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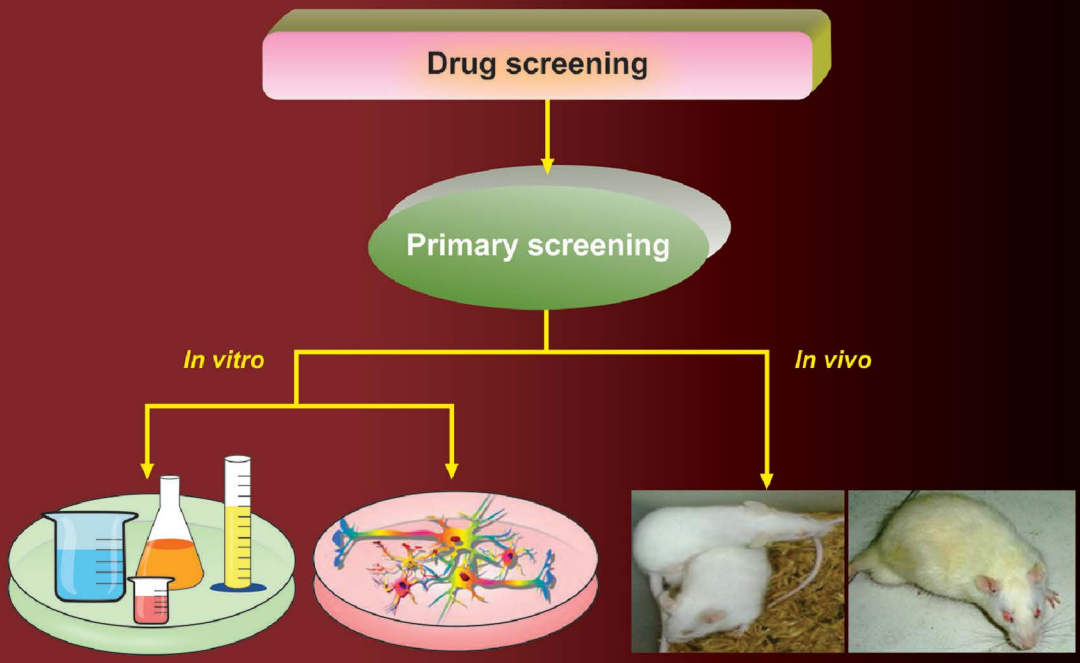
3rd Edition

DRUG SCREENING METHODS



SK Gupta

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CHAPTER

2

High Throughput Screening for Drug Discovery

INTRODUCTION

The increasing burden of new diseases like cardiovascular disorders, diabetes, immune system related disorders, diverse microbial infections are demanding new drugs to control them in an improved manner. The rise in the occurrence of treatment failure has also increased the strain of the drug discovery scientists for the discovery of novel compounds with better treatment outcomes. As in the case of antibiotics, an innovation gap of almost 50 years came from the rapid discovery era of beta lactam class of antibiotics, still patients are being treated with the congeners of the antibiotics discovered almost 80 years ago. Rapid discoveries of novel drug targets have increased the use of high throughput screening in a potential manner to meet the pace with biochemical or pathological findings.

In the domain of drug research target identification, purification and assay development constitute the initial step. In the next step, the potential compounds are screened against the identified target. Conventionally this has been a slow and tedious manual process requiring huge investment of manpower, time and money. Traditional techniques like test tube analysis and chemical analysis of the intermediate biomolecules may lead to the loss of the precious samples and may not be sensitive enough to capture the minute information that may help in the due course. Consequently, the conventional drug discovery program has been called as a slow process.

WHAT IS HIGH THROUGHPUT SCREENING (HTS)?

The last two decades have seen astonishing innovations in technology that have helped the manual low speed screening to evolve into an automated, microprocessor controlled robotic process called 'High Throughput Screening (HTS)'. This recent process is a synergy of chemistry, biology, engineering and informatics. HTS has helped to speed up conventional languid process and now over 50,000-1,00,000 compounds can be screened per week, against the validated biological target. Further advancements are making it possible to screen 10,000-100,000 compounds within 24 h time and the process is called as ultra-high throughput screening (uHTS). High throughput synthesis of large number of test compounds in a lesser time is also a reality now. Simultaneous development in other areas such as drug synthesis, toxicity screening, drug metabolism and pharmacokinetics studies (DMPK) are helping the process to achieve its ultimate speed in new drug development (NDD). After the fundamental

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biological research, the effective target is identified and validated for its function. Then the method development takes a few months to initiate screening process. In HTS, the trend is to replace radio labeling by simple luminescence and fluorescent techniques. Since the yield of target protein or purified biological target is generally low (often obtained only in a few milligrams), technologies that permit screening with reduced volumes (3-20 µl) and reduced protein/ligand have played a pivotal role in facilitating HTS. Development of detection techniques having ultra high precisions are used in these assays to give more valuable information about the ligand-protein interaction. The conventional 96-well plates have been replaced with 384-well plates and subsequently by 1536-well plates with low internal volume to make the screening possible at a high speed. Nowadays, rapid development of negative screening method is also in place for earlier detection of the ligand affinity with known targets leading to adverse reactions, drug interaction, altered intestinal permeation, etc. Revolution in every individual process towards drug development is bridging the gap between older concepts with newly optimized robust and technology enabled methods for its quick and economic implementation in drug discovery platform.

HTS—POSITIVE AND NEGATIVE SCREENING

In the drug development screening **Hit** and **Lead** are two frequently used terminologies. **Hit** is explained as: "a molecule with confirmed activity from primary HTS assay with a good profile in secondary assays and with confirmed structure," and **Lead** is explained as**:** "a hit series exhibiting the Structure Activity Relationship (SAR) and demonstrating activities both *in vitro* and *in vivo*."

IN VITRO INTERACTION STUDIES (ARRAY BASED)

In vitro assays have now been miniaturized from the test tubes to well plates having capacity of assaying samples starting from traditional 96 well format to 1536 wells. Microfluidics has impacted the interaction study area in a surprising way and has drastically decreased the need of both reagents and samples. Chemical analysis on the microarray plate for the quick and economic identification of the biological target is evolving as a good tool.[1] The accuracy and speed of the system has made these techniques a great success. Still the system is not completely free from errors

1. Different chemicals have different functional groups
2. Difference in the diffusivity of the samples

Chemical microarray technology has come up with great successes in the evaluation of chemical–protein interactions, enzyme activity inhibition, target identification, signal pathway elucidation and cell-based functional analysis. Their success can easily be seen from the Genomic and proteomic studies where almost 10,000 targets for the drug activities have been found out; however, less than 500 of these targets have turned up in approved drug candidates.

Chip based microarray: Chip based microarrays have started becoming popular these days. They are made in the same way as semiconductors. The microfluidics helps in the mixing of samples with the targets bound on the chip, finally signals are read by the analyzers that can be fluorescent or simple UV or visible signals.

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Other microarrays: So many other microarrays are also available in the market. These arrays are specific for some of the important biomolecules in the physiological or pathological pathways, e.g. Cytokine based kits, angiogenesis responsive bimolecular kits, transcription factor analysis kits, etc. They work on the principle of the binding of the biological samples with the antibodies bound to the array membrane, incubation, binding to the secondary antibodies and finally tagging of the secondary antibody with the signal emitting molecules such as dyes.

FLUORESCENCE TECHNIQUES IN HTS

Fluorescence (FU) based detection of the compounds, analytes or genetic materials has now become a well-established technique (Table 2.1). In HTS the interaction of ligand with the biological compartment is elucidated by luminescence-based binding assays. Various fluorescence techniques like Fluorescence Anisotropy (FA), Fluorescence Correlation Spectroscopy (FCS), Fluorescence Intensity (FI), Fluorescence Lifetime Imaging Microscopy (FLIM), Fluorescence Resonance Energy Transfer (FRET), Total Internal Reflection Fluorescence (TIRF) and Time Resolved Resonance Anisotropy (TRRA) are used. Along with these techniques, certain specific nano bead-based techniques like Scintillation Proximity Assay (SPA), Amplified Luminescence Proximity Homogeneous Assay (ALPHA) are also used (Fig. 2.1).

Detection system for the fluorescent based assays has also developed hand in hand with the techniques. Currently the instruments are available which are capable of screening a single reaction well for different modes starting from UV, Visible to FU and chemiluminescence. These instruments are known as multimode readers (Fig. 2.2). These instruments are cartridge based and having the advantage of replacing the cartridge as per the need. FU microscopes

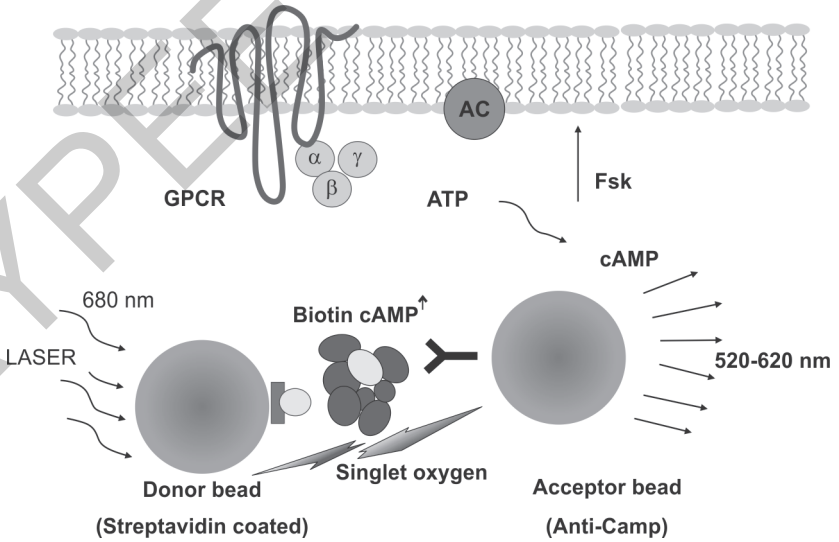

Figure 2.1: cAMP detection using ALPHA screen principle. The ALPHA screen kit includes streptavidin coated donor beads and acceptor beads conjugated with an antibody to cAMP. Biotinylated cAMP is also included as a positive control and for competition with unlabeled cAMP (*For color version see Plate 1*)

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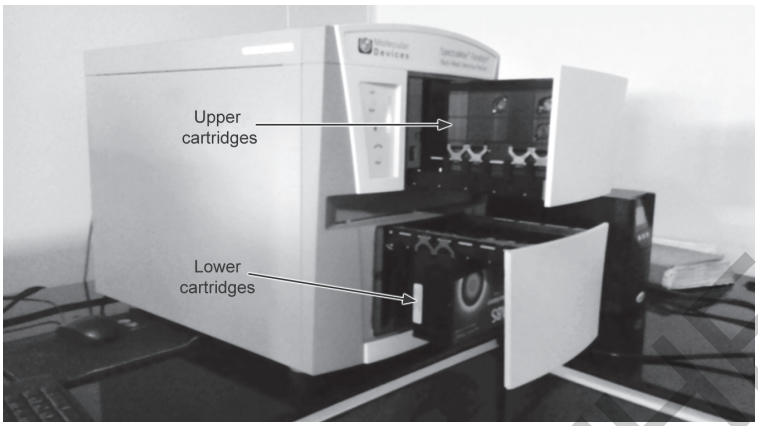

Figure 2.2: Photograph showing multimode reader (*Courtesy* – Ocular Pharmacology & Pharmacy; Dr RP Centre, AIIMS)

are also playing a big role in the drug development. FU microscopes help in the identification of the entry of the fluorescent tagged compound at the desired site in the cell. FU microscope also helps in the identification of the drug effect on to a particular cellular component or biomolecule. Immunofluorescent technique depends on the FU microscope for the detection of the fluorescent bound antibody on the tissue sectioned glass slide.

Table 2.1: Fluorescent techniques used in drug discovery process

Technique	*Principle of working*	*Area of application*
Fluorescence anisotropy (FA) or fluorescence polarization (FP)	Fluoroprobe is exited using plane polarized light and emission is recorded parallel to plane polarized light. Binding of the fluoroprobe with receptor would restrict the rotational mobility of it and would exhibit a higher polarization.	Receptor ligand binding assay[2,3]
Time resolved fluorescence anisotropy (TRFA)	Records the change in the rotational diffusion of phosphorescent-labeled membrane receptor when they bind to a ligand. Also delivers information on protein structural fluctuations	Screening of chemical compounds[4]
Fluorescence correlation spectroscopy (FCS)	Used for mass-Dependent and independent fluorescent assays. Can be used to study the protein-ligand interactions on the membrane bound receptors in live cells	Screening of chemical compounds
Fluorescence resonance energy transfer (FRET)	Works on the interaction of two different chromophores via dipole-dipole mechanism, excited donor chromophore transfers its excitation energy to a closely located acceptor chromophore	Interaction of the chemicals or drugs with biomolecules[5]
Time resolved Fluorescence resonance energy transfer (TR - FRET)	Combines the advantage of fluorescence resonance energy transfer along with the time	Interaction of the chemicals or drugs with biomolecules[6,7]
Fluorescence microscopy	Based on the excitation of the fluorescent molecule bound to the cellular surface and visualizing under a magnifying object lens	Cellular or tissue structural analysis
Real time PCR	Works on the ability of the dyes to emit fluorescence signals via binding to a double stranded DNA	Gene expression analysis
Fluorescence Lifetime Imaging Microscopy (FLIM)	Sample is illuminated with a pulsed laser and the lifetime of the fluorescent probe is determined from the phase shift between the modulation of the excitation light and the emission of fluorescence	Analysis of the cellular components[8]

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FLOW CYTOMETRY FOR CELL-BASED ASSAY

A flow cytometer (FCM) is an instrument which illuminates the cells or particles as they flow in form of droplets in front of a light source and then reads the illumination. FCM was first described as an analytical tool in 1960. After that FCM changed a lot for it's utilization in the drug discovery and diagnosis.

Principle of working: FCM has several components which work in tandem to produce desirable results.

1. ***Light source:*** Nowadays most of the FCMs contain laser light as a source for the illumination. Lasers are preferred because of their intense and narrow beams. They may be gas lasers (e.g. argon ion laser or helium neon laser) or solid state lasers (e.g. red or green diode laser, new blue and violet lasers). These lasers would be generating defined and fixed type of wavelengths (e.g. 488 nm for argon ion laser). Usually a flow cytometer would be having one laser light source but advance machines may have more as well.
2. ***Fluidics:*** In FCMs particles need to be suspended in the fluid so that a single particle can be analyzed at a time. Concentrated cell suspension may form aggregates of the cells, even so called single cell suspension cells may form clumps. Hence fluidics is designed in a way to decrease the chances of aggregation and to facilitate similar illumination of each cell. The strategy used by Crosland-Taylor for the confinement of the cells in a focused, narrow flow stream (known as hydrodynamic focusing) is applied to achieve this. The sample stream is injected into the rapidly flowing stream (sheath stream) in a "flow cell" which has a narrow exit orifice. This difference in the diameter increases the velocity of the entire stream and confined the path of the cells tightly to the center of the laser beam.
3. ***Detectors:*** Photodiode is fitted in the line of the illuminating laser beam with an obscuration bar in front of it. The laser light which has been bent by the cell can only reach the detector after avoiding the obscuration bar, it is known as forward scatter. The forward scatter light is not well defined in the terms of biology or chemistry of the cells because of the confusion in the refractive indices of the live or dead cells and the medium. One lens is fitted to the right angle to the direction of the laser beam and light collected by it is known as side scatter light or 90° light scatter.

- **Applications**: Since the development of the first FCM in 1960s, a tremendous work has been carried out in the hematological discoveries. One or two color FCMs helped in the identification of cellular (T-cells) and humoral (B-cells) cells lineages, followed by CD3, CD4 and CD8 identification by multicolor flow cytometer.[9,10] These days flow cytometry has also been utilized in the vaccine development by intracellular cytokine staining assays and further cell sorting for their genomic as well as transcriptional analysis.[11] In previous 10 years possibilities of the *in vivo* flow cytometry has also been tried by several research groups from around the world. By using very high speed, high resolution, continuous CCD or CMOS cameras, images have been taken from the rat mesenteric blood and lymphatic vessels.[10] Several compounds have been tested in the *in vivo* flow cytometric analysis of the blood and lymphatic vessels. Natural property of the lymph vessel and valve function has also been studied by the *in vivo* flow cytometry.[12]

THERMAL SHIFT ANALYSIS FOR HTS

Thermal shift analysis of the proteins has now become a good tool for the identification of the ligand interaction with the proteins in a rapid and high throughput manner. This method

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needs a fluorescent dye which associates and dissociates from the protein with the change in the temperature of the reaction. It is based on the principle of binding of the ligand to a protein and stabilizing or destabilizing its structure which ultimately changes the melting temperature (Tm) of the protein. Sypro orange is such a dye which binds to the protein in a temperature dependent manner. Fluorescence of sypro orange is quenched in the aqueous environment but when protein unfolds and expresses its hydrophobic core then dye binds to it and starts emitting fluorescence. Finally fluorescence is monitored and plotted versus temperature. Thermal shift analysis can be used to assess the following:

1. Role of inhibitor in the binding affinity of the ligand with protein.
2. Stability of the protein at different pH and salt conditions.
3. Ligand screening for the binding with the protein for the lead identification.

Thermal shift assay has helped in the identification of the inhibitors of the carbonic anhydrase enzyme.[13,14] Carbonic anhydrase is a zinc metal containing enzyme which is involved in the dehydration of the bicarbonate. This enzyme is also involved in the several pathologies like glaucoma, epilepsy, Alzheimer's and Parkinson's disease hence serves as a potential drug target. Thermal shift assay has also helped in the identification of the estrogen receptor antagonists (Toremifene and tamoxifen) as anti-cryptococcal agents. The assay showed that these drugs directly bound to the calmodulin (cam1) which was purified from *Cryptococcus neoformans* and prevented it from binding to its substrate calcineurin (Cna1) and blocked its activation.[15] Further thermal shift assay helped in the evaluation of the mitogen activated protein kinase inhibitor 4 (MAP2K4) which activates pro-invasion signaling pathways in the prostate cancer.[16]

ROLE OF MASS SPECTROMETRY IN THE HIGH THROUGHPUT SCREENING

Mass spectrometry is an important tool in the clinical and medical development area. Almost hundred years have passed when Nobel laureate Sir J. J. Thomson first described about mass spectrometer in Cambridge Philosophical Society. From the time of its initial development, mass spectrometer changed a lot but became suitable for the biological field only after the development of the electrospray ionization (ESI) technique by John Fenn which fetched him a Nobel Prize in 2002. After this development mass spectrometer evolved as an important tool in the field of biology. Coupling the separation capabilities of the liquid chromatography with the structural analysis abilities of mass spectrometer gave a third dimension for the quick and accurate identification and quantification of the compounds. Information dependent acquisition (IDA) available with some of the mass spectrometers gives the freedom of the identification of the unknown from the pool of several molecules by available mass spectras of the known one. Thus the mass spectrometer has now become an essential tool for the drug development in a rapid and accurate manner.

Phytochemical extract analysis: Analysis of the natural product extracts has always been a tedious task. This process needs the identification of the gross chemical group, purification, enrichment and structure elucidation of the final compound. Once the process is done, the compound is again screened pharmacologically. This process is having one drawback that compound recovered at the end may fail to show any pharmacological activity. The reason behind this is that the separated and enriched compound might not be responsible for the pharmacological activity found with crude extract at the beginning. Mass spectrometry has given a solution for the above problem—information dependent scans of the extract

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and identification of the hit. It works on the basis of the available structural and functional (pharmacological) information for the similar natural product group compounds. Once the pharmacological activity on the crude extract is done, the library is constructed for the compounds similar in the pharmacological activity and from the similar natural product group. The extract is screened for the diverse mass ranges (from few Daltons to almost one thousand Dalton) and the fragmentation pattern of the similar molecular mass compounds (hit) is matched with the available mass spectra of the known compounds. The same technique was used by Kyadari et al for the identification of lyngbyatoxin-A and malyngamide-J in the marine algae extracts.[17]

Purity analysis of the drugs or synthesized compounds: Mass spectrometry can very well be used for the purity analysis of the compounds. Purity analysis of the drug formulation or the synthesized molecules gives important information regarding the improvement in the formulation or synthesis process. In case of drug formulation impurities may lead to therapeutic failure or even adverse events due to toxic metabolites or contaminants. The stability and impurity profile of latanoprost drug formulation was evaluated by Velpandian et al using LC-MS/MS.[18]

IN VIVO IMAGING OF DRUG ACTION (NEAR INFRARED IMAGING)

It is a newly added method to evaluate the drug action and quantification without killing the animal. Rapid development in this technology is expected to revolutionize the *in vivo* studies that warrant the killing of the experimental animals. Fundamental of this technique is using molecules capable of emitting light in higher wavelength near IR region thereby, it gains the capability of crossing biological membrane and to be detected by sensitive camera turned for near IR region. For this application an exogenous contrast agent like indocyanine green (ICG) is used. *In vivo* near IR fluorescence imaging has been successfully utilized in vascular mapping, and tissue perfusion studies, to image vasculature of brain, for conducting angiograms of eye, imaging tumors, biodistribution studies, gastrointestinal tract (using endoscopic optics), inflammation, atherosclerosis, cell death, and osteoblastic activity, etc.[19] For all the studies excitation was achieved by using pulsed diode laser having different fluence rate above 700 nm and emission was recorded using powerful cooled CCD capable of monitoring near IR emission radiation.

Recently, GFP (Green fluorescent protein) or Ds Red2 genes are making a rapid entry in the biological research. For tumor imaging Ds Red2 expressing CR8 Lewis-lung carcinoma cells were injected into nu/ne mice. Seven to ten days after inoculation of cells Cy-annexin V conjugate was injected intravenously to the mice bearing tumors and submitted for imaging near infrared signal. Using cyclophosphamide, Petrovsky et al[20] evaluated the usefulness of active Cy-annexin that can be used as a near infrared probe to image apoptosis from outside an intact living animal and facilitate the antiproliferative drug screening.

Other Whole Body Imaging Techniques

Single photon emission computed tomography (SPECT) is a highly sensitive nuclear medicine tomographic imaging technique using gamma rays. It is very similar to conventional nuclear medicine planar imaging using a gamma camera. However, it is able to provide true

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3D information. This information is typically presented as cross-sectional slices through the organism, but can be freely reformatted or manipulated as required.

SPECT uses gamma-emitting nuclides, which undergo a radioactive decay with the emission of gamma radiation. In order to attain a nuclear stability, the nucleus of an unstable atom undergoes a process of transition in which neutrons are converted to protons and vice versa. This process leads to the formation of daughter nucleus in an excited state. This state is unstable; therefore, it reverts back to the ground state and loses some form of energy in terms of electromagnetic radiation or gamma radiation, e.g. Technetium-99m (^{99m}Tc), Iodine-123 (^{123}I), cobalt-60 (^{60} Co).

SPECT imaging involves the administration of radiotracers into the biological system and the radiations emitted from the radiotracer in the body are captured by the gamma cameras with sodium iodide crystal detector and photomultiplier tube (PMT) in which the radiations are converted into scintillating photons and amplified and the images at different angles are reconstructed by the computer in such a way that the imaging of the organ of interest is achieved.

Recently, SPECT has been utilized in the study of involvement of drug efflux transporters like multidrug resistance associated protein (MRP), P-glycoprotein (P-gp) and lung resistance protein (LRP) as a contribtuting factor for multidrug resistance in cancer therapy in patients.[21] In research, due to the ease of small animal imaging with SPECT, it is been utilized to evaluate and to visualize the modulation of P-gp or MRP using (99m) Tc - sestamib as a model substrate.

For the first time from our laboratory, SPECT imaging has been utilized to explore the intraocular uptake of fluoroquinolones following systemic administration by such drug efflux transporter (Fig. 2.3) in the eye using ciprofloxacin labelled^{99m}Tc as a radiotracer in rabbits.[22] This study was carried out in presence of GF 120918, which is a P-gp modulator and verapamil, a known P-gp as well as OCT inhibitor.

VIRTUAL SCREENING (*IN SILICO* DRUG DEVELOPMENT OR DRY LAB)

It is totally an indirect approach using advanced computer technology to screen newer compounds based on virtual coordinates of receptor and ligands. Computer-aided molecular design (CAMD) approach involves computational analysis of large data set in order to highlight those compounds most likely to be active in the actual assay, so that a focused

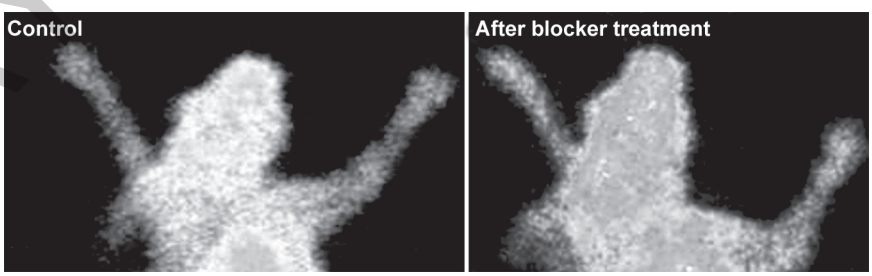

Figure 2.3: Pictures of SPECT-CT showing the intraocular uptake of radiolabeled ciprofloxacin after blocker (verapamil) treatment. Dual head SPECT–CT with collimator-low energy (*Courtesy*: Dept. of Ocular Pharmacology & Pharmacy, AIIMS and INMAS, New Delhi) (*For color version see Plate 1)*

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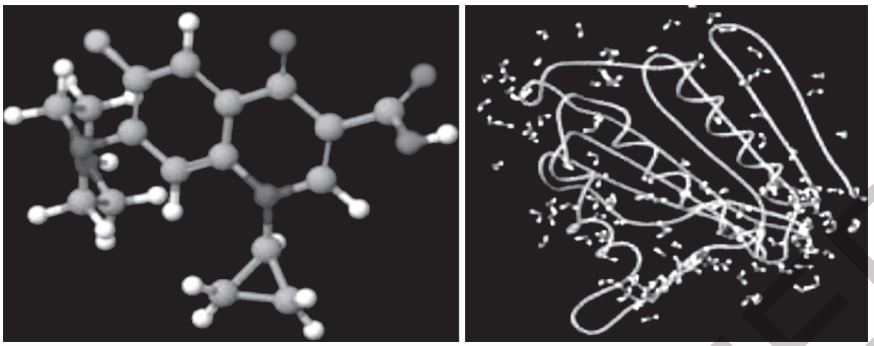

Figure 2.4: Computer-aided molecular design approach (*For color version see Plate 2*)

subset of compounds can be selected. CAMD covers a wide range of technologies leading to very fast property predictions through more computationally elaborate modeling of drug-receptor binding. Using receptor-based properties, such as binding affinity and receptor selectivity, CAMD calculates to propose a broad range of properties that are likely to be useful in drug design—from physical properties like molecular size and solubility to indicators of developmental issues like metabolic fate and toxicity, etc. Therefore, virtual screening can filter out undesirable compounds on the basis of a wide variety of criteria, depending on the problem in hand. Virtual screening needs the 3D molecular structure of the receptor along with the 3D structure of ligands to perform docking. However, this approach is highly applicable for the lead optimization process since for many newly isolated receptor's conformational data may not be available. Moreover, the techniques like X-ray crystallography and NMR studies are necessary for the information about spatial arrangement and virtual coordinates of targets and ligands.[23] Figure 2.4 showing the docking of ciprofloxacin on DNA gyrase using CaChe (Ver.6.1, Fujitsu, Japan).

The applicability of computer models has also used completely empirical and statistical model like the Rule of Five or Lipinski's rule. According to this rule, a drug like compound looks like a molecule with a molecular weight less than 500, OH and NH groups less than 5, the sum of N and O atoms less than 10 and log P value less than 5 for a better absorption in the intestine.

HTS-SCINTILLATION METHODS IN DRUG SCREENING

Apart from fluorescent techniques, the advancements in using the radioactive compounds to understand molecular mechanisms are also very interesting. One of such attempts is named as scintillation proximity assay (SPA). This system uses the principle that an antibody or a receptor molecule, which is bound to a bead, emits light when beta emission from an isotope occurs in close proximity, i.e. when a radiolabeled ligand binds to the bead with receptor or antibody (Fig. 2.5). Amersham has introduced several advancements in these techniques leading to make it as a successful high throughput screen. The basic technology of the SPA is based on the fluorescent signal produced by a scintillant-dyed polystyrene or polyvinyl toluene microsphere that could be excited by the proximity of a radiolabeled molecule. The scintillant-dyed microspheres are in the size range of < 2 μm in diameter. The recent improvements in this technique are yttrium silicate and yttrium oxide beads which enable the use of higher number

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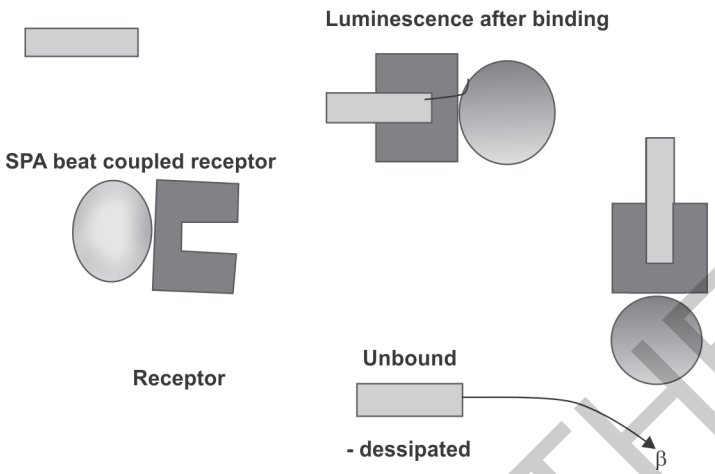

Figure 2.5: Principles of scintillation proximity assay (SPA) (*For color version see Plate 2*)

of wells in which the signal can be picked up by highly sensitive readers containing CCD camera instead of classical photomultiplier tubes. So far, this technology is applied in several attempts in drug discovery processes like evaluation of kinetics of protein kinase inhibitors, to evaluate neurotransmitter transporter inhibitors, to evaluate novel farnesyltransferase inhibitors, to identify poly (ADP-ribose) polymerase-1 inhibitors (enzyme involved in DNA repair), evaluation of HIV-I reverse transcriptase inhibitors, etc. SPA is a homogeneous, rapid, versatile and amenable to automation, which can also simplify the screening protocol in the drug discovery process.

UNCONVENTIONAL RAPID PHARMACOLOGICAL AND TOXICITY TESTING MODELS

Zebrafish (Danio rerio) and Gold Fish (Carassius auratus auratus)

Drugs working on the central nervous system (CNS) are having a choice of being tested on the marine organisms like fishes. Antipsychotic class of drugs have been discovered without having any target approach. Still mechanism is unknown for many of them. For these classes rational approach may not work but behavioral approaches may work for the screening of newer compounds. Gold fishes have mainly been used for the CNS depressant and stimulant drug analysis. It was an attempt of the Vijayakumar et al[24] to develop a model for the screening of the drugs for their effect on CNS. Gold fishes were exposed to the low concentration of known CNS depressant and stimulant drugs and their swimming pattern (optomotor response) was assessed along the gyro-dots of different colors. Similarly, toxicity of the ethambutol on the eye was also evaluated using the gold fishes.

Zebrafishes are used mainly for the angiogenic and anti-angiogenic effects of the compounds on the vasculature of this marine organism. The reason of the extensive use of the zebrafish is the development of optically clear embryo provides the opportunity of the visual inspections and very high egg laying capacity of the female zebrafish.[25] Zebrafish has been utilized for the better understanding of the Alzheimer's disease.[26] Zebrafish has been used in the hematology

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research because of the conserved genetic factors regulating blood development and visualization of the circulating erythrocytes with only a dissecting microscope. Their organ regeneration capacity made them popular model in the regeneration experiments like heart regeneration.[27] As zebrafish express cytokines, macrophages, neutrophils, dendritic cells, mast cells, eosinophil, T-cells, and B-cells; it has also been used in the host pathogen interaction studies as well.[28]

Chick (Gallus gallus domesticus)

Use of chick for the drug screening is now a well applied model in the drug development. Extraembryonic membrane serving gas exchange known as Chorioallantoic membrane has a network of blood vessels (Fig. 2.6A). Chorioallantoic membrane can be used for the evaluation of the angiogenic or antiangiogenic potential of the compounds. One important factor for the use of chick for these studies is the non-requirement of the ethical approval. Fertilized egg can be obtained from the hatchery and can further be developed as per need in the incubator at the laboratory. Several marine isolates have been studied for their anti-angiogenic effect on the chorioallantoic membrane of the chick.[29]

The size of the lens and the time taken for the development of the model has made hydrocortisone-induced cataract in the chick a good model (Fig. 2.6B). This model has been used for the screening of the larger groups of the compounds for their anti-cataract activity on the lens. Several polyherbals have also successfully been studied using the same technique.[30,31]

Caenorhabditis elegans

Currently in toxicity and pharmacological studies *C. elegans* are being utilized extensively. Availability of whole Genome sequence, transparency of the tissues at all the developmental stages, availability of large mutant species and shorter life span has made *C. elegans* a very good model for biological as well as disease and drug effect research. *C. elegans* was used for the evaluation of the Genistein for its life span enhancement ability by Lee et al (2015). Genistein is a dietary phytoestrogen present in the seeds of Vigna angularis cultivated in East Asia. The phytoestrogen was tested on the nematode *C. elegans* in both normal and stress conditions

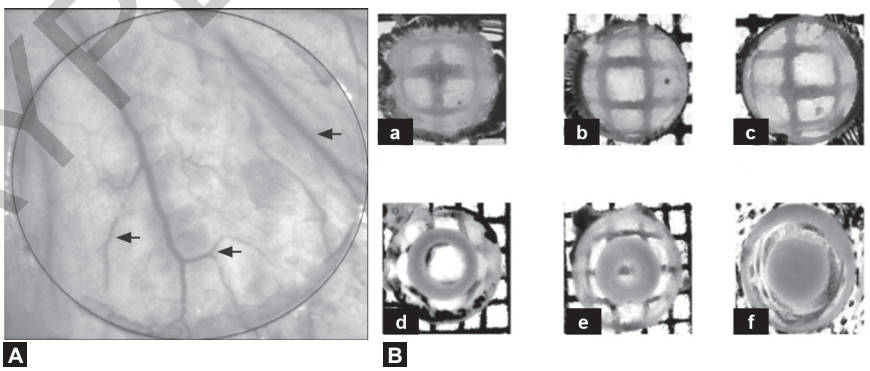

Figure 2.6: Photographs showing use of chick for pharmacological screening of compounds: (A) Black arrows showing developing blood vessels in the chorio allantoic membrane of the chick under a coverslip (black circle). (*Courtesy*: Gupta et al (2014); (B) Figures a,b and c are showing normal lenses; d—stage 2 cataract; e—stage 3 cataract; f—stage 5 cataract; *Courtesy* Velpandian et al (2003)

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produced by heat and oxidation. Genistein could able to enhance the life span of the nematode in both the conditions.[32] In another study the growth retardation effect of caffeine on the fetus was assessed on the larvae of the *C. elegans* by Min et al. They found that the caffeine was able to hinder development at most of the stages in a dose dependent manner.[33]

HTS PHARMACOKINETIC STUDIES

HTS-absorption Studies

Oral route is a preferred way of drug administration. In the drug development process it is very important to screen the drugs for gastrointestinal absorption. Conventionally, it is a very lengthy and time-consuming process. Moreover, this process also requires a large number of animals. Intestinal absorption is mainly due to the concentration dependent diffusion. If the drug passes through paracellular route, then there is not much of a hurdle. Some compounds, like cyclosporine and digoxin, are substrates of well recognized P-gp transporters, which belong to the ATP Binding Cassette (ABC) transporters having Walker's motives. P-gp substrates were found to have variable bioavailability. Therefore, it is necessary to screen the drug candidates during the developmental stage to ensure that they are not substrates for P-gp. Colon cancer cell lines (CaCo) grow confluently and form a monolayer upon polycarbonate support or collagen coated polycarbonate support.[34] They are quite suitable for performing intestinal permeation studies and to elucidate the drug candidates susceptible for P-gp efflux mechanism in the intestine. Moreover, they also express CYP3A4 enzyme allowing prediction of intestinal metabolism. This process can be automated to examine the intestinal absorption characteristics of drugs in a shortest possible period to decrease the incidence of failure in the Phase I clinical trial in human volunteers.

PARALLEL ARTIFICIAL MEMBRANE PERMEABILITY ASSAY (PAMPA)

In order to assess the intestinal permeation, apart from cell-based assays like CaCo monolayer cells, PAMPA is an alternative. It is a passive-permeability screen with focus on the simulation of transcellular processes. It is reported to be an excellent compliment to cellular models in absorption, distribution, metabolism, excretion (ADME) screening of research compounds. It is a fast, versatile and low-cost method that is compelling and biologically relevant model of transport. Its principle is based on the application of physicochemical properties of molecules to predict intestinal permeability.[35] In PAMPA, a 96 well microtiter plate completely filled with aqueous buffer solutions is covered with a hydrophobic filter coated with lipids in an organic solvent solution in a sandwich construction. Typically**,** Hwang et al[36] prepared the membrane by wetting with 5 µl of the artificial membrane solution (0.8% egg lecithin in n-dodecane). The donor and recipient solutions consisted of phosphate-buffered saline (pH 5.5 or 7.4) with 2% DMSO (donor contained 200 µM test compound in each well). These plates were incubated for 2 hours at room temperature with gentle shaking, following which drug concentrations in the receiving solutions were assayed by HPLC. Percentage transport across the lipid bilayer (%T) was calculated by %T= 100 x Ar/Ad (Ar and Ad correspond to the HPLC peak areas of the receiving solution and initial donor solution). Using this method they have predicted their model drug supposed to be absorbed via passive diffusion across the human gastrointestinal

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