High-Throughput and *In Silico* Screening in Drug Discovery

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Abstract

The process of drug discovery involves multiple branches of science. Discovery of novel molecule with biological modulation activity is a time-consuming and expensive process. High-throughput and *in silico* tools can reduce time and cost in drug discovery. The aim of high-throughput screening is to identify bioactive molecule from large compound collection and further development of active compounds to leads. There are two types of assay in high-throughput drug discovery: biochemical- and cell-based assays. Choice of assay depends on nature of target and assay feasibilities. Assay method should detect active compound from chemical library. Assay optimization and validation steps reduce false-positive and false-negative results. The assay results must be statistically validated to ensure reliability of results. The good assay design and implementation will give optimal results. *In silico* tools in drug discovery facilitate hit identification, hit to lead development, and optimization of druggability (improvement absorption, distribution, metabolism, excretion, and toxicity properties). High-throughput and *in silico* screening can be streamlined for hit identification and lead development. Streamlining of these methods reduces cost and time of drug discovery process. The wise use of these high-throughput methods can lead to discovery of drug with good potency and low toxicity profile.

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11.1 Drug Discovery and High-Throughput Screening (HTS)

Drug discovery is a complicated and interdisciplinary process to identify novel drugs. First step of drug discovery is identification of competent drug target. Proteins are the main target class in drug discovery. Mostly proteins such as enzymes, receptors, and ion channels are targeted in drug discovery. Second step is target validation. Target validation is attained through biochemical assays and animal model experiments. After target validation, compounds modulating target will have to be identified. Next phase is assay development to screen modulators. Modulators that bring about dose-dependent target modulation are called lead compounds. Common pharmacophore can be developed from lead compounds showing common chemical properties. Structural activity relationship can be accessed and molecular descriptors can be optimized to improve selectivity and drug likeness of lead compounds; this process is known as lead optimization. Optimized lead compounds become potential candidates for drug development. These compounds are evaluated using animal models, and the successful compounds are selected for clinical trials (Carnero 2006).

The drug discovery process usually takes 10–12 years and costs over 1.5 billion USD (http://cen.acs.org/articles/92/web/2014/11/Tufts-Study-Finds-Big-Rise.html.). Among 1 million compounds screened, only 250 lead compounds enter into preclinical testing, 10 of them advance to clinical trial, and only 1 will be approved as drug by Food and Drug Administration (FDA).

High-throughput screening (HTS) involves testing of large number of chemical substances (natural products or synthetic compounds) against biological target. HTS identifies chemical compounds called hits that modulate drug target. Hit compounds are starting point of drug discovery. High-throughput screening is broadly defined as the testing of 10,000 to 100,000 compounds per day (Carroll et al. 2004; Wölcke and Ullmann 2001). HTS analyzes catalytic activity of enzymes, receptor ligand binding, opening and closing of ion channels, and phenotypic changes in cell (McDonald et al. 1999; Seville et al. 1996; Verma et al. 2004). HTS uses 96-well microtiter plate designed for serological studies (Sever 1962). Usually, HTS assays are carried out in 96-, 384-, or 1536-well microtiter plates.

In the early 1990s, advancement in combinatorial chemistry, genomics, and development of technologies like homogenous assays, high-density microplate, high-performance microliter dispensers, and imaging and laboratory automation was the driving force of HTS. In this period, colorimetry was used for enzymatic assay. ATP consumption readouts and bioluminescence technology were also developed. In the late 1990s, combinatorial chemistry focused on the enrichment of privileged motifs to create libraries of pure drug-like compounds, and scientists focused on assay development and quality controls. In the twenty-first century, availability

Fig. 11.1 Sequential steps involved in the high-throughput screening

of the human genome sequence provided opportunity to identify potential human drug targets (Carnero 2006).

The HTS process is a subset of the drug discovery process and can be described as the phase from target to lead (Fig. 11.1). The target of choice must be biologically relevant, and assay method must be robust. Compound showing desired biological activity should be detected by screening methods from the chemical library. False negatives and false positives can be minimized in the assay by setting low variability and high signal to background. There must be sufficient amount of cost-effective reagent available to run the entire screening campaign. Otherwise, it will impasse HTS operation (Macarron and Hertzberg 2011).

HTS assays can be divided into two categories, namely, biochemical assays and cell-based assays (An and Tolliday 2010). Biochemical assays include enzyme inhibition and receptor-ligand binding assays. The biochemical assay evaluates binding affinity or specific binding of compounds against biological target in an artificial environment. Purification is not possible for some biological targets. This limits application of biochemical assay in HTS. Besides, biochemical assays can't represent precise cell-specific responses against small molecules. Cell-based assays monitor drug effects on biochemical pathways or more specific targets (Sharma et al. 2010). Cell-based methods provide more biologically relevant microenvironment and direct information about cell permeability of compounds as well as acute cytotoxicity associated with the compounds.

11.2 Detection Methods in HTS

The most common assay readouts used in biochemical assay for HTS are optical, including absorbance, fluorescence, luminescence, and scintillation (Ramm 1999). Fluorescent or luminescent mechanism is highly amenable, which can be modified to different fluorescent assays (Inglese et al. 2007). Fluorescence happens on a timescale ranging from 10^{-9} s to 10^{-4} s, thereby allowing for light's many optical properties to be exploited by a number of detection methods (Lakowicz 2006).

Fluorescence Polarization or Fluorescence Anisotropy It is a solution-based homogeneous technique that allows rapid and quantitative analysis of diverse molecular interactions and enzymatic activities. FP uses polarized light to excite molecules in solution. Free fluorescent molecule shows fast tumbling, and it will be reoriented to prior light emission condition and consequently will show a low polarization value. But receptor-bound fluorophore that tumbles slowly will show a high polarization value (Trinquet and Mathis 2006). The fluorescent polarization

approach has been adapted to almost every protein class like GPCRs, nuclear receptors, and enzymes. It can be used for the analysis of molecular interactions studies including protein-ligand, protein-protein, and protein-DNA binding events. It is also used for monitoring enzymatic reaction progress (Alpha et al. 1987).

Fluorescence Resonance Energy Transfer (FRET) It is an energy transfer between a fluorophore donor and a suitable fluorophore energy acceptor (Stryer 1978). The absorption spectrum of the acceptor should overlap with the emission spectrum of the donor. Two fluorophores are in close molecular proximity of each other to allow significant FRET. Most common FRET readouts are based on fluorescence variation between donor and acceptor. In cell-based HTS, genetically incorporated fluorescent indicators are used to understand signal transduction dependent on protein phosphorylation (Sato et al. 2002).

Bioluminescence Detection method is prevalent in HTS assays because of convenient detection and high sensitivity. In bioluminescence assay, ATP derived from enzyme-coupled reaction is detected by a reporter enzyme (luciferase from firefly *Photinus pyralis*) that acts on luciferin substrate to create a luminescent output. This method is free from compound interference and 1000 times sharper than equivalent fluorescence-based assays (Fan and Wood 2007). Luminescent-based assays have been used to access the activity of cytochromes P450, proteases, and monoamine oxidases (Sato et al. 2002). Luciferase can be mutated to develop multiple luminescent sensors in an assay. The development of dual luciferase reporter assays with different kinetics or emission maxima and substrate specificities has been used for identification of activities specific to the signaling pathway of interest (Davis et al. 2007). Luciferase reporters can be clumped with other detection formats, for example, combining green fluorescent protein (GFP) reporter (Bandyopadhyay et al. 2006), with β-galactosidase or alamarBlue (O'Boyle et al. 2005) to estimate cytotoxicity.

Bioluminescence Resonance Energy Transfer (BRET) It is a hybrid system involving the donor and acceptor (Pfleger and Eidne 2006). In BRET donor is a luminescent molecule excited by the enzyme (Renilla luciferase), and acceptor can be a fluorescent protein like a green fluorescent protein or yellow fluorescent protein. The use of an enzyme as an excitation source eliminates interferences from autofluorescent compounds and inner filter effects. BRET has been using as tool for the study of GPCRs by investigating receptor oligomerization or activation (Trinquet and Mathis 2006).

Disassociation-Enhanced Lanthanide Immunoassay (DELFIA) This method uses lanthanide ion chelates (europium or terbium), as fluorescent probe (Hemillä et al. 1984). The chelate, having longer fluorescent life, forms non-covalent association with a lanthanide ion and protects it from potential quenching by its environment. It acts as an antenna by transferring energy to lanthanides from the excitation

source. UV-excited fluorescent probe emits their fluorescence 500 and 700, depending on lanthanide used, which has fluorescence more than 100 μs. Time-resolved fluorescent detection by pulsed excitation source rejects background produced by chemical compound, biological media, and instrumentation components.

Fluorescent lanthanide chelates are not stable in biological media. To overcome this limitation, two step-based heterogeneous assays were developed. In the initial step, nonfluorescent chelates associated with biomolecule conjugates were used to carry lanthanides during the biological reaction. In the second step, lanthanide ions are removed from nonfluorescent chelates by adding huge amount of different fluorescent chelating agents (Terpetschnig et al. 1995). DELFIA is free from fluorescent background, so very low detection limits can be achieved (Trinquet and Mathis 2006).

Homogenous Time-Resolved Fluorescence (HTRF) It combines FRET with time-resolved fluorescence detection. HTRF uses long lifetime FRET donor, cryptic lanthanide, which is formed by incorporating rare earth ion (e.g., europium) into ligand. This is called cryptand, it protects lanthanide ion from potential quenching by the environment and transfers energy to lanthanide ion from excitation source (Alpha et al. 1987). UV-excited europium has longer fluorescence (100–1000 μS) having wavelength range between 550 and 710 nm. Usually cross-linked allophycocyanin (XL665) is used as acceptor. Long lifetime of europium donor allows time-resolved fluorescence measurement. Clear distinction can be made between long-lived FRET signal of europium-associated acceptor and short-lived signal of freely diffusing acceptor, which allows separation of FRET signal from background.

Cryptic signal is independent of optical properties of test media at excitation wavelength. The signal ratio between acceptor signal and europium cryptate depends only on biological events under probe. High efficiency obtained in HTFR allows monitoring variety of biological interaction like enzyme activities and protein-protein interaction. It is independent of compound interference. It can be easily automated and miniaturized to wells format for the HTS to screen large chemical compound library (Kon et al. 2004; Mathis 1993).

Fluorescent Correlation Spectroscopy (FCS) It measures intensity fluctuations. The detection volume of this technique is around one femtoliter. Confocal microscope is needed to observe the reaction (Pope et al. 1999). In this assay, the fluorescent molecules are passed through signal and only through detection volume measured; therefore, signal is related to the diffusion coefficient of the sample. Autocorrelation techniques analyze fluorescent intensity fluctuations, which give information regarding the concentration of fluorescent molecule, their diffusion through detection volume, and the average brightness per molecule (Moore 1999). Receptor-bound fluorescent molecules show around tenfold increase in variation in diffusion time. FCS is free from compound interference. It is suitable for miniaturization in HTS (Trinquet and Mathis 2006).

Alpha (Amplified Luminescent Proximity Assay) Screen It uses a donor bead and acceptor bead (Seethala and Prabhavathi 2001). Both beads are bearing a biological partner under examination. When both beads under investigation come close together, the laser excitation of photosensitizer in the donor bead results in the generation of the singlet oxygen, which reacts with thioxene derivatives in the acceptor bead and generates chemiluminescence. This activates fluorophore, and activated fluorophore subsequently emits fluorescence. It can be used to investigate large molecule interaction and large number of targets. Low detection limit can be achieved in Alpha Screen. It can be easily miniaturized because of its homogenous format. Antioxidants and metal ions can affect measurement.

Beta-Lactamase Reporter System It can be used for study of mammalian expression system. It makes use of TEM-1 beta-lactamase which lacks 23 amino acids at the N-terminal end. This enzyme uses CCF2/CCF4 as substrate composed of dyes, 7-hydroxycoumarin-3-carboxamide and fluorescein, bridged by cephalosporin (Zlokarnik et al. 1998). De-esterified CCF2/4 trapped in the cell can easily be detected by FRET, which generates green-colored fluorescence. Cleavage CCF2/4 by beta-lactamase causes loss of FRET, which results in blue-colored fluorescence. CCF2/4 substrate enables ratiometric data analysis (the net blue fluorescence signal intensity and the net green fluorescence signal intensity ratio; em. 460/em. 530) that helps minimize variation in cell number or substrate concentration because the emission maxima of the cleaved and intact beta-lactamase substrates are distinct. Cytotoxicity can be assessed using uncleaved substrate (Qureshi 2007)

11.3 Assay Formats in HTS

There are two assay formats for HTS: biochemical and cell-based formats. Biochemical assays are designed to find compounds that interact with an isolated target *in vitro* environment. Cell-based assays assess phenotypic effect of compounds on the cell. HTS-compatible technology has developed to measure G protein-coupled receptor (GPCR) (Schroeder 1996) and ion channel function (González et al. 1999); confocal imaging platforms for rapid cellular and subcellular imaging and the continued development of reporter gene technology lead to development of user-friendly cell-based assays. For most drug discovery programs, both cell-based and biochemical assays are required for hit discovery, characterization, and subsequent lead optimization. If technical conceivability, expense, and throughput are equitable, cell-based assays are often preferred for HTS because compounds tested will be interacting with a more realistic mix of protein target conformations in their physiological milieu, i.e., with the right companions at the right concentration. Further, cell-based assays tend to avoid some common artifacts in biochemical assays such as aggregators (Shoichet 2006). Cell-based assays may wrongly identify hits that do not act on targets and may miss hits with low cell membrane permeability. If a cell-based assay is chosen for primary screening, a

biochemical assay will often be used as a secondary screen to characterize hits and guide lead optimization. An effective HTS strategy considers both the primary and subsequent secondary assay designs carefully.

11.4 *In Vitro* Enzyme-Based HTS Assays

Developing HTS assays for enzymes necessitates recognition of accurate enzyme and substrate forms, purification methods, precise measurement of kinetic parameters, characterization of cofactors, choice of detection technology, and mode of action. HTS enzymes have been developed using detection of substrate consumption, product formation, and enzyme-ligand binding. Development of enzyme-based HTS begins with demonstration of catalytic activity on substrate *in vitro* environment. Available literature will provide information about initial test conditions. Preliminary experiments provide the Michaelis-Menten constant that is critical for assay optimization (Copeland 2003).

11.4.1 Components of Enzyme-Based Assays

Enzyme *In vitro* biochemical assays use enzyme isolated from cell. Absence of native *in vivo* conditions can significantly affect enzyme activity and stability. Enzymes may be expressed as truncated variants, or it may be expressed in alternatively tagged species. These artificial conditions may give compounds irrelevant in physiological conditions or miss compounds showing activity in physiological conditions. The choice of protein construct for HTS assay depends on stability and activity of enzyme construct. Besides, compounds with known modes of inhibition can be used for evaluation of constructs to use in a HTS based on the mode of inhibition. When multiple constructs of enzyme are used for same substrate, the activity can be compared by determining in kcat/Km. Very large difference in kcat/Km or subtle difference in Km value between constructs is indicative of difference in structure and/or stability. Comparison between different preparations of enzyme constructs is also possible using specific activity (Acker and Auld 2014).

Impure enzyme may lead to aberrant result. Mass spectrometry can be used to analyze enzyme purity. Enzymatic purity analysis is done by analyzing IC50 curves of known inhibitor or by determination of Michaelis-Menten parameters and comparing with previous results (Scott et al. 2004). Sometimes, vehicle-carrying compound may impair enzyme function. Vehicle sensitivity is estimated by titrating the vehicle in different concentrations against enzyme and analyzing its activity. Besides, poor interaction of enzyme with tubing and surfaces of dispensing unit results in a decrease of effective enzyme concentration. This can be prevented by adding BSA or small amount of detergent in the reaction buffer.

Substrate Concentration of substrates comparative to their Km will have influence on inhibitor type identified. In the case of competitive inhibitor, substrate concentra-

tion above Km value decreases the capability of inhibitor to bind enzyme active site. In uncompetitive inhibitor screening, high substrate concentration relative to Km value improves the binding of uncompetitive inhibitor. Allosteric (noncompetitive) inhibitors bind independent of the substrate molecule. Thus, it is unaffected by substrate concentration (Copeland 2003; Yang et al. 2009). While choosing an ideal substrate concentration, there must be correct balance between signal window and desired mode of inhibition (Iversen et al. 2006). When optimizing signal, it is better to keep substrate turned over low for identification weak inhibitors (Inglese et al. 2007). Insolubility of the substrate in assay buffer limits highest concentration possible. The substrate stability assays must be carried out to pinpoint stability effects, and modification should be made to resolve issues identified.

Cofactors Many enzymes require cofactors for structural integrity or to assist in the enzyme reaction. The enzyme may be purified with cofactor, or additional cofactor may need to be added to maximize enzymatic activity. The affinity of the cofactor will also influence whether a compound that competes with cofactor binding can be identified. The effects external of cofactor on biochemical enzyme assays can often be treated like substrate addition; the amount required depends on the level of activity needed and the necessity of the cofactor for the enzyme form one chooses to inhibit. It is usually best to use a saturating concentration of additional cofactor in the assay, when not specifically screening for cofactor-competitive compounds. The best possible signal to background ratio is identified by titration of cofactor. Finally, stability of a cofactor needs to be considered for the time and environment that the cofactor will be exposed to during an HTS run. For example, some cofactors are light sensitive (iron guanylylpyridinol), while others can change redox state in common buffers without reducing agents (iron salts). The timing of these modifications must be considered and tested to assure compatibility with the HTS process (Acker and Auld 2014).

Buffers *In vitro* biochemical assays are performed at near physiological pH in an attempt to mimic the intracellular environment of the native enzyme. For cytosolic proteins, pH=7.4 can be maintained by a number of buffers including Tris, HEPES, MOPS, and sodium or potassium phosphate buffers. However, simply because an enzyme is found in the cytosol does not guarantee that the activity will be optimal at pH=7.4. A range of pH values encompassing pH=7.4 should be tested in enzymatic activity assays, taking into account that differences in the local environment *in vitro* versus *in vivo* or changes in the protein construct from the native form could alter the optimum pH for reaction (Acker and Auld 2014).

The buffer choice also has significant influence on biochemical reaction because each buffer can have particular and compelling effects on a given enzyme target. Components of reaction mixture can interact poorly with certain buffers resulting in suboptimal assay conditions and affecting the reproducibility of an assay. For instance, phosphate buffer forms insoluble phosphate salt with Mg^{2+} and Ca^{2+} cofactors, this makes essential component unavailable for the reaction. Such an insoluble complex may result in poor liquid dispension in the automation equipment affecting

the high-throughput screening. Free amine group in the tris buffer react with enzymes and/or substrates, altering the equilibrium of the system. Tris is also able to chelate metal ions, which could have deleterious effects on the activity of enzymes requiring metals for catalysis or structure (Desmarais et al. 2006).

11.5 Receptor-Ligand Binding-Based HTS Assays

There are two types of assay format used to investigate receptor-ligand binding: radioactive method and fluorescent-based methods. Radioactive methods like a filtration and scintillation proximity assay (SPA) are widely used for receptor binding assay. Both these methods use radiolabeled ligand and membrane. Reagents used for binding assays should have high specific activity, indicated by radioactivity/molecule of ligand, and its unit is Curies per millimole. Nonspecific binding of reagents used in binding assays can be reduced by coating filters with bovine serum albumin (BSA). Radiochemical purity of ligand should be above 90%. The purity of radiolabeled ligand decreases with time. Highly selective ligand will provide more reliable result. ^{125}I-labeled ligands will be stable for 1–2 months and tritated ligands have 3–6 months of stability. Beta energy released by ^{3}H produces proton on interacting with scintillates which can be measured by the detector. ^{125}I releases both gamma energy and beta energy. For flash plate assay ^{125}I will be ideal candidate. ^{3}H- or ^{125}I-labeled ligands can be used for SPA (Auld et al. 2012).

11.5.1 Assay Formats

SPA Ligand Binding Assay SPA ligand binding assay is a homogenous assay, which can be run in 96- or 384-well format. In this format, cell membranes are attached to SPA beads. When radio ligand binds to receptor, the proximate radio ligand transfers beta energy to scintillant in the bead and produces a signal that can be measured using microplate scintillation counter. The appropriate settling time needs to be determined experimentally (Auld et al. 2012).

Filtration Ligand Binding Assay In filtration assay, a separation of free radio ligand and radio ligand bound to the receptor is required for measurement. Filtration binding assay is carried out first in assay plate. After adding and incubating assay components, unbound ligand is removed by applying vacuum, while the bound ligand remains attached to the filter. The liquid scintillation cocktail or liquid scintillator is added to dried filter. Result is measured using microplate scintillation counter. It is more efficient than SPA (Auld et al. 2012).

Flash Plate Ligand Binding Assays In flash plate binding assay, a target receptor or receptor membrane is bound to wall of flash plate. Necessary compounds for flash plate assay were added. Energy from radio ligand interacts with scintillator bound in the wall of flash plate, producing signal. Unbound ligand far from plate

scintillant can't produce signal, which distinguishes bound and unbound ligand (http://www.perkinelmer.com/Technologies/Assays-and-Reagents/Receptor-Ligand-Binding/default.xhtml.).

Fluorescent Polarization Assay for Receptor Binding Assay Receptor-ligand binding fluorescent polarization assays use a fluorescein-labeled ligand (tracer) and a large unlabeled receptor. Bound ligand will show high polarization value.

11.5.2 Assay Optimization

Ligand Concentration Bound ligand concentration can be determined using the equation given below:

$$BL = Bmax \times L / (Kd + L)$$

where BL = bound ligand concentration, Bmax = maximum binding capacity, L = total ligand concentration, and Kd = equilibrium affinity constant also known as dissociation constant.

IC_{50} for competitive binders can be calculated by the formula

$$IC_{50} = (1 + L / Kd) \times Ki$$

Functional assays should be performed for finding uncompetitive inhibitor. Keeping ligand concentration equal to dissociation constant (Kd) is ideal way to attain a good signal. This method is ideal for finding competitive binders (Auld et al. 2012).

Receptor Concentration The optimal concentration can be determined by calculating the proportion of bound ligand at the disassociation constant (Kd). Linearity of ligand binding with respect to membrane concentration must be evaluated. In radio filtration assay, it is better to keep membrane concentration at 10% of bound ligand at Kd concentration (Burt 1986). Determination of nonspecific binding (NSB) will give optimal assay result.

Preincubation and Equilibrium For slow receptor binders, preincubation is necessary. Maintaining equilibrium in HTS binding assays ensure a proper calculation of displacement by putative inhibitors.

11.6 Cell-Based HTS Assays

Cell-based assays for HTS can be divided into three types: reporter gene assays, second messenger assays, and cell proliferation assays (Sundberg 2000). Cell-based assays differentiate agonists and antagonists, pinpoint allosteric modulators, and provide direct information about intracellular permeability and stability compounds and cytotoxicity of the compound (Kunapuli et al. 2006). Besides, cell-based assays

have been performed in a more biological relevant environment. They have been successfully used for early drug discovery in identifying high-quality leads. The main constituents of a cell-based HTS assays are cells, cell-culturing device, and detection system.

11.6.1 Cell Types Used for Cell-Based HTS Assays

Immortalized cell lines are inexpensive, easy to grow, dependable, and reproducible. Various types and sources of cells have been used in cell-based assays. Primary cells are capable of giving characteristic responses; even so, they are difficult to grow and transfect (Ebert and Svendsen 2010). Human cancer cells are widely used for anticancer drug screening assays. Recent advancements like cancer stem cells, which can differentiate, self-renew, and regenerate a phenocopy of the original tumor (Sabisz and Skladanowski 2009), are promising models in anticancer drug screening.

Embryonic stem cells (ESCs) can serve as better models for both drug efficacy and toxicity screening than primary or immortalized cells lines. ESCs are isolated from embryo and have unlimited capacity to self-renewal and can be differentiated into any cell type *in vivo*. IPSCs are pluripotent cells artificially derived from somatic cells by inducing a small set of powerful pluripotency genes. As IPSCs can be derived from patients with specific diseases, they have been considered as a new tool in drug discovery. Cell-based assays are mostly carried out in microtiter wells. It can be easily miniaturized to carry out assays at high-throughput scale and handled with a robotic system for automation (Sundberg 2000).

Common cell culture methods include single cells, monolayer cells on a two-dimensional (2D) surface, and multilayer cells or aggregate clusters in a 3D scaffold (Yang et al. 2008). 2D cell-based assays are generally used in drug screening because they are cheap and easy for operation. But the 3D cell culture generally shows similar *in vivo* morphology with intimate cell-cell and cell-extracellular matrix interactions, which are absent in 2D cell culture. Cell-based assays are widely used to assay the effects of compounds on cellular activities, cell number, intracellular calcium levels, nuclear size, mitochondrial membrane potential, and membrane permeability (Hughes et al. 2008).

11.6.2 Detection Methods in Cell-Based Assay

Online detection methods like electrochemical and optical techniques are used in HTS. Generally, optical sensation is effortless for miniaturization than electrochemical sensing (Kumar et al. 2004).

Electrochemical Methods Living cells act as an electrochemical system. Electrochemical changes in living cells have been used to define cell viability in a homogenous solution (Nonner and Eisenberg 2000). Metabolism in cells presided

by modification in metabolic products or substrates. These changes can be detected by electrochemical methods based on cellular activities including potentiometry and amperometry (Zang et al. 2012). Conventional potentiometry cell-based sensors encompass gas-sensing electrode (GSE) or an ion-selective electrode (ISE) coated with a layer of cells (May et al. 2004). However, it requires very stable reference electrode, which limits its application.

Amperometric electrochemical method has been used for the determination of pH, DO, or glucose. The acidification rate in the proximity of cells can be calculated using a microphysiometer (Rabinowitz et al. 1997). Cellular biochemical reactions resulting from the accumulation of lactic acid and carbon dioxide can be relatively monitored using the pH value. Besides, heterogeneous pO2 distributions around tissues could be detected using a miniaturized system (Braun et al. 2001). Even so, many uncontrollable environmental factors affect metabolic activities which limit the applications of this approach in high-throughput cell-based assays.

Electrochemical Method Based on Barrier Behavior Electronic impedance significantly increases in the presence of cell. This property can be used for monitoring of biological status of the cell. Using electrochemical impedance spectroscopic techniques, real-time assessment of cytotoxicity and acute toxicity can be attained. Bioelectrical signal from electrogenic tissues can be used to test drugs against critical diseases such as cardiac arrhythmia, hypertension, Parkinson's disease, diabetes, depression, and neuropathic pain (Hogg et al. 2006). Single-wall carbon nanotubes (SWCNTs) are used for detection of membrane potential change of axon (Pui et al. 2010). This technique can be used to noninvasively track cellular activities for electrogenic cells with high throughput, high sensitivity, easy use, and the capacity of long-term cell culture.

Optical Methods Colorimetric methods are based on color change of the growth medium after cell metabolites react with chemical agents. Tetrazolium salts can differentiate living and nonliving cells based on the reduction of a tetrazolium salt by actively growing cells to a colored formazan product that can be quantified with a spectrophotometer. There is spectrum of HTS detection methods available using tetrazolium salts. Colorimetric methods are invasive, time-consuming, and laborious. Besides, they usually can only provide end point data, which can't provide more details about the effects of drugs on cells comparing to dynamic data. Automation of colorimetric end point assays is costly (Zang et al. 2012).

The detailed description of fluorescent and luminescent methods has been given in previous section.

Green fluorescent protein (GFP) has enabled online, noninvasive detection and quantification of cell proliferation and specific cellular functions. GFP-based cellular assays are inclinable to real-time, automated, and noninvasive analysis cellular events (Yang et al. 2008). Specific cellular function can be observed using reporter gene and can be expressed under control of promoter gene or regulatory DNA sequence. Activation of signal transduction pathway can be detected in this method

(Mahajan et al. 1999; Xu et al. 1998). Laser-scanning imaging systems with fluorescence microscopy can be used to investigate the context of living cells, quantify, intracellular proteins, and monitor the trafficking of proteins fused with fluorescent reporters and some subcellular structures (Abraham et al. 2004). Howbeit, their high costs and relatively low capacity limit their uses to the late phase compound characterization (Haney et al. 2006). Moreover, these imaging systems are limited to read planar images of cells cultured on 2D surfaces and are not suitable for 3D cell cultures (Zang et al. 2012).

11.7 Drug Target in HTS Assays

The major considerations for cherry picking therapeutic target for HTS are target validity, chemical tractability, and screen ability. The target must be disease relevant. There is reciprocal association between target novelty and validity. Some targets will have a high degree of validation, but low novelty and others will be highly novel but poorly linked to disease. Target validity can be evaluated by genetic method and/or compound-based experiments. Gene knockouts or RNA interference methods can be time engaging and sometimes lead to false inferences, but it is less expensive when comparing to compound-based experiments. Compound-based target validation approaches can be used for less-validated targets and to screen for tool compounds, followed by *in vivo* experiments. Most organizations are using combination of these methods (Macarrón and Hertzberg 2011).

The chemically amenable target increases the probability to find out drug-like compound producing therapeutically relevant effect. Certain target classes, like G protein-coupled receptors (GPCRs), ion channels, nuclear hormone receptors, and kinases, are more chemical amenable than others. About half of experimental and marketed drugs target five main protein families: G protein-coupled receptors (GPCRs), kinases, proteases, nuclear receptors (NRs), and ion channels (Hopkins and Groom 2002). The drawback of this approach is that it may entirely eliminate target classes that would otherwise be extremely attractive from a biological point of view.

A final factor to consider when choosing targets is screenability – the technical probability of developing a robust and high-quality screening assay. GPCRs, kinases, proteases, nuclear hormone receptors, and protein-protein interactions are relatively easy targets to establish screening assay. Ion channels are more difficult, although new technologies are being developed which make these more approachable from an HTS point of view (González et al. 1999). Approximately two thirds of therapeutic targets are comprised of enzymes and receptors (Zheng et al. 2006).

Phenotypic assays can be used for HTS if the target is inamenable, which measure cellular properties like a secretion of protein factors, chemotaxis, apoptosis, and cell shape change in response to test compound. Multiple targets have been screened in phenotypic assays. But these assays are difficult to configure and expensive (Macarrón and Hertzberg 2011).

All these factors should be evaluated before commencement of a HTS to make a choice to go forward. Chemically amenable, technically easy, inexpensive, and biologically relevant targets are ideal for drug screening, but these kinds of targets are rare (Macarrón and Hertzberg 2011).

11.7.1 Common Targets in HTS Assays

Protein Kinases Protein kinases are enzymes that phosphorylate the hydroxyl group present on serine, threonine, or tyrosine residues (Glickman et al. 2004) during posttranslational modification in cells and signaling pathways. The biochemical approaches to measure protein kinase activity can be divided into two categories: generic assays independent of subfamily and antibody-based formats that detect an epitope within the phosphorylated product. Measurement of ATP depletion via luciferase is an example of a generic assay format (Koresawa and Okabe 2004; Singh et al. 2004). The drawback of this assay is large quantity of enzyme required for sufficient signal to background ratio (Auld et al. 2013). ADP produced during the enzyme action can be detected using ADP specific antibody (Lowery and Kleman-Leyer 2006). Phosphorylated peptide is detected by radiometric filter binding assay. Immobilized metal-ion affinity-based fluorescence polarization (IMAP) is a homogeneous antibody-free method. The IMAP utilizes immobilized transition metals on nanoparticles as a "binding reagent" to make complexes with phosphate groups on phosphopeptides generated in a kinase reaction. Such a binding results increase in FP value (Sportsman et al. 2004). Antibody-based technologies and ALPHA, FP, and HTR-FRET are developed to detect phosphorylated peptide. These assays are ideal for kinase cascades and hierarchical phosphorylation.

Proteases Proteases are well-established drug targets (Leung et al. 2000). Proteases are measured using FRET-based or profluorescent substrates. The probes coupled to a protease cleaving site become fluorescent after protease cleavage (Karvinen et al. 2004). Inefficiency of fluorescent substrate to cover the entire binding site is the limitation of this assay. The AHTRF assay for endoproteases, "carboxypeptidase B," has been developed for HTS; in this assay peptide cleavage unmasks an epitope which is then recognized by an antibody (Ferrer et al. 2005).

Nuclear Receptor Nuclear receptors (NR) are a large family of ligand-activated transcription factors that act as transcriptional switches responding to lipophilic hormones, vitamins, dietary lipids, or other intracellular signals (Nagy and Schwabe 2004). NRs are regulated by hormones and metabolites (endobiotic ligands), or through xenobiotics (Olefsky 1999). Binding assays for NRs are divided into radiometric and fluorometric assays. GFP fused with NRs has been used to measure the glucocorticoid receptor (GR) translocation from nucleus to cytoplasm (Fung et al. 2006). NRs' agonist and antagonistic compounds can be detected using coregulator recruitment assays based on TR-FRET ligand (Gowda et al. 2006). Highly sensitive

cell-based reporter gene assays fuse NR reaction components with reporter genes such as beta-lactamase, luciferase, and secreted alkaline phosphatase.

G Protein-Coupled Receptor GPCRs are the most targeted protein molecule in drug discovery. There are well-established assay methods for GPCR-targeted drug discovery (Eglen et al. 2007; Jacoby et al. 2006). HTS assays can be configured to monitor GPCR-orchestrated cellular events, such as protein phosphorylation, ion channel activity (Ferrer et al. 2003), modulation of secondary messenger (Chambers et al. 2003; Williams 2004), gene transcription (Dinger and Beck-Sickinger 2004), and cell proliferation. Intracellular Ca2+ stores activated by GPCRs can be analyzed using calcium-sensitive dyes such as fluo-3 and fura-4 and rapid inject imaging platform (Chambers et al. 2003). The GPCR secondary messengers have been analyzed by the direct measurement of inositol phosphate species (Eglen and Singh 2003; Trinquet et al. 2006) or cAMP (Williams 2004). GPCR internalization assays, independent of G protein subtype, can be applied to assess number of GPCRs using fluorescence microscopy (Inglese 2006; Taylor 2006) or microtiter plate reader (Hamdan et al. 2005).

Ion Channels Membrane-spanning ion channel proteins control the movement of inorganic ions such as Na^+, K^+, Ca^{2+}, and Cl into or out of cells. Ion channels are well-recognized therapeutic target for treating different diseases. Cell-based HTS assays have been developed for different types of ion channels using either fluorescence methods to monitor changes in membrane potential or microtiter plate readers to measure intracellular calcium levels (Inglese et al. 2007).

Ligand-receptor binding assays are established methodology for ion channel HTS. But inability to detect functional effect of compound on ion channels is the limiting factor of the binding assay. The calcium-sensing fluorescent probes such as fura-2, fluo-3, and fluo-4 can be used in HTS for ligand- and voltage-gated channels (Zheng et al. 2004). The fluorescent signal intensity of intracellular fluorophores increases proportionally with rise in intracellular free $Ca2^+$ concentration (Gee et al. 2000). Positional voltage sensors and FRET-based assays use voltage-sensing probes, which measure changes in membrane potential (González and Maher 2002). Atomic absorption spectroscopy, which has been used to measure ion transport, can be used in HTS ion flux assay. Automated patch-clamp instruments, with increase in throughput, have been used in HTS assays (Finkel et al. 2006).

11.8 Assay Validation and Evaluation of HTS

Hit and non-hit compound can be distinguished based on statistical evaluation. In enzyme-based HTS assays, blank, positive control, and negative control are used for primary evaluation. Blanks or NSB controls are prepared traditionally by adding an excess of unlabeled ligand used in ligand-receptor binding assays. Errors in sample processing and liquid handling can be distinguished by analysis of control plate.

Liquid handling errors are pinpointed by analysis of patterns. The analysis of performance starting with mean (M) and standard deviations (SD) for signal and background and combinations of these is as follows.

Signal to Background Signal to background ratio (S/B) furnishes information of the separation of positive and negative controls. S/B is useful to validate reagents in early assay development. But it is a poor indicator of assay quality (Zhang et al. 1999).

$$S / B = M\,\text{signal} / M\,\text{background}$$

Coefficient of Variation of Signal and Background It is the measure of variability, which is a function of the assay stability and precision of liquid handling and detection instruments (Macarrón and Hertzberg 2011).

$$CV = 100 \times SD / M(\%)$$

Z′ Factor The value of Z′ factor is a relative indication of the separation of the signal and background (Zhang et al. 1999). The Z factor's dimensionless scale ranging from 0 to 1 allows comparison of different assays and screens using the control wells (Z′) and sample wells (Z) of the plate (Inglese et al. 2007). Z′ must be assessed during assay development and validation and also throughout HTS campaigns on a per plate basis to assess the quality of dispensement and reject data from plates with errors (Coma et al. 2009b).

$$Z' = 1 - (3\sigma\,\max + 3\sigma\,\min) / \mu\,\max - \mu\,\min$$

Signal window and Z′ factor are used for assay validation. Signal window measures fold change in maximum and minimum output signals, but it is not as reliable as Z factor for predicting assay performance. Z′ factor measures the precision of this response within a plate and across plates. The minimum significance ratio (MSR) is used to track assay sensitivity variation between assay plates, which is often an indicator of reagent stability. The MSR can be calculated from titrations of control compounds on some or all assay plates (Inglese 2007).

After assay optimization, a pilot study must be done before starting HTS campaign. In this pilot step, representative sample must be tested from screening collection. It should be treated as the way HTS plates will be treated. The objectives of this pilot study are prediction of hit rate, assessment of screening compound interference, reproducibility of result, estimation of hit rate, and optimal sample concentration. The size of pilot screening compound collection can be 1–5% of total compound collection (Macarrón and Hertzberg 2011).

Apart from pilot screening, at least 10–20 assay plates should be run to test the HTS system in real action. Duplicates and triplicates of these samples should be run. Independently running duplicates evaluate the reproducibility of results. Triplicates access the rates of false positives and negatives (Coma et al. 2009a).

11.9 *In Silico* Screening in Drug Discovery

CADD is used to screen large compound library to identify biologically active compounds (hits), improving drug likeness of lead compound by increasing biological activity or optimizing pharmacokinetic properties and designing new compounds (Sliwoski et al. 2014).

CADD can be classified into two categories: structure or target based and ligand or pharmacophore based. Structure-based CADD (SBCADD) requires 3D structure of target molecule. Ligand-based CADD is done by superimposing active molecule to identify common chemical features or identifying interaction points between ligand and target molecule.

11.10 Pharmacophore-Based Drug Discovery

According to IUPAC, pharmacophore is defined as "the ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target structure and to trigger (or to block) its biological response". Pharmacophoric descriptors include H-bond acceptors, H-bond donors, and aromatic, hydrophobic, positive, and negative ionizable groups. In case the macromolecular structure of target protein is not available, drug designing can be done using ligand-based drug discovery method. Chemical compounds with similar molecular fingerprints can be screened using molecular fingerprints of known ligands. A pharmacophore model can be generated either in a ligand-based method or in a structure-based manner (Yang 2010).

11.10.1 Ligand-Based Pharmacophore Modeling

Ligand-based pharmacophore modeling involves two steps: creation of conformational flexibility of ligands and alignment of multiple ligands in the training set. Mainly, various automated pharmacophore generators are available such as PHASE (Dixon et al. 2006) (Schrodinger Inc.), HypoGen (Li et al. 2000) (Accelrys Inc.), DISCO (Martin 2000), GALAHAD (Tripos Inc.), MOE (Chemical Computing Group), and HipHop (Barnum et al. 1996). There are two key challenges in ligand-based pharmacophore modeling. The first challenge is generating ligand flexibility. This problem can be solved either by precomputing conformations or by carrying out conformation analysis during the pharmacophore modeling process (Poptodorov et al. 2006).

Molecular alignment of multiple ligands is another difficult issue in ligand-based pharmacophore generation. There are two methods of alignment: property-based and point-based approaches (Wolber et al. 2008). The property-based approach uses molecular descriptors, generally represented by sets of Gaussian functions for alignment generation. In point-based algorithms, alignment is generated by super imposing atom pairs, chemical features or fragments by using the least-squares fitting.

Proper selection of training set compounds is necessary to ensure the accuracy of pharmacophore models (Poptodorov et al. 2006). Different training sets give different pharmacophore models of ligands that interact with same protein generated from same program.

11.10.2 Structure-Based Pharmacophore Modeling

Structure-based pharmacophore modeling depends on protein-ligand complex. A structure-based 3D pharmacophore reflects amino acids position and type of interaction in active site of protein. Major interaction sites between ligands and macromolecule can be determined by using the macromolecule-ligand-complex-based approach. The software used for macromolecule-ligand complex pharmacophore generation are GBPM (Ortuso et al. 2006), Ligand Scout (Wolber and Langer 2005), and Pocket v.2 (Chen and Lai 2006). This method needs macromolecule-ligand complex structure. Pharmacophore model with more than seven chemical features is not fit for practical applications, such as screening of 3D database (Pandit et al. 2006; Toba et al. 2006).

Pharmacophore building has been done using IC50 or Ki50 value of 15 diverse chemical structures. Alternatively, it can be done on the basis of three or four known active compound chemical structures from different chemical scaffolds (Toba et al. 2006; Funk et al. 2004).

11.11 Structure- or Target-Based CADD

This method is based on knowledge of 3D structure of target macromolecule (biological target). It depends on the hypothesis that molecules interacting with target protein can exert specific biologic effect. Therefore, novel compound with biological modulation activity can be screened by analysis of binding site. The 3D structure of macromolecules has been resolved by X-ray crystallography, NMR, and electron microscopy. The 3D coordinates of macromolecules are available in Protein Data Bank (PDB). The output of virtual screening depends on quality of PDB structure.

11.11.1 Homology Modeling of Protein

Complex protein molecules can't be solved by NMR analysis and X-ray crystallography techniques. In the absence of experimental protein structures, computational tools can be used for predicting 3D structure of proteins. Homology model of target protein is predicted based on template amino acid sequence. It is based on principle that distantly related primary structures of protein fold into similar tertiary structures. It involves the following steps: (1) template recognition and alignment of the target and template proteins, (2) alignment correction, (3) backbone generation, (4) loop modeling, (5) side-chain modeling, and (6) model optimization and

validation. The software tools used for homology modeling are Swiss model, PSIPRED (Buchan et al. 2010), and MODELER (Marti-Renom et al. 2000). Using PSI-BLAST, template structure with similarity to target sequence can be found out (Altschul et al. 1990). Multiple alignment tools can be used for searching of template structure, which give more accurate result. Factors like template selection and resolution, alignment length and sequence identity between target and template influence output of homology model.

Gaps or insertions in sequence alignment are filled by connecting anchor residues. Missing residues are mostly seen in loop region. This can be filled by two modeling methods, either by knowledge-based approach or energy-based approach. Knowledge-based approach depends on an amino acid sequence present in target sequence. Energy-based methods create a large number of loop models, and quality has been assessed by energy-based function (Hillisch et al. 2004). Side-chain conformation prediction has been done based on Monte Carlo search (Rohl et al. 2004) and dead-end elimination (Desmet et al. 1992). These methods using side-chain conformation library are grouped by statistical methods (Krivov et al. 2009).

Models are refined by minimizing models using techniques such as Monte Carlo Metropolis minimization (Misura and Baker 2005), molecular dynamics (Raval et al. 2012), or genetic algorithms (Xiang 2006). Model evaluation is done by determining energy conformation of amino acids and by calculating root mean square difference between target and template.

11.11.2 Molecular Docking

Docking procedure predicts ligand conformation and orientation within a binding site of target macromolecule. Molecular docking studies give precise structural model and correct activity prediction. Docking is usually a multistep procedure in which one or more degrees of complication are added in each step (Brooijmans and Kuntz 2003). By applying docking algorithm, small molecule orients in the active site. Docking is a two-step process: first step is sampling conformations of the ligand in the active site of the protein, and the second is ranking these conformations via a scoring function. Ideal sampling algorithms should be reproducing the experimental binding mode, and the scoring function should also rank it highest among all generated conformations.

In molecular docking, three methods represent protein-ligand binding: atomic, grid, and surface (Halperin et al. 2002; Kitchen et al. 2004). Atomic method is used as a function of potential energy field. It is often used only during ranking. Surface methods work based on the topography of the molecule. These methods are guided by alignment of binding site and ligand, by minimizing the angle between the surfaces. It is usually used in protein-protein docking. The grid representation stores physicochemical features of receptor binding surface as energy potentials on grid points.

Docking methods have been classified as flexible docking and rigid-body docking (Halperin et al. 2002; Dias and de Azevedo 2008). Rigid-body docking

considers physicochemical or geometrical complementarities as stationary state. Flexible docking considers a multiple conformations of a ligand and/or target. Treatment of ligand flexibility can be divided into three categories: systematic methods, random methods, and simulation methods. In systematic method algorithms try to investigate all possible conformation of the molecules. In this method ligands search the active site in stepwise manner. Docking programs, such as DOCK (Ewing et al. 2001), FLEXX (Rarey et al. 1996), GLIDE (Friesner et al. 2004), and FLOG (Miller et al. 1994), use this method. Stochastic or random methods operate by materializing arbitrary changes to either a single ligand or a population of ligands. The ligand evaluation is based on predefined probability function. The algorithms using random search methods are AutoDock (Morris et al. 1998) and GOLD (Jones et al. 1997). Molecular simulation methods calculate the system trajectory by the applying Newtonian mechanics.

11.11.3 Scoring Function in Molecular Docking

In molecular docking experiments, hundreds of thousands of macromolecule-ligand structures are generated. Incorrect conformation should be sorted out from correct macromolecule-ligand conformations. Reliable scoring function is necessary to distinguish valid binding conformation. Essentially, four types of scoring functions are currently applied: (1) force-field-based scoring functions, (2) empirical scoring functions, (3) knowledge-based scoring functions, and (4) consensus scoring functions (Sliwoski et al. 2014).

For force-field or molecular mechanics-based scoring functions, classic molecular mechanics are used. It usually calculates the internal ligand energy and the ligand-receptor interaction energy. These calculations are the sum of van der Waals and electrostatic interactions. AutoDock and DOCK use AMBER force field in ranking.

Empirical scoring functions fit parameters to experimental data, such as conformations and/or binding energy. The coefficients for various parameters are attained from regression analysis using experimentally calculated binding energies and X-ray crystallography information. Empirical scoring function terms are often easy to assess and are based on approximations. Empirical functions are used in several docking programs such as FLEXX (Rarey et al. 1996), SURFLEX (Jain 2003), and LUDI (Böhm 1992).

Information from experimentally determined structure is used in knowledge-based scoring function. In knowledge-based scoring function, protein-ligand complexes are modeled using interatomic distance. Several knowledge-based scoring functions are used in programs like DRUGSCORE (Velec et al. 2005), SMOG (DeWitte and Shakhnovich 1997), and BLEEP (Mitchell et al. 1999). Consensus scoring approach combines different scoring functions to balance the error in single method and improve scoring function.

11.12 QSAR and Drug Likeness Prediction

"Quantitative structure-activity relationships" (QSAR) relates chemical property of compound with its biological activity. Mathematical model can be constructed based on activity of set of ligands. Structure and activity can be quantified based on set of chemical properties called "molecular descriptors." QSAR modeling uses molecular descriptors as independent variables and activity as a dependent variable. QSAR model can predict biological activity of novel compounds from their molecular descriptors. Active compound can be screened from chemical database using QSAR model.

In silico drug likeness properties can be predicted using Lipinski rule of five. Lipinski rule of five is used to predict absorption, distribution, metabolism, and excretion (ADME) properties of lead compound. Lipinski's rule states that, in general, an orally active drug has no more than one violation of the following criteria:

- Not more than 5 hydrogen bond donors (nitrogen or oxygen atoms with one or more hydrogen atoms)
- Not more than 10 hydrogen bond acceptors (nitrogen or oxygen atoms)
- A molecular mass less than 500 daltons
- An octanol-water partition coefficient log P not greater than 5 (Lipinski 2000)

Prediction of drug likeness saves cost of pharmaceutical company for drug discovery. Lead compound can be optimized for improved pharmacokinetic properties.

11.13 Conclusions

The process of drug discovery can be accelerated by combining high-throughput and *in silico* screening. Using in silico screening, the number of compound screened for *in vitro* activity can be narrowed down. Compounds without biological activity and nondrug likeness can be eliminated *in silico* screening stage. This method reduces cost and time for HTS screening. Drug likeness of lead compound can be improved using *in silico* tools.

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