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Antibodies and venom peptides: New therapeutic modalities for ion channels

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Abstract

Ion channels play fundamental roles in both excitable and non-excitable tissues and therefore constitute attractive drug targets for a myriad of neurological, cardiovascular and metabolic diseases as well as for cancer and immunomodulation. However, achieving selectivity for specific ion channel subtypes with small molecule drugs has been challenging and there currently is a growing trend to target ion channels with biologics. One approach is to improve the pharmacokinetics of existing or novel venom derived peptides. In parallel, after initial studies with polyclonal antibodies demonstrated the technical feasibility of inhibiting channel function with antibodies, multiple preclinical programs are now using the full spectrum of available technologies to generate conventional monoclonal and engineered antibodies or nanobodies against extracellular loops of ion channels. After a summary of the current state of ion channel drug discovery, this review discusses recent developments using the purinergic receptor channel P2X7, the voltage-gated potassium channel $K_V1.3$ and the voltage-gated sodium channel $Na_V1.7$ as examples of targeting ion channels with biologics.

Introduction

Ion channels are pore-forming transmembrane proteins that allow the regulated flow of cations or anions across membranes. The IUPHAR (International Union of Basic and Clinical Pharmacology) *Guide to Pharmacology*[1] currently lists 145 genes for voltage-gated-like ion channels[2], 82 genes for ligand-gated ion channels[3], and 52 genes for "so-called" other channels[4] like aquaporins, connexins or store-operated channels in humans. However, since many ion channels form homo- or heteromers of two, three, four, or five subunits - which additionally may interact with auxiliary proteins - the total number of possible ion channels that can be assembled from these genes to serve very specific physiological functions is much larger. Ion channels currently constitute important drug targets for the treatment of type-2 diabetes, hypertension, epilepsy, cardiac arrhythmia, and anxiety, and many of the classical drugs on the WHO´s list of essential medicines, like nifedipine ($Ca_V1.x$ inhibitor), amiodarone (mixed K_V channel inhibitor), phenytoin (Na_V inhibitor), or diazepam ($GABA_A$ activator) are ion channel modulators. A recent comprehensive analysis of molecular targets[5] estimated that 18% of small-molecule drugs exert their therapeutic effects through ion channels (see Supplementary Table 1 for a complete list of clinically used drugs targeting human ion channels). It is noteworthy that these clinically successful examples are all small molecule drugs that were developed long before their molecular targets were identified, solely by exploiting *ex vivo/in vivo* approaches. Following the initial registrations of these "classics" between the early 50s and early 80s the number of ion channel targeting drugs increased quickly, arguably often because of "me too" drug development around the original chemical structure and "incremental" property adjustments (most clearly illustrated by the dihydropyridine anti-hypertensives, the sulfonylurea type-2 antidiabetics, and the benzodiazepine anxiolytics shown in Supplementary Table 1).

Based on electrophysiological experiments, and especially with the introduction of patch-clamp technology[6], the mode-of-actions of the most important ion channel targeting drug classes (Na_V inhibitors, K_{ATP} inhibitors etc.) were quite well established around 1990[7]. At the end of the cloning era in the late 90s, the majority of ion channels were identified revealing a complex picture with respect to subtypes, stoichiometry and pharmacology[8]. The exceptions were voltage-dependent H^+ channels (Hv), Ca^{2+}- and volume regulated Cl^- channels (CACL, VRAC), the stretch- and voltage activated Piezo channel, and the Ca^{2+} release activated Ca^{2+}-channel (Orai), which were only cloned a decade later. It specifically became apparent that even closely related subtypes like members of the K_V1 family can have very different physiological functions, and thus can provide highly variable therapeutic and adverse effect profiles. This realization initiated an "explosion" in the number of ion channel drug discovery programs[9, 10, 11, 12, 13], dedicated towards development of subtype-selective small molecule drugs, tailormade for improving the therapeutic index (i.e.: $Ca_V2.2/3$ inhibitors for neurological indications like stroke or pain without effects on Ca_V1 and thus without cardiovascular side effects; $GABA_A$ α2/α3 selective activators for anxiety without effect on α1 containing channels and therefore without sedative properties). Hand-in-hand with this progress, cloning, molecular, and cell culturing techniques improved significantly, high-throughput automated ion channel assays were developed, and all large pharmaceutical companies upgraded (often to several millions chemical entities) and refined their

chemical libraries[14, 15]. In the most streamlined versions a total screening campaign, including reporting on one or more subtypes, could be completed in just 2-3 months.

This tremendous and long-lasting effort across essentially the entire pharmaceutical industry should have been a leap into the future with respect to improved ion channel medicines, but very few registrations have resulted from these efforts. For example, out of the K_V channel modulator programs we reviewed in 2009[10] only one compound, the K_V7 activator retigabine (known as ezogabine in the USA) for treatment of pharmacotherapy-resistant partial epilepsies made it to market in 2011 and then was withdrawn in 2017 by GlaxoSmithKline because of skin discoloration and suspected eye toxicity related to slow accumulation of a colored metabolite. It is especially sobering that even recent registrations are either based on long-known compounds (e.g. the K_V channel blocker 4-aminopyridine for multiple sclerosis[16], 2010, the $GABA_A$ activating allopregnanolone registered in 2018 for postpartum depression) or were discovered in low-throughput phenotypic screening by combining organ preparations with animal model work (the I_f (HCN) inhibitor ivabradine[17], 2015). The only notable exception we could find is the CFTR potentiator ivacaftor (VX-770), which originated out of a high-throughput membrane potential assay screen and which increases the open probability of wild-type and mutant CFTR[18]. For a list of the ion channel targeting drugs approved in the last 10 years, see Table 1.

Inspecting the reported clinical pipelines from major pharmaceuticals and biotech companies does not change the picture significantly (Table 2): One topical polyclonal antibody, three peptides and 33 small molecule clinical programs are currently reported; most are in phase I, a handful are in phase I/II, and only two, SAGE-217, a synthetic $GABA_A$ activating neurosteroid for major depression, and Gefapixant, a P2X3 inhibitor for chronic cough, are in phase III. One compound, mirogabalin, an improved gabapentenoid targeting Ca_V channels, is under registration in Japan for peripheral neuropathic pain. To underline the case further, two of the phase II examples are "repurposed" compounds (senicapoc, a $K_{Ca}3.1$ inhibitor, for hereditary xerocytosis and Alzheimer's disease and gaboxadol, a δ-subunit preferring $GABA_A$ agonist for Angelman's/Fragile-X syndrome), and XEN496 is retigabine now redeveloped for the orphan indication $K_V7.2$-mediated epileptic encephalopathy. A trend is that many of these development programs originate from small/intermediate pharmaceuticals and biotech companies, which may indicate a decline in the popularity of ion channels with the major pharmaceutical companies.

Even though small molecules will continue to be important for ion channel drug development in years to come, we are convinced that the role of ion channel targeting biologics will increase and help "revitalize" ion channels as targets for drug development. Following on the heels of the G-protein coupled receptor field[19], where several dozen antibody programs have advanced into clinical trials and CCR4 and CGRP targeting antibodies are on the market, the ion channel field is presently particularly excited about antibody approaches. This article will discuss the current state of research into antibodies and peptides that target this complex and diverse group of molecules.

Challenges in targeting ion channels

Ion channels have long been regarded as difficult drug targets and the reasons that have traditionally been given for this with respect to small molecule drug discovery[10] were 1) the technical difficulties in ion channel high-throughput screening; 2) the lack of crystal structures enabling "true" structure based drug design; 3) the challenges of achieving subtype selectivity, also taking into account stoichiometry of heteromeric channels and possible interactions with auxiliary subunits (a point often ignored in drug discovery), and ideally state/conformation selectivity. All these issues are also applicable to ion channel targeted biologics. The first issue has been somewhat addressed with the development of ultra-high-throughput membrane potential or flux assay systems and automated electrophysiology platforms that are currently capable of running high quality giga seal electrophysiological recordings in 48, 96 and 384 wells. However, we would like to posit that the specialized biophysical expertise that is necessary to expertly execute and analyze ion channel screens constitutes a real or sometimes perceived barrier to committing to an ion channel drug discovery program, which together with the fact that overall more resources in industry and academia have been dedicated to GPCRs than to ion channels, is probably responsible for the overall slower progress of the ion channel than the GPCR field. The 2nd point, the lack of structures, is a deficiency that is currently being addressed by advances in structural biology (see Box 1), but again somewhat more slowly than in the GPCR field. The 3rd point, obtaining relevant subtype and state/confirmation selectivity, remains a challenge for both the ion channel and the GPCR field (although voltage-clamp electrophysiology with its high temporary resolution and voltage control combined with fast application should give ions channels an advantage), and requires very detailed understanding of the exact role and context of the target protein in the chosen disease indication.

Target specificity

Since nearly all efforts within ion channel drug discovery/development previously focused on small molecules, it is worth considering the one key challenge for the field in addition to the general issues all small molecule medicinal chemistry programs face, namely optimizing pharmacokinetic properties[20], avoiding toxicity due to toxic metabolites[21], and finding chemical matter that ideally allows one to obtain "strong" composition of matter patents. Obtaining selectivity within ion channel families can be difficult to achieve, since subtypes are often highly homologous. This is especially challenging for small molecule drugs due to their small sizes and therefore limited number of interaction points with the target. The field of Na_V inhibitors is particularly instructive: Antiepileptics, class I antiarrhythmics, and local anesthetics target voltage-gated Na_V channels at a highly conserved site within the pore lumen formed by transmembrane segments S6 in the third and fourth domains[22]. Thus, despite many years of drug-development by many companies, all classical Na_V drugs are essentially unselective and their therapeutic value as systemically administered drugs relies solely on their strong state- or use-dependency, which favors binding to channels in pathologically depolarized or excessively firing cells.

State-dependence in this context means that a drug preferentially binds to one of the conformational states (closed, open, inactivated) that an ion channel undergoes

during its gating cycle (Fig. 1A). For voltage-gated channels like Na_V, Ca_V and K_V channels the occupancy of these states strongly depends on the membrane potential which means that drug development programs focused on targeting neuronal ion channels must decide which state of the channel it would be most desirable to target. For example, for Na_V channels that is generally assumed to be the open or inactivated state, while it might be better to target the closed state when developing a K_V channel opener. When neuronal ion channels are expressed in HEK293 or CHO cells, their voltage-sensor domains (VSD) are exposed to more depolarized resting membrane potentials (around -40 mV) instead of the more hyperpolarized resting membrane potential (around -70 mV) they would be experiencing in a neuron. This means that in drug screening assays such as flux or binding assays, that do not allow to control the membrane potential, the VSDs will tend to be in the thermodynamically favored “up” state that is present in open or inactivated states of the channel, which makes it difficult to capture the “down” state of the VSD that is present in the closed state (Fig. 1A). One of the major advantages of using manual or HTS patch-clamp electrophysiology for ion channel drug screens is that the membrane potential can be precisely controlled to capture the desired state of the channel.

The Na_V channel family contains 9 functional subtypes[23], $Na_V1.1$ to $Na_V1.9$. Nav1.1 is expressed in fast spiking interneurons of the brain, where loss-of-function causes Dravet syndrome[23], and has recently been shown to regulate the mechanical excitability of visceral nerves in the gut[24, 25]. $Na_V1.2$ is the predominant brain isoform and, together with Nav1.6, is a major target for anti-epileptics. $Na_V1.5$ is the main cardiac channel and the target for class I antiarrhythmics, whereas the analgesic effects of local anesthetics are mediated by several channels, including $Na_V1.7$, $Na_V1.8$, and possibly $Na_V1.9$, which are preferentially expressed in sensory nerves[26]. $Na_V1.7$ and $Na_V1.8$ have clear pain phenotypes in humans as loss-of-function and gain-of-function mutations in $Na_V1.7$ result in congenital insensitivity to pain[27] and primary erythromelalgia[28], respectively, whereas gain-of-function mutations of $Na_V1.8$ may cause painful peripheral neuropathy[29]. Despite convincing preclinical effects of unselective Na_V blockers in animal pain models and the effective use of the antiepileptic carbamazepine in trigeminal neuralgia pain[30] existing Na_V channel inhibiting drugs are not optimal for general treatment of severe pain disorders due to their unselective mode-of-action. Several companies (Genentech, Biogen, Amgen, Pfizer, Bayer) are or have been developing highly subtype selective and potent small molecule inhibitors of $Na_V1.7$ by targeting binding to a site in the domain IV voltage sensor responsible for channel inactivation rather than the classical local anesthetic site in the pore lumen[31, 32, 33]. Based on the currently published structures, this is achievable with a series of close analogues all with a conserved aryl-sulfonamide core structure, often dramatically called the “warhead”. For example, Genentech has published an x-ray structure[31] of the aryl-sulfonamide GX-936 bound to a receptor site within the $Na_V1.7$ fourth voltage-sensing domain (Fig. 1B). Unfortunately, one of these small molecules, the clinical phase II compound PF-05089771 (Fig. 1B), recently failed in patients suffering from painful diabetic neuropathy[34], which led Pfizer to stop further development activities. Bankar et al. suggested this failure possibly reflected insufficient $Na_V1.7$ targeting in the clinical study and recommend the evaluation of acyl-sulfonamides with better physiochemical and pharmacological properties[35], including a longer residence time on the target[36].

Since PF-05089771 is intentionally peripherally restricted, compounds with improved blood brain barrier (BBB) penetrability in humans, might better engage NaV1.7 channels expressed close to or on the bouton of dorsal root ganglion (DRG) neuron dorsal horn synapse. Another reason for the apparent failure of some $Na_V1.7$ blockers could be interaction with β-subunits for the native channel in DRG neurons, which was not recaptured in heterologous screening systems using the "naked" α-subunit alone. Sokolov *et al.* recently demonstrated that co-expression of $Na_V1.7$ with the glycosylated form of the sodium channel β3, an auxiliary subunit that is upregulated in injured human sensory neurons[37] and in DRGs in rat pain models[38], makes $Na_V1.7$ less sensitive to several state-dependent Na_V blockers[39]. Whether this would also be the case for the aryl-sulfonamides was not investigated in the study but it is interesting to speculate based on the recently solved structures of the human and electric eel $Na_V1.4$-β1 complexes[40, 41], that β–subunits could possibly prevent the binding of small molecules or antibodies to $Na_V1.7$. Finally, it is possible that a slightly broader selectivity comprising also the other DRG channels, $Na_V1.8$ and Nav1.9, may be needed. The Biogen compound, vixotrigine (a.k.a Raxatrigine), which is currently still active in Phase II, despite a recent failure in painful lumbosacral radiculopathy, has a different structure (Fig. 1B), passes the BBB, and is reportedly less subtype selective than the aryl-sulfonamides[42].

An interesting and highly challenging "spin-out" of the selectivity issues with small molecules is that considerable domain homology also exists between even remotely related ion channel families, which can lead to the apparently paradoxical finding that highly subtype-selective compounds may still have off-target effects on other ion channels like the cardiac delayed rectifier $K_V11.1$ (hERG), inhibition of which may cause ventricular fibrillation and sudden death[43, 44]. Unfortunately, hERG is remarkably promiscuous with respect to binding of many different small molecule chemotypes[43, 45] and thousands of otherwise useful drug candidates have been "filtered out" due to hERG activity[43, 45].

Another aspect that is worth mentioning, when discussing the problem of selectivity, is species specificity. While it has been incredibly challenging to achieve subtype selectivity between closely related human channels like within the Na_V or Ca_V family or for certain $GABA_A$ or nACh receptor subtypes, species selective small molecules seem to be more easily attainable as demonstrated by the extremely low acute mammalian toxicity of neuroactive insecticides like the Na_V channel blocking pyrethroids, the nicotinic receptor channel activating neonicotinoids and the $GABA_A$ channel blocking polychlorocyclohexanes and fiproles[46]. Examples of species specific ion channel modulators used in human medicine are the antivirals amantadine and rimantadine, which target the M2 proton channel, a member of the so-called viroporin ion channel family specific to viruses, with very little homology to prokaryotic or eukaryotic ion channels[47]. However, species differences in sensitivity to drugs among mammals can also be very problematic for translational drug discovery, as exemplified by the insensitivity of the rat isoform of $Na_V1.7$ towards inhibition by the aryl-sulfonamide inhibitor, AMG-8379[33].

Biologics versus small molecules

Even though biologics have their own well-known challenges, such as poor membrane permeability and the risk of triggering adverse immune reactions, they constitute an attractive alternative to small molecules, first-of-all because of their much higher binding selectivity (approaching true specificity), both with respect to subtypes and off-targets, than is generally possible to achieve with small molecules. Additionally, antibodies and peptides are metabolized as part of the body´s normal protein dynamics and thus do not show the drug-drug interactions, and metabolism-mediated toxicity that are always a risk with small molecules. Taken together, these two advantages are often considered to be responsible for the overall higher success rate of new molecular entity biologics (13.2%) versus small molecules (7.6%) for progression from Phase-1 to gaining FDA approval[48].

The feasibility of targeting ion channels with biologics has been amply demonstrated by Nature. Venomous animals have developed a myriad of highly potent and selective peptides that can both inhibit and activate ion channels and that have been incredibly useful in probing ion channel structure-function relationships. Similarly, autoantibodies developed by a patient's own immune system can inhibit ion channel function and acutely transfer disease to experimental animals. For example, most cases of myasthenia gravis, a disease leading to skeletal muscle weakness, are caused by pathogenic antibodies that bind to nicotinic acetylcholine (nACh) receptors at the neuromuscular junction[49], while a related condition, Lambert-Eaton myasthenic syndrome is triggered by antibodies against P/Q-type voltage-gated Ca^{2+} channels on presynaptic nerve terminals[50]. In both cases, some autoantibody clones have been shown to directly block the respective channels in electrophysiological experiments, while other antibodies can activate complement or induce channel internalization[49, 50]. Another instance of a neurological disease characterized by the presence of ion channel specific autoantibodies is neuromyelitis optica, where most patients test positive for aquaporin-4 antibodies[51].

Ion channel-targeted peptides as therapeutics

Since the development of exenatide[52], a 39-amino acid glucagon-like peptide-1 agonist isolated from the venom of a lizard, and its approval in 2005 as an injectable treatment for type-2 diabetes, peptides are increasingly being considered as viable therapeutics. The FDA has approved over 60 peptide drugs (predominantly targeting GPCRs for the treatment of metabolic disease and for oncology[53]), around 140 peptides are in clinical trials, and another 500 are in pre-clinical development [53]. The global peptide therapeutic market is estimated to be close to US$25 billion in 2018[54].

Venoms are a rich source of bioactive peptides with therapeutic potential [55, 56, 57, 58]. Over eons, more than 100,000 venomous creatures – arthropods (scorpions, spiders, bees, centipedes, wasps), cnidarians (sea anemones, jellyfish), mollusks (cone snails), annelids (fire worms, parasitic worms) and vertebrates (snakes, lizards, frogs, mammals) – have used their toxic cocktails to engage important biological targets. These cocktails are deployed as weapons to immobilize or kill prey, or as defense to deter predators or microbial invaders. Over 10 million bioactive peptides and proteins are estimated to be present in animal venoms, and their immense chemical diversity is unrivalled by synthetic libraries. The ability of these animals to kill with tiny amounts of

powerful venom has inspired both fascination and fear in humans, and snakes and scorpions were deified in the ancient world[59]. Traditional medicines in China, India, Greece and the Middle East have for centuries used this vast bioactive resource for medicines. In modern times, there has been an increasing interest in exploiting this extensive and relatively untapped pharmacopeia using proteomic and genomic approaches[55, 58]. As of today, six venom derived peptides, including the $Ca_V2.2$ blocking Ziconotide, have been approved by the FDA, about a dozen are in clinical trials, and several more are in pre-clinical development[55, 58]. Below we will discuss the ion channel targeting peptides in more detail by focusing on peptides that have entered clinical trials or started preclinical development in the last 10 years.

Ion channels are frequently targeted by peptide toxins. Starting in the 1960s, animal toxins were used as molecular tools to investigate ion channels[60, 61 62]. Many of these toxins have disulfide-constrained architectures that enhance their stability and protease resistance and permit tremendous variations in primary sequence without perturbation of the three-dimensional fold (Supplementary Figure 1). These characteristics make peptides with disulfide-rich scaffolds attractive as therapeutics. Figure 2A highlights the channel-modulating peptides that are discussed below. Many peptides are pore blockers[56, 57, 58] that bind at the extracellular entrance to the channel's pore (external vestibule) and occlude the ion conduction pathway (Fig. 2B). Others bind to the voltage-sensor of the channel and impact channel-gating (Fig. 2B).

Channel-modulating peptides for the management of pain

The opioid crisis in the United States of America has spurred efforts to develop non-opioid drugs for chronic pain, but many of these efforts have failed. A notable exception is Ziconotide (Prialt®), a peptide derived from the venom of fish-hunting cone snails[63], which was FDA-approved in 2004. In the 1980s, ω-conotoxin GVIA from *Conus geographus* and ω-conotoxin MVIIA from *Conus magus* (Fig. 2A) were shown to block voltage-gated calcium channels in the nervous system but not muscle[64, 65, 66]. The neuronal calcium channel was later defined as the N-type $Ca_V2.2$ channel. These peptides decrease neurotransmitter release from nociceptive afferents that terminate in the dorsal horn of the spinal cord. The peptide that advanced to the clinic was native MVIIA (Ziconotide)[63]. It is administered intrathecally through an implanted pump because it does not cross the BBB. Ziconotide is efficacious when administered as a single therapeutic, but is increasingly combined with an intrathecal opioid for the management of refractory chronic and cancer pain. Recent guidelines also recommend Prialt® as a first-line agent for neuropathic and nociceptive pain. Prialt®'s wider clinical use is impeded by its relatively high cost, narrow therapeutic window, and by the requirement for an intrathecal pump[63, 67].

Another ion channel that has been suggested as a pain target based on genetic studies is the sodium channel $Na_V1.7$[27, 28]. μ-theraphotoxin-Pn3a, a peptide from the South American tarantula *Pamphobeteus nigricolor* (Fig. 2A*)* blocks $Na_V1.7$ with picomolar affinity and selectivity over other Na_V channels[68]. The peptide is analgesic in rodent pain models when co-administered with sub-therapeutic doses of opioids, but displays no analgesic activity when administered on its own[68]. A broad lack of analgesic activity was also found for the selective $Na_V1.7$ inhibitors PF-04856264 and phlotoxin I[68]. Amgen recently engineered AM-8145 and AM-0422, two peptides based on JzTx-V

toxin that selectively inhibit Nav1.7[69]. As mentioned above, in a recent human trial the peripherally restricted, $Na_V1.7$-selective small molecule PF-05089771 did not significantly reduce pain scores compared to placebo[34]. Taken together, these results might suggest that selective $Na_V1.7$ inhibitors may need to be administered with a sub-therapeutic dose of an opioid to achieve adequate analgesia[70, 71, 72].

The $\alpha9\alpha10$ nictonic acetylcholine (nACh) receptor is also considered a therapeutic target for pain[73]. The α-conotoxins Vc1.1 and RgIA from the cone snails *Conus victoriae* and *Conus regius* respectively (Fig. 2A), antagonize $\alpha9\alpha10$ nACh receptors[74, 75]. Agonism of $GABA_B$ receptors and resulting inhibition of $Ca_V2.2$ channels via G-protein βγ interaction is a second mechanism of action that has been suggested for these peptides[76]. Vc1.1 was effective in rodent models of pain[77], and advanced to human phase-2 trials where it failed, possibly because it was less potent on human than rat $\alpha9\alpha10$ nACh receptors[58, 63, 78], another example of the translational challenges of mammalian species differences. RgIA-4, an analogue of RgIA with high affinity for both rodent and human $\alpha9\alpha10$ nACh receptors and no activity on $GABA_B$ receptors, suppresses rodent cancer chemotherapy-induced neuropathic pain[58, 63, 79] and is currently in pre-clinical development.

P2X3 receptors are being targeted with small molecules for endometriosis-associated pain (Table 2). These receptors are also inhibited by purotoxin-1 (PT1), a 35-amino acid residue peptide from the venom of the wolf spider *Geolycosa sp.,* that slows recovery from desensitization following channel activation with ATP and reduces hyperalgesia in rat models of inflammatory pain[80]. Analogues of PT1 or related peptides, if optimized for specificity, potency and stability, could be advanced for pain indications.

Channel-modulating peptides for cancer therapy

Chlorotoxin (Fig. 2A), a peptide from the death stalker scorpion *Leiurus quinquestriatus hebraeus,* was initially reported to inhibit small-conductance chloride currents in epithelial cells, astrocytomas and gliomas[81, 82, 83, 84] but later found to also bind to matrix metalloproteinase-2 (MMP2) on glioma cells[85] and not block volume-, cATP- or Ca^{2+}-activated chloride channels[86]. The molecular identity of the putative chloride channel blocked by chlorotoxin is currently undetermined. Human phase-1 trials a decade ago showed that a ^{131}I-radiolabeled analog was safe following intravenous administration for high-grade glioma, but the compound was not advanced further (NCT00733798). However, there are currently efforts underway to use chlorotoxin-based fluorescent dye conjugates as so-called “tumor paint” to help visualize gliomas[87], and one analog, BLZ-100 [88], was tested in a Phase-1 study (NCT02234297) in 2016, with a new Phase-2/3 study about to start (NCT03579602) in pediatric patients with CNS tumors where fluorescent-labeled chlorotoxin will be assessed with an imaging system.

TRPV6 channels are over-expressed in ovarian, breast, prostate, colon and thyroid cancers, and have been implicated in tumor progression[89]. SOR-C13, a C-terminal truncation of a longer, 54-residue paralytic peptide called Soricidin (accession number POC2P6) from the short-tailed shrew (*Blarina brevicauda*), blocks TRPV6 at low nanomolar concentrations and suppresses tumors in xenograft models of ovarian and breast cancer[58, 90]. Fluorescent-labeled and super-paramagnetic iron oxide-conjugated analogues are able to visualize ovarian tumors *in vivo* in mouse models[90]. In

a phase-1 trial in 23 patients with cancers of epithelial origin, SOR-C13 stabilized disease suggesting antitumor activity[91] but caused grade 2-3 dose-related hypocalcemia and atrial fibrillation in a quarter of patients[91]. Soricimed, the company developing SOR-C13, currently seems to be focusing on 2nd generation peptide drug conjugates.

Channel-modulating peptides for neurological diseases

The acid-sensing ion channel 1a (ASIC1a), a key mediator of acidosis-mediated neuronal damage in cerebral ischemia, is widely regarded as a potential therapeutic target for the treatment of ischemic stroke[92]. π-hexatoxin-Hi1a, a peptide from the Australian funnel-web spider *Hadronyche infensa* (Fig. 2A), delays ASIC1a channel-activation by binding to an acidic pocket critical for proton gating of the channel[93]. In rodent stroke models, Hi1a attenuates brain damage and improves behavioral outcomes even when administered intracerebroventricularly 8 hours after stroke onset[93]. Intravenous and intranasal routes are currently being trialed in mice.

$Na_V1.1$ is localized in fast-spiking inhibitory neurons in the brain, and epilepsy in Dravet's syndrome, a loss of function mutation of $Na_V1.1$, is thought to be due to reduced inhibitory neurotransmission. The peptide Hm1a from the spider *Heteroscodra maculate* (Fig. 2A), activates and slows inactivation of $Na_V1.1$ at nanomolar concentrations[24]. In mice carrying the human R1407X nonsense mutation, Hm1a rescued the collapse of action potentials in inhibitory interneurons without affecting excitatory neurons[94]. Intracerebroventricular delivery of Hm1a reduced seizures and post-ictal mortality in Dravet syndrome mice[94]. Further development of Hm1a would require microinfusion pumps like those used for Prialt® delivery or microfluidic ion pumps that electrophoretically pump ions across an ion exchange membrane and thereby deliver the peptide "dry" without fluid[95].

Channel-modulating peptides for treatment of autoimmune diseases

Potassium channels were discovered in T lymphocytes in 1984[96, 97]. Two potassium channels, the voltage-gated $K_V1.3$ and the calcium-activated $K_{Ca}3.1$ channel, promote calcium Ca^{2+} entry into lymphocytes through store-operated CRAC (Orai/Stim) by providing a counterbalancing cation efflux[98, 99]. Blockade of these channels therefore suppresses T lymphocyte activation. Differential expression of these channels allows preferential suppression of terminally-differentiated effector memory T cells (T_{EM}), which contribute to the pathogenesis of many different autoimmune diseases, with specific $K_V1.3$ inhibitors[100]. The ShK peptide from the sea anemone *Stichodactyla helianthus* (Fig. 2A) blocks $K_V1.3$ with picomolar affinity but also displays high affinity for neuronal potassium channels[101, 102]. An extensive structure-activity-relationship program led to the development of ShK-186 (Dalazatide), an analogue with picomolar affinity for $K_V1.3$ and 100-1000-fold selectivity over related channels[103, 104]. Sustained high picomolar levels of ShK-186 are achieved in plasma following subcutaneous injection. Due to its long circulating-half-life, ShK-186 is effective in rodent models of multiple sclerosis, rheumatoid arthritis and atopic dermatitis when administered once every 2-3 days[104, 105]. In phase-1 trials in healthy human volunteers, no ECG changes were observed, and no severe or life threatening adverse effects were noted. In a Phase-1b trial in patients with plaque psoriasis, Dalazatide administered twice weekly by subcutaneous injection

significantly reduced the psoriasis area and severity index in nine of ten patients[106]. It caused temporary mild grade 1 hypoesthesia and paresthesia involving the hands, feet, or perioral area in the majority of patients[106], possibly because Dalazatide is cleaved into a product with decreased K_V1.3-specificity[103, 104]. Plans for a phase-2 trial in the orphan disease inclusion body myositis[107] are on hold due to financial constraints.

Other efforts have focused on improving *in vivo* pharmacokinetic properties of K_V1.3-blocking peptides. PEGylation of the scorpion peptide HsTx1[R14A] prolonged plasma circulating half-life in rodents and resulted in sustained efficacy in rodent models of multiple sclerosis and rheumatoid arthritis[108]. By screening a combinatorial ShK peptide library, novel analogues were identified that, when fused to the C-termini of IgG1-Fc, retained picomolar potency, effectively suppressed *in vivo* delayed-type hypersensitivity and exhibited a prolonged circulating half-life[109]. Other approaches included the engineering of a scorpion toxin into a humanized antibody to achieve picomolar affinity for K_V1.3 and long plasma half-life[110], and the development of novel formulations that achieved satisfactory blood levels of peptide inhibitors following buccal or pulmonary delivery[111, 112]. Scientists at Amgen generated the derivative ShK[Q16K] by "brute-force" structure-activity analoging and then demonstrated sustained inhibition of plasma cytokine levels in primates with weekly administration of a PEG-conjugated version as well as efficacy in a rat model of multiple sclerosis[113], while a group at Janssen created half-life-extending Fc or albumin fusion proteins[114] with the α-KTx3 scorpion toxin OsK1 (α-KTx3.7) and tested them in minipigs. However, both companies subsequently seem to have dropped K_V1.3 as a target based on the previously made observation[100], that the efficacy of K_V1.3 blockers depends on the strength of T-cell stimulation and that Kv1.3 inhibition is therefore immunomodulatory rather than immunosuppresssive[115, 116].

Other channel-modulating peptides

The μ-conotoxin CncIIIC from the cone snail *Conus consors* (Fig. 2A), a blocker of Na_V1.4 sodium channels with myorelaxant and analgesic properties[117], is marketed by Activen as XEP-018, a topical cosmetic cream to reduce periocular wrinkles[58, 118]. SYN-AkeTM, an analogue of the peptide Waglerin-1 from the Southeast Asian Temple viper *Tropidolaemus wagleri*, blocks muscle nicotinic acetylcholine receptors and modulates $GABA_A$ receptors[119], and is being developed as an alternative dermaceutical to BoTox™ for wrinkles[58].

Lastly, αCT1 (ACT1), a peptide based on the last nine amino acids of the C-terminus of connexin 43, a gap junction protein often found at the edge of wounds and associated with poor wound healing[120], has been shown in multiple Phase-2 trials to reduce mean ulcer area for chronic neuropathic foot[121] and venous leg ulcers[122]. Granexin® gel is currently being tested in a Phase-3 trial (NCT02667327) for diabetic foot ulcers, which affect roughly 15% of the diabetic population globally.

Extending the plasma half-life of peptides

One weakness of peptide-based drugs is their short *in vivo* half-life due to their instability and their rapid renal elimination. The first drawback can be overcome by increasing the effective molecular mass of the peptide via conjugation to create so called "biobetters"[123]. As discussed for several examples above, ion channel-targeting

peptides have been conjugated to polyethylene glycol (PEG), to large proteins (e.g. human serum albumin), or protein domains (e.g. antibody Fc domain) or engineered into the complementary-determining region of humanized antibodies to generate fusion proteins[124]. Strategies to enhance peptide stability include peptide backbone cyclization, disulfide-bridge modification, residue substitutions, peptide stapling[125], and computational design of hyper-stable constrained peptide scaffolds[126]. Backbone cyclization of the α-conotoxin RgIA enhanced its stability in human serum without perturbing the structure or function of the peptide[127, 128, 129, 130]. However, cyclization of APETx2 decreased its activity on the ASIC3 channel while enhancing stability[131]. Modification of disulfide bridges is another approach to enhance stability. In the α-conotoxin Vc1.1, disulfide bridges have been replaced by non-reducible dicarba linkages, or have been eliminated and the core of the peptide stabilized by residue-substitutions near the removed bridges[132, 133]. Both strategies improved stability and oral bioavailability of Vc1.1, but reduced activity. Stapling technologies have also been successful for stabilizing peptides that target N-methyl-D-aspartic acid receptors[134]. Thus, several post-translational methods can be used to augment stability, improve circulating half-life, and even achieve oral bioavailability. The need for half-life extension, however, will depend on a number of factors, including the route of administration, nature of the disease, and whether the drug is intended for acute or chronic use. Some venom peptides are exceedingly stable in plasma, while others depot after cutaneous injection (e.g. Dalazatide[104]). For chronic diseases such as diabetes and persistent pain, daily injectables are likely to be tolerated by patients and therefore half-life may not be as critical. Exenatide with an elimination half-life of 140 minutes requires twice daily injection in diabetic patients and has peak sales approaching $1 billion/year.

Using peptide-channel interactions to guide the design of peptidomimetics or small molecule inhibitors

Many groups have exploited the understanding of peptide-channel interactions to design channel-modulating peptidomimetics. The earliest attempt to design a peptidomimetic of the sea anemone ShK peptide resulted in a million-fold loss in potency against $K_V1.3$[135]. More recently developed peptidomimetic 4-arm ethylene glycol-conjugated star polymers based on scorpion toxins have achieved nanomolar potency against $K_V1.3$[136]. Peptidomimetics have also been designed to target connexins, neuronal $Ca_V2.2$ channels[137, 138] and the $Ca_V\beta2$ subunit of cardiac L-type ($Ca_V1.2$) calcium channels[139].

In the 1990s, high-throughput screens using [^{125}I]-charybdotoxin competitive binding assays led to the discovery of several small molecule inhibitors (WIN-17317-3, CP-339,818) with nanomolar potency against the Kv1.3 channel[140, 141, 142]. However, while charybdotoxin bound in the outer vestibule of the channel, these small molecules likely bound in the inner chamber below the selectivity filter. Displacement of the radiolabeled peptide by these small molecules was due to a transpore effect. Similar transpore inhibition of [^{125}I]-charybdotoxin competitive binding to the large-conductance $K_{Ca}1.1$ channel was seen with indole diterpenes[143]. Radiolabeled-peptide toxin binding assays have subsequently fallen out of favor for screening and more recent high

throughput screens have used thallium flux or membrane potential- or calcium-sensing dyes.

Ion channel targeted antibodies and nanobodies

There are currently more than 60 approved therapeutic antibodies on the market, predominantly in the areas of oncology, autoimmunity and inflammatory disease, and approximately 550 antibodies are estimated to be in various stages of clinical development[144]. However, ion channel antibody discovery and development has lagged far behind with only one antibody, a sheep polyclonal (BILO10t) targeting a non-functional form of P2X7 from Biosceptre formulated as a topical ointment for the treatment of basal cell carcinoma having reached human clinical trials thus far[145].

While venom peptides have long been recognized to be superior to small molecules in terms of their potency and selectivity for ion channels, targeting channels with equally selective antibodies has more recently generated intense interest, mostly driven by the highly desirable pharmacokinetic characteristics of immunoglobulins compared to peptides resulting in a much lower dosing frequency. However, antibodies also have unique properties in terms of their mode of action. Small molecules and peptides usually act by directly blocking ion flux through the open channel or by interfering with the gating processes, which can be both positive (activators) or negative (inhibitors), and therefore affect channel function on the micro- to millisecond time scale (e.g. use-dependent inhibition of Na_V channels by classical small molecule anti-epileptics). Antibodies can also work by occlusion of the ion channel pore or induce allosteric-induced gating effects, but in addition they can be engineered to either lack or possess enhanced Fc-mediated functions, such as antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP), and FcRn-mediated transcytosis. Antibodies can further carry toxic or radioactive payloads to target disease cells[144, 146]. Thus, antibodies can potentially exert their modulating effect through one, or a combination of mechanistic actions, including, Fc-mediated depletion, internalization of ion channel-antibody complexes[147], and/or modulation of ion channel behavior with a conjugated peptide or small molecule toxin - features that may also be enhanced by bivalent avidity effects.

The above mentioned role of anti-ion channel autoantibodies in autoimmune diseases[148] and success in generating functional polyclonal antibodies against multiple ion channel targets[149] provided strong evidence for the feasibility of generating functional monoclonal antibodies (mAbs). However, overall progress in ion channel mAb discovery has been slow due to significant technical challenges that appear to be somewhat target specific. Indeed, ion channels and other multi-pass membrane proteins have recently been ranked among the “high-hanging fruit” in terms of antibody drug discovery[144]. For example, while many ion channels share common structural motifs based around transmembrane and pore-forming domains, the surface topography of ion channels where antibodies would be predicted to bind and exert their effect (the extracellular epitope target area) can be quite different amongst ion channel families. For example, members of the acid sensing ion channel (ASIC) and the purinoceptor P2X family contain extracellular amino acids that constitute approximately 68% or 48%, respectively, of the total protein[3]. Conversely, voltage-gated ion channels[2]

such as Na_V family members have far fewer amino acids exposed to the extracellular space (approximately 15%). Therefore, it is not surprising that reports of successful monoclonal antibody programs have tended to describe the discoveries of antibodies targeting the former and not the latter. Further complicating antibody discovery against these targets is the relative lack of immunogenicity of small surface loops, conservation amongst orthologs leading to tolerance in host immune animals and a relative lack of robust sources of recombinant ion channel protein to enable large scale antibody discovery programs.

Nevertheless, several strategies have been successful in generating monoclonal antibodies that inhibit ion channel function with the simplest approaches using antigenic peptides derived from ion channel extracellular (ECL) domains. A particularly popular tactic has been targeting the pore-forming E3 loop of 6-transmembrane domain K_V, Na_V, or TRP channels using peptide antigens with the rationale that antibody binding to this region will inhibit ion flow. This approach has been mostly successful in generating polyclonal antibodies that block channel function[149] although it has also been used to generate functionally blocking mAbs targeting $K_V10.1$[150] or the T cell calcium influx channel Orai1. Using a peptide from the second extracellular loop of Orai1 a group at Amgen generated mAbs that inhibit CRAC current[151], while a group at Novo Nordisk produced mAbs that inhibit calcium influx in T cells, suppress T cell proliferation and cytokine production, and showed efficacy in a graft-versus-host disease mouse model induced by human T cell transfer presumably by inducing Orai1 internalization[152]. Channel internalization also is the mechanism of action of a mAb generated against purified peptide antigens from the first ECL domain of $hK_{2P}9.1$ (KCNK9) that inhibits tumor growth and metastasis in mouse cancer xenograft models[153]. However, there appear to be clear limitations for isolating mAbs using peptide-derived strategies presumably due to the fact, that peptide antigens, while abundant and inexpensive, typically are not representative of native structures. This challenge can be mitigated by employing immunization strategies that use ion channel antigens that contain a native fold. For example, mAbs with blocking properties have been generated by immunizing host animals with DNA (ASIC1[154], $K_V1.3$[155]), cells expressing target ion channels (P_2X_7[156], Orai1[151]), ion channel containing virus like particles (P2X3[157]), and recombinant purified ion channels (P2X3[147], $K_V1.3$[158]). In an alternative approach, Quiang *et al.* generated a mAb that blocks ASIC1a currents with an IC_{50} of 85 nM and reduces infarction in a rodent stroke model by panning a human scFv combinatorial antibody phage library with nanodiscs containing reconstituted truncated ASIC1a protein and then converting into a full-length IgG1 form[159].

The difficulty in discovering clinically relevant conventional ion channel mAbs has led academic and industry investigators to explore alternative modalities. For example, the variable domain of heavy chain only camelid antibodies, or nanobodies[160], are small, 12-15 kDa, modular immunoglobulins that can offer distinct advantages over conventional mAbs such as access to epitopes that otherwise would be difficult to reach with conventional immunoglobulins[161, 162]. Additionally, the modular nature of their small singular domains allows for relatively straightforward engineering[161] of homo- and heteromeric molecules that can increase their potency, avidity, bispecificity and lead to half-life extension (HLE). Several examples of ion channel modulating nanobodies,

including bispecific ones, have now been described including those targeting P2X7 and $K_V1.3$ (see below for further details).

Other modalities that are garnering considerable interest include KnotBodies, which incorporate cystine-knot proteins like venom toxins[110] into the complementary-determining region (CDR) of the variable domain of an antibody light chain and antibodies complexed with warheads or small molecule moieties. While these fusion molecules differ from traditional antibodies where target binding is solely CDR driven, they nevertheless, represent a step-forward in the development of potential ion channel therapeutics by combining the potency of toxins with the therapeutic advantages afforded by mAbs. Another, largely unexplored group of biologics in terms of targeting and modulating ion channel function are various non-immunoglobulin protein-binding scaffolds characterized by their relatively small mass (~6-20 kDa). These include DARPins (designed ankyrin repeat proteins), affilins (ubiquitin), anticalins (lipocalin), atrimers (C-type lectin), monobodies (fibronectin type III), Kunitz domains (serine protease inhibitor) and affibodies (staphylococcal protein A domain Z)[163]. As of 2015 more than 20 scaffold-derived candidates were in preclinical or clinical development with one product derived from a Kunitz domain, the kallikrein inhibitor Ecallantide (Kalbitor®), on the market for the treatment of hereditary angioedema[163]. While none of the identified clinical candidates target ion channels, there is evidence suggesting that these large and diverse scaffold libraries could provide fertile ground for identifying novel ion channel binders. For example, monobodies selected from combinatorial libraries were shown to bind and block two bacterial Fluc-type fluoride channels[164], while high-affinity DARPins have been co-crystallized in complex with AcrB, the inner membrane pump of the *E. coli* multi-drug resistance tripartite complex AcrAB-TolC[165].

<u>Case study:</u> P2X7 as a target for cancer therapy and inflammation

The lone ion channel targeting immunoglobulin formulation that is currently in clinical development is BIL010t[145] from Biosceptre. BIL010t is a topical therapy for basal cell carcinoma (BCC) that contains sheep polyclonal antibodies directed against a non-functional form of the purinergic receptor channel P2X7. This form, called nfP2X7, represents a distinct conformation with a non-functional pore that is upregulated in response to high-ATP concentrations in tumor micro-environments and is required for tumor cell survival[166]. Moreover, the P2X7 E200 peptide sequence (G^{200}HNYTTRNILPGLNITC^{216}) whose conformation is distinct and exposed in nfP2X7 but not in WT P2X7 has enabled the generation of polyclonal and monoclonal antibodies that selectively bind nfP2X7 on the surface of tumor cells[166]. A PEG-based topical ointment containing polyclonal antibodies purified from sheep repeatedly immunized with the E200 peptide conjugated to keyhole limpet hemocyanin caused significant reduction in B16F10 tumor growth in an orthotopic mouse model of melanoma[145]. In a Phase-I clinical trial (NCT02587819) an ointment containing 10% BIL010t was applied to primary BCC lesions twice daily for 28 days. The treatment was well-tolerated, did not result in systemic penetration of sheep polyclonal antibodies and resulted in a decrease in lesion size in 65% of the patients with 20% showing no change and 15% showing an increase in size[145]. In addition to BIL010t, Biosceptre is developing

BIL03s, an anti-nfP2X7 human monoclonal antibody for treatment of solid and hematological tumors.

A monoclonal antibody targeting human P2X7 was generated following immunization of mice with a mouse myeloma cell line, XS63, expressing P2X7 and screening hybridoma cell line supernatants by flow cytometry using transfected and non-transfected XS63 cells[156]. One mAb that specifically reacted with HEK cells expressing P2X7 but not cells expressing P2X1 or P2X4 also recognized native P2X7 in human monocytes that had been differentiated into macrophages with LPS or γ-IFN. The anti-P2X7 mAb was shown to block BzATP-induced inward currents in HEK cells transfected with human P2X7 (IC_{50}~5 nM), but not mouse or rat orthologues[156].

Anti-mouse P2X7 nanobodies with antagonistic (13A7, IC_{50} = 12 nM) or potentiating activity (14D5, EC_{50} = 6 nM) were isolated from phage libraries derived from llamas immunized with either HEK cells stably expressing P2X7 or cDNA[167]. The potencies of the blocking and the enhancing nanobodies increased upon multimerization. A dimeric-HLE version of 13A7 that additionally contains the albumin-binding nanobody Alb8 to extend serum half-life was effective in ameliorating both allergic contact dermatitis and experimental glomerulonephritis in mice[167]. Additionally, another nanobody called Dano1, that specifically recognizes human P2X7, blocked ATP-induced Ca^{2+} influx and pore formation in P2X7-expressing HEK cells with dimerization leading to increased potency (IC_{50} = 0.2 nM). Dano1 also inhibited inflammasome assembly by LPS-primed human monocytes as well as the shedding of CD62L and the externalization of phosphatidylserine by T cells. Interestingly, Dano1 was significantly more potent (20-50 fold) than the previously described conventional anti-P2X7 mAb[167].

<u>Case study:</u> K_V1.3 as a target for autoimmune disease

In addition to the validation accompanying K_V1.3 as a therapeutic target for autoimmune disease[100, 106, 168], its accessibility to antibodies in autoreactive effector memory T-cells makes it a particularly strong ion channel target for therapeutic intervention with immunoglobulins. This, may explain, in part, why K_V1.3 has been a popular target for the discovery and development of immunoglobulin-based molecules amongst academic and biotech researchers with examples describing polyclonal and monoclonal (conventional and nanobody) antibodies as well as KnotBodies (Fig. 3), that inhibit ion channel function and alleviate T-cell mediated autoimmune disease in animal models. For example, a polyclonal antibody (E314) purified from the sera of rabbits immunized with a 14-amino acid E3 peptide antigen directed against the hK_V1.3 S5-S6 loop specifically bound to HEK cells expressing K_V1.3 but not cells expressing other K_V family members[169]. The E314 antibody was also shown to inhibit K_V1.3 currents by approximately 90% at a concentration of 300 nM in transfected HEK cells and Jurkat T cells but showed no significant effect on K_V1.1, K_V1.2, K_V1.4, K_V1.5 or K_V11.1 (hERG)[169]. Interestingly, a K_V1.3 vaccine was recently shown to induce high titers of anti-K_V1.3 antibodies in mice and rats that lessened clinical symptoms and decreased pathological CNS damage in a model of experimental autoimmune encephalomyelitis-presumably through the action of polyclonal anti-K_V1.3 antibodies[170].

Ablynx, now a Sanofi company, have described the generation of anti-K_V1.3 nanobodies isolated from llamas using a genetic prime-boost immunization strategy.

K_V1.3 nanobodies were found to be selective (10,000-fold over Kv1 family members), state-dependent and demonstrated varying functional profiles. The fast onset of functional effects strongly indicated that they were due to channel inhibition and not to internalization mechanisms[155]. Construction of bivalent hetero- and homodimers resulted in molecules with mixed functional properties in the case of the former, namely increased potencies, target residence time, avidity effects and duration of blockade. Additionally, a trimeric nanobody displayed higher potency in T cell based assays. A nanobody comprising two identical anti-K_V1.3 nanobody monomers fused to an anti-albumin moiety to increase half-life was effective *in vivo* in reducing ear thickness in a rat delayed hypersensitivity model[155]. More recently, conventional full-length anti-K_V1.3 mAbs have been isolated from chickens and llamas using a similar prime-boost immunization and screening strategy[158]. Chickens were immunized and boosted with purified recombinant K_V1.3 reconstituted into liposome formulations and anti-K_V1.3 antibodies identified and cloned following B-cell screening using a gel encapsulated microenvironment (GEM) assay incorporating various K_V1.3 containing formulations including liposomes and oriented channel adhered to magnetic beads[158]. In the case of llamas, initial immunizations were carried out with DNA and boosts with K_V1.3 liposomes. Phage libraries derived from immunized llamas were screened using K_V1.3 bound to magnetic beads. In all, 69 specific anti-K_V1.3 scFv-Fc antibodies were isolated from both host-animal platforms with 10 antibodies (9 chicken and 1 llama) demonstrating functional block of K_V1.3 current. Characterization of select scFv-Fc clones demonstrated that current block was time and concentration dependent with the most potent clone having an IC_{50} of 6 nM. Antibodies showed no activity against related family members (K_V1.1, K_V1.2, K_V1.5), hERG or Na_V1.5. Epitope binning analysis revealed that while most (7 of 10) functional antibodies segregated to one bin suggesting a dominant functional epitope, 3 antibodies segregated to different bins indicating that functional block could be achieved through distinct epitope binding events.

In an alternative approach IONTAS, a company developing a KnotBody platform based on incorporating peptide toxins with a conserved inhibitory cysteine knot (knottin) structural motif into peripheral antibody CDR loops (Fig. 3), have generated a ShK-KnotBody with a reported K_V1.3 inhibitory IC_{50} of 8.6 nM[171]. Using a similar approach, a group at the Scripps Research Institute previously incorporated the K_V1.3 inhibitory scorpion peptide toxins Moka1 and Vm24-toxin into the CDR3H domain of a humanized anti-lysozyme antibody with high structural similarity to a bovine antibody and into the CDR2H (Moka1) and CDR3L (Moka and Vm24) domains of the humanized RSV-neutralizing antibody Syn[110]. The syn-Vm24-CDR3L antibody fusion demonstrated subnanomolar potency (IC_{50} = 0.59 nM) in a K^+ flux assay, inhibited activation of αCD3 stimulated T_{EM} cells with an IC_{50} of 1 nM, displayed a long serum half-live of approximately 2 days, and showed dose-dependent inhibition of delayed type hypersensitivity reactions *in vivo* in rats.

<u>Case study:</u> Na_V1.7 as a target for pain

A monoclonal antibody, SVmab1, with purported nanomolar potency (IC_{50} 31 nM) was generated in mice using a peptide targeting the Na_V1.7 S3-S4 extracellular loop region in the domain II (DII) voltage-sensor[172]. Using antibody purified from hybridoma

cultures, SVmab1 exhibited state-dependent inhibition of $Na_V1.7$, 400-1500-fold selectivity over most $Na_V1.X$ family members, efficacy in both inflammatory and neuropathic pain models in mice and reduced scratching in both acute and chronic itch models[172]. However, a subsequent study using a recombinant form of the antibody (rSVmab) generated from published light and heavy chain antibody sequences was unable to bind neither the S3-S4 antigen peptide, a soluble and purified DII voltage sensor protein nor the mammalian cell expressing $Na_V1.7$. Neither was rSVmab able to specifically block $Na_V1.7$ current in transfected HEK293 cells[173]. Interestingly, the discoverers of SVmab confirmed the apparent discrepancies between the functional hybridoma-derived and non-functional recombinant forms of the mAb and speculated that the difference may have resulted from incorrect disclosure of antibody sequences or possibly differences in post-translational modifications of antibodies produced in hybridomas compared to transfected mammalian cells[174]. It will be of considerable interest to learn if other researchers utilizing a similar DII voltage-sensor targeted strategy can identify mAbs with similar functional properties to SVmab.

In an approach distinct from the CDR-targeted KnotBody strategy[110], a $Na_V1.7$ toxin-antibody conjugate was recently described that maintained the selective potency of the toxin warhead while conferring significant half-life extension and biodistribution to nerve fibers[175]. In their study, Biswas et al. describe assembling a conjugate comprised of a nontargeting anti-2,4-dinitrophenol human IgG1 with a cysteine mutation at a surface residue (excluding CDR, effector binding domains, proline and glycine residues) attached to the tarantula venom GpTx-1 peptide toxin via a PEG_{11} linker[175]. In a combinatorial approach several factors including cysteine mutation sites of the carrier antibody, linker chain-length, conjugation chemistry and peptide loading were evaluated for their effect on $Na_V1.7$ current inhibition. One antibody conjugated at substituted cysteine E384C exhibited a 30-fold loss in potency compared with naked peptide (IC_{50} 250 nM vs 8.5 nM), but had a serum half-life of 80 h, about 130-fold longer than the naked peptide. Following intravenous administration, the GpTx-1 mAb conjugate biodistributed to mice dorsal root and sciatic nerve better than the parent mAb[175]. Additionally, distribution of the conjugate to dorsal root and sciatic nerve was significantly more pronounced in WT mice compared to $Na_V1.7$ knockout mice indicating that both the presence of $Na_V1.7$ and the peptide toxin were required for distribution to peripheral nerve elements across the so-called blood nerve barrier (BNB). While the conjugate was not effective *in vivo* in a mouse histamine-induced pruritis model, most likely due to plasma concentrations that were not sufficiently greater than measured IC_{50}s, the data demonstrate the effective combination of Na_v inhibitory properties of a toxin with the desired pharmacokinetic characteristics of an antibody.

Ion channel antibodies going forward

In many respects the ion channel antibody field is playing “catch-up” with its GPCR cousin with the latter able to boast three marketed antibodies, the anti-CCR4 antibody mogamulizumab for the treatment of Adult and Peripheral T cell lymphoma and two anti-CGRP antibodies (erenumab and fremanezumab) for migraine; there are also at least fifteen other immunoglobulins in various phases of clinical development and more projects at the preclinical stage[19]. The challenges associated with developing GPCR

antibodies have been described in detail elsewhere[19, 176, 177]; suffice it to say, that in cases where functional antibodies are desirable, the GPCR and the ion channel field face the same difficulties of developing antibodies that recognize specific conformational states of the target protein (e.g. the active versus the inactive state of a GPCR or the open versus the inactivated state of an ion channel, see Fig. 1A). However, attributing the discrepancy in the current development status of GPCR and ion channel antibodies to the comparative difficulty of targeting ion channels versus GPCRs would not sufficiently acknowledge the significant advancements made in the GPCR field. These include technologies like StaR® (Stabilized Receptor), where a small number of point mutations are introduced into a GPCR to improve its thermostability without disrupting its pharmacology[178, 179]. Heptares Therapeutics is using this technology not only to generate protein for x-ray crystallography and small molecule screening, but also to enable purification of high-quality, functional and monodisperse protein that can be incorporated into effective antigen and screening formulations for both *in vivo* immunization and *in vitro* antibody discovery platforms. Instead, the proliferation of clinical antibody candidates during the last 10 years against a difficult class of membrane proteins like GPCRs should provide a level of optimism that similar advancements in ion channels are also within reach.

Overcoming the aforementioned challenges for advancing the ion channel antibody pipeline will undoubtedly involve integrating next generation platforms into the discovery process. For example, limitations of traditional approaches such as hybridoma screening, where non-efficient fusion events and subsequent loss of rare B cells can impact immune diversity[180], can be mitigated by technologies that incorporate direct B-cell cloning allowing the identification of rare clones and the recovery of natively paired light and heavy chain genes[119]. Similarly, traditional display technologies such as phage and yeast-display are being further enhanced by next-generation sequencing that allows up to 10,000-fold more sequences than the Sanger method and is enabling deeper interrogation of library diversity and identification of rare clones[181, 182]. Parallel advances in recombinant ion channel production using alternative expression hosts (e.g. *Tetrahymena thermophila*), cell-free systems and non-detergent based purification methods and formulations leading to increases in the quality (native-fold) and quantity (>mg) of purified protein or following the development of protocols enabling the production of possibly even stable, state-specific channels will likely complement newer approaches to antibody discovery and therefore increase the chances of successfully recovering antibodies with desired properties. With nanobodies it might even be feasible to target intracellular ion channel domains (see Box 2).

Overall perspective and concluding remarks

As seen from the foregoing paragraphs, ion channel drug discovery/development is approaching a cross road where future breakthroughs and new market introductions are likely to increasingly rely on biologic modalities comprising the plethora of technical opportunities based on peptides, antibodies and hybrid molecules. Given the diversity of solutions and the current speed of innovation across academia and the pharmaceutical

industry, it is difficult to pinpoint exactly which therapeutic areas will gain the most and the fastest from this development, but a conservative view is that it will largely follow the accessibility of the cellular targets. Since injected peptides and antibodies usually have quite low “volumes of distribution”, meaning that they tend to stay in the blood stream rather than penetrating deeply into tissues and cells, it is likely that hematological diseases, certain cancer forms, and immune diseases are straightforward therapeutic areas, whereas CNS diseases are much more challenging due to the tight blood brain barrier. (This view is supported by a parallel development in the field of therapeutic antibodies targeting extracellular proteins, where peripheral anti-cytokine therapy is very established and successful for treatment of autoimmune diseases, whereas attempts to treat Alzheimer’s disease with antibodies against amyloid beta are lacking behind). That said, between these opposite poles there are many opportunities defined by no or less tight endothelial barriers towards other organs (e.g. bone marrow, liver), by accessibility via local administration (e.g. lungs, skin), by administration via specific routes (e.g. intrathecal) or even by uptake across disease damaged barriers, such as the blood brain barrier in stroke and the intestinal epithelium in inflammatory bowel diseases. Considering the ion channel targets themselves it is a common view that channels with prominent extracellular loops (like $GABA_A$, nicotinic acetylcholine and P_2X receptors or two pore domain K^+ channels) are more tractable by antibodies than channels with restricted extracellular exposure, However, as discussed here it is indeed possible to generate function-blocking antibodies against ion channels with limited extracellular epitopes (e.g. $K_V1.3$ and Orai).

While we believe that the field of ion channel targeted biologics, which at the moment can only boast the intrathecally administered $Ca_V2.2$ blocking peptide Ziconotide in the clinic, and a polyclonal antibody and three peptides in clinical trials, is very likely to develop and expand in the next decade, it is currently hard to predict whether venom-derived peptides or antibodies will be more successful. Peptide design is currently benefitting tremendously from the advances in ion channel structural determination and molecular modeling which together are enabling structure-based or at least structure-assisted peptide design. However, “naked” peptides typically have short circulating half-lives and, depending on whether they are intended for acute or chronic use, might require some of the half-live extending modifications or formulation approaches described here to be considered suitable for clinical development. For traditional mAbs the main challenges in our opinion will be to select the most suitable ion channel targets and then tailor the antibody characteristics to the desired therapeutic effect (e.g. inhibition or activation of channel function, channel internalization, induction of target cell apoptosis) by engineering the antibody to fit the pathophysiology of the target disease. The above discussed BIL010t antibody against a non-functional form of P2X7 that is required for tumor cell survival[166] is a perfect illustration of a very clever topical use of a polyclonal sheep antibody for basal cell carcinoma where the characteristic of the antibody seem to meet the medical need.

Text Box 1

Recent progress and challenges for ion channel structure-based drug design

In the last 5 years an increasing number of ion channel structures has become available through advances in cryoelectron microscopy (cryoEM) such as improvements in microscope design and imaging hardware, and enhanced image processing which allow the reconstruction of 3D structures from a large number of single particle 2D projection images even even in the presence of structural and conformational heterogeneity[183]. While many cryo-EM structures are not high enough resolution for "true" structure-based drug design because they do not allow the possible location of hydrogen bonds or salt bridges to be seen, Rosetta computational structure refinement can be used to improve atomic details of cryoEM structures with 3-5 Å resolution[184]. For example, in the Na_V channel field both traditional x-ray and cryoEM structures now provide high-resolution structural templates for structure-based design of novel small molecules, peptides, and antibodies. However, while many of the structures contain fully resolved extracellar loops, a remaining challenge that needs to be further explored is how to stabilize the extracellular loop regions in native conformations for immunization without having to produce large quantities of full-length protein.

- An x-ray structure of a human Nav1.7– bacterial Na_VAb chimera[31] with a picomolar affinity drug bound to the domain IV (DIV) voltage sensor constitutes a template for targeting the voltage sensor, which plays a key role in stabilizing Na_V channels in an inactivated state, with small molecules or peptides.
- All human Nav channels have unique sequences in the extracellular loop regions within the voltage-sensing and pore-forming domains. CryoEM structures of the human Nav1.4[41] and *American cockroach* Na_VPaS[185] with a small molecule and or a peptide toxin bound have all extracellular loops resolved in the pore and voltage sensor domains.
- The CryoEM structure of the *electric eel* Nav1.4[40] has all extracellular loops in the pore domain resolved.

Text Box 2

Targeting ion channel cytoplasmic epitopes with nanobodies

Immunoglobulins are not able to passively access the cell cytoplasm. Therefore, ion channel antibody discovery programs, like those directed towards other cell surface molecules, are generally designed to identify antibodies that recognize extracellular epitopes to exert their functional effect, be it steric or allosteric block, or removal of a channel from the cell surface by internalization mechanisms. Consequently, the intracellular epitope space, which is considerably larger than that displayed on the surface for many voltage-gated like ion channels (e.g. ~70% of the sodium activated K^+ channel $K_{Na}1.1$), is left untargeted. In a recent review, Ingram *et. al.*[162] highlighted the attributes of nanobodies (Nbs) that may allow exploiting the cytosolic side of membrane

proteins such as GPCRs or ion channels for basic research, immunodiagnostics and potentially therapeutic intervention.

Nbs are single domain antibodies comprised of the variable domain of VHH camelid IgGs. Nbs are small (~15kDa) and modular in nature, bind their targets with affinities similar to conventional antibodies[186] and tend to recognize epitopes in clefts of protein surfaces or at protein-interaction interfaces[187]. Like conventional antibody light and heavy chain variable domains, Nb antigen binding is driven by three complementarity-determining regions (CDRs). However, unlike conventional antibodies that require disulfide-paired light and heavy chain variable domains to form a paratope[188], this is achieved in Nbs in a single domain. Therefore, for Nbs where intrachain disulfide bonds are not necessary for maintaining target specificity this attribute imparts a significant tolerance, compared to conventional antibodies, to reducing environments like the cytoplasm where Nbs can be expressed in functional form, bind cytosolic epitopes and exert a functional effect.

Cytosolic expression of Nbs has been exploited in a number of phenotypic screens to identify functional hits and further explore cellular processes. In one example, a randomized VHH-CDR3 intracellular antibody library was introduced into highly metastatic human fibrosarcoma derived cells followed by iterative rounds of cell migration assays to identify clones associated with non-migrating cells[189]. Using this approach one nanobody clone was used to identify heterogenous nuclear ribonucleoprotein K (hnRNP-K) as having a role in metastasis[189]. In another instance VHH coding sequences derived from alpacas immunized with inactivated Influenza A virus (IAV) or vesicular stomatitis virus (VSV) were cloned into a lentiviral vector and used in a phenotypic screen to identify intracellularly expressed Nbs that protected human A549 cells from lethal infection with either IAV or VSV[190]. In addition to phenotypic screens, Nbs have been engineered to selectively degrade target molecules via ubiquitination by fusion to ubiquitin ligase F-box subunits[191, 192] and have served as chaperones that bind to the intracellular surface of GPCRs to stabilize active conformations such as the β2-adrenergic and M2 muscarinic receptors that enabled structural determination by x-ray crystallography[193, 194].

It remains to be determined whether targeting intracellular domains of ion channels with nanobodies will provide the same molecular insights into stabilized state-dependent structures as they have with GPCRs or whether therapeutic applications like gene therapy that result in the expression of anti-ion channel Nbs for functional disruption or degradation of targeted ion channel will move beyond speculation. Nevertheless, the propensity for Nbs to recognize epitopes in protein-protein interfaces raises the intriguing possibility of investigating the biological mechanisms of ion channel complexes. The alpha subunit comprising the ion channel pore typically represents only one of a number of regulatory and auxiliary proteins in a complex that influence channel function, trafficking, distribution and signaling. Indeed, several channelopathies are associated with mutations that disrupt ion channel complex formation and cause cardiac arrhythmias[195]. Induced expression of Nbs raised against ion channel interaction domains may help de-convolute complex formation under varying physiological conditions, illuminate the precise temporal and spatial role of complex components and possibly identify novel targets for therapeutic intervention. New insight into the cytosolic

face of ion channel biology may simply await the design of elegant phenotypic screens utilizing intracellular nanobody libraries as described above.

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Competing interests statement
The authors declare competing financial interests: see web version for details.
Palle Christophersen is a full-time employee of Saniona A/S. Paul Colussi, is a full-time employee of TetraGenetics Inc.

Table 1: Ion channel targeting drugs approved in the last 10 years.

Table 2: Ion channel targeting drug development candidates currently in clinical trials.

Figure Legends

Figure 1. ***A)*** Cartoon representation of the Na_V channel gating cycle in closed, open and inactivated states. The voltage sensing domain (VSD) segments are colored blue (S1-S3) and red (S4). The pore-forming domain segments are colored sand (S5, P1-helix, P2-helix, and N-terminal part of S6) and green (C-terminal part of S6). The gating charge carrying arginines in the S4 segment are represented by "+" signs. Key conformational changes in the Na_V channel during gating between closed, open, and inactivated states are highlighted by transmembrane movement of the S4 segment (colored in red) and lateral movement of the C-terminal part of S6 segment (colored in green). Inactivation in Na_V channels can be either fast and involve one of the cytoplasmic inter-repeat loops "plugging" the inner vestibule[8] or slow and involve a rearrangement of the selectivity filter[8]. ***B)*** Small molecule receptor sites within Na_V channels. Transmembrane view of Na_V channel interaction with pore bound (lidocaine) and voltage-sensor bound (GX-936) small molecule drugs. The Na_V channel structure is based on the electric eel $Na_V1.4$ channel structure[40] (pdb: 5XSV) and is shown in ribbon representation. The pore domain is shown in beige and the voltage-sensors are shown in blue (domain I), green (domain II), yellow (domain III), and red (domain IV). Lidocaine is shown in space-filling representation and colored purple. GX-936 is shown in space-filling representation and colored light blue. The chemical structures of

vixotrigine, lidocaine, GX-936, and PF-05089771 are shown in 2D representation next to the channel.

Figure 2. ***A)*** Structures of peptide toxins targeting ion channels. Ribbon representation of peptide toxin structures colored individually and labeled. Disulfide bonds are colored in yellow. Ziconotide (pdb: 1TTK), μ-theraphotoxin-Pn3a (pdb: 5T4R_A), JZTX-V (pdb: 6CGW), Rg1A (pdb: 2JUT), chlorotoxin (pdb: 5L1C), π-hexatoxin-HI1a (pdb: 2N8F), Hm1a (pdb: 2N6O), ShK (pdb:2K9E_A), HsTx1 (pdb: 1QUZ_A), μ-CNIIIc (pdb: 2YEN). ***B)*** Peptide toxin receptor sites on ion channels. *Left,* Pore blocking peptide receptor site in a K_V channel illustrated by the transmembrane view of charybdotoxin in the pore of the K_V1.2-K_V2.1 chimera structure[196] (pdb: 2R9R) and shown in ribbon representation. The pore is shown in beige and the voltage-sensors are shown in blue. Charybdotoxin is shown in orange. Potassium ions within the selectivity filter region are shown in sphere representation and colored purple. *Right*, Voltage sensor binding peptide receptor site in a Na_V channel illustrated by the transmembrane view of the voltage-sensor bound peptide toxin Dc1a. The Na_V structure is the American cockroach Na_VPaS structure[197] (pdb: 6A90) and shown in ribbon representation. The pore is shown in beige and voltage-sensors are shown in blue (domain I), green (domain II), yellow (domain III), and red (domain IV). The Dc1a toxin is shown in orange.

Figure 3. Figure illustrating the size relation of a full-length immunoglobulin G (IgG2a, pdb: 1IGT, ribbon presentation) colored in orange, an antigen-binding fragment (Fab, pdb: 1K4C) colored in orange, a KnotBody Fab colored in green, and a nanobody (pdb: 6C5W) colored in pink. The *Ecballium elaterium* Trypsin Inhibitor (EETI-II) structure within the KnotBody is shown in space-filling representation. The K_V channel structure is based on the K_V1.2-K_V2.1 chimera[196] (pdb: 2R9R) and shown in ribbon representation and colored in beige. Potassium ions within the selectivity filter region are shown in sphere representation and colored in purple.

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Compound	Modality	Indication	Route of administration	Target/Mechanism	Approval/ Withdrawal	Company
Brexanolone	Neurosteroid	Post partum depression	PO	$GABA_A$ δ preferring PAM	2018	Sage Therapeutics
Clevidipine	Small molecule	Acute hypertension	IV	$Ca_v1.x$ inhibitor	2008	The Medicines Company
Lacosamide	Small molecule	Epilepsy	PO	Na_v inhibitor	2008	UCB
Amifampridine	Small molecule	Lambert–Eaton myasthenic syndrome	PO	K_v inhibitor	2009	BioMarin Pharmaceutical
Dronedarone	Small molecule	Arrhythmia	PO	Multi K channel inhibitor	2009	Sanofi-Aventis (Sanofi)
Eslicarbazepine	Small molecule	Epilepsy	PO	Na_v inhibitor	2009	Bial
Fampridine	Small molecule	Multiple sclerosis	PO	K_v inhibitor	2010	Acorda Therapeutics
Retigabine	Small molecule	Epilepsy	PO	$K_v7.2$-$K_v7.5$ activator	2011/2017	GSK
Clobazam	Small molecule	Anxiety, epilepsy	PO	$GABA_A$ activator	2011	Maestretti Research Laboratories (Sanofi)
Clobazam	Small molecule	Anxiety, epilepsy	PO	$GABA_A$ activator	2011	Maestretti Research Laboratories (Sanofi)
Perampanel	Small molecule	Epilepsy	PO	GluA1-4 inhibitor	2012	Eisai
Ivacaftor	Small molecule	Cystic fibrosis	PO	CFTR potentiator	2012	Vertex; Cystic Fibrosis Foundation
Ivabradine	Small molecule	Heart failure	PO	I_f (HCN) inhibitor	2005 EMA 2015 FDA	Servier Amgen
Lumacaftor/ ivacaftor	Small molecule	Cystic fibrosis	PO	CFTR chaperone plus potentiator	2015	Vertex

IV = intravenous; PO = per os

compound	company	indication	Target or mechanism	clinical phase
Granexin gel (αCT1)	FirstString Research	Diabetic foot ulcer, cutaneous radiation injury	Cx43 c-terminal mimetic peptide	III
Dalazatide (ShK-186)	Kv1.3 Therapeutics	Psoriasis, IBM	K_v1.3 inhibitor	Ib/IIa
Tozuleristide (BLZ-100)	Blaze Bioscience	Imaging of glioma ('tumour paint')	Metalloprotease inhibitor, possible chloride channel inhibitor	I/II
SOR-C13	Soricimed Biopharma	Cancer	TRPV6 inhibitor	I
Antibodies				
BILO10t	Biosceptre	Cancer	Non-functional P2X7	I/II
Small molecules and neurosteroids				
Mirogabalin	Daiichi Sankyo	Peripheral neuropathic pain	Ca_V inhibitor ($\alpha_2\delta$ subunit	Registration phase
XEN007	Xenon	Hemiplegic migraine	Ca_v2.1 inhibitor	I
Tetrodotoxin (Halneuron)	Wex Pharmaceuticals	Cancer and chemotherapy induced pain	Na_v inhibitor	III
Vixotrigine (BIIB074)	Biogen	Painful lumbosacral radiculopathy, trigeminal neuralgia	Na_v1.7 inhibitor	II
BIIB095	Biogen	Pain	Na_v1.7 inhibitor	I
CC-8464	Chromocell, Astellas Pharma	Neuropathic pain	Na_v1.7 inhibitor	I
DSP-2230	Sumitomi Dainippon Pharma	Neuropathic pain	Na_v1.7, Na_v1.8 inhibitor	I
DSP-3905	Sumitomi Dainippon Pharma	Neuropathic pain	Na_v1.7 inhibitor	I

RG-6029 (GDC-0310)	Roche	Pain	$Na_v1.7$ inhibitor	I
Xen901	Xenon	Epilepsy	$Na_v1.6$ inhibitor	I
Gefapixant	Merck, Roche	Chronic cough	P2X3 inhibitor	III
BAY-1902607 [a]	Bayer	Persistent chronic cough	P2X3 inhibitor	II
BAY-1817080 [a]	Bayer	Endometriosis, persistent chronic cough	P2X3 inhibitor	I
P2X4 inhibitor [a]	Bayer	Endometriosis	P2X4 inhibitor	I
JNJ-55308942	Johnsson & Johnsson	Neuroinflammation, anhedonia	P2X7 inhibitor	I
Intravenous glibenclamide (BIIB093)	Biogen	Stroke	SUR1–TRPM4 inhibitor	III
Basmisanil (RG-1662)	Roche	Cognitive impairment associated with schizophrenia	$GABA_A$ α5-subunit-targeting NAM	II
Sage-217 [b]	Sage Therapeutics	Major depression	$GABA_A$ δ-subunit-preferring PAM	II
Gaboxadol (OV-101)	Ovid Therapeutics	Angelman syndrome, Fragile X syndrome	$GABA_A$ δ-subunit-preferring agonist	I/II (repurposed)
RG-7816	Roche	Autism spectrum disorder	$GABA_A$ α5-subunit-targeting NAM	I
Evt201	Evotec	Insomnia	$GABA_A$ PAM	II
BIIB104	Biogen	Cognitive impairment associated with schizophrenia	AMPA activator	IIa
TAK-653	Takeda	Depression	AMPA activator	II

PF-04958242	Pfizer	Schizophrenia	AMPA activator	II
BAY-2253651 [a]	Bayer	Obstructive sleep apnoea	$K_{2P}3.1$ inhibitor	II
ASP-0819	Astellas Pharma	Fibromyalgia	$K_{Ca}3.1$ activator	II
Senicapoc	SpringWorks Therapeutics and University of California, Davis	Hereditary xerocytosis, Alzheimer's disease	$K_{Ca}3.1$ inhibitor	II (repurposed)
AP-30663	Acesion Pharma	Atrial fibrillation	$K_{Ca}2$ inhibitor	I/II
CAD-1883	Cadent Therapeutics	Ataxia or essential tremors	$K_{Ca}2$ activator	I/II
XEN496 (retigabine)	Xenon	KCNQ2 epileptic encephalopathy	K_V7 activator	II
GSK-2798745	GlaxoSmithKline	Heart failure	TRPV4 inhibitor	II
SB705498	GlaxoSmithKline	Rhinitis/Pain/Cough	TRPV1 inhibitor	II
Galicaftor (ABBV-2222)	Abbvie	Cystic fibrosis	CFTR	II
GLPG-1837	Galapagos, Abbvie	Cystic fibrosis	CFTR potentiator	II
ABBV-2451	Abbvie	Cystic fibrosis	CFTR	I
ABBV-2737	Abbvie	Cystic fibrosis	CFTR	I
ABBV-3067	Abbvie	Cystic fibrosis	CFTR	I
Sage-324 [a,b]	Sage Therapeutics	Essential tremor Parkinson's disease	$GABA_A$ PAM	I
Sage-718 [a,b]	Sage Therapeutics	NMDA hypofunction	NMDA activator	I

TrpC4/5 inhibitor	Hydra/Boehringer Ingelheim	Anxiety disorder, depression	TRPC4, TRPC5 inhibitor	I
Xen1101	Xenon	Epilepsy	K_V7 activator	I
HBI-3000	HUYA Bioscience International	Atrial fibrillation	Multi channel inhibitor	I
DWJ-208	Daewoong Pharmaceutical	Neuropathic and cancer pain	$Na_V1.7$	I

Table 2: Ion channel targeting drugs currently in development

Cx43, connexin 43; IBM, inclusion body myositis; NAM, negative allosteric modulator; PAM, positive allosteric modulator.
AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor. Ca_V, voltage-gated calcium channel. CFTR, cystic fibrosis transmembrane conductance regulator. GABA, γ-aminobutyric acid. GluA, ionotropic glutamate receptor. HCN, hyperpolarization-activated cyclic nucleotide-gated channel. K_{2P}, two-pore domain potassium channel. K_V, voltage-gated potassium channel. K_{Ca}, calcium-activated potassium channel. Na_V, voltage-gated sodium channel. NMDA, *N*-methyl-*D*-aspartate receptor. P2X, purinoreceptor. SUR, sulfonylurea receptor. TRPC, transient receptor potential canonical channel. TRPM, transient receptor potential melastatin channel. TRPV, transient receptor potential vanilloid channel.
[a] Presumed to be small molecules. [b] Neurosteroids.

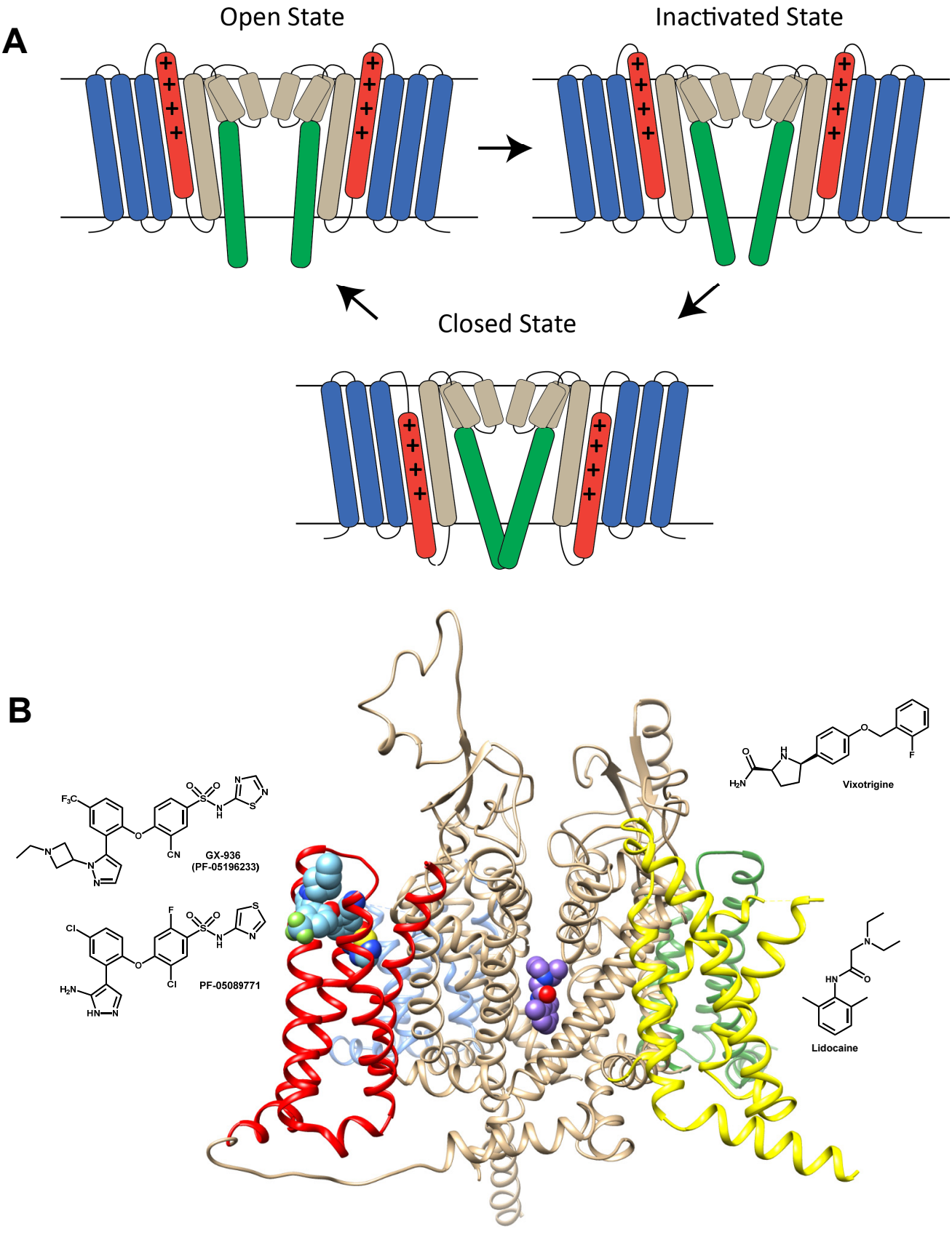
A
Open State
Inactivated State
Closed State
B
GX-936
(PF-05196233)
PF-05089771
Vixotrigine
Lidocaine

Figure 1

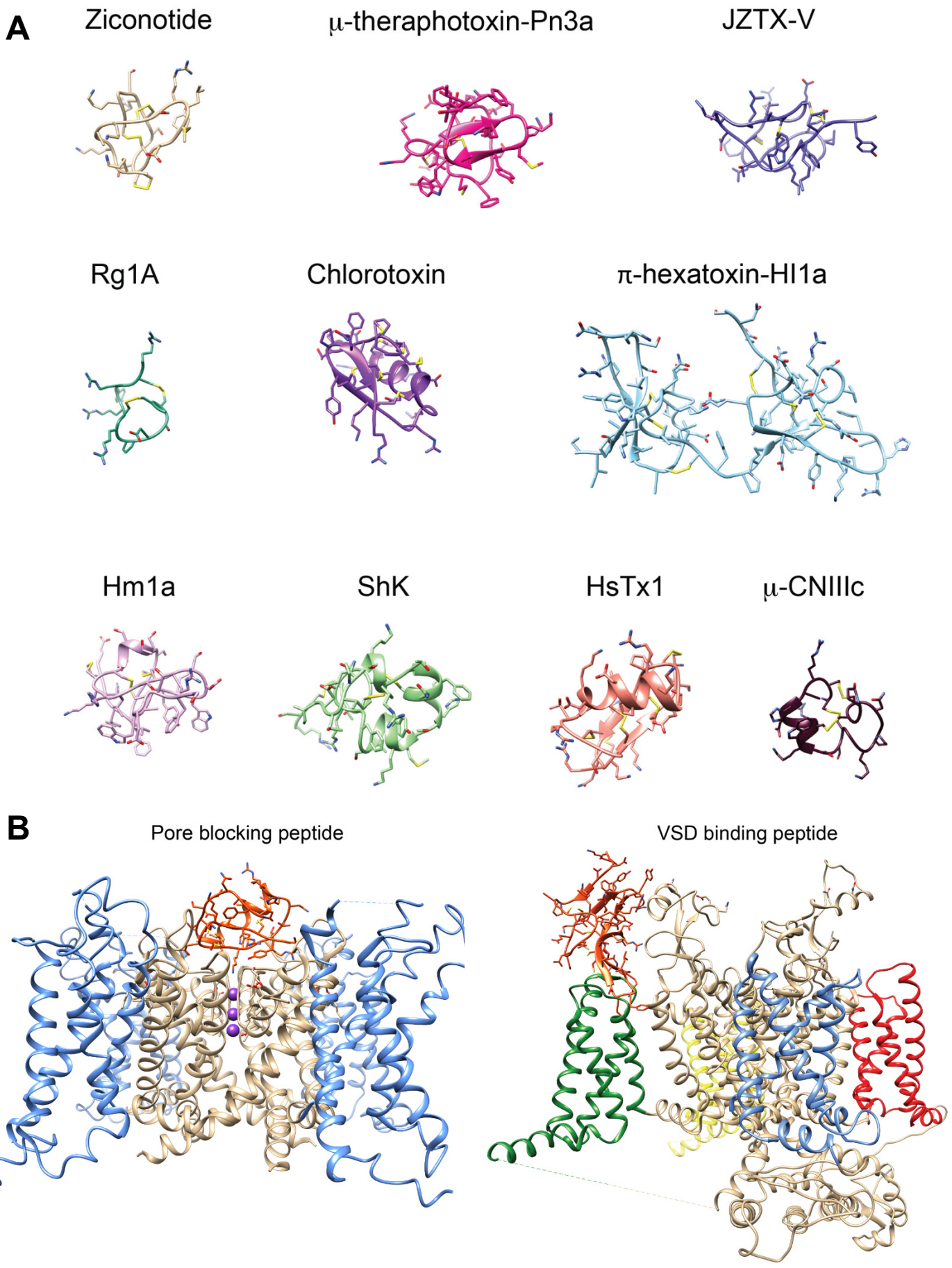
A
Ziconotide
μ-theraphotoxin-Pn3a
JZTX-V
Rg1A
Chlorotoxin
π-hexatoxin-HI1a
Hm1a
ShK
HsTx1
μ-CNIIIc
B
Pore blocking peptide
VSD binding peptide

Figure 2

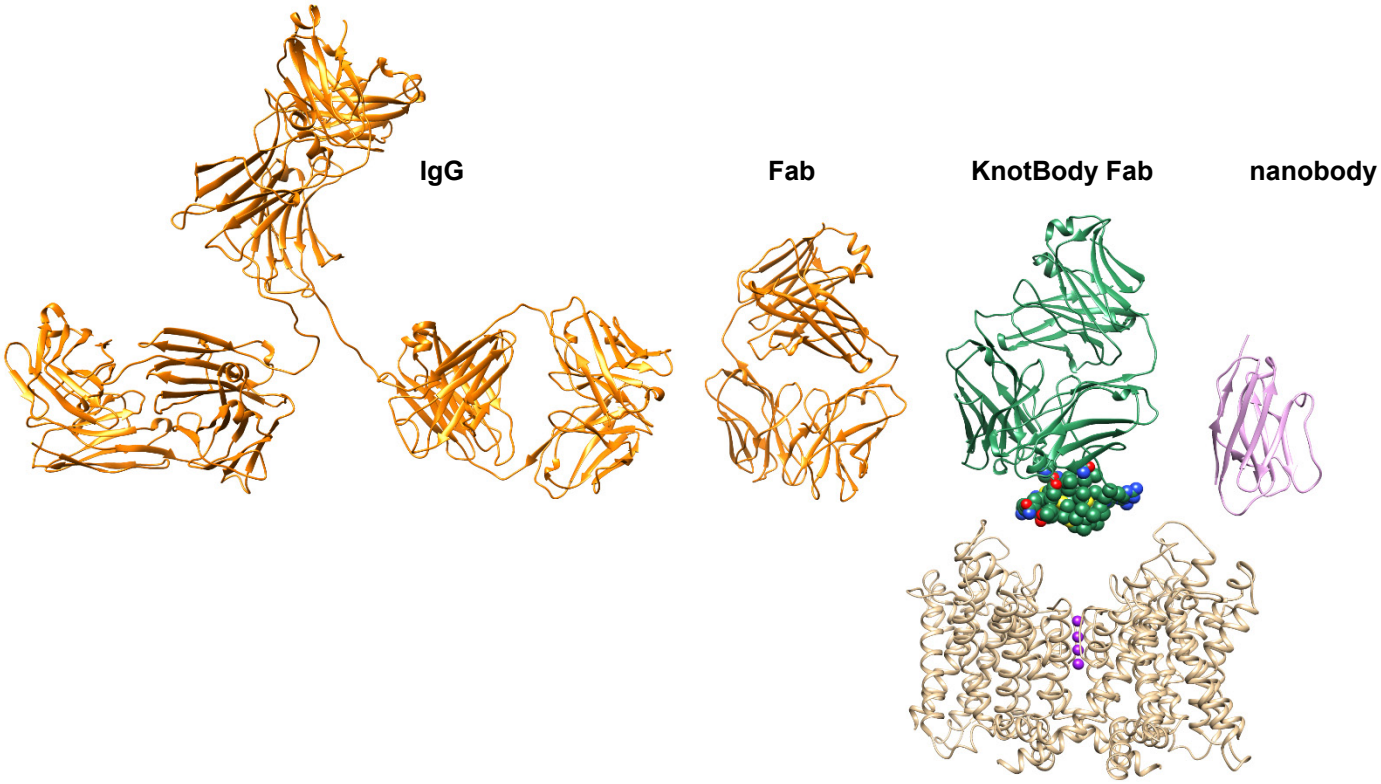

Figure 3

Supplementary Table 1: Ion Channel Drugs

	Medicine	Modality	Indication	Route of adm.	Target/Mechanism	Approval/ Withdrawal	Company	Essential Medicine (WHO)
	Ziconotide	Peptide	Chronic pain	Intrathecal	$Ca_V2.2$ inhibitor	2004	Elan Corporation	
	XEP-018	Peptide	Periocular wrinkles (cosmetic)	Topical	$Na_V1.4$ inhibitor		Activen SA	
Dihydro-pyridines	Nifedipine	Small molecule	Hypertension	PO	$Ca_V1.x$ inhibitor	1981	Bayer	Yes
	Amlodipine	Small molecule	Hypertension	PO	$Ca_V1.x$ inhibitor	1990	Pfizer	Yes
	Cilnipidine	Small molecule	Hypertension	PO	$Ca_V1.x$ ($Ca_V2.2$) inhibitor	1995	Fuji Viscera Pharmaceutical Company/Ajinomoto	
	Clevidipine	Small molecule	Acute hypertension	IV	$Ca_V1.x$ inhibitor	2008	The Medicines Company	
	Efonidipine	Small molecule	Hypertension	PO	$Ca_V1.x$ ($Ca_V3.x$) inhibitor	1995	Shionogi & Co	
	Felodipine	Small molecule	Hypertension	PO	$Ca_v1.x$ inhibitor	1991	Hässle (AstraZeneca)	
	Isradipine	Small molecule	Hypertension	PO	$Ca_V1.x$ inhibitor	1990	Sandoz Pharmaceuticals (Novartis)	
	Lercanidipine	Small molecule	Hypertension	PO	$Ca_V1.x$ inhibitor	1997	Recordati S.p.A.	
	Nicardipine	Small molecule	Hypertension, Angina	PO	$Ca_V1.x$ inhibitor	1988		
	Nimodipine	Small molecule	Hypertension, vasospasm	PO/IV	$Ca_V1.x$ inhibitor		Bayer	
	Verapamil	Small molecule	Hypertension, angina	PO/IV	$Ca_V1.x$ inhibitor	1981		Yes
	Diltiazem	Small molecule	Hypertension, angina	PO	$Ca_V1.x$ inhibitor			

Supplementary Table 1: Ion Channel Drugs

	Gabapentin	Small molecule	Pain, Epilepsy	PO	Ca_V inhibitor (α2,δ)	1993	Parke-Davies (Pfizer)	
	Pregabalin	Small molecule	Pain, Epilepsy	PO	Ca_V inhibitor (α2,δ)	2004	Parke-Davies (Pfizer)	
	Flunarizine	Small molecule	Migraine	PO	Ca_V inhibitor	1968	Jansen Pharmaceutica (J&J)	
	Cinnarizine	Small molecule	Vertigo, motion sickness, sea sickness	IM	Ca_V inhibitor	1955	Jansen Pharmaceutica (J&J)	
	Fluspirilene	Small molecule	Schizophrenia	PO	Ca_V inhibitor (unselective)		Jansen Pharmaceutica (J&J)	
	Pinaverium bromide	Small molecule	IBS	PO	Ca_V inhibitor	1975	Solvay (Abbvie)	
	Mibefradil	Small molecule	Hypertension, angina	PO	Ca_V3.x inhibitor	1997/1998	Roche	
	Dantrolene	Small molecule	Malignant hyperthermia	IV	RyR inhibitor	1974		
	Capsaicin	Small molecule	Muscle pain (topical)	Topical	TRPV1 activator	2009 FDA		
	Repaglinide	Small molecule	Type II diabetes	PO	Direct K_{ir}6.2 inhibitor	1997	Novo Nordisk	
	Nateglinide	Small molecule	Type II diabetes	PO	Direct K_{ir}6.2 inhibitor	2001	Novartis	
	Riluzole	Small molecule	ALS	PO	K_{Ca} activator Na_V inhibitor	1995	Rhone Poulenc Rorer (Sanofi)	
	Chlorzoxazone	Small molecule	Spasm	PO	K_{Ca} activator	1958	McNeilab Inc (Johnson & Johnson)	Yes
	Zoxazolamine	Small molecule	Spasm	PO	K_{Ca} activator	1955/1961	McNeilab Inc (Johnson & Johnson)	
	Ivabradine	Small molecule	Heart failure	PO	I_f (HCN) inhibitor	2005 EMA 2015 FDA	Servier Amgen	

Supplementary Table 1: Ion Channel Drugs

	Ivacaftor	Small molecule	CFTR	PO	CFTR potentiator	2012	Vertex; Cystic Fibrosis Foundation	
	Lumacaftor/ivacaftor	Small molecule	CFTR	PO	CFTR chaperone plus potentiator	2015	Vertex	
Sulfonyl-ureas	Glibenclamide	Small molecule	Type II diabetes	PO	$K_{Ir}6.x$ inhibitor (SUR)	1966	Boehringer Manheim/Hoechst (Novartis, Sanofi)	
	Tolbutamide	Small molecule	Type II diabetes	PO	$K_{Ir}6.x$ inhibitor (SUR)	1956	Upjohn Company (Pfizer)	
	Acetohexamide	Small molecule	Type II diabetes	PO	$K_{Ir}6.x$ inhibitor (SUR)			
	Carbutamide	Small molecule	Type II diabetes	PO	$K_{Ir}6.x$ inhibitor (SUR)		Servier	
	Chlorpropamide	Small molecule	Type II diabetes	PO	$K_{Ir}6.x$ inhibitor (SUR)	196?	Pfizer	
	Tolhexamide	Small molecule	Type II diabetes	PO	$K_{Ir}6.x$ inhibitor (SUR)			
	Metahexamide	Small molecule	Type II diabetes	PO	$K_{Ir}6.x$ inhibitor (SUR)			
	Tolazamide	Small molecule	Type II diabetes	PO	$K_{Ir}6.x$ inhibitor (SUR)	196?	Pfizer	
	Glibornuride	Small molecule	Type II diabetes	PO	$K_{Ir}6.x$ inhibitor (SUR)		Meda Pharma	
	Gliclaside	Small molecule	Type II diabetes	PO	$K_{Ir}6.x$ inhibitor (SUR)	1972		Yes
	Glipizide	Small molecule	Type II diabetes	PO	$K_{Ir}6.x$ inhibitor (SUR)	1984	Pfizer	
	Glisoxepide	Small molecule	Type II diabetes	PO	$K_{Ir}6.x$ inhibitor (SUR)			
	Glycopyramide	Small molecule	Type II diabetes	PO	$K_{Ir}6.x$ inhibitor (SUR)			
	Glimepiride	Small molecule	Type II diabetes	PO	$K_{Ir}6.x$ inhibitor (SUR)	1995	Hoechst Marion Roussel	
	Diazoxide	Small molecule	Acute hypertension	PO	$K_{Ir}6.x$ activator			

Supplementary Table 1: Ion Channel Drugs

	Pinacedil	Small molecule	Hypertension	PO	$K_{Ir}6.x$ activator	1989	Leo Pharma	
	Minoxidil	Small molecule	Hypertension, hair loss	PO, topical	$K_{Ir}6.x$ activator	1979	Upjohn Company (Pfizer)	
	Nicorandil	Small molecule	Hypertension, angina	PO	$K_{Ir}6.x$ activator		Upjohn Company (Pfizer)	
	Retigabine	Small molecule	Epilepsy	PO	$K_V7.2$-$K_V7.5$ activator	2011/2017	GSK	
	Flupirtine	Small molecule	Pain	PO	$K_V7.2$-$K_V7.5$ activator	1984	Asta-Medica	
	Fampridine	Small molecule	Multiple sclerosis	PO	K_V inhibitor	2010	Acorda Therapeutics	
	Amifampridine	Small molecule	Lambert–Eaton myasthenic syndrome	PO	K_V inhibitor	2009	BioMarin Pharmaceutical	
	Amiodarone	Small molecule	Arrhythmia	IV, PO	Multi K channel inhibitor	1961	Labaz company (Sanofi)	Yes
	Dronedarone	Small molecule	Arrhythmia	PO	Multi K channel inhibitor	2009	Sanofi-Aventis (Sanofi)	
	Sotalol	Small molecule	Arrhythmia	PO	Multi K channel inhibitor	1974	Mead-Johnson Pharmaceutica	
	Ibutulide	Small molecule	Arrhythmia	IV	Multi K channel inhibitor	1999	Pfizer	
	Dofetilide	Small molecule	Arrhythmia	PO	$K_V11.1$ inhibitor	1999	Pfizer	
Benzo-diazepines	Clorazepate	Small molecule	Anxiety	PO	$GABA_A$ activator	1972		
	Estazolam	Small molecule	Anxiety	PO	$GABA_A$ activator	1990	Upjohn Company (Pfizer)	
	Flurazepam	Small molecule	Anxiety	PO	$GABA_A$ activator	1970		
	Halazepam	Small molecule	Anxiety	PO	$GABA_A$ activator	1981		
	Lorazepam	Small molecule	Anxiety	PO	$GABA_A$ activator	1977	Wyeth Pharmaceuticals (Pfizer)	Yes

Supplementary Table 1: Ion Channel Drugs

	Midazolam	Small molecule	Anxiety	PO, IV ,IM	$GABA_A$ activator	1985	Hoffman LaRoche (Roche)	Yes
	Nitrazepam	Small molecule	Anxiety	PO	$GABA_A$ activator	1965		
	Oxazepam	Small molecule	Anxiety	PO	$GABA_A$ activator	1965		
	Quazepam	Small molecule	Anxiety	PO	$GABA_A$ activator	1985		
	Temazepam	Small molecule	Anxiety	PO	$GABA_A$ activator	1981		
	Triazolam	Small molecule	Anxiety	PO	$GABA_A$ activator	1982		
	Alprazolam	Small molecule	Anxiety	PO	$GABA_A$ activator	1981	Upjohn Company (Pfizer)	
	Zolpidem	Small molecule	Sleep disorders	PO	$GABA_A$ activator	1992	Synthelabo (Sanofi)	
	Flumazenil	Small molecule	Benzodiazepine antidote	IV	$GABA_A$ binder (neutral)	1987	Hoffman LaRoche (Roche)	
	Diazepam	Small molecule	Anxiety	PO	$GABA_A$ activator	1963	Hoffman LaRoche (Roche)	Yes
	Chlordiapoxide	Small molecule	Anxiety	PO	$GABA_A$ activator	1960		
	Clobazam	Small molecule	Anxiety, epilepsy	PO	$GABA_A$ activator	2011	Maestretti Research Laboratories (Sanofi)	
	Clonazepam	Small molecule	Anxiety	PO	$GABA_A$ activator	1975	Hoffman-la-Roche (Roche)	
	Phenobarbital	Small molecule	Epilepsy	PO, IV, IM	$GABA_A$ opener	1912	Bayer	Yes
	Alpidem	Small molecule	Anxiety	PO	$GABA_A$ activator	1991/1995	Synthélabo (Sanofi)	
	Eszopiclone	Small molecule	Insomnia	PO	$GABA_A$ activator		Sunovion	
	Zopiclone	Small molecule	Insomnia	PO	$GABA_A$ activator	1986	Rhône-Poulenc (Sanofi)	
	Propofol	Small molecule	General anaesthesia	IV	$GABA_A$ activator	1989		Yes

Supplementary Table 1: Ion Channel Drugs

	Valproate	Small molecule	Epilepsy, bipolar, migraine	PO	$GABA_A$ modulator	1967		Yes
	Ondansetron	Small molecule	Chemotherapy associated nausea	PO	5HT3 antagonist	1991	GSK	Yes
	Palonosetron	Small molecule	Chemotherapy associated nausea	IV, PO	5HT3A antagonist	2003 FDA 2009 EMA	Helsinn Healthcare	
	Alosetron	Small molecule	IBS associated diarrhoea	PO	5HT3A antagonist	2000/2000 2002 FDA	GSK	
	Granisetron	Small molecule	Chemotherapy associated nausea	PO, IM and transdermal	5HT3A antagonist	1993 FDA 2012 EMA	Beecham (GSK)	
	Phenytoin	Small molecule	Epilepsy	PO	Na_V inhibitor	1953	Parke-Davies (Pfizer)	Yes
	Phosphenytoin	Small molecule	Epilepsy	IV	Na_V inhibitor (prodrug)	1996	Parke-Davies (Pfizer)	
	Carbamazepine	Small molecule	Epilepsy	PO	Na_v inhibitor	1962	J.R. Geigy AG (Novartis)	Yes
	Eslicarbazepine	Small molecule	Epilepsy	PO	Na_V inhibitor	2009	Bial	
	Oxcarbazepine	Small molecule	Epilepsy	PO	Na_V inhibitor	1990	Geigy (Novartis)	
	Lamotrigine	Small molecule	Epilepsy, bipolar	PO	Na_V inhibitor	1994	GSK	Yes
	Lacosamide	Small molecule	Epilepsy	PO	Na_V inhibitor	2008	UCB	
	Primidone	Small molecule	Epilepsy	PO	Na_V inhibitor $GABA_A$ modulator	1950	Imperial Chemical Industry (Astra Zeneca)	
	Ethosuximide	Small molecule	Epilepsy	PO	Ca_V inhibitor Na_V inhibitor	1960	Parke-Davis (Pfizer)	Yes
	Mesuximide	Small molecule	Epilepsy	PO	Ca_V inhibitor Na_V inhibitor		Pfizer	

Supplementary Table 1: Ion Channel Drugs

	Phensuximide	Small molecule	Epilepsy	PO	Ca_V inhibitor Na_v inhibitor		Pfizer	
	Amiloride	Small molecule	Diuretic	PO	ENaC inhibitor	1981	MSD (Merck)	Yes
	Triamterene	Small molecule	Diuretic	PO	ENaC inhibitor	1964	Smith Kline & French (GSK)	
	Tetracaine	Small molecule	Local anaesthesia	IM	Na_V inhibitor	1931		
	Procainamide	Small molecule	Arrhythmia	IV, PO, IM	Na_V inhibitor	1951	Bristol-Meyers Squibb	
	Disopryamide	Small molecule	Arrhythmia	PO	Na_V inhibitor	1977		
	Flecainide	Small molecule	Arrhythmia	PO	Na_V inhibitor	1982		
	Propafenone	Small molecule	Arrhythmia	PO	Na_V inhibitor	1994	Knoll Pharmaceuticals (Abbvie)	
	Moracizine	Small molecule	Arrhythmia	PO	Na_V inhibitor	1990/2007	DuPont (Bristol-Myers Squibb)	
	Ranolazine	Small molecule	Chronic angina	PO	Late Na_V inhibitor	2006	Gilead	
	Mexiletine	Small molecule	Arrhythmia	PO	Na_V inhibitor	Still used in US	Boehringer Ingelheim	
	Lidocaine	Small molecule	Local anaesthesia Arrhythmia	IM	Na_V inhibitor	1948		Yes
	Bupivacaine	Small molecule	Spinal anaesthesia	IM	Na_V inhibitor	1963		Yes
	Etidocaine	Small molecule	Local anaesthesia	IM	Na_V inhibitor	1972		
	Levobupivacaine	Small molecule	Local anaesthesia	IM	Na_V inhibitor	1995		
	Mepivacaine	Small molecule	Local anaesthesia	IM	Na_V inhibitor	1957		

Supplementary Table 1: Ion Channel Drugs

	Prilocaine	Small molecule	Local anaesthesia	IM	Na_V inhibitor	1960		
	Ropivacaine	Small molecule	Local anaesthesia	IM	Na_V inhibitor	1997		
	Cisatracurium	Small molecule	General anaesthesia Neuromuscular block	IV	Chrn $\alpha_1\beta_1\gamma/\varepsilon\delta$ inhibitor	1995	Burroughs Wellcome (GSK)	
	Rocuronium	Small molecule	General anaesthesia Neuromuscular block	IV	Chrn $\alpha_1\beta_1\gamma/\varepsilon\delta$ inhibitor	1994	Schering-Plough (Merck)	
	Succinylcholine	Small molecule	General anaesthesia Neuromuscular block	IV	Chrn $\alpha_1\beta_1\gamma/\varepsilon\delta$ activator	1951		Yes
	Varenicline	Small molecule	Smoking cessation	PO	Chrn $\alpha_4\beta_2$ activator	2006	Pfizer	
	Nicotine	Small molecule	Smoking cessation	Topical (skin)	Chrn activator			
	Perampanel	Small molecule	Epilepsy	PO	GluA1-4 inhibitor	2012	Eisai	
	Felbamate	Small molecule	Severe epilepsy	PO	GluN inhibitor $GABA_A$ modulator	1993	Carter Wallace Laboratories	
	Ketamine	Small molecule	Anaesthesia	IV	GluN inhibitor	1970		Yes
	Memantine	Small molecule	Alzheimer's	PO	GluN inhibitor	1968	Eli Lilly and Company	
	Amantidine	Small molecule	Parkinson's disease (historical for flu)	PO slow release	GluN inhibitor	2017/2018 (formulations)		

ω-MVIIA CKGKGAKCSRLMYDCCTGSCRSGKC Ziconotide, Prialt^{R}

m-TRTX-Pn3a DCRYMFGDCEKDEDCCKHLGCKRKMKYCAWDFTFT

α-conotoxin Vc1.1 GCCSDPRCNYDHPEIC

α-conotoxin RgA GCCSDPRCRYRCR

α-conotoxin RgIA4 GCCTDP-Cit-CR93-I-Tyr)QCY KCP-400

Chlorotoxin MCMPCFTTDHQMARKCDDCCGGKGRGKCYGPQCLCR Tozuleristide
Tumor Paint

SOR-C27 EGKLSSNDTEGGLCKEFLHPSKVDLPR

SOR-C13 KEFLHPSKVDLPR

Pi-hexatoxin-HI1a NECIRKWLSCVDRKNDCCEGLECYKRRHSFEVCVPIPG

Pi-hexatoxin-HI1a FCLVKWKQCDGRERDCCAGLECWKRSGNKSSVCAPIT

Kappa theraphotoxin Hm1a ECRYLFGGCSSTSDCCKHLSCRSDWKYCAWDGTFS

ShK-186 (Py)(AEEA) RSCIDTIPKSRCTAFQCKHSMKYRLSFCRKTCGTC Dalazatide

HsTX1[R14A] ASCRTPKDCADPCAKETGCPYGKCMNRKCKCNRC

Vm24 AAAISCVGSPECPPKCRAQGCKNGKCMNRKCKCYYC

OsK1 GVIINVKCKISRQCLEPCKKAGMRFGKCMNGKCHCTPK

μ-conotoxin CnCIIIC ZGCCNGPKGCSSKWCRDHARCC

Waglerin 1 SLGGKPDLRPCHPPCHYIPRPKPR

Cx43 mimetic peptide Gap27 SRPTEKTIFII-hexadecyl

Cx43 mimetic peptide 5 VDCFLSRPTEK