

# Validation of ion channel targets

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A prerequisite for a successful target-based drug discovery program is a robust data set that increases confidence in the validation of the molecular target and the therapeutic approach. Given the significant time and resource investment required to carry a drug to market, early selection of targets that can be modulated safely and effectively forms the basis for a strong portfolio and pipeline. In this article we present some of the more useful scientific approaches that can be applied toward the validation of ion channel targets, a molecular family with a history of clinical success in therapeutic areas such as cardiovascular, respiratory, pain and neuroscience.

## Overview

A key early strategic decision for any discovery program is the selection of high confidence therapeutic targets. Given the amount of time and resource that will ultimately be invested in a program, teams need to consistently provide information on the validity of these targets, the scientific rationale of the biological pathway, and the confidence that pre-clinical *in vivo* efficacy and safety will translate to man. In many ways, a thorough validation strategy for ion channels is similar to those for other target classes. However, the weighting of the validation whether it be more for efficacy, safety, preferred pharmacological mechanism, or translatability can differ based on the role of the channel in normal physiology and the opportunity for therapeutic intervention in the diseased state.

To minimize downstream risk, a robust validation data set is best built throughout the life cycle of the program using a variety of scientific methodologies. For ion channels validation data from human genetic association, tissue distribution, phenotype of genetically engineered animals, and *in vivo* effects of selective probes can provide key decision making data. In this article we discuss these approaches and their successful application to ion channel validation. Even with the best scientific evidence, it is important to keep in mind that a target can only be fully validated after success in the clinic and even then questions may remain. Thus, an overall strategy that maximizes chances of

success and minimizes target selection risk is important even when working with ion channels with strong initial validation.

## Human Genetics

Human genetic association studies have been and continue to be critically important for identification of ion channel targets. Historically these studies have been focused on severe or very well defined phenotypes that can be readily followed in familial linkage studies. The discovery of Kv7.2 opens for the treatment of epilepsy is a success story with origins in human genetics. Kv7.2 are voltage-dependent and non-inactivating potassium channels important in the stabilization of neuronal resting membrane potentials. Kv7.2 loss of function mutations genetically associate with a rare epilepsy, benign familial neonatal convulsions (BFNC)<sup>1,2</sup> Individuals affected with BFNC have recurrent, brief, generalized seizures beginning on about the fourth day of life and ceasing after 1–3 months. Although infants otherwise develop normally, affected persons carry a 10–16% risk of seizures later in life. The underlying deficit in BFNC are Kv7.2 mutations with impaired gating resulting in destabilization of the resting membrane potential and neuronal hyperexcitability. The human genetics combined with a strong mechanistic understanding provided the rationale for extending the therapeutic indication to treatment of broader spectrum epileptic disorders. First in class success was fully recently realized with the FDA approval of retigabine (Ezogabine), a potent and selective first in class Kv7 channel opener for the treatment of partial drug resistant seizures, reinforcing the value of genetic linkage in selection of ion channel targets.<sup>3</sup>

Advances in the treatment of cystic fibrosis (CF), a common genetic disorder for which fluid transport across epithelial tissues is impaired, are rooted in an understanding of the underlying genetic mutations. Affected individuals suffer from impaired respiratory, gastrointestinal, hepatic and reproductive function resulting from loss of function mutations in CFTR, a chloride channel expressed in the apical membrane of epithelial cells. Over a thousand unique CFTR mutations are linked to CF with the most common variant,  $\Delta F508$ , unable to fold properly resulting in inability to traffic to the plasma membrane. Another common variant, G551D, folds and traffics correctly but channel gating is severely impaired in response to secretagogues. Because CFTR loss of function variants are mechanistically distinct, a successful CF drug therapy requires a personalized medicine so that appropriate therapy is linked to individual genotype.<sup>4</sup> In 2012 the personalized medicine concept

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in treating a channelopathy was validated with the approval of Ivacaftor, a CFTR modulator for the treatment of CF patients afflicted with gating mutants such as G551D.<sup>5,6</sup> Trafficking correctors also hold promise for CF linked to the  $\Delta F508$  mutation as evidenced by the recent approval of Lumacaftor in combination with Ivacaftor for patients homozygous for the  $\Delta F508$  mutation.<sup>7</sup>

Both loss and gain of function human genetic data can increase confidence in target validation as provided in the case of a voltage-dependent sodium channel, Nav1.7, which is predominantly expressed in sensory ganglia. Loss of function mutations in Nav1.7 have been mapped to a rare genetic disorder known as congenital indifference to pain (CIP).<sup>8</sup> In this disorder, affected individuals are unable to sense painful stimuli despite normal sensory innervation and ability to sense non-noxious stimuli. In contrast, Nav1.7 gain of function mutations are associated with erythromelalgia, a rare pain disorder manifesting as extreme burning pain and redness in the extremities.<sup>9</sup> Since both Nav1.7 loss and gain of function mutations are linked with pain phenotypes, confidence in the potential analgesic efficacy of selective Nav1.7 modulators is increased.

The availability of target validation data from human genotyping will continue to rapidly grow, becoming more accessible. Ongoing efforts to mine the massive amounts of human genetic data being collated by independent organizations offer researchers the promising ability to assess mutations and variants in target populations.<sup>10</sup> By comparing phenotypic data from surveys with genotyping results from an ever growing population of individuals, potential genes of interest within oncology, neurology, and immunology have been identified. This in turn could lead to a better understanding of clinical results and further open the door to personalized medicine approaches for the treatment of channelopathies.<sup>11</sup>

### Distribution and Function in Human Tissue

An understanding of ion channel target tissue distribution can provide a map for future critical path studies on efficacy, site of action, mechanism of action and liability. Transcriptome level data can provide some of the most valuable distribution data due to the higher specificity and throughput of these techniques compared to protein based methods. Critical questions such as whether the channel is expressed in tissue consistent with therapeutic rationale, analysis of critical organ distribution, desired CNS profile, and similarity of distribution between pre-clinical species and human can be addressed. A specific antibody can be used in follow-up protein based studies to answer questions on sub-cellular distribution as these cannot be addressed with transcriptome based methodologies. In sensory neurons, for example, the subcellular ion channel distribution can help in forming testable hypotheses on the role in transduction and/or transmission and whether CNS penetration should be considered for efficacy. For example Nav1.6 channels are subcellularly distributed in axon initial segments and nodes of ranvier suggesting a role in action potential propagation rather than in dendritic generator potentials or synaptic transmission.<sup>12</sup>

Often, disease or injury can result in either up or down regulation of channels of interest. *In situ* hybridization and immunohistochemistry techniques allow for analysis of channel levels/distribution within in normal and diseased tissue. Such data when combined with functional electrophysiological studies can add significant weight to target validation. Continued refinement of high-throughput, single cell RNA-seq techniques have allowed for the analysis of tissue cell populations on a single transcriptome level.<sup>13</sup> This opens the possibility of directly comparing expression and distribution patterns within relevant heterogeneous tissue samples from healthy and disease samples. This technique may also give researchers the ability to identify disease relevant splice variants/mutations and determine their relative prevalence in healthy and disease populations.

### Transgenic Mice

The phenotype of genetically engineered mice often provide the first direct *in vivo* evidence for the role of an ion channel in normal and modeled disease states. These data can help in refinement of therapeutic indication. *Ex vivo* experiments comparing wild type and transgenic animals can help in elucidating the cellular mechanism underlying the transgenic phenotype. Advances in the field of molecular biology have resulted in more sophisticated conditional and tissue-specific gene modification approaches. These techniques, several which are presented below, have addressed concerns with traditional global knock-outs regarding plasticity and genetic compensation

KCa2.3 channels were the first to be studied using conditional knockout techniques.<sup>14</sup> These  $Ca^{2+}$  activated  $K^{+}$  channels had been shown *ex vivo* to be involved in neuronal afterhyperpolarization, adaptation of burst firing and maintenance of smooth muscle tone. To test for a phenotype consistent with these observations, the KCa2.3 gene promoter was modified so that transcription could be driven by recombinant tetracycline transactivator (tTA) protein. In this way KCa2.3 transcription could be controlled by dietary inclusion of doxycycline (dox) to interfere with tTA binding to the promoter. Using this methodology, the authors could bypass concerns of developmental gene compensation by knocking down expression only in adult animals. By comparing the phenotypes of dox treated and untreated animals, combined with quantitative measurements of gene expression, the authors could directly correlate KCa2.3 gene expression with observed phenotype. Although this technology is a significant step in surmounting concerns with traditional knockouts, normal regulation of gene expression throughout development is compromised because of the altered promoter. In this case, a 3-fold overexpression of KCa3.2 was observed in the transgenic animals before administration of dox compared to wild type counterparts. Surprisingly, no overt phenotype was observed with knockdown of channel expression in dox treated animals. However, the overexpression phenotype had deficits in smooth muscle contraction which added validity to the potential for KCa2.3 channel openers as smooth muscle relaxants.

More recent advances have utilized the Cre-loxP transgenic approach for tissue-specific gene modulation. With this transgenic method, Cre recombinase is expressed under the promoter of a tissue specific protein. In a separate transgenic strain, an

exon of the targeted channel is flanked by 34 bp lox P sites which when crossed with the Cre recombinase mice is excised resulting in tissue specific deletion.

An example of this utility has been in understanding the role of Nav1.7 channels in pain as global Nav1.7 null mutants mice were found to die shortly after birth. Transgenic animals were designed such that Cre recombinase expression was driven by the Nav1.8 channel promoter to target this subpopulation of nociceptive DRG neurons. In another strain exons 14, 15 which encode most of domain II of Nav1.7 were flanked with loxP sites to target for excision. Thus crossing these strains of mice resulted in functional knockout of Nav1.7 channels in a subset of Nav1.8 expressing sensory neurons. In these animals a role for Nav1.7 channels in inflammatory pain, specifically in Nav1.8 expressing nociceptors, was demonstrated.<sup>15</sup>

Another example is the use of this technology in both conditional and pathway selective transgenic animals. These data can be particularly useful in understanding the physiology of the cellular pathway for which the targeted ion channel is expressed. In pain research, for example, this information can be very helpful in understanding the specific pain modalities that would be attenuated with analgesic ion channel modulators. These data can ultimately guide the selection of the most relevant proof of mechanism experiments in the clinic. An example of the application of this technology is transgenics in which the diphtheria toxin receptor (hDTR) is driven by the promoter for the ion channel of interest such that intraperitoneal injection of diphtheria toxin will selectively ablate cells expressing the target ion channel. By further crossing these mice with an Advillin-Cre strain, Advillin being a sensory neuron specific protein, this technique was used to define the specific contribution of CGRP $\alpha$  expressing sensory neurons in pain signaling.<sup>16,17</sup> In these mice peripheral responses to noxious heat but not cold or mechanical stimuli was attenuated.<sup>18</sup> The authors were then able to study the GFP labeled CGRP $\alpha$  neurons in *ex vivo* experiments to build a mechanistic understanding of this phenotype and understand the ion channels linked to the pathway and the phenotype.

Humanized transgenic mice are animals in which the gene of interest has been replaced with the human variant. These mice can be used to provide evidence for pre-clinical target engagement and efficacy for drug candidates that are species-selective or for which there are subtle differences in mechanism of action, such as in state and/or use-dependent interaction. With advances in antibody therapies and more selective agents to improve therapeutic index, this is becoming increasingly common. Data from humanized mouse efficacy can aid in linking pharmacokinetics with efficacy, information that can be used to model human dose predictions.<sup>19</sup>

### Probe Molecules

A key step in validating any new drug target is building confidence that the *in vitro* data generated will translate to *in vivo* activity in man. Genetics, expression and distribution data may build a strong case for the role of an ion channel in physiology or disease however these data are insufficient in validating the pharmacological strategy being pursued. RNA knockdown techniques

can be very valuable in developing a correlation relationship between target modulation and *in vivo* efficacy.<sup>20</sup> However for a small molecule therapeutic approach, potent and selective tool compounds are necessary for building confidence in the pharmacological strategy. Typically initial discovery efforts identify such molecules that may be undevelopable as drug candidates but useful as tools for validation studies. Selectivity against other targets is important as the potential for off-target activity can complicate the interpretation of the probe data. In most cases, this will expand beyond closely related gene family members to proteins that could confound early efficacy and safety readouts. The ideal probe molecule would also have bioavailability such that free drug concentrations reach multiples of target potency. Depending on where the channel of interest is located, CNS penetration may be required. Efforts should also be made to understand the metabolic stability of the compounds and any potential metabolites should be profiled for activity.

Once adequate probe molecules have been identified, efficacy can be assessed in a variety of manners. Primary cell/tissue recordings *in vitro* or *ex vivo* experimentation can provide insight into mechanism of action. *In vivo* studies can help refine therapeutic indication, flag potential safety concerns, and provide information on the correlation of target potency and free drug concentration with efficacy in pre-clinical disease models. If available, transgenic animals can also be utilized to verify that the activity of a compound is a direct result of channel modulation and not an off-target effect. Because of the known preference of compounds to interact with specific channel states, the general mechanism by which the probe molecule affects function should be understood. To off-set the risk of misinterpretation from unknown off-target effects or having a probe compound with the wrong mechanism of action, at least 2 structurally distinct molecules should be used for validation if possible.

### Conclusions

The major goal of validation studies is to identify the most promising drug discovery targets. During the life cycle of a project these initial data sets are extended to increase the confidence in mechanism, therapeutic strategy and translation of efficacy to man. A multi-disciplinary validation approach is required given the importance of selecting the right targets and because of the limitations in data interpretation from any single technique. In recent years much progress has been made in mapping human genetic variation to disease increasing confidence in translation to man and bolstering the potential of personalized medicine. A target is not fully validated until efficacy and safety can be demonstrated in man over time. Even then questions usually remain providing opportunity for improvement in the next generation of therapies through alternate mechanisms of interaction and improvement in overall compound profile.

#### Disclosure of the Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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