IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

SCREENING METHODS FOR ANTI-CANCER DRUG: IN VITRO AND IN VIVO TESTING

1Monali Parbhane, 2Divya Lande, 3Prajakta Shelke, 4Neha Gaikawad

1Teacher, 2Student, 3Student, 4Student

1SPPU,

2SPPU,

3SPPU,

4SPPU

ABSTRACT:

Anticancer drug development is a tedious process, requiring several in vitro, in vivo, and clinical studies. In order to avoid chemical toxicity in animals during an experiment, it is necessary to envisage toxic doses of screened drugs in vivo at different concentrations. Several in vitro and in vivo studies have been reported to discover the management of cancer.

This study focused on bringing together a wide range of in vivo and in vitro assay methods developed to evaluate each hallmark feature of cancer

These underlying changes are the cause for cancer development, progression, and drug resistance. An in vitro model system which mimics the in vivo cancer is essential to study the various genetic, epigenetic and biochemical changes and also for screening anticancer drugs. The implications of in vitro tumor models in cancer research have been appreciated from early 1900s.

The pharmaceutical industry presently relies on several widely used in vitro models, including two-dimensional models, three-dimensional models, microfluidic systems, Boyden's chamber and models created using 3D bioprinting.

KEYWORDS: MTT, SULPHORHODAMINE B ASSAY, HEMOCYTOMETER, TRYPHAN BLUE DYE, SCREENING METHODS, ANTI-CANCER DRUG, IN VITRO TESTING, IN VIVO TESTING.

INTRODUCTION:

Most chemotherapeutic drugs for cancer treatment are molecules identified and isolated from plants or their synthetic derivatives. currently used for cancer treatment have been isolated.

Approximately 60% of drugs from natural products and the plant kingdom has been the most significant source. Various methods are used for screening of anticancer activity.

The number of patients diagnosed with cancer is increasing worldwide and one of the most important challenges remains the development of effective, safe and economically viable antitumor drugs. Clinical approval for drugs tested in preclinical studies enabling them to enter phase I clinical trials is essential. Currently, potential anticancer drugs have a very low rate of gaining clinical approval at around 7%, much lower than drugs for other diseases.

In vitro tumor models are a necessary tool in not only the search for new substances showing antitumor activity but additionally for assessing their effectiveness. Realistic in vitro models of tumors enable more detailed primary screening of potential antitumor drugs thus preventing drugs with insufficient antitumor activity from entering preclinical animal testing. Pharmacological testing on animal models is carried out to assess bioavailability, toxicity at specific doses and therapeutic efficacy of compounds. According to industry standards, any novel drugs must undergo preclinical trials using animal models before being admitted to human clinical trials. However, the use of animal models can cause a number of problems including high cost, differential responses due to physiological variations between species, and limitations in test availability and feasibility.

The need for novel drugs for the treatment of different cancers is still under great pressure. For a drug to be successful in treating the target population it requires competent preclinical studies using animal models and in-vitro studies which can exactly simulate the disease in patients. There are various types of models developed for studying the anticancer property which include both in-vitro and in-vivo models. Meticulous use of these methods specific to the cancer subtype can help to decrease the rate of failure of drug action in clinical phases. This chapter highlights the various in-vitro and in-vivo models for anticancer drug discovery with due mention of their strengths and limitations.

SCREENING METHODS FOR ANTI-CANCER DRUG: -

IN VITRO:

1. Tetrazolium salt assay.
2. Sulphorhodamine B assay.
3. 3H-Thymidine uptake.
4. Dye exclusion test.
5. Clonogenic test.
6. Cell counting assay.
7. Morphological assay

IN VIVO:

1. Carcinogen induced models
2. Viral infection models
3. Transplantation Models
4. Genetically Engineered Mouse Models
5. In vivo hollow fibre

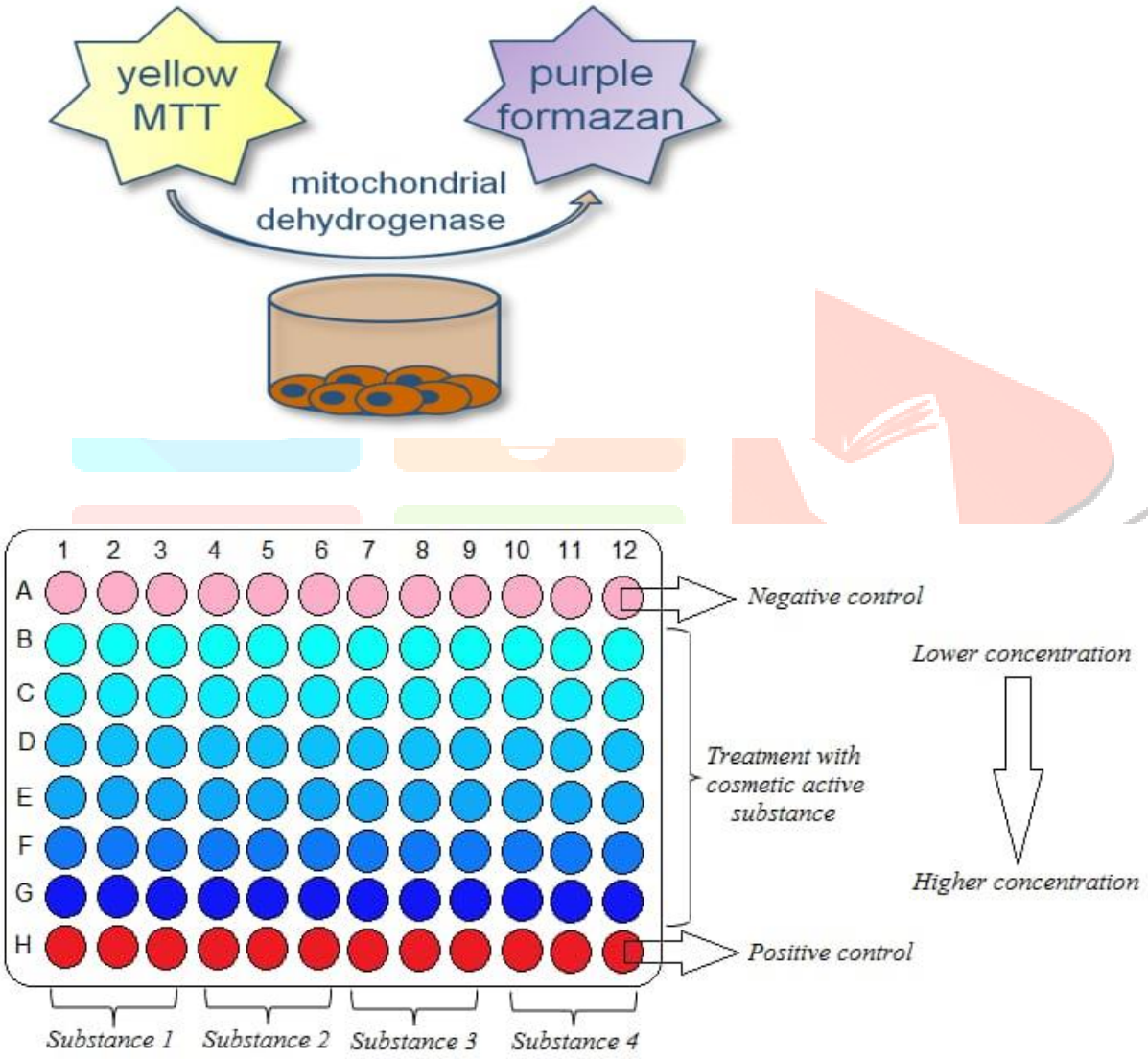
IN VITRO METHODS:

1.Tetrazolium salt assay

This assay is a sensitive, quantitative and reliable colorimetric assay that measures viability, proliferation and activation of cells.

The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue formazan product which is insoluble in water.

The amount of formazan produced is directly proportional to the cell number in range of cell lines.



The cell lines were prepared and cryopreserved using reagents such as DMSO which preserve the cell during freezing. DMSO is toxic at room temperature. The freezed ampoule is brought to room temperature by slow agitation (thawing).

The freezed cryovials plunged into the water bath and is rapidly thawed until it get liquified. Solution, centrifuged with saline for 10 mins to remove the DMSO. The saline is discarded and aliquot is taken for cell counting, cell viability and for sub-culturing.

MTT assay is a quantitative colorimetric assay for measuring cellular growth, cell survival and cell proliferation based on the ability of living cells.

The assay was carried out using (3-(4, 5-dimethyl thiazol-2yl) 2. 5-diphenyl tetrazolium bromide (MTT).

MTT is cleaved by mitochondrial enzyme dehydrogenase of viable cells, yielding measurable purple product formazan.

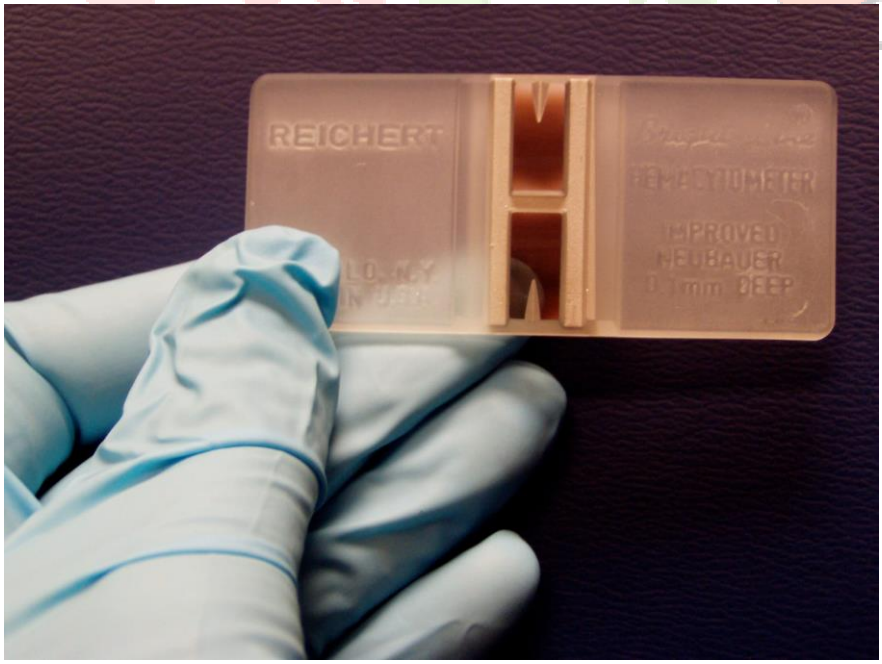
This formazan production is directly proportional to the viable cell number and inversely proportional to the degree of cytotoxicity.

ASSAY PROCESS:

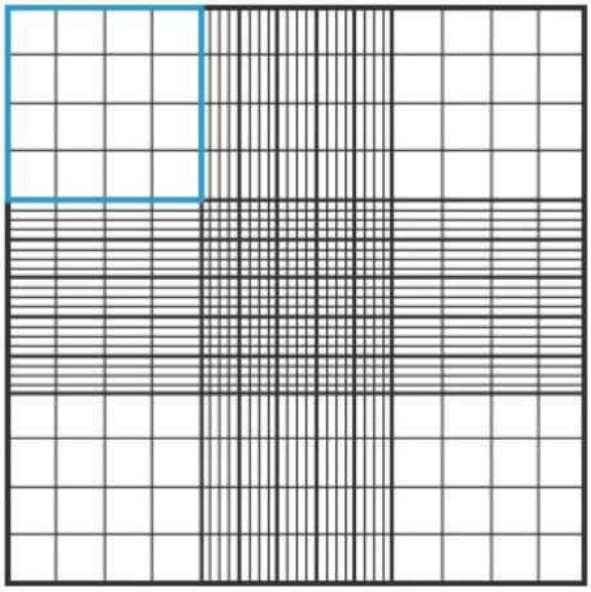
- After incubation, the medium from the well was aspirated carefully and then discarded.
- Each well was washed with Eagle's Minimum Essential Medium (EMEM) without Fetal Calf Serum (FCS).
- 200 µl of MTT solution (5mg MTT/ ml of PBS, pH 7.2) will be added to each well.
- The plate was incubated for 6-7 h at 37°C in a CO2 incubator with 5% CO2. After incubation 1 ml of DMSO was added to each well and mixed with pipette and left for 45s at room temperature. Purple formazan was formed in the wells.
- Cell control and solvent controls were included in each assay to compare the full cell viability in cytotoxicity and antitumor activity assessments.
- The suspension was transferred to a spectrophotometer cuvette and the optical density (OD) was measured at 540nm using DMSO as blank.
- The % cell viability was calculated with the following formula: Cell viability % = Mean OD of wells receiving each plant extract dilution/Mean OD of control wells x 100.

HEMOCYTOMETER CELL COUNTS

• Hemocytometer

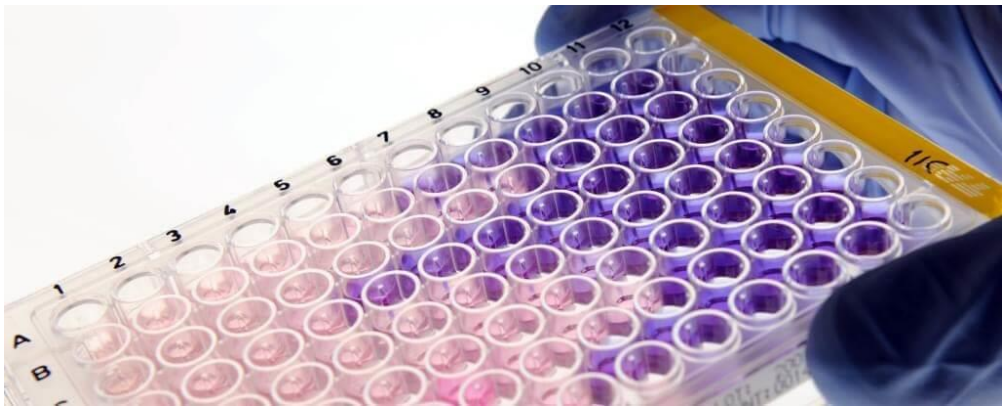


The most common routine method for cell counting which is efficient and accurate is with the use of a hemocytometer % cell viability (OD of treated cells/ OD of control cells) 100.



Sulphorhodamine B Assay

- The Sulphorhodamine B assay measures whole-culture protein content, which should be proportional to the cell number.
- The antiproliferative SRB assay was performed to assess growth inhibition. This is a colorimetric assay which estimates cell number indirectly by staining total cellular protein with the SRB dye Skehan. The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. For present screening experiment, cells were inoculated into 96 well microtiter plates 90 µL/well at appropriate plating densities, depending on the doubling time of individual cell lines.
- After cell inoculation, the microtiter plates were incubated at 37 °C, in 5 % CO2, 95% air and 100 % relative humidity for 24 hrs prior to addition of experimental drugs. After 24 hrs, cells from one plate of each cell line were fixed in-situ with TCA (trichloro acetic acid), to represent a measurement of the cell population for each cell line at the time of drug addition (Tz).
- Experimental extracts were solubilised in appropriate solvent at 400-fold the desired final maximum test concentration and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed and diluted to 10 times the desired final maximum test concentration with complete medium containing test article at a concentration of 100, 200, 400 and 800 µg/ml. Aliquots of 10 µl of these different dilutions were added to the appropriate microtiter wells already containing 90 µl of cell suspension, resulting in the required final drug concentrations of 10, 20, 40 and 80 µg/ml.



- **Endpoint measurement:**
  After compound addition, plates were incubated at standard conditions for 48 hrs and assay was terminated by the addition of cold TCA. Cells were fixed in-situ by the gentle addition of 50 µl of cold 30 % (w/v) TCA (final concentration, 10% TCA) and incubated for 60 minutes at 4°C. The supernatant was discarded; the plates were washed 5 times with water and air dried.
- Sulforhodamine B (SRB) solution (50 µl) at 0.4 % (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 20 minutes at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing 5 times with 1 % acetic acid. The plates were air dried.Bound stain was subsequently eluted with 10 mM Trizma base, and the absorbance was read on an Elisa Plate Reader at a wavelength of 540 nm with 690 nm reference wavelength.
- Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells.
- Percent growth was expressed as the ratio of average absorbance of the test wellto the average absorbance of the control wells* 100. Using the 6 absorbancemeasurements [time zero (Tz), control growth (C), and test growth in thepresence of drug at the 4 concentration levels (Ti)]; the percentage growth wascalculated at each of the drug concentration levels

- Percentage growth inhibition was calculated as follow.

  [(Ti - Tz)/(C - Tz)] *100 for concentrations for which r >> Tz(Ti - Tz) positive or zero

  [(Ti - Tz)/(Tz)] *100 for concentrations for which Gamma*i < Tz (Ti - Tz) negative

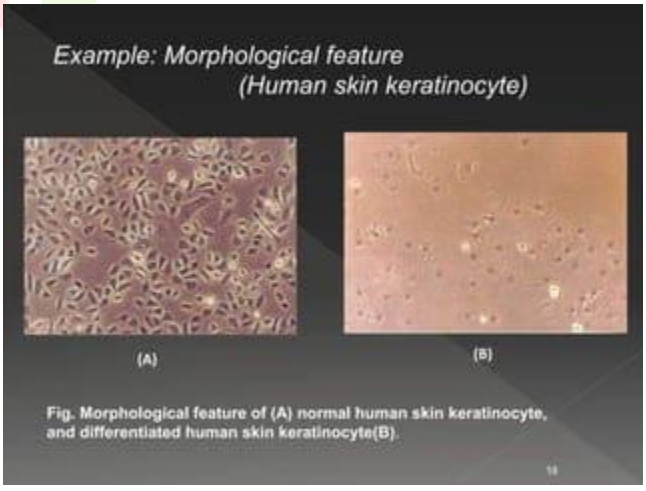
3. Morphological assay:

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 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.



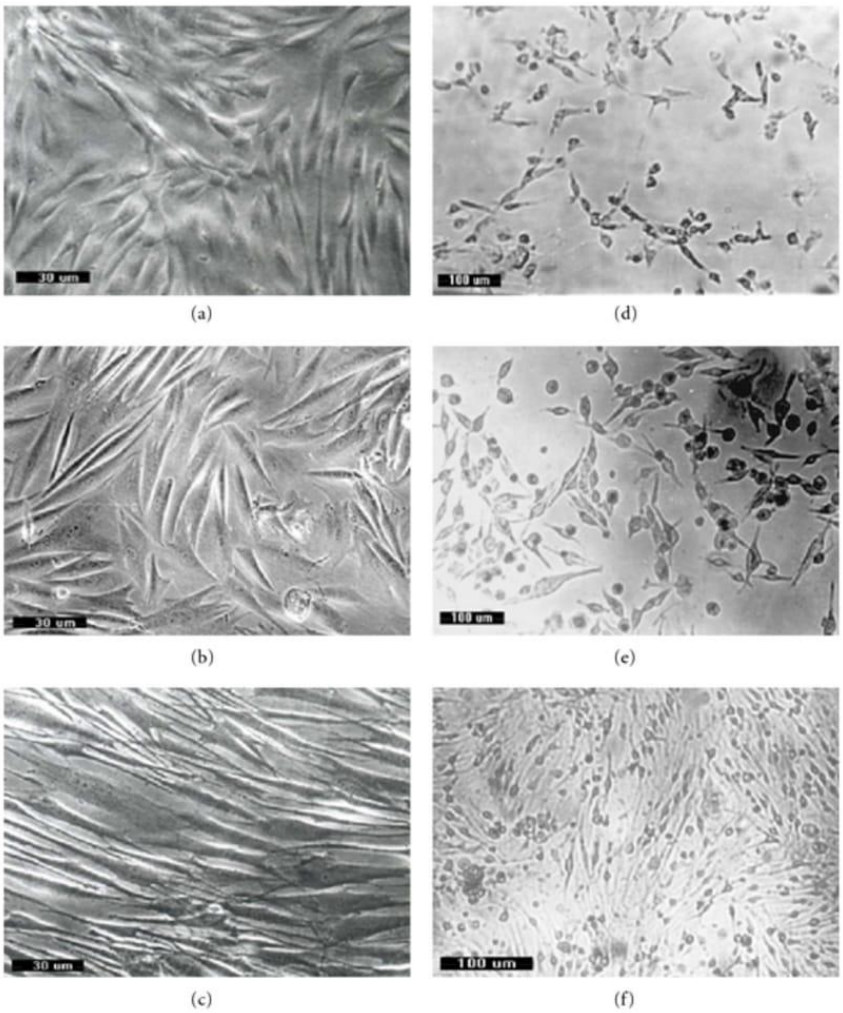


Fig. Human skin fibroblasts

4.Dye exclusion test:

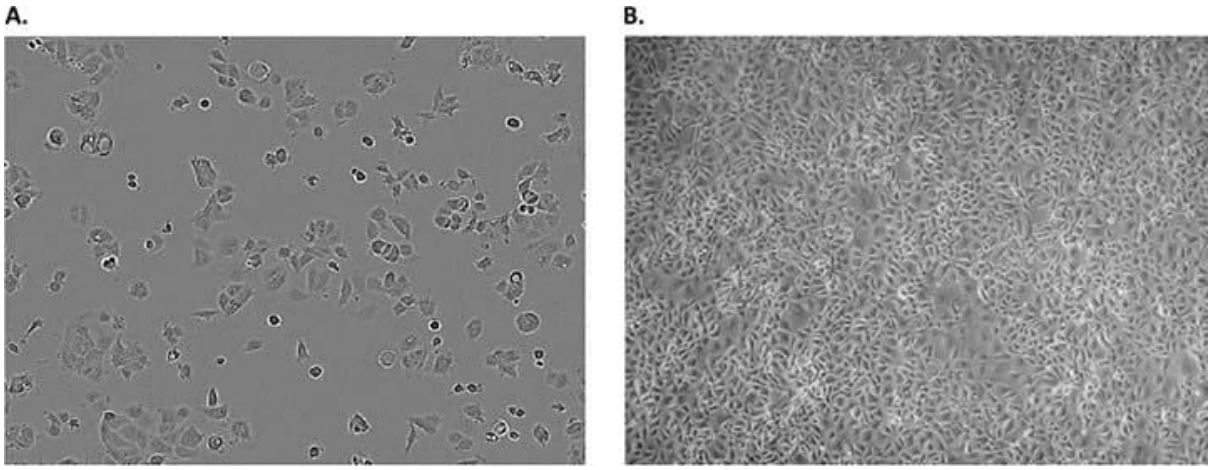
This assay is based on the structural integrity of the cells.

Live cells possess intact cell membranes that exclude certain dyes, such as tryphan blue, Eosin, or propidium, whereas dead cells would have lost membrane integrity.

Hence they would take up the dyes while the live cells exclude it.

METHOD:

1. Cell lines are counted, cultured and innoculated in 96 well plates as above.
2. Cells were incubated with different concentrations of test compounds for 4days.
3. Number of cultured cells in different wells were counted using haemocytometer after staining with suitable dyes.



Untreated Live Cells

Treated Cells

%Cell Viability = [No. Of Viable Cell / Total No.Of Cells (Viable+Dead)] X 100

TRYPHAN BLUE DYE:

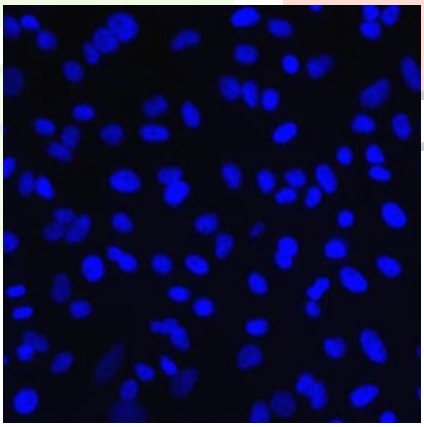


Fig. Hoechst Dye

ADVANTAGES:

- Reduce the usage of animals.
- Less time consuming, cost effective & easy to manage.
- Able to process a larger number of compounds quickly with minimum quantity.
- Range of concentrations used are comparable to that expected for in vivo studies.

DISADVANTAGES:

- Difficulty in Maintaining of cultures.
- Show Negative results for the compounds which gets activated after body metabolism and vice versa. Impossible to ascertain the Pharmacokinetics

IN VIVO

1. CHEMICAL CARCINOGEN MODEL

DMBA induced mouse skin papilloma

1. Two stage experimental carcinogenesis
    - Initiator - DMBA (dimethylbenz[a]anthracene),
    - Promotor - TPA (12-O-tetradecanoyl-phorbol-13- acetate)
2. Mice: Single dose - 2.5 µg of DMBA, 5 to 10 µg of TPA in 0.2 ml of acetone twice weekly.
3. Papilloma begins to appear after 8 to 10 wks - Tumor incidence & multiplicity of treatment group is compared with DMBA control group.

i. Mice are topically applied a single dose of 2.5 µg DMBA in acetone, followed by 5-10 µg of TPA in 0.2 ml acetone twice weekly on the same site starting one week after DMBA application.
ii. Percent tumor incidence and multiplicity of treatment groups is compared with DMBA control group.
iii. Drug under test can be administered either topically or oral route.
iv. The tumor incidence in this model is usually about 100% DMBA controls.
v. In repeated topical application of DMBA Alone has also been shown to induced carcinogenesis.
vi. Drug efficacy is measured as percent reduction in carcinoma incidence, compared with that of carcinogen control.

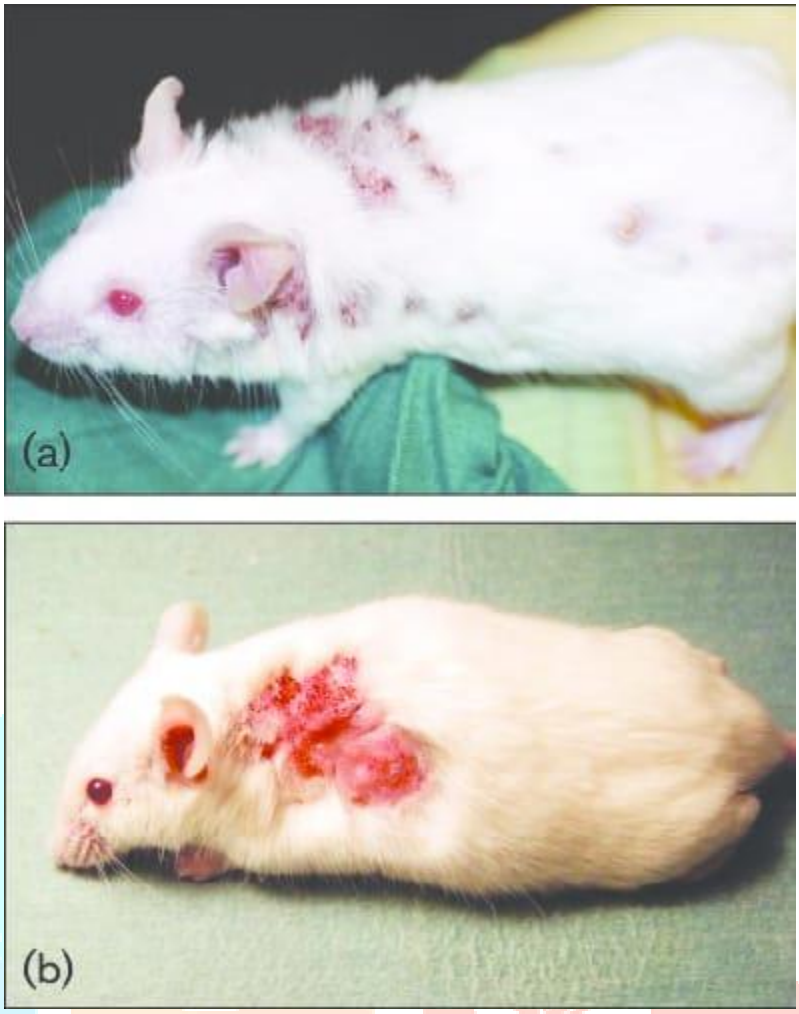


Fig.Mouse Skin Papilloma

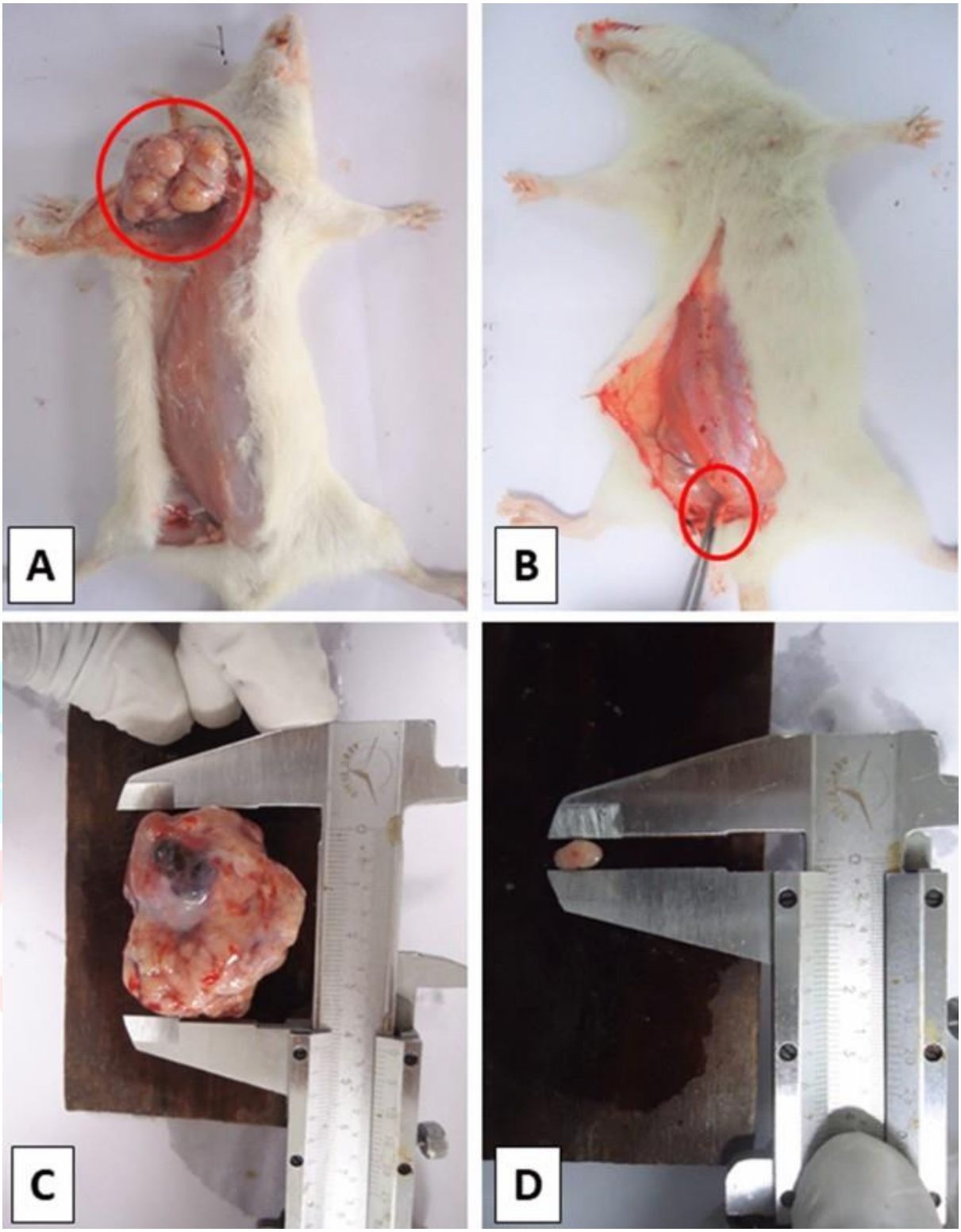


Fig.Rat Mammary Gland Cancer

2. VIRAL INFECTION MODELS

- Mouse Mammary Tumor Virus (MMTV) was the first mouse virus, isolated at Jackson labs as the "non- chromosomal 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
  - ✓ Abelson murine leukemia virus – Abl
  - ✓ Moloneymurine sarcoma virus – R
- Engineered viruses now used routinely in the laboratory to induce cancer.

3. TRANSPLANTATION MODELS

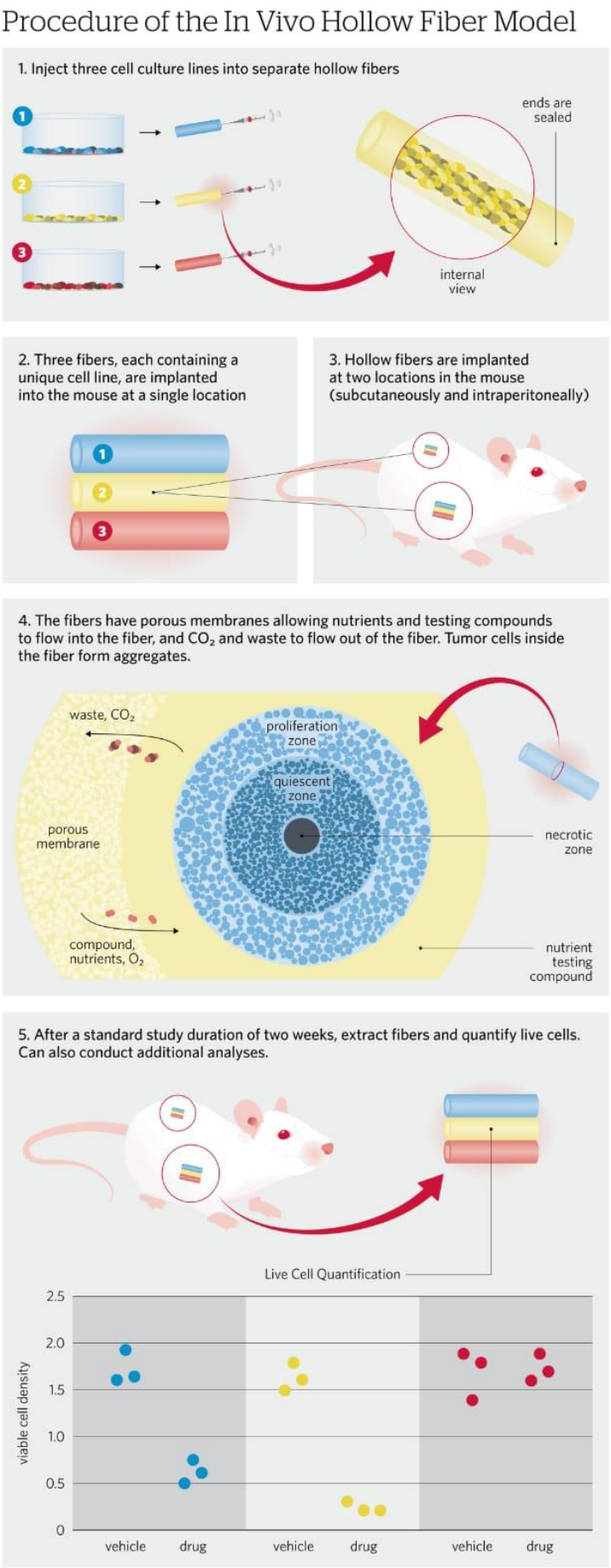
- ❖ Tumor cells or tissues (mouse or human) transplanted into a host mouse.
- ❖ Ectopic - Implanted into a different organ than the original (typically subcutaneous or kidney capsule)
- ❖ Orthotopic - Implanted into the analogous organ of the original tumor.

Advantages:

- ✓ Typically cheap, fast & easy to use.
- ✓ Not covered by patents.
- ✓ In Vivo Hollow Fibre Assay.

IN VIVO HOLLOW FIBRE ASSAY

- 12 human tumor cell lines (lung, breast, colon, melanoma, ovary, and glioma.
- Cells suspended into hollow polyvinylidene fluoride fibers implanted IP or SC in lab mice
- After in vivo drug treatment, fibers are removed and analyzed in vitro
- Antitumor (growth inhibitory) activity assessed
-
- 2-D and 3-D Cell-based Assays in Drug Screening.


Procedure of the In Vivo Hollow Fiber Model
1. Inject three cell culture lines into separate hollow fibers
ends are sealed
internal view
2. Three fibers, each containing a unique cell line, are implanted into the mouse at a single location
3. Hollow fibers are implanted at two locations in the mouse (subcutaneously and intraperitoneally)
4. The fibers have porous membranes allowing nutrients and testing compounds to flow into the fiber, and CO_2 and waste to flow out of the fiber. Tumor cells inside the fiber form aggregates.
waste, CO_2
proliferation zone
quiescent zone
porous membrane
necrotic zone
compound, nutrients, O_2
nutrient testing compound
5. After a standard study duration of two weeks, extract fibers and quantify live cells. Can also conduct additional analyses.
Live Cell Quantification
viable cell density
2.5
2.0
1.5
1.0
0.5
0
vehicle
drug
vehicle
drug
vehicle
drug

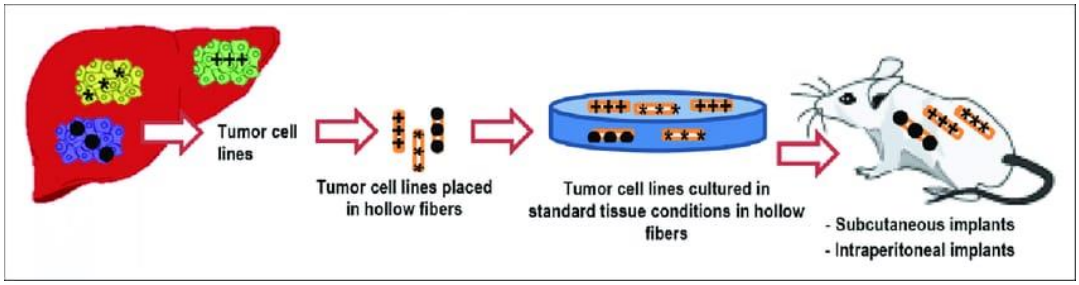


Fig. Subcutaneous Hollow Fibre

2-D and 3-D Cell-based Assays in Drug Screening

- Currently, pharmaceutical firms spend a large amount of money on the compound efficacy and cytotoxicity test.
- There is still a 78% failure rate for all drugs, which may be devastating to developing companies.
- Effective compounds in vitro may be non-effective in vivo for many reasons, including differences between in vitro and in vivo target biology, interrelated biochemical mechanism, metabolism, poor penetration into solid tissues, etc.
- Currently, almost all cell-based assays or biosensors are developed in 2-D culture systems, although conventional 2- D cultures usually suffer from contact inhibition and a loss of native cell morphology and functionality.
- In comparison with 2-D cultures, 3-D cell models create a more realistic representation of real human tissues, which is critical to many important cell functions, including morphogenesis, cell metabolism, differentiation and cell-cell interactions. gene expression.

REFERENCE:

1.Ashish A., Sonia SY, Mark AH, and Minas TC., Pressure Related apoptosis in Neuronal Cel Lines., Journal of Neuroscience Research 2000 60: 495-503

2.Essentials of medical pharmacology by KD Tripathi, 7th edition page no :857

3.https://en.wikipedia.org/wiki/Chemotherapy

4. https://oncologypro.esmo.org/education-library/esmo-books/esmo-handbooks/clinical-pharmacology-of-anti-cancer-agents/in-vitro-evaluation-of-new-anti-cancer-agents

5.https://www.frontiersin.org/articles/10.3389/fbioe.2020.00322/full

6. https://images.app.goo.gl/zxAa6BHdtcLQ6PzV7

7. https://images.app.goo.gl/kR3LP7unAb1rot4Q9

8. https://images.app.goo.gl/vpgC4sDsheCyQyuP6

9.https://journals.indexcopernicus.com/api/file/viewByFileId/745045.pdf

10. https://www.nature.com/articles/s41598-019-45523-2