WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES
SJIF Impact Factor 7.421
Volume 8, Issue 1, 481-494 Review Article ISSN 2278 – 4357

REVIEW: THE IN-VIVO SCREENING METHODS OF ANTICANCER DRUG

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Article Received on
10 Nov. 2018,

Revised on 29 Nov. 2018,
Accepted on 20 Dec. 2018

DOI: 10.20959/wjpps20191-12881

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ABSTRACT

Cancer is one of the major life threatening diseases in world**.** The available anticancer drugs have distinct mechanisms of action which shows varying effects on different types of normal and cancer cells. Screening methods are routinely and extensively used as concerned with cost and time reduction in drug discovery. The traditional anticancer drug screening methods, including animal experiments and cell-based screening assays. The screening and evaluation procedures for the development of anticancer agents indicated that the entire process which is a 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. 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 screening, highlighting several outcomes that have contributed to advances in cancer chemotherapy. Finally, we discuss primary and secondary in vivo evaluation in experimental chemotherapy.

KEYWORDS: Cancer is one tumor xenografts chemotherapy.

1. INTRODUCTION[1,2]

Pharmacology is the science which deals with the study of drugs. The word “pharmacology” is derived from the Greek word Pharmakon (a drug or poison) and logos (discourse). Drugs, again, are chemical substance used for the purpose of diagnosis, prevention, relief or cure of a disease in man or animals. The word, “drug” is derived from the French word “drogue” meaning herb.

Experimental pharmacology is relatively the youngest branch of basic medical science. Although this discipline was started in Europe and England in nineteenth century it has been developed to its present status only during last few decades or so. today experimental pharmacology has tremendously drifted from the conventional approach to molecular and biochemical aspects. The advancements in the field of electrophysiology, biochemistry, molecular biology and analytical chemistry have enriched and broadened the horizon of experimental pharmacology.

The main aims of experimental pharmacology are to

1. Find out a therapeutic agent suitable for human use.
2. Study the toxicity of a drugs and
3. Study the mechanism and site of action of drugs.

Since experimental pharmacology involves the discovery of new drugs or to study the actions of existing drugs it is done in two main stages, i.e.

1. Preclinical experimental pharmacology which involves the identification and optimization of novel chemical lead structures and testing on animal tissues or organs for their biological action, and
2. The second stage, clinical pharmacology where testing of drugs is done on human volunteers and patients for assessing the pharmacokinetics, safety and efficacy in human.

Since pharmacist come in contact with drugs at every stage of its development, right from synthesis, pharmacological testing, formulation of drugs to dispensing, it is quite apperant that a student of pharmacy to have an adequate exposure and background to experimental pharmacology during his undergraduate curriculum. This will help the student to understand the mechanism of action, receptor action and drug interactions. With this view in mind, this laboratory manual has been prepared wherein some basic background on experiments, their relevance to the study of drugs, and detailed procedure have been descried. The experiments are selected and designed for pharmacy students. The experimental data given in some exercises is based on the experiments actually done in the author's laboratory. Relevant references are given at the end of the exercise for additional information.

Cancer chemotherapy is a relatively young discipline of oncology. It has only been pursued with scientific vigor and multinational collaborations since the mid-twentieth century. To date, over 100 monographs of drugs used for the treatment of more than 200 different tumour

types exist. Over the past decade, cancer has become a large therapeutic market, third only after central nervous system and cardiovascular drugs, and it is continuously growing. The number of blockbuster anticancer drugs with sales of $1 billion or more increased from 19 in 2007 to 24 in 2008. Nonetheless, the cure rate of 4% for cancers that require systemic treatment remains very low. Thus, the need for novel drugs is still pressing Public institutions, pharmaceutical industry, small business, and biotech companies create hundreds of thousands of compounds with potential anticancer activity. Only a certain number of drugs and concepts, however, can be evaluated clinically because of cost and ethical considerations. A preselection, called the screening process, is therefore required. The aim of screening efforts is to identify products that will produce antitumor effects matching the activity criteria used to define which compounds can progress to the next stage in the preclinical development program. Anticancer drug screening can be performed using various types of in vitro and in vivo tumor models. The ideal screening system, however, should combine speed, simplicity, and low costs with optimal predictability of pharmacodynamic activity.

2. History of Anticancer Drug Screens[2]

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 of dyes or respiratory poisons on tumor growth. 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 Latter in Germany, who studied colchicine derivatives and other mitotic poisons in tissue culture and ascites tumors. 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 established 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 still provide the mainstay of available drugs for systemic treatment of cancer and encompass alkylating agents (cyclophosphamide, bis(chloroethyl)nitrosourea [BCNU], 1(2-chloroethyl)-3-cyclohexyl-l-nitrosourea [CCNU], antimetabolites (methotrexate, 5-fluoroura-cil [5-FU], 6-mercaptopurine), antitumor antibiotics (mitomycin C, adriamycin), and mitotic spindle poisons (*Vinca* alkaloids, taxanes).

- **The NCI Screen**

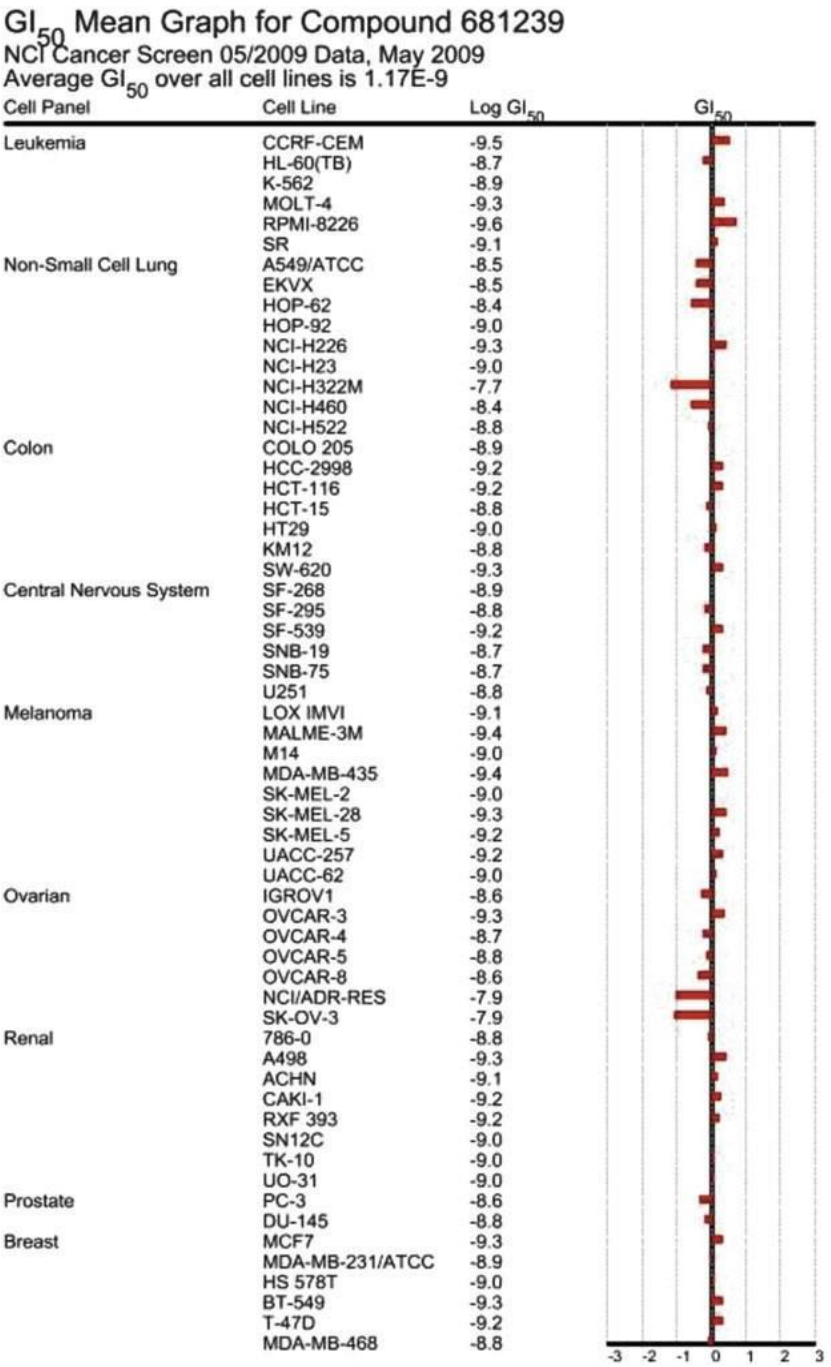
The NCI Developmental Therapeutics Program (DTP) anticancer drug screen has undergone several changes since its inception in 1955. It has become the foremost public screening effort world-wide in the area of cancer drug discovery, not the least because the experimental screening models were always adapted to novel emerging knowledge and technologies. The early philosophy from which the NCI endeavour proceeded was that the elucidation of empirically defined antitumor activity in a model would translate into activity in human cancers. The choice of specific screening models was guided by sensitivity to already identified clinically active agents and in the early period was exclusively focused on in vivo testing procedures. Initially, three transplantable murine tumors were employed, namely, the sarcoma 180, the carcinoma 755, and the leukemia L1210. The found to be the most predictive rodent model among the available panel and was retained in 1975, when the NCI screening process was changed in that the P388 murine leukemia model was utilized as a pre screen and followed by a panel of tumors now also including human xenografts (breast MX-1, lung LX-1, colon CX-1). The human xenografts were utilized with the intent to achieve a better prediction for clinical response against solid human malignancies as compared to haematological malignancies. For the same reason, starting in 1985, the human tumor cell line panel comprised of 60 different cell types, including mainly solid malignancies, was introduced and replaced the P388 in vivo leukemia pre screen in the 1990s. This project has been designed to screen up to 20,000 compounds per year for potential anticancer activity. Selection criteria for preclinical drug candidates are cytotoxic potency and differential activity against particular tumor types and/or a few specific cell lines. The screen is unique in

that the complexity of a 60-cell-line dose response produced by a given compound results in a biological response pattern that can be utilized in pattern recognition algorithms. Using these algorithms, it is possible to assign a putative mechanism of action to a test compound or to determine that the response pattern is unique and not similar to that of any of the standard prototype compounds included in the NCI database. Such agents are then tested against the sensitive cell line grown as subcutaneous xenografts in nude mice in vivo. Because of the vast number of molecules emerging from the in vitro screen for nude mouse testing, in 1995 the preclinical development cascade was amended to include the hollow fibre (HF) assay. The HF assay is a short-term in vivo assay combined with in vitro culture methods. It has been proven as a rapid and efficient means of selecting compounds with the potential for in vivo activity in conventional xenografts.

In parallel with the implementation of the HF "in vivo filter system," a pre screen preceding the 60-cell-line screen was established in early 1995 as it became obvious that many agents were completely inactive under the conditions of the assay. Initially, the pre screen comprised three cell lines (MCF-7 breast, H460 lung, and SF268 brain cancer lines) tested against a range of drug concentrations. Currently, the pre screen assesses a new drug at one concentration of 10^{-5} M in all 60 cell lines. Only compounds which satisfy predetermined threshold inhibition criteria will progress to the five-dose screen. The threshold inhibition criteria for progression to the 5-dose 60-cell-line screen were designed to efficiently capture compounds with anti-proliferative activity and are based on careful analysis of historical DTP screening data. The actual NCI preclinical anticancer drug screening process is summarized in Fig. 2. Although the NCI drug development scheme is still empirical as it is based on selection of in vitro and in vivo anti-proliferative activity, a number of new agents that are now in clinical use have been identified based on their unique patterns of and/or activity in the in vitro screen such as bortezomib (Velcade®, NSC 681239), romidepsin (depsipeptide, NSC 630176), a histone deacetylase (HDAC) inhibitory agent, and tanespimycin (17-AAG, NSC 330507).

Recent insights into the molecular basis of human cancer and high-throughput profiling of the genome and proteome of the NCI 60-cell-line panel initiated a transition to rational molecular targeted discovery and development of anticancer agents in vitro and also in vivo. New programs such as the NCI Chemical Biological Consortium (CBC) have therefore been implemented. The CBC will select targets, actively screen for agents that affect these targets,

and optimize the "drug-like" properties of hits, rather than focus on developing new agents submitted by outside investigators. The CBC drug discovery process is divided into four distinct stages including Exploratory Screen Development (ESD), Screening/Designed Synthesis (SDS), Lead Development, and Candidate Seeking with the goal to test the latter in phase 0/I trials. The CBC will mobilize a cancer drug discovery group on the scale of a small biotechnology concern, with an R&D pipeline linked to the academic community.



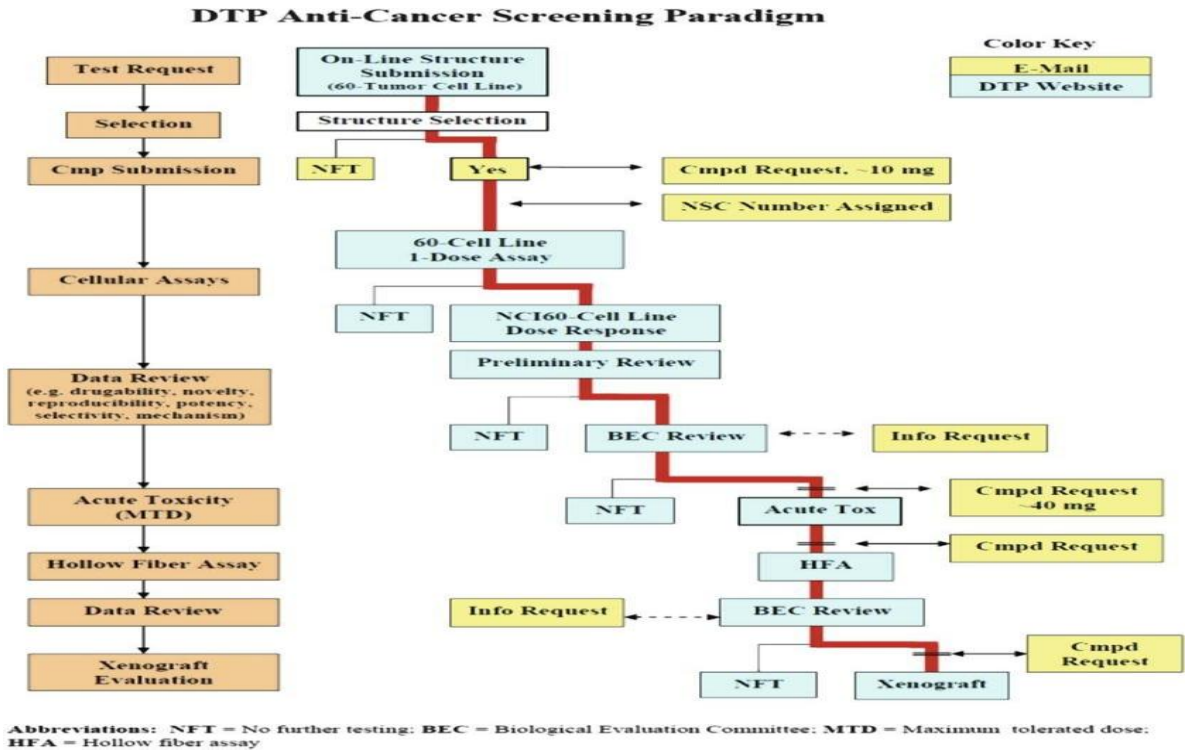


Fig. 1: Example of NCI 60-cell-line screening data. Shown is the sensitivity profile of bortezomib (681239) in 9 different tumor histologies on the basis of the 50% growth inhibition (GI50). Bars to the *left* indicate more resistant and bars to the *right*, more sensitive cell lines.

In- Vivo Methods

An enormous variety of different tumor systems for *in vivo* evaluation of new anticancer agents is available. Mostly murine host systems are used for experimental tumor therapy because of the availability of in-bred lines at relatively low costs, the ease of obtaining tumor models and established, widely accepted experimental endpoints. Spontaneous or transplanted murine tumors can be studied in immunocompetent mice whereas investigation of human tumors requires an immunodeficient host, *e.g.* nude mice, to avoid tissue rejection. Spontaneous tumor models offer some advantages over transplanted tumor cell lines, *e.g.* genetic diversity, growth in the original environment, angiogenesis more likely to resemble the situation in patients, but are rather difficult to obtain and maintain. Genetically engineered mice may help to improve this situation. Experimental data show that tumor characteristics such as growth rate and potential to metastasize depend on implantation site. Tumors injected orthotopically, *i.e.* into the organ of origin, apparently behave more similarly to the clinical situation. Also, the response to anticancer drugs may depend on the implantation site. For practical reasons, scientists mainly use ectopically-implanted, subcutaneously-growing

tumor models. Most pre- clinical data on new anticancer drugs were obtained using transplanted tumors in mice, frequently as xenografts of human origin. Considerable scepticism about the value of fast growing, ectopic tumors arose when some new drugs in clinical trails were not as effective as in the pre-clinical setting. However, detailed comparison of pre-clinical results and clinical data reveals that ectopically-implanted tumor models can be remarkably predictive when experiments are performed under clinically relevant conditions. Thus, these models can provide proof of principle, but the magnitude of effect does not necessarily correspond to the clinical situation.

Animal tumor systems have to meet several requirements to be suitable for experimental tumor therapy. It is very important that the tumor precisely reflects treatment response, and that the natural history of the host allows the study of the experimental endpoint, *e.g.* a sufficient life-span for follow-up to assess local tumor control. Stable biological characteristics of the tumor system such as expression of the molecular target, growth rate, differentiation and immune response are also required to assure the high quality of experiments. To avoid undetected changes of characteristics of tumor systems, it is indispensable that each researcher follows strict quality assurance protocols. The results obtained from quality assurance measures should always be reported. In our laboratory, we determine, for each experiment, the tumor identity by microsatellite assay, the human origin of the xenograft by LDH isoenzyme pattern, the growth rate of control tumors, histology and DNA index by flowcytometry.

- **Tumor growth delay assay**[7,8]

This functional assay is robust, standardized, widely accepted and used in most experiments to study anticancer agents *in vivo*. A delay in tumor growth would provide a benefit to cancer patients and is, therefore, an experimental endpoint of clinical relevance. A large body of experimental data clearly shows that the growth delay assay is a valuable tool to evaluate new anticancer agents in animals. The tumor growth delay assay provides evidence for further drug evaluation in clinical trials. Moreover, results from pre-clinical investigations in animal tumor models may help to design clinical trials, while detailed *in vivo* experiments may help in understanding the results from clinical trials. The results from pre-clinical and clinical studies on inhibitors of EGFR and angiogenesis demonstrate that animal models can prove the principle of a new therapeutic approach.

Knowledge and experience is required to correctly perform and interpret the growth delay assay. Usually tumors are allocated to two experimental groups. Animals of the

first group are treated with the anticancer agent. Animals receiving the so-called vehicle, *e.g.* the compounds and solutions that were used to prepare the drug solution, are controls. There is no consensus about the minimal group size to perform the tumor growth delay assay. Of course, the group size to detect a difference in tumor growth times depends on the magnitude of effect and intertumoral heterogeneity. Unfortunately, both factors usually are unknown when the experiment is designed. To account for intertumoral heterogeneity, it is useful to randomize the animals over the experimental matrix and to treat both experimental groups in parallel. As many tumor characteristics such as growth rate, cell loss, hypoxia, angiogenesis and response to anticancer agents may change with increasing tumor volume, it is necessary that the tumors in both experimental groups be enrolled into the treatment protocol at a similar tumor volume. Apparently most anticancer agents are more effective in smaller than in larger tumors. This is an important caveat because in clinical trials often patients with advanced stages and tumor masses are treated.

To determine tumor growth delay, the tumor volume is repeatedly measured, and for each individual tumor the time to reach a multiple of the starting volume, *e.g.* two, five or ten times the starting volume, is recorded. The calculated growth delay (tumor growth time of treated tumors minus tumor growth time of control tumors) is a direct measure of the drug effect on tumor growth. To generalize the data for comparison with other tumor models and drugs, the so-called specific tumor growth delay (ratio of growth delay to growth time of control tumors) is calculated. It is important to note that the endpoint of the tumor growth delay assay is a *time* to reach a volume but not a *volume* at a given time point. For many drugs the tumor growth delay increases with increasing endpoint sizes, because, in experiments with multiple drug administrations, the tumor growth delay increases with time because of the accumulation of drug effect. There is no consensus about the optimal endpoint size to report data from growth delay assays. If tumor cell kill is the major mechanism of action of an anticancer agent, dead and doomed cells and their clearance will contribute more and more to the tumor volume. Especially in slow shrinking tumors, this may mask the rapid regrowth of surviving tumor cells. Therefore, it appears that the smaller the endpoint size the more closely this will reflect the actual anticancer effect of the drug. Multiple administrations of antiproliferative agents probably result in an increasing tumor growth delay with time. In fast growing tumors, the effect on tumor growth rate is detectable only after some drug administrations and, thereby, at later time points. Thus, for antiproliferative agents, larger

endpoint sizes seem preferable. As the mechanisms of action of new drugs are usually unknown before the experiment, it is reasonable to analyze and report tumor growth delay with multiple endpoints.

Anticancer drugs may prolong tumor growth by several mechanisms. Agents may affect tumor cells directly or indirectly, *e.g. via* targeting stromal cells by inhibiting angiogenesis. Both directly and indirectly acting anticancer agents can reduce the tumor growth rate by inhibition of tumor cell production, increased tumor cell death, or improved clearance of dead and doomed cells. Determination of the mechanism underlying the anticancer effect of an anticancer agent by a simple tumor growth delay assay is impossible and requires more detailed *in vitro* and *in vivo* experiments. Whether a new drug affects proliferation or survival is of particular significance for designing more complex *in vivo* experiments and clinical trials.

- **Tumor control assay**[7,8]

In contrast to tumor growth delay, the results from the tumor control assay solely depend on the therapeutic effect on clonogenic cells. Permanent tumor control is the most relevant experimental endpoint for testing of potentially curative settings. In practical terms, after therapy tumors are followed-up and regrowth of the recurrent tumor is recorded. This requires sufficient follow-up times to detect virtually all recurrences. An alternative to this time- consuming procedure is the tumor-excision assay. For this, tumors are excised after treatment, a single cell suspension is prepared and cells are seeded into flasks or multi-well plates. After incubation, the fraction of surviving clonogens can be determined and compared with control tumors without treatment. Although this assay has the limitation that the survival of clonogenic cells is not determined in their original environment, the tumor-excision assay is less expensive than the tumor control assay because no follow-up is necessary and the number of animals required is smaller. Many of the new anticancer drugs reduce tumor growth but do not eradicate the tumor. Combination of new anticancer agents with potentially curative therapy modalities, such as radiotherapy, can improve the results compared with radiotherapy alone. For example, inhibitors of the EGFR or VEGF- dependent angiogenesis are not curative as a monotherapy. However the combination of these inhibitors with irradiation in animal models consistently resulted in longer tumor growth delay than either treatment alone. Administration of the VEGFR2 mAB DC101 to tumor bearing animals exposed concomitantly to fractionated irradiation improved the results of the tumor

control assay. However, results from tumor growth delay and tumor control assays are not necessarily consistent. BIBX1382BS is an potent inhibitor of the receptor tyrosine kinase of EGFR, resulting in clear-cut effects on tumor cell proliferation *in vitro* and *in vivo* using the human squamous cell carcinoma FaDu, which shows membranous expression of the molecular target, *i.e.* the EGFR. In combination with fractionated irradiation, tumors treated with BIBX1382BS showed a longer tumor growth delay than irradiated tumors or tumors treated only with BIBX1382BS (Figure 2). This clearly shows that the drug is also effective on the growth of irradiated tumors. However, BIBX1382BS did not improve the tumor control probability in the same tumor model.

The underlying reason for the discordance of the growth delay assay and the tumor control assay is unclear. From this example, it is quite obvious that an extrapolation of results from non-clonogenic assays to predict response of clonogenic cells can be misleading and may cause incorrect conclusions with far-reaching consequences for clinical trials. In our opinion, tumor control is the most relevant endpoint for pre- clinical testing of anticancer agents. Alternatively, large growth delay studies using different dose levels may yield results similar to those obtained from tumor control assays.

Monoclonal antibodies against EGFR have been shown to improve tumor control after radiotherapy in patients with head and neck cancer. Interestingly, xenografted FaDu tumors also showed a higher local tumor control rate after anti-EGFR antibody therapy with C225 and irradiation. Comparison of pre-clinical and clinical data of EGFR inhibition and radiotherapy corroborates the importance of detailed *in vivo* studies with suitable, well-characterized tumor models in a clinically relevant setting. Neglect of clonogenic endpoints might result in misleading strategies for further clinical testing. Although failure of new approaches in the clinic cannot be prevented by *in vivo* animal studies, consideration of data from carefully performed *in vivo* studies on efficiency, curative potential and optimal regimen are valuable for the design of clinical trials and the investigation of mechanisms of action.

- **NCI Analysis of Activity in Preclinical Models and Early Clinical trials[10,17,20]**

Xenografts: 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. 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 anti-cancer 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 development candi- dates from large pools of compounds with in vitro antiproliferative activity for expensive and time- consuming subcutaneous xenograft testing.

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.

Of 564 compounds tested in the HF model and that were also tested in in vivo xenografts, 20% showing HF activity also responded in xenograft models. This response was most likely if the intraperitoneal fiber activity was found in more than six intraperitoneal fibers. While a positive HF result could correctly predict in vivo xenograft response in one-fourth of the cases, 60-cell-line screening activity was able to predict correctly HF response in the order of 50%. Significant HF activity in more than six intraperitoneal fibers was likely if the mean IC_{50} for in vitro growth inhibition of a compound was below $10^{-7.5}$ *M*. These analyses showed that the HF assay is a very valuable, rapid model system with predictive value.

- **Relationship Between Clinical Response and Patient Explants in Nude Mice**[3,4,6]
- **The Freiburg Experience:** Unlike the NCI in vivo screen, the Freiburg xenograft panel is derived directly from patient explants and not established from permanent human tumor cell line material. By comparing drug efficacy in patients and their tumors grown in nude mice, a total of 21 patients reached a remission. The same result was observed in 19 tumors growing as xe- nografts. 59 patients did not respond to treatment and the same result was found in 57 cases in the nude mouse system. Overall, xenografts gave a correct prediction for resistance in 97% and for tumor responsiveness in 90%.

Although most analyses of predictivity and usefulness of in vitro and in vivo screening procedures indicate clearly a high value of anticancer drug screens, particularly if validated by employing agents that have made it to the clinic, it remains uncertain how the new molecular targeted agents with no prior defined clinical activity will translate into patient benefit. It also seems further to be certain that pure in vitro screening methodology will not be sufficient to delineate potential clinical activity, particularly because pharmacokinetics have a major impact on pharmacodynamic activity. Data derived from in vivo model systems deem necessary to ensure that drug concentrations inhibiting the target and in vitro cell growth to 100% or at least 50% can be reached.

2. CONCLUSION

Preclinical experimental pharmacology which involves the identification and optimization of novel chemical lead structures and testing on animal tissues or organs for their biological action, and the second stage, clinical pharmacology where testing of drugs is done on human volunteers and patients for assessing the pharmacokinetics, safety and efficacy in human.

Preclinical screening is necessary to prioritize compounds for further development. In the era of target-oriented molecular therapeutics, screening procedures are tailored toward the desired mechanism.

Standardized, well-established *in vivo* methods are available for experimental evaluation of new anticancer agents. A step-wise procedure from *in vitro* to *in vivo* seems reasonable to reduce the large quantity of potential drugs to a few promising agents for further clinical testing. The clinical application for which the drug is aimed, *e.g.* palliative, curative, tumor entity, or combination with other modalities, needs to be considered in the experimental evaluation. For evaluation of new anticancer agents, we advocate *in vitro* and *in vivo* experiments with at least two or three different tumor cell lines, applying functional non-clonogenic and, if applicable, clonogenic assays.

This review includes summary of In-vivo screening methods of Anticancer drugs which are obtained from many reference sources. It might be helpfull for as a reference source during many screening processes, Drug development Processess.

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