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REVIEW ARTICLE

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Overcoming challenges of HERG potassium channel liability through rational design: Eag1 inhibitors for cancer treatment

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Abstract

Two decades of research have proven the relevance of ion channel expression for tumor progression in virtually every indication, and it has become clear that inhibition of specific ion channels will eventually become part of the oncology therapeutic arsenal. However, ion channels play relevant roles in all aspects of physiology, and specificity for the tumor tissue remains a challenge to avoid undesired effects. Eag1 (K_V10.1) is a voltage-gated potassium channel whose expression is very restricted in healthy tissues outside of the brain, while it is overexpressed in 70% of human tumors. Inhibition of Eag1 reduces tumor growth, but the search for potent inhibitors for tumor therapy suffers from the structural similarities with the

Abbreviations: 14-3-3θ, θ isoform of 14-3-30 family of proteins; BD-C1, binding domain at C-terminus 1; BD-C2, binding domain at C-terminus 2; BD-N, binding domain at N-terminus; bEAG, bovine EAG; BK_{Ca}, large conductance calcium-activated potassium channels; CaM, calmodulin; CHO, Chinese hamster ovary cell line; CNBHD, cyclic-nucleotide binding homology domain; CNS, central nervous system; COS-7, CV-1 in Origin with SV40 genes cell line; cryo-EM, cryogenic electron microscopy; CTTN, Cortactin; DEAG, *Drosophila* EAG; ER, endoplasmic reticulum; FDA, The United States Food and Drug Administration; GOPC, Golgi-Associated PDZ And Coiled-Coil Motif-Containing Protein; HEK293, human embryonic kidney 293 cells; HUGO, Human Genome Organisation; ICK, inhibitor cystine knot; IUPHAR, The International Union of Basic and Clinical Pharmacology; KCNE1, potassium voltage-gated channel subfamily E member 1; KCR1, K^+ channel regulator 1; K_V, voltage dependent potassium channels; LQT, long QT syndrome; mAb, monoclonal antibody; MCF7, Michigan Cancer Foundation-7, breast cancer cell line; MD, molecular dynamics; mEAG, mouse EAG; MPF, mitosis-promoting factor; nH, Hill coefficient; Orai1, Calcium release-activated calcium channel protein 1; p38 MAPK, p38 mitogen-activated protein kinases; p53, tumor protein p53; PAS, (Per–Arnt–Sim) domain; PD, pore domain; PDB, Protein Data Bank; PIP2, phosphatidylinositol 4,5-bisphosphate; pRb, retinoblastoma protein; rEAG, rat EAG; SAR, structure–activity relationship; SHH, Sonic hedgehog; SPCA2, Golgi secretory pathway Ca^{2+}-ATPase; TB, Temple–Bareitser syndrome; TdP, *torsade de pointes*; TGF, transforming growth factor; VSD, voltage sensor domain; ZL, Zimmermann–Laband syndrome.

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cardiac HERG channel, a major off-target. Existing inhibitors show low specificity between the two channels, and screenings for Eag1 binders are prone to enrichment in compounds that also bind HERG. Rational drug design requires knowledge of the structure of the target and the understanding of structure–function relationships. Recent studies have shown subtle structural differences between Eag1 and HERG channels with profound functional impact. Thus, although both targets' structure is likely too similar to identify leads that exclusively bind to one of the channels, the structural information combined with the new knowledge of the functional relevance of particular residues or areas suggests the possibility of selective targeting of Eag1 in cancer therapies. Further development of selective Eag1 inhibitors can lead to first-in-class compounds for the treatment of different cancers.

KEYWORDS
arrhythmia, cancer, Eag1, HERG, potassium channels, rational drug design

1 | INTRODUCTION

Potassium channels play key roles in cellular excitability, but they also have many other functions and are often critical for many cellular processes.[1] It is not surprising that they are also crucial in a pathological context, like for tumor progression.[2] Eag1 is a paradigmatic example of this tumor relevance, as its expression is dysregulated in a majority of human cancers.[3] At the same time, normal tissues outside of the brain do not express the channel. Eag1 would thus be an excellent candidate for tumor therapy, but it belongs to the same family (*KCNH*) as the cardiac channel HERG. Besides their radically different electrophysiological properties and functions, both channels share a similar structure, and therefore affinity for almost all known compounds. The importance of HERG for cardiac physiology poses a severe safety concern. In this review, we will describe in detail the structure, function, and pharmacology of Eag1, and highlight which aspects that distinguish Eag1 and HERG can serve to design compounds that block Eag1 while preserving HERG.

The *KCNH* family of voltage-gated potassium channels was first described in *Drosophila ether à go-go* mutants, which show altered excitability, resulting in leg shaking under ether anesthesia.[4] The founding member of the family was termed after the mutation, *eag*.[5–7] Initial screening of the mouse and human genomes identified an*eag* ortholog in the mouse, but in humans, only a paralog with a sequence distant enough to be classified as a related but independent gene was found.[8] This second gene was named human *eag*-related gene (HERG). The discovery that mutations in HERG cause a form of congenital long QT syndrome (LQT),[9,10] a malignant arrhythmia that can result in torsade de pointes (TdP), reentry, and sudden cardiac death, shifted most of the interest on the family towards this member in particular. Moreover, a large fraction of episodes of drug-induced LQT, frequently in carriers of mutations in other ion channels that prolong the QT interval, are due to the inhibitory effects of drugs on HERG. HERG inhibitors are so numerous that authorities started requesting proof of lack of activity on a molecule in the approval process.[11–13] In preclinical safety assessment, the drug safety threshold is usually expressed as the ratio of the IC_{50} value for HERG inhibition to the maximum therapeutic free concentration (C_{max}^{free}) of the drug.[14] To reduce the risk of TdP and LQT, Gintant proposed as appropriate a safety

margin threshold of 30 and 45, respectively.[15] These safety margin predictions have been studied several times in recent years. They have been shown to be reasonable, but definitely not perfect predictors of TdP appearance.[16] When C_{max}^{free} is not available, i.e. in the early stages of drug discovery, an absolute HERG potency of IC_{50} values less than 10 μM is suggested to have a 63% probability that the compound would cause a QT elongation in clinical trials. On the other hand, compounds with IC_{50} values larger than 10 μM are suggested to have 87% probability of not prolonging the QT interval.[17]

Later on, it became evident that the human Eag1, besides its activity in the central nervous system, plays a relevant role in tumor development and progression in virtually every oncological indication.[18,19] Importantly, in tumors showing ectopic expression of the channel, inhibition of Eag1 leads to reduced tumor growth, turning the channel into an attractive therapeutic target.[20,21] Unfortunately, most of the known blockers of Eag1 have an even higher affinity for HERG, leading to cardiac risk. Although this risk could be minimized and made assumable, the only genuinely safe blockers would be those that leave HERG unaltered. The knowledge acquired in the last years of the structure and function of both Eag1 and HERG makes it feasible to reach this goal through rational design.

The terminology of ion channels is often confusing, and the KCNH family is an extreme case. The gene family consists of eight members in three subfamilies, initially named Eag, Herg, and Elk (eag-like). The IUPHAR recommended terms,[22] probably the least equivocal, are K_V10.1 and K_V10.2 for the Eag,s, K_V11.1 to 3 for the Herg,s, and K_V12.1 to .3 for the Elk,s. The HUGO nomenclature[23] assigned the name *KCNH* (voltage-gated potassium channels, subfamily H) to the genes for all members. *KCNH1* encodes for Eag1 and *KCNH2* for HERG1. *KCNH5* encodes for Eag2, while *KCNH6* and *7* encode HERG2 and 3, and ELK channels are *KCNH8*, *KCNH3*, and *KCNH4*. The subjects of this review are K_V10.1 and HERG, that is, Eag1 and HERG1. We will use the "classical" nomenclature throughout the text, Eag1 and HERG because those names are most familiar for the community and least prone to generating confusion. We will only use their gene names, *KCNH1* and *KCNH2*, when necessary and refer to the whole family as *KCNH*.

2 | EAG1 POTASSIUM CHANNEL: AN OVERVIEW

2.1 | Structure

Although the *Drosophila eag* channel's amino acid sequence was immediately recognized as compatible with a potassium channel monomer,[6] with an array of six transmembrane segments (S1–S6), several substantial differences with known potassium channels became evident already at the time. On the one hand, the large intracellular domains displaying a PAS domain in the N-[24] and a cyclic nucleotide-binding domain in the C-terminus (which is occupied by a ligand from the same protein and not by cyclic nucleotides[24]) represent a characteristic feature of *KCNH* channels.[25] On the other hand, although the channels are reasonably selective for K^+, the "signature sequence" at the selective filter of *Shaker*-type channels (GYGD)[26] is different in this family (GFGN). Moreover, the "kink" (residues PVP) at the S6 segment,[27] which are essential for the gating of Shaker-type channels, is also absent in*KCNH* channels. This structural difference, along with the very short S4–S5 linker, already suggested substantial mechanistic differences in the voltage-dependent gating of this channel family.[28,29] In other voltage-gated channels, it is assumed that the movement of the S4 segment in response to depolarization conveys through the S4–S5 linker to the gating machinery acting as a rigid mechanical lever. This is possible because of the close interaction between S4–S5 and the S6 segment of a different subunit, a feature known as "domain swapping." In channels of the *KCNH* family, the voltage sensor of a subunit interacts with the pore domain of the same subunit (thus, they are non-domain-swapped channels). This notwithstanding, the intracellular domains are "swapped," and the N-terminus of one subunit interacts with the C-terminal domain of a neighboring one example, Barros et al.[30] These differences result in a very complex gating process whose description exceeds the scope of the present review.

Although divergent from other families of voltage-gated K^+ channels, the amino acid sequence within the transmembrane segments is conserved among the family members (see e.g., Barros et al.[30] and

Bauer and Schwarz[31]). The two channels that we intend to compare show moderate homology between them, although their functional properties are dramatically different. HERG shows larger intracellular domains, but the family's major hallmarks can be recognized in both Eag1 and HERG. The distal *N*-terminus displays a flexible stretch of 11 residues that are relevant for gating.[32] Downstream of this stretch, a PAS (Per–Arnt–Sim) domain, typically related to redox processes in other proteins, is conserved in the family. It was the first PAS domain identified in eukaryotic cells,[33] but its function is still incompletely understood. The core of the channel, S1–S6 segments, shows, in general, the classical structure of voltage-gated channels, except for the lack of domain swapping and a very short S4–S5 linker, as described above. The C-terminal cytoplasmic part of the channel presents a cyclic-nucleotide binding homology domain (CNBHD) and the segment between this domain and the end of S6 is called C-linker, which is relevant for gating; close to the end of the sequence, a tetramerizing coiled-coil participates in selective tetrameric assembly.[34] Finally, the C-terminus of the channel presents a ciliary localization signal (see below). Initially proposed as a nuclear localization signal (both types of signals are exchangeable), because functional Eag1 channels are also detected at the inner nuclear membrane,[35] where the channel traffics from the plasma membrane. However, the localization at the nuclear envelope was independent of the nuclear localization signal and happened in channels devoid of such a signal.

The structures of homotetrameric Eag1 channels were solved using cryo-EM.[36,37] The first structure (PDB: 5k7l, resolution: 3.78Å) was a rat Eag1 (Figure 1) truncated in the C-terminus (removal of 114 residues) and bound to the inhibitor complex of calmodulin and Ca^{2+} (Ca^{2+}–CaM). The electrical field in the experimental setup was 0 mV, and the channel should be open at that potential; the voltage-sensing parts of the protein are in the active conformation. Nevertheless, the channel was in a nonpermeant conformation because binding of four Ca^{2+}–CaM to the cytoplasmic domains induces the closing of the channel. The exact mechanism of channel closure by Ca^{2+}–CaM is not entirely understood. Still, a recent cryo-EM structure of a mutant Eag1 (PDB: 6PBY, resolution: 3.67Å) lacking residues 3–13 suggests that Ca^{2+}–CaM stabilizes interactions between cytoplasmic domains and voltage sensor domain (VSD) that maintain the channel closed.[32]

Analogously to other K_V channels, four VSDs form the body for sensing changes in the membrane potential. Each VSD comprises four transmembrane alpha-helices (segments S1–S4) (Figure 1, VSD in orange). S4 carries six positively charged residues responsible for sensing the membrane potential and is linked to S5 and S6, which constitute the pore domain (PD, Figure 1 in yellow) through the S4–S5 linker. The PD shows a water-filled cavity (central cavity) and selectivity filter (SF) (Figure 1C). SF is the narrowest part of the pore and responsible for the selective flow of potassium ions. The surface of the SF is lined with oxygen atoms that coordinate K^+ ions during permeation[37] (Figure 1C).

In the cryo-EM structure, S4 is in the "up" or activated position (displaced towards the extracellular side) corresponding to a membrane potential at which the channel is in the open state. A similar S4 voltage sensor position is observed in the solved cryo-EM structure of the HERG channel (PDB: 5va1, resolution: 3.7Å). HERG channel structure was obtained using a truncated construct that is missing 249 residues in the N-terminus and 134 residues in the C-terminus.[36] The truncation results in a 20-mV shift in the threshold voltage activation compared to wild-type HERG, and the structure shows the voltage sensor in the activated position with the channel pore open, while that of the Eag1 structure is closed. The most evident difference between the cavities in Eag1 and HERG structures is the appearance of a hydrophobic pocket just below the SF that extends out of the pore cavity (length ~11Å, diameter ~8Å, Figure 1C). In the closed Eag1 structure, those pockets are tightly sealed.[36,37] None of the channels with known structures had those pockets, and therefore they could not be modeled into homology models of Eag1 and HERG before their structures were described.[40,41] This is relevant because the cylindrically shaped pocket could accommodate a substituted aromatic ring, a known feature of most pharmacophore models reported for HERG blockers (Figure 1E,F). Another common element in the inhibitors is a positively charged moiety, usually represented as a (substituted) amino group in Eag1 and HERG inhibitors.[38,39,42] A positively charged group could be accommodated in the negative electrostatic potential located in a cross-section of all four pockets right below the entrance into the SF, or it could form cation-π interactions with aromatic rings lining the cavity (Figure 1C, in red mesh).[43,44] The residues surrounding the pocket are in both channels the same, and their

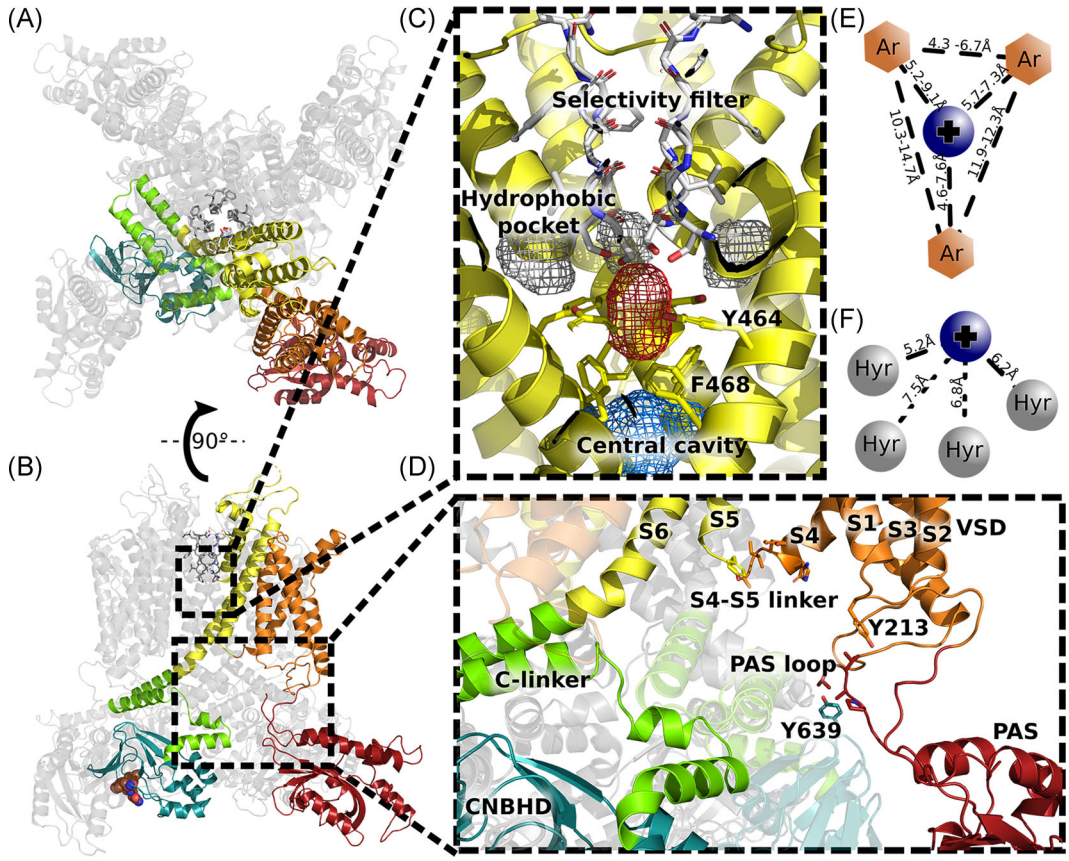


FIGURE 1 Structure of Eag1 (PDB: 5k7l). (A) Top view with one subunit colored: PAS domain in red, VSD in orange, PD in yellow, SF in gray, C-linker in green, and CNBHD in blue. (B) Side view with intrinsic ligand represented in brown spheres. (C) Hydrophobic pockets in gray mesh and central cavity in blue mesh and red mesh below SF. The red mesh of the central cavity represents its more negative electrostatic potential. (D) PAS loop without the first nine residues oriented toward the S4–S5 linker; residues important for PAS loop interactions are represented with sticks. (E) Cavalli et al.'s[38] HERG pharmacophore model. (F) Ekins et al.'s[39] HERG pharmacophore model. CNBHD, cyclic-nucleotide binding homology domain; PAS, Per–Arnt–Sim domain; PD, pore domain; SF, selectivity filter; VSD, voltage sensor domain [Color figure can be viewed at wileyonlinelibrary.com]

location varies highly only with side chains of residues F656 (HERG) or F468 (Eag1).[36,37] Whether or not the hydrophobic pocket remains closed and what is the orientation of F468 in Eag1 when the channel is in the open conformation are still unanswered questions. Yet, the existence of many common blockers and the effects of F468 mutations allow predicting that the pockets will be practicable also in the open Eag1.[40]

In addition, recent studies highlighted that the cytoplasmic domains (PAS, C-linker, and CNBHD) are also tightly involved in the modulation of *KCNH* channels gating (reviewed in Barros et al.[30]). These domains are closely bundled together (in a domain-swapped fashion) just below the VSD in the proximity of S4–S5 linker[36,37] (Figure 1D). The displacement of S4 upon depolarization would allow interaction of the N-terminal PAS loop (residues 1–9 of Eag1) with the C-terminus of S4 (Asp 342), stabilizing the depolarized conformation of the VSD, and at the same time interfering with the interaction between the C-terminus of the PAS loop (residues 10–13) and two tyrosine residues, one just below S1 (Tyr 213) and in the CNBHD (Tyr 639). These interactions would play mainly a regulatory role of gating since an Eag1 channel where most of the cytoplasmic N- and C-termini have been deleted is still able to gate in a voltage-dependent manner.

The CNBHD does not bind cyclic nucleotide as the cyclic nucleotide-binding domain of other channels. Instead, the binding site hosts the side chains of residues Y699 and L701 (called the "intrinsic" ligand) that imitate the binding of cyclic nucleotides (Figure 1D). In the closed Eag1 and open HERG structures, the intrinsic ligand is in close proximity to the PAS domain of the neighboring subunit, possibly representing the "bound" state.[36,37] At least in ELK channels, binding of the intrinsic ligand appears to be a dynamic process, since a peptide with the same sequence competes for the binding with the native intrinsic ligand. Mutagenesis studies on the intrinsic ligand of Eag1 revealed its importance in promoting activation of the voltage sensor and stabilizing the open pore. However, how the intrinsic ligand communicates with the VSD is still not completely understood.[45,46]

As stated above, the available cryo-EM structures of Eag1[28,32] were determined with calcium–calmodulin (Ca^{2+}–CaM) bound, which overrides the activation of the voltage sensor and induces closure of the gate. Ca^{2+}–-CaM binding induces a rotation of the intracellular domains of the channel,[28] which distorts the structure and makes a comparison between HERG and Eag1 in this area very difficult, since we cannot distinguish what differences are due to Ca^{2+}–CaM and what are true structural differences between the channels. A structure of Eag1 in the absence of inhibition by Ca^{2+}–CaM should allow for such studies, and predicting which areas and residues can allow selective binding to either one of them. It is reasonable to assume, however, that the cytoplasmic domains of both channels are functionally similar, since a chimeric channel carrying the PAS domain, C-linker, and CNBHD of HERG on an Eag1 background results in behavior not massively different from wild type Eag1.[28]

The mature Eag1 is core glycosylated on Asn-388 and complex glycosylated on Asn-406. Proper glycosylation is required for efficient transport to the plasma membrane, but also enzymatic removal of glycosylation causes a large reduction of the peak current, indicating that the presence of sugar moieties impacts the functional properties of Eag1 channels at the plasma membrane.[47] Whether or not this is also the case for its pharmacological properties remains to be investigated.

2.2 | Biology and physiological function

2.2.1 | RNA expression and role

KCNH1 is abundantly expressed in the central nervous system (CNS), while undetectable in most peripheral tissues except testes and adrenal gland.[18,19] In contrast, the expression patterns of the other members of the *KCNH* family are much less restricted. In the CNS, abundant *KCNH1* RNA and Eag1 protein have been described in the olfactory bulb, cerebral cortex, hippocampus, hypothalamus, and cerebellum of adult rats and humans.[48] Potassium channels in general play key roles in many neural functions, such as regulating the resting membrane potential, generation and shaping of action potentials, regulation of spiking frequency, modulation of backpropagation in dendrites, control of synaptic potentials, and their integration in dendrites.[48] Voltage-dependent potassium channels of the KCNH family on the other hand play roles in cardiac repolarization, cellular proliferation, tumor growth, and cellular excitability. More specifically, membrane depolarization causes the opening of these channels and conduction of potassium ions outside the cell, resulting in repolarization and hyperpolarization of the membrane.[49] The physiological role of Eag1 in mammalian neurons remained elusive for a long time. Knockout mouse models indicated that Eag1 is not crucial for development in mice.[50] Animals expressing a truncated form of Eag1 that lacks the transmembrane domains and cannot act as an ion channel showed no detectable anatomical or histological changes in the brain, nor alterations in lifespan, reproduction, or development. The fetal human brain does not express detectable RNA,[18] and also in rodents, expression starts only at birth and rises until adulthood. In zebrafish, however, Eag1 knockout disrupts early embryonic development and causes early mortality.[51] This striking difference could be explained by the fact that the knockout mice still conserve the intracellular domains of the channel. There are at least four *KCNH1* transcripts generated by alternative splicing in mammals.[52] The full-length Eag1a is the most common splice variant and differs from Eag1b by 81 base pairs, resulting in a shorter exon 6. Both isoforms have similar electrophysiological properties.[53] E70 is a variant that can also be found in the normal brain

but lacks all transmembrane segments and a part of the PAS domain. E65, another splice variant, can be found in neuroblastoma and melanoma cells and also lacks all transmembrane segments and a part of the PAS domain. None of the variants can form an ion channel, but both can interact with Eag1a, reducing the whole-cell current. In the absence of Eag1a, E65 can activate cyclin-dependent kinase 1 when expressed in *Xenopus* oocytes. In knockout mice, both short splice variants could still be present.

Along with the lack of anatomical changes, knockout mice showed only mild hyperactivity and longer-lasting haloperidol-induced-catalepsy. In the parallel fiber-Purkinje cell synapse of Eag1 knockout mice's cerebellum, repetitive stimulation caused accumulation of calcium and consequent increase in a synaptic release that resulted in robust potentiation.[54] Therefore, the physiological function of Eag1 would be to limit excitability at high frequencies of stimulation in mature neurons. This function would fit the peculiar electrophysiological properties of the channel (see below) and explain the hyperexcitability in behavioral experiments.[55]

Besides its expression in the CNS, Eag1 is also to a limited extent- expressed in peripheral tissues, such as myoblasts, the female and male reproductive system, and the adrenal gland. In myoblasts, Eag1 is transiently expressed during differentiation. Myoblasts are precursor cells that will fuse during myogenesis to form adult muscle cells. Myoblast fusion is a complex process involving cell-cycle withdrawal, cell–cell interactions, adhesion, alignment, and final membrane fusion to form the multinucleated skeletal muscle fiber.[56] There is little or no Eag1 expression in undifferentiated myoblasts, but the expression is activated and reaches a peak immediately before fusion.[57] Activation of Eag1 current coincides with the cell-cycle arrest.[56] Eag1 can also be detected in the female reproductive system, specifically in epithelial cells in the endocervix and endometrium, particularly in secretory activated endometrial glands. In the normal human placenta, Eag1 was found to be strongly expressed in syncytiotrophoblast and the vascular endothelium. Similar to its transient expression in myoblasts, also here Eag1 is likely involved in the proliferation and/or fusion of trophoblasts.[18,19,58] As described above, Eag1 can be detected in the female reproductive system, more specifically in epithelial cells in the endocervix and endometrium. During pregnancy, the cervix undergoes significant remodeling by an increase of proliferation and decrease of apoptosis. Ramírez et al.[59] reported the presence of Eag1 in the cervical cells of 100% of the tested pregnant women and 26% of the tested nonpregnant women. As human pregnancy provides an estrogen and progesterone-enriched environment for cervical cells, this indicates a possible regulation of Eag1 by these hormones.

Besides expression in myoblasts and the female reproductive system, Eag1 was also detected in the adrenal gland and the male reproductive system, specifically in the spermatogenic cells.[19] Finally, Eag1 was also detected in mesenchymal stem cells. A reduction of adipogenesis and osteogenesis was observed in human mesenchymal stem cells infected with *KCNH1* short hairpin RNA, suggesting that Eag1 participates in the regulation of adipogenic and osteogenic differentiation and might be important in maintaining bone marrow physiology.[49,60]

The limited detection outside of the CNS can be due to the channel's tightly controlled time of expression. All cases described in the last paragraph correspond to cellular events restricted in time and closely related to cell-cycle control. Other normal cells show the channel's expression, but only during a limited period through the G2 and M phases of the cell cycle.[61] The master regulator of cell cycle progression, the transcription factor E2F1, binds to the promoter of KCNH1 and induces its expression at the start of G2. Quiescent cells or cells in G1 or S are the vast majority of cells in tissues, and therefore the channel is not detected at the tissue level, but it can be demonstrated in cells rapidly proliferating (like in the colon crypts) or arrested in G2 (such as secondary spermatogonia). For example, this temporal regulation is lost in tumor cells, which exhibit sustained expression (although oscillating quantitatively along the cell cycle).[61]

2.2.2 | Biophysical properties of Eag1

Eag1 is electrophysiologically characterized by a slow voltage-dependent activation which results in apparently noninactivating currents.[55,62] The activation properties of Eag1 are reminiscent of the Cole-Moore shift observed in for instance squid axons[63]; although the original description probably corresponds to a different phenomenon,[64]

the term Cole–Moore shift is still the usual one in the field for describing the activation behavior of Eag1. Upon mild depolarizations, Eag1 activates slowly. However, the time constant of activation is strikingly dependent on the prepulse potential in which a more depolarized membrane potential gives rise to a faster Eag1 activation. The activation process of most K_V channels displays such a holding potential dependency of the activation rate, but in other channels what is observed is a shorter delay to activation, rather than faster kinetics. Notwithstanding, the orders of magnitude change in activation rate for Eag1 is so distinctive that it allows characterizing this channel in native preparations.[18,65] For the family of K_V channels, gating is the resultant of a sequence of structural changes in response to depolarization. In Eag1, these conformational changes do not require a direct connection between the pore domain and the voltage sensors.[29] Indeed, a range of voltage-dependent transitions between non-conducting channel states contributes to the activation characteristics of Eag1. Individual subunits independently influence the time constant of activation for this channel.[66]

The distinct electrophysiological properties of Eag1 provide an understanding of the physiological function of Eag1 in neurons. Although the specific function of Eag1 has been unknown for a long time, it is now clear that this channel plays an important role in the control of neuronal excitability. A single action potential is insufficient to activate the Eag1 channel since it requires profound depolarizing potentials over an extended time. Despite this, as described above, this first single action potential will function as a prepulse and does alter the activation rate for the subsequent stimulus.[54] Thus, repetitive stimuli enhance the speed of activation and as such precondition Eag1 closer to activation, eventually resulting in channel activation. Eag1 hereby counterbalances the depolarization since the Eag1-mediated potassium flux will contribute to hyperpolarization of the neurons. This indicates that Eag1 functions as a regulator for neuronal hyperexcitability during high-frequency presynaptic transmission but remains silent at lower frequencies.[54,62]

2.2.3 | Endogenous regulation Eag1 channels/signaling pathways

Many factors regulate Eag1, its trafficking, and its involvement in signaling pathways. Some of such interactions are specific for Eag1 and have not been described for HERG, opening a possibility for differential intervention.

The best known and probably most relevant Eag1 interactor is *Calmodulin* (CaM), a highly conserved soluble cytoplasmic protein that translates the rise of intracellular Ca^{2+} into a physiological response by modulation of a plethora of target proteins. CaM consists of two N- and C- terminal Ca^{2+}-binding lobes connected by a flexible helix. The flexibility of the helix allows it to adopt different conformations that depend on whether CaM is bound to Ca^{2+} or bound to the target protein. The rise of intracellular Ca^{2+} above basal levels markedly reduces Eag1 current[67] through CaM,[68] which binds to Eag1 via three sites, one binding domain at the N-terminus (BD-N) and two binding domains at the C-terminus (BD-C1 and BD-C2). Mutations of the three sites disrupt inhibition in the presence of high Ca^{2+}.[68–71] In in vitro binding experiments, BD-C2 and BD-N are more crucial for calmodulin binding to Eag1, while BD-C1 is more dispensable.[69,71] BD-N lies after the PAS domain, while BD-C1 & BD-C2 are located after the CNBHD. The available cryo-EM structure of Eag1 includes bound CaM and shows the intricacy of the interaction.[37] (Figure 2). Four CaM molecules form a complex with the ion-conducting subunits, in which the N-lobe of CaM interacts with BD-N of one subunit, and the C-lobe is positioned close to BD-C2 from the opposite subunit. All three binding sites for CaM are located in the proximity of the intracellular domains that form a ring below the gate of the channel, formed by the four N-and C-termini, which interact with each other in a "domain-swapped" fashion. The N-terminal PAS domain of one subunit interacts with the C-terminus of the neighboring subunit, and this interaction determines the gating behavior of the channels. Therefore, in the presence of CaM, the intracellular domains of three of the four ion-conducting subunits interact with each other either directly or through CaM. Ca^{2+}–CaM Calmodulin cross-bridges opposite subunits, pulling together the PAS domain and the CNBHD. This induces a rotation in the C-linker and S6, closing the pore and obstructing the potassium flow, and locks the channel in a closed state. Deletions in the intracellular domains of Eag1 either abolish the inhibition or

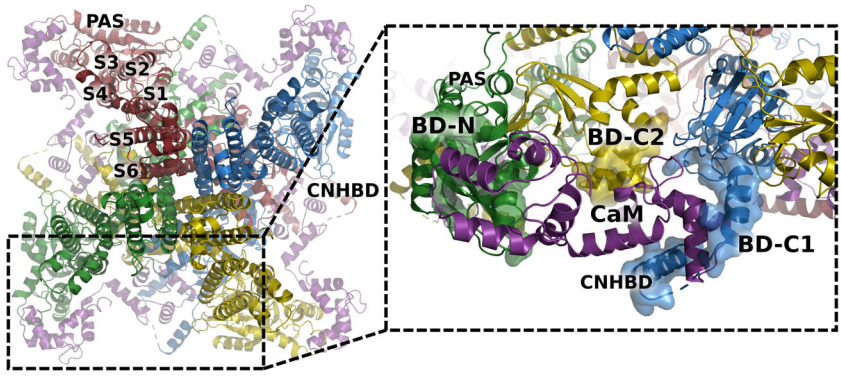


FIGURE 2 CaM interaction with Eag1 (viewed from the cytoplasmic side). Calmodulin interacts at the boundary between Eag1 subunits with the N-terminus of one subunit (green) and the C-terminus of the opposing subunit (blue). Notice that the intersubunit interaction occurs between the N-terminus of one and the C-terminus of the adjacent subunit (yellow). CNBHD, cyclic-nucleotide binding homology domain [Color figure can be viewed at wileyonlinelibrary.com]

even result in a potentiation of the current in response to Ca^{2+}.[72] The inhibition of Eag1 current can act as a positive or negative feedback signal. In neurons, the rise of Ca^{2+} inhibits K^+ efflux through Eag1, hence maintaining the depolarized signal at the presynaptic membrane.[73] In cancer cells, Eag1 promotes the entry of Ca^{2+} through Orai1 to support cell survival, while the rise of Ca^{2+} inhibits Eag1 in a self-terminating feedback loop.[62]

The interaction of 14-3-3Θ with Eag1 is reminiscent of that of CaM. It happens through both the N- and C-termini (the PAS domain and the CNBHD, respectively).14-3-3Θ suppresses Eag1 currents without altering protein expression, surface trafficking, voltage dependence, gating kinetics or single-channel conductance.[74] 14-3-3 isoforms also interact with HERG. It prevents internalization by protecting protein kinase A phosphorylation sites from dephosphorylation, and thereby enhanced HERG current.[75]

K^+ channel regulator 1 (KCR1) is a membrane protein believed to constitute a regulatory subunit of Eag1, as its C-terminus interacts with the C-terminus of the rat Eag1. This association leads to an increase in Eag1 expression and facilitation of the channel activation.[76] KCR1 also shows functional interaction with HERG, though not by direct interaction but by control of glycosylation (KCR1 is an α-1,2-glucoystrnaferase).[77] Activation of mitosis-promoting factor (MPF), a complex of the regulatory cyclin B and catalytic subunit $p34^{cdc2}$ in *Xenopus* oocytes induces a reduction of Eag1 current expressed in oocytes. Still, no direct interaction has been documented.[78]

The expression of Eag1 at the plasma membrane is very dynamic, with an internalization rate of 2%/min.[79] Several proteins participate in the trafficking of Eag1. Epsin, a protein present in presynaptic nerve terminals and involved in endocytosis and cell cycle progression, binds directly to Eag1 and slows down the opening of the channel, resulting in a decreased open probability.[80] Rabaptin-5, another binding partner of Eag1 that interacts with Rab5 and Rab4, which are involved in early endosome fusion and membrane recycling respectively, participates in the recycling rates of Eag1 and maintaining Eag1 current density, suggesting a role for Rabaptin-5 as a molecular bridge between endocytosis and recycling.[81] Golgi-Associated PDZ And Coiled-Coil Motif-Containing Protein (GOPC) interacts with the C-terminus of Eag1 and leads to an increase in surface expression of Eag1 without influencing its electrophysiological properties. GOPC could act as a molecular switch determining the fate of the channel after internalization in early endosomes, by increasing the recycling rate and leading to a net increase in membrane expression.[82] Cortactin (CTTN) interacts with the C-terminus of Eag1 and increases Eag1 current, without modifying the electrophysiological properties of the channel. The loss of CTTN reduces the abundance of Eag1 at the plasma membrane. Its overexpression does not increase the Eag1 current, indicating that CTTN is involved in channel endocytosis and not in transport to the membrane. Actin cytoskeleton remodeling is

essential for endocytosis; CTTN interacts with the actin cytoskeleton and plays a crucial role in endocytosis. Therefore, it is hypothesized that the presence of CTTN stabilizes Eag1 on the membrane, while its absence reduces the membrane residence by increasing Eag1 internalization.[83] To our knowledge, none of these factors show direct interaction with HERG, which appears to follow a different trafficking pathway towards the plasma membrane. Many nonprotein factors are reportedly implicated in the regulation of Eag1.

Arachidonic acid interacts directly with Eag1 and increases current and causes a shift in the activation curve to the left. As polyunsaturated fatty acids are the second messengers involved in many cell regulation processes, arachidonic acid could contribute to the involvement of Eag1 in neuronal signaling and excitability.[84] Arachidonic acid is in contrast a HERG blocker.[85] Phosphatidylinositol 4,5-bisphosphate (PIP_2), an important structural cofactor in many membrane proteins, is the precursor for diacylglycerol and inositol 1,4,5-triphosphate and is involved in cellular proliferation and neurological diseases. Endogenous PIP_2 in the plasma membrane causes an increase in Eag1 channel activity. Exogenous PIP_2, however, strongly inhibits Eag1 and causes a shift of the activation to more positive potentials.[86] PIP_2 is thought to bind directly to the N-terminus of Eag1 at a site overlapping CaM BD-N, suggesting a possible interaction between CaM and PIP_2.[87] PIP2 also interacts with HERG, accelerating its deactivation through interaction with the C-terminal domains.[88,89]

Extracellular magnesium, among other divalent cations, causes a long delay in Eag1 activation.[90,91] Mg^{2+} sits in a pocket between the S2 and S4 segments and would interfere with the voltage-induced rearrangement of both segments, slowing down activation.[92] Low pH also slows down the activation of the channel and competes with magnesium,[90] suggesting that magnesium binding sites need to be deprotonated to become accessible for magnesium. The voltage-and pH-dependence of the magnesium effect on Eag1 may constitute an important mechanism for neuronal regulation; more specifically, it could modulate firing frequencies by shaping the action potential.[90] Both extracellular divalent cations[93,94] and low pH[95] show also an inhibitory effect on HERG.

Reactive oxygen species (ROS) during oxidative stress can inhibit Eag1 currents by cysteine oxidation both at the N-terminus, resulting in a higher threshold of the activation, and at the C-linker with a slower time course, abolishing channel function.[96] ROS also modulate HERG currents, although this action is mechanistically less clear.[97]

2.3 | Pathophysiology

The conservation of Eag1 throughout the animal kingdom,[98] including species that do not have a nervous system, suggested that Eag1 must also serve functions less specialized than the modulation of electrical excitability. However, full knockout of the transmembrane domains of the channel did not result in detectable phenotypic alterations,[50] and its expression was not detectable in peripheral tissues.[18] There are no reports of loss of function mutations in the *KCNH1* gene that correlate to pathological conditions. In contrast, several reports in recent years established a causal relation between a gain of function mutations that originate a shift in the activation potential of Eag1 towards more hyperpolarized values and congenital diseases (Temple–Bareitser [TB] and Zimmermann–Laband [ZL] syndromes) characterized by coarse face appearance, hypertrichosis, nail, and skeletal alterations, seizures, and intellectual disability.[99–103] Although the neurological symptoms are compatible with the role of Eag1 in the CNS, the developmental alterations would require the role of Eag1 in development, but *KCNH1* is not detectable in fetal RNA. This apparent contradiction obeys to the mentioned fact that the expression of Eag1 is restricted to a short period in the cell cycle of somatic cells.[61] E2F1, one of the main transcription factors orchestrating cell division, binds to the promoter of the channel and induces its expression at the beginning of the G2 phase of the cycle. The expression is maintained during the rest of the cell division, after which the channel is rapidly downregulated.[61] Hence, at any given time, only cells in G2/M phases will display mRNA for KCNH1, which will then be diluted in the bulk of G0/G1 cells and, therefore, not detectable. The expression of Eag1 at this specific cell cycle phase impacts many cellular events.

Loss of this regulation mechanism would result in sustained expression of Eag1, which is a common finding in tumor cells. The central control factor, E2F1, is under the control of the tumor suppressors p53 and pRb, which are frequently downregulated in tumor cells. Overexpression of Eag1 has been detected in all studied tumor types, except for chronic lymphoid leukemia and glioblastoma, which poses a problematic interpretation because of the high level of expression in the normal nervous tissue.[19,104–117] Therefore, Eag1 can be considered an almost universal tumor marker and has been used to target therapeutic agents to tumor cells, preserving the surrounding normal tissue.[118–120] Moreover, tumor cells expressing Eag1 obtain a selective advantage and become rapidly dependent on the channel to proliferate and survive, in a mechanism similar to the oncogene addiction described for classical oncogenes. This has two implications: On the one hand, patients with tumors that express Eag1 show in many cases shorter survival, like documented for fibrosarcoma,[117] ovary carcinoma,[121] glioblastoma,[116] acute lymphoid leukemia,[108] gastric,[105] head and neck,[111] or colon cancers.[122] On the other hand, removing the selective advantage through inhibition of Eag1 reduces tumor progression in vitro and in animal models.[20,112,113,123–131]

Increased (or extemporaneous) expression of Eag1 results in faster proliferation that can be explained by several mechanisms that might coexist in the same cell. More complexity arises from the fact that the properties of the channel heterologously expressed in cell models change during the cell cycle. Cells that are not in the M phase show less ionic selectivity, which translates in permeability to Cs^+ ions (a classical blocker of K^+ channels) and more physiologically in a block by intracellular Na in cells in the M phase.

Excessive Eag1 activity would cause hyperpolarization of the membrane potential. The membrane potential does influence cell cycle progression. Fast proliferating cells such as cancer cells are, in general, more depolarized, while cells with a very hyperpolarized resting membrane potential (such as neurons) show no mitotic activity. Inhibition of DNA synthesis can be experimentally induced by hyperpolarization of the membrane potential of Chinese hamster ovary cells. The opposite is also true: causing depolarization in cells leads to the initiation of DNA synthesis.[132] Changes in potential during cell cycle progression are much slower, more gradual, and smaller than the fast action potential.

As a result of membrane hyperpolarization, the driving force for calcium entry would increase. Ca^{2+} is a major second messenger involved in cellular functions such as proliferation, migration, survival, and apoptosis.[133] The complexity of the Ca^{2+} signaling pathways allows every individual cell to exploit Ca^{2+} in a unique way to perform its specific function. Depletion of extracellular Ca^{2+} arrests cells at early G1 phase and G1/S phase transition. However, cancer cells can circumvent this requirement and can keep proliferating in Ca^{2+}-deficient media. The depletion of Ca^{2+} in the endoplasmic reticulum (ER) leads to cell division arrest. This inhibition of DNA synthesis, protein synthesis, and nuclear transport, causes an accumulation of cells in the quiescent state. Normal cells are thus dependent on both intracellular and extracellular calcium for their proliferation, especially during the G1 phase.[134–136] Recent evidence supports a mechanism implying Ca^{2+} homeostasis in cells expressing Eag1. The channel forms part of a complex where the calcium channel Orai1 and the calcium pump SPCA2 play pivotal roles.[137–139] Inhibition of the complex (through blockade of Orai1 or Eag1) results in a change in the frequency of calcium oscillations and decreased microtubule dynamics. Knockdown of Eag1, in contrast, changes the amplitude of calcium peaks, leaving the frequency unaltered, and enhances microtubule dynamics. In both cases, cell functions dependent on microtubule growth will be altered. Cell division or primary cilium homeostasis belongs to this group of functions, among many others, explaining the pleiotropy of Eag1 impact on cell physiology. The exact effect of Eag1 inhibition or knockdown will depend on the moment in the life of a cell and the exact location of the population of channels inhibited.

The alteration of Ca^{2+} and microtubule homeostasis is likely responsible for the observation that proper resorption of the primary cilium in dividing cells requires Eag1.[140] The primary cilium is a microtubule-based structure expressed by essentially all nonactively dividing vertebrate cells that act as a signaling hub that concentrates spatially factors implicated in relevant pathways such as TGF, SHH, or Wnt signaling.[141] The cilium is

disassembled[141] or shed[142] at the initiation of the cell cycle, to allow its basal body (the mother centriole) to nucleate the formation of the mitotic spindle. Knockdown or inhibition of Eag1 results in delayed ciliary resorption, while overexpression of the channel impairs ciliary formation after division.[140] These opposite effects do not respond to altering the same pathway in opposite directions, but rather to two different mechanisms. While the effect on ciliary resorption depends on K^+ permeation and requires ciliary localization, the impact on reciliation occurs in the absence of permeation and is even induced by constructs lacking transmembrane segments and, therefore, incapable of ion permeation.[140] This apparent contradiction could be explained if Eag1 forms part of a protein array where the channels participate in both the maintenance of the structure and the proper function of the complex. In such a scenario, knockdown of the protein would affect both functional aspects, while inhibition would change the function, but not the structure of the complex. It is not uncommon that ion channels show functions that do not require ion permeation.

In some cases, the voltage-dependent conformation changes of the channel are still necessary for the effects, while permeation itself is not. A relevant example of this phenomenon is the interaction of HERG with β1 integrin and its influence on cell adhesion and migration.[143] For the case of Eag1, nonconducting mutants expressed in heterologous systems retain the ability to produce an increase in proliferation in vitro and tumor progression in vivo, although to a lesser extent than the wild type. In the *Drosophila eag* channel, nonconducting mutants can stimulate DNA synthesis through an increase in p38 MAPK. Interestingly, a nonconducting mutant in the open conformation did not stimulate proliferation, meaning that proliferation could only be affected in a closed conformation of the channel. As described above for HERG, *Drosophila eag* channels use their voltage sensor to activate the MAPK signaling pathway. That signaling activity is linked to channel conformation, determined by the position of the voltage sensor and independent of the ion flux.[144]

Eag1 might also influence volume regulation, which is essential for the cell cycle. A dividing cell increases its volume during interphase. Subsequently, it shows a large, fast, and regulated decrease in cell volume during the transition from prophase to metaphase, in a process termed pre-mitotic condensation.[145,146] After the metaphase, the cell volume increases again, followed by cytokinesis.[147] In most cell types, these changes in cell volume correlate with volume-dependent chloride channels that allow the flow of chloride followed by water. The activity of potassium channels compensates for the charge disbalance generated.[147] Although we are not aware of any studies that have documented the role of Eag1 in volume regulation, medulloblastoma cells the close relative Eag2 during mitosis, and its downregulation precludes the cell volume reduction and induces mitotic catastrophe.[148] The possibility that Eag1 has a similar role in other cell types deserves further study. Eag1 also favors tumor progression through increased resistance to hypoxia and angiogenesis,[21] ROS signaling,[149] and by augmented cell migration,[150] but these effects are mechanistically less clear.

Due to its action on primary cilium homeostasis, overexpression of Eag1 during development would result in a phenotypic defect resembling a ciliopathy, which is compatible with the rough facies, hypertelorism, and defects in the digits that define ZL and TB syndromes. Alterations of cell proliferation match well with the gingival hypertrophy that defines ZL, as well as the hyperproliferative features of cancerous cells. However, in cancer, the alterations probably also obey to a loss of the time control of Eag1 expression because sustained expression of wild-type channels is sufficient to favor tumor progression. There is not enough data about cancer incidence in TB and ZL patients, partly because of the patients' reduced life span, attributable to the epileptic phenotype. The physiology and pathophysiology of Eag1 are summarized in Figure 3.

In summary, if Eag1 function is increased during development in somatic cells, the result is a ciliopathy-like phenotype. If there is sustained expression of the channel in time, due to promoter alterations or transcription changes secondary to alteration of p53, Rb, E2F1, miR34a,[151] and miR 296-3p,[113] this will offer a selective advantage to cancer cells. We have no evidence of the transforming activity of Eag1 on its own. The large number of factors that participate in the timely control of Eag1 explains the extraordinary incidence of Eag1 overexpression in human cancer.

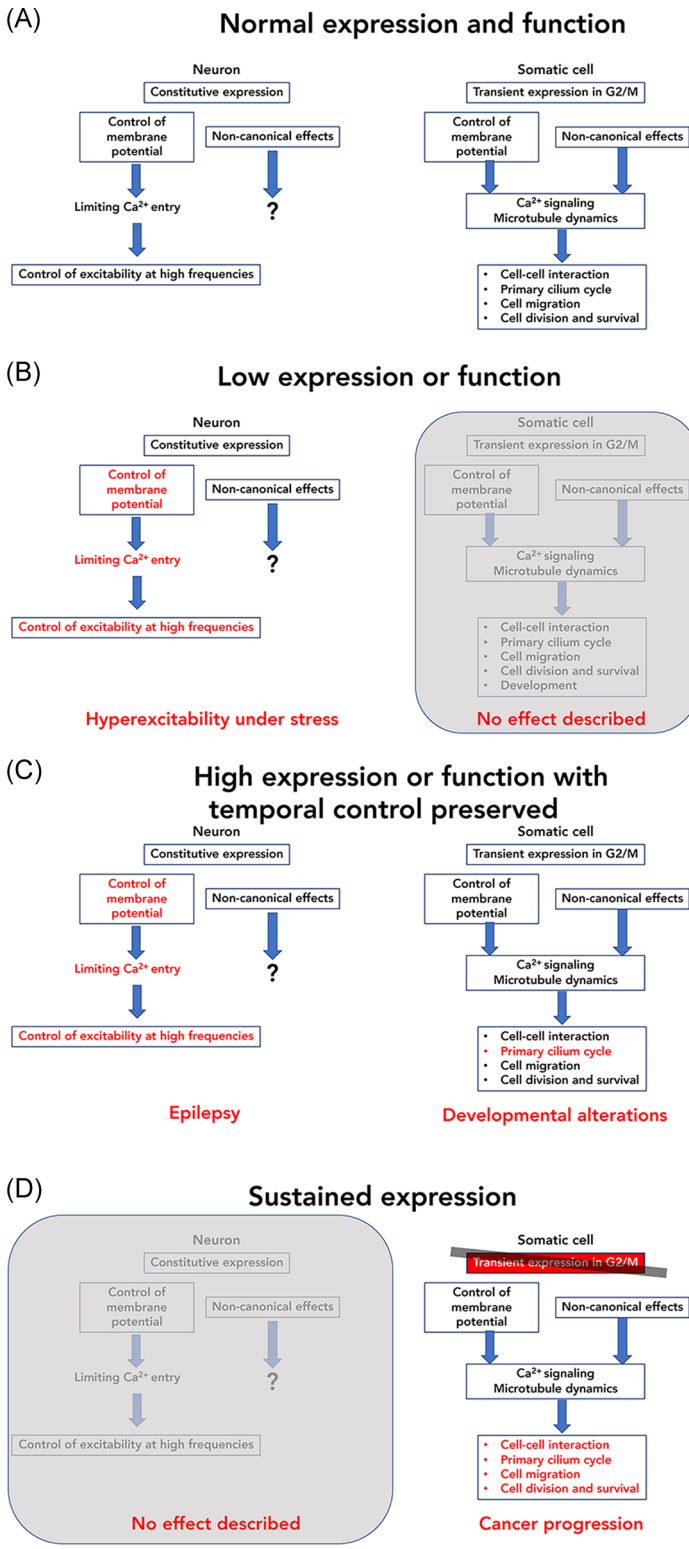


FIGURE 3 (See caption on next page)

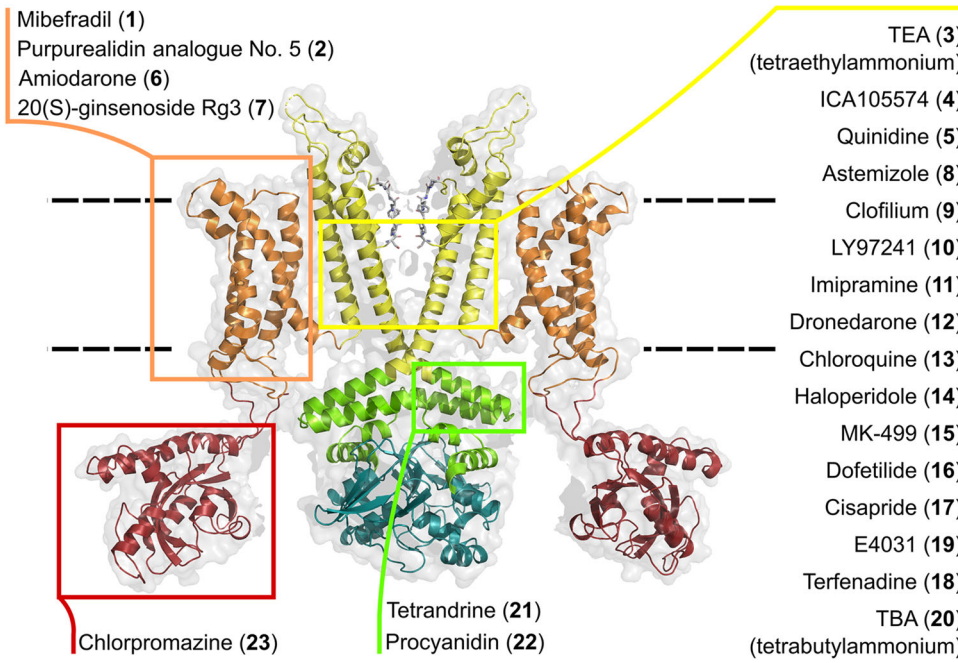


FIGURE 4 Proposed binding sites for the known Eag1 modulators. Dimeric structure of Eag1 (K_V10.1) (PDB code: 5K7L), representing two opposite subunits: PAS domain colored in red, voltage sensor domain (S1–S4) in orange, pore domain (S5–S6) in yellow with selectivity filter in gray, C-linker in green and CNBHD in blue. CNBHD, cyclic-nucleotide binding homology domain; PAS, (Per-Arnt-Sim) domain; PDB, Protein Data Bank [Color figure can be viewed at wileyonlinelibrary.com]

3 | MODULATION OF EAG1

3.1 | Small molecule inhibitors of Eag1

The discovery of Eag1 inhibitors often responds to testing on Eag1 the HERG inhibitors' activity because HERG is one of the most studied off-targets in drug discovery. The pool of compounds tested on HERG consist of more than 16,000 unique Molecular Chembl ID entries, many more than the number of compounds that were tested on Eag1.[152] Some of the HERG-active drugs that showed anticancer activity were then tested on Eag1 because Eag1 is ectopically expressed in many solid tumors.[153]

Small molecules can interact with the Eag1 channel in several different ways (Figure 4). The VSD has binding sites for small molecules that act as gating modifiers (molecules **1, 2, 4, 6**, and **7**, Figure 4). Accessibility of the VSD from the cell's outer side allows molecules to modulate the channel without crossing the membrane, like toxins do.[154–157] Most of the known small molecules that modulate Eag1 activity first cross the cell membrane and bind to the intracellular side of the channel. A majority of those compounds are pore blockers that physically occlude

FIGURE 3 Schematic depiction of the physiological roles of Eag and the pathological consequences of its altered expression and/or function. (A) Normal physiology. (B) Reduction of Eag1 expression or function. (C) Normal periodic expression of an Eag1 form with increased activity. (D) Loss of periodicity in Eag1 expression [Color figure can be viewed at wileyonlinelibrary.com]

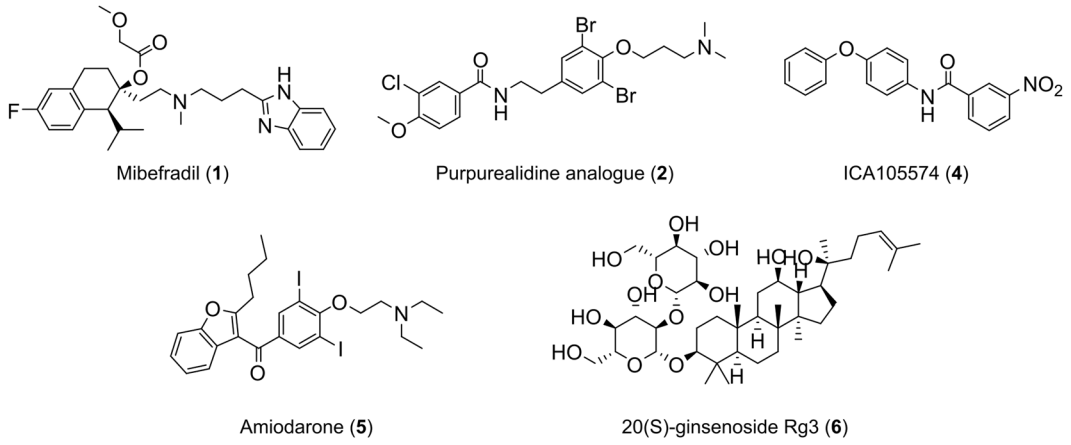


FIGURE 5 Structures of gating modifiers

the ion permeation pathway (molecules **3, 5, 8–20**, Figure 4). Recent cryo-EM studies of the channel have highlighted the importance of the intracellular domains in voltage-dependent gating kinetics, especially the interface between the intracellular part of the VSD, PAS domain, and CNBH domain[32,37]; PAS domain and C-linker were recently identified as targets of small molecules as allosteric modulators (compounds **21–23**, Figure 4).[131,158,159]

3.1.1 | Gating modifiers

Gating-modifiers (**1–5**, Figure 5) mostly change the kinetics of the channel activation, inactivation, or deactivation by binding to the PD or VSD of the channel (Figure 4). It is believed that the gating modifiers mibefradil (**1**) and purpurealidin analog no. 5 (**2**) bind to the outer part of the channel in a similar mode as peptide toxins (see below).[156,159–161]

Potassium channels show several types of inactivation, such as N-, P-, U-, and C-type.[162] C-type inactivation is a consequence of conformational changes in the selectivity filter and is slowed down by high extracellular concentration of potassium ions or tetraethylammonium (TEA) (**3**); Eag1 does not show such a phenomenon.[163–167] ICA-105574 (**4**, Figure 5 and Table 1), which acts as an activator of HERG, produces a slow voltage-dependent inactivation of Eag1, reminiscent of C-type inactivation.[164,168] ICA-105574 is the only compound reported that has opposite effects on HERG and Eag1 channels. It enhances HERG current by slowing down the rapid voltage-dependent C-type inactivation and inhibits Eag1 through C-type inactivation. A similar inactivation was found in the Eag1 (Y464A) mutant, and this effect was further increased by ICA-105574,[164] indicating separate mechanisms.

Mutational and molecular dynamics (MD) studies in both channels indicate that the binding site for ICA-105574 (**4**) is located close to the selectivity filter between the S5 and S6 segments.[172,176] The residues important for ICA-105574 binding in Eag1 are F359, L434, and Y464 in all four subunits (Figure 6). The mutations F359A and L434A/C, which produced an apparently functionally unaltered Eag1 channel, reduced the efficacy ICA-105574 (**4**). The mutant Eag1 F359L showed prominent inactivation, and ICA-105574 (**4**) acts as an activator. The triple mutation M431F/M458L/L463M of the binding site of Eag1 to mimic the sequence of the HERG binding pocket still retained the biophysical properties of the wild-type channel as well as inhibition with ICA-105574 (**4**). Therefore, the opposite effect of ICA-105574 (**4**) on Eag1 and HERG was not a consequence of the different binding sites, but of the differences in the intrinsic speed of inactivation gating.[172] In HERG channels, all four binding sites have to be occupied for maximal agonistic effect, due to cooperative subunit interactions and not to

TABLE 1 Potencies of Eag1 gating modifiers and selectivity against the HERG channels

Compound	Test system and activity on EAG1	Test system and activity on HERG
Mibefradil (**1**)	HEK293; K_d = 1.3 μM, nH = 0.8[155]	COS-7; EC_{50} = 1.4 μM[169]
Purpurealidine analog no. 5 (**2**)	*Xenopus* oocytes; IC_{50} = 7.7 ± 1.0 μM[170]	*Xenopus* oocytes; 57.6 ± 3.4%[171]
ICA105574 (**4**)	*Xenopus* oocytes; IC_{50} = 0.4 ± 0.03 μM[172]	HEK293; EC_{50} = 0.5 ± 0.1 μM[168] (activator)
Amiodarone (**5**)	HEK293; K_d = 203 nM, nH = 0.9[154]	Rabbit ventricular myocytes; IC_{50} = 2.8 μM, nH = 0.9[173] *Xenopus* oocytes; IC_{50} = 9.8 μM[174] HEK293; IC_{50} = 0.2 μM[175]
Ginsenoside 20(S)-Rg3 (**6**)	*Xenopus* oocytes; EC_{50} = 1.3 ± 0.2 μM, nH = 0.7 ± 0.1[157] (activator)	*Xenopus* oocytes; EC_{50} = 414 ± 49 nM, nH = 1.7 ± 0.1[157] (activator)

Note: Unless otherwise indicated, oocytes were recorded using two-electrode voltage clamp, and mammalian cells in whole-cell patch clamp.

Abbreviations: CHO, Chinese hamster ovary cell line; COS-7, CV-1 in origin with SV40 genes cell lines; HEK293, human embryonic kidney 293 cells; nH, Hill coefficient.

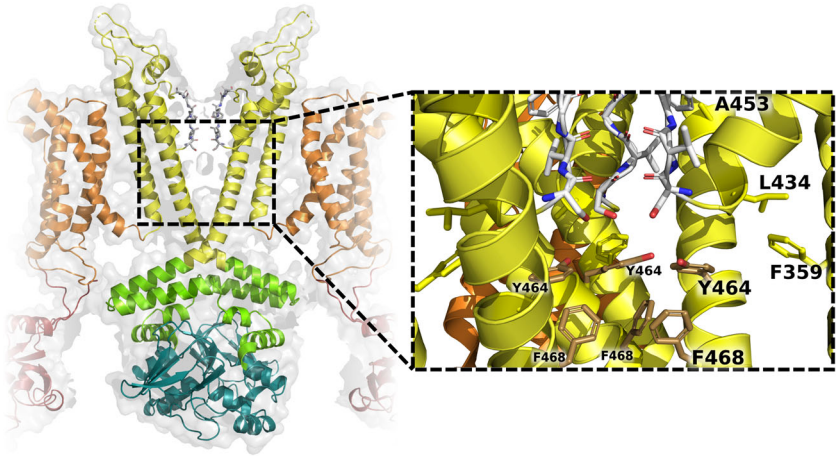


FIGURE 6 Binding site of pore blockers. Residues important for binding of different compounds are shown in sticks. Residues in light gray represent the lower part of the selectivity filter that also form interactions with some blockers [Color figure can be viewed at wileyonlinelibrary.com]

positive cooperativity of ICA-105574 (**4**) binding to multiple sites.[177] Recently, a structure–activity relationship (SAR) and MD study of ICA-105574 (**4**) activation of HERG channel suggested that ICA-105574 (**4**) does not influence the K^+ occupancy in the selectivity filter, and it does not fit into the negatively charged activator pharmacophore proposed by Schwew et al.[178,179] ICA-105574 (**4**) would stabilize F627 in the selectivity filter of HERG through an allosteric mechanism involving M645 in the S6 segment and strengthening of the hydrogen-bond network behind the selectivity, leading to an attenuated C-type inactivation. For this well-tuned modulation of the selectivity filter, ICA-105574 (**4**) geometry and especially the nitro group are crucial.[179]

Mibefradil (**1**, Figure 4) reportedly modifies the gating of Eag1 by binding to the VSD (Figure 4). Mibefradil (**1**) induced an apparent open-state inactivation when applied to the extracellular side at hyperpolarized potentials, but not from the intracellular side. In contrast to ICA-105574 (**4**), which requires all four binding sites to be occupied for a maximum effect, mibefradil (**1**) binds to the VSDs in an independent and noncooperative manner,[160,177] similarly to divalent ions that also bind to all four VSDs noncooperatively. Mibefradil (**1**) did not alter the kinetics of current deactivation, and recovery from inactivation is affected by increased extracellular concentration of potassium, supporting the idea of inactivation. Moreover, mibefradil (**1**) also inhibited the Cole–Moore shift. It also does not act as an open-pore blocker because it does not compete with quinidine, a known pore blocker (**5**). Mibefradil (**1**) also inhibits other potassium channels as well as L- and T-type calcium channels.[160]

Investigation of marine sponges as a versatile source of natural products led to the discovery of purpurealidin analogs, such as compound **2** (at 10 μM inhibition of Eag1 by 52 ± 3%, Figure 4; Table 1), that exhibited similar potency on Eag1 channels as mibefradil (at 10 μM inhibition of Eag1 by 50 ± 5%) (**1**). The natural alkaloid bromotyrosine purpurealidin J1 (**2a**, Figure 5) produced by Verongida sponges (*Pseudoceratina pupurea*) was a starting point for the synthesis of simplified analogs. Among 27 synthesized analogs, purpurealidin analog **2** was the most potent with an IC_{50} of 7.7 ± 1.0 μM. The introduced modifications are represented in Figure 7. Three parts of the scaffold were modified: the aromatic ring next to the amide (R_1), the substitution of the tyramine ring (R_2) and the basic center (R_3). Amine (R_3) is crucial for the activity because compounds with isopropyl show no activity. Also, only monomethylamines were 1,5-5 times less active than dimethylamines. If bromines are substituted with hydrogens on the tyramine ring, the activity is also completely lost. Most of the phenyl substitutions at R_1 show better activity than 3,5-dimethylisoxazole, but the substitution of phenyl is also important. Double substitutions on *para* (methoxy) and *meta* (halogen) positions are preferred compared to double *meta* or double *meta* and *para*

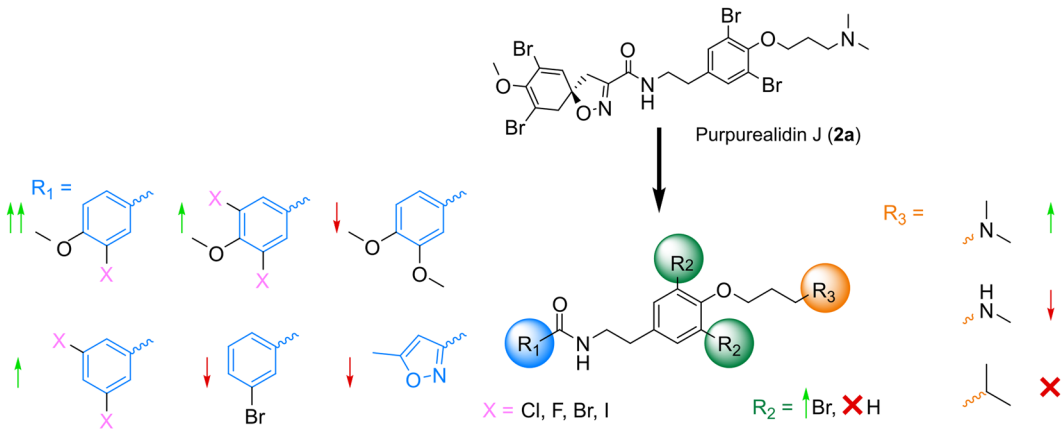


FIGURE 7 Representation of natural alkaloid bromotyrosine purpurealidin J1 (**2a**) and modifications of some synthetic analogs. Arrows represent the activity of the substitution while a cross represents a loss of activity. The most active purpurealidin analog is compound **2** with R_1 = 1-chloro-2-methoxyphenyl, R_2 = Br, R_3 = dimethylamine [Color figure can be viewed at wileyonlinelibrary.com]

substitution. As with mibefradil (**1**), there is an inhibition of the Cole–Moore shift and induction of apparent open state inactivation during prolonged depolarization, and the effect occurs at hyperpolarized potentials. Competition assay with mibefradil (**1**) confirmed that both compounds share the same binding site in the VSD. Purpurealidin analog **2** was tested in different cell lines and inhibited proliferation but also induced cytotoxicity in cell lines not expressing Eag1.[170] The effects of compound **2** in the tested cell lines can, therefore, not only be attributed to the effect on Eag1 but may indicate binding also to other targets.[144,170] A selectivity screening on different voltage-gated ion channels is still missing. However compound **2** was tested on HERG and showed inhibition by 57.6 ± 3.4%.[171]

Among antiarrhythmic drugs (**5**, **9**, **12**, **16**) that inhibit Eag1, amiodarone (**6**, Figure 5) is the only compound that inhibits the Cole–Moore shift. In addition to this effect, amiodarone (**6**) also strongly reduced the peak K^+ current, inhibited potassium current at potentials at which the K^+ permeation pathway is closed, and slowed recovery from hyperpolarized holding potentials with high external K^+ concentration. All those effects are not characteristic of the classical open-pore channel blockers. Therefore, even with the induction of a significant apparent inactivation in a voltage-dependent manner that usually results from a slow open-pore block, it is hypothesized that amiodarone (**6**) binds either to the PAS domain or to the VSD of Eag1.[154] Amiodarone (**6**) was also found to inhibit sodium and calcium channels.[175]

Ginsenoside 20(S)-Rg3 (**7**, Figure 4) is a steroid glycoside that alters the gating of multiple members of the *KCNH* family in submicromolar concentrations. Its action is not limited to K_V channels, since it modified gating of K_V1.4, K_V1.5, KCNQ1/KCNE1, BK_{Ca}, other Na^+, and Ca^{2+} as well as ligand-gated channels at micromolar concentrations. Ginsenoside Rg3 (**7**) induced Eag1, HERG, and hELK1 channels to open at more negative potentials than normal. In addition to this, it slowed HERG and hELK1 channels deactivation, and in the latter channel ginsenoside Rg3 (**7**) also enhanced inactivation. Eag1 activation was accelerated in the presence of glycoside. Due to its large size and rapid effect on the gating, it was suggested that its binding site lies in the extracellular side of the channel.[161] The residues in the outer region of VSD that reduce the effect of ginsenoside Rg3 (**7**) on HERG were identified using alanine scanning mutagenesis and MD simulations. The relevant residues were Y420, L452, F463, I521, and K525. The double mutation Y420A and L452A abolished the effects of ginsenoside Rg3 (**7**) on voltage-dependent gating, but the current amplitude increase was still present. Mutation L417A produced a channel that enhanced the effects of ginsenoside Rg3. It is proposed that ginsenoside Rg3 (**7**) stabilizes the VSD of

HERG in an activated state,[180] compared to deactivated state stabilization by divalent ions that bind to a similar location.[181]

3.1.2 | Ion channel pore blockers

A positively charged moiety (a basic center) is an essential part of all Eag1 pore blockers (Table 2). *N*-methyl-astemizole applied to the extracellular side of the channel showed significantly lower inhibition compared to astemizole (**8**, Figure 8). A similar property was reported for clofilium (**9**, Figure 8) and LY97241 (**10**, Figure 8), where a slower onset of the block by the permanently charged clofilium (**9**) was observed if applied externally. Eag1 inhibition was more potent for compounds with a permanent positively charged amine if applied from the cytoplasmic side of the channel. For external application, a non-permanently charged compound showed better inhibition because of easier passage through the lipophilic membrane in uncharged form.[40,182] The complementary effect was also seen in HERG channels with *N*-methyl-astemizole and imipramine (**11**, Figure 8), which is also an open-pore channel blocker of Eag1.[153] Tertiary or secondary amines in molecules are already well-known pharmacophore features of HERG blockers.[183–185]

The difference between various channel blockers is that they can dissociate from the binding site and therefore the channel can close or stay trapped in a closed channel conformation ("drug-trapping"[204]). Among the Eag1 pore-blockers investigated, drug trapping was reported for clofilium (**9**), LY97241 (**10**), and dronedarone (**12**, Figure 8) (partially),[182,194] while chloroquine (**13**, Figure 8), haloperidol (**14**, Figure 8), imipramine (**11**), MK-499 (**15**, Figure 8), and dofetilide (**16**, Figure 8) dissociate from the binding site.[40,198,203] The dissociation from the binding site is also observed in HERG for amiodarone (**6**), cisapride (**17**, Figure 8), and haloperidol (**14**).[205]

There are several different amino acids important for the binding of pore blockers. For binding of MK-499 (**15**), terfenadine (**18**, Figure 8), and clofilium (**9**) A453 is important. The long lipophilic tails of the compounds would be stabilized between S6 and pore helix in close proximity to A453. Additional interactions with the channels are π-stacking interactions between the compounds and residues Y464 and F468 (Figure 6).[190]

There is a significant difference between the block of Eag1 and HERG induced by methanosulfonanilides **12, 15, 16, 19** (Figure 7). Because intact C-type inactivation is essential to retain the potent inhibition in HERG channels,[202] of C-type inactivation was introduced in Eag1 by two point mutations, T432S and A443S, both residues in the pore region,[203] to test the affinity of the methanosulfonanilide dofetilide (**16**) as well as of astemizole (**8**) and imipramine (**11**). In the case of dofetilide (**16**) and astemizole (**8**) the IC_{50} values for the Eag1 mutant were 3-fold lower than in wild-type, but imipramine (**11**) showed an almost two-fold increase in the IC_{50}. F468 in Eag1 and F656 in HERG are known to be important for π-stacking interactions with several blockers (Figure 6). The F468C mutation confirmed that it is crucial for astemizole (**8**) and imipramine (**11**) but not for dofetilide binding (Figure 6). In addition to the difference in recovery kinetics, the binding site seems to be different for imipramine (**11**) compared with that of astemizole (**8**) and dofetilide (**16**).[40] Residues S436, V437 located at the beginning of the selectivity filter are also necessary for the block by clofilium.[190] TBA (**20**, Figure 8) is one of the smallest blockers of Eag1 that binds more directly into the cavity and has fewer interactions with the neighboring pore compartments, as indicated by the lack of effect of the mutation at A453 on the block by TBA (**20**).[190]

Dronedarone (**12**), an alternative antiarrhythmic drug to the structurally similar amiodarone (**6**), was recently investigated as an Eag1 channel modulator. In contrast to amiodarone (**6**), dronedarone (**12**) inhibited Eag1 with an open-pore block mechanism independent of the VSD. A fraction of dronedarone molecules being trapped in the channel pore could explain the slowed-down recovery rate of the channel and its two-exponential time course at −140 mV. Reversible block by increased extracellular K^+ concentration also supported open-channel block. Moreover, competition experiments confirmed that amiodarone (**6**) and dronedarone (**12**) bind to independent nonoverlapping binding sites, probably in the Eag1 pore. There is no shift of the Cole-Moore effect to more negative potentials, as observed with amiodarone suggesting that dronedarone (**12**) does not interact with the VSD.[194]

TABLE 2 Activities of the Eag1 pore blockers

Compound	Test system and activity on EAG1	Test system and activity on HERG
TEA (**3**) (tetraethylammonium)	CHO; block by 10% at +50 mV at 10 μM[186]	CHO-K1; K_i = 46 ± 5 mM, nH = 1.4 ± 0.1[187]
	rEAG, *Xenopus* oocytes; IC_{50} = 28 ± 13 mM[188]	
Quinidine (**5**)	CHO; IC_{50} = 1.4 ± 0.1 μM[186]	*Xenopus* oocytes; IC_{50} = 4.6 ± 1.2 μM[189]
	Xenopus oocytes; IC_{50} = 0.4 ± 0.2 mM[188]	HEK293; IC_{50} = 1.3 ± 0.5 μM[190]
	Xenopus oocytes, inside-out; IC_{50} = 2.1 ± 0.4 μM[190]	HEK293; IC_{50} = 0.4 ± 0.04 μM[191]
Astemizole (**8**)	HEK293; IC_{50} = 196 nM[184]	HEK293; IC_{50} = 0.9 nM[192]
	Xenopus oocytes; IC_{50} = 2.8 ± 0.1 μM[40]	rERG, GH3 cells; IC_{50} values in the range of 50 nM[183]
	Xenopus oocytes, inside-out; IC_{50} = 91 ± 6 nM[40]	CHO; IC_{50} = 5.1 ± 1.4 nM, nH = 1.0[185]
Clofilium (**9**)	CHO-K1; IC_{50} = 255 ± 35 nM[182]	CHO-K1; IC_{50} = 3 ± 1 nM[182]
	Xenopus oocytes, inside-out; IC_{50} = 1 ± 1 nM[182]	CHO; IC_{50} = 21 ± 2 nM, nH = 1.0[185]
LY97241 (**10**)	CHO-K1; IC_{50} = 5 ± 1 nM[182]	CHO-K1; IC_{50} = 2 ± 0.44 nM[182]
	Xenopus oocytes, inside-out; IC_{50} = 2 ± 0.17 nM[182]	HEK293; IC_{50} = 2 ± 0.44 nM[190]
	HEK293; IC_{50} = 10 ± 1 nM[190]	
Imipramine (**11**)	*Xenopus* oocytes; IC_{50} = 40.2 ± 0.3 μM[40]	CHO, perforated patch clamp; IC_{50} = 3.4 ± 0.4 μM, nH = 1.2 ± 0.03[193]
Dronedarone (**12**)	HEK293; K_d, app = .9 μM, nH = 0.9[194]	*Xenopus* oocytes; IC_{50} = 9.2 μM[195]
		HEK293; IC_{50} = 59.1 nM, nH = 0.8[196]
		HEK293; IC_{50} = 42.6 ± 3.4 nM, nH = 0.9 ± 0.1[197]
Chloroquine (**13**)	HEK293; IC_{50} = 31.1 ± 4.5 μM, nH = 0.9 ± 0.1[198]	HEK293; estimated IC_{50} = 2.5 μM[199]
		Xenopus oocytes; IC_{50} = 8.4 ± 0.9 μM[200]
Haloperidol (**14**)	CHO-K1; IC_{50} = 590 ± 121 nM[182]	CHO-K1; IC_{50} = 19 ± 10 nM[182]
	Xenopus oocytes, inside-out; IC_{50} = 752 ± 35 nM[182]	

TABLE 2 (Continued)

Compound	Test system and activity on EAG1	Test system and activity on HERG
MK-499 (**15**)	*Xenopus* oocytes; IC_{50} = 43.5 ± 4.7 μM[40]	*Xenopus* oocytes, single channels; IC_{50} = 32 ± 4 nM[201]
		HEK293; IC_{50} = 8 ± 1 nM[190]
Dofetilide (**16**)	*Xenopus* oocytes; IC_{50} = 29.6 ± 1.1 μM[40]	*Xenopus* oocytes; IC_{50} = 0.3 ± 0.04 μM[202]
	bEAG, *Xenopus* oocytes; IC_{50} = 31.8 ± 7.5 μM[203]	CHO; IC_{50} = 51 ± 1 nM, nH = 1.3[185]
Cisapride (**17**)	dEAG, *Xenopus* oocytes; IC_{50} = 11.9 ± 1.2 μM[43]	*Xenopus* oocytes; IC_{50} = 0.102 ± 0.001 μM[43]
Terfenadine (**18**)	*Xenopus* oocytes, inside-out; IC_{50} = 16 ± 3 nM[183]	CHO; IC_{50} = 61 ± 16 nM, nH = 1.2[185]
E4031 (**19**)	CHO-K1; IC_{50} = 416 ± 44 nM[182]	CHO-K1; IC_{50} = 7 ± 2 nM[182]
	Xenopus oocytes, inside-out; IC_{50} = 162 ± 17 nM[182]	
TBA (**20**) (tetrabutylammonium)	*Xenopus* oocytes, inside-out; IC_{50} = 1.2 ± 0.1 μM[190]	CHO-K1; K_i = 0.4 ± 0.1 mM, nH = 1.3 ± 0.1[187]

Note: Unless otherwise indicated, oocytes were recorded using two-electrode voltage clamp, and mammalian cells in whole-cell patch clamp.

Abbreviations: bEAG, bovine EAG; CHO, chinese hamster ovary cell line; dEAG, drosophila EAG; HEK293, human embryonic kidney 293 cells; GH3 cells, rat pituitary tumor cell line; mEAG, mouse EAG; rEAG, rat EAG; nH, Hill coefficient.

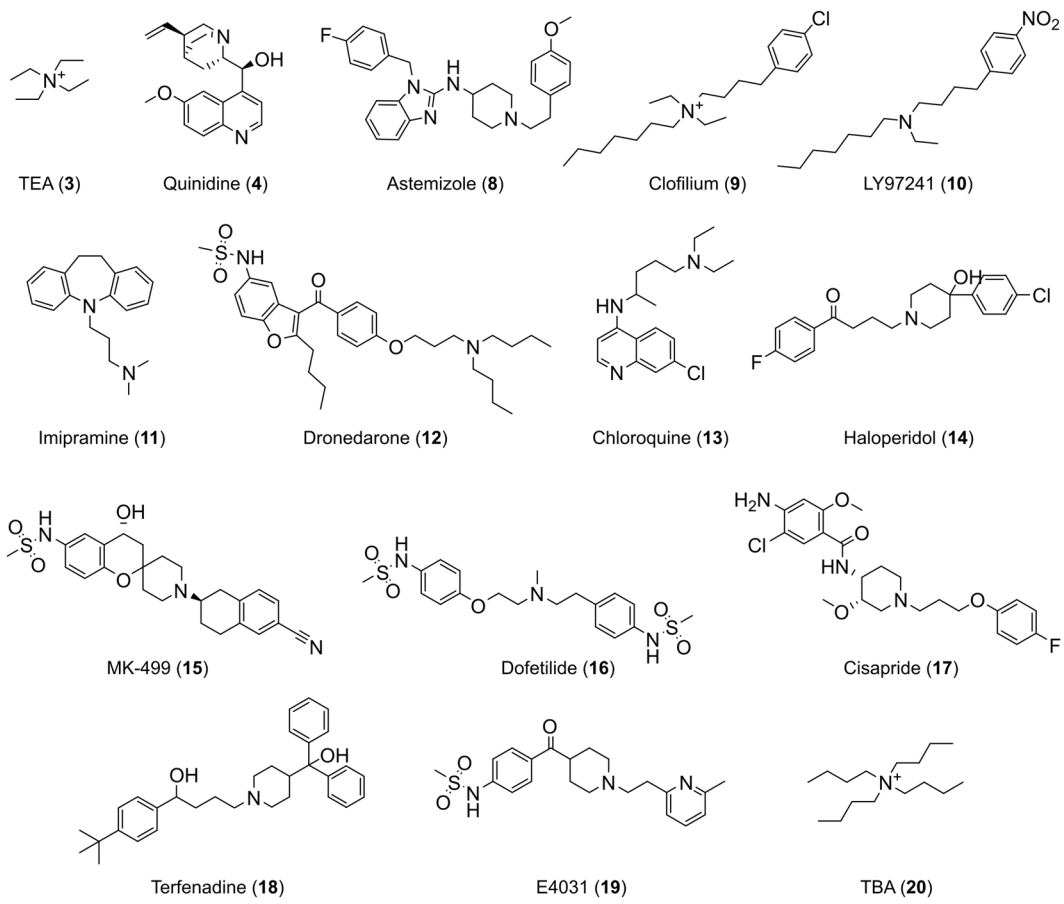


FIGURE 8 Structures of ion channel pore blockers

The antimalarial drug chloroquine (**13**) has been recently reported to inhibit Eag1. Inhibition of other potassium channels, inhibition of autophagy, and normalization of the tumor vasculature by chloroquine suggested examining the effect of chloroquine (**13**) on Eag1.[198,206,207] Electrophysiological experiments suggested that chloroquine (**13**) inhibits Eag1 in HEK293 cells with an IC_{50} value of 31.05 ± 4.5 μM as an open-channel blocker that needs to dissociate from its binding site before the channels can close (Table 2). Open channel block was inferred from the slow current deactivation and absence of block of the current if chloroquine (**13**) was applied externally. However, competition with known open channel blockers was not tested; it is still possible that the binding site is located elsewhere accessible from the cytoplasmic side. Chloroquine (**13**) blocked Eag1 in a voltage- and concentration-dependent manner.[198] The block of HERG channels is also concentration-dependent but voltage-independent with an IC_{50} value of 2.5 μM (Table 2) in HEK293 cells.[199]

3.1.3 | Allosteric modulators

Tetrandrine (**21**, Figure 9) is a natural compound used in traditional Chinese medicine that inhibited Eag1 with an IC_{50} of 69.97 ± 5.2 μM (Table 3). The inhibition of Eag1 current is concentration-dependent, but **21** did not inhibit the Cole–Moore shift. Moreover, the inhibition was independent of the intracellular calcium concentration. In silico

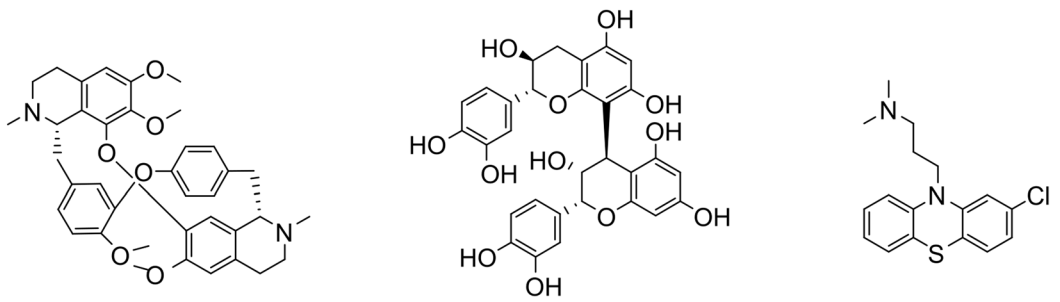


FIGURE 9 Structures of allosteric modulators

modeling supported by mutation studies were used to map the putative binding site for tetrandrine (**21**). The triple mutant Eag1 I550A/T552A/Q557A, Figure 10) in the C-linker domain showed significantly less current inhibition than wild-type channels, while the individual mutations I550A, T552A, or Q557A did not have a similar effect. Tetrandrine (**21**) specificity was also investigated on K_V 7.1, K_{ir}2.1, and HERG channels at 100 μM, a concentration that inhibits Eag1almost completely, but only slightly HERG.[131] Tetrandrine (**21**) also inhibits voltage-gated Ca^{2+} channels, large-conductance Ca^{2+}-activated K^+ channels, two-pore channels as well as intracellular Ca^{2+} pumps.[208,209] The binding site of tetrandrine (**21**) could also be the same for procyanidin B1 (**22**, Figure 9), a natural compound present in grape seeds that inhibits Eag1 in a concentration-dependent manner with an IC_{50} value of 10.4 ± 0.9 μM (Table 3). Similar to tetrandrine, 100 μM procyanidin B1 (**22**) does not inhibit K_V7.1, Kir2.1, or HERG significantly. The triple mutant Eag1 I550A/T552A/Q557A also shows a 40-fold reduction of the inhibition over wild-type Eag1.[210]

The binding site for chlorpromazine (**23**, Figure 8) in the PAS domain was identified with a combination of surface plasmon resonance (SPR) and tryptophan fluorescence. Deleting the PAS domain abolished inhibition by chlorpromazine, especially at voltages lower than 30 mV, and decreased it at higher voltages. When the voltage rises above −30 mV, current inhibition becomes constant and voltage-independent in mouse Eag1.[158] An open-pore block could explain the inhibition at higher voltages, and therefore chlorpromazine (**23**) would have two different mechanisms. At lower voltages, chlorpromazine (**23**) could inhibit mouse Eag1 by binding to the PAS domain, and when the channel opens at higher voltages, then inhibition occurs by pore block. The structural similarity between chlorpromazine (**23**) and imipramine (**11**), which is a known voltage-dependent open-pore blocker of Eag1[184] would support an open-channel pore block. Moreover, chlorpromazine (**23**) displayed a voltage-dependent open-pore block of HERG, indicating the possibility for a similar mode of

TABLE 3 Eag1 allosteric modulators

Compound	Test system and activity on EAG1	Test system and activity on HERG
Tetrandrine (**21**)	CHO; IC_{50} = 70.0 ± 5.2 μM[131]	CHO; >100 μM[131]
Procyanidin B1 (**22**)	HEK293; IC_{50} = 10.38 ± 0.87 μM[190]	HEK293; >100 μM[210]
Chlorpromazine (**23**)	mEAG, *Xenopus* oocytes, inside-out; IC_{50} = 3.7 ± 0.7 μM[158]	*Xenopus* oocytes; IC_{50} = 21.6 ± 6.9 μM; nH = 1.1 ± 0.2[211] IC_{50} = 4.9 ± 0.3 μM; nH = 1.3 ± 0.1[212]

Note: Unless otherwise indicated, oocytes were recorded using two-electrode voltage clamp, and mammalian cells in whole-cell patch clamp.

Abbreviations: CHO, Chinese hamster ovary cell line; mEAG, mouse EAG; nH, Hill coefficient.

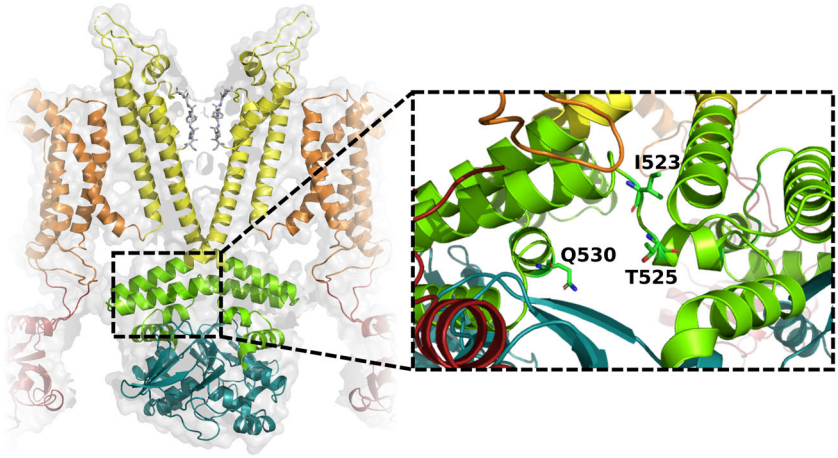


FIGURE 10 Proposed binding site for tetrandrine (**21**) and procyanidin B1 (**22**), located in the C-linker of Eag1 (PDB code: 5K7L). Amino acids T525 (equivalent to T552) and I523 (equivalent to I550) in one subunit, important for binding of tetrandrine, are shown as sticks. The Q530 (equivalent to Q557) in proximity is from the neighboring subunit. The enlarged view displays three subunits for better visualization [Color figure can be viewed at wileyonlinelibrary.com]

action in Eag1.[212] The PAS domain of the HERG channel did not bind chlorpromazine (**23**) in SPR studies. It is likely that the selective binding of chlorpromazine (**23**) to the mouse Eag1 PAS domain modulates interactions between PAS and VS or CNBH domains.[158] Chlorpromazine (**23**) inhibits also voltage-gated Na^+ channels,[212] nicotinic acetylcholine receptor channels,[213] and ATP-activated channels.[214] The binding site for chlorpromazine (**23**, Figure 9) in the PAS domain was identified with a combination of surface plasmon resonance (SPR) and tryptophan fluorescence. Deleting the PAS domain abolished inhibition by chlorpromazine, especially at voltages lower than 30 mV, and decreased it at higher voltages. When the voltage rises above −30 mV, current inhibition becomes constant and voltage-independent in mouse Eag1.[158] An open-pore block could explain the inhibition at higher voltages, and therefore chlorpromazine (**23**) would have two different mechanisms. At lower voltages, chlorpromazine (**23**) could inhibit mEAG1 by binding to the PAS domain, and when the channel opens at higher voltages, then inhibition occurs by pore block. The structural similarity between chlorpromazine (**23**) and imipramine (**11**), which is a known voltage-dependent open-pore blocker of Eag1[184] would support an open-channel pore block. Moreover, chlorpromazine (**23**) displayed a voltage-dependent open-pore block of HERG, indicating the possibility for a similar mode of action in Eag1.[212] The PAS domain of the HERG channel did not bind chlorpromazine (**23**) in SPR studies. It is likely that the selective binding of chlorpromazine (**23**) to the mEAG1 PAS domain modulates interactions between PAS and VS or CNBH domains.[158] Chlorpromazine (**23**) inhibits also voltage-gated Na^+ channels,[212] nicotinic acetylcholine receptor channels,[213] and ATP-activated channels.[214]

3.1.4 | Divalent ions

As stated before, the *KCNH* family of channels is sensitive to different divalent cations that slow the activation rate of the channels when applied from the extracellular side. Sr^{2+}, Ba^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+} slow down the activation in increasing order, from millimolar to micromolar concentration. The slowing of activation correlates with ion hydration enthalpy. The effect of Ca^{2+} is minor compared with other ions. Ba^{2+} and Zn^{2+} also induce a slow current decay with time that could be an effect of channel block or

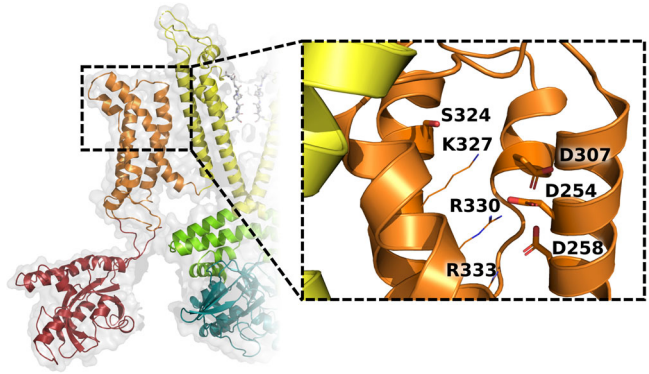


FIGURE 11 Binding site of divalent cations in the voltage-sensor domain (PDB code: 5K7L), with sticks representation of amino acids important for the binding of divalent ions. Residues K327, R330, and R333 represent positively charged residues that respond to changes in the membrane voltage [Color figure can be viewed at wileyonlinelibrary.com]

inactivation.[90] Externally applied divalent ions interact with the VSD by interactions with negatively charged residues.

The binding site for Mg^{2+} in *Drosophila eag* was investigated through the mutation of several residues in different segments of the VSD (Figure 11). Mutations D278V (Eag1 D258) and D327A/F (Eag1 D307), in which a negatively charged amino acid is replaced by a hydrophobic residue, eliminated Mg^{2+} modulation of the activation kinetics. When residue D278 was extended by one methylene group by mutation D278E, the mutated channel was insensitive to Mg^{2+}. In contrast, the D327E mutation did not affect Mg^{2+} modulation.[92] Mn^{2+} and Ni^{2+}, which have different sizes and polarizability, produced similar effects on the *eag* channel as Mg^{2+} ions. Based on the different ions and their properties, it was proposed that their coordination geometries are important for the formation of interactions with the acidic residues in S2. This was especially pronounced with residue D278, where mutation to glutamic acid would disrupt the regular octahedral geometry of the binding site.[215] S321 (Eag1 S324) in Eag2, located in S4, was suggested to form a "bridge" between S4 and acidic residues in S2 and/or S3 through the divalent cation. In *eag* channels, this bridge is not necessary for the slow activation effect of the divalent cations but, if present, the effect is significantly increased. These interactions slow down the transition of the channel from an early closed state at the hyperpolarized potential to a late closed state at more depolarized potentials, from which the channel can open.[181]

3.2 | Natural products

Nature is a very powerful, yet underexploited source of potential drugs, to which animal toxins make a particularly rich contribution. Venomous animals are found everywhere, and more than 220 000 species are described so far; however, most of their venoms have not been studied. The acquisition of venom is a life-changing event in the evolution of animals, as it transforms the predator-prey relation into a biochemical instead of physical combat. Venoms are evolutionary evolved to bind physiological targets with excellent potency and outstanding selectivity, often even distinguishing between channel subtypes. They have a unique spatial fold, and their metabolites often possess low toxicity.[216,217] Six venom-derived peptides have been approved by the FDA so far, such as an analgesic, ziconotide (Prialt) derived from the *Conus magus*, and an antidiabetic, exenatide derived from the Gila monster.[218] In terms of drug design, it would be ideal to combine the epitope responsible for the unseen potency and selectivity of peptide toxins, grafted on a template of small molecules, who offer a better bioavailability and

stability.[219] The known natural compounds with activity against Kv10.1 are described below and summarized in Table 4.

3.2.1 | Alkaloids

Sponges are among the most versatile sources of marine natural products, and their secondary metabolites show promising properties such as defensive, antibiotic, antiangiogenic, antiproliferative, hemolytic, and cytotoxic activities.[223,224] Pupurealidin analogs are alkaloids produced by Verongida sponges. Bromotyrosin purpurealidin J1 was found in the *Pseudoceratina pupurea* and served as inspiration for synthesizing simplified amide analogs as described above. Among them, purpurealidin Analog 5 was the most potent, with an IC_{50} of 7.7 ± 1.0 μM for Eag1. An electrophysiological examination of this compound revealed a shift of the activation curve to more negative potentials. This effect of Compound 5 was correlated to an induced apparent open state inactivation during prolonged depolarization, while it also affects the gating at hyperpolarized potentials. Since these effects are reminiscent of mibefradil, a competition assay with mibefradil was performed, and Compound 5 was found to compete for the same binding spot as mibefradil. Therefore, it was suggested that Compound 5 is a gating modifier that binds to the voltage sensor on a binding spot that overlaps with the one for mibefradil. Compound 5 was tested on different cell lines and inhibited proliferation but also induced cytotoxicity in all the cell lines that were tested, also cell lines not expressing Eag1. The effects of Compound 5 on the tested cell lines can therefore not only be attributed to the effect on Eag1.[170] A selectivity screening on different voltage-gated ion channels is still missing, however, Compound 5 was tested on one of the most relevant cardiac ion channels HERG and showed inhibition of 57.6 ± 3.4%.[171]

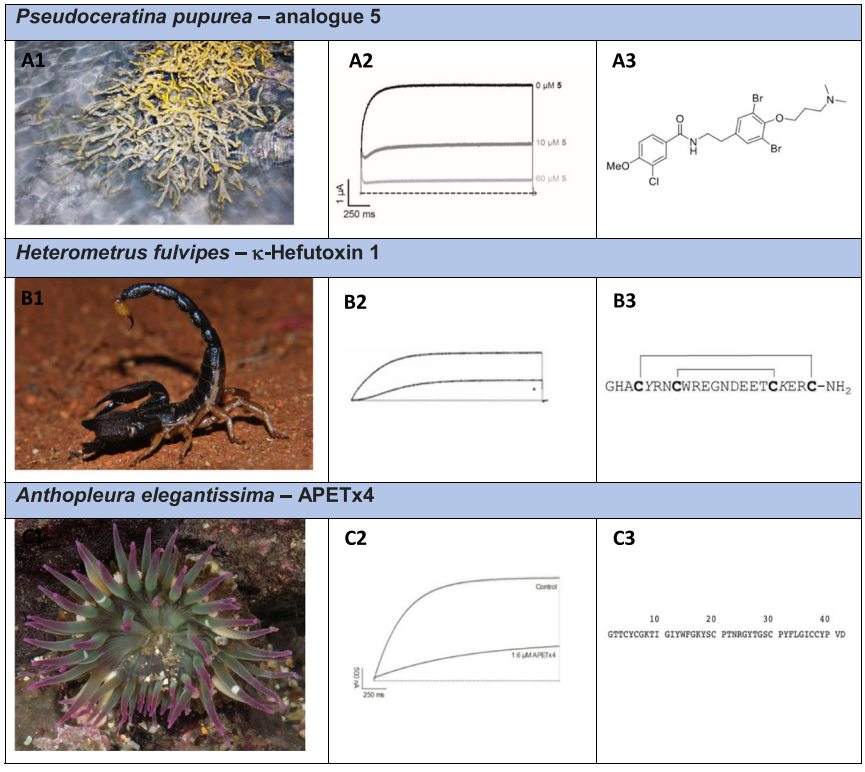
3.2.2 | Peptides

κ-Hefutoxin 1 is the first toxin described to inhibit Eag1. Isolated from the Asian forest black scorpion *Heterometrus fulvipes*, κ-hefutoxin 1 contains 22 residues and two disulfide bridges, matching the typical potassium channel scorpion toxins structure with 20–75 amino acids and 2–4 disulfide bridges.[220] Toxins blocking K channels, even with very divergent three-dimensional (3D) structures, share a common feature crucial for interaction with the channel: a functional dyad consisting of the combination of lysine and a hydrophobic residue (mostly tyrosine or phenylalanine) on an average distance of 6.52 ± 0.62 Å from each other. This allows the positively charged lysine to interact electrostatically with a negative residue in the pore, resulting in a physical obstruction for ion conduction. Also κ-hefutoxin 1, despite its unique 3D fold of two parallel helices without any β-sheets, contains such a functional dyad.[225] The first screening by Srinivasan et al.[225] revealed that κ-hefutoxin inhibits $K_V1.2$ and $K_V1.3$ with low affinity, and a different primary target was postulated. Subsequent screening by Peigneur et al.[226] showed inhibitory activity of κ-hefutoxin 1 on $K_V1.6$, but not on $K_V1.1$, $K_V1.4$, $K_V1.5$, Shaker IR, $K_V2.1$, $K_V3.1$, $K_V4.2$, or HERG, revealing an exciting selectivity profile for this toxin. Finally, Moreels et al.[220] identified Eag1 as the most potent target of κ-hefutoxin 1, with an IC_{50} of 26 ± 2 μM. Although the affinity is still relatively low, this toxin offers a beautiful starting point in the search for Eag1-inhibiting peptides as the selectivity profile shows a lack of activity on HERG, opposed to most Eag1-blocking small molecules.

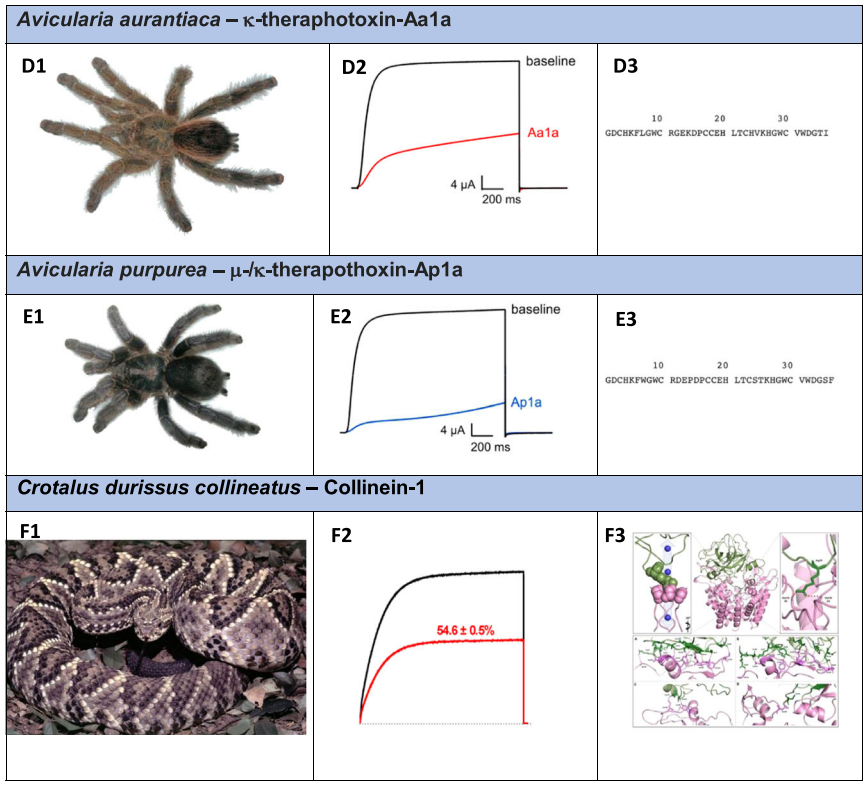
APETx4, derived from the sea anemone *Anthopleura elegantissima*, reduces the activation rate of Eag1 and shifts the steady-state activation curve to more depolarized potentials. Together with the observation that APETx4 inhibits Eag1 in the closed state, it is suspected to act as a gating modifier, binding to the S3b-E2-S4 region (the voltage sensor paddle). Just like the functional dyad that many potassium channel toxins have in common, gating modifiers often rely on a hydrophobic triad in combination with some charged amino acids on the toxin surface for their binding to ion channels. In APETx4, the hydrophobic triad is formed by Y32, F33, and L34. Amongst a panel of

voltage-gated channels, APETx4 is most active on Eag1 with an IC50 of 1.01 ± 0.01 μM, but also inhibits other channels such as K_V1.4, Na_V1.4, K_V2.1, Na_V1.5, K_V1.3, K_V1.5, and Na_V1.6 (in decreasing order of inhibition percentage). Although no inhibition on HERG was observed during a standard test protocol (pre-pulse of +40 mV, followed by a pulse to −120 mV), APETx4 showed a voltage-dependent effect on HERG with the highest inhibition of the tail current observed after a pre-pulse of −20 mV. In the low micromolar range, however, APETx4 can discriminate between Eag1 and HERG. APETx4 shows a concentration-dependent cytotoxic and proapoptotic effect on different cancer and Eag1 expressing cell lines but does not affect the proliferation of healthy fibroblast cells. Since also cancer cell lines not expressing Eag1 are affected, the effect of APETx4 is believed to be mediated not only by Eag1.[156]

TABLE 4 A1. *Pseudoceratina purpurea*, photo by Ria Tan. A2. Eag1 current traces during perfusion with ND96 (black), 10 μM of analog 5 (dark gray), 60 μM of analog 5 (light gray).[170] A3. Structure of Eag1 modulator analog 5.[170] B1. *Heterometrus fulvipes*, photo by Vivek Philip. B2. Eag1 current traces in control and after application of 40 μM of κ-Hefutoxin 1 (*).[220] B3. Amino acid sequence of κ-Hefutoxin 1.[220] C1. *Anthopleura elegantissima*, Photo by Ron Wolf. C2. Eag1 traces in control and after application of 1.6 μM APETx4.[156] C3. Amino acid sequence of APETx4.[156] D1. *Avicularia aurantiaca*.[221] D2. Eag1 current traces in control and after application of Aa1a.[221] D3. Amino acid sequence of Aa1a.[221] E1. *Avicularia purpurea*.[221] E2. Eag1 current traces in control and after application of Ap1a.[221] E3. Amino acid sequence of Ap1a.[221] F1. *Crotalus durissus collineatus*, Photo by Wolfgang Wuster. F2. Eag1 current traces in control and after application of 5 μM rCollinein-1.[222] F3. Selected docking solution for Collinein-1 and Eag1-1[222]



(Continues)



Two spider toxins, derived from the spiders' *Avicularia aurantiaca* and *Avicularia purpurea*, respectively κ-theraphotoxin-Aa1a and μ-/κ-therapothoxin-Ap1a (*Aa1a* & *Ap1a* in short), show 81% identity and are the most potent Eag1 inhibiting peptides described until now, with an IC_{50} of 637 ± 59 nM and 236 ± 21 nM respectively. Interestingly, Aa1a and Ap1a discriminate Eag1 from the cardiac-relevant ion channels HERG (13 and 35-fold) and $Na_V1.5$ (both 11-fold). However, both toxins are also active on $Na_V1.2$ and $Na_V1.7$. No selectivity screening was done on voltage-gated potassium channels, yet many of them are also involved in heart physiology, such as $K_V1.4$ and $K_V4.2$.[227] A more extensive selectivity screening would undoubtedly be of added value for these toxins. However, a detailed electrophysiological characterization of Aa1a and Ap1a was performed and revealed some complex effects of both toxins on Eag1 channel gating. First, both toxins shift the *activation* of the channel to more depolarized potentials, thereby reducing the open probability. Second, both toxins enhance the *Cole–Moore shift*, which results in an Eag1 channel that is more difficult to activate after pre-pulsing. At very depolarized potentials, a voltage- and time-dependent slow inactivation in the open state can be observed; Ap1a enhanced this *open state inactivation*, while Aa1a had no effect. Aa1a enhanced the *steady-state inactivation*, while Ap1a had no effect. None of the toxins affected channel *deactivation* significantly. Interestingly, both peptides adopt an inhibitor cystine knot (ICK) motif, which is often observed in spider toxins. This ICK provides chemical and thermal stability and protects the peptide against proteases. Aa1a and Ap1a are very stable in human serum and cerebrospinal fluid. Similar to APETx4, also these gating modifiers contain hydrophobic patches, which are very well conserved in both toxins (F6, L7, W9, W29, and W32 for Aa1a; F6, W7, W9, W29, and W32 for Ap1a).[221]

3.3 | Proteins

3.3.1 | Monoclonal antibody mAb56

Antibodies enjoy the benefit of high binding selectivity and the ability to distinguish between subtypes from highly homologous ion channel families. In addition, whole antibodies are divalent, and this not only increases avidity for the epitope but also can induce clustering and internalization of targets. Furthermore, antibodies show pharmacokinetic features that allow its metabolism as part of the body's normal protein dynamics, which reduces immunogenicity and the probability of generating toxic metabolites. The constant region of antibodies (Fc-region) enables the design of modifications to produce diverse effector functions. Although polyclonal antibodies have been in therapeutic use, monoclonal antibodies represent the practical alternative that warrants generating equally effective blockers using the same strategy. Besides, mAbs are also amenable for manipulation, modification, and production in recombinant systems, making them favorable as diagnostic tools and therapeutic approaches. Only a handful of mAbs able to inhibit the function of ion channels has been described.[228] SVmab1 targets sodium channel $Na_v1.7$ at the S3–S4 extracellular loop region of domain II, that is, the voltage-sensor paddle, and stabilizes the closed state in a state-dependent manner; this mAb showed analgesic effects in inflammatory and neuropathic pain in mice models.[229] The calcium release-activated Ca^{2+} channel Orai1 can also be inhibited by antibodies, either by direct inhibition[230] or through the induction of antibody-mediated internalization in primary T cells.[231] Against Eag1, we generated monoclonal antibodies that target though immunization using a fusion protein that consists of two segments of Eag1: the third extracellular loop (E3), between the transmembrane segments S5 and S6, which makes Eag1 distinguishable from its structural analogs Eag2 and HERG, and is the closest area to the ion permeation route from the extracellular side. E3 was fused to the C-terminal tetramerizing coiled-coil region. Only one of the antibodies able to bind Eag1 and not Eag2 or HERG (mAb56) reduced specifically Eag1 current in transfected cells and the endogenous channel in tumor cells with an EC_{50} of approximately 50 nM. mAb56 also showed the ability to inhibit the growth of tumor cells in vitro and of tumor models, including patient-derived xenografts in vivo.[20] Epitope mapping for mAb56 identified the linear sequence GSGSGKWEG in the E3 domain, relatively far from the permeation pathway, as indicated by the cryo-EM structure. mAb62, another monoclonal antibody whose epitope lies 23 residues downstream, that is, closer to the permeation path, does not produce inhibition of the ionic current.[20] Therefore, the mechanism of inhibition by mAb56 is likely not a direct interference with ion permeation. When comparing the cryo-EM structures of Eag1 and HERG, which have been studied in different conformations, with the gate closed and open respectively, the epitope faces the pore Eag1. In contrast, the homologous regions of HERG face outward. This could reflect a tilting of that region during gate opening that is inhibited by mAb56 binding.[232] Although it did not show any effects on ionic permeation through Eag1, mAb62 exhibited a promising application as diagnostic tools or/and to deliver drugs as Cy5.5-labeled mAb62 specifically bound and accumulated at tumor sites in vivo for at least 1 week, also drug-activating enzyme β-D-galactosidase conjugated mAb62-β-gal bound to the tumor site and the enzyme galactosidase remained active in vivo[233]. Constructs derived from anti-Eag1 antibodies are being developed as carriers to target the TNF-related apoptosis-inducing ligand to tumor cells. Some of those constructs have shown efficacy in vivo as sensitizers to conventional chemotherapeutic agents.[118–120]

3.3.2 | Collinein-1

Snake venom thrombin-like enzymes are a class of serine proteases with an activity similar to human thrombin. Collinein-1, derived from the *Crotalus durissus collineatus*, is such an enzyme. Besides its catalytic activity, cleaving the Aα-chain of fibrinogen, it also blocks Eag1 with an IC_{50} of 4.2 ± 0.5 μM. The recombinant derivative of collinein-1 (rCollinein-1) was used for the screening on ion channels and revealed inhibition on Eag1 (IC_{50} = 2.5 ± 0.3 μM) and

slightly on HERG, but no effect was seen on *Shaker*, $K_V1.4$, $K_V2.1$, $Na_V1.1$-1.6, or $Na_V1.8$. A mutant collinein-1 without its catalytic properties showed the same activity on the mentioned ion channels, with an IC_{50} of 4.3 ± 0.8 μM on Eag1. Moreover, collinein-1, together with phenylmethylsulphonyl fluoride, a blocker of the catalytic triad (His57, Asp102, and Ser195), blocked the Eag1 currents to the same extent as collinein-1 alone. Therefore, channel inhibition by collinein-1 is not dependent on its catalytic activity, allowing the use of the noncatalytic mutant as a potential drug targeting Eag1 and with this avoiding the possible side effects on blood coagulation that could have been caused by the native collinein-1. An electrophysiological characterization showed how the Eag1 block increases towards more negative potentials, suggesting that rCollinein-1 interacts with Eag1 in a closed formation. A docking simulation to elucidate the binding mechanism of Collinein-1 to Eag1 resulted in many interaction sites. The putative pharmacophore consists of R79, which forms hydrogen bonds to G536 of the selectivity filter, allowing a perfect fit in the pore inlet. This would agree with the electrophysiology experiments, which revealed only a slight shift of the activation curve towards more positive potentials, showing little contribution of channel gating to the channel inhibition. Moreover, the fact that two other snake venom serine proteases and chymotrypsin showed less Eag1 inhibition the less Eag1-interacting residues they had in common with collinein-1, confirmed this hypothesis. Finally, collinein-1 caused cell death in the human breast adenocarcinoma cell line MCF7, possibly via inhibition of Eag1. In contrast to other K_V channels, Eag1 contains a 40 amino acid turret between S5 and the pore helix. As this turret surrounds the pore opening, it was thought that toxins were not able to overcome this structural barrier. However, the docking simulation described reveals a mechanism by which collinein-1 is able to bind straight to the selectivity filter and thus overcoming this barrier.[222]

4 | PERSPECTIVE AND CHALLENGES OF EAG1 AS A POTENTIAL THERAPEUTIC TARGET

Because of its frequent aberrant presence in tumors and its participation in cancer progression, Eag1 is regarded as an interesting oncology target. As described above, the advances in the mechanistic understanding of its regulation and pathophysiology now allow to dissect which functional aspects of Eag1 are most relevant and therefore should be addressed in particular. Nevertheless, voltage-gated potassium channels represent a challenging field in drug discovery, because of scarce structural data, high sequence similarity between isoforms, complex high-throughput screening assays and complicated mechanisms of voltage sensing and mechanical movements of channel gating. In the case of Eag1, its high degree of structural similarity with the HERG channel makes the development of selective ligands challenging. The potential severe side effects following cardiac HERG inhibition accompanying Eag1 inhibition would represent a safety concern. Although our understanding of the important differences between Eag1 and HERG is increasing, to the best of our knowledge, no potent, selective small-molecule Eag1 inhibitor is known. To aid the design of the latter, a combination of multiple drug design methods, including molecular modeling and artificial intelligence, will be needed to identify important differences between the two channels and use them to design Eag1-selective ligands.

Ligand-based design of specific Eag1 inhibitors has been explored by pharmacophore modeling, but this method is hampered by the small number of known active and inactive compounds, which are required for the creation and validation of existing pharmacophore models. In such an in silico approach, it is also crucial to know whether the compounds bind to the same site and in the same state of the channel. This is a problem because the exact binding site has not been determined for the most known Eag1 inhibitors. Moreover, there is no x-ray or cryo-EM structure with any of those ligands bound to either Eag1 or HERG. For better creation and validation of pharmacophore models, having a series of similar compounds with different effects on activity significantly improves the predictive strength of the model. In the case of Eag1, the pool of compounds is very small. More or less, there is only one larger series of purpurealidine analogs, which contains a set of active and inactive compounds that most probably have the same mechanism of Eag1 inhibition.[170] This significantly limits the rational drug

design, but on the other hand, offers a lot of unexplored chemistry space to be investigated and to build structure–activity relationships for novel libraries of compounds.

On the positive side, the recently solved high-resolution cryo-EM channel structures allow a medicinal chemist to use the structure-based drug design methodology.[36,37] In the case of Eag1, such a method is advantageous for the design of compounds that bind to the central cavity of the channel. The Eag1 cryo-EM structure can be used for compounds that would remain trapped in the channel when the gate closes. With the help of homology modeling, it should be feasible to predict the structure of the Eag1 channel with the PD in the open conformation, because the sequence similarity between Eag1 and HERG is very high in this region. For the same reason, achieving selectivity for pore blockers poses a big problem. Nevertheless, the difference in the gating kinetics between both channels could provide the desired selectivity, as it is the case for ICA-105574 with modulation of C-type inactivation.[172] However, further research is needed to better understand the mechanism of C-type inactivation and to use it for selective modulation of Eag1.

Alternatively, small molecules may be used to interfere with the signal transmission between the detection of a change in membrane potential and the mechanical opening of the channel pore. For this kind of channel modulation, the best binding location is probably the VSD and the intracellular domains that participate in the gating process. The amino acid sequences of Eag1 and HERG differ the most in the loops connecting S1–S4 segments pointing outside of the phospholipidic bilayer. From the extracellular side, the binding site could be similar to the site in which mibefradil binds.[160] The identification of the exact binding location for mibefradil would help in the rational design of selective inhibitors. A possible starting point is also the extracellular binding site for divalent ions, where small molecules could mimic their interactions with the VSD, and stabilize it in a closed state.[181] Currently, available cryo-EM structures, unfortunately, have low resolution and lack some residues especially in the loops between segments of VSD, due to the high flexibility of these protein parts. Besides this, VSD movements (especially S4) at the change of membrane potential probably significantly modify the morphology of the potential binding surface. Therefore, the binding site could change, vanish or even a new binding site could appear based on the state of a channel. A drawback of the currently available Eag1 and HERG structures is that the conformation of VSD is only known in the up/depolarized position. This lack of different channel states along the gating path that the channel goes through when it opens inactivates and closes, increases the challenge for structure-based design. Some of these problems can be addressed with homology modeling based on the other channels, followed by molecular dynamics simulations. Previously disclosed Eag1 homology models, before the cryo-EM structures became available, were mostly built for the PD and none of them included the side pockets that are visible in the cryo-EM structure, illustrating the limitations of this method. Recently, new cryo-EM structures of ion channels were solved, increasing the template database for structure building and enabling homology modeling of different states of the channels. Nowadays, the computer power has increased to the point that even multi-microsecond molecular dynamics simulations are possible and in a combination of enhanced sampling methods could enable us a glance into ion channel gating at the molecular level.

Probably the best solution would be to combine ligand- and structure-based drug design methods. The negative effect of the similarity between Eag1 and HERG in designing selective inhibitors can be put to good use because the number of characterized compounds with HERG activity is significantly higher than for any other off-target in drug development. Given the already routine testing of compounds on HERG, these molecule datasets can be used as a filter for pre-preparation of a compound library for in silico or high-throughput screening purposes. Since compounds with potential HERG inhibition would be excluded before screening, there would be a higher probability to identify Eag1-selective hits. Moreover, because of the similarity between the two channels, compounds with known weak inhibitory activity could be tested for HERG in the search for compounds that would show greater selectivity toward Eag1. On the other hand, several different pharmacophore models have already been made for HERG channel blockers, mostly based on compounds that bind to PD. These pharmacophore models can be used in the design of compounds binding not to PD but to VSD or the cytoplasmic domains.

The same arguments apply to peptide toxins. Detailed knowledge of both binding sites and mechanism of action would be needed to design modifications to improve activity and specificity. For the case of antibodies, where the epitope is identified, we still lack enough knowledge about the mechanism of inhibition of Eag1. The location of the epitope of the currently available monoclonal antibody, in the PD but far away from the permeation pathway, makes it unlikely that it acts as an open channel blocker.[232] Studies on the mechanism of action can provide alternative approaches that could be useful also for small molecules and peptides.

The limitation of the expression of Eag1 to the CNS could also be exploited for selectively targeting cancerous cells without greater effect on the normal tissue. This could be achieved by modifying the physicochemical properties of molecules so that they could not cross the blood-brain barrier, therefore reducing the possibility of unwanted side effects originated by the inhibition of noncancerous cell in the CNS.

It is difficult to compare IC_{50} values from different experiments and experimental conditions. For example, up to 200-fold differences in IC_{50} measurements for the same compound were observed in patch-clamp measurements on HERG channels, with voltage protocol, cell system, and temperature having the greatest influence on IC_{50} values.[234] This is important when evaluating HERG selectivity for potential LQT and TdP risk assessment. Drug design of new Eag1 inhibitors should consider experimental conditions when measuring HERG potency, especially for TdP and LQT risk assessment. Inhibition of HERG even in the nanomolar range does not always lead to LQT or TdP as in the case of verapamil,[14] but a cautious approach is still required. Considering that Eag1 inhibitors are being developed for diseases such as cancer, the drug safety threshold, represented as the ratio of IC_{50} of HERG inhibition to C_{max}^{free}, could be lowered to at least 10^{14}. This would reduce the likelihood of overlooking a highly potent Eag1 inhibitor, but due to the higher likelihood of HERG inhibition, a more rigorous in vivo LQT and TdP risk assessment is therefore required.

5 | CONCLUSION AND OUTLOOK

The relevant role of Eag1 in the pathophysiology of (mainly) cancer and the specificity of its expression pattern justify the efforts to identify potent and selective inhibitors.

Although it is known that antibodies can provide sufficient specificity, they have shown limited efficacy in vivo. The current research is mostly coming from the natural toxins, therefore development is oriented to the smaller biological molecules that target the channel to achieve tumor specificity but use an alternative strategy to attack the tumor cell. Such approaches display the advantages but also the disadvantages of biological molecules, like the lower permeability, consequently a limited choice of application modes as well as the possibility of triggering the adverse immune reaction. Going smaller than this, into the field of small molecule drug discovery, can overcome the shortcomings of biological agents showing the need for potent Eag1 small molecule inhibitors that can, either alone or associated with current therapies, improve the outcome of difficult to treat cancers.

The main obstacle in the development of anti-Eag1 drugs is its remarkable similarity to HERG, which can explain why the identification of Eag1 inhibitors has up to now led to compounds that block both channels with similar affinities. The differences in the functional properties between the two channels, together with their different regulation, open a window for a new generation of compounds that either target a specific functional property or interfere with downstream pathways. Accurate information of the function and regulation of both channels, in combination with the structural knowledge achieved in the last years and studied effects of biological and small molecules, should allow the rational design of Eag1-targeted anticancer compounds.

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Structure and ligand based drug design; Design and synthesis of voltage-gated potassium channels modulators (K_v1.3 and hEAG1); Design and synthesis of novel antibacterial compounds; Toxicological testing in the early drug discovery process; in vitro studies of drug metabolism; testing compounds on nuclear receptors; metabolism of xenobiotics and activity studies of their metabolites; and bimolecular mechanism studies of toxicity.

Luis Pardo's work has been focused on the structure and function of ion channels, mainly potassium channels. After the discovery of the implication of Kv10 channels in cancer in the late 1990s, the interest of the group is centered on understanding the mechanisms ad roles of potassium channels during tumor progression and on the design of diagnostic and therapeutic tools targeting them. Luis Pardo is a Max-Planck research group leader at the Max Planck Institute of Experimental Medicine, Göttingen, Germany, since 2008. He was affiliated with the Department of Molecular Biology of Neuronal Signals, led by Prof. Walter Stühmer since 1996. Between 2001 and 2004, he served as CSO at iOnGen AG, Göttingen Germany, a company he founded, together with Walter Stühmer and the Max-Planck Society in 2001. From 1993 to 1996, he was a scientist at the Biochemistry Department, University of Oviedo, Spain. Previously, he had received post-doctoral training (1991–1993) in the Department led by Prof. E: Neher at the Max-Planck Institute for Biophysical Chemistry (Göttingen, Germany), after completing his PhD degree at the Biochemistry and Molecular Biology Department at University of Oviedo (Spain). He also received his MD from this University in 1986.

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