA (pH 8.0), and 0.5% Triton X-100] and incubated for 10 min at 4°C and then incubated with 200 ug/ml RNase A for 1h at 37°C. After centrifugation, the supernatants were incubated with 200 ug/ml proteinase K for 30 min at 50°C. Next, DNA fragments were precipitated with 0.5 M NaCl and 50% isopropanol, and the samples were loaded in 2% agarose TBE gel and stained with ethidium bromide.

**NFκB-dependent reporter gene transcription** - The effect of FZ on NFκB dependent reporter gene transcription was measured by NFκB SEAP assay as described by Shishodia et al. (Shishodia, Potdar et al. 2003). Briefly, after overnight culture, A549 cells in twelve-well plates were transfected with Secretary alkaline phosphatase (SEAP) vector carrying NFκB binding sites using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Cells were left untreated or treated with 5 uM FZ. The conditioned media were then collected at the indicated time points and analyzed for SEAP activity according to the protocol described by the manufacturer (Clontech, Palo Alto, CA). In brief, 25 ul medium was mixed with 30 ml of 5X buffer (0.5 M Tris, pH 9 and 0.5% BSA) in a total volume of 100 ul in a 96-well plate and incubated at 65°C for 30 min. The plate was chilled on ice for 2 min. Then 50 ul of 1 mM 4-methylumbelliferyl phosphate was added to each well and incubated at 37°C for 2 h. The activity of SEAP was assayed using a 96-well fluorescence plate reader (PerkinElmer Multilabel reader Victor X 3) with excitation set at 355 nm and emission set at 460 nm. The experiment was performed in triplicates.
**Glucose Uptake Assay** - Glucose uptake assay was performed using 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG; Invitrogen), a fluorescent analogue of 2-deoxyglucose, followed by detection using fluorescence microscope. After 24 h incubation in glucose free medium, cells were incubated with 2-NBDG (100 μM) for 30 min. The 2-NBDG uptake reaction was stopped by removing the incubation medium and washing the cells with pre-cold PBS.

**Glucose oxidation assay** - Glucose utilization was estimated in cells exposed to FZ with the commercially available glucose assay kit from Sigma (St. Louis, MO). After 24 h of exposure, supernatant medium was collected and centrifuged to remove any cellular debris. One milliliter of the assay reagent was mixed with 10 μl of the medium and incubated at 25°C for 30 min, after which the absorbance was recorded at 505 nm on a PerkinElmer VictorX3 spectrophotometer. The total glucose consumed was estimated and plotted.

**Cell invasion and migration assay** - Invasion and migration of H460 cells were analysed using a BD Biosciences Matrigel invasion chamber. The 8-μm pore inserts were coated with 15 μg of Matrigel (Becton Dickinson Labware, Bedford, MA). Briefly, H460 cells were added to coated filters (2 x 10⁴ cells/filter) and incubated for 24 h in 200 μl of serum-free medium in triplicate wells, and 750 μl of medium supplemented with serum was placed in lower chamber. After 48h, cells were fixed with 5% formaldehyde in PBS for 10 min and stained with crystal violet. Cells on the upper side of the membrane were then removed carefully using cotton swab. Cells that had invaded to the lower surface of the filter were counted. All invasion assays were done in triplicate. Representative fields were photographed.

The migration assay was performed likewise but without coating with Matrigel. H460 cells were loaded on transwell membrane inserts in triplicate wells. The plates were incubated for 48 h at 37°C in a 5% CO2 incubator, and then the cells on the lower side of the membrane were stained with crystal violet and counted. Each experiment was carried out in triplicate. The statistical significance of the results was determined using the Student’s t test procedure. A P-value of less than 0.05 was considered statistically significant.
Nude mice experiments – Human NSCLC A549 cells were grown in DMEM with 10% FBS. When cells were 70-80% confluent, 3-4 h before harvesting, medium was replaced with fresh medium to remove dead and detached cells. Cells were then trypsinized and collected in complete medium. They were collected by centrifugation at 1500 rpm for 2-5 min and washed twice with PBS. Cell number was determined using a Hemocytometer. Cells were suspended in a volume so that 100 μl contained required number of cells per injection i.e. 5.0 x 10⁶ cells. nu/nu mice used were 6 weeks old and they were acclimatization for 3 days before injection. Area to be injected was cleaned with 70% ethanol. 1ml syringe with 30 gauge needle was used for injection. The cells were resuspended in 100ul sterile PBS and taken into a syringe without a needle. A549 cells (5.0 x 10⁶) were subcutaneously injected into the right flank of mice. Oral dosing of FZ (1 mg/mouse) was started after 4 weeks when the tumors reached an average volume of 2–3 mm³. Tumor diameters were measured with digital calipers, and the tumor volume in mm³ was calculated by the formula: Volume = (width) 2 x length/2.

Construction of a wild type p53 cDNA clone in mammalian expression vector-

Full-length p53 was amplified by PCR using cDNA from H460 cell line as the template.

Forward primer:

\[
\text{HindIII site} \quad \text{FLAG sequence} \\
5'\text{-GTAGCTTACCATGGACTACAAGGACGACGATGACAAGATGG} \\
AGGAGCCGCG-3' \\
\]

Reverse primer:

\[
5'\text{-TCAAAGCTTGTGACAAAGTGGAGAATG-3'} \\
\]

PCR consisted of 35 cycles of denaturation at 95°C for 30 s, annealing at 51°C for 1 min, and extension at 72°C for 1 min. The PCR fragments were inserted into a pRc/CMV vector (Invitrogen) at the HindIII site.

Construction of stable and inducible p53 clones by transfection of wild type p53 construct in p53 null H1299 human lung cancer cells using the Tet-On mammalian expression system -
The **Tet-On Inducible Gene Expression System** is a tightly regulated and highly responsive system that produces conditional expression of a gene of interest in target cells. The system is established in target cells by sequentially transfecting them with the provided vectors (Clontech) and selecting stable cell lines. Target cells that express the Tet-On transactivator, and that also contain a TRE expression vector with the gene of interest will express high levels of the gene when cultured in the presence of the system’s inducer, doxycycline (Dox).

The Tet-On system consists of two vectors and allows precise dose dependent regulation of our gene of interest. The first critical component of the Tet-On System is the **regulatory protein**, based on a "reverse" Tet repressor (rTetR) which was created by four amino acid changes in TetR (Hinrichs, Kisker et al. 1994; Gossen, Freundlieb et al. 1995). The resulting protein, rTA (reverse tTA), is encoded by the pTet-On regulator plasmid, which also contains a neomycin-resistance gene. The second critical component is the **response plasmid** which expresses a gene of interest under control of the tetracycline-response element, or TRE.

Working of the Tet-On system is diagrammatically represented as follows:

![Diagram of the working of Tet-On expression system](image.png)
The Tet-On expression system consists of two vectors – the **pTet-On vector** and the **pTRE2-Hyg vector**. The pTet-On vector contains the rtTA element and the pTRE2-Hyg vector is for the cloning and expression of the gene of interest.

The vector maps of the two vectors are given below:
Towards this end, the p53 null human non-small cell lung cancer cell line H1299 was transfected with pTet-on vector and the stably expressing clones were selected using G418 selection. p53 cDNA was subcloned in the Not I-Sal I sites of the pTRE2-Hyg vector and the construct thus obtained was transfected into Tet-On stable cells. The double positive Neomycin-Hygromycin resistant clones were selected and screened for p53 expression after induction with 1μg/ml Doxycyclin.

**Statistical analysis** - All results are expressed as means ± S.D. unless otherwise mentioned. Student's t-test was used to calculate the significance accepting p < 0.05 as a level of significance.