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Review Article

A comprehensive review on preliminary screening models for the evaluation of anti-cancer agents

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ABSTRACT

Cancer refers to a category of illnesses characterised by uncontrolled growth of new cells. The screening of an anticancer drug is a time-consuming procedure that requires several in-vitro, in-vivo, and clinical investigations. Toxic dosages of screening pharmaceutical drugs in-vivo at varying concentrations need to be envisioned in order to prevent chemical poisoning in animals throughout an experiment. Cancer management has been the subject of several in-vitro and in-vivo investigations. To determine the test compound's anti-cancer efficacy, this research compiled a review of in vivo and in vitro assays. Here, the study emphasizes on the numerous preclinical approaches and processes used in anticancer research.

1. Introduction

In the past two decades, biological factors have taken the role of empiric chemical factors in the discovery and development of novel anti-cancer drugs. These factors are mostly based on compounds that operate on molecular targets. This is the outcome of increased biological understanding of the molecular pathways that have been discovered to be dysregulated and/or over-activated in various tumors, which has resulted in the identification of molecular targets such as growth factor receptor and intracellular signaling molecules against which drugs have been developed.[1] This increased biological knowledge has been associated with development of high-throughput genomics and compound screening that allow development of drug which interact with above mentioned targets.[2] Identification of new anti-cancer agents is mainly based on in-vitro methods, the in vivo models are absolutely required to assess the pharmacological activity of a potential new drug in animal models.[3]

The development of a new anti-cancer agent involves several steps including in-vitro assays, both cells based and molecular target-driven, for the identification of an active compound and in-vivo studies to evaluate the potential anticancer activity; pharmacological studies to evaluate drug absorption, distribution, metabolism, and elimination; and, toxicological studies.[4]

We understand that the conceptual distinction between traditional cytotoxic agents and targeted drugs is frequently incorrect and deceptive, given that the majority of cytotoxic agents do indeed have well-defined targets, such as DNA or tubulin, and that the majority of new agents are instead directed at non-cancer-specific targets, such as enzymes or factors expressed in both normal and cancer cells. Figure 1 elucidates the preclinical developmental steps for the screening of anti-cancer agents.

Here we presented the theoretical information on the preclinical experimental models that are available in this work to assess either drugs with non-cancer-specific targets or those that have been utilized to establish such targets.

There are number of models are available to evaluate the same and described below.

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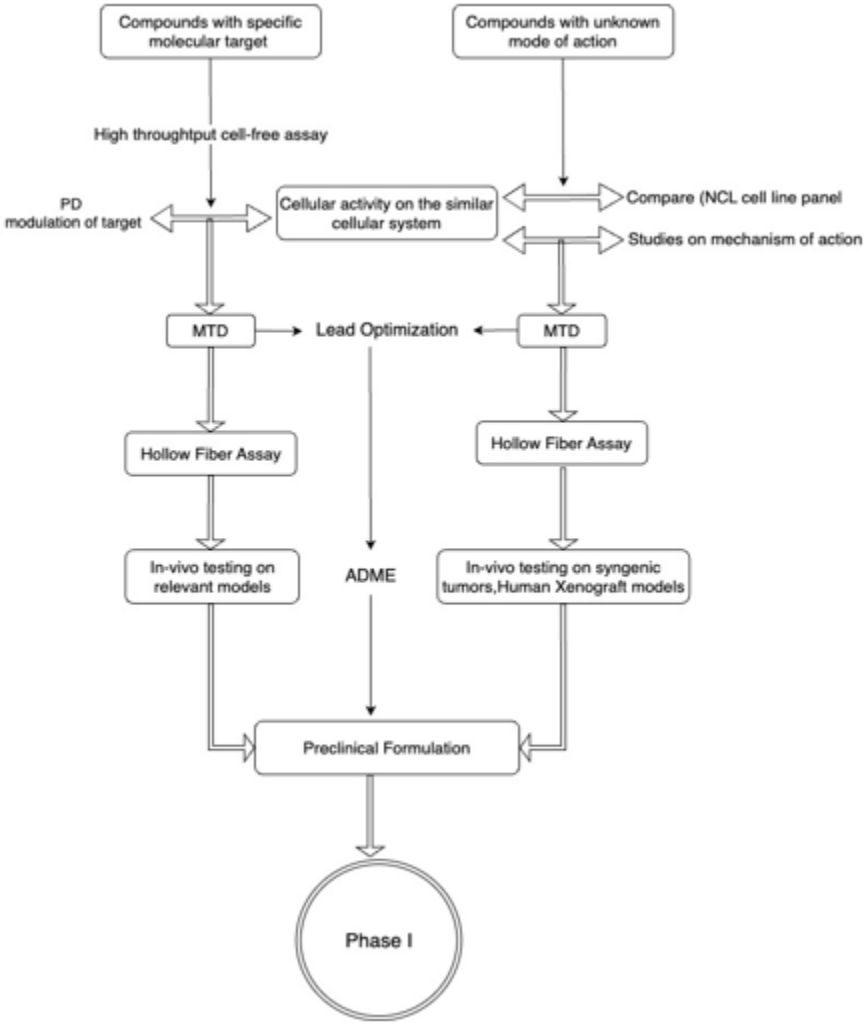

Fig. 1: Preclinical development steps of new anticancer agents

2. MTT assay/Tetrazolium Assay

Cell viability can be measured using a number of different tetrazolium compounds. MTT, MTS, XTT, and WST-1 are some of the most used chemicals. These chemicals may be roughly classified into two groups: those that are positively charged, like MTT, and those that are negatively charged, such MTS, XTT, and WST-1.[5] Mosmann created this technique in 1983, making it the oldest of its kind. First designed for a 96-well format, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction test is a homogenous cell viability assay (HTS). After cells in culture have been incubated for 1 to 4 hours, a final concentration of 0.2 to 0.5mg/ml of the MTT substrate (made in a physiologically balanced solution) is applied. Absorbance variations at 570 nm[5] may be used as a proxy for the amount of formazan (which is, theoretically, directly proportional to the number of viable cells).

It is a cytotoxicity test that uses colorimetry to determine cell viability (Loss of viable cells). Proportionally to the number of live cells, a tetrazolium salt is converted into a colourful, insoluble formazan. The formazan may be spectrophotometrically quantified after being solubilized in dimethyl sulfoxide or acidic isopropanol.[6] Because it is an electron acceptor, tetrazolium salt may be reduced to a colourful formazan with the help of NADH, NADPH, or other oxidised substrates or the right coenzymes. In this case, only living cells create a colour shift, whereas non-living ones have no effect. So, the number of living cells in a sample may be inferred from the amount of formazan produced.[6] Only metabolically active cells are able to reduce MTT, and this decrease serves as a proxy for the cells' overall viability. MTT is yellow Formazan, a purple byproduct of mitochondrial dehydrogenase reduction. It is possible to use DMSO to dissolve the water-insoluble compound known as formazan. The amount of converted

dye in a solution may be determined by measuring the absorbance of the solution using spectrophotometry. This change is triggered by the presence of live cells, which may be determined by the presence of their mitochondrial dehydrogenase enzyme. Dehydrogenase is not made by cells that are no longer alive.[6] Therefore, this will not occur due to cell death. The percentage of live cells to total amount of formazan in a sample is linear. The cell's health is shown by the degree of purple fluorescence.

2.1. Procedure

Trypsinization of cells occurs and it is followed by scrapping. Resuspend the cells to 96 well-plate. Check the cell confluency. If it lies from 90% to 100% then it is ready for use. 100~200 μl of desired drug or compound is introduced to each well. Usually, one compound or drug should have triplicates.[7] Incubate that plate for 12 to 24 hours. Dissolve 1 mg MTT in 1 ml PBS solution to prepare the solution of MTT with concentration of 2.41 mM. Add 20 μl of MTT reagent to each well and incubate for 3~4 hours. Observe the cell in well-plate to check whether it shows purple precipitation or not. If it is clearly visible, then discard the media carefully as it doesn't drain the cells. After Discarding the media, Add 100 μl DMSO/Isopropanol. Cover it with aluminum foil and mildly shake it for 10~15 mins. Measure in fluorescent reader. Measure absorbance (Optical Density) at 570 nm.[7]

3. Sulphorhodamine B assay

To quantify drug-induced cytotoxicity and cell proliferation in high-throughput drug screening, Skehan and colleagues developed the sulforhodamine B (SRB) test.[8] The SRB assay is the technique of choice for high cost-effective screens, and it has been frequently utilised to assess cytotoxicity in cell-based investigations[8]. This technique makes advantage of the fact that SRB attaches to proteins in a stoichiometric fashion under mildly acidic circumstances and can be removed under basic ones; the quantity of bound dye can be used as a proxy for cell mass, and this in turn may be extrapolated to quantify cell proliferation.[8]

3.1. Procedure

The protocol for Sulphorhodamine B assay is divided into four main steps and are as per the standard procedure.[9]

3.2. Preparation of treatment

Volume of treatment must be enough for triplicates in 96-well plates (per replicate; final volume in well 100 μl) and consider the pipette variation.[9] Treatment solution (Test solution) is prepared in aqueous solution or other solvents such as DMSO.

3.3. Cell preparation

Take the cell monolayers out of their media and give them a quick PBS wash (Phosphate Buffer Solution). Take away the phosphate buffer solution and replace it with 0.25 percent (Wt/vol) trypsin. For 5 minutes at 37 °C, or when cells begin to separate,[9] incubate the sample. In order to achieve a homogenous cell suspension,[9] scatter cells from the culture surface using a glass pipette and 10 volumes of culture media containing FBS. The cell suspension should be transferred to a clean falcon tube (polypropylene tube). Before seeding, assess cell concentration using a hematocytometer and a solution of trypan blue dye at 0.4 percent (Wt/vol) in a 1:1 cell suspension. If trypan blue staining of the cells indicates cell death, do not continue. Adjusting the cell concentration using growth medium (10 percent FBS) allows us to seed cells at the optimal density in a 50 L volume per well (96-well format). To make the cell suspension more manageable for use with a multichannel pipette, transfer it to a sterile reagent reservoir for long-term storage.[9]

3.4. Treatment exposure

Mix the treatment solution which is prepared in step A with quantity of 50 μl to each well. Mix cell suspension which is prepared in Step B and add 50 μl to each well which is already having treatment solution. Set aside three wells in the plate containing only cell suspension and solvent of choice for no growth control (Day 0). Incubate that plate at 37 ° with 5% CO2 until the cell attachment completes. For most of the cell lines, this time varies from 2~4 hours. Then procced to next step to fix the cell monolayer.[9]

3.5. Cell fixation and staining

Without removing the cell culture supernatant, add 25 μl (96-well format) cold 50% TCA (Trichloroacetic acid) to each well. Incubate the plate incubate the plates at 4 °C for 1 h. Mixing is not required as it could lead to some cells detaching from the bottom of the well. Plates are washed four times by submerging in tub with slow running tap water. Dry the plate at room temperature. Add 50 μl of 0.04% (wt./vol) SRB solution into each well. Leave at room temperature for an hour and then wash the plate four times with 1%(vol/vol) acetic acid (200 μl). Purpose of this wash is to remove unbound dye. Keep aside this plate for air dry at room temperature. You can also use a blow dryer for drying.

3.6. Absorbance measurement

Add 50 μL of 10 mM Tris base solution (pH 10.5) to each well. Shake the plate on gyratory or orbital shaker for approximate 10 mins to solubilize the protein bound dye. Measure the absorbance at 510 nm in a microplate reader.

3.7. Evaluation

Calculate the percentage of cell-growth inhibition using a formula which is given below:

$$\%\ Cell\ growth = \frac{Absorbance\ of\ sample}{Absorbance\ of\ negative\ control} * 100\ \%$$

$$\%\ Growth\ inhibition = 100 - \%\ Cell\ growth$$

Result of SRB assay is used only if data falls in linearity range of assay. To determine the linearity range, it is suggested to perform a cell number titration experiment.

4. Trypan Blue Dye Assay

The membrane integrity test known as the trypan blue dye determination from live cells is based on this principle. One of the most used standard procedures for determining the cell quantity and percentage of viability in a population of cells is the selective staining of dead cells with trypan blue and microscopic inspection on a hemocytometer.[10]

The viability of the cells and the accuracy of the Trypan Blue Dye Assay are dependent on the cells maintaining their structural integrity. This method relies on the fact that polar dyes like trypan blue, eosin, and propidium cannot enter live cells with intact cell membranes.[10] The cytoplasm of living cells is impermeable to dyes, while the membranes of dead cells are permeable, allowing trypan blue to color them blue. The number of stained cells may be compared to the overall cell population, and this can all be seen using a light microscope. Here As a result, the proportion of dead cells in the population may be calculated based on the number of stained cells.

4.1. Procedure

Cells are washed with Hank's Balanced Salt Solution (HBSS). Centrifugate the cell suspension for 10-15 min at 10000 rpm. This process is repeated for three times. Cells are suspended in HBSS or serum-free complete medium. Cell suspension is transferred into Eppendorf tube and cells are exposed to drug dilutions. Equal quantity of drug treated cells are mixed with 0.4% trypan blue dye. Mixing can be performed in a well of a microtiter plate or a small plastic tube using 10 to 20 μL each of cell suspension and trypan blue. Incubate at room temperature for 3-5 mins. Take a drop of mixture (trypan blue and cell suspension) to a hemocytometer. Place the hemocytometer on the stage of microscope. Count the unstained cells and stained cells. Viable cells exclude dye and dead cell cause staining. Evaluation will be done by using the following equation.[11]

$$\%\ Viability = \frac{Total\ viable\ cell}{Total\ cell\ count} * 100$$

5. The Hollow Fiber Essay

The hollow fiber assay (HFA) is a fast in-vivo assay to evaluate the cytotoxicity of drugs and their pharmacodynamic effects on human tumor cells which are grown in hollow fibers that are implanted in mice or rats. The HFA has been developed at the National Cancer Institute to help bridge the gap between the in vitro cell-based assays and human xenograft models in immuno-deficient mice. The hollow fiber assay is a unique in vivo model for drug discovery as It allows the simultaneous evaluation of the efficacy of anticancer agents against multiple tumor cell lines in two physiological compartments in same animal.[12] The hollow fiber model has a shorter evaluation time and a smaller number of compounds is required as compared to traditional xenograft models. The model allows for the effective pairing of a novel compound with the appropriate cell line by its capacity to utilize multiple cell lines. In this assay, semipermeable biocompatible fibers are used. These fibers are made of polyvinylidene fluoride (PVDF) and have a diameter of 0.5–1 mm and are 2 cm long. The fiber has a molecular weight exclusion of 500,000 Da. The fibers are mostly implanted in mice, but it is also possible to implant in rats and other animals for preclinical testing of anticancer agents. One animal can support the growth of up to six cancer cell lines and, therefore, we can test more than one cell line at a time in one animal.

The hollow fiber assay requires considerable hands-on time and it consists of the following steps: preparing fibers, preparing cells, loading fibers, implanting fibers, removing fibers, assaying for cell viability by MTT (3-4,5-dimethylthiazol-2-yl-2,5-diphenyl-tetrazolium bromide) dye conversion assay, and calculating percent net growth.

5.1. Procedure

This assay is composed of in vitro and in vivo studies. In this assay, in vitro study is used for determining the percent net growth in the test and control fibers.[13] Other in vitro time studies are run in parallel with the in vivo study as controls as reference points for the dosing schedule. Fibers are loaded with cells and they can stabilize overnight before using for in vitro and in vivo studies. These fibers can be implanted into two different physiological compartments: subcutaneous [SC] and/or peritoneal [IP] sites. The cells can stabilize and grow in vivo for 3 days before starting of a dosing process. Fibers are taken from animals to determine the percent net growth at start of the treatment.[13] Animals are dosed from days 3 to 7, and the trial is finished on days 7. The endpoints discussed in this article were all chosen at random and can be changed to fit the study. Variable cell densities are typically implanted into the subcutaneous and intraperitoneal compartments throughout a 7–10-day period in order to measure the growth patterns. It is needed to

determine cell viability by the MTT dye conversion assay at day 3, 7, and 10. Based on the growth of each cell line the appropriate cell-loading concentration and study end points can be determined. [13]

6. Comet Assay

Each cell experiences DNA damage at some point during its lifetime, which is characterized as a change in the chemical structure of genomic DNA. To prove the effectiveness of a genotoxic drug, quantitative evaluation of DNA damage during cancer therapy is essential. In this study, we focus on comet assay which is also known as single cell gel electrophoresis. The comet assay is an effective and simple way to measure the amount of DNA damage which occurs. It needs less time and financial requirements and having good reproducibility.

The comet assay was first developed by 2 scientists Ostling and Johanson in 1984 under a neutral condition. Singh et al. refined this method further by demonstrating that doing the experiment under an alkaline setting greatly improved its specificity and repeatability. The alkaline comet test is often utilised for lower quantities of DNA damage, including single and double strand DNA breaks, [14] whereas the neutral comet assay is recommended for detecting double-stranded DNA breaks.

This test works on the idea that, when subjected to an electric field, DNA fragments leave the nucleoid body (also called the "comet head") and deposit themselves as a stain in the agarose gel (also called the "comet tail"). Nucleotide staining allows for a quantitative analysis of DNA damage by examining "comets" generated during this kind of single cell electrophoresis. [15]

7. Conclusion

The review was undertaken to emphasize on the available screening techniques to evaluate the anti-cancer agents. Based on the review, there are number of in-vitro screening methods are available that can provide the preliminary insight on the effectivity of the test compound which can be furtherly investigated by in-vivo methods. In-vitro screening methods for anti-cancer drugs are comparatively complex compared to the other class of drugs. Similarly, inducing cancer may result into mortality during the study period and can be considered as limitation of the method. It can be resolved by performing further investigations in selection of appropriate dose to induce cancer.

8. Conflict of Interest

The authors declare no conflict of interest

9. Source of Funding

None.

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