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A REVIEW ON INVITRO ANTICANCER EVALUATION STUDIES

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ABSTRACT

Cancer is one of the world's most pressing health care challenge with more than 14 million people diagnosing each year. New insights into cancer and inhibition of its regulators will be investigated for the development of a novel treatment for cancer. The present research aimed to Cytotoxic Evaluation of New chemical Derivatives. Cancer is leading to 13% deaths in the world, as per the World Health Organization (WHO) survey the Oncological patients count may increase to 21.7 million cases and 13 million deaths by 2030.

Keywords: *Cancer, Cytotoxic Evaluation, Oncological Patients.*

INTRODUCTION

Cancer Cells:

• Cancerous cell division goes repeatedly out of control even though they are not needed, the cancer cells crowd out other healthy cells and make them function abnormally.

• They can likewise decimate the working of significant major organs.

• Mutation of a normal gene is the cause to change in deoxyribonucleic acid sequence.

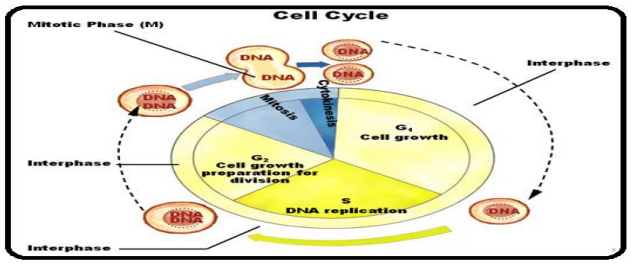

Fig: 1. The process of cancer cell division

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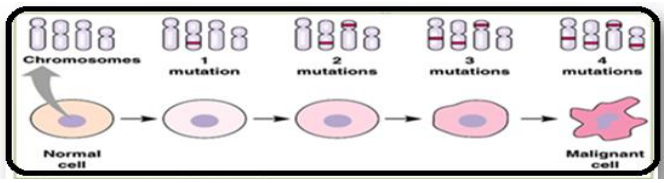


Fig: 2. The Process of the Normal gene after several mutations produces Mutated Genes (Oncogenes) and these mutated genes cause to cancer.

CARCINOGENS:

Cancer-causing is also called carcinogens and these are the following examples.

• Ionizing radiations like UV light, X Rays etc.

• Chemicals like Tar from Cigarettes, Natural or Synthetic Chemicals.

• Virus infections like Cervical Cancer caused are by Papilloma Virus.

Those tumours which do not spread from their site of origin and can crowd out surrounding cells are called Benign Tumours.

Ex: a brain tumour, warts etc.

Those tumours which can spread from the original site and cause secondary Tumours are called Malignant Tumours. These metastatic tumours are interfering with neighbouring cells and block the blood vessels, Gut, glands, lungs etc.

ANGIOGENESIS:

• The cells need huge amount of nutrients to sustain the rapid growth and division of the cells. In order to grow beyond 2mm

tumor needed a steady supply of amino acids, nucleic acids, carbohydrates, oxygen and growth factors for metastasis. Tumors must activate the angiogenesis to develop fresh blood vessels from prior blood vessels in order to get these supplements.

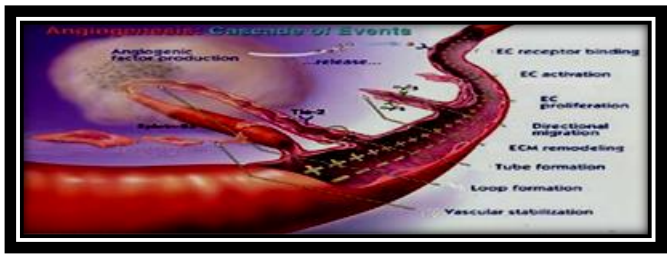


Fig: 3. The angiogenesis activation through EC activation.

History about Angiogenesis:

• In the year of 1971 Judah Folkmana estimated that tumor development would be ended on the off chance that it was denied of a blood supply.

• In the year of 1989 VEGF was identified and isolated by the Dr. Napolene Ferra.

• In the year of 1996 first clinical trials regarding VEG by Dr. Jeffery Isner.

• In the year of 2004 the Bevacizumab was used to treat colorectal cancer and approved by the FDA, sold under the trade name Avastin.

Determination of Cytotoxic concentration of samples against Cancer cell lines using by MTT Assay:

The MTT Cell Viability Assay Kit gives a supportive, quantitative, touchy and trustworthy measure for choosing the amount of active cells in given culture. This homogeneous colorimetric test relies upon the change of a tetrazolium salt, a light yellow substrate to formazan, purple shading. This cell decline reaction incorporates the pyridine nucleotide cofactors NADH/NADPH and catalyzed by living cells. The formazan thing has a low fluid dissolvability and is open as purple valuable stones. Dissolving the formazan with a solubilization supports allows the advantageous appraisal of thing improvement. The intensity of concealing was assessed at 550-620nm, is obviously identifying with the measure of living cells. Reagents in the unit have been updated and purposely made arrangements for affectability, test force and automation.

Highlights:

• It is Safe test and Non-radioactive look at.

• It is Sensitive and accurate (As low as 950 cells can be effectively assessed).

• It is Convenient and high-throughput "mix brings forth measure" type look at.

• No washing and reagent move steps are joined. Z' factors of 0.5 or more are Held.

• It is expediently automated with HTS fluid managing frameworks.

Applications:

• Cell Proliferation: impacts of cytokines, improvement factor, supplements.

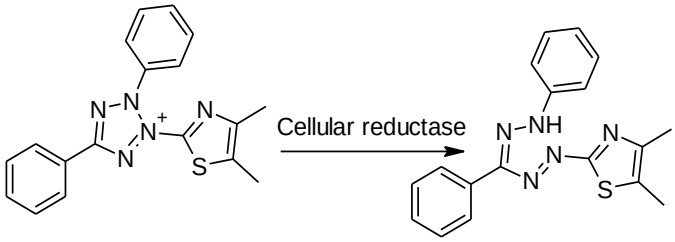
• Cytotoxicity and Apoptosis: assessment of dangerous mixes, opposing to destructive improvement antibodies, hurts, ordinary contamination and so on.

• Drug Discovery: high-throughput screen for dangerous and anticancer drugs.

Background: The examination of cell duplication and cell sensibility requires the specific assessment of the amount of useful cells in a cell culture. Right now for determining cell attainability are principal for improving cell culture conditions, surveying cell improvement factors and enhancements, discovering novel enemy of disease specialists and antagonistic to threatening development drugs, evaluating hazardous effects of biological pollutions and cell mediated lethality and examining altered cell destruction (apoptosis).

Assay Principle:

MTT Cell Proliferation Assay gives a colorimetric configuration to estimating and checking cell expansion. The unit contains adequate reagents for the assessment of 960 measures in 96-well plates or 192 tests in 24-well plates. Cells can be plated and afterward treated with mixes or operators that influence multiplication. Cells are then recognized with the expansion reagent, which is changed over in live cells from the yellow MTT to the purple formazan structure by a cell reductase. An expansion in cell multiplication is joined by an expanded sign, while abatement in cell expansion (and sign) can show the harmful impacts of mixes or imperfect culture conditions. This cell expansion reagent can be utilized to distinguish multiplication in microscopic organisms, yeast, growths and protozoa just as refined mammalian and piscine cells[1].



Chemical structures of Yellow MTT and Purple Formazan product in living cells

Assay Protocol:

1. Plate and refined A549 and A-375 cells (100 µL per well) in a sensible base 96-well culture plates. (1000 to 80000 cells / each well and 50 to 150 µL volume, albeit 100 µL).

2. Included test moieties, control and the cells were incubated for the 48hrs. A volume of 20 µL in phosphate supported saline (PBS) or culture medium is prescribed for the test compounds and controls. The Control Reagent can be advantageously reconstituted with 5 mL PBS.

3. added 15 µL (per 100 µL cell culture) of Reagent per well and incubated for 4hrs at 37°C. The volume of the reagent ought to be balanced relying upon the volume of cell culture.

4. Included 100 µL of the Solubilizer to each well and blended delicately on an orbital shaker for one hour at room temperature. The volume of the Solubilizer ought to be balanced relying upon the volume of cell culture. (In the event that precipitation happens in the Solubilizer, place the bottle in a warm water shower or at 37°C and shake to dissolve the precipitates).

5. Measured the absorbance at OD 570nm for each well on an absorbance plate reader. Maximum absorbance of the formazan color lies somewhere in the range of 560 and 590 nm. Whenever wanted, the OD estimation can be played out the next day. For this situation, it is prescribed to seal the plate to limit vanishing.

Evaluation of Apoptosis by DAPI Fluorescence:

The blue-fluorescent DAPI nucleic acid stain preferentially stains dsDNA; it appears to associate with AT clusters in the minor groove. Binding of DAPI to dsDNA produces a ~20-fold fluorescence enhancement, apparently due to the displacement of water molecules from both DAPI and the minor groove. DAPI also binds RNA, however in a different binding mode one thought to involve AU-selective intercalation. The DAPI/RNA complex exhibits a longer-wavelength fluorescence emission maximum than the DAPI/dsDNA complex (~500 nm versus ~460 nm) and a quantum yield that is only about 20% as high.

DAPI is a popular nuclear counterstain for use in multicolor fluorescent techniques. Its blue fluorescence stands out in vivid contrast to green, yellow or red fluorescent probes of other structures. When used according to our protocols, DAPI stains nuclei specifically, with little or no cytoplasmic labeling. Both DAPI and DAPI dilactate work well in these protocols. The DAPI dilactate form may be somewhat more water soluble.

The counterstaining protocols are compatible with a wide range of cytological labeling techniques direct or indirect antibody-based detection methods, mRNA in situ hybridization, or staining with fluorescent reagents specific for cellular structures. DAPI can also serve to fluorescently label cells for analysis in multicolor flow cytometry experiments. The following protocols can be modified for tissue staining or for staining unfixed cells or tissues.

Principle:

Nuclear staining with DAPI fluorescent dye: DAPI (4',6-diamidino-2-phenylindole) is a fluorescent stain that binds strongly to A-T rich regions in DNA. It is used extensively in fluorescence microscopy. As DAPI can pass through an intact cell membrane, it can be used to stain both live and fixed cells, though it passes through the membrane less efficiently in live cells and therefore the effectiveness of the stain is lower. When bound to double-stranded DNA, DAPI has an absorption maximum at a wavelength of 358 nm (ultraviolet) and its emission maximum is at 461 nm (blue). Therefore, for fluorescence microscopy DAPI is excited with ultraviolet light and is detected through a blue/cyan filter. The emission peak is fairly broad DAPI will also bind to RNA, though it is not as strongly fluorescent. Its emission shifts to around 500 nm when bound to RNA. DAPI's blue emission is convenient for microscopists who wish to use multiple fluorescent stains in a single sample. There is some fluorescence overlap between DAPI and green-fluorescent molecules like fluorescein and green fluorescent protein (GFP) but the effect of this is small. Use of spectral unmixing can account for this effect if extremely precise image analysis is required. Outside of analytical fluorescence light microscopy DAPI is also popular for labelling of cell cultures to detect the DNA of contaminating mycoplasma or virus. The labelled mycoplasma or virus particles in the growth medium fluoresce once stained by DAPI making them easy to detect2.

Fluorescence spectral characteristics:

The excitation maximum for DAPI bound to dsDNA is 358 nm and the emission maximum is 461 nm. DAPI can be excited with a xenon or mercury-arc lamp or with a UV laser. DAPI may be used in flow cytometry systems utilizing UV excitation sources.

Materials and methods:

DAPI dihydrochloride (MW = 350.3)

The following materials were used for the different applications for fluorescence microscopy.

Phosphate-buffered Saline (PBS)

Preparing the DAPI stock solution:

To make a 5 mg/mL DAPI stock solution (14.3 mM for the dihydrochloride or 10.9 mM for the dilactate) dissolve the contents of one vial (10 mg) in 2 mL of deionized water (dH2O) or DMF.

The less water-soluble DAPI dihydrochloride may take some time to completely dissolve in water and sonication may be necessary.

Note: Neither of these DAPI derivatives is very soluble in PBS.

Storage and handling:

For long-term storage the stock solution can be aliquoted and stored at –20°C. For short-term storage the solution can be kept at 2–6°C protected from light. When handled properly DAPI solutions are stable for at least six months.

Caution: DAPI is a known mutagen and should be handled with care. The dye must be disposed of safely and in accordance with applicable local regulations.

Protocol for DAPI fluorescence microscopy:

Sample Preparation

Use the fixation protocol appropriate for your sample. DAPI staining is normally performed after all other staining. Note that

fixation and permeabilization of the sample are not necessary for counterstaining with DAPI.

Counterstaining Protocol

1. Equilibrate the sample briefly with phosphate-Buffered Saline (PBS).

2. Diluted the DAPI stock solution to 300 nM in PBS. Added approximately 300 µL of this dilute DAPI staining solution to the coverslip preparation making certain that the cells are completely covered.

3. Incubated for 1–5 minutes.

4. Rinsed the sample several times in PBS. Drain excess buffer from the coverslip and mount. We recommended using a mounting medium with an antifade reagent such as our SlowFade® Gold antifade reagent or ProLong® Gold antifade reagent.

5. Viewed the sample using a fluorescence microscope with appropriate filters.

Apoptotic Evaluation: (DAPI Assay)

Cell type

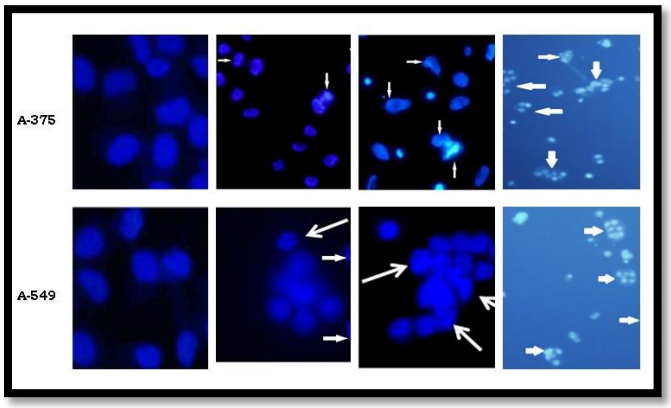


Fig: 4. Evaluation of Apoptosis by DAPI Fluorescence

Apoptotic DNA fragmentation:

4.1 Principle and Mechanism:

DNA fragmentation is a key feature of apoptosis, a type of programmed cell death. Apoptosis is characterized by the activation of endogenous end nucleases with subsequent cleavage of chromatin DNA into internucleosomal fragments of roughly 180 base pairs (bp) and multiples thereof (360, 540 etc.). This effect can be used to detect apoptosis, for example via the DNA laddering assay, the TUNEL assay or the Nicolette assay. The enzyme responsible for apoptotic DNA fragmentation is the Caspase-Activated DNase (CAD). CAD is normally inhibited by another protein, the Inhibitor of Caspase Activated DNase (ICAD). During apoptosis, the apoptotic effector caspase, caspase 3, cleaves ICAD and thus causes CAD to become activated. CAD cleaves DNA at internucleosomal linker sites between nucleosomes, protein-containing structures that occur in chromatin at ~180-bp intervals. This is because the DNA is normally tightly wrapped around histones, the core proteins of the nucleosomes. The linker sites are the only parts of the DNA strand that are exposed and thus accessible to CAD.

Degradation of nuclear DNA into nucleosomal units is one of the hallmarks of apoptotic cell death. It occurs in response to various apoptotic stimuli in a wide variety of cell types. Molecular characterization of this process identified a specific DNase (CAD, caspase-activated DNase) that cleaves chromosomal DNA in a caspase-dependent manner. CAD is synthesized with the help of ICAD (inhibitor of CAD) which works as a specific chaperone for CAD and is found complexed with ICAD in proliferating cells. When cells are induced to undergo apoptosis, caspase 3 cleaves ICAD to dissociate the CAD: ICAD complex allowing CAD to cleave chromosomal DNA. Cells that lack ICAD or that express caspase-resistant mutant ICAD thus do not show DNA fragmentation during apoptosis although they do exhibit some other features of apoptosis and die.

DNA fragmentation is a secondary consequence rather than an integral cause of apoptosis. Endonuclease involved might be similar to DNase I, a potential indication that DNA fragmentation might occur after the release of enzymes from the plasma membrane lysis, an event that would potentially occur only after the final lytic event in the apoptotic sequence. More recently the data have shown that specific proteases residing in the cytoplasm mediate the terminal events of apoptosis including those of nuclear morphology. Even so the detection of DNA fragmentation and the presence of single strand ends of DNA have continued to be used in many studies to detect apoptotic cells, particularly in intact tissues, though necrosis also produces single-strand DNA ends in cell nuclei.

Therefore the interpretation of these insitu assays of DNA fragmentation insitu nick translation (ISNT), TUNEL assay must be carefully assessed together with morphological features of apoptotic cells3.

Even though much work has been performed on the analysis of apoptotic events, little information is available to link the timing of morphological features at the cell surface and in the nucleus to the biochemical degradation of DNA in the same cells. Apoptosis can be initiated by a myriad of different mechanisms in different cell types and the kinetics of these events varies widely from only a few minutes to several days depending on the cell system[4].

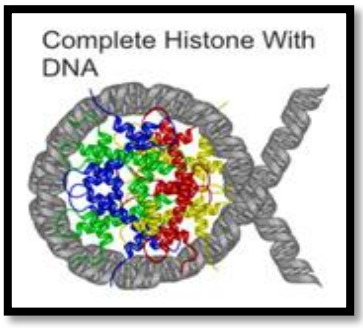


Fig: 5. DNA (grey) wrapped around a histone tetramer (coloured)

Protocol:

In 96 level wells plate, 4x106 A-549 cells (40 wells of 105 for every well) were incubated with the concentration equivalent to 3 times to that of IC50. After incubation gathered every one of the cells from test treated flask in 1.5 ml eppendorf tubes, turn down, re-suspend with 0.5 ml PBS in 1.5 ml eppendorf cylinders and include 55ul of lysis support for 20 min on ice (4oC). Axis the eppendorf tubes in cold at 12000 g for 30 min. Move the examples to new 1.5 ml eppendorf cylinders and afterward extricate the supernatant with 1:1 blend of phenol:chloroform(delicatetumult for 5 min pursued by centrifuge) and accelerate in two equality of cold ethanol and one-tenth identicalness of sodium acetic acid derivation. Turn down, empty and resuspend the accelerates in 30ul of deionized water-RNase arrangement (0.4ml water + 5ul of RNase) and 5ul of stacking cushion for 30 minutes at 37oC. Likewise embed 2ul of Hind III marker (12ul of Stock IV) on the external paths. Run the 1.2% gel at 5V for 5min before expanding to 100V.

DNA Fragmentation:

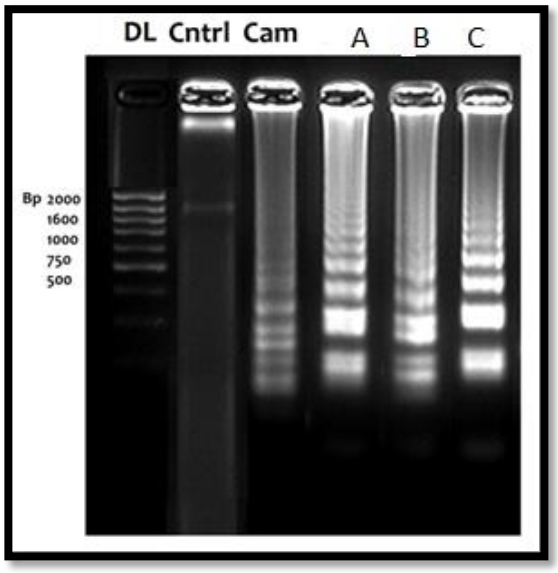


Fig: 6. DNA Fragmentation of A-375

D.L	:	DNA Ladder
Cntrl	:	Control
A8	:	test compound
B2	:	test compound
C6	:	test compound
Ca	:	Camptothecin treated

Caspase Assays:

A distinctive feature of the early stages of apoptosis is the activation of caspase enzymes which participate in the cleavage of protein substrates and in the subsequent disassembly of the cell. We offer a series of caspase assays that allow the simple detection of active caspases in living cells in real-time or in cellular lysates or extracts 5.

Caspases 3/7 Assay:

Caspase 3 Colorimetric Assay Kit provides the reagents needed for a quick and efficient detection of caspase 3 activity in cell lysates and in purified preparations of caspase 3. The Caspase 3 Colorimetric Assay Kit is based on the hydrolysis of acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase3 resulting in the release of the p-nitroaniline(pNA) moiety. p-Nitroaniline is detected at 405 nm (εmM=10.5). The concentration of the pNA released from the substrate is calculated from either the absorbance values at 405 nm or from a calibration curve prepared with pNA standards (pNA standard included with the kit).

Caspase 8:

The Caspase 8 Colorimetric Assay Kit is based on the hydrolysis of the peptide substrate Acetyl-Ile-Glu-Thr-Asp-p-Nitroaniline (Ac-IETD-pNA) by caspase-8 that results in the release of a p-Nitroaniline (p-NA) moiety. The p-NA is read at 405 nm. The concentration of the p-NA released can be calculated from a calibration curve prepared from p-NA standards (included with the kit). The kit detects caspase 8 activity in crude and purified preparations of caspase 8.

Complete kit contains: purified caspase 8 for a positive control caspase 8 inhibitor p-NA fluorescent standard.

Caspase 9:

Activation of ICE-family proteases/caspases initiates apoptosis in mammalian cells. Chemicon's Caspase 9 Colorimetric Activity Assay Kits provide a simple and convenient means for assaying the activity of caspases that recognize the LEHD. The assay is based on spectophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from the labeled substrate LEHD-pNA. The free pNA can be quantified using a spectrophotometer or a microtiter plate reader at 405 nm. Comparison of the absorbance of pNA from an apoptotic sample with an uninduced control allows determination of the fold increase in caspase 9 activity.

Assay Protocol:

A caspase colourimetric protease assay sample kit (Biovision, CA, USA) was used to determine the activity of caspases 3, 7, 8 and 9. Cells (106/ml) were seeded in a 6-well plate and incubated for 24 hrs prior to exposure to FAA in various concentrations. The cells were then transferred into the sterile test tube and lysed using the cell lysis buffer and incubated on ice for 10 min. After centrifugation at 5000 rpm for 2 min the samples (50 µl) of the lysate were aliquoted into a 96-well microplate to which 50 µl of reaction buffer containing 10 Mm DTT was then added to the sample. Substrates for each of the caspases (5 µl) were added to the appropriate wells and the plate was then incubated for 2 hrs. Absorbance at 405 nm was then read in a microplate reader. The absorbance of treated samples was compared with untreated control. Samples (50 µl) of the lysate were aliquoted into a 96-well microplate to which 50 µl of response buffer solution containing 10 Mm DTT was then added to the example. Substrates for every one of the caspases (5 µl) were added to the proper wells and the plate was then incubated for 2 hrs. Absorbance at 405 nm was then read in a micro plate reader. The absorbance of treated samples

was contrasted and untreated control. The selective substrates used were p-nitro aniline.

Western Blot Analysis

Assay Protocol:

Following treatment with 3 molecules (A8, B2 and C6) for 24hrs, A-549 and A-375 cells were harvested, washed with ice-cold PBS and lysed in lysis buffer containing a protease-inhibitor-cocktail tablet (Roche Diagnostics, Basel, Switzerland). The supernatant was obtained by centrifuging at 2000 x g for 15 min. Total protein was extracted and protein concentration was determined using a bicinchoninic acid assay kit (Thermo Fisher Scientific). For immunoblotting 30 µg protein from each sample was subjected to 10% SDS-PAGE and separated proteins were transferred onto a PVDF membrane. The membrane was blocked with 5% skimmed milk at room temperature for 1 h and then incubated with the primary antibodies against caspase-3, caspase-9, Bcl-XL (1:1000; Cell Signaling Technology, Danvers, MA, USA) PARP (1:1000; Abcam, Cambridge, MA, USA) phosphorylated (p)-JNK, p-ERK, p-p38 (1:1000; Santa Cruz Biotechnology, Inc.) and GAPDH (1:2000; Santa Cruz Biotechnology, Inc.) respectively, at 4°C overnight. After washing the membrane was incubated with anti-rabbit or anti-mouse secondary antibody (1:2000; Santa Cruz Biotechnology, Inc.) GAPDH was used as an internal control to monitor equal protein loading and transfer of proteins from the gel to the membranes for these blots were stripped with GAPDH antibody. Signals were detected using an enhanced ECL reagent, and an LAS 4000 imaging system (Fujifilm, Tokyo, Japan). The results shown are representative of three independent experiments[6].

Western Blot Analysis:

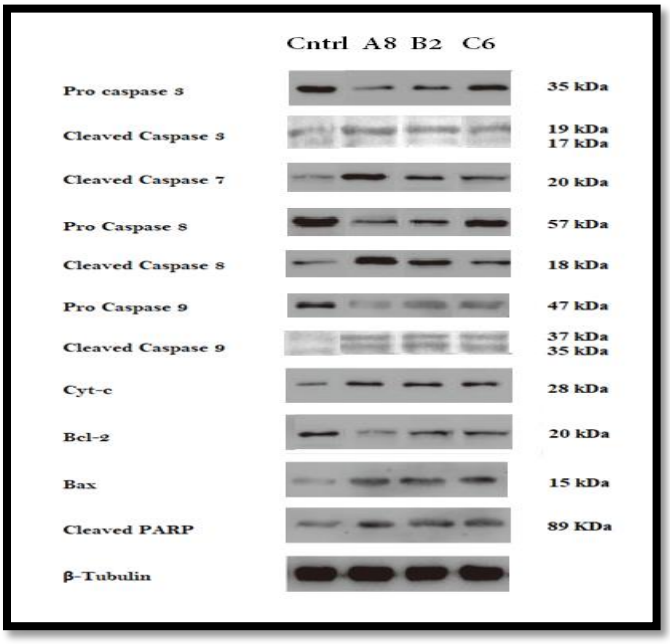


Fig: 7 Western Blot Analysis of cancer cells.

Cell culture:

Various cancer cells were used to screen anticancer activity of newly synthesized compounds. Lung (A-549 and NCI-H460), colon (HCT-15 and HT-29), prostate (DU-145) cancer cells maintained in RPMI-1640 media while normal lung fibroblasts cell line (HFL-1) were maintained in F-12 k medium supplemented with 10% fetal bovine serum (FBS) with 1% antibiotic-antimitotic solution (Sigma). Cells were maintained in 5% CO2 with 98% relative humidity at 37oC in incubator. When 80-90% of confluency is reached, they were sub-cultured using 0.25% trypsin/1 mM EDTA solution for further passage. The compounds in this series were dissolved in DMSO (1 %) to prepare the stock solution of 10 mM. Further dilutions were made accordingly with respective media to obtain the required concentrations[6].

Effect of colonies formation by Clonogenic assay:

The assay mainly tests ability to undergo "unlimited" division by every cell in the population and the hindrance of colony formation by compound 4i was determined in exponentially growing A-549 cells by culturing 100-150 cells per well. Treatment with compound 4i was given at different concentrations (1, 2.5 and 5µM), then colonies formation was observed in 7 days' time period. Then, formed colonies were fixed with glutaraldehyde (6.0% v/v) stained with crystal violet (0.5% w/v). The treatment clearly retained the capacity to produce colonies in lung cancer cell population in a concentration dependent manner. Reductions in the number of colonies were observed as shown in Figure-4.4B. Hence these results denote the importance of compound 4i in inhibiting the potential of colony formation. The total colonies were counted by molecular imaging system Vilber Fusion Fx software and the values were represented as a percent colony forming ability[7].

Flow-cytometry analysis:

Effect of compounds on mitochondrial membrane depolarization

The fluorescent orange-red staining is indicative of the presence of polarized mitochondria. On depolarization, there is usually a reduction in orange-red staining occurs. The mitochondria play a fundamental role in initiating the intrinsic pathway of apoptosis in response to many triggers, as it is the leading target of cellular oxidative stress, which impede the electron transport chain, which generate reactive oxygen species (ROS). Therefore, to test the impact of compounds on mitochondria the ΔΨm was measured. A549 cells treatment with compounds caused significant collapse in the ΔΨm compared to control cells as shown in the Figure-4.5A. The compound 4i prompted the depolarisation of ΔΨm dose-dependently leading to the disruption of electron transport.

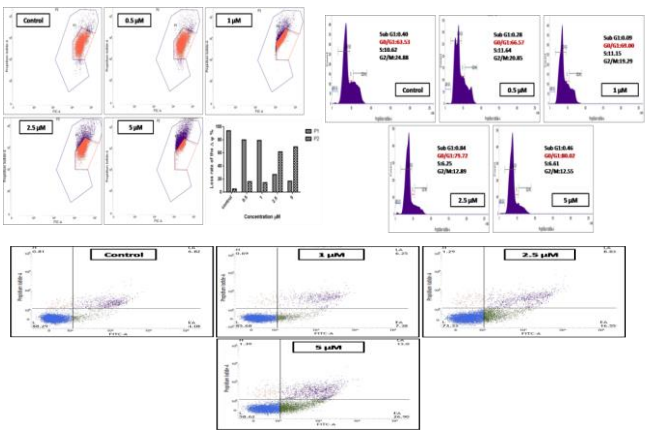


Fig 8: A) Compound reduced ΔΨm in lung cancer cells

Cancer cells were treated with various concentrations of compound for 72hrs. The control represents the cells without compound 4i treatment. P1 indicates formation of J-aggregates in healthy mitochondria whereas, P2 represents depolarized mitochondria in cells due to the presence of J-monomers; Cell cycle analysis of lung cancer cells following Compound treatment. A-549 cells were grown and treated with compound 4i at different concentrations 0.5, 1, 2.5 and 5 μM for 72hrs. The cells were harvested, stained with propidium iodide and DNA content was quantified by flow cytometry. Histogram showing the percentage of cells in the Sub G1, G0/G1, G1, S and G2/M phase of the cell cycle obtained after FACS analysis. 10,000 cells were acquired for each sample.

B) Apoptosis induction by Compound in lung cancer cells using annexin V FITC/PI staining: A-549 cells were cultured and treated with compound ranging from 0.5 to 5μM concentration and incubated for 72hrs and processed for annexin V-FITC/ PI double-staining. Quantification of cells undergoing apoptosis or necrosis was carried out with 10,000 cells from each sample was analyzed by flowcytometry. The percentage of cells positive for Annexin V-FITC and/or Propidium iodide is represented inside the quadrants. Cells in the upper left quadrant (Q1-UL; AV-/PI+): necrotic cells; lower left quadrant (Q2-LL; AV-/PI-): live cells; lower right quadrant (Q3-LR; AV+/PI-): early apoptotic cells and upper right quadrant (Q4- UR; AV+/PI +): late apoptotic cells.

Effect of compound on Cell cycle distribution:

From the in vitro screening results, it was evident that the compound shown remarkable toxicity against lung cancer cell line respectively. Hence, in order to reveal whether this cytotoxicity may be due to the phase arrest, cell cycle determination was performed which was parallel with increased percentages of cell death. Cells were treated with compound at concentrations ranging from 0.5 to 5 μM for 48hrs and then the cells were stained with propidium iodide and analyzed by using flow analyzer. The results from Figure-8.A indicated that the A-549 untreated control cells shown 63.53% cells in G0/G1 phase, whereas compound treatment resulted in significant elevation in G0/G1 population from 63.53% to 80.02% which gradually increased with increase in doses, which implies G0/G1 arrest of the cell cycle The G0/G1 phase arrest was more prominent at 2.5 and 5 μM concentrations[7].

Annexin V Dead cell apoptosis assay:

To determine whether the cytotoxicity by compound 4i treatment could induce early apoptotic or late apoptotic cell death, Annexin V-FITC/PI flow cytometry was performed. As appeared in Figure-4.5B, compound 4i fundamentally exhibited significant early apoptosis, the population of early apoptotic cells increased from 4.08 % (control) to 26.90% (5 +6969+μM) concentration. While at 2.5 and 5μM

Conclusion

By doing vitro cytotoxicity by MTT assay against the panel of human cancer cell lines, we can know the IC50 value of the new chemical entities.

After knowing IC50 value we can proceed further for find out the cytotoxicity by cytotoxic assay like MTT assay, DAPI assay, DNA fragmentation assay, western blot assay and caspase assays.

After finding some promising molecules we can proceed for othe preclinincal studies.

We can find the Cancer cells where they are arresting at the G0/G1 Phase of cell cycle.

We can find the evidence of the depolarization of ΔΨm dose-dependently leading to the disruption of electron transport chain.

Annexin V-FITC/PI flow cytometry exhibited significant early apoptosis.

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