ION CHANNELS

new opportunities for an established therapeutic target class

There is an increasing recognition within the pharmaceutical industry of the immense potential for ion channels as a drug target class. Advances in the understanding of the human genome, combined with the introduction of higher throughput electrophysiology platforms, promises a wealth of new opportunities to design potent and selective ion channel targeted therapeutics. While the recently introduced higher throughput electrophysiology technologies offer many advantages compared to conventional screening techniques, throughput still remains significantly lower than established ion flux and fluorescence-based high throughput screening systems. We discuss how the introduction of new technology means that the pharmaceutical industry can now choose from an expanded number of options for their ion channel research strategy.

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Ion channels are ubiquitous pore-forming proteins that allow the passive diffusion of ions across cell membranes[1]. They act as highly selective filters facilitating the movement of a particular ionic species (Na+, K+, Ca^{2+}, Cl^-) between cellular compartments, although a number of channels are less discriminative. Functionally, the channels exist as dynamic structures sensing external factors such as voltage gradients, endogenous ligands and mechanical forces, which are able to induce conformational transitions within the channel between non-conducting closed states to an open state where ions can flow according to the electrochemical gradient (gating). Ion channels are key proteins in all neurones and a variety of other cell types. They underlie electrical signalling in the central and peripheral nervous systems as well as controlling a diverse range of other physiological processes including proprioception, nociception, heart rate, cell volume, secretion and cell membrane potential. The pharmaceutical industry has benefited from a number of therapeutics that modify ion channel function. Examples include the long established first line anti-epileptics such as the Na+ channel blocker carbamazepine (Tegretol, Novartis), the antihypertensive dihydropyridine Ca^{2+} channel blockers (Norvasc, Pfizer; Adalat LA Bayer) and sulphonylurea potassium channel openers for diabetes (Amaryl, Aventis). Most of these established ion channel modulating drugs were discovered serendipitously at a time when knowledge of the ion channel superfamily was either non-existent or in its infancy. Despite this, recent total annual sales of ion channel targeted drugs are around $20 billion, with Pfizers' Norvasc alone earning $3.2 billion in the first nine months of 2004.

As the pharmaceutical industry made systematic attempts to exploit the range of ion channels for drug development, it became clear that these complex molecular targets presented their own set of problems. The lack of suitable high throughput screening (HTS) assay formats, the complexity of ion channel biophysics, the range of potential binding sites and binding modes for drugs, combined

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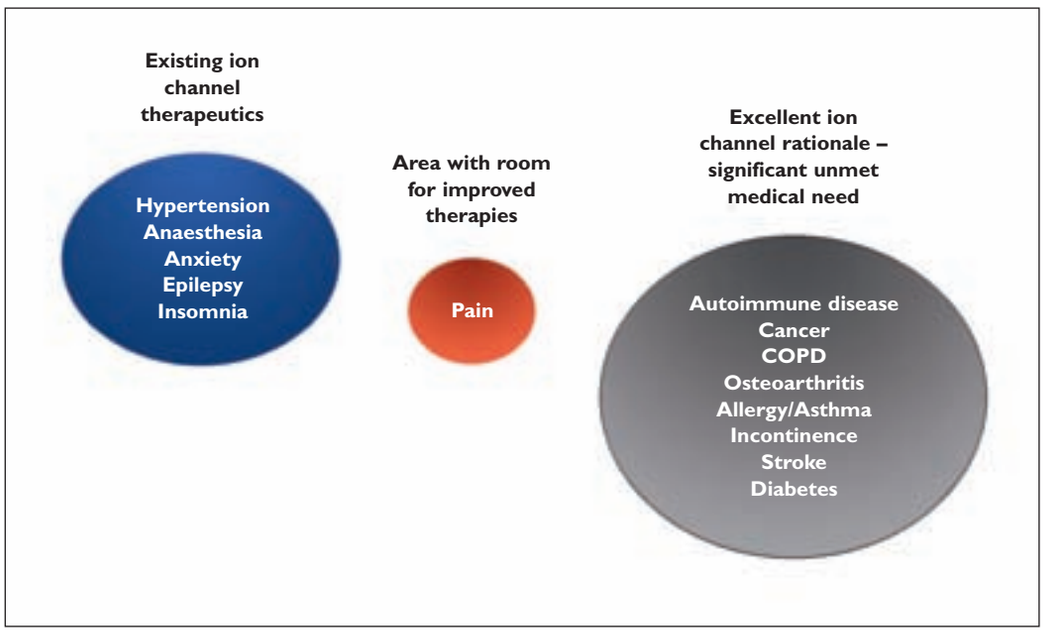

Figure 1: The potential of ion channel targets: Drugs already on the market for ion channel targets, largely in the cardiovascular and CNS areas earned around £20 billion in 2004. There is still a massive opportunity to extend the potential of ion channel drugs into other therapeutic areas, either by improving on treatments in areas such as chronic pain where channels are already being exploited, or by developing compounds in areas where the rationale for ion channel targets is very good, but the drugs do not yet exist

with serious gaps in our knowledge of the ion channel superfamily, have all held back progress in developing novel targeted therapeutics with the appropriate potency and selectivity profile. Significant progress has been made recently on a number of fronts and many pharmaceutical companies have a renewed interest in ion channels as a target class.

One of the most significant advances has been in our understanding of the molecular biology of ion channels. Over the last couple of decades academic institutions have added considerable depth to our understanding of the architecture, distribution and biophysical profile of individual ion channel subtypes. Coupled to the wealth of information available from the human genome project, it is increasingly obvious that ion channels are an underexploited area with immense therapeutic and commercial value (**Figure 1**).

Other advances have been made in the area of assay technology. Ion channel proteins are extremely complex and dynamic structures. They exist in a number of sub-states (eg open, closed and inactivated) and compounds may bind preferentially to any one of these states. The method of choice for studying ion channels is electrophysiology, which allows resolution of the interactions of candidate drug molecules in real time with very high fidelity. This type of information is essential to allow meaningful progression of hits through to optimised lead candidates. Historically, the inability of electrophysiology to accommodate the large number of compounds typical of a HTS campaign has been a major obstacle to the progress of ion channel research programmes. The established HTS technologies are extremely useful hit finding tools applicable to screening large libraries (>50,000 compounds), but they normally lack the sensitivity, temporal resolution and physiological relevance to steer medicinal chemistry programmes[2,3]. The recent introduction of high throughput electrophysiology platforms is changing screening strategies for ion channel targets, but before discussing these advances, we will review current HTS systems.

HTS of ion channel targets

The trend in ion channel HTS is not dissimilar to that seen for G-protein coupled receptors but there are several problems that are unique to the target class, particularly for voltage-gated channels. Initially radioligand binding assays were used to identify compounds that bound to specific sites on channels. The value of this type of assay is limited in that there are usually several distinct sites on any channel where a compound could act. Hence important hits could be missed in binding assays by focusing only on the site

that interacts with the radioligand[2]. In the case of voltage-gated channels where there is no endogenous ligand, the physiological relevance of binding assays is even less clear[4].

For these reasons, there has been an increasing preference for functional assays. In high throughput assays channel function is either measured through monitoring the movement of specific ions through channels (ion flux methods) or measuring gross changes in the membrane potential of the cell expressing the channel of interest. The former assay format provides a direct and specific measure of channel function, whereas the latter is a more non-specific readout of an event that occurs as a consequence of channel activation but offers a generic assay platform. Several methods are in common usage in HTS laboratories, they are summarised in **Tables 1** and **2** for their applicability

TECHNIQUE USED (DETECTION METHOD)	APPLICABLE CHANNEL TYPE	ION DETECTED	COMMENT
Radioactive Uptake or Flux			
(Scintillation Counting)	K^+	$^{86}Rb^+$	Some assays formats are homogeneous and highly amendable to automation and HTS. Lack of temporal resolution within data results in low information content. Limited data available to correlate relevance to electrophysiology[2,4,5]
	Na^+	$^{22}Na^+$, [^{14}C]-guanidinium	
	Ca^{2+}	$^{45}Ca^{2+}$	
Non-Radioactive Flux			
(Atomic Absorption Spectroscopy, AAS*)	K^+	Rb^+	Non-homogeneous assay format but samples remain stable for several days. Lack of temporal resolution leads to low information content. Compound potency values generally correlate well with those derived in electrophysiology[2,5-9]
	Na^+	Li^+	
(Fluorescence)	Ca^{2+}	Ca^{2+}	The use of calcium sensitive indicators (e.g. Fura-2, Fluo-3, Fluo-4, Indo-1) is widely utilised and described. Assays provide good temporal resolution and kinetic information but generally slower than the true channel kinetics. Robust instrumentation available eg FLIPR™, CellLux™[2,4,5,10]
	K^+	Tl^+	
	Cl^-	I^-	Uses a halide sensitive green fluorescent protein mutant yellow fluorescent protein YFP-H148Q which is co-expressed with the target of interest to give a label free detection system. Homogeneous assay with kinetic readout, specific for halides but most sensitive to iodine. Patent restrictions on use of fluorescent proteins[11]
(Colorimetric)	Cl^-	I^-	Multi-step assay utilising modified Sandell-Kolthoff reaction to quantitate I^- offering high sensitivity. End point assay[12]

* Instrumentation supplied by Thermo-electron Corporation or Aurora Biomed

Table 1: High throughput methods detecting ion flux

Table 2: Indirect measurement of ion channel activation using membrane potential sensitive dyes

DYE SYSTEM	APPLICABLE CHANNEL TYPE	COMMENTS
$DiBac_4(3)$	Wide	Slow response time to changes in membrane potential due to redistribution between extra- and intracellular compartments. Temperature sensitive. Reported to interact with some compound classes which may lead to false positives and negatives in high throughput screens. Very well established and low cost per data point[13,14]
FLIPR Membrane Potential Dye (Molecular Devices Corporation)	Wide	Homogeneous format due to the presence of additional dye to quench extracellular fluorescence but this can interfere with some channel types. Rapid change in fluorescence with changes in membrane potential. Low signal change in hyperpolarisation assays. Prone to identifying false positives resulting in poor correlation with electrophysiology compared to other high throughput methods. Throughput per day and ease of automation is good[14]
FRET – Voltage Sensor Probe (Invitrogen)	Wide	Utilises coumarin-linked phospholipids as a FRET-donor anchored to the cell membrane and a mobile voltage sensing oxonol dye. Dual dye system gives good sensitivity, rapid kinetics and allows correction for compound interference and variation in cell numbers. Assay is complex to set up and requires instrument capable of simultaneous dual emission reading e.g. VIPR™ or CellLux™. Cost per data point is high compared to other fluorescence methods. Good correlation with electrophysiology[13,15,16]

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across channel type and perceived relevance of the data when compared to conventional electrophysiology. It is clear from these tables that it is not possible to identify a single high throughput method that fulfils the needs of screening all ion channel types. However, by focusing on a few technologies most types can be covered, including both ligand and voltage-gated channels (with the caveats discussed below). As methods continue to evolve the number of channel types that are covered by the technologies expands. For example, Aurora Biomed (www.aurorabiomed.com) is currently developing an AAS method utilising silver ions for indirectly studying chloride channels and Invitrogen (www.invitrogen.com) has new fluorescent probes reportedly specific for sodium.

Within a high throughput screen the relevance of the assay format compared to the normal gating of the channel in the physiological setting is a concern, particularly for voltage-gated channels. In most assays, voltage-gated channels are opened either through depolarisation of the cell by elevation of the extracellular potassium concentration or by employing toxins to activate or delay channel inactivation leading to an accumulation of channels in the open state. For example, the application of veratridine or type I pyrethroids to cells expressing voltage-gated sodium channels leads to an influx of extracellular sodium to cause cell depolarisation[17]. Normal ion channel function is dependent on the resting membrane potential of the cell in which the channel is expressed. In neurones, under normal physiological conditions the membrane potential is close to -70mV whereas most cell lines heterologously expressing cloned channels have a more positive resting potential, typically around -20mV. For voltage-gated channels, the non-physiological mechanism of channel gating coupled with high resting membrane potential is often regarded as the reason for a lack of progression of hits from plate-based high throughput functional assays translating to active molecules in conventional electrophysiology. In order to address this several groups are investigating the use of electrical stimulation to gate ion channels via an applied electrical field within assay wells[18,19]. This advance in technology, while not currently commercialised, combines the proven FRET-based voltage sensor probe technology with the benefits of a more physiologically relevant mechanism for opening voltage-gated channels.

High throughput electrophysiology

Electrophysiology allows extremely high quality resolution of the interactions of drug molecules with ion channels. Although providing essential information regarding the potency and mechanism of action of drug binding, the technical complexity of the conventional methodology has meant that the pharmaceutical industry has not been able to obtain the full benefits from this rich source of information. The technique has traditionally required highly skilled personnel providing a limited throughput of around 30 data points per day. A number of companies have been developing new technology to simplify and automate the process of obtaining high quality electrophysiological information. Predominantly based around a technique known as planar patch-clamp it is now possible to perform multiple automated recordings in parallel, vastly increasing throughput compared to conventional methods. The most successful systems on the market achieve this by replacing the glass micropipettes used in conventional electrophysiology with pre-manufactured multi-well substrates. Typically, the plates consist of a flat substrate perforated with a small hole (~1-2µM diameter). The Molecular Devices (www.moleculardevices.com) IonWorks® platform was the first to the market and presently offers the highest throughput of the commercially available systems. Based around a 384-well format, the system uses a 48-channel amplifier to read each screening plate over eight cycles during an experiment. Electrical control of the cell membrane involves use of 'perforated' patch-clamp methodology, using an antibiotic solution to permeabilise a small section of cell membrane. While some technical considerations mean that the system does not provide the highest fidelity patch-clamp data, this compromise is acceptable when considering the increase in throughput compared to the conventional technique. With the latest generation instrument, IonWorks® Quattro™, it is possible to obtain around 2,500 data points per eight-hour working day. Furthermore, the system allows the user to gain an insight into details of the mechanism of action of a much larger cross-section of compounds at an early stage. Important considerations such as open channel block and use-dependence are both rapidly and easily resolved, allowing information rich data to be conveyed to medicinal chemists early in the programme.

Stepping down in throughput, the PatchXpress® 7000A (www.moleculardevices.com), QPatch 16 (www.sophion.dk) and NPC©-16 (www.nanion.de) allow higher fidelity recording and have the facility to enable complex recording protocols (including ligand-gated ion channels). Due to the lower throughput, these machines are

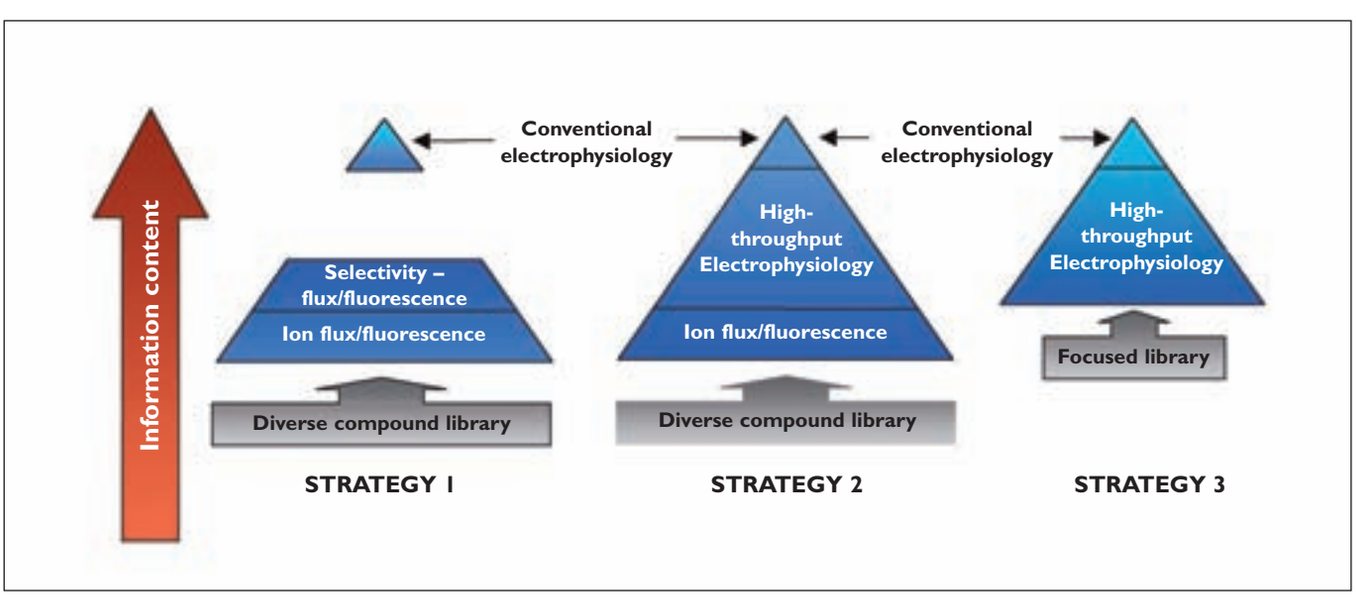

Figure 2: Three strategies for ion channel screening: Strategy 1 is the traditional approach where large diverse libraries are screened by ion flux or fluorescence methods. Conventional electrophysiology is used to characterise a few chosen compounds in detail.
Strategy 2 also involves screening of large libraries by ion flux or fluorescence methods. In this case a significant subset (2-3,000 compounds) can be profiled with high throughput electrophysiology. The best of these are characterised in more detail by conventional electrophysiology.
Strategy 3 involves design of focused ion channel libraries, each of around 1,000 compounds, which can be screened directly by high throughput electrophysiology, so that high quality information is gathered on all the compounds in the screen. The best compounds can be further profiled by conventional methods

suited to profiling high 'value compounds', making them attractive for lead optimisation or providing high quality safety pharmacology data. First to the market, the PatchXpress®, developed by Axon Instruments (www.axon.com) and Aviva Bioscience (www.avivabio.com), is an increasingly common research tool within the industry. The QPatch has been adopted by fewer companies at the time of writing, however, the 48 channel machine currently in development is a particularly attractive prospect and it could challenge the dominance of the PatchXpress® during 2006. Alternative approaches are also being pioneered by Cytocentrics (www.cytocentrics.com) and FlyIon (www.flyion.de). The Cytocentrics CytoPatch10™ is a 1-3 channel machine using microstructured chips with specialised cell positioning and recording structures. The company claims that the system can be scaled up into 20 parallel recording channels. The Flyion® 8500 (www.flyion.de) is a 2-6 channel system developed using glass micropipettes.

The high throughput electrophysiology systems are starting to fill the gap between high throughput, but low resolution screening assays and very low throughput, but high resolution traditional electrophysiology (**Figure 2**).

Screening strategies

Despite the range of high throughput assay formats and the efficiency and cost-effectiveness with which large numbers of compounds can be tested, electrophysiology remains the bench mark for confirming compound activity[5]. The traditional approach to ion channel screening has been to test large diverse chemical libraries in high throughput ion flux or fluorescence-based assays. A few of the best compounds are then characterised in detail by conventional electrophysiology (**Figure 2, Strategy 1**). In this approach, it is not possible to profile large numbers of compounds in detail, so a great deal of important information is missed and data fed into medicinal chemistry programmes are sub-optimal.

The high throughput electrophysiology systems discussed in this review remove the bottleneck associated with the evaluation of large numbers of compounds, but the cost per data point on such systems remains significantly higher than that of the ion flux or fluorescence methodologies. These costs would almost certainly prohibit testing more than a few thousand compounds in an early stage drug discovery programme for most organisations[20]. Consequently, high throughput methods that do not rely on electrophysiology still have a role in ion channel screening for many groups with large diverse screening libraries[2]. The high throughput electrophysiology methods are used to obtain high quality information on a significant subset of the hits. Conventional electrophysiology is available, if more detailed characterisation is needed for a small number of compounds (**Figure 2, Strategy 2**). In this approach high quality data can be obtained for a few thousand compounds to provide a good basis for guiding medicinal chemistry programmes. The starting point is still the large diverse libraries, where hit rates are likely to be low.

An increasingly popular strategy is to screen small ion channel focused compound libraries using high throughput electrophysiology. Here, the emphasis is switched from large diverse library sets to small libraries that are designed specifically for ion channel targets, based on the emerging knowledge of channel structures. This strategy is being pioneered by BioFocus in the UK which has a proprietary ion channel ligand design tool called Helical Domain Recognition Analysis (HDRA™) (www.biofocus.com). With small libraries, screening can be run directly by high throughput electrophysiology, which gives the most appropriate and information-rich readout, while costs are controlled by reducing the number of compounds screened compared to traditional campaigns (**Figure 2, Strategy 3**). The best compounds can be further profiled by conventional methods. With this approach, hit rates are likely to be relatively high and good quality information is available for all the compounds in the screening set. It is a very attractive option, which combines the latest strategies in ion channel screening and library design to provide the best possible data for guiding chemistry programmes. DDW

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