Preclinical screening methods in cancer

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Abstract:
Cancer, a group of diseases of unregulated cell proliferation, is a leading cause of death worldwide. More than 80% of compounds which have shown promising effects in preclinical studies could not get through Phase II of clinical trials. Such high attrition rate is due to improper or selective use of preclinical modalities in anticancer drug screening. The various preclinical screening methods available such as in vitro human cancer cell lines, in vivo tumor xenograft model, or genetically engineered mouse model have their respective pros and cons. Scrupulous use of these preclinical screening methods vis-à-vis efficacy of potential anticancer compound with diverse mechanism of action can help in bringing down the rate of failure of anticancer compound at clinical phase. This article provides an insight into the various preclinical methods used in anticancer studies along with their advantages and disadvantages.

Key words:
Cancer epidemiology, cell line prescreen, genetically engineered mouse, xenograft

Cancer is one of the leading causes of death worldwide, accounting for death of 82 lakh people in the year 2012. Top three death-causing cancers are lung cancer, liver cancer, and stomach cancer which killed 15.9 lakh, 7.45 lakh, and 7.23 lakh people, respectively, in the same year. On gender-wise, lung cancer is the leading cause of deaths in males while breast cancer in females, killing 10 lakh males and 5.21 lakh females in single year, respectively. In India, 6.82 lakh people died because of various types of cancer during the same period, of which 48,697 males and 70,218 females died because of lung cancer and breast cancer, respectively. Therefore, one can say that worldwide, lung cancer and breast cancer are the leading cause of cancer-related deaths in males and females, respectively. Worldwide, lung cancer is the foremost cause of cancer-related deaths, whereas in India, breast cancer is the topmost cause. Higher incidence of lung cancer can be accured to increased air pollution or smoking while the reasons for higher incidence of breast cancer in India are late diagnosis due mainly to lack of awareness on early detection, barriers to health services or change in lifestyle such as obesity, late pregnancy, hormone replacement therapy, and lower lifetime duration of breastfeeding.

Cancer, also known as malignant tumor or neoplasm, is a broad term used for a large collection of diseases that can affect any organ or tissue of the body. One of the defining features of cancer is the rapid generation of undifferentiated cells that grow outside their natural boundaries, and which can also invade adjacent or distant tissues or organs of the body (metastasis). Normally, when cells become old or damaged, they undergo programmed cell death, i.e. apoptosis and new cells replace them to fulfill the need of the body. Whereas in cancer case, this orderly process is disrupted and as the cells become old or damaged, instead of dying, they survive. These cells can divide into less specialized cells (tumor) and are able to ignore the signals which stop division or by which apoptosis is started in normal cells.

Depending on the potential clinical behavior, a tumor can be divided into two categories: Benign and malignant. Benign tumor, termed by attaching the suffix “-oma” to the type of cells in which the tumor arises, for example, fibroma, adenoma, and papilloma, will remain localized and the patient generally survives to local surgical procedures. Malignant tumors of solid mesenchymal tissues are called sarcomas; for example, cancer of fibrous tissue is known as fibrosarcoma while those ascending from the mesenchymal cells of the blood are leukemias or lymphomas. However, malignant tumors of epithelial cells are termed as carcinomas irrespective of the origin of tissue (as the epithelial cells are originated from three germ cell layers). Therefore, malignant tumors arising in the renal tubular epithelium (mesoderm),
skin (ectoderm), and lining epithelium of the gut (endoderm) are all carcinomas. Carcinomas are subdivided further. Carcinomas with glandular pattern, squamous cells, and undifferentiated cells are called adenocarcinomas, squamous cell carcinomas, and undifferentiated carcinoma, respectively.\[13\]

As cancer was primarily considered a disease of uncontrolled cell division, by measuring the regression in tumor size, identification of a cytotoxic or an antiproliferative compound was considered as the main objective endpoint of efficacy of a compound in preclinical and clinical anticancer drug development for decades.\[5\] For rapid screening of new anticancer compounds, murine models of rapidly growing cancer were developed, for example, sarcoma 180, carcinoma 755, and L1210 mouse leukemia model which were later replaced by the P388 murine leukemia model.\[6\] Several clinically important anticancer agents such as methotrexate, actinomycin D, 6-mercaptopurine, 5-fluorouracil were identified using these murine models; however, successes were achieved mainly in the cases of rapidly growing cancers, e.g. lymphomas, childhood leukemia, and germline tumors while relatively limited successes were seen in the treatment of the slow-growing common solid tumors of the adults, e.g. lung, breast, and colorectal cancers.\[11\]

According to Hutchinson and Kirk, the attrition rate for the development of new anticancer drugs is 95%, while in case of drugs for treatment of cardiovascular diseases, the attrition rate is 80% at the end of Phase II of clinical trials.\[12\] Improper preclinical strategies, such as selective use of rapidly growing tumor models, were implicated as the reason for the failure of number of anticancer agents clinically. Recently, several potential new drug targets have generated due to growth and advances in genomics and proteomics, leading to paradigm shift in anticancer drug discovery from cytotoxicity to targeted therapeutics at molecular levels. These new targets have led investigators to incorporate a variety of cell lines and tumor types in the prescreening and screening protocols of potential anticancer drug candidates.\[10\] The aim of screening is to identify products that will produce antitumor effects matching the certain criteria used to define which compounds can progress to the next stage in the drug development program. However, ideal screening system should be simple, fast, and low cost with optimal predictability of pharmacodynamics.\[13\]

The National Cancer Institute (NCI) Developmental Therapeutics Program for screening of anticancer drug, started in 1955, has become the leading public screening effort worldwide in the area of cancer drug discovery and it also has collaborations with other organizations which are conducting in cancer-related research, e.g., the European Organization for Research and Treatment of Cancer and Cancer Research UK.\[14\] The NCI have updated its preclinical screening methodologies periodically over the years on the basis of knowledge acquired from the fundamental studies of biological factors that influence the effects of a treatment such as the interaction between drug response and the kinetics of tumor cell growth and retrospective analysis of data obtained from preclinical and clinical studies and its correlation.\[15\]

**In vitro Screening Methods**

Large-scale screening using animal systems as practiced in the past is highly unethical and in certain countries such as Europe and India is strictly regulated. The Committee for the Purpose of Control and Supervision of Experiments on Animals in India regulates the screening using animals and has inculcated the credo of 4Rs: Replacement, reduction, refinement, and rehabilitation of animals used in experimentation.\[16\] Therefore, in vitro evaluation of anticancer drugs usually preceded by either cellular or target-based high-throughput assays.

**Cell Line Prescreens**

Cellular screens in cancer research mainly consist of permanent human tumor cell lines; most suitable test system, in terms of management, because of their immortal nature and reproducible growth behavior. A panel of sixty different human tumor cell lines from nine different types of cancer (leukemia, nonsmall cell lung cancer, colon cancer, brain cancer, melanoma, ovarian cancer, renal cancer, prostate cancer, and breast cancer) constitute the NCI in vitro primary screen, against which compounds are tested over a defined range of concentrations to determine the relative growth inhibition or cytotoxicity against each of the cell line.\[17\] The screen is designed and operated in such a way that, for each compound tested, the intricacy of a 60-cell-line dose response results in a characteristic profile or “fingerprint” of cellular responses that can be exploited in pattern recognition algorithms. These algorithms can help in presuming the mechanism of action of a test compound or can help in deciding whether the response pattern is unique or it is parallel to response of any of the standard compounds included in the database.\[18\]

In the year 1995, to weed out inactive agents and to find out potential agents from the pool of compounds, NCI adopted an in vitro prescreen which consists of MCF-7 breast, H460 lung, and SF268 brain cancer cell lines. This prescreen is used to check the presence of toxicity at a drug concentration of 10⁻⁴ M and could eradicate a large fraction of the inactive agents but retain active agents for multidose 60-cell-line testing. The efficiency of 60-cell-line screen was increased with limited loss of information as approximately 50% of the agents could be removed without a significant drop in the capability to identify active anticancer agents.\[13,18\]

**Cell Growth Determination**

Cell growth can be determined by various accepted methods that utilize the exclusion of certain dyes by live cell membranes. Selection of a particular method depends on factors such as minimum number of cells required, sensitivity, speed, and ease of handling. The various preferable methods for cytotoxicity studies are 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay, sulforhodamine B (SRB) assay, propidium iodide (PI) assay, and luciferase assay.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Assay
It is the earliest of all the methods and was developed by Mosmann in the year 1983, in which a colorless tetrazolium salt is metabolized into colored insoluble formazan in
The protein-bound dye is extracted in vivo in a cell line-specific manner and quantitation of drug cytotoxicity is influenced by the length of exposure to MTT. It is, therefore, needed to standardize the assay conditions for each cell line as to minimize their effects on assay results. This would include optimization of cell inoculation densities and assay length in such way that these do not result in exhaustion of nutrients from the medium, and the concentration as well as exposure duration of MTT should also be standardized.

**Sulforhodamine B Assay**

SRB assay is a rapid, sensitive, and inexpensive method, which utilizes a bright pink anionic dye, that binds electrostatically to the basic amino acids of trichloroacetic acid fixed cells. The protein-bound dye is extracted with tris base (tris (hydroxymethyl) aminomethane), after washing off the unbound dye, and thus, protein content can be quantified indirectly spectrophotometrically. This method is suitable for an ordinary laboratory as well as for a very large-scale antitumor screening. The endpoint of SRB assay is nondestructive, not time critical (stable) and comparable with other fluorescence assays. Although this labor intensive method (several washing steps) offers practical advantage with other fluorescence assays, results obtained with SRB assay are not significantly different from the results obtained with MTT assay.

**Propidium Iodide Assay**

Ethidium bromide and PI are two cationic fluorescent dyes, known to pass only through the membranes of dead or dying cells and intercalate with DNA. Binding of these dyes with the DNA increases their fluorescence (more intensely by PI); therefore, fluorescence is seen only in nuclei of dead cells. These dyes are stable after uptake, and viability can be determined even after several days and therefore are more accurate and reliable. Cells are incubated with PI, and the number of nonviable cells is assessed by the subsequent fluorescence detection (first measurement). The second measurement will be taken after freezing the cells for 24 h at −20°C. Due to freezing, PI will be intercalated into the DNA of all the cells and the difference in the two measurements will give the number of viable cells. The assay is a simple, rapid without any washing step and only 150–500 cells per well are sufficient for drug testing. One major drawback of this assay is that PI also binds with double-stranded RNA which might be present in the cytoplasm, but this can be overcome using RNase enzyme during the assay.

**Luciferase Assay**

The nucleotide adenosine triphosphate (ATP) is the principal donor of free energy as it is needed by all cells to remain alive and perform their specialized functions and levels of cytoplasmic ATP decreases in case of any injury or hypoxia. Therefore, by measuring the amount ATP, one can determine the living status of a cell. Cellular ATP, after cell lysis, is free to react with luciferin and luciferase and which results in the generation of high-quantum chemiluminescence. Intensity of emitted light is linearly related to ATP concentration with optimum conditions. Luciferase assay showed better sensitivity and reproducibility over several days when compared to MTT assay and was able to detect the viability of cells when cell count was as low as 2000 cells/well, while in case of the MTT assay, minimum 25,000 cells/well are required for above background readings. The shortcoming of this method is that quenching of the sample can influence the luminescence readout.

Other methods for the determination of cell viability are also available, but their usefulness is limited by several problems occurring with them, for example, in case of trypan blue dye exclusion assay, cells must be counted within 3–5 min as the number of dead cells increases with time, and in case of lactate dehydrogenase assay, results could be misleading if the agent under investigation affects only intracellular activities.

Monolayer cellular screens are the most convenient and frequently applied methods for cytotoxic studies but have certain disadvantages as these do not mimic heterogeneity of three-dimensional in vivo growth. Drugs such as signal transduction inhibitors, antibodies, bioreductive drugs, antiangiogenic peptides or small molecules, and anti-telomerase cannot be evaluated properly by monolayer cellular screens. Techniques such as growing cells in two-dimensions on matrices or in three-dimensions by encapsulation which mimic physical and biological properties of in vivo environment more appropriately are gradually replacing the monolayer cell screens. However, these techniques are still in their nascent phase and until fully available either specially designed cell screens or biochemical assays are best suited for above mentioned classes of drugs.

**In vivo Screening Methods**

The cell line screens, although provide faster results in a cost and time effective manner yet only cytotoxic compound can be identified by these screens. Many new anticancer agents (molecule targeted cytostatic drugs) would be considered inactive by these screens. Furthermore, in vitro cytotoxicity is only one of the many factors which play critical role in clinical efficacy of a given compound. Factors such as physiochemical properties, pharmacokinetics, and toxicological assessments are equally important along with the outright effectiveness of the anticancer agent. Regardless of an agent’s affinity to its targets, poor aqueous solubility, inadequate bioavailability, and metabolic instability can lead to the failure of the compound at clinical trial. Moreover, in vitro cell line screens are inadequate in the evaluation of the off-target effects, which may contribute to the potency or toxicity, of a novel agent. In vivo tumor models used in preclinical drug development express the targets for the new generation anticancer agents and are both disease focused and target based. In vivo tumor models include either human tumor explants/xenografts or specifically bred transgenic mice. In vivo tumor models are proven for predicting clinical...
Tumor Xenograft Model

In 1969, growth of human tumor in an immunodeficient “nude” (athymic) mouse was reported first time by Rygaard and Povelsen. A localized, well-defined tumor process was obtained which was easily accessible for observation, measurement, and biopsy procedures after simple transplant of human tumor tissue to nude mice. The human tumor tissue could be transplanted either in intact form or in form of suspension of cells obtained from the human tumor. The tumor tissue could be surgically transplanted or injected to subcutaneous tissue of trunk, muscles of flank, or orthotopically to any organ (generally similar to human organ from which tumor tissue is obtained) of nude mice.

Subcutaneous implantations are much easier to perform than orthotopic implantations and have been shown to closely maintain the histopathological, cytological, and biochemical characteristics typical of original tumor. However, they do not reproduce the primary site of the common human cancers and lack the invasive and metastatic potential. It was shown that metastasis after implantation of human tumor in nude mice depends on several factors such as site of implantation, blood supply to the implanted site, presence of fibrous capsule (mice origin) surrounding the human tumor, and the cell types of implanted human tumor. Orthotopically growing tumors have the advantage that metastases occur in much higher frequency and the invasion seems to be more pronounced compared with subcutaneously growing models. Human tumor xenografts grown subcutaneously or orthotopically in nude or in severe combined immunodeficiency mice are available for all the major tumor types and have become the major model for preclinical in vivo anticancer screening and drug development. Tumors growing in internal organs (orthotopic model) are usually not accessible for serial size measurements; therefore, the mice have to be sacrificed to measure tumor volume that allows only a one-point measurement. This drawback of this otherwise very attractive, more clinically relevant disease model can be overcome using imaging techniques now available. While the subcutaneous human tumor xenograft model is good for evaluating cytotoxic or cytotstatic drugs, the orthotopic model provides the most appropriate evaluation of specific inhibitors of metastases or invasion.

One of the main contributions of this model is that the efficacy of an anticancer drug in patient can be compared with the effects in the xenograft model (in vivo) obtained with the tumor of patient and with the well-established parallel cell lines (in vitro). Comparison of drug responses in many xenografts developed from different types of tumors and individual patient has shown that xenograft model has >90% possibility in correctly predicting the clinical response. A retrospective analysis of 39 screened compounds for which results of both Phase II and xenograft studies were available showed that at least 33% of the xenografts tested for preclinical activity predicted effect in not less than two different types of tumor in Phase II trials and some of the screened compounds are currently used in standard chemotherapies such as paclitaxel and doxorubicin. The human tumor xenograft model is a good predictor of clinical activity for anticancer drugs and is useful in assessing the drug’s pharmacokinetics and pharmacodynamics and it also provides a renewable and readily accessible source of target human tumor cells.

Despite many advantages of the xenograft model, one needs to be cautious and bear in mind the variables which affect the results of studies conducted on these models, e.g., the origin of the tumor (i.e. cell line versus patient biopsy), target/receptor status of the tumor, site of tumor implantation (e.g., s.c., i.p., orthotopic), size of tumor at the onset of agent treatment, growth rate and characteristics, dose, formulation, frequency and route of administration, and experimental endpoints. Further, xenografts should be characterized at the molecular level for a particular target before the use as chemosensitivity studies are generally performed in well-characterized models expressing the target at which the new compound should act.

Genetically Engineered Mouse Models of Cancer

Genetically engineered mouse (GEM) model of cancer is a diverse collection of genetically modified mice, in which tumor development occurs in situ in appropriate tissue compartments. In the early 1980s, cellular/viral oncogenes were introduced into the mouse germline for the first time and these transgenic mice were shown to be predisposed to cancer. Manipulation of mouse germline to the deletion of tumor suppressor genes (TSGs), expression of dominant negative versions of TSGs, and/or overexpress putative oncogenes in a tissue-specific manner leads to development of lung cancer, bone cancer, lymphoid cancer, pituitary cancer, breast cancer, and many other types of cancer mouse models.

GEM models can be divided broadly into two categories: Germline GEM models, in which cancers developed in an unregulated (spontaneous) fashion, and conditional GEM models, in which spatiotemporal control of tumor onset is possible with the use of tissue specific, ligand-regulated, and/or viral-based technologies. Germline GEM model approaches involve the replacement of endogenous embryonic stem cell chromatin. Biallelic disruptions of TSGs in mice often lead to embryonic lethality which provides the understanding of the role of these genes in normal murine development. Although the embryonic lethality could be overcome by developing heterozygous allele, the germline mutation is present throughout the mouse body which leads to developmental flaws, unwanted effects outside tissues of concern, or compensation from associated gene products or those in the same pathway. Conditional GEM models are developed by the use of site-specific recombinases (SSRs) to control the spatiotemporal mutation of the genome. These SSRs catalyze the deletion or inversion of the intervening sequence between a pair of inverted repeat DNA elements resulting in a nonfunctional or unstable gene product. Temporal control can be attained with a ligand-regulated SSR, in which the SSR remains inactive before induction of the ligand. This improves the accuracy of the model in mimicking human cancer.

The GEM models (transgenic and conditional knock-out and/or knock-in mouse) closely recapitulate human cancer-like
mechanisms of tumor initiation, progression, maintenance, and drug resistance.[43] Results of standard clinical treatments in GEM model and corresponding clinical trials predicted the outcome and interrogating mechanisms of therapeutic response and resistance in Kras-driven nonsmall cell lung carcinoma and pancreatic ductal adenocarcinoma models.[44,45]

Although GEM models imitate human disease more accurately and are more relevant in today’s target-based drug discovery and would assist investigators better in identification of optimal new drug, GEM model do have their own problems, e.g., expensive, complicated breeding schemes, restricted experience, variable penetrance, and tumor latency. Furthermore, it is very difficult to follow tumor kinetics and nonphysiological expression of mutated genes.

Conclusion

No single preclinical modality can completely provide the required data to justify the progress of a compound into the next stage of drug development program in clinical trials. In vitro monolayer cellular screens are fast but are not of much use in case of noncytotoxic anticancer agents. Tumor xenograft model is useful in assessing pharmacokinetics and pharmacodynamics of anticancer agent, but the metastasis in this model is of murine nature. GEM model closely mimic human cancer, but the technique is expensive and requires expertise. Therefore, meticulous use of these available preclinical modalities is needed to ascertain the criteria required to be matched to move into the next phase of anticancer drug development program for a compound.

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Conflicts of Interest

There are no conflicts of interest.

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