A High Throughput Screening Technology-Overcoming Bottlenecks in Ion Channel Drug Targets

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Ion Channels as therapeutic targets: Ion channels are transmembrane proteins that allow the formation of a concentration gradient between the extracellular and intracellular contents. Three major types of gated ion channels: (1) Ligand gated, (2) Mechanically gated and (3) Voltage gated channels are known to play pivotal roles in a variety of cellular processes within the human body and have been attributed to multiple diseases. Among the wide variety of ion channels, potassium channels are a diverse and ubiquitous family of ion channels present in both excitable and non-excitable cells. Members of this channel family are involved in cellular signaling processes regulating neurotransmitter release, heart rate, neuronal excitability, smooth muscle contraction, insulin secretion, electrolyte transport and cell volume. Enhanced understanding of ion channel biology has correlated gene mutations in these channels with various diseases of the heart, kidney, pancreas, and central nervous system. Moreover, the combination of genomics, proteomics and biophysical approaches have remarkably progressed our knowledge on different aspects of these diseases. This knowledge helped in rationalizing how various mutations affect channel structure and function. The contribution of such mutations to the etiology of diseases and modulating the activity of ion channels has lead to the development of novel treatment strategies called channelopathies. Thus, regulating ion channel function is proving to be a successful approach in drug therapy.

Safety Screening: Ion channels can be used for drug discovery in primary screening while looking for functional ion channel targets and for drug safety in secondary screening. It has been shown that the activity of a potassium channel encoded by the gene hERG, the human *either-a-go-go* (*eag*)–related gene is critical for the regulation of membrane potential in cardiac myocytes. This channel is also expressed in human brain, however, its physiological role in the brain remains unknown. The cardiac hERG ion channels give rise to a potassium current that re-polarizes the ventricular action potential.
Mutations leading to loss of function in this channel are associated with the potentially lethal inherited syndrome known as long QT (LQT) caused by prolonging of the QT phase of the heart beat reflected in the electrocardiogram. This state can also be caused by pharmacological blockage of this channel that leads to the development of another form of LQT called acquired LQT. In either its inherited or acquired forms, LQT can lead to life-threatening ventricular arrhythmias and sudden death. Since 1997, different classes of therapeutic agents, including antiarrhythmics, antibiotics, antihistamines, antipsychotics, and prokinetic agents that were once widely prescribed have been known to induce acquired LQT. As a result of their potential to block the hERG channel and thus causing LQT syndrome, 5 prescription drugs were withdrawn from the U.S. market in 1997. Withdrawing a drug from the market is a huge loss to the pharmaceutical company in terms of the expense associated with the development of the drug. It is financially advantageous to the pharmaceutical companies to conduct early compound safety screening to eliminate such compounds. This concern is also shared by many regulatory agencies world wide. The publication of

Points to Consider by the Committee for Proprietary Medicinal Products (CPMP) of the European Agency for Evaluation of Medicinal Products (EMEA) in 1997 is a valuable document reflecting this concern. The CPMP has elaborated a series of *in vivo* and *in vitro* assays for pharmaceutical companies to assess the LQT liability of non-cardiovascular therapeutic agents. This document has also regarded the ability of a compound to block the hERG channel as an important indication of its possible LQT liability during safety screening of the compounds.

Need for an HTS system: The pharmaceutical industry has been under pressure from competition and public policies to reduce the cost of drug discovery and development. To provide new drugs, it has almost exclusively relied upon screening of compound libraries. Therefore, the most pressing need of this industry is for development and improvement of high throughput screening technology (HTS). However, there have been many bottlenecks in the screening processes and many technology platforms are addressing such bottlenecks.

With the advent of new chemical synthesis procedures and combinatorial chemistry there has been an explosion of availability of compounds requiring HTS. Concurrently, identification of previously unknown ion channels as new pharmaceutical targets with the knowledge of genomics and proteomics has further necessitated the need for HTS. For example, in 2000, the average number of compounds tested per screen for an average pharmaceutical/ biotech company was 270,000 which is expected to swell up to approximately one million compounds per screen per average company in year 2005. The development of HTS for ion channel modulators has proven to be difficult and time – consuming especially for voltage gated ion channels. Therefore, for the want of HTS, the bottlenecks in drug discovery and drug screening have shifted towards the necessity of HTS as the rate-limiting step for drug discovery/screening.

Assessment of HTS technologies: Currently, there are several methods used for evaluating ion channel activity and screening lead compounds for important ion-channel targets. New technologies are always being developed for improved performance. Therefore, to make a worthy decision before purchasing a technology one needs to know various aspects of the technology. When evaluating a technology, some important aspects to consider are suitability of the technology for a particular application, suitability of the desired application with the technology, the trend of technology competition, and advantages and disadvantages of the technology. The judgment can further be refined by evaluating various other attributes like sensitivity, specificity, throughput, robustness, flexibility, cost and physiological relevance of the technology. Normally, all these attributes are not found in each technology as the achievement of one attribute is usually at the sacrifice of the other. For example information content has to be sacrificed to achieve higher throughput. This is true in case of patch-clamp technology, which is excellent for providing high information content, but it suffers from low throughput. Similarly antagonistic is the case of sensitivity and specificity. Because of the interdependency of the attributes, a single attribute should not be used to determine the merit of a technology. Strongly interdependent attributes, especially opposing attributes, are usually considered at the same time. One such example is sensitivity and specificity, cost and information content.

Technologies for hERG Screening: The number of methods available for measuring hERG channel activity is limited in the market. Voltage clamping of membrane patches (referred to as patch-clamping) is sensitive and reliable but labor intensive (Table 1). By applying these attributes, this

method is indisputably the gold standard for ion channel research offering the most accurate and high information content data. But it is still restricted to low throughput and requirement for advanced electrophysiologists. The ability to screen large amount of compounds using this technology is not presently available. New technology in this area involves direct coupling of ion channels to semiconductors on silicon chips. However, it is restricted to academics only.

The advances in fluorescent assays that involve membrane-sensitive fluorescent dyes have significantly enhanced its suitability for drug discovery and safety screening against ion channel targets. Indirect fluorescent assays include fluorescent voltage sensitive probes, which report membrane potential change as a result of ionic flux and fluorescent ion-sensitive probes, such as calcium-sensitive dye Flu-3 which quantify the change of the concentration of the conducted ions. One of the most widely used oxonol dyes is bis-(1,3-dibutylbarbituric acid)trimethine oxonol (DiBAC4). Recently, Molecular Devices (Sunnyvale, CA) released a new fluorometric imaging plate reader (FLIPR®) Membrane Potential Assay Kit (FMP) that has been claimed to work for Na1 and K1 channel screening. However, both dyes measure the change of membrane potential instead of channel activity. Thus they have the potential of selecting compounds that change membrane potential but that does not affect channel activity. They are also prone to dye artifacts.

New assay approaches to achieve better performance and lower cost are under development. The Lab-on-a Chip approach of this technology promises high throughput and miniaturization. However, the translocation of the dyes in response to changes in membrane potential is slow. Similarly, membrane potential sensors based on fluorescent resonance energy transfer has lead to the development of Voltage Ion Probe Reader (VIPR) to provide automated screening for ion channel modulators. This technology despite having good throughput is also prone to dye artifacts.

In addition to the above functional assays, radioligand binding assays are available to study ion-channel targets that require radio labeled probe specific for target and previous knowledge of binding sites. Due to allosterical interactions it is prone to high false negatives.

Functional assays are ideally suitable to identify modulators of ion channel activity. The Rb^{86} based radioactive flux assay and the non-radioactive flux assays are relatively reliable and direct methods to measure ionic flux at medium throughput. However, many high throughput-screening (HTS) labs are reluctant to use the Rb^{86} based radioactive flux assay format because of the highly radioactive nature of Rb^{86} to the cells during loading.

Table 1. Comparison of hERG channel assay methods.

Method	Information Content	Throughput (HT)	Sensitivity	Accuracy	Comments
ICR 8000	Medium	Medium to High	High	Medium	Currently used for K^+ channels and has potential applications for other ion channels including Na^+, Cl^- and Ca^{2+}
ICR 12000	Medium	High	High	Medium	

Patch Clamp Technology	High	Low	High	High	Less than 20 data points/day
Binding Assays	Low	High	Medium	Low	Requires radio labeled probe specific for target
Radioactive Flux Assays	Medium	Medium	Medium	Medium	Short half-life and exposure concerns
Fluorescent Imaging	Low	High	Medium	Low	Prone to dye artifacts, ½ life concerns, high expense of dyes over time and high background noise

The performances of most methods for ion channel analysis have typically fallen on the extremes of either accuracy or speed. For example, fluorescent dye assays offer unsurpassed speed, however, they suffer from low accuracy with high background to signal noise, high cost and limited capabilities for some ion channels (for example hERG channel).

The recent development of a non-radioactive Rb assay has greatly enhanced the ease of using this system. In an effort to address solutions to bottlenecks in ion channel drug discovery, Aurora Biomed Inc. has developed the Ion Channel Reader (ICR) based Flux Assays that are designed to study ion channels by chemically initiating the opening of the channel and measuring the response with the ICR. The ICR based on flame atomic absorption spectroscopy (FAAS) has the advantage of detecting very finite concentrations of ions. Aurora Biomed Inc. has patented the automation of ICR series to revolutionize the use of flux assays for high throughput screening of ion channels.

This technology is based on the tracer element. In the ion channel literature, tracer elements are widely used to detect ion channel activity in various methodologies. For example, Rb^+ is an excellent tracer ion for K^+; it is relatively similar in size, same charge, permeable to K^+ channels and not found in physiological systems. Rb^+ assays have been described for voltage-gated potassium channels including inward rectifiers, outward rectifiers, delayed rectifiers, calcium sensitive channels and ligand-gated channels. The Rb^+ Flux Assay has been well described in the scientific literature and has been widely used for the detection of ion channel activity when studying the potassium channel family. In addition, flux assays can be applied when studying ion channel activity. Several ion channel candidates including Na and Cl are now being studied to develop new flux assays compatible with the ICR series where Li is used as a tracer for Na and Cl with silver precipitation. The channels that do not produce detectable flow/amount of the desired ion through the endogenous channels are normally expressed in heterologus cell lines to express enough number of desired ion channel in the cell membrane so that a detectable signal is produced. Since expression of ion channels in cell lines may alter resting membrane potential, many factors have to be considered in optimizing these flux assays.

The ICR series are fully automated, compatible with existing robotic automation, fully programmable to allow for automatic dilution, calibration, washing, and analysis of samples in one convenient step (Table 2). The ICR series can deliver speed, precision and reproducibility to evaluate therapeutic agent effectiveness. ICR Efflux Assay for hERG ion channel has been compared with patch clamp. It has generated the same rank order of hERG blockers as given by patch clamp electrophysiology. The relationship between the two technolgies is shown graphically in Figure 1.

The ICR Efflux Assay technology is relatively inexpensive to use over time in comparison with alternative technologies. The cost per data point of this technology is eight cents / data point. Thus the ICR flux assays is economical to be used in primary screening when looking for functional ion channel targets or in secondary screening to participate in drug safety analysis. Because of such merits, the ICR series are currently used by many of the top 20 pharmaceutical leaders worldwide.

Fig 1. Relationship of ICR Rb Efflux Assay and Electrophysiology

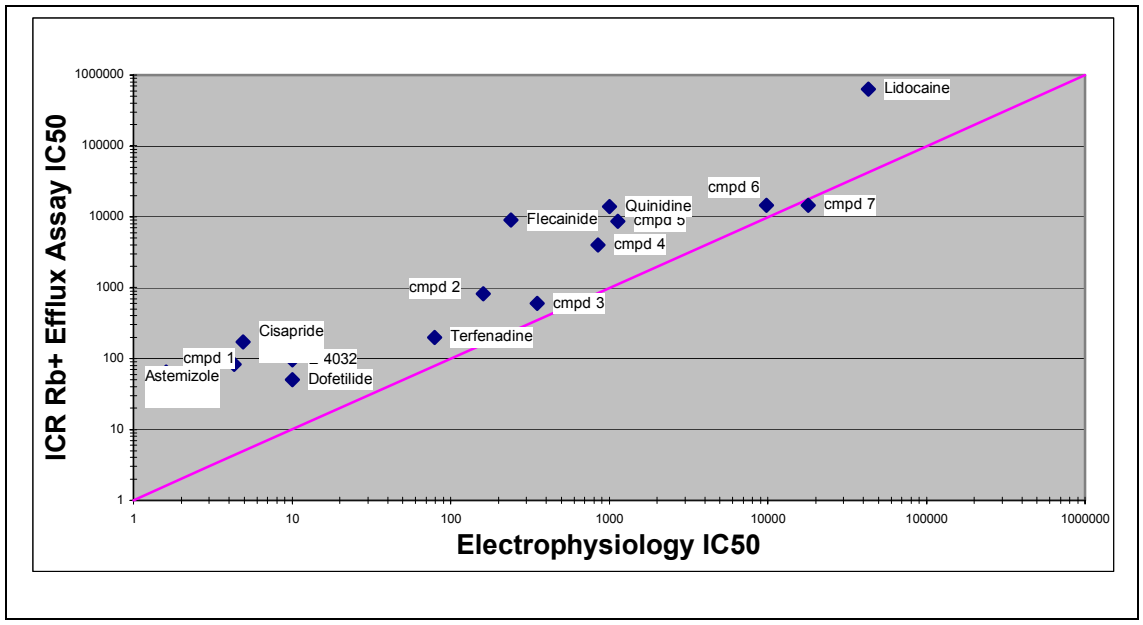

Table 2. ICR 8000 vs ICR 12000

ICR 8000	ICR 12000
Medium Throughput Up to 5000 wells/day	High Throughput Up to 60,000 wells/day
Single Channel 1 Sample at a Time	Multi Channel 12 Samples at a Time
50 µl Samples	10-20 µl Samples
Accommodates 96/384-well Plates	Accommodates 96/384-well Plates
65 cm X 55 cm X 37 cm	120 cm X 95 cm X 37 cm
Acetylene – Compressed Air	Natural Gas or Acetylene – Compressed Air
Manual with Optional Plate Stacker/ Bar Code Reader	Plate Stacker/Bar Code Reader
Sensitivity: 0.05 ppm detection limit Precision: <3% CV	

Conclusion: Recent understanding that ion channel modulators offer significant therapeutic solutions to a variety of patho-physiological conditions has lead to developing specific drugs and safety screens targeting ion channels. Among the available technologies, flux assays can be used as primary screens when looking for functional ion channel targets or as secondary screens for drug safety analysis. The ICR series is an accurate means to screen for pharmaceutical drugs that modulate ion channel activity. Drug safety concerns regarding the hERG K^+ channel are prevalent with over thirty two compounds pulled off the commercial market worldwide for hERG channel interactions leading to prolonged QT syndrome. Aurora Biomed's technology is currently being used in a number of pharmaceutical laboratories and is performing beyond the original expectations assessed for precise data recovery. The system has been very successful in analyzing hERG K^+ channel activity and is one of the most popular techniques used on the market today.