

DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

**Pharmacological modulation of voltage-gated ion channels
by small molecules**

by Geraldo Jorge Domingos

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DOCTORAL SCHOOL OF MOLECULAR MEDICINE

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Supervisor: Zoltán Varga, PhD, DSc



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LIST OF ABBREVIATIONS

VGIC: Voltage-Gated Ion channel

VSD: Voltage Sensor Domain

PD: Pore Domain

VSOP: Voltage Sensor Only Protein

Hv1: Voltage-Gated Proton Channel

Nav: Voltage-Gated Sodium Channel

Kv: Voltage-Gated Potassium Channel

Ca_v: Voltage-Gated Calcium Channel

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

NOX: NADPH Oxydase

ROS: Reactive Oxygen Specie

BCR: B Cell Receptor

CD8: Cluster of Differentiation 8

CatSper: Cation channel of sperm

pH: Potential of Hydrogen

MDSC: Myeloid-Derived Suppressor Cell

WT: Wild-type

GFP: Green Fluorescence Protein

GBI: 2-Guanidinebenzimidazole

ClGBI: 5-Chloro-2-guanidinobenzimidazole

P-region: Pore region

SF: selectivity filter

IFM: Isoleucine-Phenylalanine-Methionine

DEKA: Aspartate-Glutamate-Lysine-Alanine

Ig: Immunoglobulin

TTX: Tetrodotoxin

SXT: Saxitoxin

Kir: Inwardly rectifying K⁺ channels

K2P: Two-pore domain K⁺ channels

K_{Ca}: Calcium-activated K⁺ channels

IKCa or SK4: Intermediate-conductance KCa3.1

GPCRs: G Protein-Coupled Receptors

V_{thr}: Activation threshold

V_h: Holding potential

NMDG: N-Methyl-D-glucamine

HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

EGTA: Ethylene glycol-bis (β-aminoethyl ether)-N, N,N',N'-tetraacetic acid

ECS: Extracellular Solution

ICS: Intracellular Solution

DMSO: Dimethyl sulfoxide

CHO: Chinese Hamster Ovary

PBMCs: Peripheral Blood Mononuclear Cells

FBS: Fetal Bovine Serum

DGC: Density Gradient Centrifugation

RPMI: Roswell Park Memorial Institute

PHA: Phytohemagglutinin A

HEK: Human Embryonic Kidney

RCF: Remaining Current Fraction

E_{rev} : Reversal Potential

G_{norm} : Normalized Conductance

G-V: Conductance-Voltage

IC₅₀: Half maximal inhibitory concentration

NZ-58: N-(3-(2-amino-1H-imidazol-5-yl) phenyl)-5-(trifluoromethoxy)-1H-indole-carboxamide

PREFACE

Voltage-Gated Ion Channels (VGIC) are transmembrane proteins that form pathways for ions to cross the plasma membrane controlled by the cellular membrane potential. They are at the core of many physiological, pathophysiological and pharmacological processes involved in electrical signaling such as neurotransmission, muscle contraction and hormone secretion. Malfunctioning or changes in the expression levels of these proteins results in a variety of disorders named channelopathies. Thus, VGICs serve as targets for a broad array of pharmacological interventions, including small molecules, gene therapies and antisense oligonucleotides.

In my research, I worked with small molecules targeting voltage-gated ion channels, developed by our pharmaceutical chemist collaborators at the University of Ljubljana in Slovenia. We aimed for their in-depth biophysical characterization and to unveil their mechanisms of action for research applications and potentially for future therapeutic use.

We first searched for a novel inhibitor targeting specifically the voltage-gated proton channel, H_v1. By using the open structure of the channel, our collaborators performed virtual screening and identified hit molecules that we tested experimentally in our system. The most potent compound was fully characterized in detail to understand its biophysical parameters and mechanism of action by using standard patch clamp methods. We further found that the compound also inhibited other off-target channels at higher concentrations. However, when tested at lower concentrations, the compound unveiled an interesting mechanism of action that was further characterized on Nav1.5 and K_v1.3 channels, which have some similar but also some distinct gating properties.

By using a variety of voltage protocols and patch clamping configurations, the molecule named NZ-58 was biophysically characterized and its interaction mechanism with the VSDs of the voltage gated ion channel was unveiled.

INTRODUCTION

Voltage-Gated Ion Channels (VGIC)

Voltage-Gated Ion Channels (VGIC) are transmembrane proteins with permeation pathways for ions such as Na^+ (Na_V), K^+ (K_V), Ca^{2+} (Ca_V), and H^+ (H_V) across the plasma membrane, controlled by the cellular membrane potential. VGIC are essential for generating action potentials in excitable cells, but they are also extensively expressed by non-excitabile cells where they control a plethora of cellular functions such as volume-, pH- or osmoregulation, transport mechanisms and many others via regulating the membrane potential [1-4]. In general, these channel proteins belong to the same family and are formed by a voltage-sensing domain (VSD) and a pore domain (PD), connected by a linker in each of the four subunits or domains to result in four times six transmembrane segments (S1-S6) in a functional channel. As noted later H_V1 represents an exception to this rule.

Comparison of the members of the VGIC family

Despite belonging to the same family and sharing a conserved structural framework, voltage-gated ion channels also differ in how their pore-forming units are assembled. For example, potassium channels, which are highly selective to K^+ ions are built from subunits that assemble as homo- or hetero-tetramers, arranged symmetrically around a central ion-conducting pore (Fig. 1) [5, 6]. On the other hand, channels only permeable to Na^+ or Ca^{2+} ions share the closest structural similarities, forming a single, large, and continuous polypeptide- α -subunit often referred to as the pore-forming α -subunit, that contains four homologous domains each of which corresponds to the monomeric K_V channel subunits [7-9]. The pore-forming main subunit is often co-assembled with various auxiliary subunits, which fine-tune channel properties such as gating kinetics, trafficking, and pharmacological sensitivity. Notwithstanding, the H_V1 channel (Fig. 2), deviates from this canonical architecture by having only the VSD and lacking the PD [10-12].

Full elucidation of the structure-function relationships of VGICs is essential for understanding how these proteins sense voltage, undergo conformational changes, and regulate ionic flux across membranes, which is critical for clarifying how channel dysfunctions contribute to a wide spectrum of human diseases: from cardiac arrhythmias and epilepsy to neuropathic pain and

cancer. Nevertheless, VGICs constitute one of the largest and pharmacologically most attractive family of drug targets, with a significant proportion of FDA-approved therapeutics acting directly or indirectly on these channels.

Functional features of VGIC: transitions, VSDs and PD

The fundamental features of voltage-gated ion channels including conductance, ion selectivity, pharmacology, and voltage dependence are dictated by several factors: the amino acid sequence of the α -subunit, the contribution of accessory subunits, the lipid composition of the membrane, and the intra- and extracellular ionic environment [9, 13, 14]. During gating, VGIC transition among distinct conformational states: a closed or resting state, an open or activated state, and a non-conductive inactivated state. Although functionally both are non-conducting, the resting and inactivated states represent distinct conformational states. The channels reside in the resting state in the absence of the activating stimulus, while they usually transition into the inactivated state in the extended presence of the activating stimulus to prevent significant ion concentration changes. The VSDs share a highly conserved architecture and play a central role in these transitions. They contain multiple positively charged residues, mostly arginines, interspersed with hydrophobic residues, and balanced by negatively charged acidic residues. This arrangement enables voltage sensing and coupling to pore opening [9, 15-19]. The PD in VGICs is formed by a conserved architecture in which the S4–S5 linker helix couples the VSD to the two pore-forming α -helices, S5 and S6. The four subunits/domains assemble into a central conduction pathway, through which ions can cross the plasma membrane. Ions move in a fully or partially hydrated state through the pore, but K^+ ions completely shed their hydration shell before passing through the selectivity filter, the narrowest segment of the pore [9, 15-19] .

During depolarization-induced activation, VSDs undergo significant conformational changes. As the positively charged residues within the S4 helices shift through the electric field, they generate a measurable displacement of charge, known as the “gating current,” which can be experimentally monitored to probe the kinetics and energetics of channel activation and inactivation [20-22]. In the resting or closed state, the S4 transmembrane segments, each containing 4-7 regularly spaced positively charged residues, form stabilizing ion-pair interactions with negatively charged residues located in the adjacent S1, S2, and/or S3 helices. These electrostatic interactions, together with the

strongly negative intracellular resting membrane potential, draw the S4 helices inward toward the cytoplasmic side of the membrane at rest. In response to depolarization the S4 helices undergo a rotational displacement along a helical or spiral trajectory. During this outward motion, each positively charged residue in S4 sequentially forms new ion-pair interactions with acidic residues in the surrounding helices. This biophysical mechanism, is often referred to as the “sliding-helix” model, provides a structural framework for understanding how voltage-sensor movement is coupled to pore opening and channel activation [23-25]. The movement of the VSDs is attached to the S6 transmembrane segments by a linker, and this rearrangement permits the intracellular ends of the S6 helices to splay apart, generating the open activation gate and enabling ion flux. Conversely, closure involves the re-establishment of these stabilizing contacts, restoring the constricted configuration of the S6 bundle [19].

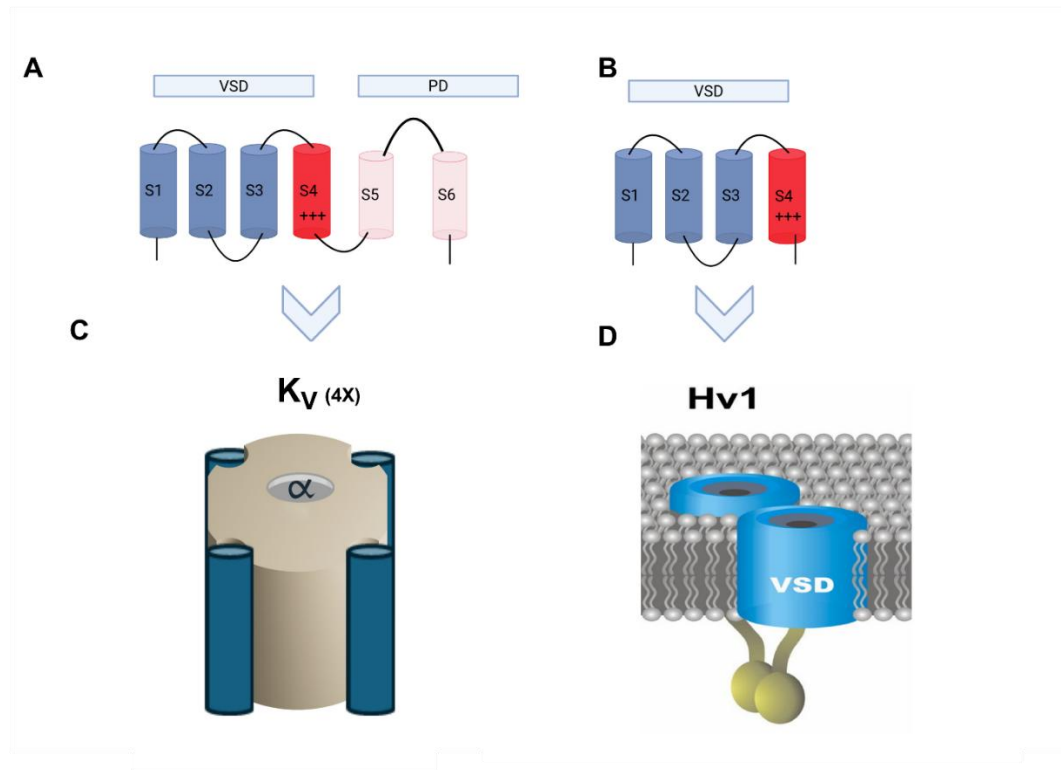


Figure 1: Illustration of the molecular structure of classical VSD-containing proteins. (A and B). Representation of monomers of voltage-gated proteins with numbered transmembrane helical segments. **(A)** A is a representation of a general K_V subunit structure, **(B)** whereas H_V1 , is shown as a voltage-sensing-only protein (VSOP) that lacks a central pore. The first four transmembrane

segments in each monomer, S1-S4, form the VSDs, which play a critical function in channel conformational changes owing to their highly conserved architecture and the presence of positively charged arginine residues in S4 (red) and a central pore formed by the S5-S6 segments (pink), which is not present in Hv1. **(C)** The gray cylinder is a representation of the general functional structure of a K_v channel built from four alpha subunits, with four VSDs (S4 highlighted in blue) and a central pore. **(D)** Hv1 functions as a homo-dimer, where each monomer contains its own conduction pathway and can work independently [26-28].

Overview of the voltage-gated proton channel, Hv1

Throughout the years, the existence of channel proteins exclusively responsible for proton extrusion was met with considerable skepticism. This uncertainty largely arose because of the exceptionally low concentration of free protons in biological systems relative to other ions, making selective proton conduction seem unlikely. Furthermore, the proposed direction of proton movement seemed paradoxical: based on the typically negative membrane potential of most cells, inward proton movement and the resulting intracellular acidification would be the energetically favored process, questioning how a regulated, proton efflux pathway could operate [29, 30]. Nevertheless, these longstanding questions began to take a new direction in 1982, when Thomas and Meech provided the first electrophysiological recordings of proton currents. Their elegant experiments in molluscan neurons demonstrated that membrane depolarization markedly enhanced a large outward proton efflux, offering the first compelling evidence that cells possess voltage-dependent mechanisms capable of mediating proton conduction [30, 31]. However, despite these early physiological insights, the molecular identity of the proton channel remained elusive for more than two decades. The discovery was made in 2006, when two independent laboratories identified the gene encoding the voltage-gated proton channel, first in mouse and subsequently its human homologue, thereby laying the molecular groundwork for a field long constrained to functional observations [11, 32].

Physiological expression of Hv1

Hv1 has a surprisingly wide tissue distribution, spanning a variety of immune cells, sperm cells, tumor cells, cells of the nervous system and other specialized tissues and now, recently found in

human chorion-derived mesenchymal stem cells (MSCs) [33]. Its physiological role is most prominently recognized for its essential contribution to innate immunity. In professional phagocytes such as macrophages and neutrophils, H_v1 serves an indispensable role in sustaining the oxidative burst required for the effective elimination of pathogens. Activation of the NADPH oxidase (NOX) complex during phagocytosis leads to rapid electron efflux across the membrane, a process that simultaneously depolarizes the membrane and acidifies the cytosol. Both depolarization and intracellular acidification are conditions that suppress NOX activity, yet they are precisely the stimuli that activate H_v1. By opening under these conditions, H_v1 mediates proton extrusion, thereby counteracting membrane depolarization and restoring cytosolic pH. This compensatory proton efflux maintains the electrochemical environment necessary for continuous reactive oxygen species (ROS) production, ensuring that the oxidative burst proceeds efficiently [34-43]. In the context of adaptive immunity, H_v1 activity has also been identified in human peripheral blood B cells, where it contributes to the regulation of B cell receptor (BCR) signaling. In T cells, H_v1 also has an immunoregulatory function, as recent studies demonstrate that H_v1 activity is required to prevent excessive intracellular acidification and to preserve mitochondrial integrity in naïve CD8⁺ T cells. During the transition from a naïve to an activated state, metabolic reprogramming and increased respiratory demand impose substantial acid-base stress on the cell. H_v1-mediated proton extrusion helps maintain a permissive intracellular pH and supports mitochondrial fitness, thereby enabling efficient T cell activation and early clonal expansion [44].

In spermatocytes, motility and fertilizing capacity are critically dependent on intracellular pH. As spermatozoa transit through the female reproductive tract, a gradual rise in intracellular pH is a key trigger for sperm capacitation within the oviduct, enabling the acquisition of hyperactivated motility and the ability to undergo the acrosome reaction [45, 46]. H_v1 interacts closely with CatSper, the principal calcium channel of the sperm flagellum. By extruding protons and thereby elevating intracellular pH, H_v1 promotes the activation of CatSper, leading to a robust influx of calcium ions. This Ca²⁺ entry is essential for the induction of hyperactivated motility, the progression of capacitation, and the initiation of the acrosome reaction. [45-48]. Concomitantly, pH homeostasis is equally critical in the airway, where the composition and acidity of the airway surface liquid are essential for mucociliary clearance, antimicrobial defense, and overall epithelial function. H_v1, expressed on the apical membrane of human tracheal epithelial cells and airway-derived cell lines, contributes directly to the regulation of this microenvironment. [49, 50].

Pathological expression of Hv1

Beyond its involvement in physiological processes, extensive research has focused on Hv1 due to its emerging roles in neurodegeneration, ischemic stroke, immune disorders, and tumor progression, conditions in which Hv1 is frequently overexpressed [36, 39, 41, 51-55]. Several studies have also identified high levels of Hv1 expression in microglia, the principal resident immune cells of the central nervous system. In these cells, Hv1 plays a key role in supporting proton extrusion during membrane depolarization, thereby sustaining the activity of the NOX complex. Continued NOX function promotes the generation of reactive oxygen and nitrogen species, which, under pathological conditions, contribute to neuroinflammation and tissue injury. In experimental models of Parkinson's disease, Hv1-dependent NOX activation has been linked to enhanced production of pro-inflammatory cytokines, microglia-mediated demyelination, and subsequent neuronal damage. These findings highlight Hv1 as an important modulator of microglial reactivity and a potential contributor to neurodegenerative processes [36, 56, 57]. Hv1-dependent regulation of reactive oxygen species is also central to the immunosuppressive activity of myeloid-derived suppressor cells (MDSCs), particularly within the tumor microenvironment. MDSCs exert potent inhibitory effects on T cell function, and a major component of this suppression involves the extracellular generation of reactive oxygen species. Studies in mouse models have demonstrated that MDSCs express functional Hv1 channels, which are tightly coupled to the activity of the NOX2 complex. By sustaining proton extrusion during NOX2-mediated electron transfer, Hv1 enables prolonged ROS production, thereby reinforcing the immunosuppressive milieu that supports tumor progression and dampens antitumor immune responses [53, 58].

In a highly regulated tumor microenvironment, cancer cells must continuously extrude excess cytosolic protons to maintain intracellular pH within a range compatible with proliferation, migration, tumorigenesis, and metastasis. This stringent pH control is essential because the elevated metabolic activity characteristic of malignant cells generates substantial acid loads. In this context, Wang and colleagues conducted an extensive analysis demonstrating markedly elevated Hv1 expression in human breast cancer tissues and cell lines. High Hv1 levels were strongly associated with poor clinical progression and unfavorable patient outcomes. Their findings further suggested that Hv1 may serve as a valuable biomarker for the diagnosis and

prognosis of colorectal cancer, underscoring the broader relevance of proton channel–mediated pH regulation in tumor biology [51, 59-61].

Structure and function of the Hv1 channel

Altogether, the remarkable diversity of Hv1 expression across immune, reproductive, respiratory, neural, and cancerous tissues underscores why pharmacological modulation of this channel with therapeutic goals has attracted substantial interest. In oncology, selective Hv1 inhibition is being explored as a strategy to counteract tumor growth, metabolic adaptation, and metastatic potential by disrupting the proton-extrusion mechanisms that malignant cells rely on to maintain intracellular pH homeostasis.

Beyond its physiological and pathological relevance, the structural architecture of Hv1 has captivated biophysical and electrophysiological research. Unlike classical voltage-gated ion channels, which typically contain four VSDs surrounding a central pore, Hv1 is composed solely of a VSD and lacks a canonical pore-forming region. Physiologically, it normally operates as a homo-dimer, in which each subunit functions both as a voltage sensor and as the conduction pathway [11, 12, 62]. Dimerization is mediated primarily by interactions between the cytoplasmic C-terminal domains, but the dimer interface is also formed by parts of the S1 and S4 helices, which enables the two subunits to gate cooperatively [51, 52, 54]. Each subunit is comprised of 273 amino acid residues, and possesses a voltage-sensor–controlled gate capable of mediating proton flux on its own [51-54]. Deleting both the N and C termini ($\Delta N/\Delta C$) simultaneously results in monomeric constructs leading to five- or sixfold faster activation [51-54].

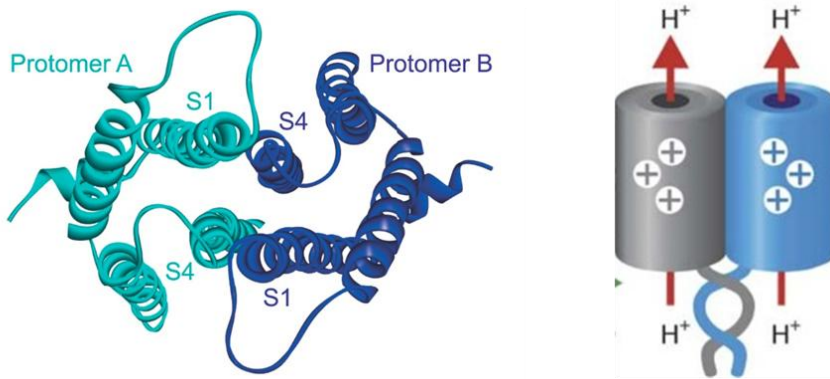


Figure 2. Structure of the Hv1 channel. The schematic on the left illustrates the helical structure of each protomer represented in 2 different colors (cyan and dark blue) whereas the schematic on the right depicts the dimeric architecture, in which each VSD subunit has its own proton permeation (red arrow) and opening of one subunit favors the opening of the neighboring subunit. The presence of positively charged arginines 205, 208, and 211 residing in the S4 are represented with plus signs in each protomer, the central components of the voltage-sensing machinery, [63, 64].

Hv1 has a very small conductance with characteristic current that is highly dependent on ΔpH , the difference between the intracellular (pH_i) and extracellular (pH_o) pH. Increasing pH_o or lowering pH_i shifts the voltage dependence of current activation to more negative potentials, a crucial mechanism evolved to allow a voltage-dependent proton flux from the inside to the outside of the cell, thus allowing these channels to serve as efficient regulators of intracellular pH [12, 65, 66]. In both natively and heterologously expressed Hv1 dimeric or monomeric structures the voltage of half maximal conductance shifts -40 mV for every 1 unit decrease of ΔpH [66].

Despite substantial advances in characterizing the conformational transitions that underlie Hv1 channel gating, the molecular principles governing proton permeation remain only partially resolved. This makes Hv1 a particularly compelling subject of investigation across structural, biophysical, and computational studies [67-70]. One prominent hypothesis for proton transfer through Hv1 is the Grotthuss mechanism, a process fundamentally distinct from the diffusion of other cations. In this model, a proton does not traverse the channel as a discrete particle; instead, it is relayed through a hydrogen-bonded network of water molecules. A single proton associates

with a water molecule to form a hydronium ion (H_3O^+). Within this dynamic structure, one of the hydronium's protons can be transferred to an adjacent water molecule that is properly oriented to accept it, thereby generating a new hydronium ion. This rapid, stepwise relay, often described as “proton hopping”, enables efficient proton conduction without requiring bulk water flow or the physical movement of a specific proton along the entire permeation pathway [30, 38, 71]. Mutagenesis studies have shown that substitution of Asp112 in human Hv1 abolishes proton permeation while leaving gating largely intact. This conserved acidic residue is positioned within a hydrophobic layer formed by two conserved phenylalanine residues, a structural arrangement that likely prevents water penetration and ion conduction in the closed state. Upon channel opening, however, the aspartate side chain consistently interacts with the arginines of the S4 segment, suggesting that its electrostatic environment is dynamically reshaped to support proton transfer. These interactions may help stabilize a water wire, acting as a proton acceptor/donor, or create an electrostatic filter that enforces strict proton selectivity [10, 11, 72-74].

Pharmacology and modulation of Hv1

Several classes of Hv1 modulators have been identified and extensively studied [75-83]. However, inorganic zinc ion remains the prototypical non-selective Hv1 blocker [38, 39, 77, 84, 85], exhibiting competitive inhibitory effects with protons for binding sites on the two histidines at the extracellular side [38, 39, 84, 85]. Organic inhibitors, such as guanidine derivatives have also been developed, most notably the 2-guanidinebenzimidazole (2-GBI) and its modified version, the 5-Chloro-2-guanidinobenzimidazole (ClGBI). In contrast to zinc ions, these bind to the intracellular side of the channel in the open state but ClGBI is able to cross the membrane, so it is also effective when applied extracellularly [61, 77, 78, 86]. The same research group identified a blocker named HIF, targeting the VSD in both open and closed states and shifting the voltage dependence of activation [77]. By using virtual screening, YHV98-4 was identified as an inhibitor of Hv1, which reduced ROS production in inflammatory pain. While the reported affinity was high ($\text{IC}_{50} \sim 0.7 \mu\text{M}$), the block saturated at about 50 % even at high concentrations [80]. Nevertheless, a recent study reported the discovery of novel, bioavailable Hv1 inhibitors, named, PNX52429 and PNX61442, demonstrating therapeutic potential through Hv1 inhibition in a myeloid sarcoma xenograft model [82]. Additionally, several antipsychotic drugs including chlorpromazine,

haloperidol, and clozapine have been reported to suppress proton currents in microglia, in the central nervous system. [61, 87, 88]. In addition, venom-derived peptide toxins such as Hanatoxin, AGAP, and Corza6 [39, 61, 79, 83] have been shown to inhibit H_V1 channels expressed in spermatocytes, [61, 79, 81]. Of note, several of the reported H_V1 inhibitors did not work in our hands, including YHV98-4, the HIF and PNX compounds and the Corza6 toxin, and their effectiveness has not been verified by other independent groups either. On the opposite end of the spectrum, activators of H_V1 have also been identified. Notably, Okamura's group demonstrated that arachidonic acid can enhance H_V1 proton current, providing insight into lipid-mediated regulation of proton channel function [89]. Other molecules stimulating H_V1 activity by enhancing its gating are the phorbol ester PMA, the chemotactic peptide, fMLF [30] and the endocannabinoid neurotransmitter anandamide [48].

Overview of voltage-gated sodium channels

Nav channels are responsible for initiating action potentials within a few milliseconds in neurons, skeletal and cardiac muscle cells, and as such, their mutations are involved in pathologies related to excitable tissues such as epilepsies and intellectual disabilities [90, 91], myotonia and paralysis [92-94], cardiac arrhythmias [95, 96] and pain disorders [97-101]. Early biochemical experiments demonstrated that sodium channels are composed of a pore-forming α subunit of 260 kDa assembled with auxiliary 30–40 kDa β -subunits [102, 103]. Further insights into the structure of voltage-gated sodium channels by cloning and sequence analysis, revealed a continuous large protein consisting of about 2000 amino acid residues with four internally homologous domains, each containing multiple transmembrane segments as described above [104].

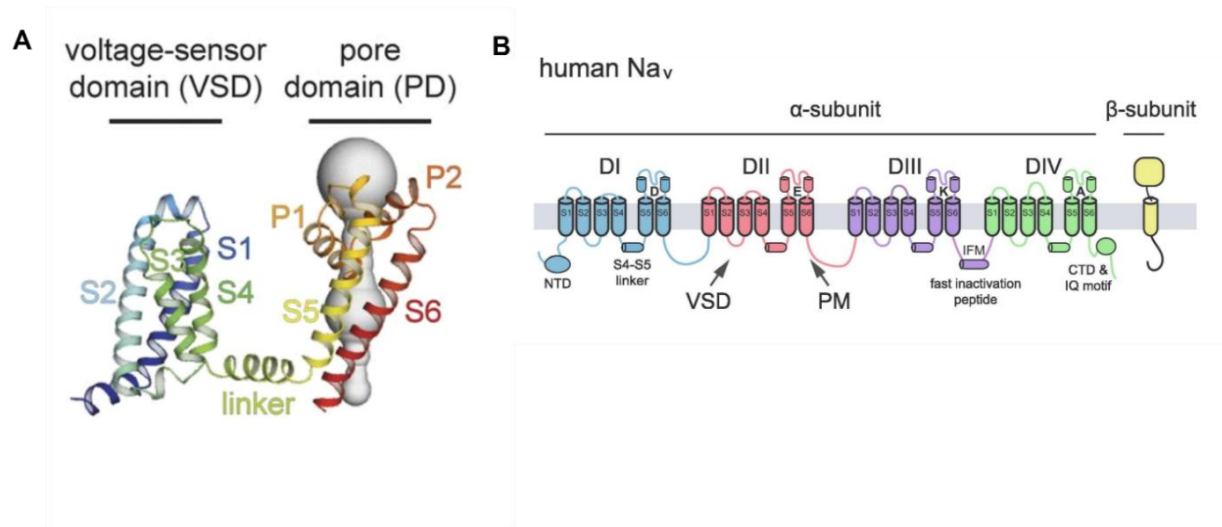


Figure 3. Overview of the structural organization of Nav channels. (A) Depiction of the VSDs, composed of transmembrane helices S1–S4, positioned adjacent to the PD, formed by S5–S6 and the P1/P2 pore helices. The diagram highlights how the S4–S5 linker couples voltage sensing to pore opening, and shows the arrangement of these structural elements as the fundamental building blocks repeated across the four domains of the Nav α -subunit. (B) Schematic structure of the human Nav channels, with the four domains color-coded (DI in blue, DII in red, DIII in purple, DIV in green). The β -subunit (yellow) is positioned peripherally, consistent with its roles in stabilizing channel conformation and influencing voltage-dependent gating [20, 105].

The nine α -subunit genes encode the mammalian voltage-gated sodium channel subtypes Nav1.1 to Nav1.9 and share around 50-80% amino acid sequence homology [26, 106-110]. They are widely expressed throughout the body where Nav1.1, 1.2, 1.3, and 1.6 are found predominantly in neurons of the central nervous system [90, 108, 110, 111], Nav1.4 is expressed in the skeletal muscles [92, 108, 110] while Nav1.5 is distributed in cardiac muscle tissues [95, 108, 110]. Nav1.7, 1.8, and 1.9 are located largely in the neurons of the peripheral nervous system [26, 106, 107, 109, 110]. In addition, voltage-gated sodium channels are also widely expressed in non-excitable cells including lymphocytes, macrophages, fibroblasts, endothelial and pancreatic β -cells [112-114], where they regulate a variety of physiological functions, as well as in cancer cells, in which they contribute to invasion [115-117]. Nav channels are divided into two major groups based on their sensitivity to the classical Nav channel blocker tetrodotoxin (TTX, Table 1).

Table 1. Summary of the human Nav channel family

SUMMARY OF hNav CHANNEL FAMILY				
Channel type	Gene symbol	Tissue expression	TTX sensitivity	References
Nav1.1,	<i>SCN1A</i>	CNS, PNS	Highly sensitive	[90, 108-110]
Nav1.2	<i>SCN2A</i>	CNS	Highly sensitive	[90, 108-111]
Nav1.3	<i>SCN3A</i>	CNS	Highly sensitive	[90, 108-111]
Nav1.4	<i>SCN4A</i>	Skeletal Muscle	Highly sensitive	[92, 108-110]
Nav1.5	<i>SCN5A</i>	Heart, Skeletal muscle	Resistant	[95, 108-110]
Nav1.6	<i>SCN8A</i>	CNS, PNS, Glia, Nodes of Ranvier	Highly sensitive	[90, 108-111]
Nav1.7	<i>SCN9A</i>	PNS	Highly sensitive	[26, 106, 107, 109, 110]
Nav1.8	<i>SCN10A</i>	PNS, DRG	Resistant	[26, 106, 107, 109, 110]
Nav1.9	<i>SCN11A</i>	PNS	Resistant	[26, 106, 107, 109, 110]

Structure and function of Nav channels

As described in the sections above, the channel is composed of six membrane-spanning α -helical segments (S1–S6) within each of the four homologous domains (I–IV), which combine to give roughly 2,000 amino acids for the entire pore-forming subunit [26, 105, 118].

The functional behavior of voltage-gated sodium (Nav) channels is governed by transitions among three principal conformational states: the closed or resting state, the open or activated state, and non-conductive inactivated states. Inactivation itself comprises at least two kinetically distinct processes: fast and slow inactivation occurring on different timescales and reflecting different

structural rearrangements within the channel. These gating transitions are driven by the movement of the voltage-sensing domains (VSDs) in response to changes in membrane potential.

Inactivation mechanisms of Nav channels

Voltage-clamp fluorometry (VCF) has been instrumental in tracking the real-time movement of individual S4 helices. VCF is a technique, in which an environment-sensitive fluorophore is attached to the channel near the VSD and its voltage-dependent conformational changes are tracked by changes in fluorescence intensity. These studies revealed that VSD-I, VSD-II, and VSD-III activate rapidly and in a strongly voltage-dependent fashion, closely correlating with the opening of the central pore. In contrast, the activation of VSD-IV occurs later and more slowly, and is the requirement for the channel to enter into its inactivated states. Together, these findings establish a sequential model in which early VSD activation initiates pore opening, whereas the subsequent activation of VSD-IV serves as the critical trigger for the transition into fast and slow inactivation [105, 119, 120].

Inactivation of voltage-gated sodium (Nav) channels is essential for terminating the inward sodium current, enabling action-potential repolarization, and maintaining controlled excitability in neurons and muscle cells. Two mechanistically distinct forms of inactivation: fast and slow are observed across Nav isoforms. Fast inactivation occurs within a few milliseconds after channel opening and is mediated by the IFM (Isoleucine-Phenylalanine-Methionine) motif of the DIII–DIV linker, which rapidly occludes the pore. Slow inactivation, by contrast, develops over much longer timescales (~100 ms to several minutes) and reflects broader conformational rearrangements within the pore domain and selectivity filter during sustained or repetitive depolarizations.

The kinetics and voltage dependence of these processes vary substantially among Nav isoforms, aligning with their specialized physiological roles. Neuronal channels such as Nav1.2 exhibit prominent faster inactivation time constants compared to those of cardiac Nav1.5 (0.27 and 0.41 ms, respectively at 20 mV). [121-123]. A critical hydrophobic triad, IFM, located within the intracellular linker connecting domains DIII and DIV is the motif that functions as the structural element required for fast inactivation. The DIII–DIV linker acts as a mobile “inactivation gate” and the IFM residues form the hydrophobic particle that rapidly occludes the pore following channel opening. Consistent with this model, fast inactivation is markedly disrupted or slowed

when this linker is proteolytically cleaved, experimentally displaced, or mutated at key positions within the IFM sequence [122].

The mechanism underlying slow inactivation in Nav channels is considerably more complex than that of fast inactivation and appears to involve extensive conformational rearrangements within the pore region. These structural transitions occur distinct from those mediating fast inactivation and remain less well understood at the molecular level [122, 123]. Notably, slow inactivation shares several mechanistic features with C-type inactivation in potassium channels, including alterations near the selectivity filter and pore constriction during prolonged depolarization. This resemblance has led to the view that slow inactivation represents a pore-based gating process that modulates channel availability over longer timescales. [122, 123].

The permeation pathway and the selectivity filter

The pore domain formed by the S5 and S6 segments from the four homologous repeats provides structural support for the selectivity filter (SF) that determines the ion selectivity. Nav channels confer a roughly 100-fold preference for Na⁺ ions passing through the SF vestibule in a hydrated or partially dehydrated state according to the dimension of the permeation pathway over other cations most notably K⁺, while Ca²⁺ ions effectively block permeation. Each domain contributes a residue to create the characteristic DEKA locus (DI-Asp, DII-Glu, DIII-Lys, DIV-Ala), which defines a narrow extracellular constriction of approximately 3.5 Å × 4 Å. This asymmetric configuration shapes the electrostatic and steric environment required for Na⁺ coordination while excluding larger or differently hydrated ions. [105, 124].

Although heterologous expression of the α -subunit alone can produce functional Nav channels, native channels in excitable tissues do not operate in isolation. Instead, they are co-assembled with a set of auxiliary β -subunits (β 1– β 4), each of which consists of a single transmembrane helix, an extracellular immunoglobulin-like (Ig-like) domain, and a short intracellular C-terminal tail. The Ig-like domain enables interactions with extracellular matrix components and cell-adhesion molecules, while the intracellular C-terminus participates in signaling and trafficking pathways.

Pharmacology of voltage-gated sodium channels

The high number of sodium-channel related channelopathies spanning epilepsy, neuropathic pain, and cardiac arrhythmias fuels efforts to develop modulator compounds with defined sodium channel pharmacology. Although some natural compounds, such as the potent neurotoxins tetrodotoxin (TTX) and saxitoxin (SXT) are known to exert their effect through sodium channels, most Nav-targeting drugs in the clinical practice are synthetic compounds.

Widely used anticonvulsants such as carbamazepine and phenytoin target Nav channels in the central nervous system, while class I antiarrhythmic agents, like mexiletine and flecainide act on the cardiac Nav channels to suppress aberrant electrical activities. The local anesthetics, most notably, lidocaine and bupivacaine are widely employed in clinical practice [19, 125]. On the other hand, Nav channel activators represent a distinct class of modulators, Among these, toxins such as batrachotoxin as well as plant-derived agents like pyrethroid and veratridine are known to activate Nav channels [125-127]. Even though loss-of-function mutations of Nav channels are also associated with certain channelopathies, such as congenital insensitivity to pain (Nav1.7), Dravet syndrome (Nav1.1) or Brugada syndrome (Nav1.5), Nav activator compounds are currently not employed in clinical practice.

Several clinical candidates have emerged, including AZD-3161, reported to have advanced to Phase I following intradermal administration, and XEN402, which demonstrated a positive Phase II outcome in acute pain when applied topically. Likewise, PF-05089771 has progressed into Phase II clinical evaluation [125, 128-131]. Just last year, a major milestone in pain therapeutics was achieved when suzetrigine (VX-548) was approved by the FDA as the first oral, non-opioid analgesic targeting Nav1.8 in peripheral neurons [19, 132].

Binding mechanisms of drugs targeting Nav channels

As recent reviews by Wisedchaisri et al. and Alsaloum et al. demonstrate, Nav channels are targets of numerous modulator compounds with several typical binding sites [106, 133]. These sites could be more accurately localized and described with the availability and precision of high-resolution cryo-electron microscopic structures. In order to facilitate the unambiguous identification of these binding sites, a systematic structural atlas was recently developed [134].

The majority of Nav-targeting drugs used in the clinic are known to bind within the central cavity, including anti-arrhythmic agents and local anesthetics, [106, 135, 136], while the best-known small-molecule Nav inhibitor, tetrodotoxin, together with several peptide blockers such as μ -conotoxin KIIIA, occludes the permeation pathway by engaging the selectivity filter at the extracellular pore entrance [137, 138]. In addition, other sites are located along the permeation pathway at and below the activation gate, as well as sites accessible via the lipid phase through fenestrations [134]. In contrast to the highly conserved central cavities of Nav isoforms, which show high homology, the VSDs show much more distinct properties and therefore giving more promising alternatives for subtype-selective targeting [106]. Nevertheless, there are currently much fewer VSD-targeting modulators known, the majority of which are gating-modifier toxins originating from animal venoms. The extracellular region of the DIV-VSD, traditionally referred to as “receptor site 3,” serves as the binding location for α -scorpion toxins. These toxins suppress fast inactivation by locking the VSD in an intermediate conformation [139]. Meanwhile, “Receptor site 4” primarily found on the DII-VSD and engaged by β scorpion and spider toxins [106]. These modulators can produce either excitatory or inhibitory effects depending on whether they stabilize the VSD in the activated or the resting state. By comparison, the VSDs of DI and DIII are much less targeted by natural toxins, likely due to reduced accessibility imposed by the presence of auxiliary β -subunits [134]. More recently, highly specific aryl sulfonamide compounds have been developed with the aim to alleviate chronic pain via Nav1.7 inhibition. They act by binding to and stabilizing the DIV-VSD in the activated conformation, keeping the channel inactivated [140].

However, despite the promising high selectivity, clinical trials have failed to demonstrate their therapeutic applicability. On the contrary, the aforementioned VX-548 became the first FDA-approved selective Nav1.8 inhibitor for the treatment of acute pain [132, 141]. This molecule acts by binding to the DII-VSD of Nav1.8 preferably in its resting state, stabilizing it and thereby inhibiting channel opening. Notably, it differs from many other Nav inhibitors in that it shows reverse use-dependence, meaning that its effectiveness declines with repetitive or strong depolarizations.

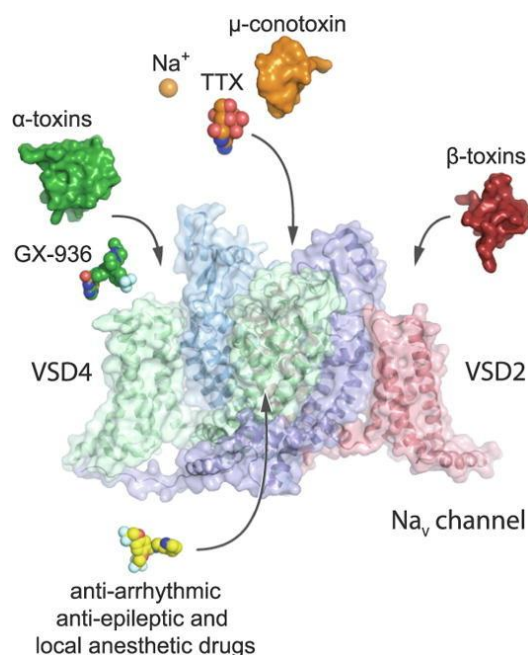


Figure 4. Schematic representation of the binding sites of Na_v channel modulators. The figure highlights representative modulators and their cognate receptor regions. For visual clarity, β -subunits and remaining VSDs are not shown [105].

Overview of voltage-gated potassium channels

Potassium (K^+) channels constitute the largest and structurally most diverse family within the voltage-gated ion channel superfamily. They mediate the highly selective efflux of K^+ ions by which they shape the repolarization phase of action potentials, stabilize the resting membrane potential, and regulate processes such as hormone secretion. While most K^+ channels reside primarily in the plasma membrane, several subfamilies are also expressed in intracellular organelles, where they contribute to organelle-specific ionic homeostasis and influence internal signaling or metabolic regulation [142-146].

Structurally, K^+ channels are traditionally classified according to the number and organization of their transmembrane (TM) domains into four major groups: inwardly rectifying K^+ (Kir) channels, two-pore domain K^+ (K_{2P}), Calcium-activated K^+ (K_{Ca}) and voltage-gated K^+ (K_V) channels [5, 142, 146-148]). With the exception of K_{2P} channels, which assemble as dimers containing two

pore-forming domains per subunit, all other K^+ channel families form functional tetramers composed of four pore-forming subunits [149].

Despite their structural and functional diversity, all known K^+ channels belong to a single evolutionary protein family defined by a conserved “signature sequence” located between two transmembrane helices; this motif forms the selectivity filter positioned in the extracellular third of the ion permeation pathway, between the central cavity and the external solution. This signature sequence is GYGD (or in some channels GFGD) in mammalian K^+ channels. Ion selectivity arises primarily from size exclusion, as the dehydrated ionic radius of K^+ (1.33 Å) closely matches the geometry of the filter, whereas the smaller Na^+ ion (0.95 Å) cannot be efficiently stabilized within the carbonyl-lined coordination sites. Notably, no other ion channel class exhibits the same degree of evolutionary conservation and ubiquity across all domains of life as K^+ channels [142, 143].

As my work only involved K_V channels and the $K_{Ca}3.1$ channel of the numerous K^+ channels, only these will be introduced below.

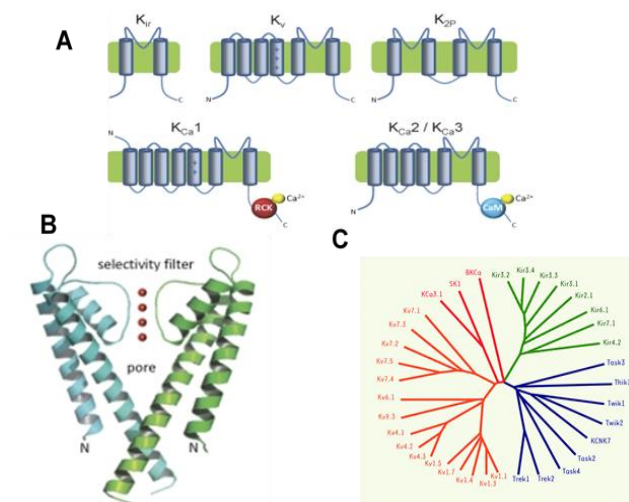


Figure 5. Structural diversity in the family of potassium channels (A) Illustration of the K^+ channel family, arranged according to their subunit structure (TMDs), functional domains and termini. Plus, signs denote charged basic residues within the voltage sensor (S4) region of K_V and $K_{Ca}1$ channels. In contrast to other K^+ channel subunits, $K_{Ca}1.1$ channel subunits have extracellular N-termini. RCK denotes a Ca^{2+} -binding regulatory domain of large conductance K^+ channels. CaM denotes calmodulin (CaM) bound to a CaM-binding site within the channel subunit. CNBD

denotes a cyclic nucleotide monophosphate (cNMP) binding site; **(B)** The crystal structure of the KcsA pore domain is shown, with only the TMDs and pore loops of two subunits depicted for clarity. Red circles represent the binding sites for K^+ ions in the selectivity filter. **(B)** A phylogenetic tree of the different classes of K^+ channels, distinguished by different colors [5, 142, 146, 147].

Calcium-activated potassium channels (K_{Ca})

Calcium-activated potassium (K_{Ca}) channels are K^+ -selective channel proteins that rely on intracellular Ca^{2+} to regulate their gating behavior. Traditionally these channels couple fluctuations in cytosolic Ca^{2+} to membrane hyperpolarization, thereby fine-tuning neuronal excitability, stimulation and release of immune cells and controlling smooth muscle tone. K_{Ca} channels are generally divided into three major families: small-conductance (K_{Ca2}), intermediate-conductance ($K_{Ca3.1}$), and large-conductance ($K_{Ca1.1}$ or BK) channels, according to their unitary conductance, biophysical features, and susceptibility to pharmacological modulation.

The intermediate-conductance $K_{Ca3.1}$ (IK_{Ca} or SK4)

The intermediate-conductance $K_{Ca3.1}$ channel (also known as IK_{Ca} or SK4), encoded by *KCNN4* gene, was first isolated from the pancreas, Chinese hamster ovary cells, and activated T lymphocytes [150-152]. $K_{Ca3.1}$ belongs to the broader SK channel family but functionally and biophysically distinct from the small-conductance SK channels. In particular, $K_{Ca3.1}$ exhibits a markedly larger single-channel conductance of approximately 20–80 pS. Like small-conductance K_{Ca2} , $K_{Ca3.1}$ is voltage-independent and activated exclusively through Ca^{2+} binding to constitutively associated calmodulin; however, its intermediate conductance and unique pharmacological profile most notably its sensitivity to TRAM-34 and clotrimazole clearly distinguish it as a separate subfamily within the KCNN gene cluster [150-152].

Functionally, IK channels regulate membrane potential and sustain Ca^{2+} influx in immune cells, thereby modulating T-cell activation, proliferation, and cytokine production. They further participate in regulating vascular homeostasis, epithelial transport, fibroblast proliferation, and disease-associated tissue remodeling mechanisms. The groundwork for elucidating the

pharmacological properties has been facilitated by the molecular identification of KCNN4 as the gene encoding IK channels [153].

Voltage-gated potassium channels (K_V)

Voltage-gated potassium channels (K_V) belong to the subgroup of potassium channels. All K_V channels play a wide range of functions including regulation of the resting membrane potential and control of the shape, duration, and frequency of action potentials. They are also involved in the secretion of neurotransmitters and hormones, tight control of apoptosis, or cell fate differentiation and growth. Genomic analyses indicate that around 40 human genes are involved in the K_V superfamily organized into two principal structural and functional categories.

The functional classical six-transmembrane (6TM) K⁺ channels, which assemble to form operational K_V channels, belong in the first category (Table 2) [146, 154, 155]. Among these are the KCNA (K_V1), KCNB (K_V2), KCNC (K_V3), KCND (K_V4), KCNQ (K_V7), and KCNH (K_V10–K_V12) gene families, the latter encompassing the *ether-à-go-go* (EAG), *ether-à-go-go-related* (ERG), and *EAG-like* channels. Members of these families assemble as functional tetramers. The second category consists of a set of electrically silent or non-conducting K_V subunits, which operate as gating modulators instead of independent ion-conducting channels. These include the KCNF (K_V5), KCNG (K_V6), KCVN (K_V8), and KCNS (K_V9) gene families. Despite being incapable to assemble into functional channels on their own, these subunits hetero-tetramerize with conducting K_V α-subunits, thereby modulating their trafficking, voltage dependence, kinetics, and pharmacological properties. This group confers an additional modulatory mechanism of diversity and regulate K_V channel function across excitable and non-excitable tissues [146].

Biophysical features of K_V channels

As described above, all K_V channels share a similar core structure, but differences in their connecting loops, and in the length and sequence of their N and C terminals, make each one of them unique [156].

Similar to the previously described Nav channels, K_V can occupy three major conformational states: closed, activated, and inactivated, each reflecting distinct protein conformations. The closed conformation can be achieved by pinching shut at the intracellular entrance. This intracellular or

S6 gate obstructs entrance from the cytoplasmic surface to the water-filled ‘cavity’ in the center of the channel protein [154]. Upon membrane depolarization, the positively charged S4 helix undergoes an outward displacement generating mechanical force that is transmitted through the S4–S5 linker that leads to rearrangements within the S5 and S6 helices, stabilizing the conductive conformation [157, 158]. Earlier studies showed that the S4 helices in all four VSDs progress through multiple closed or pre-activated states before reaching the activated conformation, and that these transitions occur in a partially cooperative manner across domains [158].

Table 2.: Summary of Kv channels

Group	Gene Families	Nomenclature	Description
Conducting Kv Subunits	KCNA	Kv1.x: Shaker family	Classical 6-TM channels; form functional tetramers; fast activation; key in neuronal excitability
	KCNB	Kv2.x: Shab family	Delayed rectifiers; major contributors to repolarization in neurons and muscle
	KCNC	Kv3.x: Shaw family	High-frequency firing; fast activation/deactivation kinetics
	KCND	Kv4.x: Shal family	A-type currents; regulate action-potential backpropagation and dendritic integration
	KCNQ	Kv7.x: KQT2	M-current channels; crucial for membrane stability and excitability control
	KCNH (EAG, ERG, ELK)	Kv10.x, Kv11.x, Kv12.x: EAG, ERG and EALK	Includes EAG, ERG (hERG/Kv11.1), and EAG-like channels; diverse gating and pharmacology; roles in cardiac repolarization and cancer biology

Nonconducting Kv Subunits	KCNF	Kv5.x: Modifiers	Do not form functional channels alone; heterotetramerize with Kv2-related subunits
	KCNG	Kv6.x Modifiers	Modulate voltage dependence, kinetics, and trafficking of partner Kv channels
	KCNV	Kv8.x: Modifiers	Alter gating and expression of Kv2 family channels; tissue-specific modulators
	KCNS	Kv9.x: Modifiers	Fine-tune excitability by modifying Kv2 channel properties; no independent conductance

Pharmacology of voltage-gated potassium channels

A wide range of endogenous and exogenous molecules can modulate K_V channel activity. A comprehensive pharmacological comparison of several delayed rectifier K_V channels revealed a broadly conserved sensitivity profile to the classical K_V blocker 4-aminopyridine (4-AP), and the channels were also inhibited by a distinct group of modulators including capsaicin, nifedipine, diltiazem, and resiniferatoxin [146, 159, 160]. They demonstrated a wide range of sensitivities to external tetraethylammonium (TEA), with IC₅₀ values ranging from 0.3 to 560 mmol/L. Most members of the K_V1.x family are also blocked by a variety of peptide toxins isolated from animal venoms in the low nanomolar or even picomolar range [161-164]. One of the most potent and selective blockers of K_V1.3, Vm24 (α -KTx 23.1) was isolated and characterized by our research group with a *K_d* of 2.9 pM [165].

Similarly to Na_V channels, modulators of K_V channels may exert their effects via several distinct mechanisms. Many compounds, act by physically occluding the channel pore and preventing K⁺ permeation [161-164], others alter the gating machinery by interacting with the voltage-sensor domain, thereby shifting activation or inactivation kinetics [166, 167]. Recently modulation of K_V1.3 with externally applied nanobodies revealed that one nanobody binds to the VSD and PD

of each subunit, which induces an inactive pore conformation [168]. Finally, additional layers of regulation arise from interactions with auxiliary subunits, which can modify channel trafficking, surface expression, and biophysical properties [159, 160].

Advances in K_V-channel directed therapeutics have led to several promising compounds such as dalazatide, a selective K_V1.3 inhibitor for autoimmune and neuroinflammatory disorders, and azetukalner BHV-7000, pyne gabine, and CB-003, all specific activators of K_V7.2/7.3 channels for neurological indications, now progressing through human clinical trials [160, 169].

Advances and critical challenges in the pharmacology of VGICs

Although G Protein-Coupled Receptors (GPCRs) remain the most heavily targeted protein family in pharmacology, up to 15% of clinically used drugs, as well as a growing number of novel experimental compounds exert their pharmacological effects through direct interactions with VGICs [19, 106, 170]. Despite this fact, the number of VGIC modulators developed into clinically useful drugs has slowed markedly over the past decades, as emphasized by López-Mateos et al. and Alsaloum et al. [19, 133]. Only a few candidates have advanced to clinical application, even with major advances in automated electrophysiology, structural biology, and computational screening. A central obstacle in the process is the high structural and functional homology within the H_V, K_V, Na_V, and Ca_V families, which makes isoform-selective modulation exceptionally difficult. Nevertheless, the vast diversity of potential binding sites and blocking mechanisms suggests that molecules with genuinely novel modes of action may open new avenues for effective VGIC-targeted therapeutics.

AIMS

Considering the widespread tissue distribution of VGICs and their substantial pharmacological relevance, our general aim in our studies was the identification of new pharmacological agents targeting VGICs and understanding their mechanisms. Due to the lack of inhibitors with high affinity and selectivity of H_v1, our initial studies focused on targeting this channel.

Accordingly, our aims were as follows:

1. To screen a diverse set of synthesized small molecules provided by our pharmaceutical chemist collaborators, with the goal of identifying modulators of the H_v1 channel.
2. To discover novel compounds that demonstrate high potency and selectivity toward the H_v1 channel.
3. To characterize the biophysical mechanisms of action of these compounds in order to gain deeper insight into H_v1 function and structural organization.

After completing the full evaluation of the compounds provided by our collaborators and identifying several active small molecules through patch-clamp electrophysiology, we found that most candidates exhibited relatively poor selectivity, blocking multiple voltage-gated ion channels. Interestingly, one molecule, NZ-58 emerged as a “pan-VGIC” modulator, affecting every voltage-gated ion channel tested while showing no activity on non-voltage-gated channels. It demonstrated a previously undescribed mode of action on these channels.

Based on these findings, we aimed to:

1. Further characterize NZ-58 to comprehensively define its interactions with the voltage-sensing domains (VSDs) across voltage-gated ion channels.
2. Explore the similarities and differences in the interaction of NZ-58 with Nav and Kv channels, exemplified by Nav1.5 and Kv1.3, and attempt to identify the key residues responsible for ligand recognition and modulation.
3. Clarify the functional selectivity of NZ-58, that is, how it interacts with various gating states, providing a foundation for rational drug design aimed at developing more selective and effective modulators of voltage-gated ion channels.

MATERIALS AND METHODS

Cell cultures and preparation

We cultured Chinese Hamster Ovary (CHO) cells in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA, Cat# 11965084) augmented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine, 100 µg/mL streptomycin, and 100 U/mL penicillin-G (Sigma-Aldrich, St. Louis, MO, USA). Cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. Cells passages were performed twice weekly and incubated no longer than 180 seconds in PBS containing 0.2 g/L of Ethylenediaminetetraacetic acid (EDTA) (Invitrogen, Waltham, MA, USA).

In conformity with the Ethical Committee of the Hungarian Medical Research Council (36255-6/2017/EKU), blood samples were collected and isolated to obtain the Human Peripheral Blood Mononuclear Cells (PBMCs), from anonymized healthy donors. PBMCs isolation were performed by Histopaque1077 (Sigma-Aldrich Hungary, Budapest, Hungary) density gradient centrifugation (DGC). Following DGC, we resuspended the isolated cells in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Cat# 11875085) with 10% FCS augmentation, (Sigma-Aldrich, St. Louis, MO, USA), antibiotics such as penicillin and streptomycin in the concentration of 100 µg/mL, and 2 mM L-glutamine amino acid. We used cell density of $5-6 \times 10^5$ cells/mL seeded into 24-well culture plates and maintained in a 5% CO₂ incubator at 37 °C for no longer than 3–5 days. To enhance K_v1.3 channel expression, Phytohemagglutinin A (PHA) (Sigma-Aldrich, St. Louis, MO, USA) was added at 8–12 µg/mL. We performed most measurements on K_v1.3 were performed on PBMCs, but the mutant channels and the inside-out experiments requiring high channel expression were performed on transfected CHO cells and described in the corresponding figure legend.

Transient transfection were carried out on CHO cells using a Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's protocol with the following ion channel coding vectors in the table 3 (below). The K_v11.1, *hERG1*, *hKCNH2* gene was a kind gift from H. Wulff, University of California, Davis, CA, USA and Nav1.4, *hSCN4A* gene, was a kind gift from P. Lukács, Eötvös Loránd University, Budapest, Hungary and currents were recorded from stably expressing Human embryonic kidney (HEK) 293 cell lines.

Table 3. Ion channel constructs used for transient transfection of CHO cells in this study

CHANNEL TYPE	GENE CODE	PLASMID NAME	PROVIDING SOURCE
hHv1	<i>HVCN1</i>	pcDNA3	A gift from Kenton Swartz, NIH, Bethesda, MD, USA
hKv1.4	<i>KCNA4</i>	pcDNA3	Heike Wulff, University of California, Davis, CA, USA
hKv1.5	<i>KCNA5</i>	pEYFP-C1	A. Felipe, University of Barcelona, Spain
hNav1.5	<i>SCN5A</i>		A gift from P. Lukács, Eötvös Loránd University, Budapest, Hungary
hKir2.1	<i>KCNJ2</i>		a gift from I. Levitan, University of Illinois, Chicago, USA
hKCa3.1	<i>KCNN4</i>	pEGFP-C1	a gift from B. Attali, Tel Aviv University, Israel

The forementioned cells (CHO cells, PBMCs, and HEK) were gently washed twice with 2 mL of extracellular solution prior to patch-clamp recordings and subsequently replated onto 35-mm polystyrene culture dishes (Cellstar, Greiner Bio-One, Kremsmünster, Austria). Nikon TS100 fluorescence microscope (Nikon, Tokyo, Japan) equipped with 455–495 nm excitation and 515–555 nm emission filters was used to identify GFP-positive transfected cells. Ionic currents were

recorded 24-36 hours after transfection, while hKV1.3 currents were measured in activated lymphocytes 4-5 days after stimulation with PHA.

Electrophysiology

By using the voltage-clamp mode, we performed standard whole-cell patch clamp configuration to record ionic currents. Micropipettes were fabricated in four pulling stages using a Flaming-Brown automatic puller (Sutter Instruments, San Rafael, CA, USA) from GC 150F-15 borosilicate glass capillaries (Harvard Apparatus, Holliston, MA, USA), yielding tip diameters of 0.5–1 μm and typical resistances were 7–10 $\text{M}\Omega$ (due to lower solution conductance) and 3–5 $\text{M}\Omega$ for $\text{Hv}1$ and $\text{Kv}1.3 / \text{Nav}1.5$, respectively. Series resistance (R_s) compensation of 45-70 % was enabled before oscillation was induced. If no stable compensation achieved and the R_s value over 20 $\text{M}\Omega$, we discarded the cell to minimize voltage errors and channel kinetics distortion.

Electrophysiological recordings were performed with Axopatch 200B amplifiers interfaced with personal computers via Digidata 1550B acquisition hardware (Molecular Devices, Sunnyvale, CA, USA). Traces were excluded when the leak current at the holding potential exceeded 10% of the peak current at the corresponding test voltage. All experiments were conducted at room temperature (20–24 $^{\circ}\text{C}$).

In general, recordings of hHv1 currents were elicited using a 1-second voltage-ramp protocol that progressed linearly from a holding potential of -60 mV to $+60 \text{ mV}$. This approach provided a continuous and stable assessment of channel activation across the full voltage range for an extended duration, which was essential for constructing the dose–response curves. In addition, this protocol allowed simultaneous monitoring of the peak current, the activation threshold (V_{thr}), and the tail current measured at -80 mV . The reversal potential was routinely assessed using a tail I–V protocol, with deviations from this approach noted in the main text. The opening voltage of hHV1 channels was determined objectively as follows: we first selected a voltage range in which the open probability of the proton channels is effectively zero and calculated the standard deviation (SD) of the leak-corrected current within this interval. As the membrane potential increased during the ramp, channel opening was defined as the point at which the macroscopic current exceeded

five times the SD for a continuous 5-ms period; the membrane potential at the start of this interval was taken as the activation threshold voltage.

Sodium currents elicited by Nav1.5 and Nav1.4, were recorded with the holding potential (V_h) maintained at -100 mV, and depolarizing pulses delivered at every 5-s unless otherwise noted. Depolarizing steps to 0 mV with durations ranging from 15 to 50 ms were applied. For $K_v1.3$, we applied voltage-step protocols with pulse durations ranging from 15 to 2000 ms. $K_v1.5$ currents were recorded using 100 -ms depolarizing steps to $+50$ mV delivered every 15 s from a holding potential of -100 mV. $K_v1.4$ currents were elicited using a 50 -ms depolarizing step from -100 mV to $+20$ mV. $K_v11.1$ currents were measured with a protocol consisting of a holding potential of -80 mV, a 1250 -ms depolarizing pulse to $+20$ mV, followed by a 2 -s hyperpolarizing step to -40 mV applied every 30 s; peak tail currents were quantified during the hyperpolarizing phase. For $K_{Ca}3.1$, a 200 -ms voltage ramp from -120 mV to $+50$ mV was applied every 10 s. $K_{ir}2.1$ currents were recorded using a 600 -ms step protocol consisting of voltage steps from a holding potential of -10 mV down to -80 mV.

Mutagenesis

QuikChange site-directed mutagenesis method (Agilent Technologies, Santa Clara, CA, USA) was used to introduce the mutations in hHv1 and hKv1.3. For the case of hNav1.5 we used overlapping extension mutagenesis. Mutated residues: V109F, V110F, D112N, F150A, and D185N, were introduced into hHv1, KOD DNA polymerase (71085, EMD Millipore; Sigma-Aldrich, St. Louis, MO). The following mutated residues: R373C and R373A mutations were introduced into the pC1-EGFP-hKv1.3-WT and the residues R1632C and R1632A introduced into the pH1TO-EGFP-Nav1.5 plasmids. Following the one-step site-directed mutagenesis protocol of Huanting Liu et al. [171], we performed two sets of mutagenesis primers in the hKv1.3. The designed primers were with an overlapping $5'$ region and a non-overlapping $3'$ extension (Integrated DNA Technologies-IDT, Leuven, Belgium). The PfuUltra II polymerase enzyme was used (Agilent Technologies, Santa Clara, CA, USA), along with dNTPs and DpnI enzyme (Thermo Fisher Scientific, Waltham, MA, USA). Finally, we designed two-sets of primers for the hNav1.5, in which the overlapping mutagenesis primers and the flanking primers contained restriction sites at the $5'$ and $3'$ ends of the mutation site. These processes were followed by FastDigest (FD) enzymes (FD-KpnI & FD-XhoI) (Thermo Fischer Scientific, Waltham, MA, USA) and, the digested products were extracted

from the gel using QIAquick gel extraction kit (Qiagen, Germany). then after ligated in a 3:1 molar ratio using rapid ligation kit (Thermo Fischer Scientific, Waltham, MA, USA). The ligated constructs were transformed into chemically competent *E. coli* Top10F' cells. All mutant and wild-type plasmids were verified by DNA sequencing (Plasmidsaurus) prior to their use in electrophysiological experiments.

Solutions

Patch clamp solutions were freshly prepared, pH were daily checked before each patch clamp experiments and osmolarities of Extracellular solutions ECS and Intra Cellular Solutions (ECS) were verified by osmometer between 302 and 308 mOsm/L and ~295 mOsm/L, respectively.

Table 4. Composition of the solutions used in this study

CHANNEL(S)	ECS IN mM	ICS IN mM
hH _v 1	75 mM NMDG; 180 mM HEPES; 15 mM glucose; 3 mM MgCl ₂ ; 1 mM EGTA	75 mM NMDG; 180 mM MES; 15 mM glucose; 3 mM MgCl ₂ ; 1 mM EGTA; pH 6.4 (titrated with CsOH) (The same for positive control)
hK _v 1.3, hK _v 1.4, hK _v 11.1, hNav1.4, hNav1.5	145NaCl; 5KCl; 2.5CaCl ₂ ; 1MgCl ₂ ; 10HEPES; 5.5glucose; pH= 7.35 (titrated with NaOH)	140 KF; 2 MgCl ₂ ; 1 CaCl ₂ ; 11 EGTA; 10 HEPES; pH 7.2 (titrated with KOH)
hK _v 1.4, hK _v 1.5 (high-K ⁺ ECS for perfusion indicator)	150KCl; 0 NaCl; otherwise, identical to standard ECS	Same as above for each channel
Nav1.4, Nav1.5	Same as standard ECS above	10NaCl; 105CsF; 10 HEPES; 10EGTA; pH 7.2 (titrated with CsOH)
Nav channels (choline-based, for positive control ECS)	145 Choline-Cl; 5KCl; 10HEPES; 5.5 glucose; 2.5 CaCl ₂ ; 1 MgCl ₂	Same as general NaV ICS above

hK _{ir} 2.1	2.5NaCl; 77.5KCl; 2.5CaCl ₂ ; 1MgCl ₂ ; 10HEPES; 5.5glucose; pH 7.35 (titrated with KOH)	40KF; 2MgCl ₂ ; 1CaCl ₂ ; 11EGTA; 1Na-ATP; 10HEPES; pH 7.2 (titrated with KOH)
hKCa3.1	145 Na-L-aspartate; 5KCl; 2.5CaCl ₂ ; 1MgCl ₂ ; 10HEPES; 5.5glucose; pH 7.4 (titrated with NaOH)	145 mM K-Asp; 2 mM MgCl ₂ ; 8.5CaCl ₂ ; 10 EGTA; 10 mM HEPES; pH 7.2 (titrated with KOH)

Perfusion indicator and positive controls were applied at a concentration equivalent to their K_d values (10 mM TEA⁺ for K_v1.3, and 20 nM TRAM-34 for K_{Ca}3.1), and ~2 μ M free Ca²⁺ to fully activate the hK_{Ca}3.1. The approximate 50% reduction in the current amplitude in the presence of these compounds or the prominent change in the current kinetics were an indicator of both the ion channel and the proper operation of the perfusion system. Bath perfusion around the measured cell with different extracellular solutions was achieved using a gravity flow micro perfusion system at a rate of 200 μ L/min. Excess fluid was removed continuously. NZ58 solutions were made fresh in ECS from 10 or 20 mM stored at –20 °C. Stock solutions were prepared from powder dissolved in water-free DMSO (Sigma-Aldrich Hungary).

Patch clamp data analysis

Data acquisition and analysis were performed using the pClamp 10.7 software suite (Molecular Devices Inc., Sunnyvale, CA, USA) and GraphPad Prism 8 (GraphPad, CA, USA). Currents were low-pass filtered with the built-in four-pole Bessel analog filters of the amplifiers and sampled at 2–50 kHz, ensuring a rate at least twice the filter cutoff frequency. Prior to analysis, current traces were digitally smoothed using a Gaussian filter and corrected for ohmic leak when required. This workflow ensured high-fidelity signal processing and consistent quantification across all recordings.

The inhibitory effect of the drug at a given concentration was calculated as remaining current fraction (RCF = I/I_0 , where I_0 is the peak current in the absence of the drug, and I is the peak current at equilibrium block at a given drug concentration).

To determine the wash in and the deactivation kinetics, we fit the curve with a standard single exponential equation $Y = A * (1 - \exp(-K * x))$ and obtained the time constant τ ($\tau=1/K$).

For fitting the dose-response curve, we used the 2 parameter Hill equation $RCF = IC_{50}^{n_H} / (IC_{50}^{n_H} + [Compound]^{n_H})$, where the IC_{50} is the half maximal inhibitory concentration and n_H is the Hill coefficient. To minimize the effect of the local proton accumulation, which is a persisting problem in the case of large currents, the G-V curves were constructed by calculating with the I_{max} at every step potential. The reversal potential was determined based on the solution composition and was checked back with tail I-V protocols.

For the steady-state activation G-V curves, the peak conductance (G) at each test potential was calculated from the peak current (I) measured at the given membrane potential (E_m) and the reversal potential (E_{rev}) using the chord-conductance equation: $G=I/(E_m-E_{rev})$. Normalized conductance (G_{norm}) was obtained by dividing each conductance value by the maximal conductance across all test potentials. For steady-state inactivation, the peak current during the depolarizing pulses was recorded after keeping the cell at increasing holding potentials. The measured current amplitudes were normalized to the peak measured at the most negative membrane potential. The resulting G_{norm} and I_{norm} values, representing the voltage dependence of steady-state activation and inactivation, were plotted as functions of the membrane potential. Quantification was performed by fitting the data with Boltzmann functions $G_{norm}=1/(1+\exp((V_{50}-V_m)/k))$ and/or $I_{norm}=1/(1+\exp((V_m-V_{50})/k))$ respectively where V_{50} is the half-activation voltage and k is the slope factor.

The activation kinetics and recovery from inactivation were fitted by a standard single rising exponential function $= A \times (1 - \exp(-K \times x)) + C$, while inactivation kinetics with a decaying exponential: $Y = A \times \exp(-K \times x) + C$, from which the time constants were obtained ($\tau=1/K$). To get the amplitude weighted effective time constants we used the equation: $\tau_{eff} = (|A_1| * \tau_1 + |A_2| * \tau_2) / (|A_1| + |A_2|)$, where we got the respective time constants and amplitudes from the double exponential fit (see above). To get the fraction of the slow component (f_{slow}) we used $f_{slow} = |A_1| / (|A_1| + |A_2|)$.

Markov model of hHv1:

As the modeling was performed by Adam Feher, its results and methods are only briefly described. A 4-state sequential Markov model was constructed in Python 3.14, based on the work of Villalba-Galea [172]. The Markov model is based on three conformational states: closed conformations and one open conformation ($C1 \rightleftharpoons C2 \rightleftharpoons C3 \rightleftharpoons O$). Voltage dependent transition rates were calculated as $k(V) = k_0 * \exp(z * \frac{V-V_{50}}{25.5})$, where k_0 is the base rate at V_{50} , z is the effective charge, V_{50} is the half-activation voltage based on the G-V curves, and 25.5 mV is RT/F at room temperature. We normalized each cell current traces to their maximum current amplitude and averaged at each test potential. Model parameters were estimated using a two-stage optimization approach.

Statistics

All data are presented as mean $\pm$ SEM, with a minimum of 3 independent experiments. In the case were, values were obtained on the same cell before and after treatment, a paired t-test was performed. For ratio comparisons, a one-sample t-test was performed using a theoretical mean of 1. For independent groups, either two-sample t-tests or one-way ANOVA were applied as appropriate. Statistical significance was defined as $p < 0.05$.

***in silico* virtual screening**

As the virtual screening was performed by our collaborators at the University of Ljubljana, its results and methods are only briefly described. We conducted two independent virtual screening (VS) campaigns: one using a fragment-based commercial library and another using our in-house collection of compounds. Each library was docked separately with Schrödinger Release 2024-3 (Schrödinger, LLC, New York, NY, USA, 2024) against the binding site previously identified for guanidine-based inhibitors, which interact with the voltage-sensing domain of the hHv1 channel from the intracellular side. To achieve this, a structural model of the open state of the human Hv1 channel [10, 73] was prepared using Protein Preparation Wizard with the default settings: bond orders were assigned using CCD database, missing hydrogens were added, termini were capped, the missing side chains were modeled with Prime, and the protonation states ($\text{pH } 7.0 \pm 2.0$) were modelled with Epik [173]. The binding site for ligand docking was prepared using MAKE RECEPTOR (Release 4.2.1.1, OpenEye Scientific Software, Inc., Santa Fe, NM,

USA; www.eyesopen.com). The grid box was centered on residues Asp112, Phe150, Ser181, and Arg211, which have previously been shown to interact with the inhibitor 2GBI.

From the commercial compound library, the top 1000 ranked molecules were retrieved, whereas all docked molecules from the in-house library were retained for further evaluation. For each library, the best-scoring FRED-generated pose of the top 100 ranked compounds was visually inspected and used for subsequent analysis. Based on this inspection and ranking, ten of the highest-scoring candidates from the in-house library were selected, and an additional ten compounds were chosen and purchased from Enamine Ltd. These twenty compounds were then tested for inhibitory activity using patch-clamp electrophysiology in cell lines expressing Hv1 channels.

RESULTS

Screening and identification of potent inhibitors of the hHv1 channel

Based on the results of our *in silico* virtual screening, we conducted experimental testing of the highest-ranked candidates, a total of 23 compounds, using patch-clamp electrophysiology in CHO cells expressing hHv1 channels. hHv currents were recorded using a 1-second ramp protocol from a holding potential of -60 mV to +60 mV. We used a ramp protocol because it provided a stable current over extended recordings, which was essential for subsequent dose-response measurements. This protocol also allowed simultaneous monitoring of the peak current, the activation threshold (V_{thr}), and the tail current at -80 mV (for more detailed description see material and methods).

We initially assessed blocking efficacy of each compound, at 50 μ M and monitored the resulting reduction in current amplitude over time. Inhibition was quantified by calculating the Remaining Current Fraction (RCF), defined as the ratio between the steady-state current measured under drug exposure and the initial control current recorded at +60 mV (i.e. $RCF = I_{drug} / I_{control}$). To verify the correct operation of the perfusion system and that current reduction was a consequence of blockade of hHv1 channels, we used lower external pH as positive control (Fig 4.), as Hv1 gating is highly dependent on Δ pH. Lowering the external pH shifts the voltage dependence of activation of the g_H to more positive potentials [30, 174]. This approach allowed us to determine the extent of channel block after saturation for each compound and identified a total of 15 molecules that produced substantial inhibition on hHv1 currents within the low-micromolar range (Fig. 6). For the most effective inhibitors such as NZ-58, NMP-132/133, GHK-21, and GHK-34 we further quantified their potency by generating dose-response curves, measuring the extent of current block across a range of concentrations (Fig. 7).

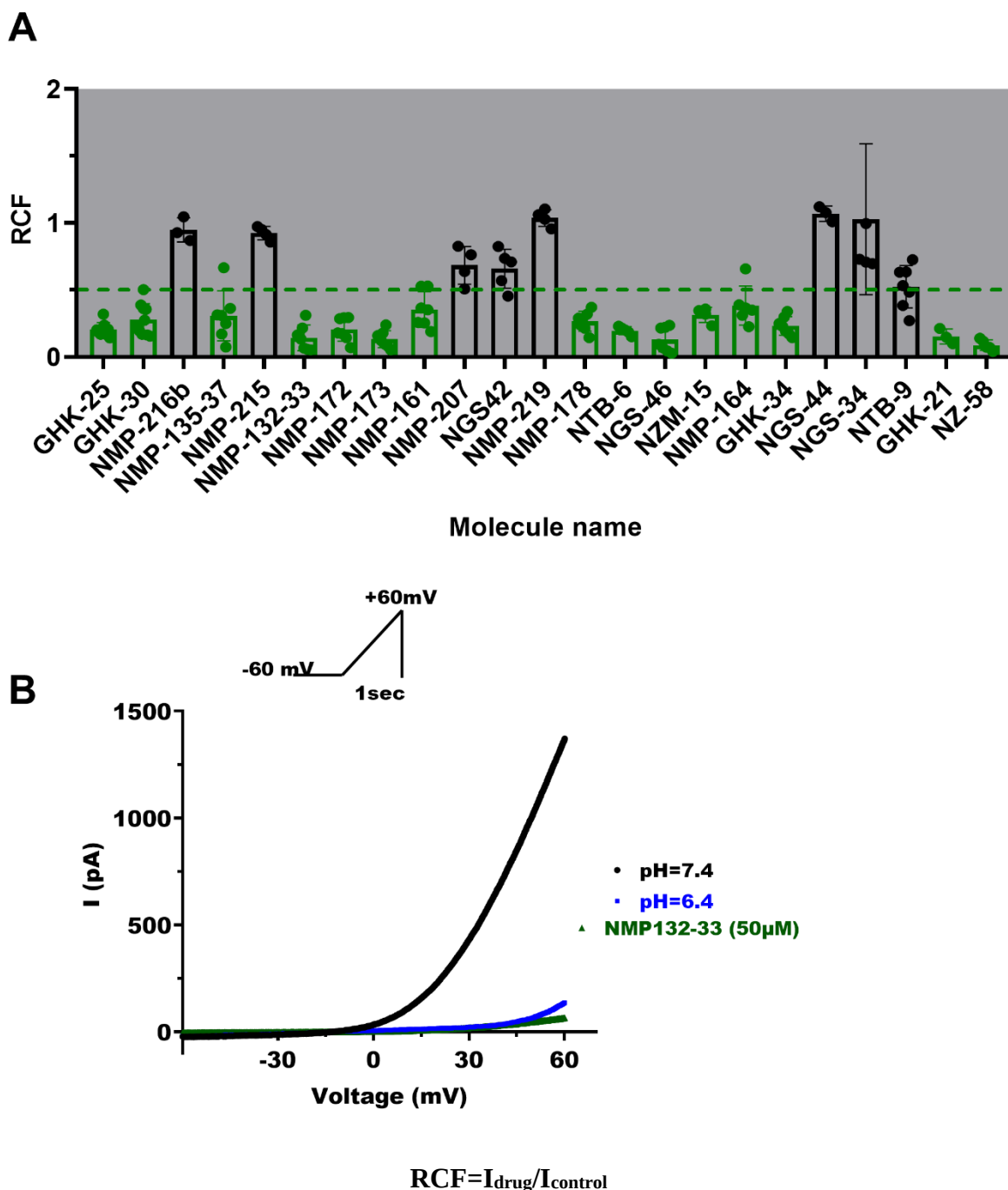


Figure 6. Screened and identified hit compounds of the human voltage-gated proton channel. (A) Each column represents one of the molecules tested, with black indicating non-effective compounds and green indicating effective H_v1 inhibitors, which were defined as RCF > 0.5 and RCF < 0.5, respectively. The dots represent the RCF values of independent measurements $n \geq 3$ and the error bars indicate the SEM. (B) Representative graph of one of the most effective

compounds tested at 50 μM , recorded with a 1-second voltage-ramp protocol from a holding potential of -60 mV to $+60$ mV as illustrated.

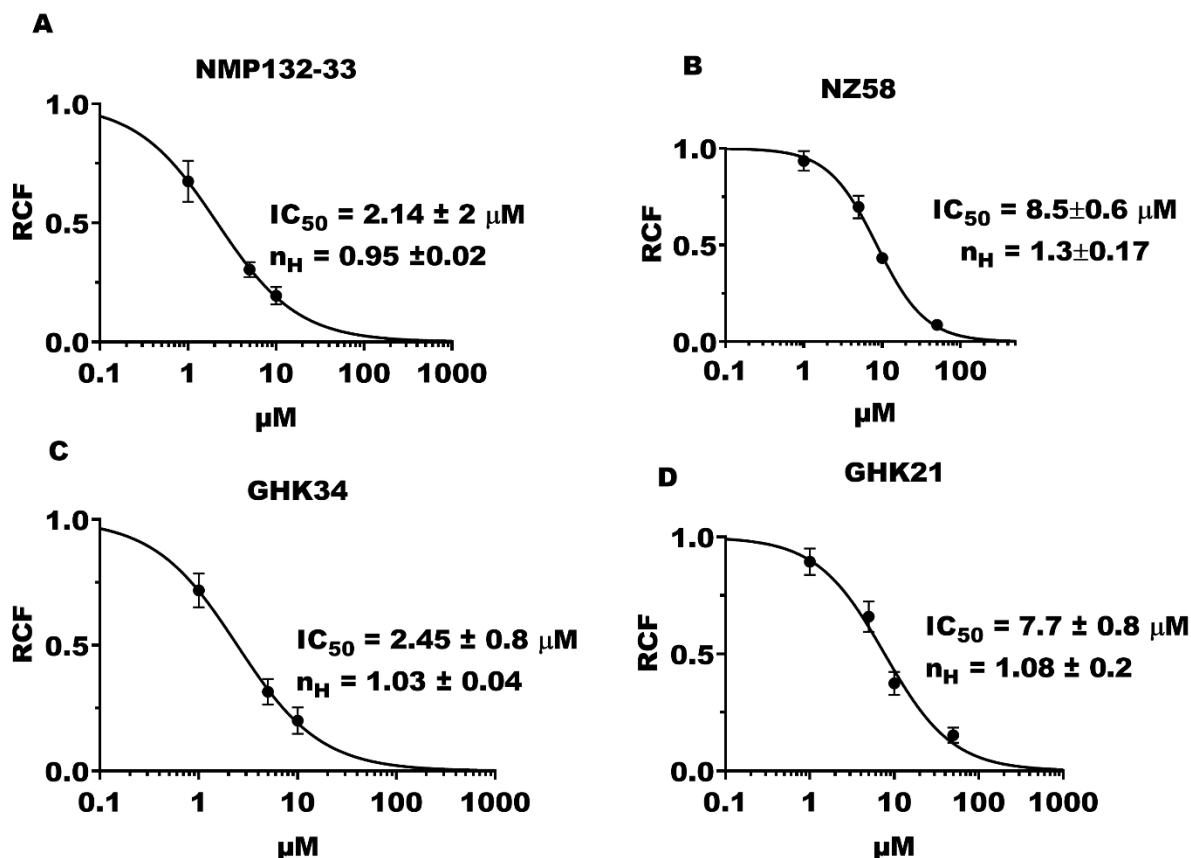


Figure 7. Dose-response curves for the most effective Hv1 inhibitors. The compounds: NMP132-33 (A), NZ-58 (B) and GHK-34 and (C) GHK21 (D). The obtained IC_{50} values were 2.14 ± 2 , 8.5 ± 0.6 , 2.45 ± 0.8 , 7.7 ± 0.8 μM respectively. The points on the dose-response curves represent the mean of 4–5 independent measurements. The data points were fitted using a two-parameter Hill equation. The Hill coefficient is labelled as n_H . The error bars represent the SEM.

NZ-58 is a potent and state-independent inhibitor of the hHv1 channel

Although we have identified several effective compounds that blocked Hv1 at low micromolar concentrations, our initial selectivity studies indicated no major differences in their effects on K_v

and Nav channels. All their IC₅₀ values were only slightly higher than the ones obtained for Hv1, thus, they could not be declared Hv1-selective inhibitors. However, we observed significant differences in the consistency of the obtained RCF values, reversibility of block, reproducibility of experiments over the course of months and the stability of the patch clamp seals among the different compounds, which most likely correlated with their lipophilicity and their stability during storage. Thus, even though some compounds showed high affinity for Hv1, we excluded them from further characterization due their inconsistent behavior. Based on these criteria we decided to further investigate the compound named NZ-58 (N-(3-(2-amino-1H-imidazol-5-yl) phenyl)-5-(trifluoromethoxy)-1H-indole-2-carboxamide) as it produced reliable and reproducible results.

Whole-cell patch-clamp measurements on CHO cells transfected with hHv1 have revealed the inhibiting properties of NZ-58 (Fig. 8 A). Currents were recorded using a 1-second ramp protocol from a holding potential of -60 mV to +60 mV. Application of 50 μ M NZ-58 almost completely inhibited the current yielding a RCF of 0.08 ± 0.02 (n=5) at +60 mV. NZ-58 also modified the voltage-dependent gating of the channels, as it shifted the opening threshold voltage toward more positive values (Fig. 8 A and C). We have tested this attribute at various concentrations and the shifts were 2.5 ± 2.8 , 10.7 ± 2.7 , 13.6 ± 4.8 and 28.8 ± 5.4 mV for 1, 5, 10 and 50 μ M concentrations, respectively (Fig. 8 C, n $\geq$ 3 for each concentration). The wash-in time constant was also determined by a single-exponential fit, and it yielded 54.6 ± 17.5 seconds at 50 μ M concentration (Fig. 8 C).

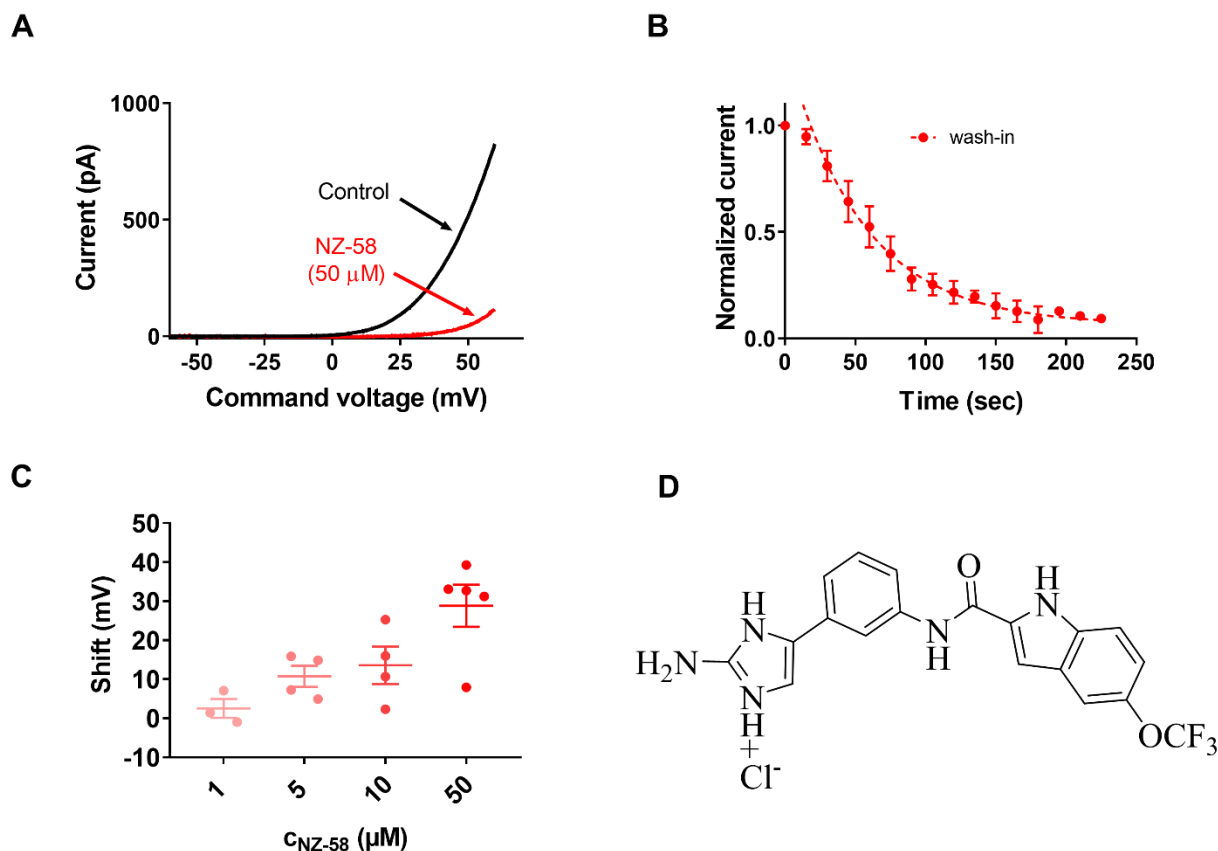


Figure 8. NZ-58 is a potent inhibitor of the hHv1 channel. (A) Representative ramp measurement of the hHv1 channel. Blue trace is control; red trace is 50 μ M NZ-58 after saturation. **(B)** Time-dependent block of 50 μ M NZ-58. The wash-in time constant was 54.6 seconds. **(C)** The concentration dependence of the V_{thr} shift (plotted on Y-axis). Tested concentrations were 1, 5, 10 and 50 μ M. The shift was calculated by the equation $Y = V_{\text{thr; NZ-58}} - V_{\text{thr; control}}$. **(D)** The structure of NZ-58.

Access of modulator compounds to the binding site may depend on the gating state of the channel, which may completely prevent binding in certain states of the channel. We therefore tested the state-dependence of binding by applying a protocol, during which channels were exposed to 50 μ M NZ-58 only in the closed state (Figure 9A). To achieve this, we have recorded the ramp protocol from Figure 8A in control conditions, then we perfused the cells with 50 μ M NZ-58 for 90 seconds at the holding potential to keep the channels closed. Before recording the second ramp the perfusion was switched back to the control solution for 15 seconds to ensure that the NZ-58 will not bind to the open channels during the second set of episodic stimulations. The perfusion

duration of NZ-58 was selected based on the wash-in kinetics (Figure 8 B). We have compared the RCF values obtained with this protocol using -60 mV holding potential (exposure in the closed state only) with those obtained with episodic stimulation (ramps applied every 15 s) during a continuous 90-second-long NZ-58 application (exposure both in the open and closed states) (Figure 9 B). The RCF values of 0.36 ± 0.04 for -60 mV holding and 0.33 ± 0.04 for episodic stimulation ($n \geq 12$ for each group) were not significantly different ($p = 0.59$, unpaired t test). These results indicate that NZ-58 is able to bind to the closed channels.

Channel modulator compounds may also exhibit functional selectivity, which means that they can bind to different channel states but have different affinities for them. For example, many Nav channel blockers are use-dependent, meaning that they preferentially bind to the open or inactivated state of the channel and therefore are more effective on hyperactive channels. Thus, we explored the use-dependence of block by varying the pulse frequency while applying NZ-58. We used a step depolarization protocol applying 1-second-long pulses to +60 mV from a holding potential of -60 mV with inter-pulse intervals (IPI) of 3 or 15 seconds. This protocol changes the ratio of the open to closed states significantly enough to detect the potential affinity differences. Single-exponential fits to the wash-in kinetics yielded time constants of 20.9-39.5 (IPI = 3 s) and 24.0-49.3 seconds (IPI = 15 s) (95% confidence intervals, $n \geq 5$ for both groups, Figure 9 C). This indicates that the rate of block was independent of the pulsing frequency ($p = 0.18$, extra-sum-of-squares F-test), thus, the binding of NZ-58 is not use-dependent.

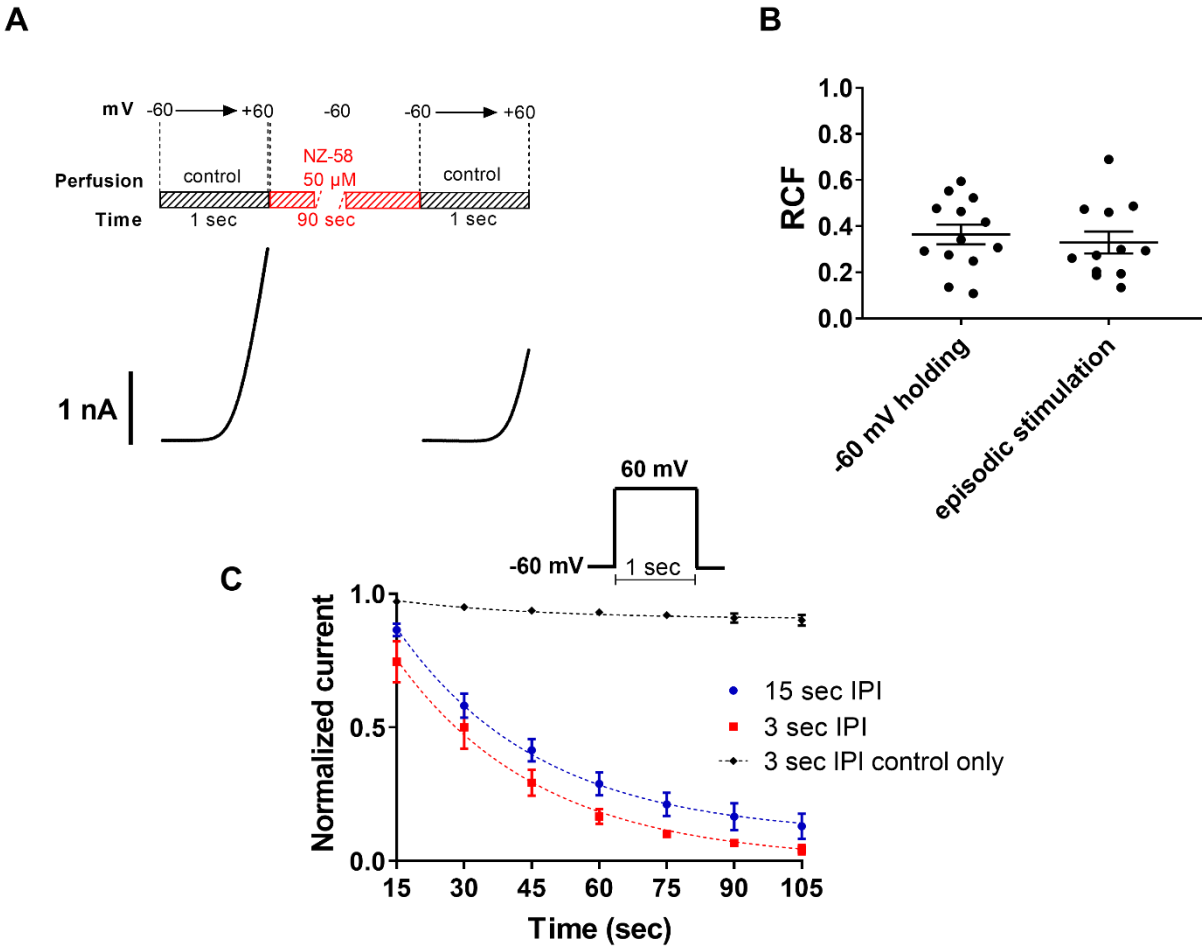


Figure 9. State- and use-dependence of NZ-58. (A) Assessment of closed-state block. After establishing maximal current ($I_{\max}$), using control external solution during the first voltage-ramp protocol, cells were held at -60 mV and exposed to $50 \mu\text{M}$ NZ-58 for 90 s to allow drug interaction with channels residing predominantly in the closed state. Before initiating the second ramp, the perfusion was switched back to control solution to minimize any contribution of open-state block during the subsequent episodic stimulation. (B) Quantification of inhibition. Left: RCFs summarizing the extent of closed-state block measured at the 90-second time point. Right: RCFs obtained during episodic stimulation, illustrating the degree of block under conditions that favor open-state interactions. (C) Use-dependence of NZ-58 block. Normalized $I_{\max}$ values are plotted as a function of time to illustrate NZ-58 inhibition under different inter-pulse intervals (IPIs). The voltage protocol used for these measurements is shown to the left of the graph. The black trace represents current stability under control conditions with a 3-s IPI. Blue and red traces depict the

progressive, time-dependent block observed with 15-s and 3-s IPIs, respectively, demonstrating the lack of use-dependence of NZ-58 inhibition.

Experimental results to confirm the binding pocket of NZ-58

According to our *in silico* docking analysis from our previous study performed by our Slovenian collaborators, NZ-58 is predicted to establish hydrogen-bond interactions with the backbone carbonyl of Val109 and with the side-chain carboxylates of Asp112 and Asp185. The ligand also engages in π -stacking with the aromatic ring of Phe150, which further stabilizes its position within the intracellular cavity of the VSD [175]. Notably, the predicted binding pose overlaps with the intracellular binding site previously proposed for guanidinobenzimidazole derivatives, including 2GBI and ClGBI (Figures 10 and 11). To support rational drug-design efforts, we experimentally tested the predicted contact residues V109, D112, and D185 as well as the neighboring residue V110. Patch-clamp recordings showed that substitutions at V109, D112, and D185 (to phenylalanine and asparagine, respectively) markedly reduced the extent of inhibition following saturation with 50 μ M NZ-58 (Figure 12), indicating that these positions contribute to ligand binding. In contrast, mutations at V110 and F150 (to phenylalanine and alanine, respectively) did not significantly alter the degree of block. The resulting RCF values were as follows: V109F (0.34 ± 0.04 ; $p=0.022$), V110F (0.096 ± 0.01 ; $p=0.99$), D112N (1.00 ± 0.13 ; $p < 0.0001$), F150A (0.092 ± 0.02 ; $p=0.99$), D185N (0.61 ± 0.06 ; $p < 0.0001$) (mean $\pm$ SEM, $n \geq 3$; one-way ANOVA with Dunnett's multiple comparison test) (Figure 12B).

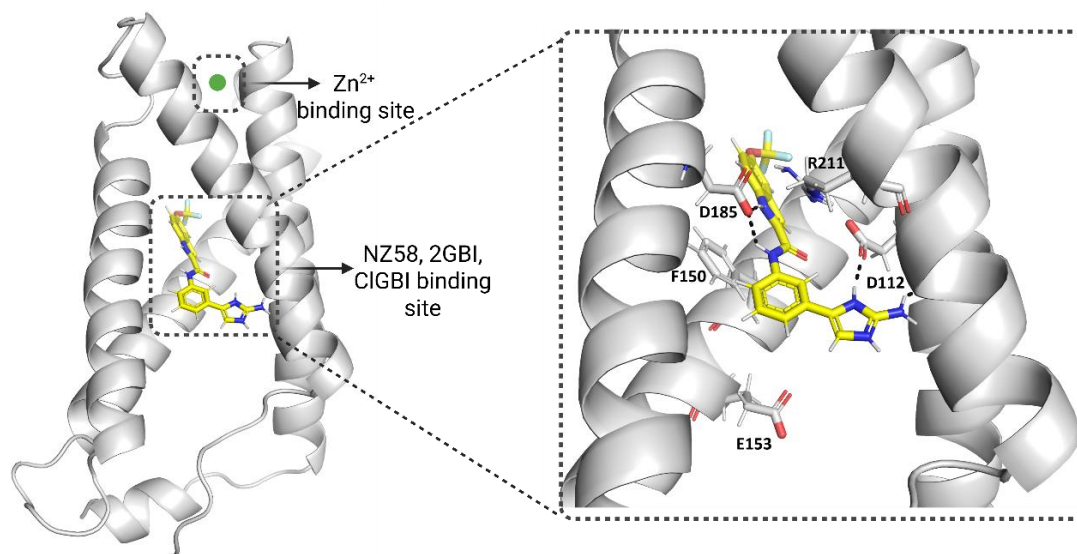


Figure 10. Binding sites of inhibitors on human Hv1. Structure of Hv1 (shown in grey cartoon representation) highlighting the intracellular binding sites for Zn^{2+} , NZ-58, and the guanidinobenzimidazole derivatives 2GBI and ClGBI (left). The predicted binding mode of NZ-58 (yellow sticks) within the proposed intracellular pocket is shown on the right, based on docking analysis. The ligand and surrounding side chains are displayed as stick models and colored by atom type (blue, N; red, O; cyan, F). For clarity, only key residues are depicted. Hydrogen bonds are indicated by black dashed lines.

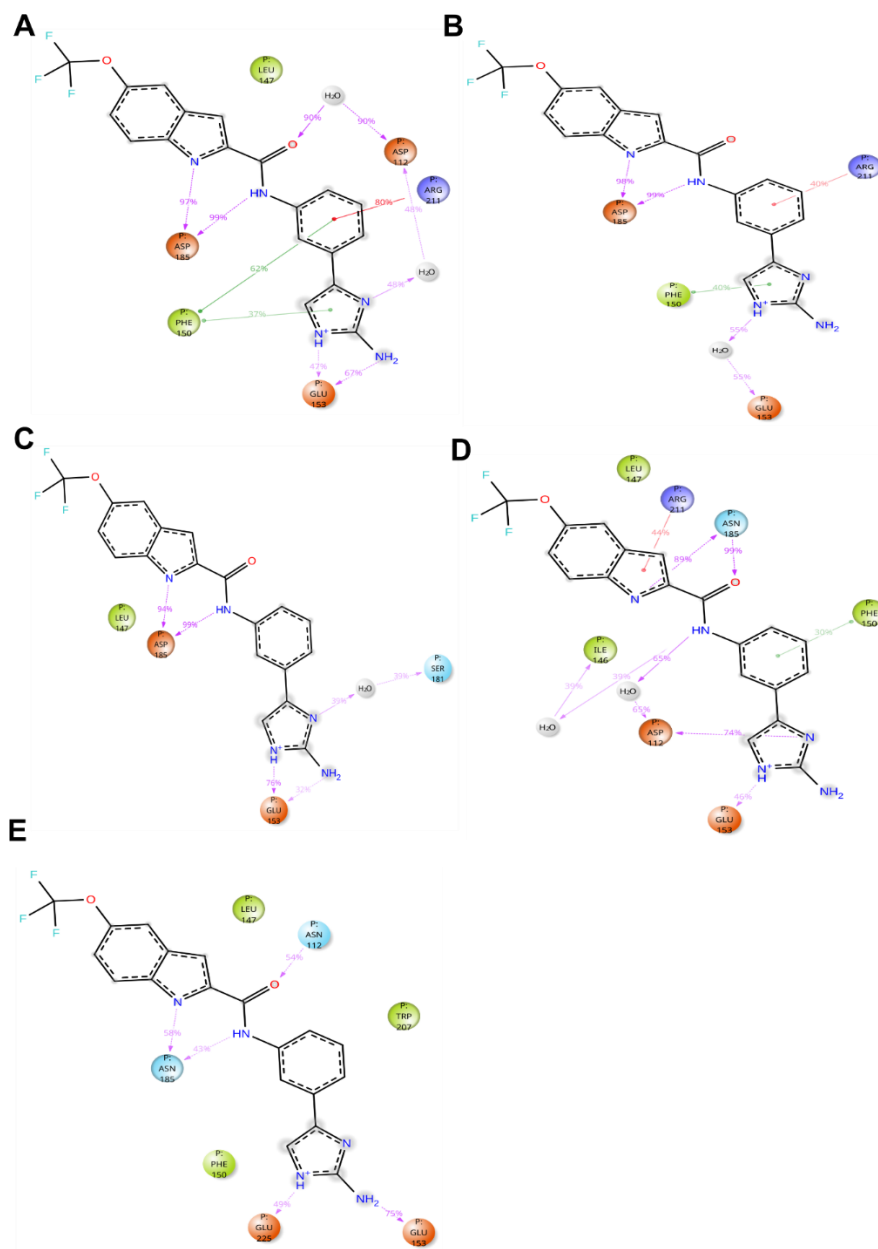


Figure 11. Interaction network between hHv1 and NZ-58 (A-E) Schematic representations of ligand atom interactions with the protein residues. Interaction network of NZ-58 and **(A)** wild-type Hv1, **(B)** D112N Hv1 mutant, **(C)** F150A Hv1 mutant, **(D)** D185N Hv1 mutant. Interactions that occur more than 30% of the MD simulation time in the trajectory of compound NZ-58 in the Hv1 binding site, are shown. The hydrogen bonds are shown as arrows (in magenta), the π -stacking is shown as a green line, and the cation- π interaction is shown as a red line.

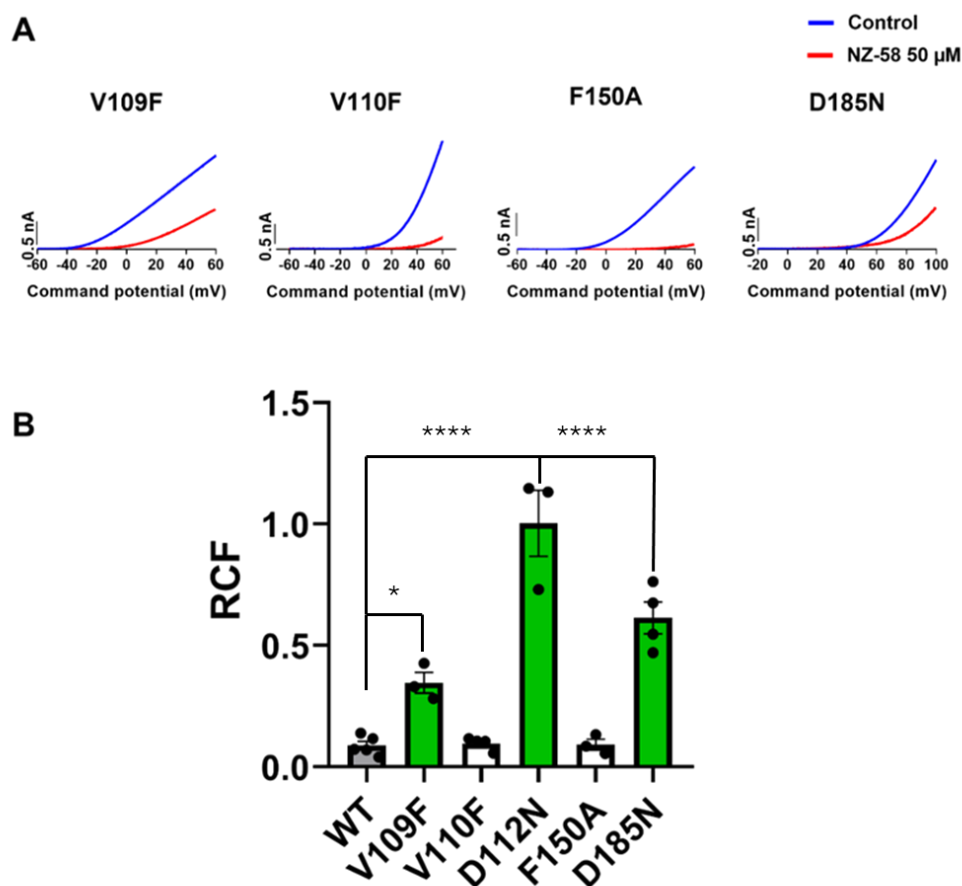


Figure 12. Experimental results confirm the binding pocket of NZ-58. (A) Representative ramp recordings for each of the examined mutants are shown. Blue traces correspond to saturated currents under control conditions, whereas red traces represent currents after perfusion with 50 μ M NZ-58. (B) Summary plot of the resulting RCF values. The grey bar represents WT, white bars indicate “miss” mutations, and green bars denote residues identified as potential interacting partners of NZ-58. Asterisks * and **** mean $p < 0.05$ and $p < 0.0001$ respectively (One-way ANOVA with Dunnett’s multiple comparisons test).

Molecular mechanism of Hv1 modulation by NZ-58

After we successfully identified the binding site, we further examined the molecular mechanism of their interaction. As shown in Fig. 8A and 8C application of NZ-58 not only inhibited the

channel but also produced a concentration-dependent shift in voltage sensitivity, which we initially assessed by monitoring the opening threshold voltage (V_{thr}) during voltage-ramp protocols. Furthermore, to disentangle effects on conductance, gating kinetics, and voltage sensing, additional experiments were necessary. Our first objective was therefore to determine whether the observed V_{thr} shift in ramps indeed reflected altered voltage-sensor behavior and not current kinetic changes. To achieve this, G-V curves were constructed under control condition and after 10 μM NZ-58 saturation (Fig. 13 A and B). A lower concentration was selected because 50 μM produces near-complete current block, precluding reliable G-V analysis. Boltzmann fits yielded V_{50} values of -2.7 mV (control) and 10.1 mV (NZ-58), confirming the V_{thr} displacement detected in the ramp experiments (Fig. 8C).

To further investigate voltage-dependent gating, we asked whether the shift in opening threshold represented the sole consequence of NZ-58 binding. Using the I-V protocols (Fig. 13B), we also assessed potential changes in channel kinetics (Fig. 13C, D). Exposure to 10 μM NZ-58 accelerated the fast component of activation, diminishing the characteristic sigmoidicity of WT hHV1 activation. However, NZ-58 reduced the deactivation time constant, producing a faster $\text{O} \rightarrow \text{C}$ transition—behavior reminiscent of a voltage-sensor-modifying compound. To reconcile this seemingly paradoxical combination of effects, we constructed a sequential four-state Markov model of hHV1 based on the previous publication by Villalba-Galea [172] (Fig. 14). The transition rates were calculated for the simulated current traces that gave the best match to the actual recorded traces in the absence and presence of NZ-58 at various voltages. Furthermore, we calculated the activation free energies ($\Delta G^\ddagger$) for the individual state transitions, enabling the construction of a free energy landscape that provides a mechanistic visualization of the effects of NZ-58 (Fig. 14). We have found that NZ-58 dramatically accelerated β_1 (60–800 $\times$), α_2 (30–90 $\times$), β_2 (470–745 $\times$), and α_3 ($\sim 4800\times$), whereas α_1 and β_3 remained relatively unchanged (NZ-58/Control ≈ 1). Because of this complexity, we investigated the fate of the channel once it reached C2 (as the first ON transition was relatively unaffected). Based on our calculations, NZ-58 creates a kinetic trap that forces the channels into the $\text{C1} \rightleftharpoons \text{C2}$ cycle. It is also important to note that once the channels reach C3, the probability of opening is visibly increased with NZ-58. The fast initial component of the macroscopic activation (Figure 13 B) can be explained by the increased $\text{C3} \rightarrow \text{O}$ transition: once the channel escapes the trap it reaches the conductive conformation faster.

Concomitantly, we performed gating current measurements independently, which supported the kinetic trap mechanism. For example, the earlier appearance of the gating current peak was consistent with rapid $C1 \rightleftharpoons C2$ equilibration. The faster decay of ON gating current reflects blocked forward progression beyond $C2$. The reduced slow component fraction (f_{slow}) indicates redistribution of state occupancies, as expected when channels accumulate in the $C1 \rightleftharpoons C2$ cycle. Finally, the reduced Q_{max} ($\sim 20\%$) is consistent with fewer channels completing the full activation pathway.

Therefore, our model captures the influence of NZ-58 on current amplitude, activation and deactivation kinetics, and the opening-threshold voltage by assuming only that the compound modifies the transition rates between conformational states. This alteration in relative state occupancy reproduces the observed gating behavior without invoking a direct physical obstruction of the permeation pathway. Within this kinetic framework, the trap-like mechanism accounts for the reduction in macroscopic conductance through a lowered open probability rather than diminished single-channel conductance. Nevertheless, a contribution from direct pore interactions cannot be definitively ruled out in the absence of single-channel measurements.

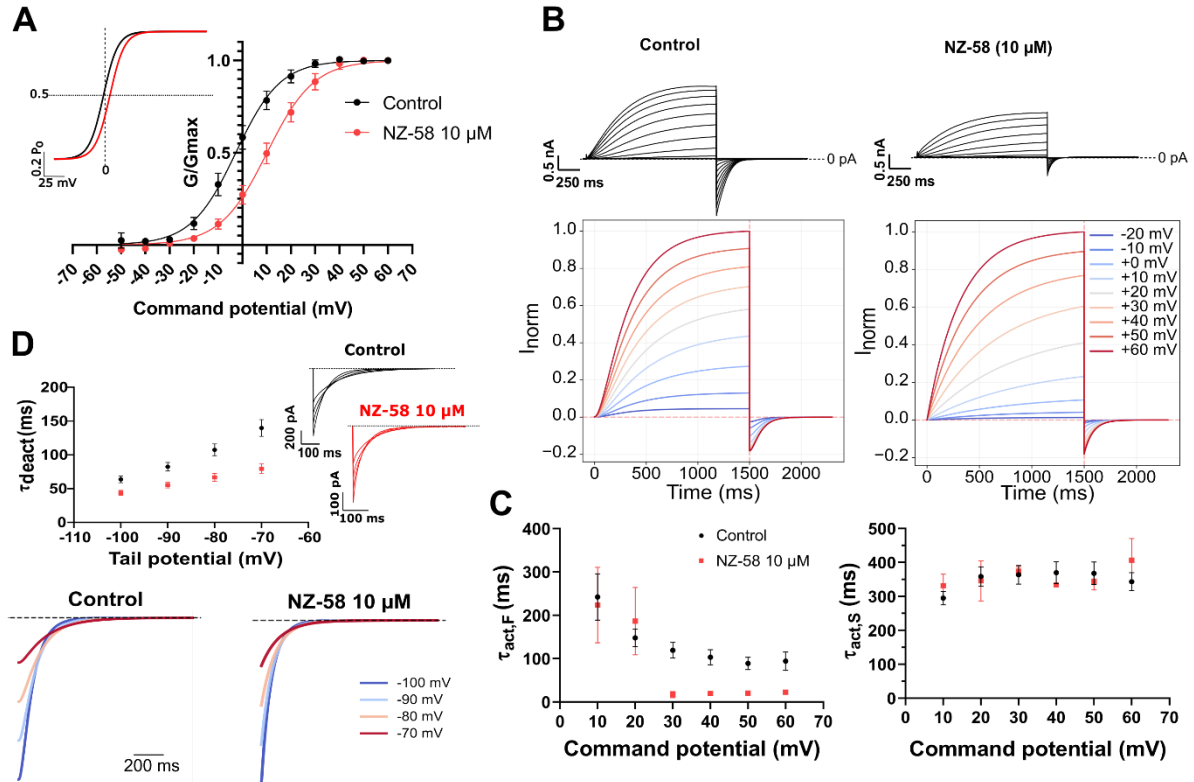


Figure 13. Examining the effect of the binding on the biophysical properties of the hHv1 channel. (A) G-V curves obtained by using the $I_{\max}$ of the different I-V steps shown on panel B. Black curve is with control perfusion while red curve is after the effect of 10 μM NZ-58 has saturated. Boltzmann fits yielded -2.7 mV and 10.1 mV V_{50} values respectively. In the inset (top left corner) the G-V curve obtained by Markov-modelling is shown. Vertical dashed line is 0 mV, while dashed horizontal line is 0.5 P_o . (B) Representative I-V measurements (top) and corresponding simulated traces based on the transition rates yielded by the 4 state-Markov-model (bottom). (C) Activation time constants determined by double exponential fit. Fast component is plotted on the left, while slow component is on the right. Black dots and red squares are control and 10 μM NZ-58, respectively. (D) Deactivation kinetics of the hHv1 channel. On the left, the deactivation time constant is plotted as a function of tail potential. Black dots are control and red squares are 10 μM NZ-58. Next to the plot, representative tail I-V traces are placed (black is control, red is 10 μM NZ-58). Below the experimental data simulated tail I-V traces are shown based on the Markov-model (Y-axis is the current normalized to the $I_{\max}$ of the preceding depolarization step).

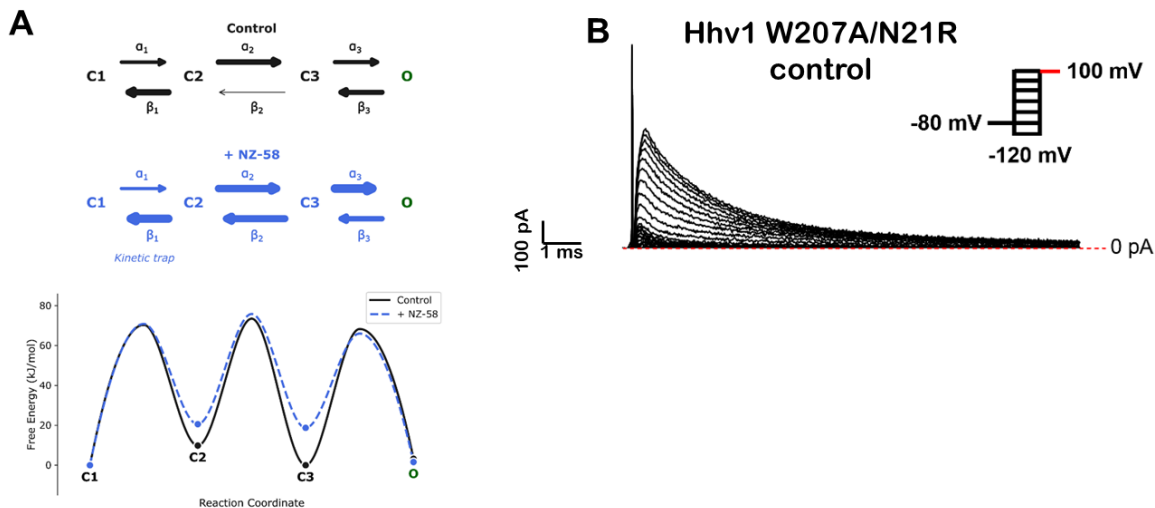


Figure 14. Modelling and the measurement of the gating current. (A) Top, kinetic scheme: Black arrows denote the transition rates at 0 mV under control conditions, whereas blue arrows indicate the corresponding rates in the presence of NZ-58. Arrow thickness reflects the magnitude

of each transition rate (thicker = faster; thinner = slower). Bottom, free-energy landscape: Energy wells derived from equilibrium constants are shown as black (control) or blue (NZ-58) points. Free-energy barriers calculated from the rate constants are represented by continuous black lines for control conditions and dashed blue lines for NZ-58. **(B)** Representative gating current measurement of the W207A/N214R double mutant hHv1 channel. Black traces represent the ON gating current elicited by +100 mV depolarization after the preceding voltage steps (V_{pp}). The voltage protocol is shown in the inset. Red continuous line in the protocol indicates the part of the voltage protocol that is displayed. Red dashed line symbolizes the current baseline (0 pA).

NZ-58 is a pan-VSD modulator, rather than selective inhibitor

For an inhibitor to be suitable for functional studies or to hold promise as a therapeutic agent, its selectivity for the intended target channel must be rigorously established, particularly against closely related off-target channels. In order to assess the selectivity of NZ-58, we evaluated its effect by whole-cell patch clamp on multiple ion channels, including VGICs Nav1.5, Nav1.2, Nav1.4, Kv1.3, fast inactivation-removed Kv1.4, Kv1.5, Kv11.1 (hERG), as well as the non-voltage-gated Kir2.1 and KCa3.1 channels. These initial experiments were performed with extracellular application of 50 μ M NZ-58, which caused nearly complete inhibition of the currents of all VGICs tested, with the exception of the non-inactivating Kv1.5, which was inhibited by a lower extent. In contrast, NZ-58 had no detectable effect on the currents mediated by the non-VGICs (Fig. 15).

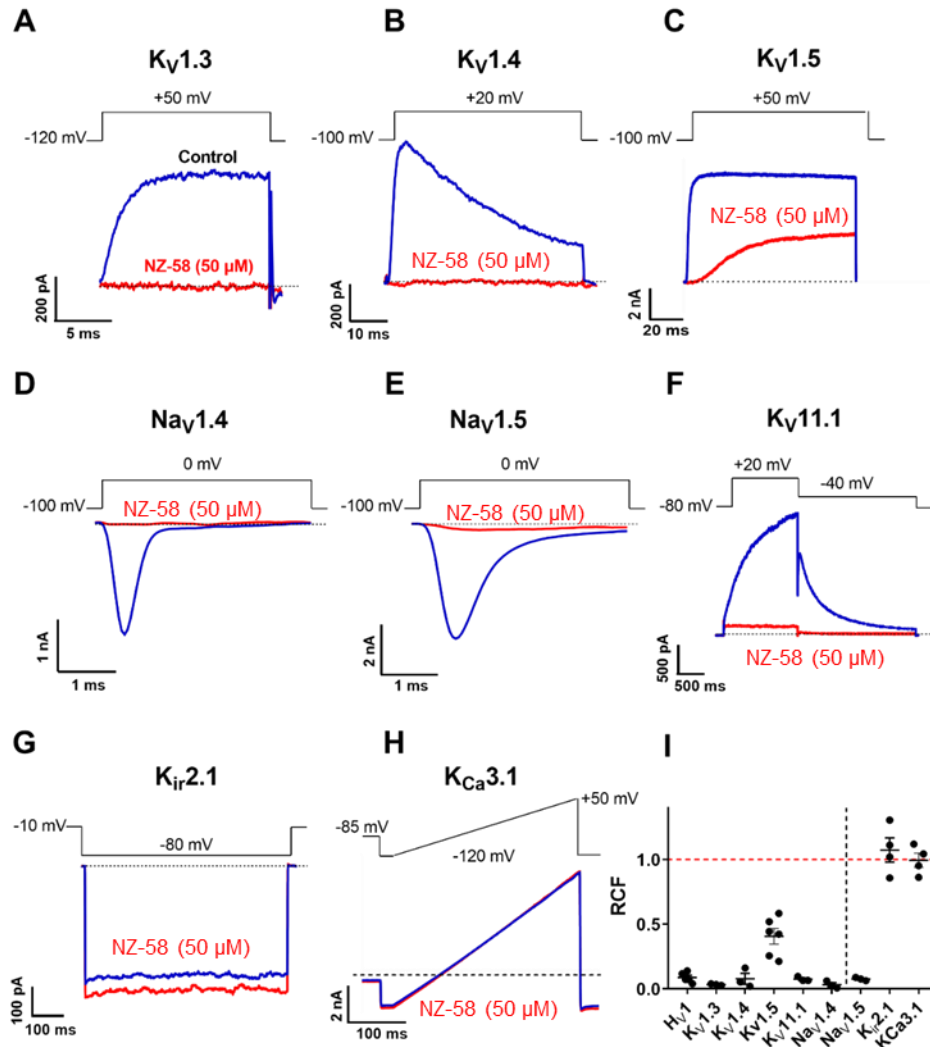


Figure 15. Effect of 50 μ M NZ-58 on voltage-gated and non-voltage gated ion channels. (A-H) In whole-cell patch-clamp experiments, NZ-58 was applied extracellularly, and channel activity was elicited using the voltage protocols shown above the current traces. Blue traces represent control recordings, whereas red traces show the currents in the presence of the inhibitor. (I) The RCF for each channel at 50 μ M NZ-58 is summarized, including the Hv1 data from our previous study. The red dashed line denotes the control current level, serving as a reference for the degree of inhibition observed.

Conversely, at 10 μ M (approximately the IC_{50} for hHv1) NZ-58 produced pronounced gating alterations across VGICs while causing only modest reductions in peak current amplitudes in most

channels. The effects varied among channel types, displaying both shared features and channel-specific, sometimes opposing, behaviors. The most consistent observation across all $K_V1.x$ and $Na_V1.x$ channels was a marked slowing of activation kinetics (Fig. 16A–E). For comparative purposes, activation was quantified using single-exponential fits, although the rising phase of many currents was visibly sigmoidal due to the multi-VSD nature of channel activation. Figure 16F illustrates the substantial deceleration of activation produced by 10 μ M NZ-58, expressed as the ratio of activation time constants in the presence versus absence of the compound. These findings point to a direct interaction of NZ-58 with the VSDs, a conclusion further supported by its lack of effect on the non-VGIC channels Kir2.1 and KCa3.1 (Fig. 15G, H).

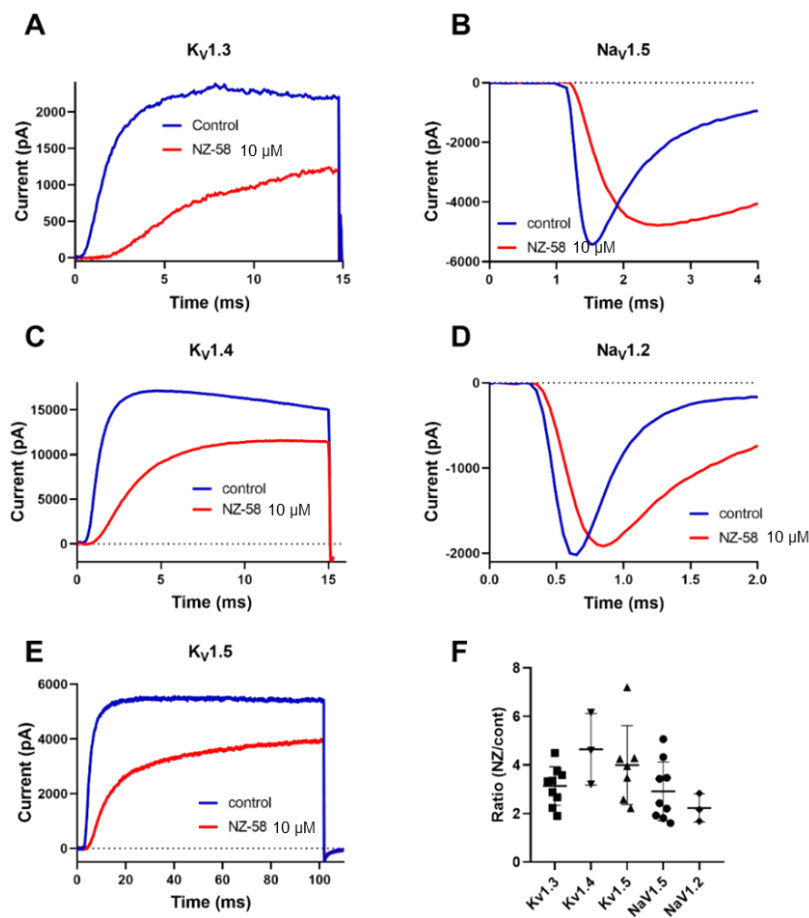


Figure 16. The impact of 10 μ M NZ-58 application on K_V and Na_V channel activation kinetics. (A–E) Extreme slowing of activation kinetics of the indicated ion channels in response to NZ-58. Currents were evoked using depolarizing steps to +50 mV for K_V channels and to 0 mV

for Nav channels. (F) The summary of NZ-58 induced changes in activation time constants, expressed as the ratio of values measured in the presence versus absence of the molecule.

The mechanism underlying delayed VSD activation suggests a process stabilizing the resting state and thus slowing of the forward rate and accelerating of the backward rate, which should manifest as faster deactivation (channel closing) at negative potentials. Despite accelerating the overall activation kinetics of H_v1, NZ-58 simultaneously caused a pronounced increase in the channel-closing rate (see above). However, no such change was observed in the tail current kinetics of K_v1.3 or Nav1.5 channels (K_v1.3: control: 6.28 ± 0.50 ms, NZ-58: 6.78 ± 0.86 ms, $n=4$, $p=0.89$; Nav1.5: control: 0.199 ± 0.059 ms, NZ-58: 0.168 ± 0.044 ms, $n=4$, $p=0.86$; Fig. 17). As the deactivation rate of Nav1.5 at -100 mV approached the temporal resolution of the voltage clamp, tail-current kinetics were instead measured at -70 mV, where deactivation is sufficiently slower and therefore suitable for reliable comparison. This behavior contrasts with H_v1, in which NZ-58 selectively increases the activation energy of the rate-limiting forward transition while leaving the backward (closing) transition largely unaffected.

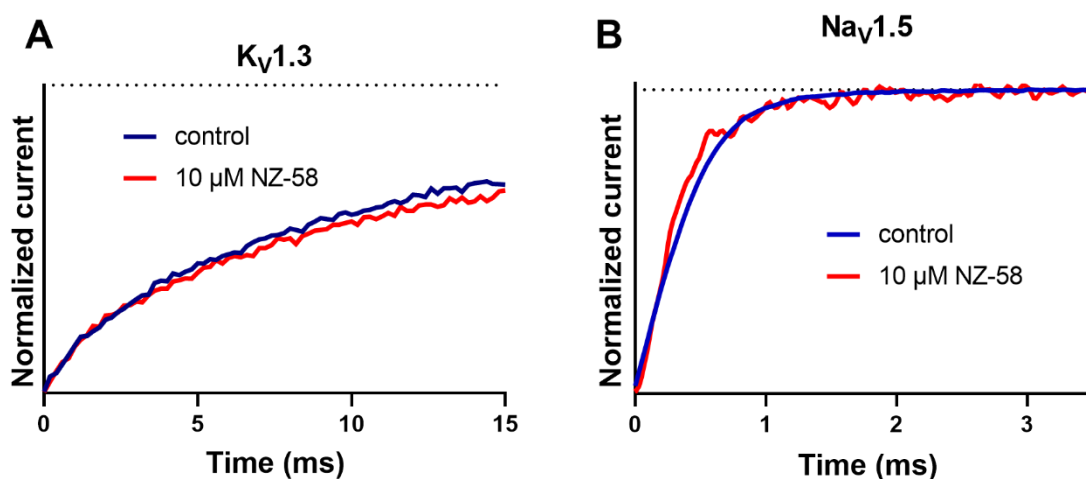


Figure 17. Lack of effect by NZ-58 on the tail currents. (A) Tail currents on K_v1.3 were measured at -100 mV after stepping back from +50 mV. (B) For the Na_v1.5 we recorded tail current

at -70 mV returning from 0 mV with Nav1.5 before significant inactivation occurred during the depolarizing pulse.

Effects on wild type Nav1.5

Motivated by these findings, we sought to explore these observations in more detail, for instance on Nav1.5. A remarkable effect of NZ-58 on Nav channels was a significant slowing of fast inactivation along with activation (Fig. 18 A). Application of 10 μ M NZ-58 produced rapid kinetic alterations in Nav1.5 currents, emerging within seconds and occurring with minimal reduction in current amplitude; however, the full effect developed gradually over several minutes. This kinetic modulation led to a marked increase in charge transfer during the depolarizing pulse (area under the current trace), translating to an overall enhancement of channel activity. Both the amplitude and the total charge displayed clear dose-dependence, as illustrated in Figs. 18B and 18C. At 10 μ M, peak current amplitude was only slightly diminished, yet charge transfer increased nearly threefold relative to control. At higher concentrations (25–50 μ M), NZ-58 initially produced a transient enhancement of charge transfer, but this effect waned over the following minutes, and at 50 μ M the charge passed ultimately fell below control levels.

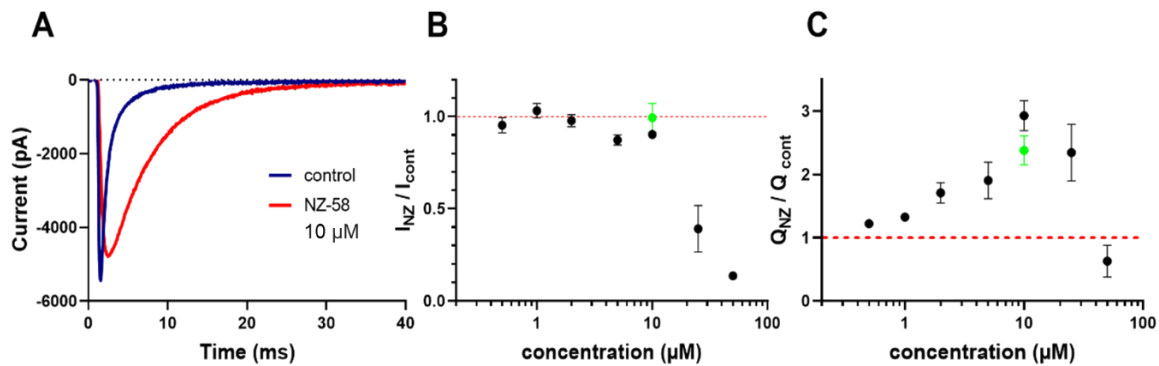


Figure 18. Changes induced by NZ-58 in the kinetics, amplitude and charge transfer of Nav1.5 currents. (A) 10 μ M NZ-58 induced a pronounced slowing of both activation and inactivation kinetics, leading to an overall increase in total charge transfer. **(B)** Changes in peak current amplitude as a function of NZ-58 concentration. Black symbols denote the wild-type channel, whereas the green symbol represents the R1632C mutant (see below). The red dashed

line indicates the control value. (C) Total charge transferred (area under the current trace) plotted as a function of NZ-58 concentration.

These variable effects can be attributed to the different rates at which NZ-58 altered activation and inactivation kinetics. As shown in Fig. 19, the inactivation rate initially slows more strongly than the activation rate, which can even produce a slight increase in peak amplitude together with enhanced charge transfer. Over time, however, the slowing of inactivation appears to plateau, whereas activation continues to decelerate, ultimately leading to reduced current amplitude and at higher concentrations a decline in total charge transfer. Because the association rate of NZ-58 scales with concentration, 50 μM rapidly and profoundly slows activation, nearly abolishing both current amplitude and charge passage. After brief exposure to low concentrations, these effects were fully reversible, but prolonged application or repeated dosing particularly at higher concentrations resulted in irreversible kinetic alterations.

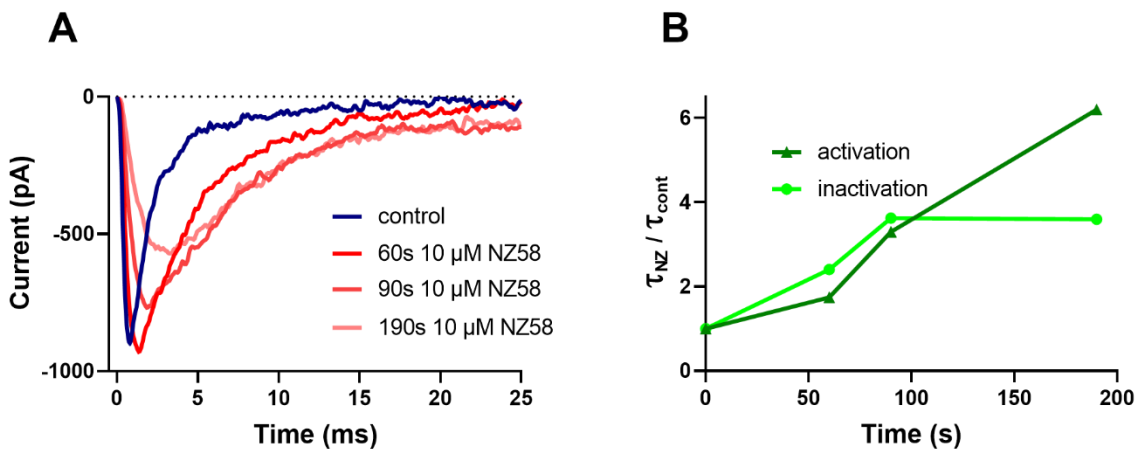


Figure 19. Time dependence of Nav1.5 changes in the activation and inactivation kinetics.

(A) Effect progression on current kinetics as a function of time after 10 μM NZ-58 application.

(B) Time-dependent changes in the ratio of activation to inactivation time constants relative to control. Time constants were extracted from the current traces shown in panel (A).

Apart from altering the activation energy barrier between conformational states, small molecule modulators may also change the energy difference between the states. In line with this, we aimed to verify the voltage-dependence of steady-state activation (G-V) and inactivation (SSI) of Nav1.5 in the presence and absence of NZ-58. We obtained current-voltage (I-V) relationships and constructed normalized conductance-voltage (G-V) relationships. For the SSI curves, the fraction of available channels was assessed by depolarizing pulses from various holding potentials. Surprisingly, neither function was affected by the compound (Fig. 20) implying that the energetics between the resting and activated states of the VSDs were unaffected (G-V: $V_{0.5, \text{cont}} = -36.0 \pm 3.4$ mV, $V_{0.5, \text{NZ}} = -32.8 \pm 4.1$ mV, $p = 0.16$; SSI: $V_{0.5, \text{cont}} = -69.6 \pm 1.0$ mV, $V_{0.5, \text{NZ}} = -70.1 \pm 2.1$ mV, $p = 0.70$).

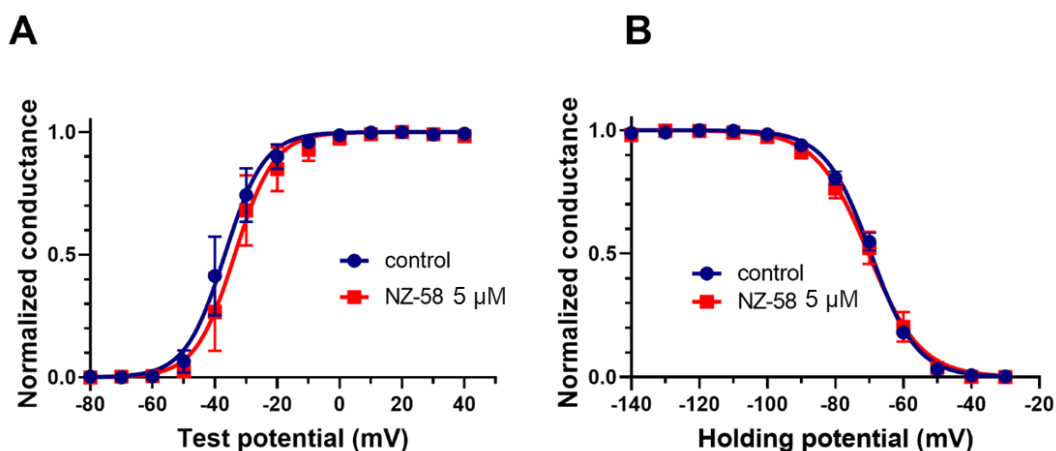


Figure 20. Voltage-dependence of steady-state activation and inactivation of Nav1.5. (A) G-V curves were constructed from I-V relationships using increasing step depolarizations in the absence and presence of 5 μM NZ-58. (B) Depolarizations to 0 mV were used to determine the fraction of available channels at various holding potentials. Blue symbols: control, red symbols: 5 μM NZ-58.

In contrast, the recovery from inactivation was significantly hindered by the compound (Fig. 21A, $\tau_{\text{cont}} = 10.9 \pm 1.9$ ms, $\tau_{\text{NZ}} = 18.4 \pm 2.3$ ms, $n=4$, $p = 0.001$). Consequently, cumulative inactivation during high frequency stimulation (200 ms-long depolarizations at 3 Hz, corresponding to a 180-bpm heart rate) was significantly enhanced, as evidenced by the faster decrease of subsequent

current amplitudes and the lower remaining current fraction in the presence of NZ-58 (Fig. 21 B, Remaining current fractions: 0.51 for control, and 0.14 for 10 μ M NZ-58).

NZ-58 is likely to interact directly with the VSDs, whose extracellular accessibility varies with the channel's gating state being more deeply embedded in the membrane when resting and more exposed upon activation. To test whether NZ-58 can bind when channels are held closed at negative potentials, we recorded a control trace, applied the compound for 20 s at -100 mV, then washed it out before eliciting the next current. NZ-58 produced the same kinetic changes under these closed-state conditions as during continuous pulsing, indicating state-independent binding (Fig. 21C).

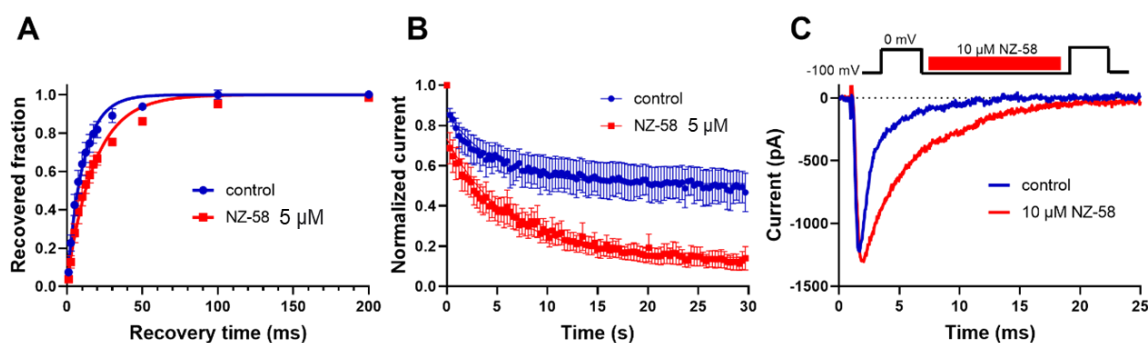


Figure 21. Nav1.5 recovery from inactivation, cumulative inactivation and state-dependence of binding. (A) Recovery from inactivation was assessed using pulse pairs separated by progressively longer intervals at -100 mV, and the fraction of recovered channels is plotted against recovery time (blue: control; red: 5 μ M NZ-58). (B) Cumulative inactivation was evaluated using 200-ms depolarizations to 0 mV delivered at 3 Hz, with normalized amplitudes plotted over time. (C) A control trace (blue) was recorded, followed by a 20-s application of 10 μ M NZ-58 at -100 mV to keep channels closed; after returning to control solution, a second trace (red) was obtained. The resulting kinetic changes indicate closed-state binding.

The effect on Brugada mutant Nav1.5-R1632C

The pronounced slowing of voltage-dependent activation across all tested VGICs, together with the absence of any effect on non-voltage-gated channels, suggested that NZ-58 interacts with a conserved region within the VSDs. Sequence comparison revealed no shared motifs other than the

highly conserved series of positive gating charges (predominantly arginines) occurring at every third position. Assuming these residues might contribute to binding, we mutated the fourth arginine (R4) in the DIV-VSD of Nav1.5, generating the R1632C variant a known loss-of-function Brugada-syndrome mutation [176]. We were able to replicate the published features of the R1632C mutant channel particularly the reduced expression, significantly left-shifted steady-state inactivation curve, and extremely slow recovery from inactivation and then tested the affinity of the mutant for the compound. The mutation had no significant effect on the affinity of NZ-58, as it induced the same kinetic changes as in the wild type channel (Fig. 22). Additionally, neither the effect on the current amplitude nor on the charge transfer was different between the two channels (Figs. 18 B and C, green symbols, amplitude ratio: WT: 0.90 ± 0.02 , mutant: 0.99 ± 0.08 , $p=0.29$; charge transfer ratio: WT: 2.93 ± 0.24 , mutant: 2.38 ± 0.23 , $p = 0.12$).

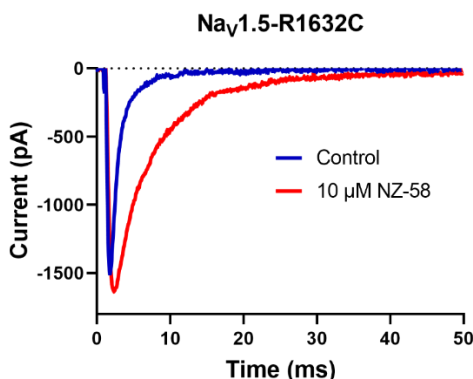


Figure 22. Effect of NZ-58 on the Brugada-mutant Nav1.5-R1632C. Application of 10 μ M NZ-58 induced similar kinetic changes in the mutant channel as in the wild type.

Effects on Kv1.3

Motivated by the similar slowing of VSD movement by NZ-58 in various VGICs and enabling direct comparison with Nav1.5, we next examined the effects of NZ-58 on Kv1.3, a representative member of the voltage-gated K⁺ channel family. As noted above, 10 μ M NZ-58 produced a pronounced slowing of Kv1.3 activation kinetics ($\tau_{act,NZ} / \tau_{act,cont} = 3.13 \pm 0.27$, $n=9$), but unexpectedly, the closing kinetics remained entirely unchanged. ($\tau_{deact,NZ} / \tau_{deact,cont} = 1.03 \pm 0.13$, $n=8$; Figs. 16 A and 17A). The observed pattern is in agreement with our observations in Nav1.5.

However, in stark contrast to Nav1.5 where fast inactivation is tightly coupled to DIV-VSD movement, the VSD-independent, slow C-type inactivation of Kv1.3 was only weakly affected, and in the opposite direction, manifesting as a slight acceleration of inactivation ($\tau_{\text{inact,NZ}} / \tau_{\text{inact,cont}} = 0.86 \pm 0.03$, $n=6$; Fig 23.). The concomitant slowing of activation and speeding of inactivation kinetics resulted in current amplitude reduction (at 10 μM : $I_{\text{NZ}} / I_{\text{cont}} = 0.58 \pm 0.04$, $n=9$; Fig. 23), which at 50 μM culminated in complete current block (Fig. 15A). It is worth noting that prolonged exposure to NZ-58 imposed clear limitations on patch stability and channel integrity. At 50 μM , application for more than 180 seconds consistently led to loss of the patch configuration, indicating a rapid onset of membrane-disruptive or seal-compromising effects at this concentration. Even at the lower concentration of 10 μM , extending the application beyond approximately 7-8 minutes resulted in irreversible alterations of the channel kinetics, suggesting that sustained exposure gradually drives the system into a non-recoverable state.

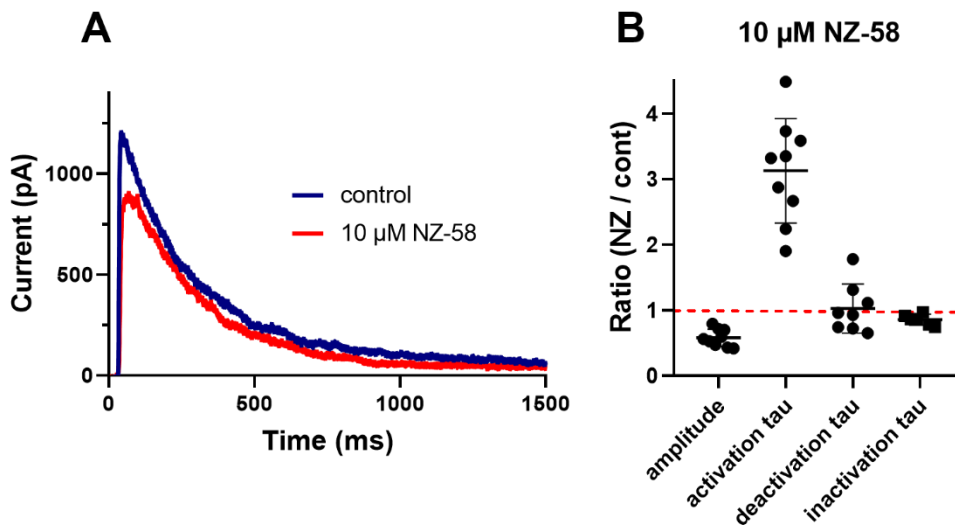


Figure 23. Effects on current amplitude and gating kinetics of Kv1.3. (A) NZ-58 slows activation kinetics and slightly speeds C-type inactivation, which result in a reduction of the current amplitude. Currents were recorded from a human lymphocyte by a depolarization to +50 mV. **(B)** Changes induced in the current amplitude, current activation, deactivation and inactivation

time constants by 10 μ M NZ-58 expressed as the ratio of the parameters measured in the presence to those in the absence of the compound. The red dashed line represents the reference control value.

The strong effect on the VSD motion implies that other aspects of voltage-dependent gating may be altered by NZ-58 binding, so similarly to Nav1.5, we examined whether the compound affects the voltage-dependence of steady-state activation. Due to the strong activation-slowing effect of NZ-58, the usual protocol had to be extended to be able to detect the peak amplitude during intermediate depolarizing pulses. To our surprise, we observed only a slight, non-significant right-shift in the G-V relationship induced by 10 μ M NZ-58 (Fig. 24 A; $V_{0.5, \text{cont}} = -11.4 \pm 2.5$ mV, $V_{0.5, \text{NZ}} = -6.3 \pm 3.7$ mV, $n=7$, $p = 0.15$). In addition, the markedly slowed activation reduces the peak current amplitude, an effect that becomes more pronounced at lower voltages where activation is intrinsically slower, thereby creating the appearance of a right-shift in the activation curve. When this is taken into account, the actual change in the voltage-dependence of activation is negligible. As for Nav1.5, we investigated the voltage-dependence of steady-state inactivation, but with a reduced protocol. This was necessary due to the slow recovery from inactivation of Kv1.3 ($\tau_{\text{rec}} \approx 15$ s) requiring long inter-pulse intervals, which result in lengthy protocols, during which slow irreversible effects of the compound and current rundown can introduce artefacts. Our results showed no significant shift in the $V_{0.5}$ induced by NZ-58 (Fig. 24B; $V_{0.5, \text{cont}} = -43.6 \pm 0.8$ mV, $V_{0.5, \text{NZ}} = -43.7 \pm 0.5$ mV, $n=4$, $p = 0.92$).

Recovery from inactivation was evaluated using a double-pulse protocol, but the sequence was shortened to three pulse pairs because the full protocol would have required prohibitively long recording times. To minimize long-term rundown and cumulative kinetic alterations, NZ-58 was applied at 5 μ M during these measurements. Time constants were extracted from single exponential fits to the data points. Unlike in Nav1.5, NZ-58 had no effect on the recovery rate of Kv1.3 (Fig. 24 C; $\tau_c = 14.44 \pm 0.78$ s, $\tau_{\text{NZ}} = 14.98 \pm 0.77$ s, $p = 0.71$, $n=3$).

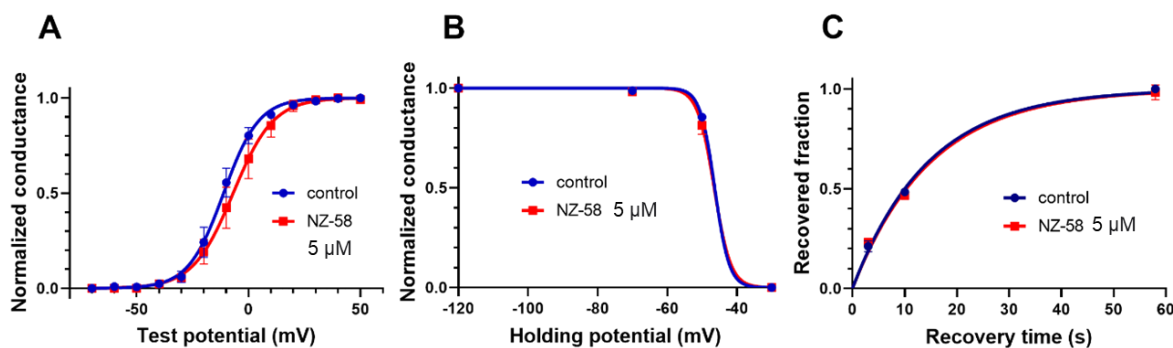


Figure 24. Voltage-dependence of steady-state activation and inactivation and recovery from inactivation in Kv1.3 (A) G-V curves were constructed from I-V relationships using increasing step depolarizations in the absence and presence of 10 μM NZ-58. Blue symbols: control, red symbols: NZ-58 (B) Depolarizations to +50 mV were used to determine the fraction of available channels at various holding potentials. To avoid rundown effects the protocol was reduced to four holding voltages and 5 μM NZ-58 was applied. (C) Recovery from inactivation was measured by pulse pairs separated by increasing recovery periods at -100 mV. The fraction of recovered channels is plotted as a function of recovery time. Similarly to (B), the number of pulse pairs was reduced to shorten the protocol and 5 μM NZ-58 was used.

Although we observed the effects described above when NZ-58 was applied extracellularly, considering its lipophilic nature ($\log D > 4.56$, [75]) we sought to explore the sidedness of channel accessibility [177]. Therefore, inside-out patch experiments were performed on Kv1.3 channels expressed in CHO cells and we applied NZ-58 from the intracellular side of the membrane. We observed the same kinetic effects as with extracellular application, demonstrating that NZ-58 can also access its binding site from the intracellular side (Fig. 26A).

As with Nav1.5, we next examined whether NZ-58 interacts with the gating arginines of Kv1.3. To this end, we generated the Kv1.3 R367C (R2) and R373C (R4) mutants and evaluated the compound's efficacy on these variants. Although the effects induced by 10 μM NZ-58 were qualitatively similar to those observed in the wild-type channel, both the slowing of activation kinetics and the reduction in current amplitude were substantially more pronounced in the mutants (Fig. 25)

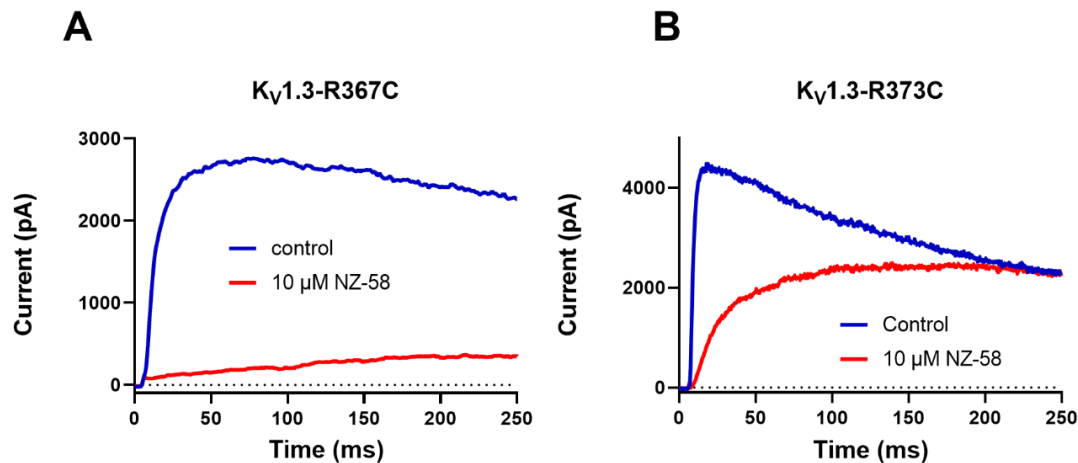


Figure 25. Binding effect of NZ-58 on mutant Kv1.3 channels. (A) Kv1.3 currents recorded from CHO cells expressing the R367C (R2) mutant. (B) Kv1.3 currents recorded from CHO cells expressing the R373C (R4) mutant. Blue traces represent control recordings, and red traces show the effects of 10 μ M NZ-58 applied from the intracellular side.

Gating current measurements

While the above findings point toward a direct action of NZ-58 on VSD movements, alternative mechanisms must also be considered. Slow current activation could, in principle, arise from the compound slowing the final gating step leading to pore opening or disrupting the coupling between the VSD and the pore. To evaluate these possibilities, we performed gating-current recordings using the non-conducting W384F mutant of Kv1.3. In the absence of ionic flux, these measurements isolate the movement of gating charge generated by VSD displacement. Application of 10 μ M NZ-58 markedly slowed the ON-gating currents associated with VSD activation during depolarization; however, strikingly, no comparable effect was observed on the OFF-gating currents that report the return of the VSDs to the resting state upon repolarization (Fig. 26B). This pattern mirrors the behavior seen in the ionic currents, where NZ-58 substantially slowed channel opening in both Nav and Kv channels but left channel closing unaffected. Importantly, the total ON and OFF gating charges remained equal in the presence of NZ-58, implying that the compound alters

the kinetics of VSD movement without reducing the overall charge displaced ($Q_{\text{OFF}} / Q_{\text{ON}} = 0.960 \pm 0.020$, $n=7$, $p = 0.095$), indicating the lack of charge immobilization. Furthermore, the total integrated gating charge remained essentially unchanged following NZ-58 application. This suggests that the compound alters the kinetics of voltage-sensor movement without reducing the overall amount of charge displaced ($Q_{\text{ON, NZ}} / Q_{\text{ON, cont}} = 1.05 \pm 0.04$, $n=7$, $p = 0.26$), implying that the VSDs complete the entire activation trajectory, only at a slower rate.

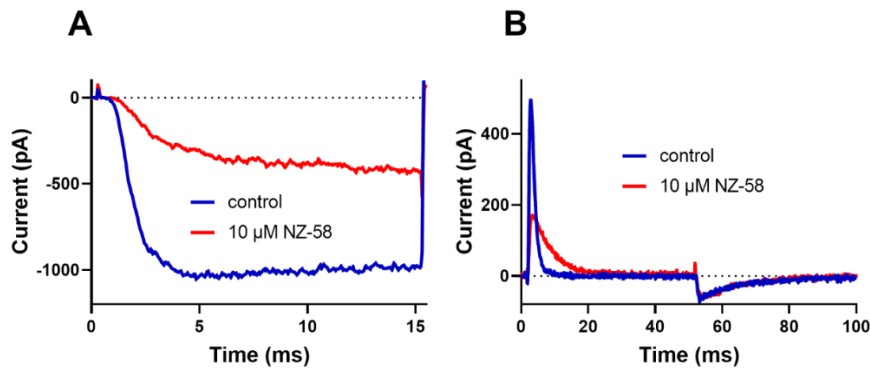


Figure 26. Inside-out configuration and gating current recordings in $K_v1.3$. (A) $K_v1.3$ currents measured in transfected CHO cells in inside-out configuration. Blue: control, red: 10 μM NZ-58 applied from the intracellular side. (B) Gating currents recorded from a CHO cell transfected with KV1.3-W384F channels, induced by depolarizations to +40 mV, from a holding potential of -100 mV and then stepped back to -100 mV. Blue: control, red: 10 μM NZ-58.

DISCUSSION

In general, VGICs share a highly homologous overall structure, in which each such subunit or domain is made up of a VSD and a pore domain. Despite these notable similarities in voltage-sensing function, all these VGICs have unique pharmacological, biophysical properties and physiological roles. Their widespread tissue distribution and essential physiological roles promoted VGICs to be attractive drug targets, and indeed, there are numerous currently used medications achieving their therapeutic effects via VGIC modulation. Well-known examples are lidocaine (local anesthetic), flecainide (antiarrhythmic) and phenytoin (anticonvulsant) acting on Nav channels, nifedipine (antihypertensive) and ziconotide (analgesic) acting on Cav channels and amiodarone (antiarrhythmic) and 4-aminopyridine (for multiple sclerosis) acting on Kv channels [7, 19, 178, 179]. Recently, the anti-arrhythmic agent ranolazine was even found to improve the survival of cancer patients, presumably via inhibiting the Nav channels in cancer cells [180].

My research dealt with small molecules targeting voltage-gated ion channels, developed by our pharmaceutical chemist collaborators in Slovenia. We first searched for a novel inhibitor targeting specifically Hv1: by using the open structure of the channel, our collaborators performed virtual screening and identified hit molecules that we further tested in our system. The most potent compounds namely: NZ-58, NMP-132/133, GHK-21, and GHK-34 were identified and their potency was further quantified by generating dose-response curves, measuring the extent of current block across a range of concentrations (Figs. 6 and 7). NZ-58 was fully characterized in detail on transiently transfected CHO expressing hHv1 to understand its biophysical parameters and mechanism of action by using standard patch clamp methods. The results clearly showed that NZ-58 modifies the voltage-sensing of the hHv1 channel and inhibits the proton current (Fig. 8). As the other published compounds that we have tested have not worked in our hands, we could only compare its effect to the other potent inhibitors, the guanidino-benzimidazoles. Within this series of compounds, ClGBI is the one that acts when applied extracellularly, thus used in functional assays. For this reason, we compared the effect of ClGBI with that of the NZ-58:

- 1.) The affinities are comparable, although NZ-58 has a slightly lower IC₅₀ value (8.5 μ M for NZ-58 and 26.3 μ M for ClGBI).
- 2.) ClGBI has a different effect, as it mostly reduces the peak current without shifting the opening voltage of the channels, i.e. it only affects the proton permeation pathway of the channel. On

the other hand, NZ-58 has a 3-in-1 effect as it modifies the apparent voltage sensing of the channel (V_{thr} and V_{50} values), alters the channel gating kinetics ($\tau_{activation}$, $\tau_{deactivation}$, τ_{eff}) (Figures 7, 11 and 14), and additionally, it reduces the macroscopic conductance.

- 3.) Intracellular access to the inner cavity can be restricted either by closure of the activation gate or by the deep membrane immersion of the VSD in the resting state, which limits the ability of hydrophilic VSD-targeting compounds to engage their binding site. Consistent with this, GBIs preferentially interact with the open conformation of the channel, NZ-58 is capable of binding even when the channel resides in the closed state (Figure 8).

Fitting macroscopic current traces with a sequential 4-state Markov-model of gating transitions revealed that the binding of NZ-58 increases the backward rates, creating a kinetic trap at C2, and that the faster initial current component can be attributed to accelerated $C3 \rightarrow O$ transitions in channels that escape the trap (Fig. 14). Calculation of Gibbs free energies for each state showed that NZ-58 raises the free energy of the C2 and C3 states relative to control, making these intermediates less stable. Furthermore, determining the activation free energies ($\Delta G^\ddagger$) allows construction of the free energy landscape which helps visualize the effect of NZ-58. The reduced backward barrier at $C2 \rightarrow C1$ creates an “open back door”, illustrating the kinetic trap. Thus, our model simply based on altered transition rates by NZ-58 and without an explicit blocked state, was able to sufficiently reproduce the observed effects on the macroscopic currents, defining NZ-58 as a gating modifier of $Hv1$.

Despite its superficial resemblance to “traditional” VGICs, $Hv1$ exhibits a very distinct structure and function. Notably, it consists of a single VSD and it lacks a pore through which other VGICs conduct the permeating ions. Accordingly, in the second phase of the study, I evaluated channel selectivity and extended the characterization of NZ-58 to additional voltage-gated channels, including $Kv1.3$, $Nav1.5$, and $Kv1.5$.

Several effects induced by NZ-58 were shared between $Hv1$ and other VGICs, yet important differences also emerged. In $Hv1$, we previously described a dual action on the two kinetic components of activation: NZ-58 accelerated the fast component, producing an overall faster activation time course, while it had no effect on the slow component. This stands in sharp contrast to the pronounced slowing of activation observed in all tested Kv and Nav channels. Deactivation kinetics also diverged: $Hv1$ exhibited markedly accelerated closing, whereas $Nav1.5$ and $Kv1.3$

showed no detectable change. Furthermore, NZ-58 induced a right-shift in the voltage-dependence of H_v1 activation, an effect absent in Nav1.5 and Kv1.3. Together, these findings point to distinct modes of action in H_v1 versus the classical VGICs.

Because H_v1 consists solely of segments S1-S4 the structural equivalent of a single VSD NZ-58 must bind within the VSD region in this channel. The effects described above suggest that a similar VSD-targeted interaction likely occurs in other VGICs as well. However, our H_v1 mutant analysis and docking studies identified a binding site involving the selectivity filter and proton-permeation pathway rather than the canonical gating arginines. In contrast, the absence of other conserved VSD motifs across Nav and Kv channels initially suggested that the gating arginines might contribute to NZ-58 binding in these channels. Surprisingly, both the R2- and R4-mutant Kv1.3 channels, as well as the DIV-R4 mutant of Nav1.5, retained their sensitivity to NZ-58. In Nav1.5, the modest effect is expected because the mutation alters only a single VSD the DIV sensor responsible for fast inactivation. In Kv1.3, where the mutations are present in all four VSDs, the NZ-58-induced effects became more pronounced, with even slower activation kinetics and, due to overlap with inactivation, a greater reduction in current amplitude compared with wild type. These results suggest that while the gating arginines may participate in the interaction, they are unlikely to be the primary determinants, and additional residues likely make more substantial contributions.

In both Nav1.5 and Kv1.3, NZ-58 produced a robust slowing of channel opening, a process that requires activation of DI–DIII VSDs in Nav1.5 and all four VSDs in Kv1.3. Thus, if NZ-58 binding slows VSD activation, a comparable effect on opening kinetics is expected in both channel types. By contrast, the consequences for inactivation differ: fast, inactivation in Nav channels depends on DIV-VSD activation and was therefore slowed, whereas the slow, C-type inactivation of Kv1.3 originating in the selectivity filter and only indirectly coupled to VSD movement was weakly affected and even shifted in the opposite direction. This slight acceleration of C-type inactivation may reflect allosteric modulation of the selectivity filter or reduced occupancy of K⁺ binding sites due to slower channel opening and diminished current. Consistent with these mechanisms, recovery from inactivation—which is tightly linked to VSD return in Nav1.5, was slowed by NZ-58, whereas recovery in Kv1.3, which is only indirectly influenced by VSD movement, remained unchanged (Fig. 27).

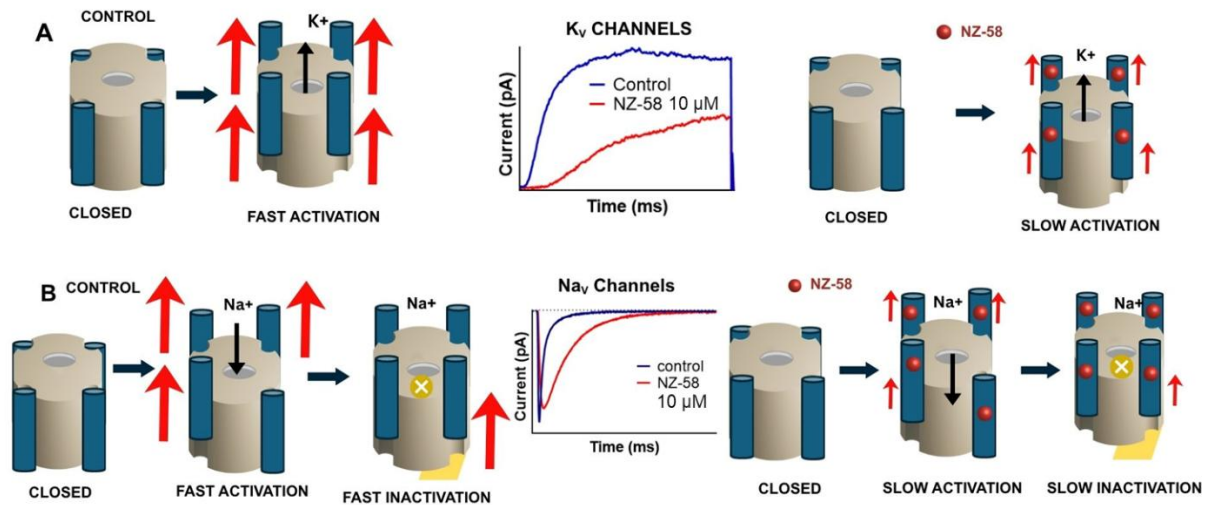


Figure 27. NZ-58 binding to VSDs and its effects on kinetics. (A) The left panel shows gating of K_v channels in control conditions, whereas the right panel illustrates NZ-58 binding, indicated by red circles on the VSDs. The accompanying red arrows represent the altered transition rates, resulting in slowed activation kinetics. (B) Effect of NZ-58 on Na_v channels. The left panel shows control conditions, and the right panel depicts NZ-58 binding. Here, the red arrows indicate modified transition rates that lead to slowed activation and inactivation kinetics.

The combined slowing of activation and inactivation in $Nav1.5$ produced a concentration- and time-dependent modulation of current amplitude that was often biphasic, and in many cases resulted in an increase in peak current. In contrast, the slowing of activation together with the acceleration of inactivation in $K_v1.3$ invariably led to a reduction in current amplitude. At $50 \mu M$ NZ-58, the extreme slowing of activation dominated in all channels, allowing only a brief occupancy of the open state before inactivation, which produced near-complete current suppression. Owing to its intrinsically slow inactivation, $K_v1.5$ retained a substantial fraction of current under these conditions (Fig. 15C).

In $Nav1.5$, the slowing of inactivation developed and saturated more rapidly than the slowing of activation (Fig. 19). This difference can be explained by the distinct VSD requirements for each process: channel opening requires activation of three VSDs (DI-DIII), whereas fast inactivation depends primarily on activation of a single VSD (DIV). Consequently, the binding sites

influencing inactivation saturate more rapidly than the threefold larger set of sites influencing activation. At high NZ-58 concentrations, all sites saturate quickly, leading to a dominant slowing of activation and the associated reduction in current amplitude.

A particularly striking observation is that NZ-58 markedly slowed channel opening in both Nav1.5 and Kv1.3, yet had no measurable effect on channel closing. This is unexpected because perturbations that slow forward transitions often accelerate the corresponding backward transitions, which would manifest as faster tail-current decay. For example, extracellular acidification produces a right-shift in the G-V relationship through surface-charge screening and consequently slows activation while speeding deactivation, as both the VSDs and the pore experience an effectively more negative membrane potential [181]. A similar effect is achieved by several known gating-modifier peptide toxins, for example hanatoxin [182], which shift the G-V curve to the right by binding to the VSD and stabilizing its resting position, thereby slowing opening and speeding closing.

The effects of NZ-58 are most parsimoniously explained by a direct modulation of VSD movement rather than by an interaction with the pore itself. In Kv channels, VSD activation normally occurs rapidly and precedes the slower, rate-limiting pore-opening transition. However, when NZ-58 sufficiently slows VSD activation as reflected by the markedly slowed ON-gating currents this step becomes rate-limiting, producing the pronounced slowing of ionic current activation. In contrast, during hyperpolarization, pore closure (which is unaffected by NZ-58) occurs before the VSDs return to their resting state. As a result, the decay of the ionic current remains unchanged regardless of which transition is rate-limiting. Because pore closure is unaltered, the subsequent charge returns and thus the OFF-gating kinetics also remain unaffected.

Although our gating-current experiments were performed in Kv1.3, the observation that the total gating charge was unchanged by NZ-58 unlike the ~38% reduction produced by lidocaine in Nav1.5 further supports the conclusion that NZ-58 modifies the kinetics of VSD motion without reducing the overall charge displaced [183]. This argues against a trapping mechanism and supports a simple slowing action of the VSDs.

Given that both the G-V and the SSI curves were unaffected by NZ-58 in both Nav1.5 and Kv1.3 channels further supports the idea that NZ-58 binding causes only the slower movement of the

VSDs locally, rather than causing larger scale conformational changes that would affect the energetics of the gating states.

As NZ-58 was equally effective in whole-cell and inside-out patch configurations, and considering its lipophilic nature and its ability to easily cross the membrane, the sidedness of the binding site accessibility is difficult to predict. Since application of NZ-58 to the closed channels induced the same kinetic changes as when channels were exposed to it in the closed, open and inactivated states due to continuous pulsing, it is reasonable to assume that it can bind to the VSD even in its resting state, when it is mostly buried in the membrane.

As discussed earlier, only a minority of Nav-modulating small molecules bind directly to the VSDs, which can in principle offer more selective sites for modulation than the central cavity. These include natural peptide toxins and synthetic aryl-sulfonamide derivatives, yet their mechanisms of action differ markedly from that of NZ-58, particularly given NZ-58's ability to enhance channel activity at low concentrations. Comparisons with known small-molecule activators likewise reveal entirely distinct binding sites, gating effects, and patterns of state-dependent interaction. To our knowledge, no other modulator has been shown to selectively alter the speed of VSD movements without measurably affecting the energetics.

NZ-58 as a prospective lead molecule?

Although NZ-58 exerts an overall inhibitory effect on most channels examined in this study primarily through a pronounced slowing of channel opening it produces a strikingly different outcome at low concentrations in Nav channels. Even at 0.5 μ M, NZ-58 enhanced Na⁺ influx by approximately 22%, demonstrating a clear potentiating action. Notably, this enhancement was also preserved in the loss-of-function Brugada-syndrome mutant, indicating that the compound can augment activity even in channels with impaired gating. Such behavior suggests considerable therapeutic potential for conditions involving Nav loss-of-function, a concept supported by prior work showing that a spider-venom peptide activator can rescue Dravet-syndrome mice by restoring activity of the loss-of-function Nav1.1 channel [184]. As demonstrated by our results, NZ-58 is far from isoform-selective; indeed, it is not even selective for channel type, as evidenced by its activity across a diverse panel of channels. Hindering factors including the inherently small interaction surfaces through which most small molecules engage their target proteins, resulting in lower selectivity compared with peptide toxins, as well as the influence of the lipophilic nature (high

logP), which can drive persistent absorption into the lipid bilayer, collectively contribute to the challenges associated with developing highly selective modulators [185]. Nevertheless, several highly isoform-selective channel modulator small molecules exist.

Unfortunately, many studies reporting newly identified ion-channel modulators either omit selectivity testing altogether or restrict it to closely related isoforms within the same family, without extending the analysis to other channel classes. Over time, this practice can lead to the mischaracterization of such compounds as “selective inhibitors”, which are then used in functional experiments and may ultimately generate misleading conclusions about the physiological roles of specific ion channels [186, 187].

Although isoform selectivity is a highly desirable property for potential lead molecules both to maximize therapeutic precision and to minimize off-target adverse effects, the reality is that most clinically used VGIC modulators are far from being selective. In practice, many of these agents function as broad-spectrum “dirty drugs,” interacting with multiple channel types and isoforms rather than a single intended target [188]. Nevertheless, despite their narrow therapeutic windows, these agents still achieve meaningful clinical benefit, which justifies their continued use. A well-known example is lidocaine, which blocks multiple Nav isoforms yet remains an effective local anesthetic, even though it also inhibits the cardiac Nav1.5 channel [183, 189]. Verapamil, a classical blocker of voltage-gated Ca²⁺ channels used in the treatment of hypertension and angina pectoris, also inhibits several Nav isoforms, including the cardiac Nav1.5 channel. [190, 191] and Kv channels [192]. The Class III antiarrhythmic drug amiodarone exemplifies this principle: as it blocks Kv channels, ligand-gated K⁺ channels, as well as Nav and Cav channels, thereby influencing virtually every phase of the cardiac action potential. [179, 193]. These examples illustrate that careful dosing and the appropriate delivery route can allow even a narrow therapeutic window to be used advantageously. Moreover, the multi-target nature of a drug can, in some contexts, become beneficial. For instance, suppression of cancer cell progression may require simultaneous modulation of multiple VGICs, as many tumors overexpress several channel types including Hv1 [61, 194, 195]. In these cases, a synergistic inhibitory effect may yield beneficial results.

Limitations and pathways for future development

Considering the lipophilic nature of NZ-58, its pan-VGIC inhibitory profile, and the highly conserved architecture of VSDs across channel families, achieving domain-specific pharmacological precision remains the central limitation of this work. Thus, due to the structurally simpler voltage-sensing-only architecture of Hv1, high-resolution cryo-EM structures captured across distinct gating states would be invaluable for revealing cryptic binding pockets and defining the anatomical features that distinguish it from other VSDs [10, 76, 79].

Furthermore, virtual screening results from our chemist collaborators underscored that an unsubstituted 2-aminoimidazole core, together with flexible linker architectures, are essential for achieving optimal Hv1 engagement [75]. These features result in a combination of hydrogen-bonding interactions, which stabilize the ligand within the VSD environment, and hydrophobic contacts, which complement the lipophilic character of the compound. Therefore, modification of these parts, while reducing lipophilicity, may offer a strategy aimed at improving selectivity while preserving pharmacological potency, as we suspect at least one binding site accessible via the membrane, and reducing or fully preventing the access to this site may make extracellular binding more specific.

SUMMARY

In this work, I systematically characterized a voltage-gated ion channel modulator using patch-clamp electrophysiology, identified by screening a chemically diverse library of synthesized small molecules provided by our pharmaceutical chemist collaborators. Through this structured and detailed discovery effort, I identified an unreported compound, NZ-58, which exhibited remarkably high potency toward the hHv1 channel. Detailed electrophysiological analysis allowed us to delineate the biophysical mechanisms underlying NZ-58 action and to clarify its influence on the structure-function relationship of hHv1, including its binding and gating mechanisms.

Beyond its activity on hHv1, NZ-58 emerged as a broad-spectrum “pan-VGIC” modulator, inhibiting every voltage-gated ion channel tested while showing no detectable activity on non-voltage-gated channels. This unique profile positioned NZ-58 as a valuable molecular probe for dissecting conserved features of voltage-sensor domain pharmacology. By comparing its interactions with representative channels, specifically Nav1.5 and Kv1.3 it was possible to identify both shared and subtype-specific effect on channel gating, providing mechanistic insight into activation, inactivation, and voltage-sensor transitions.

In summary, this research work establishes NZ-58 as a powerful tool compound with a novel mechanism of action for probing VSD-mediated modulation of channel gating that can guide the future rational design of potential therapeutic compounds targeting VGICs.

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KEYWORDS

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molecular mechanism

binding site

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Gating modulator

Nav1.5 channel activation

Brugada mutation

Enhanced charge transfer

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APPENDIX



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2. **Domingos, G. J.**, Fehér, Á., Teshome, R. T., Husain, S., Papp, F., Amna, A. O., Bangera, K. C., Vizi, Z., Elian, R., Kovács, Z. M., Szentandrassy, N., Piga, M., Tomasic, T., Zidar, N., Varga, Z.: A small molecule voltage-sensor modulator enhances the function of the cardiac NaV1.5 channel.
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List of other publications

3. Óvári, J., Kovács, Z. M., Dienes, C., Magyar, J., Bányász, T., Nánási, P. P., Horváth, B., **Domingos, G. J.**, Fehér, Á., Varga, Z., Szentandrassy, N.: Delavirdine modifies action potential configuration via inhibition of IKr and Ito in canine ventricular myocytes.
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