

Chapter 13

In-vitro Models in Anticancer Screening



Dhanya K. C., Aditya Menon, and Laxmi Shanker Rai

13.1 Introduction

Cancer is the abnormal growth of cells due to genome wide changes in gene expression. These alterations in gene expression lead to deregulation in the balance of cell proliferation and cell death. The genetically altered cells escape from the cell cycle checkpoints towards cell death and become immortal. They proliferate uncontrollably and ultimately evolve into cancer cells. These cells can invade nearby tissues and metastasize to distant sites, causing significant morbidity and, if untreated, death of the host. Even though it seems that cancer is a complex family of diseases, it is a disease caused by similar molecular defects resulting from alterations in genome from a molecular and cell biological point of view. Cancer may be considered as a genetic disease since it results from alterations within specific genes, but in most cases, it is not an inherited disease.

13.2 Treatment Modalities of Cancer

The conventional treatment modalities for cancer are surgery, radiotherapy and chemotherapy. Several new methods for specific and targeted cancer treatment such as Targeted therapies, Immunotherapy, Hormone therapy, use of Angiogenesis inhibitors, Gene Therapy, Virotherapy, etc. are now being emerged.

Dhanya K. C.

Department of Microbiology, St. Mary's College, Thrissur, Kerala, India

A. Menon

Origin Diagnostics and Research, Karunagappally, Kerala, India

L. S. Rai (✉)

Department of Mycology, Institut Pasteur, Paris, France

e-mail: laxmi.rai@pasteur.fr

As long as the growth of the tumor remains localized, the disease can be cured by surgical removal of the tumor. But malignant tumors tend to metastasize, that is, few cells break away from the original tumor mass, enter the lymphatic or vascular circulation, and spread to distant sites in the body where they establish lethal secondary tumors (metastases) that are no longer amenable to surgical removal.

Radiation therapy is used to eradicate cancer and as a palliative aid to relieve pain associated with metastases. Radiotherapy is the most common modality for treating human cancers. Eighty percent of cancer patients need radiotherapy at some time or other, either for curative or palliative purpose. The primary focus in radiotherapy is to increase DNA damage in tumor cells, as double strand breaks leads to cell death. To obtain optimum results, a judicious balance between the total dose of radiotherapy delivered and the threshold limit of the surrounding normal critical tissues is required.

Among the common therapeutic modalities of cancer, chemotherapy is the important one. In cancer chemo therapy, the chemotherapeutic agent exerts anticancer action through cytotoxic mechanisms (Gutman et al. 2000).

13.3 Need for the Discovery of Newer Anticancer Molecules

Most chemotherapeutic agents currently being used possess poor selectivity toward the target tissue and can harm normal cells as well as cancer cells. Therefore, it has several toxic side effects such as hematopoietic depression, gastrointestinal toxicity, hemorrhagic cystitis, hemopoetic suppression, nephrotoxicity, urotoxicity, cardiotoxicity and hepatic toxicity (Amudha et al. 2007). Cytotoxicity towards normal host tissue is the primary dose-limiting factor in chemotherapy that reduces quality of life and restricts treatment protocol. Cancer is one of the primary and most prevalent disease that cause morbidity and mortality in millions of people worldwide, there is undoubtedly an unmet need to discover novel anticancer drugs.

Thus there was a transition from cytotoxic chemotherapy to molecularly targeted cancer drugs. This has resulted in an increasing number of successful therapeutic agents. However, for many patients the therapeutic options are still limited and the process of bringing a new effective drug is still frustratingly slow.

However, the traditional process of identifying a new drug and its development is lengthy and expensive. Discovery and development of anticancer agents are the key focus of many pharmaceutical companies and government and non-government organizations, like the National Cancer Institute (NCI) in the United States, the European Organization for Research and Treatment of Cancer (EORTC), and the British Cancer Research Campaign (CRC).

13.4 Anticancer Screening: General Methodology and Conventional Methods

Initially cancer was recognized as a disease of uncontrolled cell division and thus all efforts to derive an anticancer drug were directed toward the identification of antiproliferative compounds. Regression of tumor size was the primary objective in preclinical and clinical testing. Rapidly growing murine models of cancer were developed and used for screening of new compounds in the drug discovery programs and resulted in identifying several clinically important anticancer compounds. Thus, treatment successes occurred mainly in the rapidly growing malignancies such as lymphomas, childhood leukemia, germline tumors, etc. and relatively fewer successes for the slow-growing solid-tumors such as lung, breast, and colorectal cancers (Suggitt and Bibby 2005). Thus the investigators had to modify the prescreening and screening protocols so that a variety of cell lines and tumor types were included.

Schwartzmann et al. (1988) noted that from over 600,000 compounds screened only less than 40 agents reached to the clinic. The recent advances in molecular sciences, genomics and proteomics have generated several potential new drug targets for anticancer drug screening.

Conventional anticancer drug screening focused and selected agents that had significant cytostatic or cytotoxic activity on tumor cells and caused tumor regression in murine tumor models and the drugs were discovered either by serendipity or as metabolic pathways inhibitors.

Although this strategy has achieved significant success, the drugs identified often possessed significant side effects and toxicity. The recent developments in molecular biology of cancer made researchers to come up with target-based drugs. They devised molecules pre-designed to inhibit and/or modify a selected molecular marker important in cancer growth and several target-based drugs have emerged.

Conventional screening models for anticancer agents aim to select cytotoxic drugs based on animal screening models via analyzing tumor regression and survival, while the human clinical trials are utilized to determine the dose. Toxicity and tumor-regression effects of cytotoxic agents are based on similar mechanism.

The molecularly targeted agents act on the extra cellular, transmembrane, or nonnuclear intracellular processes and a few examples are receptor tyrosine kinase inhibitors, matrix metalloproteinase (MMP) inhibitors, farnesyl transferase inhibitors, etc. (McKeage 2008). These agents often cause tumor growth inhibition, rather than regression, in animal models.

An important element of preclinical and clinical screening of molecularly targeted agents is the investigation on their solubility, stability in the solid state and solution, pH solubility and stability studies, identification of degradation pathways, absorption studies in cell culture models and animals, etc. The anticancer activity is prescreened in-vitro in cell culture models by cell growth inhibitory or clonogenic assays. Further studies are performed in murine allograft or human xenograft mouse models (Amundson et al. 2008).

Furthermore, high throughput preclinical screening methods and mathematical models to explain the mechanisms of drug activity are being adopted.

13.5 Strategies of Anticancer Activity Screening

Cancer patients need better anticancer drugs and for an efficient drug discovery process, reliable screening methods are necessary. These methods should not only detect the compounds with the higher therapeutic potential, but also predict whether such potential is optimal to deserve additional attention.

Large-scale screening using animal systems as practiced in the past is highly unethical and, particularly in Europe, strictly regulated. In majority of cases, either cellular or target-based high-throughput assays will precede in-vivo evaluation of potential anticancer drugs.

Preclinical screening is also important 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. Thus, rational drug design or drug discovery approaches combined with novel knowledge from genome and proteome research as well as bioinformatics are promising. The drug screening and discovery pathways have evolved into an integrated approach which combines the use of cell line and tumor xenograft models that resemble very closely the patient characteristics and response (Fiebig et al. 2007). Target-driven drug development has led to the availability of many useful cell signal transduction inhibitors and antibodies targeting growth factor receptors.

Before any potential anticancer agent is subjected to human testing, the prospective drug undergoes a series of qualifying studies. Initially, primary in-vitro screening is conducted, using the NCI60 human tumor cell line anticancer drug screening protocol, whereby a potential candidate is evaluated for its ability to inhibit the growth of tumor cells in culture. This modern pharmaceutical in-vitro screening was developed by the National Cancer Institute (NCI) and comprises a panel of 60 human tumor cell lines. Currently, the NCI60 is the most commonly used system for the preliminary screening of potential anticancer drugs (Shoemaker 2006).

Once in-vitro screening gives promising anticancer activity, then it undergoes in-vivo animal testing. This phase of evaluation is critical for understanding the in-vivo antitumor activity and is mainly conducted using inbred laboratory mice (Workman et al. 2010); however, other animals such as dogs and primates are also often utilized. Mouse tumor models are utilized for conducting cancer research and screening potential anticancer compounds due to similarity of human and mouse genomes, low cost of maintenance, short gestation period, rapid reproduction rate, and the ease of growing implanted tumors.

Higher vertebrates, such as primates, are avoided in routine toxicological studies due to high cost and ethical issues.

13.5.1 In-vivo Screening: And Disadvantages

Animal models have been indispensable to conducting further research to understand the cancer biology and develop anticancer drugs. The animal models can be divided into two classes: models in which tumors are transplanted into mice, and other where tumors develop in-situ, either spontaneously or induced.

13.5.1.1 Early in-vivo Test Models

The first in-vivo test model used for the screening of anticancer compounds was the mouse leukemia models grown as ascites tumor (Teicher 2009). In Human tumor mouse xenotransplantant models human tumor cells, cultured in-vitro, are implanted to immunodeficient mice (Rygaard and Poulsen 1969). When the tumor reaches a given size, introduction of a potential anticancer drug is made and the efficacy of the drug is determined by changes in the tumor size.

Autochthonous tumors either arise spontaneously or can be induced by carcinogens or other chemical, viral, bacterial, or physical triggers (Workman et al. 2010). Genetically engineered mouse models enable testing of drugs within an immunocompetent tumor microenvironment (Damia and D’Incalci 2009).

Due to differences in the genome of mouse and human, a question raised about relevancy of using rodents in drug screening. For example most murine cells have functionally active telomerase unlike human cells and also changes in certain genes like TP53, Rb and Ras, etc. Therefore, results obtained in laboratory testing using mouse model may not be directly extrapolated to humans. A personalized medicine screening approach where biopsied tissue are used to screen antitumor substances instead of tumor cell lines will lead closer to the actual clinical conditions (Blatt et al. 2013).

13.5.2 In-vitro Screening Model for Cancer

Discovery of a new drug and its establishment is a complex, time consuming and expensive process. Therefore, drug screening under in-vitro conditions are more advantageous as compared to in-vivo models. However, majority of the promising drug candidates identified during in-vitro studies failed under preclinical and clinical stage of drug development. This lack of success is due to the fact that the in-vitro models used in oncology, 2D in-vitro model of cancer cell lines (CCLs), are not able to represent the tumor complexity and also sensitive to chemotherapy.

Conventional in-vitro drug screening test for the analysis of viable cells may not distinguish tumor cells from normal cells (do not differentially analyse tumour cell/normal cell viability). Conventional viability (e.g. MTT, Alamar Blue) or cytotoxicity (e.g. LDH release) assays do not distinguish normal/accessory cells and cancer

cells. Assays that can distinguish tumor cells from normal cells measure cell proliferation rather than viability and require radioactivity (e.g. ^3H -thymidine incorporation, Cr release assay) or involve laborious steps (e.g. flow cytometry). Following are the in-vitro models used in various experimental setups.

1. Cell-Based Screening Assays
2. Biochemical Screening
3. Nonmammalian Model Organisms
4. In-vitro tumor models
 - (i) *Transwell based assays*
 - (ii) *Spheroids*
 - (iii) *Hybrid models*
 - (iv) *Tumour microvessel models*
5. Hollow Fiber Assay
6. Colony-Forming Assay
7. In-silico or bioinformatic methods

13.5.2.1 Cell Based Screening Assays

The human tumor cell lines have been used in cellular screens during in-vitro cancer research. How cell viability and activity of anticancer drugs are measured is an important factor.

Several procedures are employed to determine cell growth or viability. The broadly used growth inhibition assay which was developed by Mosmann and the NCI screening staff, is 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 (Mosmann 1983). It has a limitation of usage of large quantities of hazardous solvent, dimethyl sulfoxide. Thus the sulforhodamine B (SRB) assay was developed, SRB is a dye that stains protein. There are also fluorescence or luminescence detection assays where propidium iodide (PI) staining for DNA or use of a luciferase reporter can be utilized (Dengler et al. 1995).

Other methods for the determination of cell viability such as trypan blue dye exclusion assay and the lactate dehydrogenase assay can give false results when a time delay in scoring result or if the drug under study affects on intracellular activities (Kumar et al. 2016).

Currently one-dimensional or monolayer cultures are used to measure cell growth. These methods also have limitations for in-vitro evaluation of certain anticancer agents such as Selection of cytotoxic drugs, lack of extracellular matrix and angiogenesis, Gradients of oxygen tension, extracellular pH, nutrients, catabolites, etc. and drug penetration barriers. Cancer stem cell-targeted drugs and inhibitors are screened using cancer stem cells.

13.5.2.2 Biochemical Screening

Biochemical assays allow evaluation of large numbers of compounds (Aherne et al. 2002) in a fully automated manner. Suitable platforms currently under use are enzyme-linked immunosorbent assays (ELISA) or enzyme-based colorimetric methods, radiometric assays, fluorescence based methods, and luminescence detection methods. More recently, fluorescence resonance energy transfer (FRET) techniques which are suitable for cell-free and cellular systems are developed (Tian et al. 2007).

13.5.2.3 Nonmammalian Model Organisms for Screening

Non-mammalian organisms such as yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans*, or fruit fly *Drosophila melanogaster* were used for anticancer drug screening in the late 1990s since they share similar signaling and growth regulatory pathways with humans (Hartwell et al. 1997). The advantage of yeast is that the complete genome comprises only 6250 genes, and many genes involved in human tumors have homologs in this organism. For example, the p53 tumor suppressor gene has its structural homolog in RAD9, the cyclins D and E in cyclin DDm and cyclin EDm the mismatch repair genes MSH2 and MSH1 in MSH2Sc and MLH1Sc, respectively.

13.5.2.4 In-vitro Tumor Models

In-vitro tumor models have proved itself as an important tool for low-cost screening platform for anticancer drugs. Better understanding of cancer progression and treatment have been an impetus in rapid advancement in creating in-vitro models with increased accuracy and stability. This has resulted in, in-vitro models becoming progressively complex and diversification of output parameters. Advances in tumor cell biology, 3D cell culture, tissue engineering, biomaterials, microfabrication, and microfluidics have resulted in rapid development of new in-vitro models. Current model incorporates multiple cell types, extracellular matrix materials, and spatial and temporal introduction of soluble factors.

In-vitro models have been developed to provide a working insight into tumor growth, proliferation, angiogenesis and drug delivery. The variable factors of in-vitro models are cell source, biophysical properties, extracellular matrix and biochemical signals.

The molecular profiles of a large number of human cancer cell lines are available in the Cancer Cell Line Encyclopedia (Barretina et al. 2012), and these profiles can be compared to the profiles of a large number of human tumors. Using such characterised cells to develop an in-vitro model is an ideal method for recapitulating specific aspects of the tumor microenvironment.

13.5.2.4.1 Advantages of in-vitro Tumor Models

Primarily in-vitro screening is a cost effective method compared to in-vivo models. Advances in 3D cell culture, tumor cell biology, biomaterials, microfabrication, tissue engineering have increased the complexity of in-vitro tumor models. This has resulted in an increase and diversification in output parameters. The requirement of precision medicine for cancer have led to increase in adapting in-vitro models for drug screening.

13.5.2.4.2 Transwell-Based Models

This is a method to assess the ability of cancer cells to migrate and invade. This process is relevant in metastasis. Migration is the movement of cells, which may be random or influenced by matrix, soluble factors or electric field. Invasion on the other hand is the ability of the cells to traverse through an extracellular matrix (ECM) (Katt et al. 2016). There are three main adaptations of Transwell based assay,

1. Migration assays
2. Invasion assays
3. Transendothelial migration assays

Drug screening and study of migration, intravasation, extravasation and matrix remodelling can be done using transwell-based assays and this is primarily achieved by counting the cells. The applications include Studies of the influence of chemoattractants on migration and invasion, Studies of influence of other cell types (e.g., macrophages and fibroblasts), Testing the influence of knockdown, transfection, and antibody treatment on invasion and migration, Assessing drug therapies in reducing invasion (Yang et al. 2016), etc.

13.5.2.4.3 Spheroids

Spheroids are cells grown in suspension or embedded in a 3D matrix using 3D cell culture methods. Cancer cell spheroids are known as multicellular tumor spheroids (MCTS). They represent avascular tumor nodules or micro-metastases (Friedrich et al. 2009). Spheroids which bear necrotic cores mimic poorly vascularized tumors due to the establishment of oxygen and nutrient gradient. The protein and gene expressions profile of such spheroids are similar to clinical and in-vivo conditions.

There are four methods of spheroid culture

1. Suspension culture
2. Non-adherent surface methods
3. Hanging drop methods
4. Microfluidic methods

Spheroids are primarily used to study following processes.

13.5.2.4.3.1 *Cell Function*

The behaviour of tumor cells in an avascular tumor microenvironment include growth kinetics, tumor cell biology and composition of different types of cells. Invasion of cells can also be studied using spheroids (Hirschhaeuser et al. 2010).

13.5.2.4.3.2 *Drug Screening*

Studies comparing gene expression profiles of spheroids and 2D cultures revealed that spheroids closely resemble in-vivo tumor models, thus enabling it to be used in a high-throughput tool for negative selection of drug candidates to reduce animal testing (Friedrich et al. 2009).

13.5.2.4.3.3 *Angiogenesis*

Assessment of migration of endothelial cells into tumor spheroids or the formation of vascular networks in the spheroids (Timmins et al. 2004).

13.5.2.4.3.4 *Immune Cell Response*

Culturing MCTS with immune cells and observing migration and invasion. Co-culturing spheroids of tumor cells and immune cells and studying their interactions (Gottfried et al. 2006).

13.5.2.4.4 Hybrid Models

There are various types of in-vitro tumor models which cannot be classified as spheroids or transwell-based. These include,

1. Ex-vivo tumor sections
2. 3D invasion models
3. Avascular microfluidic models

The main feature of these models are that they recreate the tumor microenvironment although retaining the simplicity of in-vitro models. Various applications are as follows

1. Embedded ex-vivo Tumor Section developed from patient biopsies or resected tumor sections, can be used to probe the tumor microenvironment. This model is used for the studying fundamentals of tumor growth and invasion (Dark et al. 1997). Primary identification of individualised chemotherapeutic regimens can also be examined using this model.
2. 3D Invasion Models developed by seeding cancer cells on ECM materials reduce the complexities of tumor microenvironment. This helps in imaging of live cells to study cell morphology, cell interactions and movement (Koch et al. 2012).

3. Avascular Microfluidic Model is used to assess cancer cell migration through small channels with respect to chemotherapeutic agents (Konstantopoulos et al. 2013).

13.5.2.4.5 Tumor-Microvessel Models

Tumor vasculature is an important component of a tumor microenvironment, providing nutrients. The cells of these vessels also secrete factors promoting or suppressing tumor growth. Tumor-microvessel models recreate the complex interplay of vasculature and cancer cells under in-vitro conditions. Cancer cell movement through the vasculature can be visualized in real time and studied for various behaviors like invasion, tumor-driven angiogenesis, intravasation, and extravasation (Wang et al. 2015).

All the in-vitro models can be used for specific purposes and will have advantages and disadvantages to other models. A diagrammatic representation of in-vitro tumour culture models are given in Fig. 13.1. Advantage and disadvantage of each in-vitro model is given in Table 13.1.

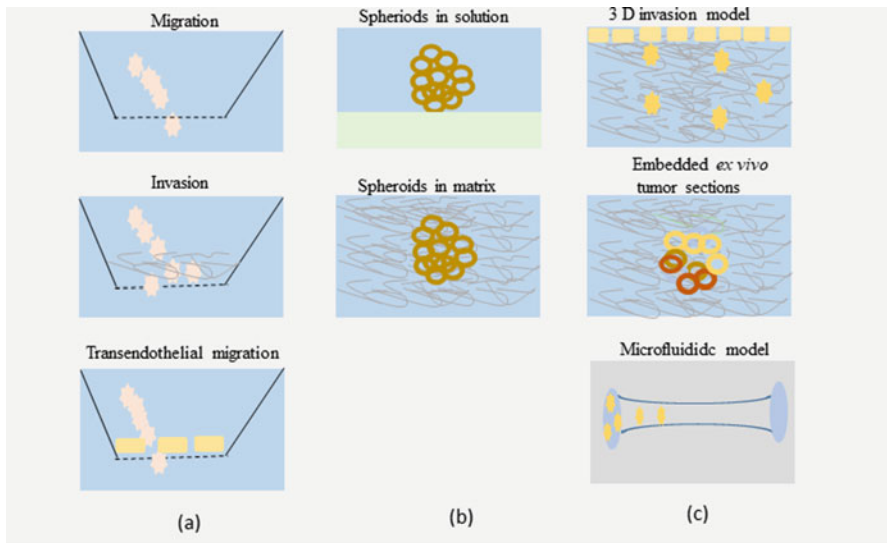


Fig. 13.1 Cartoon depict different types of transwell-based motility assays. (a) Migration, invasion, and transendothelial migration. (b) Spheroid-based assays – spheroids in media and in matrix. (c) Hybrid models – embedded ex-vivo tumor sections, 3D invasion models, and avascular microfluidic models

Table 13.1 Advantages and disadvantages of various in-vitro tumor models (Katt et al. 2016)

	In-vitro model	Advantages	Disadvantages
Transwell model	Migration	Easily implemented, low-cost assay	Low physiological relevance
	Invasion	High throughput	Can only assay single-cell motility
	Transendothelial migration	Can be used to compare metastatic potential of cells	Migration and invasion assays can result in conflicting data
Spheroid	Cell suspension culture	Simple	No individual compartments for spheroids
		Mass production	Cannot control uniformity (size, composition)
		Long-term culture	High shear force
	Non-adherent surfaces	Simple	Low throughput
		Better efficiency (MW)	Long-term culture difficult
		High throughput (MW)	
		Uniform spheroid size (MW)	
		Coculture (MW)	
	Hanging drop technique	Control of spheroid size	Low throughput
		Uniform spheroid size	Long-term culture difficult
		Allows coculture with defined cell types	Not efficient
	Microfluidic devices	Control of spheroid size	Difficulty collecting cells for analysis
		Control of spheroid growth parameters	
		Continuous perfusion	
		Faster spheroid	
Hybrid tumor microvessel model	Embedded ex-vivo tumor sections	Maintains tumor heterogeneity	Lacks flow through vasculature
		Patient-specific assay	
		Mimics outgrowth into surrounding tissues	
	3D invasion models	3D microenvironment	Lacks vasculature
		Allows real-time tracking of cells	Lacks tumor complexity
		Balance of complexity and experimental control	
	Avascular microfluidic	Simple migration assay	Lacks vasculature
		Easy to isolate effect of variables	Typically lacks 3D environment
		Allows real-time tracking of cells	

13.5.2.5 Hollow Fiber Assay

Hollow Fiber Assay (HF) is a combined in-vitro/in-vivo testing procedure. The HF assay was developed by Decker et al. (2004) at the NCI and is composed of 2 cm tubes consisting of polyvinylidene fluoride (PVDF) filled with tumor cell lines implanted into mice at two sites (intraperitoneal and subcutaneous). The mice undergo drug treatment and fibers are removed after 4–6 days from the animal and analyzed in-vitro for tumor cell growth.

The Hollow Fiber Assay Bridge the gap between in-vitro and in-vivo xenograft screening of anticancer compounds (Shnyder et al. 2005).

One disadvantage of HF assay is that tumor growth is inhibited by the inside diameter of the tube and another drawback is that the fiber wall is an artificial barrier between the tumor and its environment which hampers the diffusion of large bio-molecules, such as DNA and antibodies.

13.5.2.6 Colony-Forming Assay

Another combined in-vitro/in-vivo testing model is the soft agar colony-forming assay, it is also known as tumor clonogenic assay (TCA). The TCA can be used for sensitivity screening of patient tumor material in-vitro for predicting clinical response and also with fresh xenograft tissue for selecting the most appropriate in-vivo model for analysis (Chumsri et al. 2007). The development of the human tumor stem cell (HTSC) assay offered an approach to determine and predict a reasonable clinical response and is disease-orientated in concept.

13.5.2.7 In-silico or Bioinformatic Methods

Developing a new drug is a complex, expensive and time-consuming task. Many novel technologies and methodologies have been developed to increase the efficiency of the drug discovery process, and computational methodologies or bioinformatic techniques have become a crucial component of drug discovery programs. Techniques such as ligand- or structure based virtual screening are widely used for designing potential anticancer drugs and drug candidates (Grinter et al. 2011).

Inverse docking is computational docking of a specific small molecule of interest to a library of receptor structures. The technique can be used to identify new potential biological targets or to identify targets among a family of related receptors (Prada-Gracia et al. 2016).

The in-silico studies or computational drug design approaches and mathematical-/statistical-based modeling provide insights into the physicochemical properties associated with anticancer activity at the molecular level. These are classified mainly into Structural based and Ligand-based techniques. These are widely used for the in-silico screening of large chemical databases, whose aim is to detect novel bioactive

ligands. Models for constructing predictive molecules and separating active from inactive ligands are developed using certain optimization methods such as neural networks, genetic algorithms, and the k-nearest neighbor algorithm (Shahaf et al. 2016, Pappalardo et al. 2017). An interesting example of structure-based pharmacophore modeling is the identification of p53 upregulated modulator of apoptosis (PUMA) inhibitors (Mustata et al. 2011).

13.6 Conclusions

The limited availability of clinical samples and high cost in performing clinical trials has made in-vitro tumor models to be the premier pre-clinical tool for cancer studies. These models are relatively cheap, easily manipulated and accumulated data from these cell lines can be used in cancer pharmacogenomics studies. In-vitro models have already proved their worth in cancer research by helping to easily identify pharmacogenomics biomarkers and enabling the testing of hypothesis. By improving the pre-clinical cell line models, the translation of clinically and pharmacologically relevant markers from the research laboratory to the hospital setup can be accelerated. 3D tumor models and other hybrid models have taken cancer research to new heights. Further advancements in in-vitro tumor models will enable in developing personalized tumor research systems and therapies, and improve their clinical translation efficiency.

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