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Pharmacological modulation of voltage-gated ion channels by small
molecules

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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, Hv1. 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 Kv1.3 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, which allow ions such as Na^+ (Nav), K^+ (Kv), Ca^{2+} (Cav), and H^+ (Hv) to cross 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-excitable 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.

Comparison of VGICs

Although voltage-gated ion channels share a conserved structural framework, they also differ in how their pore-forming units are assembled. Channels selectively permeable to K^+ ions are built from subunits that assemble as homo- or hetero-tetramers, arranged symmetrically around a central ion-conducting pore. In contrast, 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 correspond to the monomeric Kv channel subunits. 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. In general, in each of the subunits or domains, VGIC contain a voltage-sensing domain (VSD) and a pore domain (PD), connected by a linker. Together, these elements form six transmembrane segments (S1–S6). Notwithstanding, the Hv1 channel deviates from this canonical architecture by having only the VSD and lacking the PD.

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 families 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. 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, which enables voltage sensing and coupling to pore opening. During depolarization-induced activation, the positively charged residues within the S4 helices shift through the electric field, and generate a measurable displacement of charge, known as the “gating current,” which can be experimentally monitored to track VSD movement. The PD in VGICs is formed by the two pore-forming α -helices, S5 and S6, and the S4–S5 linker helix couples it to the VSD. The four subunits/domains assemble into a central conduction pathway, through which ions can cross the plasma membrane.

Overview of the voltage-gated proton channel, H_v1

In 1982, Thomas and Meech provided the first electrophysiological recordings of proton currents in molluscan neurons, despite of which, the molecular identity of the proton channel remained elusive for more than two decades. In 2006, two independent laboratories successfully identified the gene encoding the voltage-gated proton channel, first in mouse and subsequently its human homologue.

Physiological expression of H_v1

The H_v1 channel expression is surprisingly broad, spanning a variety of immune cells, sperm cells, tumor cells, cells of the nervous system and several other specialized tissues and recently also found in human chorion-derived mesenchymal stem cells. Its most prominently recognized function is sustaining the oxidative burst in immune cells required for effective pathogen elimination. 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 thereby counteracting these processes.

H_v1 activity in human peripheral blood lymphocytes contributes to the regulation of B cell receptor signaling required to prevent excessive intracellular acidification and to preserve mitochondrial integrity in naïve CD8⁺ T cells. 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.

Intracellular pH also serves as a critical determinant of sperm motility and fertilizing capacity. H_v1 promotes the activation of CatSper, leading to a robust influx of calcium ions essential for the induction of hyperactivated motility, the progression of capacitation, and the initiation of the acrosome reaction. 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.

Pathological expression of H_v1

Beyond its involvement in physiological processes, extensive research has focused on H_v1 due to its emerging roles in neurodegeneration, ischemic stroke, immune disorders, and tumor progression, conditions in which H_v1 is frequently overexpressed. In experimental models of Parkinson's disease, H_v1-dependent NOX activation has been linked to enhanced production of pro-inflammatory cytokines, microglia-mediated demyelination, and subsequent neuronal damage. These findings highlight H_v1 as an important modulator of microglial reactivity and a potential contributor to neurodegenerative processes. H_v1-dependent regulation of reactive oxygen species is also central to the immunosuppressive activity of myeloid-derived suppressor cells (MDSCs), particularly within the tumor microenvironment.

In a highly regulated tumor microenvironment, cancer cells must continuously extrude excess cytosolic protons to maintain an intra- and extracellular pH compatible with proliferation, migration, tumorigenesis, and metastasis. High H_v1 levels were strongly associated with poor clinical progression and unfavorable patient outcomes and suggested that H_v1 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.

Structure and function of the Hv1 channel

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. Deleting both the N and C termini simultaneously results in monomeric constructs leading to much faster activation. Each subunit is comprised of 273 amino acid residues, and possesses a voltage-sensor-controlled gate capable of mediating proton flux on its own. Hv1 has a very small conductance with a characteristic current that is highly dependent on ΔpH , the difference between the extracellular (pH_o) and intracellular (pH_i) pH.

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.

Pharmacology and modulation of Hv1

Several classes of Hv1 modulators have been identified and extensively studied. However, inorganic zinc ion remains the prototypical non-selective Hv1 blocker. 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). A blocker named HIF, targets the VSD in both open and closed states besides shifting the voltage dependence of activation. By using virtual screening, YHV98-4 was identified as an inhibitor of Hv1, which reduced ROS production in inflammatory pain. 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. Additionally, several antipsychotic drugs including chlorpromazine, haloperidol, and clozapine have been reported to suppress proton currents in microglia, in the central nervous system. In addition, venom-derived peptide toxins such as Hanatoxin, AGAP, and Corza6 have been shown to inhibit Hv1 channels expressed in spermatocytes. 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, arachidonic acid, phorbol ester PMA, the chemotactic peptide, fMLF and the endocannabinoid neurotransmitter anandamide can enhance H_v1 proton current.

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. Mammalian voltage-gated sodium channels encompass nine α -subunit subtypes Nav1.1 to Nav1.9 and share around 50-80% amino acid sequence homology. 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, Nav1.4 is expressed in the skeletal muscles, while Nav1.5 is distributed in cardiac muscle tissues. Nav1.7, 1.8, and 1.9 are located largely in the neurons of peripheral nervous system. In addition, voltage-gated sodium channels are also widely expressed in non-excitable cells including lymphocytes, macrophages, fibroblasts, endothelial and pancreatic β -cells, where they regulate variety of physiological functions, as well as in cancer cells, in which they contribute to invasion. Nav channels are divided into two major groups based on their sensitivity to the classical Nav channel blocker tetrodotoxin.

Structure-function and biophysics of Nav channels

Sodium channels are composed of a pore-forming α subunit of 260 kDa assembled with auxiliary 30-40 kDa β -subunits. 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.

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. Pharmacological agents used for peripheral nerve targeted analgesia, most notably the local anesthetics lidocaine and bupivacaine are widely employed in clinical practice. On the other hand, Nav channel activators toxins such as batrachotoxin as well as plant-derived agents like pyrethroid and veratridine are known to activate Nav channels. 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.

Growing insights into the physiological roles of specific sodium channel subtypes have stimulated significant progress in drug discovery. A notable example is lacosamide, approved in 2008 as an anticonvulsant and for the treatment of diabetic neuropathic pain. Other clinical candidates have emerged, including AZD-3161, reported to have advanced to Phase I following intradermal administration, XEN402 demonstrated a positive Phase II outcome in acute pain when applied topically. Likewise, PF-05089771 has progressed into Phase II clinical evaluation. 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.

Binding mechanisms of drugs targeting Nav channels

Nav channels are targets of numerous modulator compounds with several typical binding sites, which could be more precisely defined with the advent of high-resolution cryo-electron microscopy structures. Most clinically used drugs targeting Nav channels, such as anti-arrhythmic agents and local anesthetics, bind in the central cavity. However, in contrast to the central cavities of Nav isoforms, which show high homology, the VSDs are much more distinct and therefore are more suitable for specific targeting. Yet, currently much fewer modulators are known to target the VSDs, and most of those are gating modifier toxins from animal venoms.

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.

Structurally, K^+ channels are traditionally classified according to the number and organization of their transmembrane (TM) domains into four major groups: inwardly rectifying K^+ (K_{ir}) channels, two-pore domain K^+ (K_{2P}), Calcium-activated K^+ (K_{Ca}) and voltage-gated K^+ (K_V) channels. 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 subunits.

As my work only involved K_V channels and the non-voltage-gated $K_{Ca3.1}$ and $K_{ir2.1}$ channels with negative results, only K_V channels will be introduced in detail.

Voltage-gated potassium channels (K_V)

The human genome contains 40 genes associated with the K_V superfamily comprised of two major structural and functional groups. The first group consists of genes encoding classical six-transmembrane (6TM) K^+ channel subunits, four of which form the functional tetrameric K_V channels. The second group comprises a set of structurally similar, but electrically silent or non-conducting K_V subunits, which function as gating modulators rather than independent ion-conducting channels. Although they lack the ability to form 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.

Biophysical features of K_V channels

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. Similar to the previously described Na_V channels, K_V can occupy three major conformational states: closed, activated, and inactivated, each reflecting distinct protein conformations.

Traditionally, K_V currents are classified into showing A-type (fast inactivation) or delayed rectifier behavior (slower or no inactivation). Notable members of A-type channels are K_V1.4, K_V3.3, and K_V3.4 and K_V4.1, K_V4.2, and K_V4.3. These typically inactivate by N-type inactivation, which is a rapid process occurring within a few milliseconds. This occurs via a tethered N-terminal inactivation domain occluding the open pore according to the classical ball-and-chain model. Delayed rectifiers, such as K_V1.3, K_V1.5 or K_V7.x channels usually inactivate by C-type inactivation involving slower structural rearrangements near the external pore mouth and selectivity filter.

Pharmacology of voltage-gated potassium channels

A comprehensive pharmacological comparison of several delayed rectifier K_V channels specifically K_V1.1, K_V1.2, K_V1.3, and K_V1.5, revealed a broadly conserved sensitivity profile across this subfamily. All four channels displayed sub-millimolar sensitivity to the classical K_V blocker 4-aminopyridine (4-AP), and were also inhibited by a distinct group of modulators including capsaicin, nifedipine, diltiazem, and resiniferatoxin with potencies in the tens-of-micromolar range. They also demonstrate a wide range of sensitivities to external tetraethylammonium (TEA), with IC₅₀ values ranging from 0.3 to 560 mmol/L in K_V1.1 and K_V1.2, respectively. 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. 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.

Binding mechanisms of drugs targeting K_V channels

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, others alter the gating machinery by interacting with the voltage-sensor domain, thereby shifting activation or inactivation kinetics. 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. Finally, additional layers of regulation arise from interactions with auxiliary subunits, which can modify channel trafficking, surface expression, and biophysical properties.

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, pynegabine, and CB-003, all specific activators of K_v7.2/7.3 channels for neurological indications, now progressing through human clinical trials.

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. Despite this fact, the number of VGIC modulators developed into clinically useful drugs has slowed markedly over the past decades, as emphasized by previous studies. 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 for 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 Na_V and K_V channels, exemplified by Na_V1.5 and K_V1.3, 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

Chinese Hamster Ovary (CHO) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA, Cat# 11965084) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 2 mM L-glutamine, 100 µg/mL streptomycin, and 100 U/mL penicillin-G (Sigma-Aldrich, St. Louis, MO, USA). Cultures were kept at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. Cells were passaged twice weekly following a 2–3 min incubation in PBS containing 0.2 g/L EDTA (Invitrogen, Waltham, MA, USA).

Human peripheral blood mononuclear cells (PBMCs) were isolated from venous blood obtained from anonymized healthy donors. Blood samples were collected by having approval from the Ethical Committee of the Hungarian Medical Research Council (36255-6/2017/EKU). PBMCs were isolated by Histopaque1077 (Sigma-Aldrich Hungary, Budapest, Hungary) density gradient centrifugation. The isolated cells were resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Cat# 11875085) supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich, St. Louis, MO, USA), 100 µg/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine. They were then seeded into 24-well culture plates at a density of $5\text{--}6 \times 10^5$ cells/mL and maintained in a 5% CO₂ incubator at 37 °C for 3–5 days. Phytohemagglutinin A (PHA, Sigma-Aldrich, St. Louis, MO, USA) was added at 8–12 µg/mL to enhance K_v1.3 expression. 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.

The ion channel constructs were obtained from the following sources: hK_v1.4-IR, the inactivation ball deletion mutant of K_v1.4 (a gift from D. Fedida, University of British Columbia, Vancouver, Canada); hK_v1.5 in the pRcTAL C1 YFP plasmid (a gift from A. Felipe, University of Barcelona, Spain); hK_v11.1 (a gift from H. Wulff, University of California, Davis, USA); hNav1.5 in pH1TO-EGFP plasmid and hNav1.4 (gifts from P. Lukács, Eötvös Loránd University, Budapest, Hungary); mK_{ir}2.1 in CMV pA CMV mRFP pA AV39 plasmid (a gift from I. Levitan, University of Illinois, Chicago, USA); and hK_{Ca}3.1 in the pCMV6 AC GFP plasmid (a gift from B. Attali, Tel Aviv University, Israel). CHO cells were transiently transfected using a Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's protocol with the above ion channel coding vectors. hK_v1.4 was transiently co-

transfected with a plasmid encoding the green fluorescent protein (GFP) at a molar ratio of 10:1. For the $K_{V11.1}$ and $Na_{V1.4}$ currents were recorded from stably expressing HEK cell lines CHO cells were transiently transfected using a Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's protocol with the ion channel coding vectors (table...). $K_{V11.1}$, *hERG1*, *hKCNH2* gene (a kind gift from H. Wulff, University of California, Davis, CA, USA) and $Na_{V1.4}$, *hSCN4A* gene, (a kind gift from P. Lukács, Eötvös Loránd University, Budapest, Hungary) currents were recorded from stably expressing Human embryonic kidney (HEK) 293 cell lines.

CHO cells, PBMCs, and HEK cells were gently washed twice with 2 mL of extracellular solution, before patch-clamp experiments, and replated onto 35-mm polystyrene culture dishes (Cellstar, Greiner Bio-One, Kremsmünster, Austria). GFP-positive transfected cells were identified using a Nikon TS100 fluorescence microscope (Nikon, Tokyo, Japan) with 455–495 nm excitation and 515–555 nm emission filters. In general, ionic currents were recorded 24–36 hours after transfection, whereas $hK_{V1.3}$ currents were measured in activated lymphocytes 4–5 days after stimulation.

Electrophysiology

Using voltage-clamp mode, the standard whole-cell patch clamp configuration was used to record ionic currents. Micropipettes were pulled in four stages using a Flaming Brown automatic pipette puller (Sutter Instruments, San Rafael, CA, USA) from GC 150F-15 borosilicate glass capillaries (Harvard Apparatus Co., Holliston, MA, USA) with tip diameters between 0.5–1 μ m and typical resistances were 7–10 M Ω (due to lower solution conductance) and 3–5 M Ω for H_{V1} and $K_{V1.3}$ / $Na_{V1.5}$, respectively. Series resistance (R_s) compensation of 45–70 % was enabled before oscillation was induced. All measurements were carried out using Axopatch 200B amplifiers connected to personal computers using Digidata 1550B data acquisition hardware (Molecular Devices Inc., Sunnyvale, CA, USA). Records were discarded when a leak at the holding potential was more than 10% of the peak current at the given test potential. Experiments were conducted at room temperature, which ranged between 20 and 24 °C.

In our standard recordings of hH_{V1} currents were elicited using a 1-second voltage-ramp protocol that progressed linearly from a holding potential of –60 mV to +60 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, it enabled simultaneous monitoring of the peak current, the activation threshold (V_{thr}), and the tail current measured at -80 mV. The reversal potential was routinely evaluated using a tail I-V protocol; exceptions to this procedure are described in the main text. The opening voltage of the hHv1 channels was objectively determined by the following: we selected a membrane potential range, where the opening probability of the proton channels is close to zero, then we determined the standard deviation (SD) of the leak corrected current within that interval. By increasing the membrane potential with the ramp protocol, when the macroscopic current was five times higher than the SD for 5 ms, then the beginning of that 5 ms interval was defined as the time of channel opening, and the corresponding membrane potential was taken as the opening threshold voltage.

To record sodium currents mediated by Nav1.4 and Nav1.5, the holding potential (V_h) was maintained at -100 mV, and depolarizing pulses were delivered at 5-s intervals unless otherwise specified. Depolarizing steps to 0 mV with durations ranging from 15 to 50 ms were applied. For the potassium channel Kv1.3, voltage-step protocols with pulse durations between 15 and 2000 ms were used. Recordings of Kv1.5 currents were obtained using 100-ms depolarizing steps to 50 mV, applied every 15 s from a holding potential of -100 mV.

For the measurement of Kv1.4 we used a 50 ms step protocol from a holding potential of -100 mV to $+20$ mV. The Kv11.1 current was recorded using a voltage-step protocol consisting of a holding potential of -80 mV, followed by a 1250-ms depolarizing pulse to $+20$ mV, and subsequently a 2-s hyperpolarizing step to -40 mV delivered every 30 s. Peak tail currents were quantified during the hyperpolarizing step. For K_{Ca}3.1 currents, a 200-ms-long voltage ramp to $+50$ mV from -120 mV was applied every 10 s. The Kir2.1 current was measured using a 600-ms voltage-step protocol, applying steps from a holding potential of -10 mV 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 R373C and R373A mutations were introduced into the pC1-EGFP-hKv1.3-WT and the R1632C and R1632A into the pH1TO-EGFP-Nav1.5 plasmids, respectively. For Kv1.3, the two sets of mutagenesis primers were designed following the one-step site-directed mutagenesis protocol of Huanting Liu et al [173]. The primers were designed

with an overlapping 5' region and a non-overlapping 3' extension (Integrated DNA Technologies-IDT, Leuven, Belgium). PfuUltra II polymerase was used (Agilent Technologies, Santa Clara, CA, USA), as well as dNTPs, DpnI enzyme (Thermo Fisher Scientific, Waltham, MA, USA). For Nav1.5, two-sets of primers were designed, the overlapping mutagenesis primers and the flanking primers containing restriction sites at the 5' and 3' ends of the mutation site. FastDigest (FD) enzymes (FD-KpnI & FD-XhoI) (Thermo Fischer Scientific, Waltham, MA, USA) were used, the digested products were extracted from the gel using QIAquick gel extraction kit (Qiagen, Germany), then 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'. All the mutants and wild-type constructs were confirmed by DNA sequencing (Plasmidsaurus) before electrophysiological experiments.

Solutions

Solutions were freshly prepared, pH checked before patch clamp recordings and osmolarities of the extracellular solution (ECS) and intracellular solution (ICS) were measured between 302 and 308 mOsm/L and ~295 mOsm/L, respectively. For hK_v1.3, hK_v1.4, hK_v1.5, hK_v11.1, hNav1.4, and hNav1.5, the ECS (bath) contained 145 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1 mM MgCl₂, 10 mM Hepes, and 5.5 mM glucose (pH = 7.35 titrated with NaOH), and the ICS (pipette) for K_v channels contained 140 mM KF, 2 mM MgCl₂, 1 mM CaCl₂, 11 mM EGTA, and 10 mM Hepes (pH = 7.2 with KOH). For potassium channels, a high K⁺-based ECS was used as an indicator of the proper solution exchange, whose composition was identical to standard ECS except that it contained 150 mM KCl and 0 mM NaCl. For Nav current recordings, Cs⁺-based ICS was used to avoid the recordings of endogenous K⁺ currents; thus, the ICS consisted of (in mM) 10 NaCl, 105 CsF, 10 HEPES, and 10 EGTA (pH = 7.2 titrated with CsOH). For the K_{ir}2.1 measurements, the extra and intracellular solution consisted of (in mM) 72.5 NaCl, 77.5 KCl, 2.5 CaCl₂, 1 MgCl₂, 10 HEPES, and 5.5 glucose (pH = 7.35 titrated with KOH) and 140 KF, 2 MgCl₂, 1 CaCl₂, 11 EGTA, 1 Na-ATP and 10 HEPES (pH = 7.2 with KOH). For hK_{Ca}3.1, the ECS contained 145 mM L-aspartic acid with Na, 5 mM KCl, 2.5 mM CaCl₂, 1 mM MgCl₂, 10 mM Hepes, and 5.5 mM glucose (pH = 7.4 with NaOH), and the ICS contained 145 mM K-Asp, 2 mM MgCl₂, 8.5 mM CaCl₂, 10 mM EGTA, and 10 mM Hepes (pH = 7.2 with KOH), giving ~2 μ M free Ca²⁺ to fully activate the hK_{Ca}3.1 current. For Nav channels, a choline-based ECS was used for perfusion control, whose composition was (in

mM) 145 choline-Cl, 5 KCl, 10 HEPES, 5.5 glucose, 2.5 CaCl₂, and 1 MgCl₂. The same ECS was used for gating current measurements to avoid the interference from endogenous Nav channels in CHO cells. 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 stocks stored at -20°C . Stock solutions were prepared from powder dissolved in water-free DMSO (Sigma-Aldrich, Budapest, Hungary).

As 10 μ M NZ-58 produced significant gating changes, we aimed at performing all measurements at this concentration. However, protocols lasting over several minutes (especially in the case of Kv1.3), or after multiple rounds of compound application and washout, irreversible changes occurred, which distorted the data. In these cases, we applied shortened protocols by reducing the number of recorded sweeps and / or performed the experiments with 5 μ M concentration. This concentration still produced significant changes, so the obtained results are relevant and comparable with the 10 μ M data.

Perfusion indicator and positive controls were applied at a concentration equivalent to their K_d values (10 mM TEA⁺ for Kv1.3, 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

The pClamp 10.7 software package (Molecular Devices Inc., Sunnyvale, CA, USA) and GraphPad Prism 8 (GraphPad, CA, USA) were used for data acquisition and analysis. In general, currents were lowpass-filtered using the built-in analog four-pole Bessel filters of the amplifiers and were sampled (2–50 kHz) at least twice the filter cut-off frequency. Before analysis, current traces were digitally filtered with a Gaussian filter and were corrected for ohmic leakage when needed.

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 [174]. The model consists of three closed states and one open state ($C1 \rightleftharpoons C2 \rightleftharpoons C3 \rightleftharpoons O$). 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. Current traces from each cell were normalized to their maximum current amplitude and averaged at each test potential. Model parameters were estimated using a two-stage optimization approach.

Statistics

Data are given as mean $\pm$ SEM, for a minimum of 3 independent experiments. In most cases, values were obtained on the same cell before and after treatment, so a paired t-test was used. For ratio comparisons, a one-sample t-test was used with a theoretical mean of 1. For independent samples, 2-sample t-tests or one-way ANOVA were used. A change was deemed significant at $p < 0.05$.

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 [26, 75] 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 [175]. 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 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, 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. 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. 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.

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

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.

Application of 50 μ M NZ-58 almost completely inhibited the proton 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. 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, ($n \geq 3$ 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.

We further 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. To achieve this, we have recorded the ramp protocol 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 channel during the second set of episodic stimulation. The perfusion duration of NZ-58 was selected based on the wash-in kinetics.

Channel modulator compounds may also exhibit functional selectivity, which means that they can bind to different channel states but have different affinities for them. In line with this, 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). 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.

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. Notably, the predicted binding pose overlaps with the intracellular binding site previously proposed for guanidinobenzimidazole derivatives, including 2GBI and ClGBI. 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, 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

≥ 3 ; one-way ANOVA with Dunnett's multiple comparison test). These results thus confirm the residues interacting with NZ-58.

Molecular mechanism of Hv1 modulation by NZ-58

After we successfully identified the binding pocket, we further investigated the molecular mechanism of the binding. To properly separate conductance, channel kinetics and voltage-sensing, additional experiments were required. Thus, our first effort was to confirm that the above described V_{thr} shift came from the altered voltage sensing. To do so, we constructed the G-V curves under control condition and after 10 μ M NZ-58 saturation. We selected a lower concentration of NZ-58 because 50 μ M causes nearly complete current block, hindering the possibility of obtaining a reliable G-V curve. Fitting the curves with the Boltzmann equation yielded -2.7 mV and 10.1 mV V_{50} values for the control and the NZ-58 perfusion respectively, thus confirming the V_{thr} shift we have seen with the ramps.

To further investigate voltage-sensitive gating, we examined if the shifted opening threshold was the sole consequence of NZ-58 binding. With the recorded I-V protocols, we could also check the possible alterations in channel kinetics. Application of 10 μ M NZ-58 accelerated the fast component of the activation reducing the sigmoidicity of the classic WT hHv1 channel activation. On the other hand, NZ-58 reduced the deactivation time constant, which yields faster O $\rightarrow$ C transition, mimicking the inhibitory property of a typical voltage-sensor modifier. In order to untangle this seemingly contradictory phenomenon we constructed the sequential 4-state Markov-model of hHv1 based on the previous publication by Villalba-Galea. 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. 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 $\approx$ 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 C1 $\rightleftharpoons$ 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 can be explained by the increased C3 $\rightarrow$ O transition: once the channel escapes the trap it reaches the conductive conformation faster.

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. However, applying at 10 μ M (close to the IC₅₀ on Hv1), NZ-58 caused significant gating changes in VGICs with only a small current reduction in most channels. The observed effects were variable throughout the channels with some common features and some more channel-specific and opposing effects.

The most notable effect on all Kv1.x and Nav1.x channels was the robust slowing of activation kinetics. This was assessed by single exponential fits for easier comparison, although, due to the multi-VSD nature of current activation, the rise of the current traces was often clearly sigmoidal. The dramatic slowing of current activation by 10 μ M NZ-58 in some VGICs, expressed as the ratio of the activation time constants in the presence and absence of the compound suggests a direct interaction of NZ-58 with the VSDs, which was further supported by the lack of effect on non-VGICs Kir2.1 and KCa3.1.

Slowing of VSD activation may indicate a process stabilizing the resting state and thus slowing of the forward rate and speeding of the backward rate, which should manifest as faster deactivation (channel closing) at negative potentials. However, we observed no such change in the tail current kinetics of Kv1.3 or Nav1.5 channels (Kv1.3: 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, $n=4$, $p=0.86$). Since the rate of deactivation of Nav1.5 at -100 mV was comparable to the clamping speed, tail kinetics was measured at -70 mV, which made it significantly slower and therefore reliable for comparison. This implies a mechanism distinct from that on Hv1, in which only the activation energy of the rate-limiting forward transition is increased, while that of the backward transition remains unaffected.

Effects on wild type Nav1.5

A remarkable effect of NZ-58 on Nav channels was a significant slowing of fast inactivation along with activation. 10 μ M NZ-58 induced kinetic changes on Nav1.5 within a few seconds without much effect on the current amplitude, but the full effect developed over the course of a few minutes. This resulted in a significant increase in the charge transferred through the channels during the depolarizing pulse, corresponding to an activating effect, which had a dose-dependence on the amplitude and the charge transferred. At 10 μ M the amplitude was barely reduced but the charge transfer almost tripled compared to the control trace. High concentrations (25 and 50 μ M) induced a quick temporary increase in the charge passed, but within a few minutes this value declined, in the case of 50 μ M even below the control value. These variable changes may be explained by the different rates at which activation and inactivation kinetics were altered. Initially, the inactivation rate slows more than the activation rate, which can result even in a slight amplitude increase accompanied by increased charge passage. However, with time, the slowing of inactivation seems to slow and saturate, while the activation keeps getting slower resulting in amplitude reduction and, in the case of high concentrations, charge transfer reduction. As the association rate of the compound is proportional to the concentration, 50 μ M NZ-58 quickly and dramatically slows activation to nearly completely suppress the current amplitude and the charge transfer. Following short term application of low concentrations, the effects were fully reversible, however, application over several minutes or repeated applications, especially with higher concentrations, resulted in irreversible kinetic changes.

Voltage-dependence of steady-state activation and inactivation

We also checked the voltage-dependence of steady-state activation (G-V) and inactivation (SSI) of Nav1.5 in the presence 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 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, $p = 0.70$).

In contrast, the recovery from inactivation was significantly hindered by the compound ($\tau_{\text{cont}} = 10.9 \pm 1.9$ 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 RCF in the presence of NZ-58 (RCF: 0.51 for control, and 0.14 for 10 μ M NZ-58).

State-dependence of binding

NZ-58 presumably binds directly to the VSDs, whose external accessibility may depend on the gating state of the channels, being more deeply buried in the resting state and more exposed in the activated state. We therefore tested whether NZ-58 could bind the channels when they were kept in the closed state at a negative membrane potential. After recording a control trace, we applied the compound for 20 seconds at -100 mV, then switched back to the control solution before recording the current and assessing its effect. We found that NZ-58 induced the same changes during closed state application as during continuous pulsing, indicating the state-independent binding.

The effect on Brugada mutant Nav1.5-R1632C

The similarly drastic slowing of voltage-gated opening on the tested voltage-gated channels and the lack of effect on the non-voltage gated ones, suggested that NZ-58 likely binds to a region in the VSDs conserved across these channels. Sequence comparison did not reveal common segments other than the highly conserved signature positive gating charges (mostly arginines) in every third position. Assuming that these residues may be influential for binding, we mutated the 4th arginine (R4) in the DIV-VSD of Nav1.5 and generated the R1632C mutation as it is a known loss-of-function Brugada mutant. We could confirm the published properties of the mutant channel such as 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, and neither the effect on the current amplitude nor on the charge transfer was different between the two channels (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 , $p = 0.12$).

Effects on Kv1.3

Motivated by the similar slowing of VSD movement by NZ-58 in various VGICs, we decided to investigate the effects on Kv1.3, a representative of the voltage-gated K⁺ channel family in more detail, for comparison with Nav1.5. As mentioned above, 10 μ M NZ-58 drastically slowed Kv1.3 channel opening kinetics ($\tau_{\text{act,NZ}} / \tau_{\text{act,cont}} = 3.13 \pm 0.27$, n=9), but surprisingly it did not affect the rate of channel closing at all ($\tau_{\text{deact,NZ}} / \tau_{\text{deact,cont}} = 1.03 \pm 0.13$, n=8. This agrees with the observations made on Nav1.5. However, in stark contrast with Nav1.5, where fast inactivation is linked to DIV-VSD movement, the non-VSD-linked slow, C-type inactivation of Kv1.3 was weakly and oppositely affected, which manifested as a slight speeding of inactivation ($\tau_{\text{inact,NZ}} / \tau_{\text{inact,cont}} = 0.86 \pm 0.03$, n=6. The concomitant slowing of activation and speeding of inactivation kinetics resulted in current amplitude reduction (at 10 μ M: $I_{\text{NZ}} / I_{\text{cont}} = 0.58 \pm 0.04$, n=9), which at 50 μ M culminated in complete current block. It should be noted that application of NZ-58 at 50 μ M for more than 2-3 minutes resulted in the loss of the patch configuration and even at 10 μ M, application beyond 7-8 minutes resulted in irreversible kinetic changes

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. Interestingly, we observed only a slight, non-significant right-shift in the G-V relationship induced by 10 μ M NZ-58 ($V_{0.5, \text{cont}} = -11.4 \pm 2.5$ mV, $V_{0.5, \text{NZ}} = -6.3 \pm 3.7$ mV, n=7, p = 0.15). Furthermore, the markedly slower activation reduces current amplitude, which is more significant at lower voltages accompanied by slower activation rates, and this can cause an apparent right-shift. Considering this, the change in the voltage-dependence of activation is negligible. As for Nav1.5, we assessed the voltage-dependence of steady-state inactivation, albeit with a shortened 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 ($V_{0.5, \text{cont}} = -43.6 \pm 0.8$ mV, $V_{0.5, \text{NZ}} = -43.7 \pm 0.5$ mV, n=4, p = 0.92).

The recovery from inactivation was assessed by a double pulse protocol, but also reduced to three pulse pairs due to the long duration of the full protocol, and NZ-58 was applied at 5 μ M

to avoid long-term rundown effects. 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 ($\tau_c = 14.44 \pm 0.78$ s, $\tau_{NZ} = 14.98 \pm 0.77$ s, $p = 0.71$, $n=3$).

Although we observed the effects described above when NZ-58 was applied extracellularly, considering its lipophilic nature ($\log D > 4.56$), we examined the sidedness of channel accessibility. We performed inside-out patch experiments on Kv1.3 channels expressed in CHO cells and applied the compound from the intracellular side of the membrane. We observed identical effects to those seen with extracellular application, confirming access of NZ-58 to the binding site from the intracellular side as well.

Similarly to Nav1.5, we intended to test the potential interactions of NZ-58 with the gating arginines in Kv1.3. We therefore generated the Kv1.3 R367C (R2) and R373C (R4) mutants, and tested the efficacy of the compound on them. While the induced effects at 10 μ M were qualitatively similar, the changes in the activation kinetics and amplitude reduction were more robust in the mutants than the wild type.

Gating current measurements

While the above results suggest a direct effect of NZ-58 on the VSD movements, other potential explanations also exist. Slow current activation may be the result of the compound slowing the final pore-opening transition or altering the communication between the VSD and the pore. In order to investigate these alternatives, we carried out gating current measurements with the non-conducting W384F mutant of Kv1.3. In the absence of ionic current through the pore, these measurements directly report on gating charge movement originating from the displacement of the VSDs. Application of 10 μ M NZ-58 significantly slowed the ON gating currents produced by the activation of the VSDs during depolarization, but surprisingly, no such effect could be observed on the OFF-gating currents produced by the return of the VSDs to the resting state upon repolarization. This is in accordance with the effects observed on the ionic currents, where channel opening was significantly slowed by NZ-58 in both Nav and Kv channels, but channel closing was not. The ON and OFF gating charges were equal in the presence of NZ-58 ($Q_{OFF} / Q_{ON} = 0.960 \pm 0.020$, $n=7$, $p = 0.095$), indicating the lack of charge immobilization. In addition, the total integrated gating charge did not significantly change upon the application of the compound ($Q_{ON, NZ} / Q_{ON, cont} = 1.05 \pm 0.04$, $n=7$, $p = 0.26$), implying that the VSDs complete the entire activation trajectory, only at a slower rate.

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. 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.

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 H_v1: 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. NZ-58 was fully characterized in detail on transiently transfected CHO expressing hH_v1 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 hH_v1 channel and inhibits the proton current. 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:

The two compounds have similar affinities, but ClGBI reduces the peak current without shifting the opening voltage of the channels. In contrast, 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 ($\tau_{activation}$, $\tau_{deactivation}$, τ_{eff}), and additionally, it reduces the macroscopic conductance. Moreover, NZ-58 is capable of binding even when the channel resides in the closed state, while ClGBI binds in the open state.

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→O transitions in channels that escape the trap. 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→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 H_V1.

Despite its superficial resemblance to “traditional” VGICs, H_V1 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 K_V1.3, Na_V1.5, and K_V1.5.

At 50 μ M, the extreme slowing of activation was dominant in all channels, which allowed a direct transition to the inactivated state via a brief occupancy of the open state, resulting in almost complete current suppression. Due to its very slow inactivation rate, K_V1.5 maintained a sizeable current fraction.

Some of the observed effects induced by NZ-58 in H_V1 (as described above) were similar in other VGICs, but there were also substantial differences. In all the tested K_V and Na_V channels the drastic slowing of activation kinetics was observed. Moreover, deactivation kinetics were not significantly affected in either Na_V1.5 or K_V1.3, similarly to the voltage-dependence of channel activation. These observations suggest different modes of action in H_V1 and the other VGICs. As H_V1 only consists of segments S1-S4, corresponding to the VSD of classical voltage gated channels, NZ-58 must bind to the VSD in H_V1 and, based on the effects described above, is likely to bind to it in the other VGICs as well. However, our mutant analysis and docking calculations for H_V1 indicated a binding site involving the selectivity filter and proton permeation pathway, but not any of the gating arginines, whereas the lack of other obvious conserved VSD regions in the other VGICs suggested the involvement of these residues in binding. In spite of this, both the R2- and R4-mutant K_V1.3 and the DIV-R4 mutant Na_V1.5 channels maintained their affinity for NZ-58. We did not observe any major change in Na_V1.5,

however, in this channel, the mutation affects only one VSD, the one responsible for inactivation, so a weaker effect is expected. In the $K_v1.3$ mutants, where the mutations were present in all four VSDs, the effects became more prominent, manifested as even slower activation kinetics and due to the overlap with inactivation, consequently more reduced amplitudes than in the wild type. Therefore, the arginines may indeed be involved in the interaction with NZ-58, even if weakening it rather than enhancing, and other residues are likely to have greater contributions considering the modest change brought on by the mutations. In both $Na_v1.5$ and $K_v1.3$ channels, NZ-58 induced a robust slowing of channel opening, which requires the activation of DI-DIII VSDs in $Na_v1.5$, and that of all four VSDs in $K_v1.3$. Thus, if NZ-58 binding slows VSD activation, this is expected in both channel types. By contrast, the fast inactivation of $Na_v1.5$ was similarly slowed, but the slow, C-type inactivation of $K_v1.3$ was oppositely and much less affected. This is not surprising, as fast inactivation in Na_v channels is linked to DIV-VSD activation, while C-type inactivation in K_v channels occurs in the selectivity filter with a much less direct connection to VSD movement. Therefore, if binding of NZ-58 slows VSD activation, slower inactivation is expected in Na_v , but not in K_v channels. The slight acceleration of slow inactivation in $K_v1.3$ may be due to allosteric modulation of the selectivity filter by the compound or simply the result of reduced occupancy of the K^+ ion binding sites controlling inactivation due to the slow opening of the channel and concomitant decreased current. Along the same lines, recovery from inactivation linked to VSD-movements was slowed in $Na_v1.5$, but not affected in $K_v1.3$ where recovery is also allosterically modulated by the VSDs.

The simultaneous slowing of both activation and inactivation in $Na_v1.5$ resulted in a variable concentration- and time-dependent change in the current amplitude, often causing an increase, while the slowing of activation and acceleration of inactivation in $K_v1.3$ always resulted in amplitude reduction.

The effects of NZ-58 on gating current can be explained by a direct modulation of VSD movement rather than an interaction with the pore. During opening of K_v channels, activation of the VSDs is faster and precedes the rate-limiting pore opening. However, sufficient slowing of VSD activation by NZ-58, manifested as slow ON gating currents, can make this step rate-limiting and thus result in slower channel opening, manifested as slow current activation kinetics. In contrast, during hyperpolarization, the pore closure, unaffected by NZ-58, precedes the return of the VSDs to the resting state, meaning that the decay of the ionic current is not affected, regardless of which transition is rate-limiting. Since the pore closure is unaffected by

NZ-58, the subsequent charge return and consequently OFF gating kinetics are not affected either.

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.

NZ-58 as a prospective lead molecule?

Although NZ-58 has an inhibitory effect on most of the tested channels in this study via slowing channel opening, at low concentrations it significantly enhances the activity of Nav channels, the increase in Na⁺ influx amounting to about 22% even at 0.5 μ M. This enhancement was also observed in the loss-of-function Brugada mutant. Such activity has significant therapeutic potential in the case of Nav loss-of-function related pathologies, as demonstrated by the rescue of Dravet-syndrome mice by a spider venom peptide activator of the loss-of-function mutant Nav1.1 channels.

Limitations and pathways for future development

As shown by the above results, NZ-58 is far from being isoform selective. It is not even selective for channel type, as revealed by testing it on a number of different channels.

Although isoform selectivity is a highly desirable trait of potential lead molecules in order to avoid side effects due to off-target actions, the reality is that the majority of clinically used VGIC modulator compounds are “dirty drugs” affecting multiple targets. Furthermore, 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. Therefore, reducing lipophilicity and/or Hv1 high-resolution cryo-EM structures could offer a strategy aimed at improving selectivity while preserving pharmacological potency,

Nevertheless, despite their narrow therapeutic windows, their administration still achieves therapeutic goals, justifying their use. For example, lidocaine blocks multiple Nav isoforms and is used as a local anesthetic despite its inhibitory effect on the cardiac Nav1.5 channel. Verapamil, which targets voltage-gated Ca²⁺ channels to treat hypertension and angina pectoris, also blocks Nav and Kv channels. The Class III antiarrhythmic drug amiodarone blocks Kv, ligand-gated K⁺, Nav and Cav channels and as a result affects almost every phase of the cardiac action potential. These examples illustrate that careful dosing and the appropriate delivery route can beneficially exploit even a narrow therapeutic window. In addition, the multi-target nature of a drug molecule may even become advantageous, e.g. in suppressing cancer cell progression, which can rely on the overexpression of multiple VGICs, including e.g. Hv1 for pH control and specific others for setting the appropriate membrane potential or ion concentrations. In these cases, a synergistic inhibitory effect may yield beneficial results.

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.

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 determinants of VSD engagement, providing mechanistic insight into activation, inactivation, and voltage-sensor transitions.

In summary, this research work establishes NZ-58 as a powerful tool compound for probing VSD-mediated modulation and highlight fundamental principles that can guide the rational design and introduces a novel molecule of broad relevance that lays the conceptual groundwork for future therapeutic development targeting VGIC pharmacology.

NEW SCIENTIFIC FINDINGS

- ✓ We have identified several compounds that significantly block the voltage-gated H_V1 proton channel at the low micromolar range
- ✓ We have carried out a detailed electrophysiological characterization of an unreported compound, named NZ-58 and unveiled its biophysical mechanisms of action on H_V1 function
- ✓ We have found NZ-58 to be a “pan-VGIC”, rather than a selective modulator, affecting every voltage-gated ion channel tested while showing no activity on non-voltage-gated channels
- ✓ We fully characterized NZ-58 interaction with VSDs of both Na_V and K_V channels;
- ✓ We have unveiled the similarities and differences in the interaction of NZ-58 with Na_V1.5 and K_V1.3, and tested the possible key residues responsible for ligand recognition and modulation
- ✓ We have established NZ-58 as a powerful tool compound for probing VSD-mediated modulation to guide rational drug design for future therapeutic development targeting VGIC pharmacology

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List of publications related to the dissertation

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J. Gen. Physiol. "Accepted by publisher", 2026.
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Biomed. Pharmacother. 200, 1-14, 2026.
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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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