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A Review on Methods of Anticancer Activity Evaluation of Synthetic and Natural Compounds

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Abstract

Cancer is a major public health burden in both developed and developing countries. Anticancer activity is the effect of natural/synthetic/biological/ chemical agents to reverse, suppress or prevent carcinogenic progression. Several synthetic and natural origin compounds are used to cure the disease but they also show toxicity and hence the research is going on to find new compounds and/or to improve upon the existing anticancer agents. Therefore an attempt has been made to review different in vitro and in vivo methods for estimating anticancer properties of synthetic and natural compounds.

Keywords: Tumor; Carcinoma; Assay; MTT Assay; In-Vitro; In-Vivo; Dye.

Introduction

Cancer can occur due to an abnormal uncontrolled growth of cells. Both external (tobacco, chemicals, radiation and infectious organisms) as well as internal factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism) are responsible for the occurrence of cancer [1]. Due to the lack of widespread and early detection methods, cancer is a major worldwide health problem. Due to its increasing incidence on a global scale, it can be associated with poor prognosis of patients and is diagnosed in the later stages of the disease [2]. One of the greatest challenges of mankind is to combat cancer. About 35,000 plant samples from 20 countries were collected by The National Cancer Institute and around 114,000 extracts have been screened for anticancer activity. About 3000 species of plants have been reported with antitumor properties [3,4]. As per WHO and the World Cancer Research Fund, the cancer is occurred and still increasing especially due to diet, environment and carcinogenic virus infections. Drugs which are obtained from natural origin have a share of 60% and 75% respectively, and it is approved by FDA [5-7].

The interference with tubules, the biological function can be clinically proven approach for the treatment of various types of tumours. Taxies and the Inca alkaloids are the compounds that bind to b-tubule will interfere with polymerization of tubule and depolymerization of microtubule and therefore will disrupt the normal cell division and will commit the cell to apoptosis [8]. To control malignancy, Chemoprevention is recognized as an important approach and on the search for desirable chemo preventive agents. In creating new chemo preventive agents, Natural products, particularly dietary substances, have played an important role [9-11].

Over 50% of the drugs in clinical trial were isolated from natural sources or are related to them. According to Crag and Newman as chemotherapeutic agents, several natural products of plant origin have potential value [12]. As a source of drug discovery the areas of cancer and infectious diseases have a leading position in utilization of medicinal plants. Against the panel of cancer cells lines, most common

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screening methods employed are cytotoxicity tests for the anticancer drugs. There are various screening methods available in vitro as well as in vivo for the evaluation of anticancerous activity of the drugs [13, 14]. This review discusses different anticancer screening methods with some details.

Screening Methods For Anticancer Activity

(A.) In Vitro Methods

i. Membrane integrity assay

- Exclusion dyes
- Fluorescent dyes
- LDH leakage

ii. Functional assay

- MTT and XTT assay
- Crystal violet/Acid phosphatase assay
- Alamar blue oxidation reduction assay
- Neutral red assay
- [3H]- thymidin/ BrdU incorporation
- Suforhodamine B Assay
- Protein Kinase Asssay

iii. DNA labeling assay

- Fluorescent conjugates

iv. Morphological assay

- Microscopic observation

v. Reproductive assay

- Colony formation assay

i. Membrane Integrity Assay

In term of plasma membrane integrity, this method is the easiest one for cell death measurement. It is based on the fluorescent dye retaining ability in the cell. Some dyes are: DAPI, propidium iodide, 7ADD and ToPro-3.

1. Dye Exclusion Assay

Viability assays calculate the % of cell suspension that is viable. This is done by a dye exclusion stain. In this assay, cells are able to exclude the dye which has intact membrane whereas the coloring agent was taken up in the cells without an intact membrane [3].The most commonly used assay is tryphan blue dye exclusion assay which is utilized for the cell viability. The cells are washed with HBSS (Hank's Buffered Salt Solution) and then centrifuged for about 10-15 min at about 10,000 rpm [15]. The procedure is repeated thrice after that. In known quantity of HBSS, the cells are suspended and the cell count was adjusted to 2 x 10^6 cells /ml. Into Eppendorf tubes (0.1 ml containing 2 lakhs cells), the cells are suspended. The cells are incubated at 37 °C for 3 h and are exposed to drug dilutions. Equal quality of the drug treated cells were mixed with tryphan blue (0.4 %) (Dye exclusion test) and left for 1 min. In a haemocytomete, it is then loaded. Within 2 min, viable and non-viable counts are recorded. Non-viable cells take up colour while viable cells do not take up the colour. However, live cells also generate and take up color if kept it for a longer time. Using the following formula, at last, the percentage of growth inhibition is calculated [16].

Growth inhibition (%) = 100-(Total cells-Dead cells)/Total cells ×100

Automated Trypan Blue Method:

For the measurement of cell viability, the trypan blue dye exclusion assay is usually used and conventional method. It gives the direct measure of cell viability and depends on the modification in membrane integrity as estimated by the uptake of dye by dead cells. The assay gives precise cell viability and cell-density estimation. The sample suspension cell densities and the difference between viable and dead cells functions were executed automatically. The modern pattern recognition methods not only permit cell identification but also differentiate between viable cells, dead cells and debris [15,17].

2. LDH (Lactic Dehydrogenate) Assay

In the culture medium, Lactic dehydrogenate activity is spectrophotometrically measured at 340nm in the cellular lysates during the pyruvate lactate transformation by analyzing NADH reduction. Cells are lysed with 50mM. By sonication and centrifuged at 13,000 X g for 15 minutes Tris- HCl buffer, pH7.4 + 20mM. EDTA + 0.5% Sodium Dodecyl Sulfate (SDS) are disrupted. For the enzymatic analysis, the assay mixture (1ml final volume) consists of 33µl of the sample in 48 mM PBS, pH 7.5 + 1 mM pyruvate and 0.2 mM NADH. As a percentage of the total amount, that is the sum of the enzymatic activity present in the cellular lysate and that in the culture medium the percentage of LDH released is calculated [18,19].

Pitfalls

- The release of LDH activity can be related to the total No. of dead & lysed cells.

- The stability of LDH can vary considerably, ranging from the loss of a few percent per day to a half-life of 12h depending upon the cell type.
- Assumed that the release of LDH occurs rapidly after damage to the cell membrane. This assumption is not necessarily correct.
- The release of LDH can be complete in cells which are considered dead by dye exclusion methods.
- Complete release may only occur upon cell lysis.

Modified Released LDH Release Assay

In the modified LDH assay the activity is estimated by an enzymatic test. Firstly reduction of NAD+ to NADH/H+ by the LDH catalysed and result into pyruvate from lactate. Then catalyst diaphorose transfers H/H+ from NADH/H+ to the tetrazolium salt 2- (4-iodophenyl)-3-(4-nitrophenyl)-5-phnyltetrazolium chloride (INT), as a result, it reduced to a red formazan which can be quantitatively measured at 490nm [20].

3. Fluorescent Dyes

Ethidium bromide (EtBr) and propidium iodide (PI) is used. PI is impermeable to the intact plasma membrane and binds to nucleic acids upon membrane damage PI is ideally suitable for the rapid evaluation of the permeability properties of large numbers of cells while sustaining superior accuracy. On intercalates with DNA or RNA it results red in colour.

Fluorescein diacetate (FDA) is a nonpolar ester which passes through plasma membranes and is hydrolyzed by intracellular esterases to produce free fluorescein, the polar fluorescein is confined within cells which have an intact plasma membrane and can be observed under appropriate excitation conditions. Fluorescent fluorescein dye represents an undamaged cell and weakly fluoresce represent damaged cell [21].

ii. Functional Assay

It evaluates the viability by examining the metabolic components that are necessary for cell growth, on the premise that cellular damage will certainly result in the loss of capability to sustain and provide energy for metabolic function and growth

1. Colorometric Assay

It gives a rapid and accurate measurement of viable cell number. It is miniaturized into 96 well plates and through microplate reader used for measurement. It allows rapid analysis of many samples and hence cost effective with low plastics costs. On an ELISA plate reader, the assay is read at 570 nm by the standard wavelength of 620nm [3, 22].

2. MTT Assay

MTT Assay is the most consistent method for the evaluation of anticancer activity. This assay is mainly employed for quantitative estimation. This assay is colorimetric based method and helps to determine the number of feasible cells that is based on mitochondrial dehydrogenase activity measurement. We used bromide salt of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium at 37 C which is bioreduced with the help of dehydrogenase within living cells and hence reduces the colourless tetrazolium salt into a coloured aqueous soluble formazan dye. Furthermore, for dissolving the insoluble formazan dye, solubilising agents are added [23-25].

Procedure for MTT Assay: Firstly, cells are coated on to 96 well plates with a cell density of 2×10^5mL per well in 100µL having RPMI 1640 and allocated to nurture in CO_2 incubator for 24h at 37 C (5% CO_2). This medium is isolated and substituted by a clean medium having distinct sample concentrations for about 48h. Cells are incubated for 24-48h at 37 C (5% CO_2). Afterwards, 20µL MTT ([3-(4, 5-dimethyl-thiazol-yl)-2, 5-diphenyltetrazolium bromide]) from stock solution i.e. 5mg/mL is added to every well and incubated for 5h. The medium is eliminated and for the dissolution of MTT product, 200µL DMSO added to every well. Subsequently, a plate is dazed for 5min at 150rpm. Therefore, optical density is calculated at 560nm. Untreated cells or we can say basal cells are utilized for the purpose of viability control (100%) and lastly results are articulated as % viability (log) relative to the control [26].

Pros of MTT Assay [24, 27]

- Cost-effective
- Rapid, flexible and reproducible.
- Consistent
- Convenient
- Sensitive and toxicity prediction is very early

Cons of MTT Assay [26, 28]

- Production of the MTT product is dependent on the MTT concentration in the medium. The kinetics and degree of saturation are dependent on cell type.

- Assay is less effective in the absence of cell proliferation.
- MTT cannot distinguish between cytostatic and cytocidal effect.
- Individual cell numbers are not quantitated and results are expressed as a percentage of control absorbance.
- Test is less effective if cells have been cultured in the same media that has supported growth for a few days, which leads to underestimation of control and untreated samples.

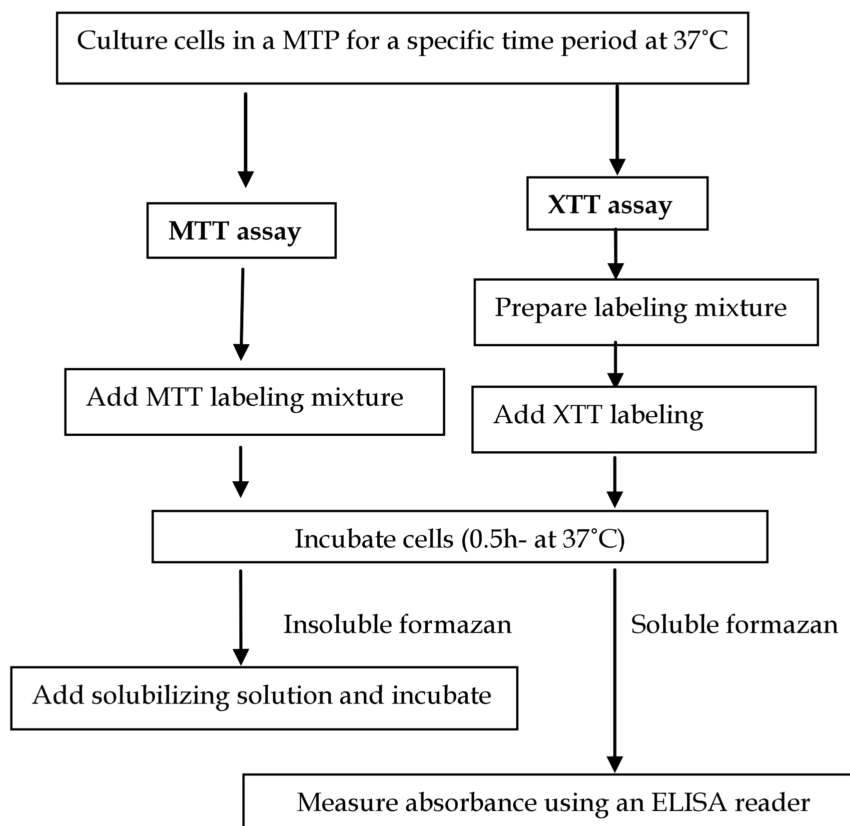
3. XTT Assay

XTT Assay is intended for the spectrophotometric quantification of cell growth and viability that devoid of radioactive isotopes. Yellow tetrazolium salt, XTT reduces to form Orange formazan dye with the help of metabolically active cells is the basis of XTT Assay. The internal salt i.e. (2,3bis[2-Methoxy-4-nitro-5-sulfophenyl]-2*H* tetrazolium-5-carboxyanilide is used for the evaluation of proliferation response [29, 30].

Procedure for XTT Assay

Cells are allowed to grow in growth medium and 10% FBS in 96-well plate's upto 70-80% as they are flowing together. Then, a suitable drug sample is added for about 24h. Now, by pouring the labelling mixture solution to every well and then incubation is done for cells for 4h at 37 C. With the help of screening multiwall spectrophotometer Enzyme Linked Immunosorbant Assay (ELISA) reader, optical density at 450nm is compared with that of control wells, having a reference wavelength of 650nm [31].

MTT V/S XTT [26, 30, 32]



4. Crystal Violet Assay

Its principle is based on:

Dye Elution: Cell up-taken dye was measured colorimetric method after acetic acid dye elution.

Procedure: After removal of medium, rinse 96 well plates with 100 µl well of PBS and stain with 100 µl 0.25% (g/10ml) aqueous crystal violet for 10 min. Further, rinse plats four times in tap water and dry the outsides of the plates with paper to help avoid

water stains. Then dry the plates at 37⁰C. When dry, add 100 µl per well of 33% glacial acetic acid (33 ml/ 100ml) and mix the contents of each well before reading at 570 nm.

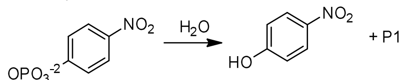
Nuclei Counting: Incubation of cell samples in a mixture of citric acid and crystal violet causes cells to lyse and the released nuclei to stain purple

Procedure: Allow microcarriers from a culture sample (1ml) to settle to the bottom of a centrifuge tube and removed clear supernatant by aspiration. Further, add 1ml of crystal violet reagent and incubate at 37⁰C at least for 1 h. Introduce a sample into the hemocytometer chamber and count the purple-stained nuclei as for whole cells [33].

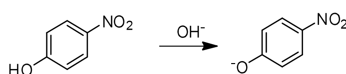
5. Acid Phosphatase Assay

The action of this enzyme in many of tissue is to cleave a waste product called pyrophosphate and effectively convert it to a useable phosphate. P-nitrophenyl phosphate will be the substrate and nitrophenol is the product of this reaction. Nitrophenol is colorless but when the pH of the reaction solution is alkaline, it appears yellow. The pH of the reaction solution will be changed by the addition of NaOH [34].

Phosphatase catalyzed reaction



Color reaction: add NaOH



Procedure: At end of cell growth period, remove medium and rinse wells in 100 µl PBS. Add 100 µl substrate-containing buffer to each well. Further incubate for 2 h in incubator and read plates at 405 nm. Either reincubate for a further time if increased sensitivity is required, or stop with the addition of 50 µl well of 1 M NaOH to cause an electrophilic shift in the p-nitrophenol chromophore and thus develop the yellow color, giving greatly increased sensitivity [35].

6. Alamar Blue Oxidation Reduction Assay

It is based on the principle that in the presence of cellular metabolism the color of Alamar Blue (ALB) changes from a fully oxidized, non fluorescent blue to a fully reduced, fluorescent red. ALB will be reduced by a variety of enzymes and small molecules, including the cytochrome system, FMN, FAD, NAD, and NADP. The ALB assay is faster, simpler, and less artefact prone than the MTT assay.

Procedure: At the end of an experimental incubation period, per 25 vol of growth medium in each well add 1 bol of ALB stock solution. Incubate plates at 37⁰C for 3 hrs to allow metabolic dye reduction. Equilibrate plates to room temperature for 30 min in the dark. Measure the relative fluorescence at 530-560 nm excitation and 590 nm emission wavelength. Fluorescence is temperature sensitive either equilibrate plates in a warm room at the culture incubation temperature. For better sensitivity measure the fluorescence in bottom reading rather than top reading mode. The ratio of test to control fluorescence values at 590 nm measures the effect of a treatment on cell growth or metabolism [36].

7. Neutral Red Assay

It is based on the principle that the incorporation of NR into the lysosomes of viable cells after their incubation with test agents. It is used widely as it is Simple, faster, economic, and sensitive.

Procedure: Resuspend cells of actively growing the culture and count cells and accurately allocate appropriate number suspended in the medium. Seed 0.2 ml containing desired number of cells to each well of 96 well plate and incubate at 37⁰C for 24 h or longer. Removed the medium and add fresh medium containing graded dilutions of the test agent. Incubate for the desired length of time. Examine at least 4-8 wells per concentration of test agent and keep serum concentration as low as possible during this step. After incubation for the desired time interval, remove medium with a test agent and incubate cells with fresh medium containing 40 µg/ml NR dye. Continue incubation for 3h to allow for incorporation of vital dye into survival cells. Remove medium by inverting the plate and rapid rinse with a mixture of 1% $CaCl_2$ / 0.5% formaldehyde. Extract dye into supernatant with 0.2 ml of the solution of 1% acetic acid/50% ethanol. After10 min at room temperature and rapid agitation for a few seconds on a micrometre plate shaker, scan the plate with an ELISA-type spectrophotometer equipped 540 nm filter [37, 38].

8. [^{3}H] Thymidine and BrdU Incorporation Assay

The rate of DNA synthesis is a reflection of proliferation under many conditions. To measure the proliferative rates by [3H]-thymidine uptake, cells are cultured in microtitre wells, thymidine is added,

and the uptake by DNA is measured, after lysing and washing on, by scintillation counting. Bromodeoxyuridine (BrdU) can be incorporated instead of [3H]-thymidine and the incorporation can be assayed with antibodies to BrdU in a nonradioactive assay [31, 33].

Labeling Index with [^{3}H]-Thymidine

Set up the culture at 2x104 cells/ml~ 5x104 cells/ml in 24 well plates containing cover-slips. Grow to the desired cell density. Add [3H]-thymidine to the medium. 100KBq/ml(~5μCi/ml)and incubate for the cultures 30 min. Remove the labeled medium, and discard it into a designed container for radioactive waste. Wash the cover-slips three times with PBSA. Add 1:1 PBSA: acetic methanol, 1ml per well, and remove it immediately. Add 1ml of acetic methanol at 4 to each well, and leave the cultures for 10min and remove the cover-slips, and dry them with a fan. Further mount the cover-slip on a microscope slide with the cells uppermost and leave the mountant to dry overnight [39].

DNA Synthesis by [^{3}H]-Thymidine

Grow the culture to the desired density. [^{3}H]-TdR, 40 KBq /ml(~1.0μCi), 2 MBq/mol(~50 μCi/mol) in HBSS. Incubate the cell for 1-24 h and remove the radioactive medium carefully. Wash the cell carefully with 2 ml of HBSS, PBSA, and 2 ml ice-cold 0.6 M TCA for 10 min. Wash the cell with TCA twice 5 min each time. 0.5 ml of 2 M perchloric acid, a hot plate at 60ÚC for 30min and allow the solution to cool. Finally, add 0.5ml SLS in NaOH incubate the solution at 37 ^{0}C for 30 min or overnight at room temperature. Collect the solubilised pellet and determine the radioactivity [40].

9. Suforhodamine B Assay

A bright pink- aminoxanthene dye is Sulforhodamine B Assay. In mild acidic conditions, it binds to basic amino acids and then dissociates under basic conditions. In 96-well flat bottom plates at 5000-10000 cell/well, cells were placed. The samples are added to the triplicate wells in serial 3-fold dilutions or differences in the growth rates of the various cell lines. To the wells, Cells are allowed to adhere [41]. To the control wells; water is added at a dilution of 1:10 in the medium. At 37C, 0.5% CO_2 for 3 days, these plates is incubated and using sulforhodamine B (SRB), assayed for growth inhibition. By adding cold 50% trichloroacetic acid to a final concentration of 10%, the cells are fixed. The cells are washed five times with de-ionized water, after 1hr incubation is done at 4^{0}C. For 15-30 min, the cells are then stained with 0.4% SRB (sigma) dissolved in 1% acetic acid to remove the unbound stain. Plates are then air dried at room temperature and after solubilization of bound dye with 10nm base, the plates are then analyzed on a micro plate reader (molecular devices) at 595nm [42]

10. Protein Kinase Assay

Testing of 14 samples can be done at the same plate with the help of this assay. Every sample operated at 100μg/L two times and sometimes three times also. By this, we can get the %inhibition reading six point data. On the other hand, at two distinct concentrations i.e. 100μg/mL and 1μg/mL 14 samples can be operated in triplicate. Similarly, by this we can get the % inhibition reading three data points [43].

Procedure: For the preparation of final concentration of 100μg/mL sample in 20%DMSO, take 1mg/mL stock solution of test compound 5μL in wells A/F1-12 of 96-well plate. Now, add 5μL of 20% DMSO solution to the positive (G1-6) as well as negative (H1-6) control wells.5 μL internal Standard is added to wells (G/H7-12). For making the concentration of 125 units/mL, melted the enzyme contained in a tube then make up the volume with kinase reaction buffer upto 2mL. By this, we obtained the final concentration having 50units/mL (2.50 units/well). Take 20μL reaction mixture that contains a 2.5×optimal concentration of kinase in kinase reaction buffer in the sixth positive control wells (G1-6) supplied with 100% luminescence and still 1880μL of this reaction mixture remained. To the above remaining 1880μL of kinase mixture, 20μL of kemptide (PKA peptide substrate 10mg/mL) is added. By this, we obtained 200μg of kinase substrate in 1900mL for 140μM substrate/enzyme solution in buffer, and it will be satisfactory for 1×96-well plate, and hence will give 56μM in each well per 50μL reaction as final concentration. 20μL of above reaction mixture that contains 2.5×optimal concentration of kinase and kinase substrate in 1×kinase reaction buffer to the wells provided with 0%luminescence. 25μL ATP solution (20μM, stock 3 ATP solution) is added to all wells. By this, we obtained 10μM in each well per 50μL of reaction as final concentration. Shake the plate with the help of plate shaker and incubate at room temperature for favourable amount of time. Kinase-Glo® reagent preparation: First stored kinase buffer in the freezer and then melted at room temperature. Then, to the Kinase-Glo® buffer, Kinase-Glo® substrate is added and from this mixture 50μL

is added to each well. The plates are mixed and incubate at room temperature for about 10minutes. Note down the luminescence and this tells that if luminescence is increasing, the percentage inhibition to the control is also increases and vice versa [44-47].

iii. DNA Labeling Assay (by fluorescent probes assay)

Detection of DNA synthesis in proliferating cells relies on the incorporation of labeled DNA precursors into cellular. DNA during the S phase of the cell cycle. The labeled DNA precursors, usually pyrimidine deoxynucleosides, are added to cells during replication, and their incorporation into genomic DNA is quantified or visualized after incubation and sample staining.

The same labeled deoxynucleosides can be injected into experimental animals to assay cellular proliferation in specific organs and tissues [32,48].

iv. Morphological Assay

Large-scale, morphological changes that occur at the cell surface, or in the cytoskeleton, can be followed and related to cell viability. Damage can be identified by large decreases in volume secondary to losses in protein and intracellular ions of due to altered permeability to sodium or potassium. Necrotic cells: nuclear swelling, chromatin flocculation, loss of nuclear basophilia. Apoptotic cells: cell shrinkage, nuclear condensation, nuclear fragmentation [49].

v. Reproductive Assay

The cell with the capacity for sustained proliferation is called as clonogenic cell. It has undergone a minimum of 5-6 doublings to give rise to colonies containing at least 50 cells. The ability to form colonies is used as a measure of reproductive integrity. It is often referred to as plating efficiency (PE).

Colony forming efficiency = Number of colonies formed/Number of cell plates * 100

Procedure: Trypsinize monolayer cultures or use cell suspension cultures and determine the viable cell count. Dilute cells in growth medium to 1000, 2000 and 5000 cells/10ml. Inoculate nine replicate Petri dishes with 4 ml growth medium plus 1ml cell suspension. Place plates in a humidified 5% CO2 plus air incubator are normal growth temperature and rock shelf or tray gently to and fro three times. The plates must not be moved now until colonies are stained. Stain and count three replicate per cell density at 1,2 and 3 weeks (murine lines) or 2 , 3 and 4 weeks (human lined). Calculate the optimum cell densities for seeding and duration of incubation [50, 51].

Pro of in-Vitro Methods: [52-54]

1. In-vitro methods are less expensive and cost effective.
2. These methods are less time consuming hence
3. In this, cell models are much simpler thereby allowing easy interpretation.
4. The strength of in-vitro methods is that, these methods were designed to check, which type of drug does cancer patients need.
5. They make our preclinical tools better
6. With less quantity, more number of compounds can be processed
7. Reduces usage of animals

Cons of in-Vitro Methods: [55, 56]

1. These methods required long term exposure and maintenance.
2. Oxygen supply is less in these methods as the dissolved oxygen is consumed in fresh culture medium during the first few hours resulting in anaerobic respiration.
3. In-vivo conditions are not represented faithfully by the in-vitro conditions, so the drugs which work in-vitro may not work as in-vivo and vice-versa.
4. To assess the anticancer activity, these tests would not be suitable for the drug candidates that are not able to kill cancerous cells via a direct mechanism of cytotoxicity, like the compounds that will stimulate the immune system
5. It is almost impossible to establish the pharmacokinetics data.

In Vitro Testing

- *Cell Line Panel*

The preliminary panel included 60 distinct human tumor cell lines arise from seven types of cancer that include melanoma, renal, ovarian, brain, lung, colon, leukemia. Selection of cell lines is generally done on the basis of least requirement of growth medium, highly drug sensitivity and has minimum quality assurance criteria. The temporary panel for cell lines

doesn't require any full detail regarding specialized characterization such as ultra structure, immunocytochemistry and histopathology [57].

- *Standard Operating Procedures for Sample preparation for NCI60 screen*

  NCI60 testing is carried out in two parts. Firstly, a single dose of 15µg/mL or 10^{-5} molar is tested on 60 cell lines. If the results are obtained according to the selection criteria, only then the compounds are again tested on 60 cell lines in 5×10 fold dilutions having dose 150µg/mL or 10^{-4} molar. Compounds accepted for NCI60 testing are prepared for both 1-dose and 5-dose testing at the same time [58].

(B.) In-Vivo Models

1. Induction of Ehrlich Ascites Carcinoma

Using Ehrlich Ascites Carcinoma (EAC) tumor model in mice, the antitumor activity of the test compounds is determined. For the in-vivo study, the ascetic carcinoma bearing mice (donor) are used, after 15 days of tumor transplantation. Based on the groups of 12 animals each, animals are divided. (a) Normal mice (b) Tumor-bearing mice (c) Tumor bearing mice treated with the standard drug (d) Tumor bearing mice groups treated with test drug. Using an 18-gauge needle with a sterile syringe, ascetic fluid is drawn out. For testing the microbial contamination, a small amount is taken out. By tryphan blue exclusion test, the Tumor viability is determined and using haemocytometer cells are counted. In normal saline to get a concentration of 106 cells/ml of tumor cell suspension, the ascetic fluid is suitably diluted and to obtain ascetic tumor this is injected intra peritoneal. On the day of tumor inoculation, the mice are weighed and then once in three days after that. On the tenth day of tumor inoculation, treatment is started. On tenth day, Standard (one dose) is injected intraperitoneally. From the tenth day, drug is administered for 5 days intra peritoneal. Followed by 18h, after the administration of last dose, six mice are sacrificed from each group for studying the hematological parameters and antitumor activity. The remaining animals are kept from each group to check the Mean Survival Time (MST) of the tumor bearing hosts [2, 59, 60]. By observation of following parameters, antitumor effects of the drug are assessed:

i. Increase in Percentage weight as compared t day-0 weight.

ii. Increase in lifespan [%ILS] and Median survival time.

i. Hematological parameters.

2. Tumor Growth Delay Assay

Tumor growth delay assay, is a functional assay which is widely accepted, standardized, robust and is needed in many experiments for in vivo study of anticancer agents. The tumor volume is measured frequently for determining the tumor growth delay, it is also used for the calculation of the individual tumor, and the starting volume is recorded. Then by using suitable formulae, growth delay has been calculated [61]

Calculated growth delay = Tumor growth time of treated tumors – Tumor growth time of control tumors

The endpoint of the tumor growth delay assay is a time to reach a volume but not a volume at a given time point is an important point. The tumor growth delay also increases as the end point sizes increase for many drugs. As because of accumulation of drugs, the tumor growth delay increases as we give multiple drugs. The dead cells will give the more tumor volume, if the tumor cells were killed is the main mechanism of anticancer agents. The rapid regrowth of surviving tumor cells will get decreased in slow shrinking tumors. The anticancer effect of the drug will be equal to the size of end point [62]. With multiple administrations, Antiproliferative agents with time will results in increased tumor growth delay. After some drug administrations, the effect on tumor growth rate is detectable in fast growing tumors and, thereby, at later time points. Larger endpoint sizes seem preferable, for antiproliferative agents. Before the experiment, as we don't know the mechanism of new drugs tumor growth delay with multiple endpoints can be reported. By several mechanisms, anticancer drugs may prolong tumor growth. Tumor cells can be affected directly or indirectly by anticancerous agents, e.g. by inhibiting angiogenesis, stoma cells can be targeted. By inhibition of tumor cell production, increased tumor cell death, or improved clearance of dead and doomed cells, the anticancer agents can reduce the tumor growth rate both directly and indirectly. About in-vitro and in-vivo experiments, this method requires more details. For designing more complex in-vivo experiments and clinical trials, it is tested whether a new drug affects proliferation or survival [63]

3. Tumor Control Assay

On the therapeutic effect on clonogenic cells, tumor control assay depends. For testing of potentially curative settings, the most applicable experimental

end point is the most permanent tumor control. As the therapy of the tumor is completed in practical terms, the reoccurrence of tumor is recorded after that. To detect virtually all recurrences, this requires so much time. After treatment tumors are excised in this, cells are seeded into a flask or multiwall plates and a single cell suspension is prepared. After incubation, the fraction of surviving clonogens can be determined and then compared without treatment with control tumors [64-66].

4. Genetically Engineered Cancer Model

From the last 20 years, GEM models have been particularly used for the oncogenes and tumor suppressor genes in case of carcinogenesis. GEM Models summarizes the genetic changes appeared in human tumors, so they used for the novel anticancer therapies and hence by this, successful compounds are obtained [32, 67].

	Genetically Engineered Model	
Transgenic Mice	**Engineered grafts in mice**	**Knock-out Knock-in models**
Inducible/Reversible tumor models	Reversible expression of target genes (tetO/tetTr)	Humanized mice

5. Chemical Carcinogen Model

Initially, DMBA (dimethylbenz [a] anthracene) is used as initiator and then TPA (12-O-tetradecanoyl-phorbol-13- acetate) used as the promoter. In mice single dose – 2.5 μg of DMBA f/b 5 to 10 μg of TPA in 0.2 ml of acetone twice weekly was given. Papilloma begins to appear after 8 to 10 wks. Tumor incidence & multiplicity of treatment group is compared with DMBA control group [68].

6. Viral Infection Model

Mouse Mammary Tumor Virus (MMTV) was the first mouse virus, isolated at Jackson labs as the "nonchromosomal factor" that caused mammary tumors in the C3H strain of mice. Some viruses cause cancer via random integration in certain cells. Some viruses carry cellular oncogenes [69]:

- Abelson murine leukemia virus – Abl
- Moloneymurine sarcoma virus – Raf

Engineered viruses now used routinely in the laboratory to induce cancer

7. Transplantation Model

Transplantation model is typically cheap, rapid and feasible. Tumor cells or tissues (mouse or human) transplanted into a host mouse [70].

- Ectopic – Implanted into a different organ than the original (typically subcutaneous or kidney capsule)
- Orthotopic – Implanted into the analogous organ of the original tumor.

8. Hollow Fiber Assay

In this assay, tubes/fibres having 2 cm length are packed with tumor cell lines. Then, these fibres embedded into mice at two sites (subcutaneous and intraperitoneal).

After 4-6 days, fibres are eradicated from animals and processed in vitro for computing the tumor cell growth. We can also analyse drugs administered by means of distinct routes are bioavailable or not and also whether these cells attained tumor sites or not and this is established by means of Net cell kill. By means of HFA, the pharmacological capability of compounds is determined thereby it achieved 2 physiological compartments within the nude mouse and shows a practical means of calculated viable tumor cell mass.

The in vivo HFA was analyzed at NCI for the improvement that it assists in the bridge the gap between in vitro cell-based assays and human xenograft models in immunodeficient mice. Its main objective is to build an assay which can best calculate those compounds which are effective in the 60-cell line panel and also it has to show the highest activity in xenograft models. High and Low dose levels are established by means of MTD, for the standard HFA [32, 58].

High Dose= [MTD*1.5]/4

Low Dose= 0.67*high dose

The procedure can be illustrated as:

A. Human tumour cells →PVDF fibre →Heat seal the ends and cell grow in hollow fibres → Subcutaneous implant/ I.P implants in mice (after 24-48 h) → Drug or vehicle I.P injection (3-4 days) → Count cells in hollow fibres in drug treated compared with vehicle treated mice.

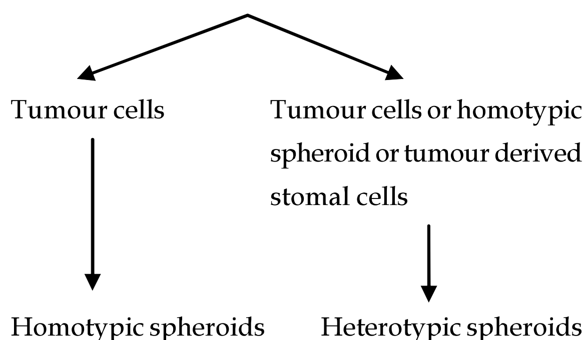
B. Tumour cell → Enzymatic or mechanical dissociation → Single cell suspension

↓

Seeding Conditions

↓

- Mechanical methods that prevent attachment eg: roller flask, spinner flask
- Mechanical methods to promote aggregation eg: centrifugal compression into a cell pellet
- Coating tissue culture surface with non-adhesive surfaces eg: agarose, poly lysine
- Hanging drop method: as for embryoid bodies.



Pros of in-Vivo Methods: [1, 22, 55]

- The homeostasis is maintained throughout as the living organism maintained the composition of body fluids.
- These models cover ADME (Adsorption, Distribution, Metabolism and Excretion).
- These models require less expensive equipment.

Cons of in-Vivo Methods: [24, 40, 70]

- Keeping the animals under quality controlled conditions and also the production is expensive.
- Lots of differences are found between species or even between strains of species.

Assays Based on Cell Screening

1. Conventional Cellular Screens

It utilizes permanent human tumor cell lines, their everlasting nature and therefore convenient reproducible growth behavior accomplished them into appropriate test systems. Sensitivity and cell number are thus the two factors on which the detection method is based. Cell growth is verified by several procedures and is used in screening laboratories.

MTT (methylthiazodiphenyl tetrazolium) assay was the first largely used growth inhibition assay and was given by Mosmann and the screening staff of NCI. This yellow dye is reduced to purple formazan via mitochondria and is analyzed with the help of ultraviolet/visible light scanners. At present, Sulforhodamine B (SRB) assay is the NCI 60-cell-line screen used, and this dye stains mainly proteins. Luminescence or Fluorescence detection systems are mainly used by industrial-scale cellular screens. For computing cell growth, monolayer cultures or one-dimensional method is used. [32,71-75].

2. Tailored Cellular Screens

Phillips et al reported the in vitro models of a tumor environment by means of multicellular spheroids or post confluent multi cell layers for selection of bioreductive agents. Agents that have need of bioactivation by the microenvironment like mitomycin C or EO9 were differentially chemoselective in plateau-phase multilayered cells in contrast to exponentially increasing monolayers. By this process, we found the two novel structures RSU 1069 and SR4233. Since last many years, the enzyme named telomerase has been used as the potential anticancer target [76-78].

3. Biochemical Screening Assays

These assays are used for assessing a large number of compounds as compared to the cellular assays. These are mainly utilized in the pharmaceutical institutions and industries for investigation of novel agents [32, 79].

Additional Technologies used are as:

- *Radiometric assays* by the use of scintillant-coated beads in microtiter plates, scintillation proximity counting is utilized [80].
- *Time-resolved fluorescence* extremely fluorescing rare-earth metal-ligands chelates (samarium, terbium, europium) is the basis of this [81].
- Fluorescence polarization and luminescence detection comprises chemiluminescence or electro chemiluminescence [82,83].

4. Target and Cell Screens Combination

Screening procedures based on both cell and target have advantages and disadvantages, as cell

based methodology will skip agents that have specific action like telomerase inhibitors not have cytotoxic potency in short-range assays. Although, they are known to analyze detection of novel modes of action and illumination of their interaction in specific pathways [84]. NCI 60-cell-line screen is the spicamycin analogue KRN5500 is the example of this. Compounds recognized in cellular screens are their confirmed cell permeable properties, which may be absent in cell-free systems. Additionally, ligand interactions may be extra suitable in the biological environment [85]. The most favorable method in novel cancer drug discovery is the combination of rational biochemical and more empirical cellular screening systems and this is useful to the recognition of novel CDK inhibitory agent E224 (5-methyl indirubin). Generally, for 23 standard anticancer agents, several toxicity data and x-ray behavior were monitored represent the DNA type and cell cycle check point mutations in individual tumors possibly will affect the conclusion of a precise regimen. Anticancer agents used nowadays were made by chance (nitrogen mustard and cisplatin) or by screening programs (paclitaxel, vinblastine)[86-88].

Conclusion

The uncontrolled proliferation of cells is known as cancer. It is caused by the effects of carcinogens, such as tobacco smoke, radiation, chemicals or infectious agents. Although several approved anticancer drugs are available today for the treatment of different types of cancers, effective therapies for most of these cancers are lacking. The available drugs are mostly cytotoxic in nature and act by a very limited number of molecular mechanisms. Thus, the need for novel drugs to treat malignant disease requiring systemic therapy is still pressing. A pre-selection, called the screening process, is therefore required which can be in vitro and in vivo. The aim of screening efforts is to identify products that will produce antitumor effects matching the activity criteria used to define which compounds can progress to the next stage in the preclinical development program. These screening methods based on different principle and mechanism have been discussed.

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