Recent Trends in Screening and Evaluation Methods of Anticancer Drugs
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ABSTRACT
Cancer is one of the major life threatening diseases worldwide. The available anticancer drugs have distinct mechanisms of action which may vary in their effects on different types of normal and cancer cells. Screening methods are routinely and extensively used to reduce cost and time of drug discovery. The traditional anticancer drug screening methods, including animal experiments and cell-based screening assays. Screening methods for the detection of anticancer activity are of importance in order to find solid tumor-specific agents. The screening and evaluation procedures for the development of anticancer agents indicated that the entire process is a rather difficult task. Presently, active compounds are selected by prescreening and screening against transplanted mouse tumors and human tumor xenografts as well as by the in vitro systems. The US National Cancer Institute (NCI) 60 human tumour cell line anticancer drug screen (NCI60) was developed in the late 1980s as an in vitro drug-discovery tool intended to supplant the use of transplantable animal tumours in anticancer drug screening. This screening model give information about the mechanisms of growth inhibition and tumour-cell kill. Recently, its role has changed to that of a service screen supporting the cancer research community. Target-based and cell-based screenings for new anticancer drugs in the molecular targeting period are methods of identifying more selective anticancer drugs. Here I review the development, use and productivity of the screen, highlighting several outcomes that have contributed to advances in cancer chemotherapy. Finally, we discuss primary and secondary in vivo evaluation in experimental chemotherapy.

Keywords
Screening, Anticancer agents, Chemotheropy, Tumor, Cell lines.

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1. INTRODUCTION:
Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. If the spread is not controlled, it can result in death. Cancer is caused by both external factors (tobacco, infectious organisms, chemicals, and radiation) and internal factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism). These causal factors may act together or in sequence to initiate or promote carcinogenesis. Cancer is treated with surgery, radiation, chemotherapy, hormone therapy, biological therapy, and targeted therapy etc.

2. Types of Cancers:
Cancer is a broad term used to describe hundreds of malignant diseases that can develop in the body. It can develop just about every part of the body, from the eyes to the heart. Each type of cancer is unique, possessing different signs, symptoms, and treatments.

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3. Anti-Cancer Drugs:
i) Introduction:
The available anticancer drugs have distinct mechanisms of action which may vary in their effects on different types of normal and cancer cells. A single "cure" for cancer has proved elusive since there is not a single type of cancer but as many as 100 different types of cancer. In addition, there are very few demonstrable biochemical differences between cancerous cells and normal cells. For this reason the effectiveness of many anticancer drugs is limited by their toxicity to normal rapidly growing cells in the intestinal and bone marrow areas. Although 92 approved anticancer drugs are available today for the treatment of more than 200 different tumors, effective therapies for most of these tumors are lacking (3). A final problem is that cancerous cells which are initially suppressed by a specific drug may develop a resistance to that drug.

ii) History of anticancer drug screens
Initial screening and drug development programs were small in scale and directed toward the evaluation of antitumor activity of small numbers and specific types of potential drugs. Stimulated by the approaches of Ehrlich and Warburg, studies were conducted on the effects on tumor growth of dyes or respiratory poisons, respectively (1). In the 1930s several researchers engaged in systematic studies of certain classes of compounds such as Boyland in the United Kingdom, who tested aldehydes in spontaneous tumors in mice, and Lettre in Germany, who studied colchicine derivatives and other mitotic poisons in tissue culture and ascites tumor. In the United States, Shear, first at Harvard and then at the National Cancer Institute (NCI), inaugurated a screening program for testing and isolation of bacterial polysaccharides employing mice bearing sarcoma 37 as test systems for necrosis and hemorrhage. The program was quickly extended to plant extracts and synthetic compounds. In the early 1950s the program had evaluated more than 300 chemicals and several hundreds of plant extracts. Two of these materials were tested clinically. Larger-scale screens emerged around 1955, stimulated by the discovery that chemical agents, such as nitrogen mustard and folic acid antagonists, were capable of producing remissions of malignant lymphomas. As a result, the program of Shear at the NCI was extended to incorporate the evaluation of synthetic agents and natural products for antitumor activity. Further institutions that engaged in screening programs were Sloan-Kettering in New York, the Chester Beatty...
Research Institute in London, and the Southern Research Institute in Alabama. In addition, screening, evaluation, and development programs were instituted at chemical and pharmaceutical companies, research institutions, medical schools, and universities in various countries in the world. As a result of these efforts, several agents were found with clinical activity, particularly against leukemias and lymphomas. Currently, they provide the battery of available drugs for systemic treatment of cancer and encompass alkylating agents (cyclophosphamide, bis(chloroethyl)nitrosourea [BCNU], 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea [CCNU], antimetabolites (methotrexate, 5-fluorouracil [5-FU], 6-mercaptopurine), antibiotics (mitomycin C, Adriamycin), and hormones (androgens, estrogens, corticoids).

iii) Cancer Chemotherapy: Chemotherapy drugs are sometimes feared because of a patient’s concern about toxic effects. Their role is to slow and hopefully halt the growth and spread of a cancer. There are three goals associated with the use of the most commonly-used anticancer agents.
1. Damage the DNA of the affected cancer cells.
2. Inhibit the synthesis of new DNA strands to stop the cell from replicating, because the replication of the cell is what allows the tumor to grow.
3. Stop mitosis or the actual splitting of the original cell into two new cells. Stopping mitosis stops cell division (replication) of the cancer and may ultimately halt the progression of the cancer.

Unfortunately, the majority of drugs currently on the market are not specific, which leads to the many common side effects associated with cancer chemotherapy. Because the common approach of all chemotherapy is to decrease the growth rate (cell division) of the cancer cells, the side effects are seen in bodily systems that naturally have a rapid turnover of cells including skin, hair, gastrointestinal, and bone marrow. These healthy, normal cells, also end up damaged by the chemotherapy program.

iv) Categories of Chemotherapy Drugs:
In general, chemotherapy agents can be divided into three main categories based on their mechanism of action.

a) Stop the synthesis of pre DNA molecule building blocks:

These agents work in a number of different ways. DNA building blocks are folic acid, heterocyclic bases, and nucleotides, which are made naturally within cells. All of these agents work to block some step in the formation of nucleotides or deoxyribonucleotides (necessary for making DNA). When these steps are blocked, the nucleotides, which are the building blocks of DNA and RNA, can not be synthesized. Thus the cells can not replicate because they can not make DNA without the nucleotides.

Examples of drugs in this class include 1) methotrexate (Abitrexate®), 2) fluorouracil (Adrucil®), 3) hydroxyurea (Hydrea®), and 4) mercaptopurine (Purinethol®).

b) Directly damage the DNA in the nucleus of the cell:
These agents chemically damage DNA and RNA. They disrupt replication of the DNA and either totally halt replication or cause the manufacture of nonsense DNA or RNA (i.e. the new DNA or RNA does not code for anything useful).

Examples of drugs in this class include cisplatin (Platinol®) and 7) antibiotics - daunorubicin (Cerubidine®), doxorubicin (Adriamycin®), and etoposide (VePesid®).

c) Effect the synthesis or breakdown of the mitotic spindles:
Mitotic spindles serve as molecular railroads with "North and South Poles" in the cell when a cell starts to divide itself into two new cells. These spindles are very important because they help to split the newly copied DNA such that a copy goes to each of the two new cells during cell division. These drugs disrupt the formation of these spindles and therefore interrupt cell division. Examples of drugs in this class include: Vinblastine (Velban®), Vincristine (Oncovin®) and Pacitaxel (Taxol®).
Screening Methods or Techniques:

1. **In Vitro Testing:**
   i. NCI-60 Tumor Cell Line Screen
   ii. Sample Handling and Preparation

2. **In Vivo Testing:**
   i. Acute Toxicity Determination
   ii. Hollow Fiber Assay
   iii. Tumor Xenograft Models

3. **Cell-Based Screening Assays:**
   i) Conventional cellular screens
   ii) Tailored Cellular Screens
   iii) Biochemical Screening Assays
   iv) Combination of Target and Cell Screens

**1. In Vitro Testing:**

i) **Cell Line Panel:**

The initial panel incorporated a total of 60 different human tumor cell lines derived from seven cancer types, including lung, colon, melanoma, renal, ovarian, brain, and leukemia. Selection of lines for inclusion in the panel required that they adequately met minimal quality-assurance criteria (testing for mycoplasma, MAP, human isoenzyme, karyology, in vivo tumorigenicity), that they were adaptable to a single growth medium, and that they showed reproducible profiles for growth and drug sensitivity. All cell lines in the interim panel were nevertheless subjected to detailed, specialized characterizations (e.g., histopathology, ultrastructure, immunocytochemistry) to verify or determine tissue and tumor type. Moreover, parallel projects were launched for the acquisition of better and more diverse candidate cell lines, and for the development of new lines directly from surgical specimens or from nude mouse xenografts for which the corresponding clinical backgrounds were more complete.

**Cell line names and Panel names:** They are 60 Cell line names, and in correspond 9 panel names and shown in the following table:

ii) **Standard Operating Procedures for Sample Preparation for NCI60 Screen:**

NCI60 testing is performed in two parts: first a single concentration is tested in all 60 cell lines at a single dose of 10^{-5} molar or 15 µg/ml. If the results obtained meet selection criteria, then the compound is tested again in all 60 cell lines in 5 x 10 fold dilutions with the top dose being 10-4 molar or 150 µg/ml. Compounds accepted for NCI60 testing are prepared for both 1-dose and 5-dose testing at the same time.

2. **In Vivo Testing:**

i) **Acute Toxicity Determination:**

Generally, the determination of maximum tolerated dose (MTD) is performed in a way that conserves compound and minimizes the number of animals sacrificed. Thus, a single mouse is given a single injection (IP, IV, SC, IM or PO) of 400 mg/kg (or lower if the compound is anticipated to be extremely potent, e.g. natural products); a second mouse receives a dose of 200 mg/kg and a third mouse receives a single dose of 100 mg/kg. The mice are observed for a period of 2 weeks. They are sacrificed if they lose more than 20% of their body weight or if there are other signs of significant toxicity. If all 3 mice must be sacrificed, the next 3 dose levels (50, 35 and 12.5 mg/kg) are tested in a similar manner. This process is repeated until a tolerated dose is found.

ii) **Hollow Fiber Assay:**

The HF assay was developed by Hollingshead et al. at the NCI and is composed of 2-cm tubes filled with tumor cell lines. These fibers are implanted into mice at two sites (intraperitoneal and subcutaneous). The fibers are removed after 4–6 d in the animal and processed in vitro for quantification of tumor cell growth. By determining net cell kill, one can examine whether drugs administered via different routes are bioavailable and can reach the tumor sites (17). The HFA assesses the pharmacologic capacity of compounds to reach two physiologic compartments within the
nude mouse and shows a practical means of quantifying viable tumor cell mass. The in vivo hollow fibre assay was developed at the NCI to help bridge the gap between the in vitro cell-based assays and human xenograft models in immunodeficient mice. The goal was to develop an intermediate assay that could better predict which compounds found active in the 60-cell line panel would be active in subsequent xenograft models. This was necessary due to the high cost of the traditional xenograft assay in terms of number of animals required, time for assay completion, and financial commitment necessary.

For the standard hollow fiber assay (HFA), the high and low dose levels are determined using the formula below.

\[
\text{High dose} = \frac{\text{MTD} \times 1.5}{4} \\
\text{Low dose} = 0.67 \times \text{high dose}
\]

The standard vehicle used for both acute toxicity testing and HFA is 10% DMSO in saline/0.05% Tween 80. Primary Anti-cancer Drug Screening Activities.

### iii) Xenografts Models:

The review of NCI in vitro and in vivo screening efforts based on the 60 human cell line panel and xenograft testing in the 1990s has recently been published. The methods of the NCI procedures were mainly empirical during this time period and disease rather than target based (10,14). Data were available on 39 agents with both xenograft data and Phase II trial results. The analysts found that histology of a particular preclinical model showing in vivo activity did not correlate with activity in the same human cancer histology. However, drugs with in vivo activity in a third of the tested xenograft models did correlate with ultimate activity in some Phase II trials. This and the fact that none of the currently registered anticancer drugs was devoid of activity in preclinical tumor models, but showed activity in the clinic, led to the conclusion that activity in in vivo models of compounds demonstrating in vitro activity remains desirable. The hollow fiber assay has proven a valuable interface for selecting

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development candidates from large pools of compounds with in vitro antiproliferative activity for expensive and time-consuming subcutaneous xenograft testing.

a) **In vitro models:**

In vitro models to define the mechanisms of action of a given compound. Once a compound has demonstrated robust cytotoxic activity against a panel of human cancer cell lines and deserves further investigation in in vivo models, it is important to clarify its mechanism of action and to identify its exact molecular targets. An example of the importance of the in vitro studies aimed at clarifying the mechanism of action of a given drug is represented by PARP inhibitors. The initial panel of cell lines incorporated a total of 60 cell lines representing nine distinct tumour types (leukaemia, colon, lung, CNS, renal, melanoma, ovarian, breast and prostate cancer). To date, more than 85,000 compounds have been screened against this in vitro panel of short term assay. Compounds are tested over a 5-log concentration range against each of the 60 cell lines for their ability to inhibit the growth of, or to kill, the cells in a 2-day assay generating 60 dose-response curves.

b) **In vivo models:**

The available in vivo models used to select compounds for further clinical development will be herein briefly summarised.

I. Murine tumours

P388 and L1210 leukaemia cell lines have been used for many years as the major preclinical models to screen new compounds. Therefore, in the early 1970s, the B16 melanoma and Lewis lung carcinoma were also incorporated to try to identify compounds potentially active in human solid tumours. These tumours also induced the formation of lung metastases and thus were of potential use for investigating both the antitumor and the anti-metastatic activity of new compounds.

II. Genetically engineered cancer models (GEM)

Over the past 20 years GEM models have been instrumental not only in our understanding of the molecular pathways responsible for the initiation and progression of tumours but also because they have highlighted the importance of specific oncogenes and tumour suppressor genes in carcinogenesis. As GEM models have been shown to partially recapitulate the genetic/molecular changes occurring in human tumours, the challenge now is to use these models to test novel anticancer therapies in an attempt to better select clinically effective compounds. GEM models have increased our understanding of the molecular pathways responsible for the initiation and progression of human cancer, and have highlighted the importance of specific oncogenes and tumor suppressor genes (TSG) in particular types of cancer. GEM models possess well-validated molecular/genetic characteristics (e.g., gene mutations) which ultimately facilitate the rational design of small molecule therapeutics.

The main aim of GEM models is to recapitulate genetic/molecular changes in human cancer and use these to test novel anticancer therapeutics in an attempt to accurately predict clinical response (15). The first strains of genetically engineered mice predisposed to cancer were transgenic mouse models whereby cellular/viral oncogenes were introduced to the mouse germ line. One of the first transgenic cancer models involved the constitutive expression of the c-myc oncogene under the control of the mouse mammary tumor virus promoter leading to the development of mammary tumors.

3. **Cell-Based Screening Assays:**

i) Conventional cellular screens

Cellular screens in cancer research employ mainly permanent human tumor cell lines; their immortal nature and hence manageable, reproducible growth behavior make them suitable test systems. Of critical importance, however, is the detection method, the choice of which depends on the cell number used and thus the desired sensitivity. Various procedures to determine cell growth are employed in screening laboratories. The earliest broadly used growth inhibition assays were developed by Mosmann and the NCI screening staff, namely, the methylthiazoldiphenyl tetrazolium (MTT) assay. The yellow MTT dye is reduced by mitochondria into a purple formazan, which can be read with ultraviolet/visible light scanners. Its limitations are the use of large quantities of a hazardous solvent, dimethyl sulfoxide, which is required to dissolve the resulting formazan crystals and the varying number of mitochondria in cells.
Currently employed in the NCI 60-cell-line screen is the sulforhodamine B (SRB) assay; SRB is a dye that stains protein (2).

Most industrial-scale cellular screens prefer the use of fluorescence or luminescence detection systems. The latter include, for example, the propidium iodide (PI) assay staining for DNA content or use of a luciferase reporter. They appear to offer the most advantages, such as high sensitivity and easy handling. The use of one-dimensional or monolayer cultures to measure cell growth is the most convenient and frequently applied method. Owing to tumor heterogeneity and three-dimensional in vivo growth, however, currently employed monolayer assays of human tumor (epithelial) cells are oversimplistic and have some disadvantages for the in vitro evaluation of certain anticancer agents:

1. Short-term culture conditions (2–6 d) may select for cytotoxic drugs.
2. Tumor cell growth can continue despite the fact that clonogenic cells are reduced, missing certain classes of cytostatic agents (e.g., inducers of cellular senescence).
3. Extracellular matrix and blood vessel targets (angiogenesis) are absent.
4. Gradients of oxygen tension, extracellular pH, nutrients, catabolites, and cell proliferation rate are a function of distance in solid tumors from blood vessels and are also not possible to mimic by monolayers.
5. Drug penetration barriers occur only in multilayered solid tumors.

Drugs that are encompassed by this list include signal transduction inhibitors, antibodies, bioreductive drugs, anti-angiogenic peptides, small molecules, or antitelomerases. These classes of drugs therefore might best be examined in either specially designed cell systems and tailored screens or biochemical assays.

ii) Tailored Cellular Screens:

Examples of successful in vitro models of a tumor environment using multicellular spheroids or post confluent multi cell layers for screening of bioreductive agents have been reported by Phillips et al. The latter showed that agents requiring bioactivation by the microenvironment such as mitomycin C or EO9 were differentially chemosensitive in plateau-phase multilayered cells as compared to exponentially growing monolayers. The novel structures RSU 1069 and SR4233 were found by this procedure. Over the past couple of years, the enzyme telomerase has been causally linked to immortalization and cancer and thus has arisen as a promising anticancer target. Telomerase acts at the end of chromosomes termed telomeres by synthesizing telomeric repeat sequences (3). Telomeres are important noncoding sequences that protect chromosomes from end-to-end fusions and maintain chromosomal stability. In each round of cell division telomeric sequences (approx 30–100 bp) are lost owing to the end replication problem.

iii) Biochemical Screening Assays:

Biochemical assays are compared to cellular assays “target-driven” and provide the means for evaluating high numbers of compounds. These screens are primarily employed in the pharmaceutical industry and institutions that harbor large compound libraries for systematic search of novel agents. Figure 3 summarizes the procedure for such an approach. An important advantage of biochemical screens is that they can be fully automated; thus, most steps can be performed by robot or computer systems such as dispensing of targets, addition of drugs and detection reagents, as well as compound library storage and management. Key requirements for target-oriented screening are:

1. The molecular target must be validated, shown to be causally linked to disease initiation or progression.
2. The target required for in vitro assays must be made available in large quantities, for example, by recombinant DNA techniques.
3. Defined, pure compound libraries comprising hundred of thousands of structures derived from combinatorial approaches or collections of natural substances should be available.
4. Simple, cost-effective, highly reproducible assay and detection systems, which can be performed in microplate formats.

Suitable platforms have been proven to be enzyme linked immunoassorbent assays (ELISA) and other enzyme-based colorimetric methods. Further technologies that are frequently used are:

1. Radiometric assays dependent on scintillation proximity counting by employing scintillant-coated beads in microtiter plates;
(2) **time-resolved fluorescence** based on highly fluorescing rare-earth metal–ligand chelates (europium, samarium, terbium);
(3) fluorescence polarization and luminescence detection including chemiluminescence or electrochemiluminescence.

**iv) Combination of Target and Cell Screens:**
Both cell- and target-based screening procedures have clear advantages and disadvantages, while cell-based approaches will miss agents with certain defined modes of action such as, for example, specific telomerase inhibitors owing to lack of cytotoxic potency in short-term assays. They might, on the other hand, identify compounds as active with previously unknown targets and hence allow for identification of novel mechanisms of action as well as the elucidation of their interplay in certain pathways. An example of this from the NCI 60-cell-line screen is the spicamycin analog KRN5500. Another **advantage** of compounds identified in cellular screens are their proven cellpermeable properties, which might be missing in cell-free systems. In addition, ligand interactions might be more appropriate in the biological environment. Considering these facts, a combination of rational biochemical and “more” empirical cellular screening systems would therefore be the most optimal methodology in new cancer drug discovery. We have recently applied this combination successfully in the identification of the novel CDK inhibitory agent E224 (5-methylindirubin). Widely different toxicity profiles were observed for 23 standard anticancer agents and X-ray treatment, indicating that the type of DNA repair and cell cycle checkpoint mutations in individual tumors could strongly influence the outcome of a particular chemotherapeutic regimen. Most anticancer agents in current use were discovered either by chance (e.g., cisplatin and the nitrogen mustards) or through screening programs (e.g., vinblastine and paclitaxel [TaxolTM]). Only recently has a more detailed knowledge of the fundamental biochemical differences between normal and tumor cells allowed a truly rational approach to the drug design process (e.g., the kinase inhibitors GleevecTM and gefitinib [Iressa™]). New lead agents are nearly always evaluated initially in **in vitro** tumor cell lines.

**Application of NCI screening method**

1. COMPARE has become an integral part of the NCI’s evaluation of newly screened compounds.
2. Even though the mean graph every compound tested by the NCI is inspected by eye, most newly screened compounds do not show sufficient pattern to warrant a COMPARE analysis.
3. Those that exhibit a detectable pattern are analyzed with a version of the COMPARE program that uses the standard agent database. This database is small enough to permit an interactive analysis.
4. Thus, it can be determine a matter of seconds whether a compound acts by a mechanism of action similar to that of one of the standard agents.
5. The screen, developed over a period of several years by the Developmental Therapeutics Program (DTP), employs 60 human tumor cell lines that have been grouped in disease subpanels including leukemia, non-small-cell lung, small-cell lung, central nervous system, colon, melanoma, ovarian, and renal tumors or cell line.

**CONCLUSIONS AND PERSPECTIVES:**
Screening is necessary to prioritize compounds for further development. In the era of target-oriented molecular cancer therapeutics, screening procedures are tailored toward the desired mechanism of tumor inhibition. They require, however, careful design and validation. In the past, empirical screens designed to find highly potent cytotoxic agents produced an arsenal of clinically used drugs with low selectivity and efficacy in solid tumors (20). Validation of any past, present, or future application of the NCI 60 cell screen as an effective tool for discovery of clinically useful new antitumor drugs, must expect definitive clinical evaluation of new investigational agents whose discovery was uniquely dependent on the screen. Realistically that may require another decade into the future.

**SUMMARY**
As we have discovered more about the underlying mechanisms responsible for the initiation and progression of human cancers, we
have experienced a move away from the development of classic cytotoxic agents to the rational design of small molecule anticancer therapeutics. This has prompted a transition from empirical compound-orientated preclinical screening to target-orientated drug screening. The use of uncharacterized tumor models (s.c. xenografts/syngeneic models) has been continuously replaced by more clinically relevant and molecularly characterized models along with the integration of pharmacodynamic and pharmacokinetic approaches.

It is emphasized that this preclinical information needs to be used appropriately in clinical trials. In particular, if a novel target-directed agent is to be used to treat a patient, the presence of its respective target must initially be confirmed.

REFERENCES


16) Marko D, Schatzle S, Friedel A, et al. Inhibition of cyclin-dependent kinase 1


