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Ion channels as therapeutic antibody targets

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ABSTRACT

It is now well established that antibodies have numerous potential benefits when developed as therapeutics. Here, we evaluate the technical challenges of raising antibodies to membrane-spanning proteins together with enabling technologies that may facilitate the discovery of antibody therapeutics to ion channels. Additionally, we discuss the potential targeting opportunities in the anti-ion channel antibody landscape, along with a number of case studies where functional antibodies that target ion channels have been reported. Antibodies currently in development and progressing towards the clinic are highlighted.

Introduction

The human genome encodes at least 400 ion channel family members (~1.5%[1]), representing the second largest class of membrane proteins for drug discovery after G protein-coupled receptors (GPCRs) (Figure1(a)).[2-5] Roughly 18% of small molecule drugs listed in the ChEMBL database are targeted towards ion channels,[5] with global sales estimated to be $12 billion.[6] Although it is well validated that ion channels are at the core of many diseases, approved drugs are available for only a small percentage of this protein class (approx. 8%) despite focused drug discovery efforts over the past 30 years.[7] Ion channels function by transporting ions across cell membranes and play important roles in a broad range of physiological and pathophysiological processes. Mutations of single ion channel proteins have been demonstrated to be the cause of genetic diseases, collectively known as channelopathies.[8] For example, mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) lead to cystic fibrosis, whereas various pain syndromes, including congenital indifference to pain and paroxysmal extreme pain disorder, are associated with either loss or gain of function mutations, respectively, in the *SCN9A* gene encoding the voltage-gated sodium channel Na$_v$1.7. Along with direct effects on the functionality of ion channel subunits or the proteins that regulate them, channelopathies can also result from autoimmune responses to channel proteins.[9]

To date, most ion channel drug development has focused on identifying and developing small molecule and peptide modulators, mainly through serendipitous discovery due to a lack of information on structure and function. Many ion channel modulators have been discovered from studies of naturally occurring substances, such as toxins from plants and venomous animals.[10] The conotoxin family is the most well-known of the animal-derived toxins,[11] with ziconotide, a selective Ca$_v$2.2 antagonist, a frequently cited example of a synthetic peptide analogue of cone snail ω-conotoxin used for the treatment of severe chronic pain.[12] Despite the initial successes in identifying ion channel modulators, only two novel ion channel drugs have been approved by the

US Food and Drug Administration (FDA) since the 1990s, despite vastly improved screening tools for small molecule/compound libraries.[13] The most recently approved drugs are ivacaftor (Kalydeco), which potentiates the cystic fibrosis CFTR chloride channel[14] and crofelemer (Mytesi), a proanthocyanidin oligomer, which inhibits both CFTR and the calcium-activated chloride channel TMEM16A.[15] As with the vast majority of other drugs targeting ion channels, ivacaftor and crofelemer are both small molecule chemical entities.[16]

Alternative modalities for targeting ion channels have recently included monoclonal antibodies (mAbs), but their therapeutic potential has been vastly underexploited.[17] An in-house analysis using information gleaned from the public domain revealed that only one antibody drug (a polyclonal or pAb) is in early clinical study among the > 650 ion channel targeting drugs under active development in the global pipeline (Figure 1(b)).

Advantages of targeting ion channels with antibodies

Although therapeutic antibodies are typically more expensive to develop, they generally attain higher approval success rates compared with their small molecule counterparts.[18] As with antibodies targeting GPCRs,[19,20] antibodies directed towards ion channels have the potential to offer many additional advantages relative to selectivity, bioavailability and effector function as summarized below.

Selectivity

Obtaining target selectivity in small molecule drug discovery is one of the foremost technical hurdles for drug development, regardless of the route from which the molecule is derived, i.e., rational design or random screening of large compound libraries. With respect to ion channels, this has been particularly challenging as ion channels within a given family often share high levels of
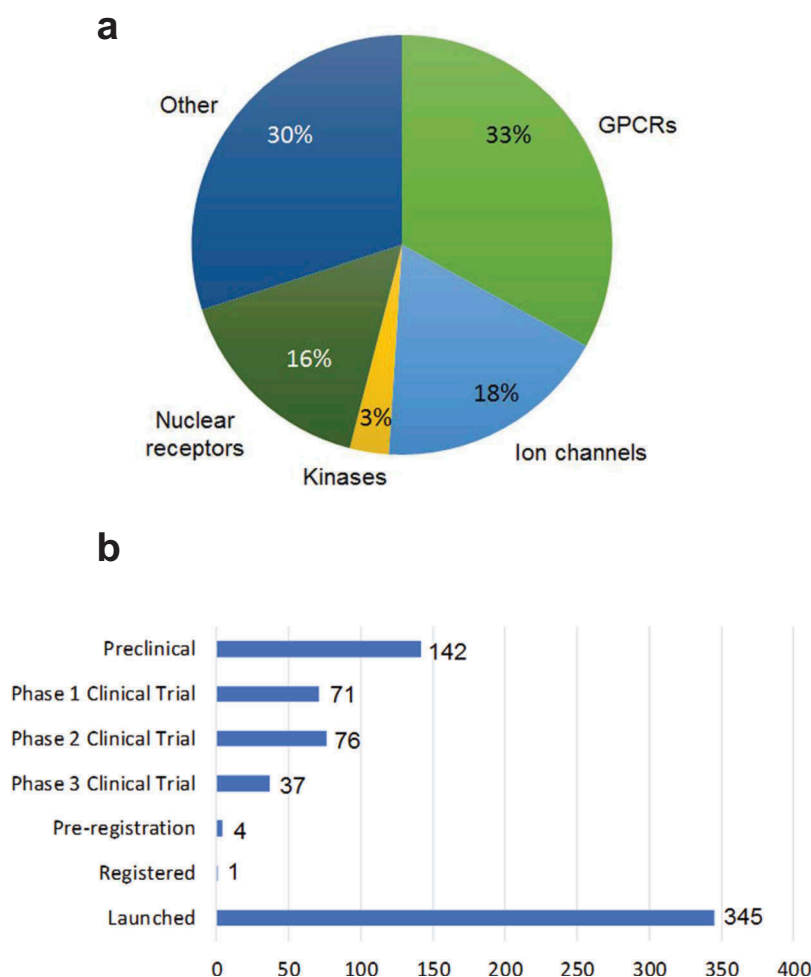
Figure 1. Market opportunities and global clinical pipeline for ion channel drug targets. (a) Market opportunities for targeting ion channels which represent the second largest membrane protein target class after GPCRs, adapted from Santos *et al* 2017.[5] (b) Ion channel drugs in development and the clinical pipeline (sourced from Pharmaprojects as of March/April 2016).

homology, notably within the pore-forming domains where many channel blockers exert their effect, but have vastly different physiological roles. For example, the sodium channel isoforms $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ have been identified as targets in nociceptor neurons where modulation ameliorates different pain states. However, stringent counter-screens are required to characterize potential modulators of these channels for effects on other Na_v family members, such as $Na_v1.5$, which initiates the cardiac action potential. Superior specificity and selectivity compared to small molecules are particularly relevant when the desire is to target specific ion channel isoforms, for example, the non-functional variant of P2X7 (nfP2X7),[21] the neo-natal splice variant of $Na_v1.5$ (nNa_v1.5),[22] or isoforms of $K_v11.1B$ that are up-regulated in certain tumors.[23,24] An obvious alternative to small molecule promiscuity is the development of mAbs, where high levels of specificity would be expected to mitigate off-target effects, and therefore generate safer classes of drugs.

Biodistribution, half-life and effector functions

MAbs offer a number of potential benefits beyond selectivity, including 1) limiting central nervous system (CNS) penetration (when targeting a therapeutic to the periphery); 2) low variability in patient pharmacokinetics; and 3) longer duration of action leading to reduced dosing. The half-lives of native antibodies can be further extended through alterations to the variable domain that enhance FcRn-mediated recycling[25] and for antibody fragments via modification with polyethelene glycol (PEG) (i.e., pegylation)[26] or binding to human serum albumin.[27] Other types of protein engineering apply approaches directed to the Fc domain that can be used to ablate or increase antibody-mediated effector functions, such as antibody-dependent cell-mediated cytotoxicity (ADCC), complement mediated cytotoxicity, or antibody-dependent cell-mediated phagocytosis,[28] which are relevant in the case of autoimmune diseases and cancer. MAbs can also be conjugated to radioisotopes or toxic compounds, or linked to the T-cell receptor (so-called CAR-T technology) to directly kill tumors or elicit T-cell mediated tumor cell destruction, respectively. Given their exquisite specificity, it may also be possible to generate mAbs that recognize different conformational states of an ion channel, such as a depolarization-induced conformational change that may render an epitope more accessible to antibody binding.[29] In addition, affinity and potency against a target can be further enhanced by well-established protein engineering methodologies for lead mAb optimization.

The challenges for ion channel antibody drug discovery

Despite considerable interest, only one polyclonal antibody, BIL010t (Biosceptre, Cambridge, UK), which recognizes a non-functional form of P2X7 and is formulated as a topical ointment for the treatment of basal cell carcinoma, has completed Phase 1 clinical trials.[30] The lack of success in generating such antibodies, particularly mAbs, is attributable to a number of important challenges. For example, for many of the voltage-gated ion channels (VGICs), extracellular loops (where mAbs are most likely to elicit a modulating effect) are short and contain few potential epitopes (Figure 2). Additionally, these loops tend to be highly conserved at the primary amino acid sequence level, and thus lack sufficient immunogenicity to generate robust antibody responses in mammalian hosts. Even in cases where the extracellular domains are large, the proteins themselves are either poorly expressed or difficult to purify from conventional platforms used for recombinant protein production. This, in turn, can limit the starting material available for large-scale immunization and screening campaigns.

Despite these challenges, autoantibodies that bind ion channels (and presumably alter their activity) have been identified in patients with a number of diseases, including myasthenia gravis (nAChR),[35] multiple sclerosis (Kir4.1),[36,37] Lambert-Eaton Myasthenic Syndrome or LEMS (VGCC),[38] neuromyotonia (K_v1 family) (voltage-gated potassium channels),[39] melanoma retinopathy (TRPM1),[40] autoimmune encephalitis (NMDA),[41] progressive encephalo-myelitis with rigidity and myoclonus, also known as PERM (glycine receptor)[42] and Morvan's syndrome (the K_v1 family of voltage-gated potassium channels).[43] Some autoantibodies[44] and at least one mAb[45] induce ion channel internalization, suggesting that antibody drug conjugates could also be a feasible therapeutic modality for targeting this drug class. Moreover, therapeutic antibodies generated in response to DNA immunization using an expression vector encoding the $K_v1.3$ potassium efflux channel have been shown to be effective in ameliorating autoimmune encephalomyelitis in rats, underscoring the validity of antibodies as ion channel drug candidates.[46]

Ion channel structural topology

The classification of ion channels can be based upon their ion selectivity, gating mechanism and/or sequence similarity. The ion channel gating mechanism system identifies three main groups, namely the voltage-gated channels, the extracellular ligand-gated channels and channel proteins utilizing other gating mechanisms, such as mechano-sensitive channels. The structural architecture associated with each family of ion channel has been described extensively elsewhere,[47-52] and is not reviewed here.

Among the key factors governing successful discovery of antibodies that can modulate ion channel activity are the size, complexity, immunogenicity and mechanistic properties of the extracellular domains where antibodies are expected to bind. The topology of select ion channel family members, as shown in Figure 2, demonstrates stark differences in the size of the extracellular domains and loops relative to the whole ion channel, such as those observed between acid sensing and P2X channels (large extracellular domains) and voltage-gated and TRP channels (small extracellular loops). Owing to the paucity of potential immunogenic extracellular epitopes of the latter group, perhaps it is not surprising that the single antibody drug in clinical development targets an ion channel belonging to the former group (nfP2X7), and is actually polyclonal.

The challenges noted above have led to targeting specific ion channel extracellular domains with varying levels of success. For example, the E3 re-entrant loop of ion channels comprising six transmembrane (TM) motifs has held a particular interest since this region is thought to maintain positioning of the ion selectivity filter and, at least in some cases, appears to interact with toxins and physiological modulators.[53] The length and accessibility of the E3 region between the fifth (S5) and sixth (S6) transmembrane domains (TMDs) presents a suitable targeting region for antibodies, and it is rarely post-translationally modified.[50] The amino acid sequence of channel subtypes can be varied in this region, which also offers the opportunity for isoform-specific interactions to disrupt channel function. Many antibodies reported to have been generated to this region tend to be polyclonal, namely $K_v1.2$, $K_v3.1$, $K_v10.1$, TRPC1, TRPC5, TRPM3, TRPV1, $Na_v1.5$, $Ca_v2.1/Ca_v2.2$,[50] and exhibit functional activity, such as modulation of store-operated or agonist-evoked Ca^{2+} entry,[54-59] promotion of oligodendrocyte
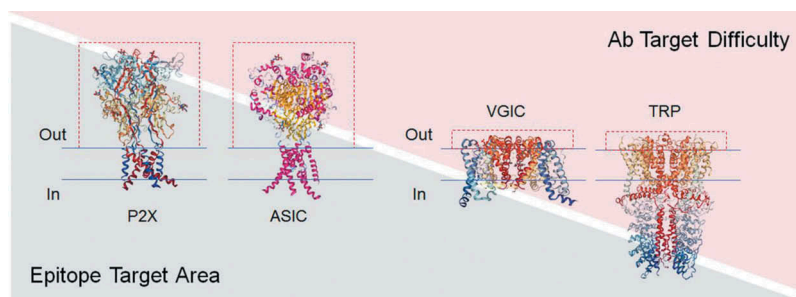

Figure 2. Ion channel extracellular domains can influence the difficulty in generating functional antibodies. A comparison of the structural topology of P2X, acid sensing (ASIC), voltage-gated (VGIC) and transient-receptor potential (TRP) ion channel families is shown with the relative mass of the extracellular domains (ECDs) highlighted by dashed red lines. Structural information was adapted from the Protein Data Bank (PDB) figures for P2X4 (3I5D)[31], VGCC (4MTO)[32], TRPA1 (3J9P)[33] and ASIC1 (6AVE)[34]. The plasma membrane is represented by blue horizontal lines. Channels with large ECDs (e.g. P2X and ASIC) are expected to display a proportionally larger epitope target area than channels with much smaller ECDs (e.g. VGIC and TRP) and would therefore present less challenging targets in antibody discovery campaigns. Conversely, VGICs and TRP channels that display much smaller epitope target areas represent more challenging targets.

proliferation and migration[60] and inhibition of tumor growth.[61,62] Additionally, NESOpAb is a polyclonal antibody that specifically recognizes a neonatal epitope presented on the second extracellular loop in Na$_v$1.5 domain I and inhibits sodium currents up to 60% with an IC$_{50}$ value of less than 25 nmol/L. Furthermore, it demonstrates selectivity, being able to distinguish between the neonatal and adult splice variant forms, which differ by seven amino acids.[63]

Sources of ion channels for generating and screening ion channel antibodies

Ion channels are typically low abundance proteins in the cells and tissues in which they are produced. Furthermore, when expressed as recombinant proteins in heterologous systems (e.g., mammalian, insect, yeast and bacterial cells) yields of purified functional protein are often low. Therefore, production of antibodies that can recognize and/or block channel activity has relied primarily on immunization of animals with either: 1) whole cells; 2) crude membrane fractions; 3) plasmid DNA expression vectors encoding channel protein subunits; or 4) peptide-based antigens that preferably mimic a targeted extracellular loop structure. As an alternative to immunization, antibody discovery can also be achieved by screening pre-existing libraries of antibody single-chain variable fragments (scFvs) from naïve or immunized animals via phage or yeast display.[64] While this latter approach can preclude immunization altogether, the need for purified, correctly folded protein is generally required for the panning and screening phases of the process. Some of the sources of protein used as antigen and to screen for antibody identification are described briefly as follows.

Purified ion channels from native sources

Previous studies have shown that ion channels can be isolated from their native source in a way that maintains functionality of the purified protein following reconstitution into proteoliposomes. For example, a number of laboratories in the 1980s and 1990s purified voltage-gated sodium ion channels from rat brains,[65] as well as from the electroplax of the electric eel, *Electrophorus electricus*,[66] and were able to reconstitute functional activity from purified components.[67-70] Given the high degree of conservation amongst ion channel orthologs, channel proteins from animal sources might therefore serve as antigens and screening reagents to identify antibodies that recognize and modulate their human counterparts. The obvious drawbacks here are the need to obtain sufficient amounts of material from sources whose channel proteins closely match their human orthologs, the typically complicated purifications required to generate that material, and the need to break tolerance in animal hosts being used for immunization.

Recombinant proteins expressed in mammalian cells

Mammalian cell lines (e.g., HEK293, CHO, U2OS) are arguably the gold standard for generating recombinant ion channels closest to their 'native' configurations and functionalities. However, as noted above, mammalian cells typically produce low levels of surface localized, functional recombinant ion channels, making them difficult to purify. The use of whole cells or crude cell fractions diminishes the antigenic load of the target protein and introduces additional contaminating proteins that are likely to be more abundant and more immunogenic than the recombinant ion channel of interest, making it difficult to generate an immune response or *in vitro* display output that is sufficiently enriched to effectively screen.

Chimeric channels expressed in E. coli

Despite the phylogenetic divergence between prokaryotes and eukaryotes, it has been possible to generate chimeric bacterial-human ion channels that facilitate protein expression and purification in bacterial host expression systems. For example, a functional chimera in which the extracellular domain (ECD) of the bacterial protein GLIC was fused to the transmembrane domain of the human α1 glycine receptor (α1GlyR) has been reported,[71] as have functional pentameric ligand-gated ion channel chimeras containing large eukaryotic intracellular domains from nAchR-α7, GABAρ1 and Glyα1 fused to the *Gloeobacter violaceus* GLIC channel.[72] Similarly, the use of bacterial Nav channels has been elegantly exemplified in structural studies of VGICs[73] and enabled crystallization of a chimeric voltage-gated sodium channel from *Arcobacter butzleri* fused to portions of the human Na$_v$1.7 voltage-sensor 4 domain bound to aryl sulphonamide antagonists.[74] Although chimeric channels could offer a possible solution to the generation of sufficient amounts of antigen and screening reagents to implement antibody discovery processes, they would nevertheless require extensive counter-screening. Moreover, the bacterial elements of the channel may present immunodominant epitopes that could overwhelm the response to the human components of the chimera.

Alternative platforms for recombinant protein expression

Cilated protozoa devote a large part of their metabolism towards membrane protein production and have expanded gene families for all four of the major classes of membrane transporters, including P-type ATPases, major facilitator superfamily members, ABC transporters and voltage-gated ion channels.[75] *Tetrahymena thermophila*, in particular, has been identified as an attractive platform for over-expression of recombinant human ion channels based on the fact that its macronuclear genome encodes approximately three times as many voltage-dependent K$^+$ channels as do human cells.[75] Although a complex eukaryote, *Tetrahymena* shares many of the features of microbial expression hosts, including ease of growth in peptone-based media at scale with relatively short doubling times of 2 to 3 hours.[76]

TetraGenetics Inc., an early-stage biotechnology company in Arlington, MA, has demonstrated expression of approximately 20 recombinant human voltage-gated, ligand-gated and mechano-sensitive ion channels in *Tetrahymena* (unpublished data). Efficient recovery of purified channel proteins (in many cases, in the order of > 1mg/L culture) has enabled the development of antigen preparations and screening tools that have recently been used to generate a panel of blocking anti-K$_v$1.3 antibodies.[77] Besides the propensity for this organism to encode hundreds of native ion channels, it remains unclear

why it bypasses the limiting factors associated with low expression yields in mammalian cells where there appears to be an upper limit on how many functional recombinant channels can reach the plasma membrane before creating a toxic metabolic environment. No such toxicity is observed in *Tetrahymena*, allowing many more recombinant channels to reach the cell surface. In mammalian cells, plasma membrane channel number is likely to be regulated by a variety of mechanisms, including manipulation of various retention signals by auxiliary subunits. *Tetrahymena* does not encode any obvious orthologs of mammalian auxiliary subunits and, in the case of Na^+-selective voltage gated channels, co-expression of the β subunits is not required for cell surface localization in the *Tetrahymena* system (unpublished data). While it is clear that *Tetrahymena* is well suited to the production of recombinant ion channel proteins, a number of other systems are being developed for this purpose, including virus-like particles,[78] cell-free lysates,[79-82] synthetic and semi-synthetic chemistries.[83,84]

Maintaining the native protein fold – SMALPs and nanodiscs

Characterization of modulating antibodies following immunization or *in vitro* display methods requires that a purified ion channel protein be maintained in its proper three-dimensional conformation regardless of its source. One approach towards stabiliizing the structure involves incorporation of detergent-solubilzed and purified membrane proteins into nanodiscs that utilize a supporting protein scaffold and lipids to generate an artificial bilayer into which the membrane protein of interest is embedded.[85-87] Potential limitations to this approach are that transfer of proteins into nanodiscs requires initial solubilization by detergent, as well as reconstition into a non-native lipid environment. In addition, solubilization of membrane proteins in detergents faces the technical challenge of maintaining a physiologically relevant conformation in addition to stability. An alternative strategy that provides a detergent-free route to membrane protein isolation with retention of the native lipid environment (as much as is possible) is to replace the detergent with amphipathic styrene-maleic acid (SMAs), where the polymer self-inserts into biological membranes and is capable of extracting small discs of the native lipid bilayer containing the membrane protein of interest, generating SMA-encapsulated lipid particles (SMALPs).[88] More recently, the tetrameric potassium channel KcsA was isolated directly from the membranes of *Escherichia coli* without the need for detergent by using SMALPs.[89] SMALPs have also been reconstituted into planar lipid bilayers directly from native nanodiscs, which enabled functional characterization of the TRPV1 channel by electrophysiology.[90] The nanodisc approach was implemented in the reconstitution of tetrameric KirBac1.1 potassium channels into lipid nanodiscs, enabling single-molecule fluorescence resonance energy transfer confocal microscopy, which permitted the elucidation of structural changes that occur upon channel activation and inhibition.[91]

DNA immunization

Plasmid expression vectors encoding ion channel proteins are likely to produce correctly folded and functional antigens following immunization of mammals. Nevertheless, the yield of protein presented at the cell surface may still be low and the resulting immune response may not be sufficiently robust to generate an antibody titer high enough to identify potential modulators. The inclusion of adjuvants and the use of tailored expression vectors with strong promoters, such as the CMV promoter, are often applied in this instance.[92] The use of T cell helper epitopes, such as PADRE, are also proving successful.[46] The DNA immunization approach has been used to generate $K_v1.3$ nanobodies using the Ablynx platform,[93] as well as in combination with purified antigen to generate conventional $K_v1.3$ antibodies.[77] These are described in a later section.

Peptides

Peptide antigens have been used to generate functional polyclonal antibodies against multiple ion channels,[50] as well as mAbs targeting select ion channels.[61] Peptides usually do not suffer from issues of quantity or purity because they can typically be produced via chemical synthesis or robust cell expression systems, such as *E. coli*, to serve both antigen and screening requirements. However, the physiological relevance of peptide-based antigens, even those that are three-dimensionally accurate representations of surface loop structures, will always be limited as they lack the context of other molecular determinants associated with the ion channel surface 'epitome'.

Ion channel antibody generation and screening – additional considerations

While the source of ion channel antigen is a critical consideration for any antibody discovery program, the approach to the generation and screening of ion channel antibodies should be scrutinized in the context of the challenges described above. The relative lack of success in the identification and clinical progression of ion channel antibodies suggests that therapeutically valuable antibodies are typically rare and difficult to identify in any given discovery program. Therefore, it would seem prudent to structure a discovery program that can either increase a specific immune response against the target ion channel and/or deeply mine an immune repertoire in an effort to capture as many potential hits as possible. In the case of the former, amino acid conservation and, depending on the ion channel, the topology of the ion channel can affect the generation of a robust immune response. Options that may mitigate this challenge include the use of phylogenetically diverse immune hosts; immunization strategies that break immune tolerance, such as DNA immunization; inclusion of T-helper cell epitopes; transgenic animals overexpressing the neonatal Fc receptor;[94-96] and, related to this, transgenic animals where the target gene has been deleted. Of course, the latter approach will be dependent on the viability of the knock-out animal. With regards to mining the immune repertoire, a number of platform technologies, e.g., direct B-cell cloning and/or deep sequencing,[97-99] have been developed recently that increase the probability of identifying rare antibodies and avoid standard hybridoma-based technologies where valuable antibodies may be missed owing to inefficient fusion events or the loss of rare B cell clones.[100]

Following the identification of antibodies that specifically recognize the ion channel target, it is important to select extracellular binders by some means to advance these clones into functional characterization. Typically, the method most commonly used to identify extracellular binders is flow cytometry using native or transfected mammalian cell lines expressing the ion channel of interest. This, however, is not necessarily straightforward because many cell lines with confirmed electrophysiological activity may nevertheless express low surface channel numbers making definitive identification difficult. Alternatively, ELISA assays using peptide and protein fragments comprising various extracellular loops is relatively quick and simple. However, peptides applied in this manner are generally not conformational and false-negative results are likely for antibodies that recognize discontinuous or conformational epitopes.

Depending on the number of hits that are recovered from a given discovery program, it may be feasible to forego the antibody sorting described above and move directly to functional characterization. The accepted gold standard for ion channel functional characterization is patch-clamp electrophysiology, which allows real-time kinetic and pharmacological analysis of the effects of drug molecule candidates. Whilst electrophysiology is the most detailed analytical tool available for ion channel functional modulation and is key in making hit-to-lead candidate determinations, it is resource intensive and has suffered historically from low throughput. Progress has been attained with increasing screening throughput and maintaining accuracy with platforms that utilize robotic multi-patch clamp configurations (Patchliner®, Nanion Technologies; Qube384, Sophion; PatchXpress, Molecular Devices; IonFlux, Fluxion). Interestingly, some investigators and platform manufacturers have reported instances of compounds (including antibodies) that demonstrate functionality when analyzed by manual patch-clamp, but are inactive when analyzed by automated platforms (Colussi, personal communication). The reasons for this remain unclear, but the possibility of false-negative results may lead to the abandonment of potentially interesting antibodies. Moreover, antibodies may reasonably be assumed to exhibit slower binding and efficacy kinetics compared to small molecules, which should be considered when analyzing electrophysiological currents over the course of 5 to 10 minutes in which measurements are usually made.

In addition to electrophysiology, a number of other technologies are available that can offer effective screening of modulating compounds that each have their own advantages and disadvantages. These include flux-based assays that measure the cellular influx or efflux of radioactive Na^+, Ca^{2+} and Rb^+ for studying sodium, calcium and potassium channels, respectively, and fluorescence-based assays that utilize either voltage-sensitive dyes that measure cell membrane voltage changes or ion-specific fluorescent probes that measure changes in intracellular ion concentrations.[101] A recent and more detailed review of ion channel antibody screening strategies can be found in Colley et al.[102]

Ion channel therapeutic opportunities

The wide range of physiological processes involving ion channels can be broadly summarized as follows: maintenance of cell resting potential, conductance of electrical signals, synaptic transmission at nerve terminals, intracellular transfer of ions and metabolites, cell volume regulation, excitation-contraction coupling and stimulation-secretion coupling, such as that involved in the release of insulin from the pancreas.

Using information in the public domain, we analyzed the ion channel target landscape against which antibody therapeutics could have potential in the treatment of disease, excluding those that would require CNS penetration or intracellular distribution. This analysis identified over 150 potential antibody targets, with over 35 of those possessing clinical or preclinical levels of validation from small molecule and peptide studies. Many opportunities fall within the oncology, autoimmune and inflammatory/neuropathic pain (including migraine) therapeutic areas (Figure 3), but there is also significant potential in the respiratory, metabolic and rare or neglected disease areas. Several ion channel targets fall into more than one therapeutic indication.[103] Our findings are summarized in Table 1. The main subclasses of targets are voltage-gated and calcium-activated potassium ion channels, voltage-gated calcium and sodium channels, acid-sensing ion channels, transient receptor potential (TRP) channels, purinergic P2X channels, calcium-release activated channels and chloride channels, which are discussed below.

Voltage-gated potassium ion channels

There are at least 40 voltage-gated potassium channels (K_v) in the human genome with physiological roles.[172] K_v channels represent an ion channel subclass that offers substantial potential for drug development in a range of diseases, such as cancer, autoimmune diseases and metabolic, neurological and cardiovascular conditions. Their roles range from regulating calcium signalling, cell cycle progression, apoptosis and cell volume to driving cellular proliferation and migration, as well as repolarization of neuronal or cardiac action potentials,[173-178] and thus present the potential for various pharmacological strategies to target K_V channels with specific antibodies. The therapeutic potential of selected potassium channels is highlighted below.

$K_v1.3$

$K_v1.3$ is encoded by the gene *KCNA3*, expressed on human T cells and was initially recognized as a target for immunosuppression based on the observation that non-selective K^+ channel blockers can inhibit T cell proliferation and interleukin (IL)-2 secretion.[104,105] It has since been validated as a therapeutic target in numerous preclinical animal models using a variety of small molecule and peptide toxin blockers[104,106-111] and, importantly, in the clinic through the development of the potent venom peptide analog Shk-186 (dalazatide).[113]

$K_v1.3$ is the predominant potassium channel expressed on effector memory T cells (T_{EM}), which are implicated in a range of T-cell mediated diseases, such as, multiple sclerosis,[106] rheumatoid arthritis,[104] Type-1 diabetes mellitus,[104] allergic contact dermatitis[109] and psoriasis.[108] $K_v1.3$ blockers selectively inhibit Ca^{2+} signalling, proliferation and *in vivo* migration of CCR7 negative and positive T_{EM} cells; however, stronger effects are observed on CCR7$^-$ T_{EM} cells compared to CCR7$^+$ central memory CD4 T cells.[179] More recently, $K_v1.3$ knock-down in memory
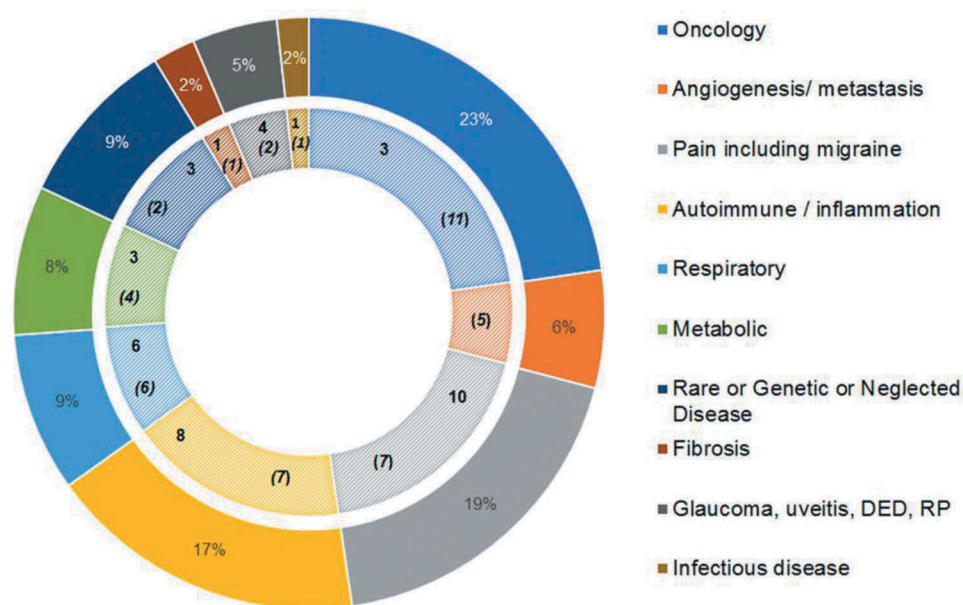
Figure 3. Therapeutic opportunities in the ion channel antibody target landscape shown by therapeutic area. The percentage values in the outer ring represent the number of ion channels implicated for that therapeutic area from the >150 potential antibody targets identified. The inner ring depicts each therapeutic area with the number of clinically (in Phase 2 or further development) validated targets in bold font and the number of preclinically validated targets indicated in bold italicized font and bracketed for distinction. In a few instances, an ion channel has presented targeting opportunities in multiple indications within a therapeutic area and therefore different levels of validation have been presented. Therefore, the highest level of validation is taken to avoid duplication, for example, P2X3 in different respiratory conditions. However, where there are ion channels representing a targeting opportunity in multiple therapeutic areas these have been treated separately and accordingly can demonstrate different levels of validation, for example, $K_v1.3$ (implicated in autoimmune conditions, such as type 1 diabetes, psoriasis, cutaneuous lupus; respiratory indications (asthma); inflammatory conditions (uveitis and dry eye disease), $K_{Ca}3.1$ (implicated in autoimmune condtions, such as IBD, multiple sclerosis, rheumatoid arthritis; oncology (glioma, renal cancer, NSCLC), respiratory indications (asthma), sickle cell anemia) and TRPC6 (pain; respiratory; metabolic; autoimmune/inflammation; oncology). For further details of the role of each of these ion channels in disease, refer to the main text. There are at least 35 ion channels with clinical or a preclinical level of validation provided by small molecule or peptidic approaches that are suitable for targeting with therapeutic antibodies. Abbreviations: DED dry eye disease; RP retinitis pigmentosa.

T cells was shown to suppress CD40L expression and memory phenotype.[180] CD40L is also a target for autoimmune disorders, and this finding provides further validation of the therapeutic potential of $K_v1.3$ blockade in immunomodulation.[123]

There are no significant intracellular calcium stores in T cells due to their small size, therefore Ca^{2+} influx through CRAC is paramount for NFAT translocation to the nucleus to elicit cytokine secretion and T cell proliferation. The T cell needs to retain a negative membrane potential through a counterbalancing K^+ efflux via $K_v1.3$ and/or the other T cell K^+ channel, Ca^{2+}-activated channel $K_{Ca}3.1$, in order to be fully activated.[172] Thus, pharmacological inhibition of $K_v1.3$ activity blocks activated T-cell proliferation and cytokine production by disrupting the driving force of sustained Ca^{2+} influx via CRAC.

$K_v1.3$ is also expressed in breast, prostate and colon cancer and is linked to resistance to apoptosis as observed by the upregulation of $K_v1.3$ expression in diffuse large B-cell lymphoma and glioma.[181] Nevertheless, the role $K_v1.3$ plays in proliferation and apoptosis appears to be complex and context dependent, and further studies are required to validate $K_v1.3$ as a potential cancer target and biomarker.[182]

$K_v10.1$

A comprehensive overview of the biophysical and pharmacological roles of $K_v10.1$ and its potential mechanisms in disease has been described elsewhere.[183,184] $K_v10.1$, also known as ether-a-go-go-related gene 1 (EAG1), is encoded by the gene *KCNH1* and

expression is predominantly restricted to the CNS in health. Most of the interest in $K_v10.1$ as a therapeutic target originates from the observation that it is aberrantly expressed in up to 70% of tumor cell lines and human cancers,[185] including colon cancer,[186] gastric cancer,[187] breast cancer,[188,189] soft tissue sarcoma,[190] acute myeloid leukaemia (AML),[62] adenoma,[191] hepatocarcinoma,[192] head and neck cancer,[193] brain metastases and glioblastoma multiforme.[194] Hence, $K_v10.1$ presents a good opportunity as an antibody target, in the context of disease association where targeting would be restricted to the periphery due to the inability of antibodies to cross the blood-brain-barrier. $K_v10.1$ expression also has potential as a biomarker in several of these tumor types and can correlate with a poor prognosis.[187,195,196]

As such, $K_v10.1$ has been extensively studied in terms of its role in aberrant cell proliferation and tumor growth, where expression has been reported to be activated by epidermal growth factor receptor (EGFR) tyrosine kinase[197] and regulated by p53 and E2F1 that are also often altered in cancer.[198] As well as presenting a potential therapeutic target, $K_v10.1$ is being explored as a diagnostic marker through tumor xenograft imaging *in vivo* studies.[199] $K_v10.1$ also plays a key role in cytoskeletal organisation, which in turn affects cell viability, angiogenesis, migration and invasion,[200] thereby conferring an advantage to tumor growth through increased vascularization and resistance to hypoxia. It has also been shown that $K_v10.1$ is constitutively and rapidly internalized by endocytosis and lysosomal sorting,[201] and that recycling contributes to maintaining $K_v10.1$ surface level expression. This property is an important

Table 1. Examples of ion channel therapeutic opportunities with level of validation attained by different drug entities, or associated biology, including genetic evidence, knock-out models, etc.

Ion channel	Therapeutic Area/Indication	Modality &/or Entity	In vitro validation	In vivo validation/preclinical	Clinical validation	Reference
$K_v1.3$	MS, RA, T1D, atopic dermatitis, uveitis, DED, psoriasis myositis, cutaneous lupus, psoriatic arthritis, IBD, allergic asthma	Antagonist – peptide analogs of ShK toxin, e.g., dalazatide	Inhibition of T_{EM} cell proliferation and migration, IL-2 secretion, Ca^{2+} signalling, inhibition of $K_v1.3$ currents, inhibition of CD3-antibody- and alloantigen-induced proliferation	Inhibition of T_{EM} cell proliferation, blocking $K_v1.3$ in psoriasiform SCID mouse model, efficacy in DTH and EAE rat models. Clears viral and bacterial infections	Validation in DED from T cells isolated from patient tissue; suppression of chemokine-induced migration of peripheral blood T cells isolated from healthy donors Dalazatide Ph1 & Ph2	77,93,104-112
	Atopic dermatitis, psoriasis	Antagonist – small molecule, e.g., PAP1	Blocking of $K_v1.3$ currents. Significant dose-dependent inhibition of proliferation and suppression of IL-2 and IFN-γ production	Potent suppression of oxazolone-induced inflammation by inhibiting the infiltration of CD8 + T cells in rat allergic contact dermatitis model; significant clinical and histological improvement of plaques in SCID mouse-psoriasis skin xenograft model with reduction in T_{EM} cells	Patient psoriatic plaques enriched in T_{EM} cells Ph1 (inactive)	108-110,113
$K_v10.2$	Brain cancer, lung and cervical	Antagonist – small molecule, e.g., TDZ	Induction of caspase-dependent apoptosis and cell cycle arrest	Reduction in xenografted MB growth and metastasis, inhibition of balbc/c nude mouse xenografts established using A549 sphere cells	Case report of MB patient demonstrated therapeutic efficacy although not without side effects	114-116
$K_v11.1B$	Some cancers (leukemias, gastric, colon)	Antagonist – small molecule, e.g., CD-60,130	Reduction in cell proliferation of tumor cells and tumor cell invasiveness, reduction in VEGF secretion	Reduced leukemic cell infiltration in NOD/SCID and higher survival rates	Epigenetically silenced in ovarian cancer	24,117-122
$K_{Ca}3.1$	Autoimmune, e.g., IBD, MS, RA, asthma, fibrosis, sickle cell anemia	Antagonist – small molecule, e.g., TRAM-34, NS6180, Senicapoc	Genetic knockdown of $K_{Ca}3.1$ suppresses T cell activation	No toxicities observed. $K_{Ca}3.1$ blockers validated in a number of animal models, e.g., rodent EAE and experimental colitis models	Restores corticosteroid sensitivity in cytokine-treated ASM cells from COPD and asthmatic patients Ph2 (inactive)	123-133
	Breast, prostate, pancreatic, endometrial, GBM, HNSCC, leukemia, ICC, melanoma	Combined activation of $K_{Ca}3.1$ and inhibition of $K_v11.1$ – small molecule, e.g., Riluzole	Cisplatin-resistant CRC cells express higher levels of $K_{Ca}3.1$ and $K_v11.1$ channels; $K_{Ca}3.1$ activators and $K_v11.1$ inhibitors have a synergistic action with cisplatin in triggering apoptosis and inhibiting proliferation; TRAM-34 also potentiates response of TMZ	In nude mice xenografted with human NSCLC, Senicapoc reduced tumor growth. In SCID mice xenografted with human GL-15 glioma cells, TRAM-34 reduced tumor infiltration and astrogliosis surrounding the tumor	Ph1 NCT01303341 Ph2 NCT00866840	134-144
$Ca_v3.1$	Breast and prostate cancer	Agonist	Tumor suppressor function	Expression inhibits proliferation and apoptosis of MCF7 cells. Inhibition of prostate cancer cell proliferation	Mutations in $Ca_v3.1$ confer gain-of-function in adenomas	145-147
$Na_v1.7$	Pain	Antagonist – peptide	Blocking of $Na_v1.7$ currents but also acts at $Ca_v2.2$	Synthetic peptides based on spider-derived venom have reversed pain behaviours in mouse models of peripheral spontaneous pain	Genetic evidence provided by loss-of-function and gain-of-function channelopathies.	9,148
		Antagonist – small molecule, e.g., PF-04856264, PF-05089771, CNV1014802/BIIB074	Bind preferentially to slow inactivated state of $Na_v1.7$, blocks TTX-induced current in DRG neurons	Less selective but more potent with respect to analgesia OD1 mouse model of $Na_v1.7$ mediated pain	Ph2 NCT01529346 NCT0156102, NCT02935608	52,149-151
ASIC1	Pain	Antagonist – small molecule, e.g, PPC-5650	Inhibition of ASIC1 mediated currents	Preclinical cancer models demonstrate nociceptive neuronal expression of ASIC receptors, that respond to a significant increase in an acidic cancer-induced environment within the bone	Ph1 (inactive) NCT01818570 NCT01449487 Reduction of IBS-related pain	152-154

(Continued)

Table 1. (Continued).

Ion channel	Therapeutic Area/Indication	Modality &/or Entity	In vitro validation	In vivo validation/preclinical	Clinical validation	Reference
TRPA1	Pain and inflammation	Antagonist	Small molecule in vitro inhibition of AITC-induced Ca2+ uptake	Functional upregulation in OVA-sensitized mice challenged with fine particulate matter. Inhibition of TRPA1 eliminates the mechanical and cold allodynia induced by cisplatin and oxaliplatin. Endogenous agonists known to cause pain sensation. TRPA1 knockout mice showed higher tolerance to heat	Ph2 NCT01726413 Genetic evidence demonstrated by TRPA1 gain-of-function mutation linked to FEPS	155-158
TRPC3	AP, SS, hypertension, atherosclerosis, COPD	Antagonist – small molecule, e.g., Pyr3, SalB	Mutated TRPC3 channels on Jurkat cells show decreased Ca2+ influx after TCR stimulation, which can be rescued by overexpression of wild-type TRPC3	Rat model of sepsis demonstrated upregulation of TRPC3 in T cells enhancing T cell apoptosis. TRPC3 inhibition protects salivary glands and pancreas cells from Ca2+ mediated toxicity by inhibiting the TRPC3-mediated component of Ca2+ influx	Genetic evidence provided by TRPC6 mutations in FSGS resulting in excessive Ca2+ influx and subsequent injury or loss of podocytes	159-162
TRPV3	Skin health, including inflammation and pain	Antagonist – small molecule, e.g., FTP-THQ, GRC15300	Inhibits agonist-induced release of ATP and GM-CSF in m308 keratinocytes	Dose-dependently blocks histamine-induced itch in mouse models. Reduction of CFA-induced thermal hypersensitivity	Gain-of-function TRPV3 mutations identified in rodent and man that are associated with pain and Olmsted syndrome. Ph2 (inactive)	163,164
P2X3 and P2X2/3	Pain, fibrosis, chronic cough	Antagonist -small molecule, e.g., gefapixant, AF-219	Block homo- and hetero-trimer forms	Blocks peripheral action in afferent neurons when ATP is released causing sensitization to pain signals	Ph2 and Ph3 NCT02477709	165
P2X4	Pain	Antagonist – small molecule, e.g., NC-2600, ivermectin	Inhibition of ATP-evoked intracellular Ca2+ influx	Efficacy demonstrated in CCI nerve neuropathic pain model and EAN. Dose-dependent inhibition of ATP-induced BDNF release	Ph1 https://adisinsight.springer.com/drugs/80008339	166-168 WO2010/093061
P2X7		Antagonist – small molecule, e.g., EVT-401	Blocks ATP-induced IL-1β release from monocytes. Blocks P2X7 mediated currents	Efficacy demonstrated in CGN-induced model of inflammation, DNBS-induced model of distal colitis, EAE rodent model and CIA model	Ph1 https://adisinsight.springer.com/drugs/80002672	US2011018287
nfP2X7	Basal cell carcinoma	Antagonist – antibody, e.g., pAb BIL010t	Expression of nfP2X7 in basal cell carcinoma confirmed by IHC	Lesion size reduced in a mouse model of melanoma	Ph1 NCT02587819	30
Orai1 (CRAC)	Autoimmune/inflammatory disease, e.g., psoriasis, AP	Antagonist – small molecule, e.g., CM-4620	Inhibits increase in intracellular Ca2+ in pancreatic acinar cells that leads to enzyme activation, mitochondrial dysfunction, ER stress and necrosis	Inhibits CRAC pathway in T cells, blocking the release of IL-2 and TNFα and reduces neutrophil activation	Ph1 NCT03401190	169
		Antagonist – mAb	Inhibition of T-cell effector function, T cell proliferation and cytokine release. Triggers internalization of Orai1	Demonstrates efficacy in rodent T-cell mediated GvHD model	Loss-of-function mutations cause severe immunodeficiency with recurrent infections due to impaired T cell function	170,171

Abbreviations and acronyms used in table:

AITC, allyl isothiocyanate; AP, acute pancreatitis; ASM, airway smooth muscle; ATP, adenosine triphosphate; BDNF, brain-derived neurotrophic factor; CCI, chronic constriction injury; CFA, complete Freund's adjuvant; CGN, carrageenan; CIA, collagen-induced arthritis; COPD, chronic obstructive pulmonary disease; CRC, colorectal cancer; DED, dry eye disease; DNBS, 2,4-dinitrobenzene sulfonic acid; DTH, delayed type hypersensitivity; DRG, dorsal root ganglion; EAE, experimental autoimmune encephalitis; EAN, experimental neuritis; ER, endoplasmic reticulum; FEPS, familial episodic pain syndrome; FSGS, focal segmental glomerulosclerosis; GBM, glioblastoma; GM-CSF, granulocyte-macrophage colony-stimulating factor; GvHD, graft versus host disease; HNSCC, head and neck squamous cell carcinoma; IBD, inflammatory bowel disorder; IBS, irritable bowel syndrome; ICC, intrahepatic cholangiocarcinoma; IFN-γ, interferon gamma; IHC, immuno-histochemistry; IL-2, interleukin-2; MB, medulloblastoma; MCF-7, Michigan Cancer Foundation-7 breast cancer cell line; MS, multiple sclerosis; NOD, non-obese diabetic; NSCLC, non-small-cell lung cancer; OD1, mouse model of Na$_v$1,7-mediated pain based on intraplantar injection of the scorpion toxin OD1; OVA, ovalbumin; Ph1, Phase 1 clinical trial; Ph2, Phase 2 clinical trial; Ph3, Phase 3 clinical trial; RA, rheumatoid arthritis; SalB, salvianolic acid B; SCID, severe combined immunodeficiency; ShK, Stichodactyla toxin; SS, Sjögren's syndrome; TCR, T cell receptor; T1D, type 1 diabetes; T$_{EM}$, effector memory T lymphocytes; TNFα, tumor necrosis factor alpha; TDZ, Thioridazine; TTX, tetrodotoxin; VEGF, vascular endothelial growth factor.

consideration for the potential of a drug's mechanism of action, such as an antibody-drug conjugate or prodrug format, for the targeting of tumor cells. $K_v10.1$ knock-out mice show no apparent deleterious phenotype during embryogenesis and develop normally to adulthood with no behavioural abnormalities,[202] suggesting that blockade or antagonism of aberrant ion channel expression is a feasible targeting strategy. Moreover, further validation is provided by experimental evidence generated from the specific inhibition of $K_v10.1$ by antisense technology[203] and siRNA[204] resulting in the reduction of tumor cell line proliferation *in vitro*.

Subsequently, a closely related family member, $K_v10.2$, has been identified as a potential target for brain, lung and cervical cancer where clinical proof-of-concept has been attained for the treatment of glioma using the re-purposed FDA-approved antipsychotic drug thioridazine as a $K_v10.2$ blocker achieving a reduction in tumor volume.[114]

$K_v2.1$

$K_v2.1$, encoded by the *KCNB1* gene, mediates a classical delayed rectifier current that is involved in neuronal repolarization. In addition to their role in the CNS, $K_v2.1$ ion channels are also involved in cell differentiation and growth of non-excitable cells, and inhibition of $K_v2.1$ in pancreatic β–cells enhances insulin secretion, which offers a potential therapeutic strategy for the treatment of Type-2 diabetes.[205] Over-expression and aberrant behaviour of $K_v2.1$ has also been reported in several tumor types including uterine cancers,[206] gastric cancers[207] and medullablastoma.[208] Further evidence of the potential therapeutic benefit of targeting $K_v2.1$ is provided by studies with perifosine, which is a third generation alkylphospholipid analog with anti-tumor properties. The principal mechanism of action of perifosine is the inhibition of Akt signalling by disrupting lipid rafts to which $K_v2.1$ ion channels preferentially localize. A recent study demonstrated that perifosine induces a hyperpolarizing shift in the voltage dependence of $K_v2.1$ inactivation, accelerating the kinetics of closed-state inactivation but without altering the voltage dependence of activation.[209]

$K_v11.1B$

$K_v11.1$ (or hERG) is encoded by the *KCNH2* gene and is the pore-forming α subunit of the voltage-gated inwardly rectifying potassium channel associated with cardiac arrhythmias and rhythmic excitability of the pituitary. $K_v11.1$ mediates the rapidly activating component of the delayed rectifying potassium current in heart (IKr)[210] and its properties are modulated by cAMP and auxiliary subunit assembly. It was one of the first voltage-gated channels linked to cancer, and has been shown to be abundantly expressed in several leukemias, gastric and colon cancer, whereas it is epigenetically silenced in ovarian cancer, and thus does not seem required for tumorigenesis in all tumor types.[24]

As with some other ion channels, $K_v11.1$ is associated with the sigma-1 receptor (SigR1), a stress-activated transmembrane chaperone.[211] SigR1 promotes the formation of $K_v11.1/β1$-integrin signalling complexes that trigger the activation of the PI3K/AKT pathways. The presence of Sig1R in tumor cells increases cell motility and vascular endothelial growth factor (VEGF) secretion. *In vitro* therapeutic

validation has been illustrated by several experimental observations following blockade of $K_v11.1$, such as the reduction of cell proliferation in cultured tumor cells,[117] ablation of the invasiveness of colorectal cancer cells[118] and gastric cancer cells,[119] as well as secretion of VEGF, a well-known driver of tumorigenesis and angiogenesis from glioma[120] and myeloid leukaemia cells.[117] NOD/SCID mice engrafted with acute lymphoid leukemia cells and treated with $K_v11.1$ channel blockers showed reduced leukemic infiltration and had higher survival rates, suggesting that potential therapeutic effects are relevant in an *in vivo* setting.[121,122]

However, the use of general $K_v11.1$ blockers in cancer therapy presents a risk for causing cardiotoxicity (by lengthening of the EGC QT interval), which would expose the patient to ventricular arrhythmias. The potential to circumvent this relies on the existence of at least 2 isoforms of $K_v11.1$, namely $K_v11.1A$ and $K_v11.1B$.[212] Tumor and cardiac cells express different ratios of the A and B $K_v11.1$ isoforms, thus side effects could be potentially avoided by specifically inhibiting the channel splice variant ($K_v11.1B$) that is highly expressed in certain tumors,[121] such as, AML, neuroblastoma[24] and acute lymphoblastic leukemia.[122,213] Some progress towards achieving this goal has been made using the small molecule CD-160,130,[121] which blocks the $K_v11.1B$ isoform with higher specificity than the $K_v11.1A$ isoform. MAbs targeting $K_v11.1B$ could provide the wherewithal for superior selectivity, and thus provide a safer therapeutic strategy. This is discussed further in a later section.

Calcium-activated potassium ion channels

Calcium-activated potassium channels are potassium channels gated by calcium or that are structurally or phylogenetically related to calcium-gated channels.[214] Intracellular calcium can also trigger potassium currents.[215] These channels can be broadly categorized into three types based on their unitary conductance: large-, intermediate-, and small-conductance.[216] Large-conductance channels are activated by both voltage and increases in cytosolic Ca^{2+} and represent a distinct gene family. Intermediate and small conductance K^+ channels are activated exclusively by increases in intracellular Ca^{2+} and represent a distinct gene sub-family.[217,218] Calcium-activated 6 or 7 TM K^+ channels (K_{Ca}), represent another structural sub-type in the potassium channel family where $K_{Ca}3.1$ is the most well-characterized member.

$K_{Ca}3.1$

First described in the 1950s, $K_{Ca}3.1$, encoded for by the *KCNN4* gene, is a voltage-independent potassium channel that is activated by intracellular calcium mediated by calmodulin.[214] Activation triggers membrane hyperpolarization, which in turn promotes calcium influx. $K_{Ca}3.1$ is expressed on activated T and B cells, macrophages, microglia, vascular endothelium, epithelia, proliferating vascular smooth muscle cells and fibroblasts, and therefore presents a potential therapeutic target for inflammatory and autoimmune diseases, such as inflammatory bowel disease and multiple sclerosis.[123] This ion channel is also expressed on erythrocytes, hence also has potential as a therapeutic target for sickle cell anemia.[124] Elevated intracellular calcium activates $K_{Ca}3.1$, thereby

maintaining a negative membrane potential, which is required for production of inflammatory chemokines and cytokines by T cells, macrophages and mast cells. Potassium efflux through $K_{Ca}3.1$ can be significant, resulting in efflux of > 50% of intracellular potassium content with the associated cell shrinkage being linked to apoptosis in certain circumstances.[219,220] Functional cooperation between TRPC1 and $K_{Ca}3.1$ in the regulation of Ca^{2+} entry has been suggested as both these ion channels co-localize into lipid rafts, and knockdown of TRPC1 suppresses the Ca^{2+} entry induced by $K_{Ca}3.1$ activation.[221]

Preclinical proof-of concept studies in animal models have validated the therapeutic potential of $K_{Ca}3.1$ blockers, with no toxicities observed in $K_{Ca}3.1$ knock-out mice and inhibition from developing severe colitis in two mouse models of inflammatory bowel disease,[125] a mouse model of experimental autoimmune encephalomyelitis,[126] several models of cardiovascular diseases[127] and unilateral ureteral obstruction-induced renal fibrosis in wild-type mice and rat.[128] $K_{Ca}3.1$ blockers, such as senicapoc, have been evaluated in clinical trials for sickle cell anemia and exercise-induced asthma, but have so far not shown efficacy, although the results have confirmed that targeting $K_{Ca}3.1$ is safe.[129,130] Although senicapoc did not reduce the number of painful sickling crises, which was the clinical endpoint that the sponsoring company, Icagen Inc., had selected for their trial,[130-133] the compound did demonstrate a reduction in hemolysis with increasing hemoglobin and hematocrit levels, a non-significant reduction in late asthmatic response, and it was well tolerated. In addition, significant inter-patient variation was observed in senicapoc's half-life, making dosing difficult. In a similar manner to $K_v1.3$ blockers, $K_{Ca}3.1$ blockade acts on specific T or B cell subsets, providing the wherewithal for targeted immunomodulation rather than whole-sale immunosuppression, and an antibody would provide a longer half-life, yielding a better pharmacokinetics/pharmacodynamics profile.

$K_{Ca}3.1$ has also been implicated in several cancers, presumably mediated by its role in the proliferative response of many cell types. Several reports describe successful inhibition of tumor cell proliferation and pro-invasive behaviour following $K_{Ca}3.1$ blockade in both *in vitro* and *in vivo* studies, including prostate,[134] breast,[135] pancreatic,[136] endometrial,[137] glioblastoma,[138-140] head and neck squamous cell carcinoma (HNSCC)[141] and leukemia.[142] It has also been proposed that targeting $K_{Ca}3.1$ could provide a potential adjuvant therapy for the inhibition of tumor angiogenesis and tumor progression.[139,143]

Recently, the combined activation of $K_{Ca}3.1$ and inhibition of $K_v11.1$ has been identified as a potential alternative strategy to overcome cisplatin resistance in colorectal cancer (CRC) from studies using molecular and electrophysical approaches with the cisplatin-resistant CRC cell lines HCT-116 and HCT-8.[144] Several previously characterized K^+ channel modulators were tested *in vitro* individually and in combination for their action on K^+ currents, cell viability, apoptosis, cell cycle, proliferation, intracellular signalling and platinum uptake. These effects were also analyzed in a mouse xenograft model that mimics chemoresistance. Cisplatin-resistant CRC cells express higher levels of $K_{Ca}3.1$ and $K_v11.1$ channels compared with cisplatin-sensitive CRC cells. In resistant cells, the $K_{Ca}3.1$

activator, SKA-31, and $K_v11.1$ inhibitor, E4031, revealed a synergistic action with cisplatin resulting in apoptosis and inhibition of cell proliferation. Similarly, riluzole is able to both activate $K_{Ca}3.1$ and inhibit $K_v11.1$, which suggests a combined approach or potential use of a bispecific antibody as a targeting strategy, for example, in patients with ovarian cancer where cisplatin resistance also presents a challenge in adjuvant therapy.

Voltage-gated calcium ion channels

Voltage-gated calcium channels (VGCC) are a group of voltage-gated ion channels found in the membrane of excitable cells (e.g., muscle, glial cells, neurons) with selectivity for Ca^{2+}. At resting membrane potential, VGCCs are normally closed. They are activated at depolarized membrane potentials and are key transducers of cell surface membrane potential changes into intracellular calcium influx that regulates intracellular processes such as contraction, secretion, neurotransmission and gene expression in many different cell types. There are 10 members of the voltage-gated calcium channel family that have been characterized in mammals, and these serve distinct roles in cellular signal transduction.[222]

$Ca_v3.1$ and $Ca_v3.2$

$Ca_v3.1$ and $Ca_v3.2$ are encoded by the *CACNA1G* and *CACNA1H* genes, respectively, and both belong to the T-type calcium channel subfamily. Although very closely related with similar biophysical properties, their functional effects are very different and emphasize the importance of being able to develop ion channel modulators with high selectivity. $Ca_v3.1$, but not $Ca_v3.2$, is thought to act as a tumor suppressor because it is involved in the inhibition of proliferation and promotes apoptosis in MCF-7 human breast cancer cells.[145,146] Whereas overexpression of $Ca_v3.1$ suppresses cell proliferation and siRNA knockdown or treatment with ProTx-I, a selective inhibitor for $Ca_v3.1$, promotes cell proliferation of MCF-7 cells, gene knockdown or overexpression of $Ca_v3.2$ exhibits no effect on cell proliferation in this cancer cell line. Moreover, $Ca_v3.1$ expression has been shown to correlate with sensitivity to apoptosis[145] and inhibition of prostate cancer cell proliferation.[147]

$Ca_v3.2$ has been suggested to promote a constitutive calcium entry influx due to the influence of $K_{Ca}3.1$.[223] It is thought that $Ca_v3.2$ is responsible for the neuroendocrine differentiation associated with the increase in calcium-dependent secretion of mitogenic factors in prostate cancer,[224] and has been nominated as a biomarker for breast cancer progression and treatment.[225] Thus, a different mode of action would be required for modulating cancer drugs targeting each of these calcium channels,[226] namely, one as a channel agonist ($Ca_v3.1$) and one as an antagonist ($Ca_v3.2$).

Voltage-gated sodium ion channels

The voltage-gated sodium channel family has 9 members (Na_v 1.1 to $Na_v1.9$) that are encoded by the genes *SCN1A* to *SCN11A*. Na_v sodium channels have key roles in the initiation and propagation of action potentials in excitable neuronal

cells, muscles and heart tissues, and as such have historically been regarded as therapeutic targets for pain, arrhythmia and epilepsy. A range of inherited disorders affecting skeletal muscle, heart rhythm and the central and peripheral nervous systems have been linked to mutations in the Na_v genes[227] that confer loss-of-function or gain-of-function properties.[228,229]

Nav1.7, Nav1.8 and Nav1.9

Na_v1.7, Na_v1.8 and Na_v1.9 are expressed in peripheral sensory neurons and hereditary gain of function mutations have been identified as the cause of pain disorders, including hereditary erythromelagia and paroxysmal extreme pain disorders (Na_v 1.7), as well as other painful peripheral neuropathies (Na_v1.8, Na_v1.9). Conversely, loss of function mutations in Na_v1.7 lead to a congenital insensitivity to pain.[230] It is not surprising then that these Na_v isoforms, particularly Na_v1.7, have generated substantial interest as targets for the development of non-opioid pain therapeutics. Additionally, recent evidence is growing that implicates Na_v1.7 in cancer. Functional Na_v1.7 expression has been found to be involved in EGF-mediated tumor cell invasion in non-small lung cancer cells[231] and has also been reported to be abundantly expressed in prostate cancer[146] and breast cancer.[232]

Nav1.5 and nNa$_v$1.5

In metastatic breast cancer cells, Na_v1.5 is upregulated and found to potentiate tumor cell migration and invasion in both *in vitro* and *in vivo* experimental models,[233] whereas stable down-regulation of Na_v1.5 expression significantly reduces tumor growth, local invasion into surrounding tissue and metastasis to liver, lungs and spleen, thus providing a further body of evidence for its role in metastasis. Furthermore, a neonatal splice variant (nNa$_v$1.5) has been shown to be upregulated and associated with metastasis and breast cancer progression *in vitro, in vivo* and in clinical samples of patient lymph node tissue.[234] In developmentally regulated D1:S3 splicing of the Na_v1.5 gene, *SCN5A*, there are 31 nucleotide differences between the 5′-exon ('neonatal') and the 3′-exon ('adult') forms, resulting in seven amino-acid substitutions in the S3/S4 extracellular region of Domain 1 (D1:S3-S3/S4 linker). Functional activity of nNa$_v$1.5 can be suppressed by both siRNA and a specific polyclonal antibody, NESO-pAb.[22] The siRNA rapidly reduced the level of nNa$_v$1.5 mRNA by ∼ 90%, but not adult Na_v1.5 mRNA; however, the effects on protein reduction were considerably less. NESO-pAb reduced metastatic activity of the breast cancer cell line, MDA-MB-231, in a dose-dependent manner. Other studies from the same group demonstrated that blockade of the Na_v1.5 channel with small molecules or siRNA also inhibited the invasiveness of endocrine-resistant breast cancer cells.[235] Recent work has shown that increasing the level of nNa$_v$1.5 cell surface expression increased the metastatic behavior of breast cancer cells[236] due to a reduction in cell adhesion, and suggested a possible interaction with the Sig1R transmembrane chaperone. Further work is necessary to elucidate the nature of this interaction and provide further understanding of the role of nNa$_v$1.5 function in an oncology setting. The adult

form of Na_v1.5 is responsible for propagating the action potential in cardiac muscle, and therefore, like K_v11.1, targeting isoforms of these channels as a therapeutic strategy necessarily stresses the importance of selectivity.

ASIC channels

The acid-sensing ion channel (ASIC) family, encoded by *ASCI1-5* genes, are part of the epithelial sodium channel superfamily and are voltage independent. Instead, ASICs are activated in response to reduced extracellular pH,[237] particularly tissue acidosis and are Na^+ permeable with an isoform, ASIC1a, showing low Ca^{2+} permeability. ASICs are expressed in the peripheral and central nervous system and are potential therapeutic targets for neurological conditions.[238]

ASIC1a

ASIC1a mediates Ca^{2+} overload and has been reported to contribute to ischemic nerve cell death and inflammation in multiple sclerosis.[239] It has also been implicated in pain,[240] migraine,[241] pain associated with bone cancer,[152] glioblastoma[232,242] and breast cancer.[243] Additionally, ASIC1 inhibitors have been shown to cause a significant reduction of tumor growth and load.[243]

TRP channels

The mammalian TRP channel family consists of six gene families comprising 28 members of cation channels activated by different stimuli and ligands with diverse physiological functions that range from pain and thermal perception to Ca^{2+}-mediated cell processes and homeostatic reabsorption of calcium and magnesium. The mammalian TRP channel family can be broadly sub-divided into 6 sub-families that consist of TRPA (ankyrin), TRPV (vanilloid), TRPM (melastatin), TRPC (canonical), TRPP (polycystin) and TRPML (mucolipin). Known naturally occurring compounds that act as ligands include capsaicin (TRPV1) and menthol (TRPM8). Trp channels are thought to play a key role in several diseases encompassing inflammation, allergy, autoimmune, fibrosis, oncology and pain indications.[244] Calcium entry through Trp channels may inhibit apoptosis, and is an effect that has been partly attributed to the stimulation of NF-kB.[245] Examples of Trp channels in various disease settings are described below and have been reviewed in depth elsewhere with regards to targeting pharmacology strategies.[155,246]

TRPM7

TRPM7 is associated with proliferation, motility and metastasis of cancer cells. Inhibition of TRPM7-regulated PI3K/AKT and MEK/ERK signalling pathways has been demonstrated to suppress glioblastoma cell proliferation and migration *in vitro*.[247,248] This inhibition is thought to enhance apoptosis induced by TRAIL,[249] and induce replicative senescence and enhanced cytotoxicity with gemcitabine in pancreatic adenocarcinoma.[250] Aldehyde dehydrogenase (ALDH1), which is a cytoplasmic stem cell marker in many malignancies, has recently been suggested to be a tumor stem cell marker in glioblastoma. The Notch signalling pathway plays

a key role in cancer stem cell (CSC) survival, proliferation and maintenance of the CSC population. TRPM7 gene silencing down-regulates both the Notch and STAT3 pathways in glioma stem cells, whereas increased ALDH1 expression and activity is induced by TRPM7.[251] Moreover, phosphorylated STAT3 binds and activates ALDH1 promoters in glioma cells. Thus, the authors concluded that these findings demonstrate that TRPM7 activates the Notch and STAT3 pathways leading to activation of ALDH1 and subsequent increases in cell proliferation and migration.[251]

TRPM8

TRPM8 is a receptor-activated non-cationic ion channel. In prostate epithelial cells, expression of TRPM8 is regulated by androgen, is elevated in androgen-sensitive cancerous cells compared with normal cells and has been confirmed as an ionotropic receptor for testosterone.[252] As such, TRPM8 has been identified as a novel target for androgen-regulated prostate cancer,[253] where overexpression is androgen-dependent and required for tumor cell survival. Although the precise mechanism involved is unknown,[254] the influx of Ca^{2+} and Na^+ in prostate cancer cells has been shown as necessary for survival and function,[255] and it is well established that Ca^{2+} signaling regulates proliferation and apoptosis in cancer cells. In androgen-sensitive cell lines, such as LNCaP, testosterone activation of TRPM8 elevates basal Ca^{2+} levels[252] whilst TRPM8 inhibition with a small molecule antagonist or siRNA results in cell death.[255] Anti-androgen therapy also significantly reduces the expression of TRPM8.[256]

The tissue-specific function of TRPM8 in prostate physiology and carcinogenesis remain unknown. This is complicated by the different cellular locations for TRPM8, namely the cytoplasm (in the endoplasmic reticulum) and plasma membrane. Testosterone-induced plasma membrane TRPM8 activity elicits calcium uptake causing apoptotic cell death. In addition, the promoter region of *trpm8* possesses a consensus p53 binding site, suggesting that TRPM8 may serve as a downstream target of tumor-suppressor genes potentially providing a protective role.

Conversely, TRPM8 expression is significantly lower in androgen-independent and metastatic prostate cancer.[257] The androgen independent pathways do not require androgens, but can be activated by growth factors acting through kinase pathways. These initial observations suggested that TRPM8, as a therapeutic target, may only be suitable for androgen-sensitive prostate cancer. However, application of an adjuvant therapy that rescues TRPM8 expression or enhances its activity or acts as an agonist could pose a potential strategy for the treatment of androgen-independent prostate cancer.[252,258,259] Such a hypothesis is substantiated by the observations that, whilst TRPM8 may not be essential for the survival of the androgen-independent prostate cancer cell line PC3, overexpression of this ion channel mediates a reduction in proliferation and migration, as well as facilitating apoptosis.[258]

Thus, androgen sensitivity would need to be taken into consideration when selecting the desired mechanism of action of an antibody targeting TRPM8 in prostate cancer. That is, an antibody with ADCC effector function targeting plasma membrane-associated TRPM8 might be preferred in the case of androgen-dependent prostate cancer, while an antibody with agonist activity combined with an adjuvant therapy to enhance expression might be more effective for androgen-independent prostate cancer.

TRPV1

TRPV1 is overexpressed in many tumor types, including endometrial,[260] thyroid,[261] breast,[262] astrocytoma,[263] prostate,[264] pancreas,[265] colon,[266] melanoma[267] and bladder.[267] TRPV1 is activated by capsaicin and is probably the most well-known TRP channel targeted for pain with marketed products, such as Qutenza® (Acorda Therapeutics) and Zuacta™ (Sanofi), both of which are capsaicin-based. As an agonist, administration of capsaicin causes an initial enhanced stimulation of TRPV1-expressing nociceptors that may be associated with painful sensations, but this is followed by pain relief thought to be mediated by a reduction in TRPV1-expressing nociceptive nerve endings. However, there may be a gradual re-emergence of painful neuropathy over time, and this is thought to be due to TRPV1 nerve fibre reinnervation. This potentially could be circumvented by the use of an antagonist antibody. TRPV1 also plays a key role in deep tissue pain,[268] joint pain in arthritis[269] and bone cancer pain.[152]

TRPV3

The clinical significance of TRPV3 in non-small cell lung cancer (NSCLC) was recently reported[270] where it was observed to be overexpressed in ~ 68% of lung cancer cases, correlating with the differentiation and tumor node metastasis stage of the tumor. Significantly, TRPV3 expression was associated with short overall survival. Blocking or knockdown of TRPV3 has been shown to inhibit lung cancer cell proliferation and arrest the cell cycle at the G1/S transition stage.[270] The rate of proliferation of epithelial cells in TRPV3 knockout mice is less than that in wild-type mice and TRPV3 up-regulation has been shown to be associated with a high risk for development of CRC.[271] TRPV3 has been proposed as a potential companion drug target for NSCLC.[270]

TRPV6

TRPV6 demonstrates higher calcium selectivity over other TRP channels and plays an important role in regulation of calcium homeostasis in the body. In cancer, evidence points to its upregulation and correlation with the advanced stages in prostate, colon, breast, thyroid and ovarian carcinomas where it translocates to the plasma membrane via an Orai1-mediated mechanism and promotes tumor cell survival by enhancing proliferation and conferring apoptosis resistance.[272]

TRPA1

TRPA1 is implicated in inflammatory pain and naturally derived compounds from plants, such as mustard oil, act as agonists on TRPA1 causing pain by excitation of nerve fibres.[273] The closed-state structure of TRPA1 was recently solved,[33] and further study using molecular dynamics simulations, in parallel with mutagenesis and functional evaluation by electrophysiology, explored conformational changes on the

proposed open state for an informed understanding on the structure and function of this ion channel.[274] However, selection of appropriate animal disease models requires careful consideration because cross-species variations in metabolic mechanisms and signal transduction pathways can lead to species-specific differences in TRPA1 function. Paclitaxel-induced neuropathy is thought to trigger the release of mast cell tryptase, which activates the protease-activated receptor 2 that in turn sensitizes TRPA1 (as well as TRPV1 and TRPV4) through the PLC, PKC and PKA signalling pathways.[275] TRPA1 expression can be modulated by other GPCRs, including the bradykinin receptor, the bile acid receptor TGR5 and the MAS-related GPCR.[156] Inhibition of TRPA1 eliminates the mechanical and cold allodynia induced by cisplatin and oxaliplatin, which are commonly used chemotherapies.[155] In addition, selective blockade of TRPA1 attenuates pain without altering body temperature regulation or the ability to feel cold.[275] TRPA1 also presents a therapeutic opportunity in the treatment of migraine.[276] Similar observations have been made for TRPM8 where it plays a major role in cold hypersensitivity[277,278] and presents a therapeutic opportunity both in the treatment of pain and migraine.[279]

In addition to its role in pain signalling, TRPA1 is found in nerve fibers that innervate the respiratory tract, in the peripheral nervous system, as well as on non-neuronal cells, such as fibroblasts and epithelial cells,[280] and it is an emerging target for respiratory conditions such as cystic fibrosis, asthma, allergic rhinitis, chronic cough and itch. A large body of evidence accumulated from *in vitro* experiments, animal disease models and patient data indicates that TRPA1 functions as a chemosensor for exogenous irritants and endogenous mediators of inflammation. Additionally, the presence of fine particulate matter in OVA-sensitized mice has been demonstrated to upregulate TRPA1 expression.[157] Based on these observations, a therapeutic strategy targeting TRPA1 in respiratory disease would require blockade of this important ion channel.[158]

TRPC3

Excessive Ca^{2+} influx regulates cytotoxic processes associated with immune-mediated diseases, such as acute pancreatitis and Sjögren's syndrome causing dry mouth and/or dry eye. Inhibition of TRPC3, and therefore Ca^{2+} influx, has been shown to protect pancreatic and salivary gland secretory cells from damage caused by Ca^{2+} cytotoxicity.[159] TRPC3 also plays a role in airway smooth muscle proliferation associated with airway remodelling, a histological characteristic of chronic respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD).[160] Inhibition or knockdown of TRPC3 blocks increased activity of TRPC3 and membrane depolarization in OVA-sensitized/-challenged cells[281] and suppresses airway smooth muscle cell proliferation and airway remodelling in mouse models of disease.[282]

TRPC6

The classical or canonical TrpC family (formerly short-TRPs, STRPs) encompasses channels presenting a large number of different activation modes. Some are store-operated, whereas others are receptor-operated channels activated by the production of diacylglycerol or redox processes. TrpC6 is amongst the latter subgroup of TrpC channels.[283]

Of clinical relevance, TrpC6 channels are upregulated in a wide range of cell types across a broad spectrum of disease indications, such as focal segmental glomerulosclerosis (FSGS),[161] pulmonary fibrosis,[284] cancer,[285-290] hypertension[291,292] and allergic asthma.[293] Expression or over-expression of TrpC6 has been shown to have a pro-proliferative effect. For example, the presence of TrpC6 has been determined to be essential for the progression of gastric cancer in studies comparing normal and cancerous epithelial cells of humans, and TRPC6 inhibitors, and a dominant negative TRPC6 channel mutant, have been shown to promote cell cycle arrest in gastric cancer cell lines.[294] There are no specific TrpC6 inhibitors in development that effectively suppress these processes, and therefore this ion channel presents a potential therapeutic opportunity. In the kidney, TrpC6 resides in podocyte membranes where it plays a role in maintaining glomerular function by acting as a non-selective cation channel that primarily transports Ca^{2+}. TRPC6 has also been implicated in renal disease, such as primary forms of FSGS where circulating factors cause dysfunction or loss of podocytes via TrpC6 channel activation. TRPC6 mutations from both familial and sporadic cases of FSGS map to the N- and C-termini, often resulting in excessive calcium influx and subsequent injury or loss of podocytes.[161] This in turn promotes glomerular mesangial cell apoptosis via calcineurin/NFAT and FasL/Fas signaling pathways.[295] Additionally, mice with podocyte-specific overexpression of TrpC6 recapitulate many of the pathological features of FSGS.[296] Despite the clear association of TrpC6 with FSGS, there are currently no clinical trials of therapeutics targeting TrpC6 for any condition.

Ligand-gated purinergic P2X channels

There are seven members of the P2X purinergic ligand-gated ion channel family (P2X1-P2X7) reported to date, which are encoded by the genes *P2RX1-P2RX7* that have dispersed chromosomal locations.[297] Of these, the homotrimers of P2X3, P2X4, P2X7 and heterotrimers of P2X2/3 offer the greatest potential for therapeutic targeting.[298] On activation by ATP released by damaged cells, these ion channels open to allow the influx of calcium, leading to cell membrane depolarization.[299] Whilst P2X channels have a wide tissue distribution, they are activated by ATP with differing affinities that trigger distinct physiological functions, such as central synapse transmission smooth muscle cell contraction, platelet aggregation, macrophage activation, cell proliferation and cell death.[300]

P2X3

Activation of P2X3 homomers contributes to acute nociception and activation of P2X2/3 heteromers that modulate nociceptive sensitivity associated with nerve injury or chronic inflammation. P2X3 receptors undergo rapid turnover that is dependent on their activity status (i.e., agonist-stimulated internalization), where expression and function are modulated by phosphorylation of intracellular domains in response to extracellular pain-causing molecules.[301] Calcium/calmodulin-dependent serine protein kinase has been demonstrated to regulate purinergic nociceptive signalling of P2X3.[302]

P2X4

P2X4 has also been shown to play a role in neuropathic pain where knockdown, knockout and inhibition of P2X4 in rodent *in vivo* models reverses neuropathic pain.[303] Studies show that the P2X4 channel expressed in the satellite glial cells of dorsal root ganglia is associated with neuropathic pain.[304] Additionally, activation of P2X4 (and P2X7) on microglia is thought to maintain nociceptive sensitivity through neural-glial cell interactions as antagonists to these receptors have been observed to reduce neuropathic pain. These mechanisms are reviewed in depth elsewhere.[305,306]

P2X7

P2X7 is expressed on T cells and is implicated in the immune response where it is upregulated on macrophages and micro-glial cells following stimulation by IFNɤ or LPS driving T cell activation and proliferation with the release of IL-1β.[307-310] P2X7 has also been directly implicated in systemic lupus erythematosus,[311] arthritis[312] and diabetic neuropathic pain.[313]

In cancer, P2X7 is thought to act as a danger "sensor" for high local ATP in inflammation[314] and the tumor microenvironment,[315] with overexpression of P2X7 linked to tumor growth and metastasis.[316,317] It was recently demonstrated that the anti-parasitic agent ivermectin can modulate sensitivity of both P2X4 and P2X7 receptors to extracellular ATP in association with the pannexin-1 channel, resulting in the induction of a non-apoptotic and inflammatory form of cancer cell death.[318] A non-functional conformational form of P2X7 (nfP2X7) has been described that is unable to form the large pore conformation associated with driving cell death and is specifically expressed on the surface of cancer cells sourced from a wide range of tumors.[21] It is thought that this variant of P2X7 could result from rare splice isoforms or single nucleotide polymorphisms,[319] and the resulting molecule only has one functional ATP binding site of the three available, thus providing a unique targeting epitope on nfP2X7 that is not present on functional P2X7.[21] To date, this is the only ion channel target with an antibody (BIL010t) in clinical study, as described below in the "Antibodies in research and development" section.

Additionally, several P2X7 splice variants have been described, with the full-length and a truncated version (P2X7RB) specifically studied in osteosarcoma.[320] In the case of glioma, Fang and co-workers have reported that suppression of P2X7 promotes tumor growth via the EGFR signalling pathway.[321] The same group evaluated the potential of transplanted endothelial progenitor cells (EPCs) as a therapeutic strategy and imaging probe to overcome anti-angiogenic resistance of gliomas in the brain. This approach takes advantage of P2X7 receptor activation on EPCs prior to transplantation, which promotes their proliferation and homing to gliomas *in vivo*.[322] P2X7 has also been shown to act as an upstream modulator of the VEGF signalling pathway with proof-of-concept demonstrated in experimental neuroblastoma[323] using ACN human NB cells. *In vivo* studies also demonstrated ACN-derived tumor growth could be reduced in nude/nude mice, and an even stronger effect of P2X7 blockade was obtained in a syngeneic immune-competent neuroblastoma model where Neuro2A cells were injected in albino mice.

Lastly, it has been reported that CD40 upregulates P2X7 in retinal endothelial cells, rendering them susceptible to ATP/P2X7–mediated programmed cell death.[324]

Calcium-release activated channels

Calcium-induced calcium release is a process that occurs in many cells and tissues whereby an increase in concentration of Ca^{2+} in the cytosol causes a further increase as Ca^{2+} is released from intracellular stores. In this case, Ca^{2+} release from the sarcoplasmic/endoplasmic reticulum occurs following activation of Ca^{2+} release channels in response to elevated levels of cytosolic Ca^{2+}, inositol triphosphate (IP3), or changes in membrane potential.

On the other hand, Ca^{2+} release-activated Ca^{2+}(CRAC) channels represent one of the main Ca^{2+} entry pathways into the cell. They are fully reconstituted via two proteins, the stromal interaction molecule 1 (STIM1), a Ca^{2+} sensor in the endoplasmic reticulum, and the Ca^{2+} ion channel Orai in the plasma membrane. After Ca^{2+} store depletion, STIM1 and Orai couple to each other, allowing Ca^{2+} influx.[325]

CRAC (Orai, STIM)

Calcium release-activated calcium (CRAC) channel protein 1 is a calcium selective ion channel that is encoded by the *ORAI1* gene in humans. Four Orai1 subunits comprise the CRAC channel, which is indirectly activated by low intracellular Ca^{2+}, whereby decreased calcium concentration in the endoplasmic reticulum is sensed by STIM1, which in turn aggregates and relocates near the plasma membrane, where it activates Orai1 via direct interaction with the ion channel.[326,327] Sig1R inhibits store-operated Ca^{2+} entry (SOCE) by attenuating coupling of STIM1 to Orai1.[328]

CRAC channels are critical to the activation of T lymphocytes, mast cells and other hematopoietic cells, as they provide the primary route for the influx of calcium into these cells.[329,330] CRAC channel inhibition could therefore provide a direct way of modulating the immune response for the treatment of multiple diseases and disorders. In addition, growth factors can stimulate CRAC channels (CRACs) to mediate Ca^{2+} entry and subsequent Ca^{2+} oscillations in proliferating cells. For example, tumor cell migration and metastases require Ca^{2+} influx and Orai and STIM have been shown to have a role in apoptosis resistance associated with proliferating and migrating tumor cells.[331-334] In the context of metastatic CRC, where only 10–20% of patients receive a clinical benefit from the use of anti-EGFR mAbs, studies suggest that efficacy of these mAbs could depend on their ability to reduce SOCE that is known to promote cancer cell growth.[335] Subsequent studies revealed that a lipid raft-ion channel complex, consisting of $K_{Ca}2.3$, TRPC1 and Orai1, regulates SOCE-dependent colon cancer cell migration,[335] in agreement with previous observations.[336] The formation of this lipid raft is triggered by STIM1 phosphorylation by EGF and activation of the AKT pathway. Additionally, evidence of an association of Orai and STIM with TRPC1 has been implicated in tumorigenesis.[337]

Chloride channels

Chloride channels are a superfamily of ion channels with diverse structures (e.g., voltage-gated and ligand-gated channels),[338] but their biology is still poorly understood and as such remain a relatively under-exploited target class for drug discovery.[339] These channels may conduct many different ions, but they are named for chloride (as this is the most abundant). They are involved in a wide range of biological functions, including epithelial fluid secretion, cell-volume regulation, neuroexcitation, smooth-muscle contraction and acidification of intracellular organelles. Mutations in several chloride channels cause human diseases, including cystic fibrosis, macular degeneration, myotonia, kidney stones, renal salt wasting and hyperekplexia. Chloride-channel modulators have potential applications in the treatment of some of these disorders, as well as in secretory diarrhoeas, polycystic kidney disease, osteoporosis and hypertension. The chloride ion channel family comprises CLCs (that contain 10 or 12 transmembrane helices), CLICs (comprising two TMDs linked by a large central pore loop that may be glycosylated and can switch from a soluble state to a membrane bound state), CFTR (an ABC transporter) and CACC (calcium activated chloride channels, e.g., TMEM16A). A number of chloride channels are intracellular in distribution, e.g., CLICs, and would therefore not be suitable for targeting with antibodies unless an "intrabody" approach could be successfully applied.

CLCs

CLCs have been implicated in osteopetrosis (CLC-7)[340] and glioblastoma (CLC-2, CLC-3 and CLC-5),[341] where in normal brain tissue only CLC-2 is expressed on the plasma membrane.

CACCs

CACCs, such as CLCA1 and CLCA2, are thought to have an apoptotic role, but are downregulated in cells resistant to detachment-induced apoptosis (knowns as anoikis), as shown in mammary gland cells,[342] suggesting a role in cancer cells where reduction in expression is thought to contribute towards tumor cell survival.[343,344] Moreover, the reduction in expression of CLCA2 by lentiviral shRNA causes cell overgrowth and focus formation, enhanced migration and invasion with an increased risk of metastasis.[344] Overexpression of CLCA2, on the other hand, inhibits cell proliferation with increases in chloride current at the plasma membrane and accompanying reduced intracellular pH.[345] CACCs are also thought to have a role in asthma, COPD, cystic fibrosis and neuropathic pain.[346,347] Currently, there are no antibodies in discovery or development, and only 2 peptides targeting chloride channels, including lancovutide (duramycin) for the treatment of COPD and cystic fibrosis, were reported to be in development, but both have been discontinued (LanthiBio, TransMolecular).

Ion channel targeting antibodies in development

Given the advances made in targeting GPCRs over the past decade,[20] there is now substantial interest from the biotechnology and pharmaceutical industries to extend these capabilities to therapeutic mAbs that target ion channels.[348-351] Several efforts are ongoing to succeed with this target class, and, whilst the pipeline is in its infancy, the ion channel-antibody pipeline in 2016 was reminiscent of the early stage GPCR-antibody pipeline 10 years ago (Figure 4(a)). A review of information available in the public domain that includes company websites, publications, conferences and searches on commercial databases, such as Pharmaprojects, has identified over 20 ion channels that are the focus of research and development activities. However, the majority of these are in early discovery or preclinical development, with only one antibody that has recently completed Phase 1 studies for basal cell carcinoma. Nevertheless, it is evident that there is a continued and burgeoning interest in ion channels as therapeutic antibody targets (Figure 4(b)), where substantial progress has been made in this field recently, as reflected by the increase in the number of preclinical stage programs in 2018 (Figure 4(c)). The range of ion channel targets under investigation has broadened with 37 programs listed in 2016 directed to at least 17 ion channel targets compared to 56 programs directed to at least 23 ion channel targets in the research and development pipeline for 2018. This and several other ion channel-targeting antibodies are discussed below as case studies of antibodies with commercial interest.

Antibodies in research and development

Clinical development

nfP2X7

Biosceptre recently published Phase 1 study results for BIL010t, a polyclonal antibody that targets a non-functional form of P2X7 (nfP2X7) for the treatment of basal cell carcinoma.[30] BIL010t is the first ion channel-targeting antibody to enter the clinic with the potential to become a first-in-class therapy. The company has built a pipeline focused on targeting nfP2X7 with various modalities in development (http://www.biosceptre.com/pipeline/) as outlined in further detail below.

P2X7 is an ATP-sensing pore-forming channel that can drive apoptosis by allowing rapid Ca^{2+} influx and downstream caspase activation.[352] Non-functional variants of this channel exist, in particular, nfP2X7, which allows residual calcium flux but fails to form an apoptotic pore. This variant is expressed at high levels in many cancers, including melanomas, and presents a unique epitope, E200, at the cell surface which is not present on normal healthy cells.[353] Selective exposure of this epitope makes it an ideal target for the development of therapeutic antibodies against a variety of different cancers.

BIL010t was generated by immunization of sheep using the E200 peptide sequence conjugated to keyhole limpet hemocyanin and then immunoglobulin G (IgG) was purified from the resulting sera. The disadvantage to this approach is that immune responses can differ from one individual host animal to another, leading to at least some batch-to-batch variation. Nevertheless, in vivo studies of BIL010t in a mouse melanoma preclinical model have demonstrated significant inhibition of tumor growth. BIL010t is being investigated as a topical therapy and has demonstrated safety and tolerability in this first clinical study,[354] as well as providing an indication of efficacy that was confirmed by histopathological analysis of post-treatment
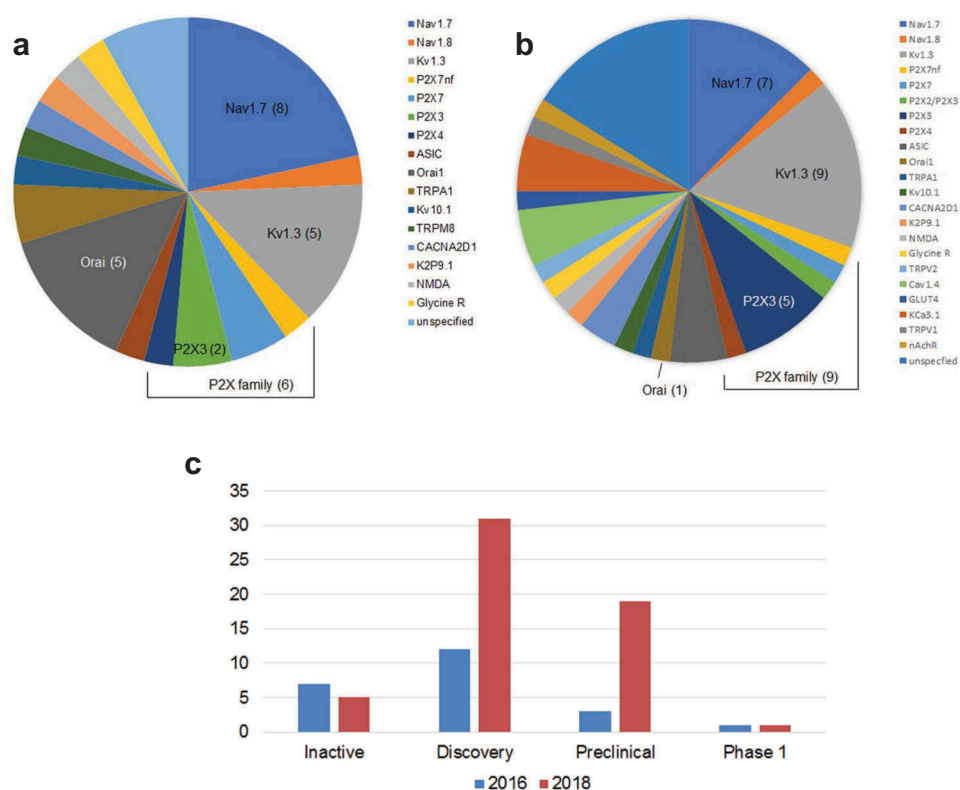
Figure 4. Ion channel targeting antibody programs in the R&D pipeline. Shown is a comparison between 2016 (a) and 2018 (b). Several ion channel targeting programs are undisclosed, such as Integral Molecular, Merck, Amgen, MedImmune, Theranyx, Ablynx and Innovative Targeting Solutions. The range of ion channel targets under investigation has broadened with 37 programs listed in 2016 compared to 56 programs in 2018. These antibody programs are directed to at least 17 targets in 2016 compared to at least 23 targets of interest in 2018, as can be observed by the increase in number of pie sectors. Selected targets of interest are denoted within the piechart layout with the number of programs indicated in brackets as the color coding of the pie sectors shifts due to the delisting of TRPM8 and the emergence of P2X2/P2X3. Since 2016, the number of programs underway for targeting Orai1 has decreased; there is a noticeable Increase in activity for $K_v1.3$ and P2X3; whereas $Na_v1.7$ and $Na_v1.8$ activity remains at a similar level. The P2X family is indicated by the black bracket line. Each target is color coded as depicted in the key to the right-hand side of each piechart. Information sourced from the public domain, such as scientific literature, company websites, etc. c. Shown are the ion channel antibodies in the R&D pipeline by stage depicting progress since 2016 to date. There is only 1 antibody program in clinical development (Ph1): nfP2X7 for basal cell carcinoma (Biosceptre). Some ion channel targets have more than one program for different therapeutic indications (for example, $K_v1.3$, P2X7 and CACNA2D1). Inactive programs include TRPA1 (Juno Therapeutics) and nAchR (NIH) and are listed as inactive (but not as terminated, unlike TRPM8 which is not currently listed).

biopsies.[30] Biosceptre is building a pipeline of nfP2X7-targeting modalities: BIL03s is a human single domain antibody that has been developed for systemic administration for a number of solid tumors and will enter a Phase 1 trial imminently in Australia; a vaccine is in Phase 1 study for solid tumors (BIL06v), and an antibody-drug conjugate has progressed to preclinical development. The company's pipeline presents the opportunity to target a broad range of other cancers, including breast, lung and prostate cancer.

Preclinical development

P2X7

P2X7 has been closely studied in cells of the hematopoietic lineage, particularly innate immune cells and lymphocytes. A collaboration between the University Medical Center Hamburg-Eppendorf and Ablynx has reported the generation of antagonist nanobodies (another type of single domain antibody) that can either block or potentiate P2X7 on T cells.[355] ATP-induced gating leads to shedding of CD62L on T cells and IL-1β release, and antagonizing P2X7 may provide an alternative or complementary strategy to blocking IL-1β. With excellent specificity for P2X7, antagonist nanobodies in modular bivalent format significantly increased the potency of the nanobody (pM) to block the IL-1β inflammatory response in whole human blood, preventing pore formation, which in turn led to cell death in P2X7 transfected cells. In the presence of 100 μM ATP, these nanobodies demonstrate an IC_{50} of 0.1 nM with 1000-fold superior potency and efficacy over existing benchmark compounds (KN-62 and A438079). In vivo function was demonstrated using surrogate mouse nanobodies in an antibody-induced nephritis model, where administration of trivalent formated, half-life extended anti-P2X7 nanobodies could modulate P2X7 dependent pathology, as well as in a DNFB-sensitized mouse model for allergic dermatitis. The lead candidate, Dano1, is being progressed through preclinical development for the treatment of inflammation and neurological disease, such as rheumatoid arthritis, inflammatory bowel disease, COPD, multiple sclerosis, renal injury and graft-vs-host disease (GvHD), however no development has been reported since the acquisition of Ablynx by Sanofi in early 2018. It is noteworthy that agonist nanobodies were also identified, e.g., Dano5, which induced shedding of CD27 and cell death of P2X7-postive T cells, that could have utility for immuno-oncology applications.

P2X3 and P2X2/P2X3

Rinat (a subsidiary of Pfizer) recently described the modulation of P2X3 and P2X2/3 ion channels by mAbs.[356] These ligand-gated ion channels have clinical relevance in pain sensation, such as inflammatory and visceral pain, cancer pain (particularly in bone and HNSCC), as well as chronic cough. Mouse mAbs were generated using standard hybridoma technology following immunization of balb/c mice with purified recombinant full-length P2X3 expressed in mammalian cells that was solubilized in dodecyl maltoside detergent and combined with Gerbu adjuvant to enhance the immune response.[356] The resulting panel of hybridoma antibodies exhibited different functional effects depending on homomeric or heteromeric composition of the ion channel, as well as the kinetic state and the duration of antibody exposure. Binding to the native channel was confirmed by fluorescence-activated cell sorting (FACS) and immunocytochemistry with functional activity assessed by Ca^{2+} flux and electrophysiology using the whole cell voltage clamp technique. Short-term exposure with one mAb, 12D4, that bound the desensitized conformation was shown to block 80% of homomeric P2X3 αβ-meATP-activated inward currents at 0.3 μM with an IC_{50} of 16 nM, whereas the same antibody potentiated αβ-methylene ATP-evoked currents mediated by heteromeric P2X2/3. Interestingly, long-term exposure (24h) reversed the potentiating effect of 12D4 on P2X2/3 mediated current and led to potent inhibition. It was suggested that these differences are based on the composition of the channel (i.e., homomeric vs heteromeric) and the length of time for exposure, as well as differences in binding affinities and epitopes. In addition, mAb 12D4 rapidly internalizes and disappears from plasma membranes of P2X3 expressing cells. Efficacious in vivo activity was confirmed for reversing visceral pain in a 2,4,6-trinitrobenzene sulphonic acid-induced colitis rodent model, where the effects were reversible. However, no effect was seen in a complete Freunds adjuvant rodent model for inflammatory pain, nor in a 0.5% formalin test rodent model. Whilst showing promise for the therapeutic treatment of chronic pain, further work will be required to humanize mAb 12D4 and evaluate its effect in other models, including non-human primate.

Shark-derived VNAR (another type of single domain antibody) antagonists to P2X3 (OSX300) are being developed by Ossianix in collaboration with Lundbeck A/S for the treatment of chronic pain. The Ossianix discovery platform encompasses both semi-synthetic phage display libraries based on specific VNAR isoforms and immunized repertoires derived from nurse shark lymphocytes.[357] Integral Molecular in collaboration with Crystal Bioscience (acquired in late 2017 by Ligand Pharmaceuticals for their transgenic chicken platform) presented data in 2016 indicating that they had also successfully raised chicken antibodies to P2X3, achieving sub-nanomolar affinities, potent inhibition of Ca^{2+} flux and in an ex vivo model for pain inhibition (90% dorsal root ganglion inhibition).[358]

$K_v1.3$

$K_v1.3$ is a well-validated therapeutic target for immunemodulation, and a number of companies have described preclinical data of Ig-based drug candidates in their pipelines.

Ablynx has reported generating highly potent and selective nanobodies with in vivo proof-of-concept[93] where the first extracellular loop (ECL1) has been shown to be essential for binding. Binding affinities are sub-nanomolar in bivalent/biparatopic format with greater than 10,000-fold selectivity for $K_v1.3$ than other related ion channels. Electrophysiology studies confirmed the lack of measurable off-target current blocking over the closest-related K_v1 family members and hERG, as well as functional activity that is comparable to the benchmark ShK toxin. Bivalent nanobody constructs demonstrated a fast onset of blocking activity on T_{EM} cells, thereby halting T-cell activation, as well as an increased duration of effects. Both bivalent and trivalent constructs (anti-human serum albumin improved serum half-life) were assessed in vivo using a rodent delayed type hypersensitivity model and found to be comparable to ShK in efficacy. Whilst these nanobodies show promise as potential therapeutics, no further development has been reported recently.

The rational design of a bovine antibody has been used to generate a selective immunosuppressive mAb targeting $K_v1.3$.[359] This was achieved by grafting the toxin peptide sequences for Moka-1 toxin and Vm24-toxin into the ultralong bovine heavy chain complementarity-determining region 3 (CDR3). The resulting mAb, SVN-001, demonstrated good selectivity and potency against effector human T_{EM} cells, a significantly improved plasma half-life and serum stability compared with the parent peptide, as well as potent in vivo efficacy.[359] By targeting a unique subset of immune cells, SVN-001 is not broadly immunosuppressive, which improves the safety profile compared to typical immunosuppressants.

TetraGenetics, in collaboration with Crystal Biosciences (acquired by Ligand Pharmaceuticals) and argenx, has generated the first conventional light and heavy chain antagonist anti-$K_v1.3$ mAbs to be advanced into preclinical studies.[77] Recombinant $K_v1.3$ was used for the immunization of chickens and llamas, as well as for antibody screening. The resulting panel of lead candidate antibodies show a high degree of potency ($IC_{50} < 10$ nM) in blocking $K_v1.3$ currents and the desired selectivity over related K_v family members that would be expected for mAbs. These antibodies are currently being developed for the treatment of Type 1 diabetes, although, as is common for $K_v1.3$ antagonists, the potential treatment of other autoimmune diseases by targeting $K_v1.3$ overexpression in T_{EM} cells is being explored.

$K_v10.1$

The aggressive behavior of pituitary tumor cells has been shown to correlate with high expression levels of HER2 and the $K_v10.1$ channel.[191] Therefore, it may be possible to target these cells via combination antibody therapy or possibly even a bispecific antibody format. Furthermore, $K_v10.1$ shares some homology with $K_v11.1$ in the inner vestibule area of the ion channel,[360] and so antibodies with superior selectivity compared to small molecules (where cardiac safety is a major concern) would presumably present a significant advantage.

Until very recently, no specific $K_v10.1$ peptide toxin has been reported; however, a novel specific $K_v10.1$ inhibitor from the sea anemone Anthopleura elegantissima has now been identified.[361] Prior to this, there was only one group (at the Max-Planck Institute of Experimental Medicine) who had

described an antibody (mAb56) that demonstrates exquisite specificity for $K_v10.1$ mediated by binding to the E3 region[61] and does not block $K_v11.1$ or the sub-family member $K_v10.2$. mAb56 was generated by immunization of mice using a fusion protein that incorporated the E3 region and tetramerizing domain of $K_v10.1$, followed by standard hybridoma methodology.[185] Current inhibition in $K_v10.1$-expressing HEK293 and neuroblastoma cells has been demonstrated with mAb56 showing dose-dependent effects and yielding an IC_{50} value of 73 ± 47 nmol/L in HEK293 cells. Additionally, the antibody's ability to inhibit tumor cell growth was confirmed both *in vitro* using anchorage-independent cancer cell growth assays and *in vivo* in both MDA-MB-435S human breast cancer and PAXF1657 human pancreas carcinoma xenograft models.[61] It is not known, however, if this mAb will progress into clinical development.

The same group has also demonstrated the induction of tumor cell-selective apoptosis by targeting $K_v10.1$ via a bifunctional antibody that is a fusion protein comprising an anti-$K_v10.1$ scFv antibody fragment and the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).[362] This antibody entity, scFv62-TRAIL, has also been reported to sensitize prostate cancer cells and other cancer cells to chemotherapy drugs, such as doxorubicin,[362,363] which could provide a potential means to overcome drug resistance. The full-length IgG from which scFv62 was derived has been used for *in vivo* imaging of tumor xenografts, providing further evidence of the utility of these antibodies in targeted cancer therapy.[199]

$K_v11.1B$

$K_V11.1$ (also known as hERG1) channels are often overexpressed in human cancers, but targeting them risks cardiotoxicity. CD-160,130 is a small molecule compound that blocks $K_v11.1$ channels with a higher efficacy for the $K_v11.1$ isoform B,[121] and shows anti-tumor effects without the cardiovascular toxicity induced by $K_v11.1$ blockade. This validates the strategy to explore selective targeting of this isoform with antibodies for therapeutic benefit without the associated risk of cardiac arrhythmia, for example, by using a tumor-specific bifunctional antibody (similar to the scFv62-TRAIL strategy) or tumor-targeted nanoparticles.[24,364] In the latter case, by linking antibodies that recognize tumor-specific cell surface receptors to nanoparticles, cellular uptake (and drug delivery) is dramatically enhanced,[365] which could further improve the safety and efficacy of targeting $K_v11.1$ on cancer cells.[24,366] Indeed, a proof-of-concept study has been described for the conjugation of a $K_v11.1$ mAb (specific against the S5 pore of $K_v11.1$)[367] to dicarboxylic acid-terminated pegylated titanium oxide (PEG TiO2) nanoparticles for the targeting of pancreatic adenocarcinoma cells.[364] Recent preclinical studies have demonstrated that antibody-targeted nanoparticles have better anti-tumor activity compared to non-targeted nanoparticles, due to more efficient localization and penetration into the tumor.[365] It is also worth noting that neoplastic cells are often depolarized and their changes in membrane potential slow, even when these changes oscillate in phase with the cell cycle stages. Therefore, the proportion of time spent by a voltage-gated channel in a given state can be very different in tumors than in excitable cells.[368] In another study, *in vitro*

proof-of-concept was attained using doxorubicin-loaded, PEGylated gold nanoparticles conjugated to a commercially available anti-$K_v11.1$ polyclonal antibody preparation for the targeting of PANC1 cells.[369] Preliminary results suggest that this approach has the potential to significantly enhance the therapeutic index of doxorubicin. The next logical step would be *in vivo* evaluation of these strategies using a mAb specific to the $K_v11.1B$ isoform in order to assess targeting efficacy and safety, functional potency, biodistribution and bioavailability.

Discovery

Sodium channels, particularly members of the Na_v family, have been the focus of intense drug discovery efforts. Notably, the key role that $Na_v1.7$ plays in pain pathways and obtaining a selective subtype targeting molecule that avoids off-target related functions, such as cardiovascular side effects,[52,370] has made this a priority in many neuroscience departments. Progress has been impeded due to the high sequence similarity with other Na_v channels, especially in the pore region where the binding sites of small molecules are located.[149] A selective neutralizing mAb to $Na_v1.7$, SVmab1, was reported to have been generated by immunization with a peptide sequence corresponding to the loop between S3 and S4 using Abmart's SEAL™ technology with standard hybridoma methodology.[371] Subsequent characterization determined that SVmab1 bound to the voltage-sensor paddle of $Na_v1.7$ and inhibited $Na_v1.7$ in transiently expressing HEK cells as determined by whole cell voltage clamping.[371] SVmab1 was also found to suppress acute and chronic itch in mouse models and inhibit chronic itch-potentiated synaptic transmission in spinal cord slices.[371] However, following the excitement surrounding the initial discovery, subsequent work was unable to replicate the previously reported effects using a recombinant version of the mAb generated using published sequences.[372] The lack of effectiveness of recombinant SVmab1 was recently confirmed by the group who initially discovered the antibody, but who also reaffirmed the activity of SVmab1 derived from two different batches of hybridoma-derived SVmab1, albeit with batch differences observed.[373] The reasons offered for the apparent discrepancy in functionality between the different mAb preparations were potential differences in post-translational modifications between hybridoma- and HEK293- derived antibodies, batch-to-batch variability in hybridoma-generated antibody material and possible errors in antibody sequencing, although the authors indicate the latter possibility is remote.[373]

Whilst many small molecules have been investigated for their potential use for pain control, an interesting observation has been reported that the more specific the molecule, the less analgesic the effect.[156] It remains to be seen if antibodies that target specific members of the Na_v family can overcome this conundrum and provide an advantage, for example, by modulating channel activity in a therapeutically beneficial way. It appears the biopharmaceutical industry is not yet convinced, as many antibody programs highlighted only a few years ago have since been discontinued.[374]

At the same time, this challenging subclass of ion channel is still the target focus of many efforts with various modalities under investigation, including the insertion of toxin peptides or knottins (Cysteine knot mini-proteins) into the peripheral CDR loops of an IgG structure to gain specificity and potency, as shown by IONTAS in 2017.[375] Similar proof-of-concept experiments that demonstrate the viability of this approach have been achieved with $K_v1.3$ (ShK toxin), $K_{Ca}3.1$ (kalio-toxin) and ASIC1a (psalmatoxin). A similar warhead approach using toxin analogs has been reported whereby the $Na_v1.7$ GpTx-1 peptide toxin was tethered to antibodies to generate bifunctional molecules and utilizes FcRn-based antibody recycling to target $Na_v1.7$ function.[376]

In the absence of purified ion channel protein that maintains a biologically relevant conformation, another approach that is under assessment by Visterra Inc. for the generation of mAbs directed to the voltage-sensing domain of $Na_v1.7$ harnesses several strategies, namely, the use of yeast surface display and immunization using a chimeric format of the voltage-sensing domain fused to the prokaryotic NavAb channel, as described in 2017.[377] The chimeric ion channel was purified and reconstituted into nanodiscs for use as antigen. Interestingly, in this study, single domain antibodies were found to engage the ion channel more efficiently. Immunizations of multiple host species that implement both DNA and nanodiscs with and without adjuvant are underway. The resulting immune repertoire will be deep-mined using microfluidics to identify specific mAbs, so further progress remains to be reported.

More encouragingly for antibody discovery, Amgen has reported the successful targeting of TRPA1 with antagonist murine mAbs that can block multiple modes of TRPA1 activation.[378] Rather than the use of peptides as antigen, various other formats were utilized for immunization, including DNA, whole cells stably expressing TRPA1 and recombinant adenoviral vector expressing TRPA1 (which incorporated immune-modulating modifications to enhance the immune response). Standard hybridoma methodology was employed, and resulting stable clones were screened for binding to TRPA1 by FACS, followed by evaluation of the purified IgG molecules for functional activity in calcium uptake assays and blocking of TRPA1 activation. A panel of mAbs was identified that demonstrated dose-dependent inhibition of TRPA1 with the most potent mAb exhibiting IC_{50} values of 260 nM in the agonist (allyl isothiocyanate) blocking assay and 90 nM in the cold activation assay. Although not confirmed, it has been suggested that these mAbs bind to the pore region of TRPA1, which would be in keeping with other observations that antagonist ion channel targeting antibodies often bind to the third extracellular loop that forms the pore in many ion channels.[50,348] The study also notes that only partial inhibition was achieved. Perhaps other antibody formats, such as nanobodies or Cowbodies that have an ultra-long heavy chain CDR3 loop, would bind epitopes otherwise inaccessible to full-length IgG antibodies and thereby achieve better inhibition.

Novo Nordisk has described the generation of antagonist antibodies to the Orai1 pore-forming subunits of CRAC in a study to assess antibody-mediated effects for SOCE in

T cells.[170] They have successfully identified a specific anti-human Orai1 mAb directed to the second extracellular loop that was generated by using a peptide sequence from the extracellular loop conjugated to bovine serum albumin for immunization and standard hybridoma generation. An ELISA- and FACS-positive antibody, 10F8, was able to inhibit T cell effector function *in vitro*, possibly through the contribution of antibody-mediated internalization of Orai1. Inhibition of effector function was also demonstrated *in vivo* by using a humanized GvHD mouse model, which confirmed a reduction in T cell proliferation and pro-inflammatory cytokine production. In addition, 10F8 was used to characterize Orai1 expression on immune cell subsets from blood and rheumatoid arthritis synovial fluid providing further validation of Orai1 as a target for autoimmune disease. Whilst the efficacy of this mAb has been demonstrated both *in vitro* and *in vivo*, no further information is currently available as to the progress of this molecule.

Amgen has described the targeting of Glycine Receptor α3 (GlyRα3), a Cys-loop receptor class ion channel implicated in pain. Glycine binding to the ECD triggers a conformational change, opening the ion channel to chloride ions. A specific antibody, AM-3607, directed to GlyRα3 was shown to enhance the response to exogenous glycine and was used in structural studies in complex with the pentameric GlyRα3 to further understand potentiator mechanism. The resulting crystal structure was resolved to 2.6Å, revealing that the mAb binding site was 10Å above the agonist binding site, suggesting a novel positive allosteric binding site.[379] Whilst this provides valuable insights for structure-based drug discovery, no further therapeutic development is planned for this mAb.

Finally, Regeneron has generated mAbs to the ASIC1 ion channel using a DNA immunization approach and the VelocImmune transgenic mouse platform, as presented in 2016.[380] Binders to cells expressing ASIC1 were identified from hybridoma clones and antibody diversity was evaluated by differential antigen disruption,[381] where the effect of chemical disruption of the cell surface antigen is assessed by FACS analysis to produce a heat map that indicates the diversity of epitope coverage. Twelve individual mAbs were identified from 106 binders for further profiling.[382] Select mAbs were shown to inhibit the pain response *in vivo* in a model of carrageen-induced muscle hyperalgesia, using a dose range of 10–40 mg/kg. However, no further development has recently been reported. More recently, the Shanghai Institute for Advanced Immunochemical Studies in collaboration with the Scripps Research Institute reported the successful isolation of ASIC1a antagonist mAbs by using the nanodisc antigen format. These mAbs were selective and potent in both *in vitro* testing and an *in vivo* middle cerebral artery occlusion (MCAO)-induced ischemia stroke model.[383]

Future directions

Currently, there are no marketed mAb-based therapeutics that target an ion channel, with only one polyclonal antibody targeting a non-functional form of P2X7 (nfP2X7) in Phase 1 clinical development for the treatment of basal cell

carcinoma.[30] Ion channels still remain significantly under-exploited as antibody drug targets[17] due mainly to the challenges of expressing sufficient protein in a biologically relevant conformation for antibody discovery purposes, as well as epitope accessibility and screening approaches. However, advances made in generating crystal structures and, more recently, cryo-electron microscopy structures, coupled with the deepening knowledge of ion channel gating and target biology validation now provide an informed base on which to progress and streamline antibody-based approaches in targeting ion channels to treat a variety of diseases.

Conclusions

Ion channels are widely recognized as important therapeutic targets in a range of diseases, but remain a challenge for drug discovery. There is still a paucity of functional ion channel mAbs described in the literature, and several of the examples cited here are drawn from recent conferences reflecting the most current advances and innovations enabling the discovery and development of therapeutic mAbs directed towards this important drug target subclass. Despite the significant focus on Na_v channels, in particular Na_v1.7, very little success has been reported. However, a review of the therapeutic pipeline suggests that the ligand-gated P2X family and K_v1.3 are likely to yield the initial successes for ion channel-targeting mAbs. The P2X family possesses a larger extracellular region than many other ion channels, and therefore is easier to target. The increasing number of ion channel structures being published will assist our understanding of this drug class and enable a deeper knowledge of the biology involved. Progress has been made in overcoming some of the technical challenges associated with ion channel expression and antibody screening, which are likely to facilitate the identification of new functional antibodies moving forward.

Abbreviations

ADCC	antibody-dependent cell-mediated cytotoxicity
ALDH1	aldehyde dehydrogenase
AML	acute myeloid leukaemia
ASIC	acid-sensing ion channel
CACC	calcium activated chloride channel
Ca_v	voltage gated calcium channel family member
CDR	Complementarity-determining region
CNS	central nervous system
CFTR	cystic fibrosis transmembrane conductance regulator
CRAC	calcium release-activated calcium channel
CRC	colorectal cancer
CSC	cancer stem cell
ECD	extracellular domain
E. coli	Escherichia coli
EPCs	endothelial progenitor cells
FSGS	Focal segmental glomerulosclerosis
GPCR	G protein-coupled receptor
HNSCC	head and neck squamous cell carcinoma
K_v	voltage-gated potassium channel family member
mAb	monoclonal antibody
Na_v	voltage-gated sodium channel family member
NSCLC	non-small cell lung cancer
pAb	polyclonal antibody
scFv	single-chain variable fragment
ShK	Stichodactyla toxin
SigR1	sigma-1 receptor
SMALPs	styrene-maleic acid lipid particles
SOCE	store-operated calcium entry
STIM1	stromal interaction molecule 1
TM	transmembrane
TMD	transmembrane domain
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
VGIC	voltage gated ion channel
VGCC	voltage gated calcium channels

Disclosure statement

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