Auxiliary subunits control biophysical properties and response to compound NS5806 of the Kv4 potassium channel complex

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Abstract
Kv4 pore-forming subunits co-assemble with β-subunits including KChIP2 and DPP6 and the resultant complexes conduct cardiac transient outward K⁺ current (Ito). Compound NS5806 has been shown to potentiate Ito in canine cardiomyocytes; however, its effects on Ito in other species yet to be determined. We found that NS5806 inhibited native Ito in a concentration-dependent manner (0.1–30 μM) in both mouse ventricular cardiomyocytes and human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs), but potentiated Ito in the canine cardiomyocytes. In HEK293 cells co-transfected with cloned Kv4.3 (or Kv4.2) and β-subunit KChIP2, NS5806 significantly increased the peak current amplitude and slowed the inactivation. In contrast, NS5806 suppressed the current and accelerated inactivation of the channels when cells were co-transfected with Kv4.3 (or Kv4.2), KChIP2 and another β-subunit, DPP6-L (long isoform). Western blot analysis showed that DPP6-L was dominantly expressed in both mouse ventricular myocardium and hiPSC-CMs, while it was almost undetectable in canine ventricular myocardium. In addition, low level of DPP6-S expression was found in canine heart, whereas levels of KChIP2 expression were comparable among all three species. siRNA knockdown of DPP6 antagonized the Ito inhibition by NS5806 in hiPSC-CMs. Molecular docking simulation suggested that DPP6-L may associate with KChIP2 subunits. Mutations of putative KChIP2-interacting residues of DPP6-L reversed the inhibitory effect of NS5806 into potentiation of the current. We conclude that a pharmacological modulator can elicit opposite regulatory effects on Kv4 channel complex among different species, depending on the presence of distinct β-subunits. These findings provide novel insight into the molecular design and regulation of cardiac Ito. Since Ito is a potential therapeutic target for treatment of multiple cardiovascular diseases, our data will facilitate the development of new therapeutic Ito modulators.

Abbreviations: AP, action potential; APD, action potential duration; DPP, dipeptidyl peptidase-like protein; ECG, electrocardiogram; HEK, human embryonic kidney; HF, heart failure; hiPSC-CMs, human-induced Pluripotent Stem Cell-derived cardiomyocytes; Ito, slow component of delayed rectifier K⁺ currents; Ito, transient outward K⁺ current; Ito,f, fast component of Ito; Ito,s, slow component of Ito; KChIP, K⁺ channel-interacting protein; QTc, heart rate–corrected QT intervals; siRNA, small interfering RNA.
1 | INTRODUCTION

Kv4 channels are the primary subunits of the rapidly activating and inactivating K⁺ channels, contributing to the cardiac transient outward K⁺ currents (Iₒ). Due to its unique kinetic features, Kv4 channels play a central role in controlling cardiac excitation and shaping the cardiac action potentials (AP). The reduction of the Iₒ density and the consequent prolongation of the action potential duration is a consistent finding in many pathological conditions such as cardiac hypertrophy and heart failure (HF) and may contribute to the arrhythmias. Hence, the pharmacological Iₒ activation may have therapeutic value in HF.

It is generally accepted that there are two Iₒ components with distinct recovery kinetics: the fast (Iₒ,f) and slow (Iₒ,s). The Kv4.2 (KCND2) and Kv4.3 (KCND3) conduct Iₒ,f, while Kv1.4 (KCNA4) forms Iₒ,s channels. In human and canine ventricular myocytes, functional Iₒ,f channels are likely to be assembled from Kv4.3 homotetramers because Kv4.2 is not expressed. In mice, Kv4.2 is believed to be the principal α-subunit of Iₒ,f. Auxiliary subunits, such as K⁺ channel-interacting proteins (KChIPs) or dipeptidyl peptidase-like proteins (DPPs), are known to interact with and modulate the properties of Kv4 channels. Within these, KChIP2 and DPP6 have been proposed as most likely candidates that co-assemble with Kv4 subunits in the human heart. KChIP2 is a cytosolic Ca²⁺-binding auxiliary subunit that interacts with the amino terminus of the Kv4 α-subunit; it facilitates channel trafficking, slows the Kv4 current inactivation kinetics, accelerates the recovery from inactivation, and shifts the voltage dependence of steady-state inactivation to more negative potentials. DPP6 is a single-membrane-spanning protein that accelerates current inactivation and recovery kinetics and shifts both activation and inactivation voltage dependencies of Kv4 channels to more negative potentials. Co-expression of Kv4.3 with KChIP2 and DPP6 in heterologous systems results in currents with kinetics similar to those of the Iₒ in human ventricular myocytes. Thus, Kv4-KChIP-DPP complex could be a valuable target for therapeutic approaches to cardiac diseases.

A small molecule sulfonylurea compound, NS5806, has been found to potentiate Iₒ in canine cardiac myocytes by slowing the current inactivation. Further studies have shown that current potentiation by NS5806 depends on the presence of KChIPs in the recombinant Kv4 channel complex. NS5806 was shown to reverse the HF-associated reduction of Iₒ density in canine ventricular cardiomyocytes and, thus, NS5806 was put forward as a prototypic Iₒ opener and a candidate template for antiarrhythmic drugs. However, its effects on Iₒ in other species yet to be determined. Given its proposed therapeutic value, a better understanding of the molecular mechanisms underlying the modulation of Kv4 channels by NS5806 is urgently required.

Iₒ is the major component of the cardiac AP repolarization in rodents and the mice have become an important model for the molecular studies of Iₒ. In addition, human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) have recently become another important system for modeling human cardiac disease and drug screening. In this study, we examined the effect of NS5806 on Iₒ in native mouse ventricular cardiomyocytes and in the hiPSC-CMs. Unexpectedly, we discovered that in contrast to its effect in canine cardiomyocytes, NS5806 decreased the amplitude of native Iₒ in both cell types with a significant acceleration of the current inactivation. We used primary cardiac cells from different species, hiPSC-CMs, expression system, and modeling to probe the intricate molecular mechanisms underlying the species-specific differences in pharmacological responses of Iₒ to NS5806.

2 | MATERIALS AND METHODS

2.1 | Animals

Male C57BL/6 mice (weight 20-25 g, 7- to 8-week old) and male Beagle dogs (weight 6-8 Kg, 1- to 2-year old) were purchased from the Vital River Laboratory Animal Technology Company (Beijing, China). Animal care standards and all experimental procedures were approved by the Animal Care and Ethical Committee of Hebei Medical University (Shijiazhuang, China). Mice were housed under the specific pathogen-free (SPF) conditions with five mice per cage and dogs were raised in a conventional animal room. All animals were kept under controlled environmental conditions (12 hours light/12 hours dark cycle, room temperature 21-23°C and humidity 50%-60%) with free access to standard laboratory food pellets and water.

2.2 | Ventricular cardiomyocytes isolation

Single ventricular myocytes were enzymatically isolated from five mouse and three canine hearts as described previously. Briefly, animals were injected with heparin (1.0 U·kg⁻¹) and anesthetized with sodium pentobarbital.
(35 mg·kg\(^{-1}\)). Hearts were rapidly removed, and a wedge from canine left ventricular free wall supplied by a descending branch of the circumflex artery was excised. Intact mouse heart or wedge preparation from canine heart were mounted on a Langendorff apparatus (Radnoti Inc, Monrovia, CA, USA), and retrogradely perfused through the aorta with Ca\(^{2+}\)-free Tyrode’s solution (mM): NaCl 140, KCl 5.4, MgCl\(_2\) 1, HEPES 10, and glucose 10 (pH 7.4 with NaOH). After 5 minutes of perfusion, the same solution supplemented with Type II collagenase ( Worthington Biochemical Corporation, Lakewood, NJ, USA; 0.4 mg·mL\(^{-1}\)) was applied for 10-15 minutes. Once ventricular tissue was softened, hearts were removed from the apparatus. The left apex ventricles of mouse heart or canine epicardium ventricles were sliced into small pieces and incubated in KB solution (in mM): KOH 80, KCl 40, KH\(_2\)PO\(_4\) 25, MgSO\(_4\) 3, Glutamic acid 50, Taurine 20, HEPES 10, EGTA 1, and Glucose 10 (pH 7.4 adjusted with KOH). Cells were then harvested and were used for patch-clamp recordings within 4-6 hours after isolation.

2.3 | In vitro electrocardiographic (ECG) recordings

The Langendorff hearts from five mice were prepared with the above method and were retrogradely perfused (2-2.5 ml·min\(^{-1}\); 37 ± 0.5°C) through the aorta with oxygenated Tyrode’s solution (in mM): NaCl 140, KCl 5.4, MgCl\(_2\) 1, CaCl\(_2\) 2, HEPES 10, and glucose 10 (pH 7.4 adjusted with NaOH) using peristaltic pump. The hearts were put in the thermostatic chamber and allowed to equilibrate for a minimum of 30 minutes to ensure the stable ECG recordings before drug testing. The in vitro equivalent lead II ECG waveforms were recorded by the Biopac 150 System (Biopac Systems, USA) at 5 kHz. The QT interval was defined as the time between the first deviation from the isoelectric line during the PQ interval until the end of the T wave, and was averaged from 30 consecutive beats between 5 and 10 minutes after the presence of test compound. The heart rate was defined by RR intervals. The heart rate–corrected QT intervals (QTc) were calculated according to a parabolic equation $QT = (333/RR)^{0.601} \times 20$.

2.4 | cDNA constructs and mutagenesis

cDNAs coding for human Kv4.3 (NM_172198), Kv4.2 (NM_012281), KChIP2 (NM_173192), DPP6-L (long isoform; NM_130797), and DPP6-S (short isoform; NM_001936) were cloned into pcDNA3.1 by Youbio (Changsha, China). Alanine substitution at positions R7, P33, D36, G38, L44 in DPP6-L was performed by Youbio. Validity of all cDNA constructs was confirmed by sequencing.

2.5 | Cell culture, transfection, and siRNA treatment

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal calf serum (Gibco) with 5% CO\(_2\) at 37°C. The HEK293 cells were transiently co-transfected with Kv4.3 (or Kv4.2), KChIP2, and DPP6 plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The green fluorescent protein (GFP) was used as a reporter. The human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs, 50-60 days of maturity; Cellapybio, Beijing, China) were maintained in serum-free medium (Cellapybio) on 24-well dishes coated with Matrigel Matrix (BD-Biocat, Corning, USA) with 5% CO\(_2\) at 37°C. The small interfering RNA (siRNA) duplex against the human DPP6 gene (NM_130797, 5’-GGTCCATCATCGGCTTCTTTT-3’) was designed and constructed by Ribobio (Guangdong, China). hiPSC-CMs were transfected with siRNA using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer’s instruction at a final siRNA concentration of 100 nM. A non-matching siRNA was used as a negative control. Cells were used for experiments 48 hours after transfection.

2.6 | Patch-clamp recordings

The conventional whole-cell patch-clamp recordings were performed at room temperature (22-25°C) using an EPC-10 amplifier in combination with Patchmaster software (V2x73.2, HEKA, Lambrecht, Germany) and data analysis was performed using Origin 8.6 software (Wavemetrics, Microcal, USA). The access resistance was typically within 5 MΩ. Whole-cell membrane capacitances were cancelled and series resistance compensated by 80%. For native $I_{to}$ recording, the external solution contained (in mM) N-methyl-D-glucamine (NMG) 130, KCl 5, MgCl\(_2\) 1, CaCl\(_2\) 1, HEPES 10, and glucose 10 (pH 7.4 with HCl) and the pipette solution contained (in mM) KCl 140, Mg-ATP 4, MgCl\(_2\) 1, EGTA 10, and HEPES 10 (pH 7.4 with KOH). The CdCl\(_2\) (300 nM) was added in the bath solution to block voltage-gated Ca\(^{2+}\) channels. For recording from HEK293 cells, the extracellular solution contained (in mM) NaCl 140, KCl 5, CaCl\(_2\) 1.8, MgCl\(_2\) 1.2, HEPES 10, and Glucose 10 (pH 7.4 with NaOH) and the patch pipette solution contained (in mM) KCl 140, Mg-ATP 4, MgCl\(_2\) 1, EGTA 10, and HEPES 10 (pH 7.4 with KOH). To isolate distinct K* current components, a rapidly activating and inactivating current, $I_{to,t}$; a rapidly activating but slowly inactivating current, $I_{to,s}$; and a slowly activating non-inactivating current, $I_{to}$, the decay phases of the currents evoked during long (4.5 seconds) depolarizing voltage
steps to various test potentials were fitted by the sum of two exponents. The following equation was used: \( y(t) = A_1 e^{-(t/\tau_1)} + A_2 e^{-(t/\tau_2)} + A_{ss} \), where \( t \) is time, \( \tau_1 \) and \( \tau_2 \) are, respectively, the time constants of decay of \( I_{to,f} \) and \( I_{k,slow} \); \( A_1 \) and \( A_2 \) are, respectively, the amplitudes of \( I_{to,f} \) and \( I_{k,slow} \), and \( A_{ss} \) is the amplitude of \( I_{ss} \).\(^{21}\)

2.7 Western blot and immunoprecipitation assays

Immunoblots were performed as previously described.\(^{22}\) Proteins were prepared from the left ventricular free wall of five mouse hearts, epicardial tissue of three dog hearts, and three batches of hiPSC-CMs (50-60 days of maturity). Protein concentration was measured by BCA protein assay (Pierce, USA). Denatured samples (60 μg/lane) were separated on precast 10% SDS-PAGE gels and transferred to PVDF membranes. Quantification of the signals was performed by Odyssey Infrared Imaging System (LICOR 9120, Li-COR, Lincoln, NE, USA). Densitometry was used for quantification; the protein band pixel intensities were normalized to the GAPDH band in each sample. The values were then averaged from all the different sets of experiments. The following primary antibodies were used: anti-DPP6 (a polyclonal antibody raised in rabbit against a purified peptide corresponding to amino acid residues 400-550 of human DPP6, Abcam, UK); anti-Kv1.4 (a polyclonal antibody raised in rabbit against a purified peptide corresponding to amino acid residues 589-655 of rat Kv1.4 located in intracellular C-terminus); anti-Kv4.3 (a polyclonal antibody raised in rabbit against a purified peptide corresponding to amino acid residues 451-468 of human Kv4.3 located in intracellular C-terminus); anti-KChIP2 (a polyclonal antibody raised in rabbit against a purified peptide corresponding to amino acid residues 2-15 of human KChIP2 located in intracellular N-terminus) (Alomone Labs, Israel); and anti-GAPDH (Proteintech, Wuhan, China).

2.8 RT-PCR

Total RNA from hiPSC-CMs was extracted using Trizol Reagent (Takara, Japan). cDNA was synthesized using the PrimeScript RT Reagent Kit (Takara, Japan). Real-time PCR was performed using ABI Prism 7300 Real-Time PCR System (ABI, Wilmington, USA). The following primers (synthesized by Sangon Biotech, Shanghai, China) were used: GAPDH forward: 5′-AAAGAGAGAAAGCACAGCCAGAG-3′; GAPDH reverse: 5′-CCAAATCCGTTGACTCCGAC-3′. DPP6 forward: 5′-AAGACCTTGGGTGGCCAGACTTT-3′; DPP6 reverse: 5′-CTTCTGGGGGTCAGACTT-3′. The amplification curves to provide Ct values were normalized to the reference gene GAPDH; the changes in expression were calculated using the \( 2^{-\Delta\Delta CT} \) method.

2.9 Computational prediction of interaction between KChIP2 and DPP6

Computer simulations and analyses were performed by Beijing Abace Biotechnology Co., Ltd. BLAST and HHBlit were used with default parameters to identify evolutionary related structures matching the KChIP2 sequence (UniProtKB accession number Q9NS61). Kv channel-interacting protein 4 (KChIP4a; PDB ID: 3DD4) was identified as the highest ranked homolog. A known structure of KChIP4a,\(^{23}\) which had 80% sequence identity with KChIP2, was thus used as a template to generate a homology model of KChIP2. No appropriate templates were identified for DPP6-L in the Protein Data Bank. Therefore, \textit{ab initio} protein prediction approaches were employed for structure modeling of DPP6-Lin. The docking experiment was performed using the program GRAMM. A global search on the surface of KChIP2 was done to identify potential binding sites for DPP6-Lin. All the predicted binding positions were ranked based on their docking scores. As a result, DPP6-Lin was successfully docked into KChIP2 and a total of 10 models were generated. The interactions between DPP6-L and KChIP2 were analyzed using the PDBePISA web server.

2.10 Chemicals

NS5806 (Tocris, UK) was prepared as a 20 mM stock solution in DMSO and stored in −20°C. The highest final concentration of DMSO in external solution was ≤0.1%, a concentration that had no effect on the current recording. Phrixotoxin-2 (Abcam, UK) was prepared as a 1 mM stock solution in water and stored at −20°C. Other common chemicals were from Sigma.

2.11 Data and statistical analysis

All data are presented as Mean ± SEM. Data analysis and statistics were carried out using Origin 8.6 software (OriginLab Corporation, USA). Group comparisons were performed with paired Student’s \( t \) tests (for comparisons before and after drug treatment) and unpaired Student’s \( t \) tests (for two-group comparisons) and ANOVA with Dunnett’s post hoc tests (for multiple-group comparisons). The “n” and “N” were used to represent the number of cells and animals/samples,
respectively. Post hoc tests were only performed when $F$ achieved $P < .05$ and there was no significant variance in the homogeneity. The differences were considered significant at $P < .05$.

3 | RESULTS

3.1 | Effect of NS5806 on outward potassium current in mouse left ventricular myocytes

We first tested the effect of NS5806 (Figure 1A, inset) on the outward potassium currents in mouse ventricular myocytes. The currents were recorded using a series of long (4.5 seconds) depolarizing voltage steps from −40 to +60 mV from a holding potential of −80 mV. NS5806 significantly suppressed the outward potassium currents; the peak current density was significantly decreased at all potentials positive to −10 mV (Figure 1A, inset). The inhibition was concentration-dependent (Figure 1C). Current inactivation was well fitted with a double-exponential equation providing rapid and slow inactivation time constants, $\tau_1$ and $\tau_2$, respectively. $\tau_1$ and $\tau_2$ were 12.5 ± 0.2 and 41.8 ± 3.0 ms, respectively. NS5806 induced significant acceleration of the current inactivation; at +60 mV $\tau_1$ changed from the control value of 53.8 ± 5.5 to 41.8 ± 3.0 ms and $\tau_2$ from 1279.8 ± 60.3 to 906.3 ± 53.7 ms (Figure 1D). The effect of NS5806 on cardiac electrophysiological activity was further tested in isolated perfused mouse hearts. Representative in vitro ECG waves in the presence of NS5806 at different concentrations are shown in Figure 1E. Analysis of ECG recordings revealed that NS5806 significantly prolonged the heart rate–corrected QT interval (QTc) in a concentration-dependent manner (Figure 1F). These results indicated that NS5806 suppressed repolarizing currents and thus prolonged QT interval in mouse heart.

The outward potassium currents in adult mouse myocytes from free left ventricular wall has three distinct components: a rapidly activating and inactivating, $I_{to,1}$; a rapidly activating but slowly inactivating current, $I_{k,slow}$; and a slowly activating non-inactivating current, $I_{ks}$. To isolate these components, we used two-exponent fit of current inactivation (see above and Methods) and selective pharmacology. Phrixotoxin-2, a specific Kv4 channel blocker, selectively blocked the $I_{to,1}$ in mouse ventricular cardiomyocytes in a concentration-dependent manner; at 1 μM Phrixotoxin-2 almost completely suppressed the outward potassium currents; the peak current amplitude (with the sustained component subtracted) was shown to represent “pure” $I_{to,1}$. Figure 2C shows representative current traces before and after the application of 10 μM NS5806 (top panel), as well as the NS5806-sensitive current fraction (bottom panel). The inactivation phase of the NS5806-sensitive current fraction was well fitted by a single-exponential equation, which gave time constants within the same range as $\tau_1$ provided by double-exponential fitting under long depolarization pulse (data not shown).

3.2 | Effect of NS5806 on $I_{lo}$ in hiPSC-CMs

To identify whether the inhibitory effect of NS5806 on Kv4 channels was just limited to rodents, we investigated the effect of NS5806 on $I_{lo}$ in hiPSC-CMs. The molecular correlates of $I_{lo}$ channel were first assessed using western blot (Figure 3A,B), which revealed that Kv4.3 was the dominant subunit and Kv1.4 was almost undetectable. However, we found that there were comparable expression levels of Kv1.4 in mouse and canine heart (Figure S2). Similar to murine cardiomyocytes, NS5806 induced prominent inhibitory effect in hiPSC-CMs. Representative $I_{lo}$ traces before and after NS5806 are shown in Figure 3C. The amplitude of $I_{lo}$ (peak minus steady-state current) was significantly inhibited by 10 μM NS5806, with a decrease of 64.6 ± 5.9% from 21.9 ± 1.5 pA/pF (control) to 7.6 ± 1.2 pA/pF in the
The presence of the drug (measured at +60 mV; Figure 3C). The effect was concentration-dependent with an IC$_{50}$ of 8.3 ± 1.9 μM (Figure 3E). The current inactivation phase was best fit with a single exponential equation; the $I_{\text{to}}$ inactivation kinetics was significantly accelerated over a voltage range from +10 to +60 mV, as reflected by the decrease of $\tau$.
values from 45.8 ± 3.5 to 32.6 ± 2.0 ms at +60 mV (Figure 3F). The steady-state inactivation curves were also analyzed. Consistently, NS5806 shifted the $V_{1/2}$ toward negative potentials (from −24.9 ± 0.4 to −38.5 ± 0.3 mV; Figure 3G). However, NS5806 did not significantly affect the recovery from inactivation of $I_{to}$ (Figure 3H). These results indicated...
that NS5806 significantly suppressed \( I_{to} \) conducted by Kv4 family channels through accelerating current inactivation in hiPSC-CMs.

Given the striking difference between the effects of NS5806 in canine cardiomyocytes\(^{14,15} \) and murine and human cardiomyocytes (present study), we tested the effect of NS5806 on the \( I_{to} \) in canine cardiomyocytes to confirm its potentiating effect under our experimental conditions. Indeed, the \( I_{to} \) amplitude was enhanced and the current inactivation was delayed in the presence of 10 μM NS5806 (Figure S3), which concurs with the previous reports.

**3.3 Effect of NS5806 on the cloned Kv4 channels in heterologous expression system**

To explore the molecular mechanism(s) underlying strikingly different effects of NS5806 on the cardiac \( I_{to} \) in different species, we next tested the effect of the compound on cloned Kv4.3 channel complex by transiently co-expressing the pore-forming α-subunit with auxiliary subunits KChIP2 and DPP6 at different stoichiometry in HEK293 cells. At least two DPP6 isoforms have been reported: DPP6-L (long) and DPP6-S (short). These isoforms differ in the sequence and length of the cytoplasmic N-terminal domain but share an identical transmembrane domain and a long C-terminal extracellular domain.\(^{26} \) Two isoforms have qualitatively similar regulatory effects on Kv4.2 channels.\(^{27} \) DPP6-S has been used to test the effect of NS5806 on recombinant channel complexes in previous reports, which have demonstrated that NS5806 enhances the current amplitude and concomitantly slows the inactivation kinetics of Kv4.3/KChIP2/DPP6-S\(^{12} \) or Kv4.2/DPP6-S associated with either KChIP2, KChIP3, or KChIP4 channel complexes.\(^{28} \) A similar response was observed in this study when the DPP6-S was co-expressed with Kv4.3 and KChIP2 (Figure S4). While consistent with previous observations, this recombinant Kv4.3/KChIP2/DPP6-S complex did not recapitulate the response to NS5806 of native \( I_{to} \) from mouse cardiomyocytes or hiPSC-CMs. To test whether different isoforms of DPP6 confer differential responses to NS5806, the DPP6-L was co-transfected with Kv4.3 and KChIP2 in HEK293 cells. As shown in Figure 4A (left panels), the different instantaneous inactivation kinetics were observed when pore-forming subunit Kv4.3 was co-assembled with auxiliary subunits KChIP2 or DPP6-L at different expression ratios. Evidently, DPP6-L accelerated the current inactivation while...
KChIP2 slowed inactivation. These findings were consistent with the previous reports. Representative current traces at +40 mV in the absence and presence of NS5806 are shown in Figure 4A (right panels). NS5806 had no effect on the current amplitude of $I_{\text{Kv4.3}}$ and $I_{\text{Kv4.3/DPP6-L}}$, but it enhanced the peak current of $I_{\text{Kv4.3/KChIP2}}$ at test voltages positive to +10 mV (Figure 4A; Table S1). Intriguingly, NS5806 significantly decreased the peak current of $I_{\text{Kv4.3/KChIP2/DPP6-L}}$, and the effect was more prominent when the expression of DPP6-L was increased by changing $I_{\text{Kv4.3/KChIP2/DPP6-L}}$ transfection plasmid ratio from 1:1:1 to 1:1:3 (Figure 4B; Table S2). These results clearly demonstrated that NS5806 significantly enhanced the current amplitude of Kv4.3/KChIP2 channels while inhibiting the current of Kv4.3/KChIP2/DPP6-L channels. Interestingly, Kv4.3/DPP6-L channels were unaffected by NS5806, suggesting that an interplay between KChIP2 and DPP6-L is necessary to confer the inhibition.

Similarly, complex effects of NS5806 on the inactivation kinetics of the cloned channels with the different subunit stoichiometry were observed. As shown in Figure 4C, NS5806 did not affect inactivation time constants of $I_{\text{Kv4.3}}$ or $I_{\text{Kv4.3/DPP6-L}}$, but it significantly delayed the inactivation...
kinetics of \(I_{Kv4.3/KChIP2}\). In contrast, NS5806 accelerated the current inactivation in the presence of DPP6-L subunits and the effect was stronger at the 1:1:3 plasmid ratio of Kv4.3/KChIP2/DPP6-L, as compared to that at 1:1 ratio. NS5806 produced a very similar pattern of effects on Kv4.2/KChIP2/DPP6-L channels (Figure S5 and Table S3). Taken together, our data suggest that DPP6-L/KChIP2 levels control the modality of Kv4 channel response to NS5806.

To further test whether the effect of NS5806 in the heterologous system can be closely matched to that in the native cardiomyocytes, we further analyzed its actions on the cloned Kv4.3/KChIP2/DPP6-L channels at 1:1:1 transfection ratio. These experiments revealed that NS5806 significantly inhibited the currents elicited by voltages positive to \(-10\) mV (Figure 4D). The steady-state inactivation was left-shifted with a change of \(V_{1/2}\) from \(-28.8 \pm 0.4\) to \(-42.5 \pm 0.2\) mV (Figure 4E), and the recovery from inactivation was accelerated from 30.9 \pm 5.1 to 14.4 \pm 2.7 ms in the presence of NS5806 (Figure 4F).

3.4 | The role of DPP6 in the inhibitory action of NS5806 on native \(I_{Io}\)

To gain better understanding of the role of DPP6 in the pharmacological responses of Kv4 channels, we determined the protein abundance of DPP6 in ventricular myocardium of dog and mouse, as well as in human hiPSC-CMs. An antibody that binds with both DPP6-L and DPP6-S was used. The brain tissue was used as a positive control for the western blots since both DPP6-L and DPP6-S are present in the brain.29 As shown in Figure 5A1, two protein bands were detected in the brain, the smaller molecule was more abundant than the larger one. This finding is consistent with higher abundance of DPP6-S, as compared to DPP6-L, in the brain.29 Thus, we assumed that two bands seen in our western blot experiments correspond to DPP6-S and DPP6-L, respectively. Intriguingly, a different expression pattern of DPP6 isoforms was found among the species tested. Thus, DPP6-L was dominantly expressed in both mouse ventricular myocardium and hiPSC-CMs, while it was almost undetectable in canine ventricular myocardium (Figure 5A1). In addition, DPP6-S was virtually undetectable in canine heart preparation (Figure 5A1). Specificity of the DPP6 antibody was tested by western blot after pre-incubation with the blocking antigen, which eliminated both bands in brain and heart preparations (Figure 5A2). Notably, the expression level of KChIP2 protein was comparable among these species (Figure S6). Based on the above results we hypothesized that the dominant expression of DPP6-L might be responsible for the inhibitory effect of NS5806 on native \(I_{Io}\) in mouse cardiomyocytes and hiPSC-CMs.

3.5 | Analysis on the association between KChIP2 and DPP6

To test this hypothesis, we knocked down DPP6 expression in hiPSC-CMs using a small interference RNA approach (the siRNA we used recognized both DPP6-L and DPP6-S mRNAs but since DPP6-L is the predominant isoform in hiPSC-CMs, we assume that the main effect of the knockdown would be mediated by the downregulation of DPP6-L). The validity of siRNA DPP6 (SiDPP6) was confirmed by RT-PCR (Figure 5B1) and western blot (Figure 5B2), in which scramble siRNA (SiNC) was used as a control. After the knock down of DPP6 in hiPSC-CMs, the \(I_{Io}\) displayed significantly slower inactivation kinetics, further evidencing a successful knockdown of DPP6 expression (Figure 5B3). Knockdown of DPP6 significantly reduced the response to NS5806, albeit the compound still produced a modest suppression of the current in hiPSC-CMs (Figure 5C1, C2). Similarly, NS5806-induced acceleration of the current inactivation was antagonized by DPP6 knockdown (Figure 5C3). These data provide further evidence that DPP6 subunits play a key role in the inhibitory action of NS5806 on native \(I_{Io}\).

To further test whether the effect of NS5806 in the heterologous system can be closely matched to that in the native cardiomyocytes, we further analyzed its actions on the cloned Kv4.3/KChIP2/DPP6-L channels at 1:1:1 transfection ratio. These experiments revealed that NS5806 significantly inhibited the currents elicited by voltages positive to \(-10\) mV (Figure 4D). The steady-state inactivation was left-shifted with a change of \(V_{1/2}\) from \(-28.8 \pm 0.4\) to \(-42.5 \pm 0.2\) mV (Figure 4E), and the recovery from inactivation was accelerated from 30.9 \pm 5.1 to 14.4 \pm 2.7 ms in the presence of NS5806 (Figure 4F).

3.4 | The role of DPP6 in the inhibitory action of NS5806 on native \(I_{Io}\)

To gain better understanding of the role of DPP6 in the pharmacological responses of Kv4 channels, we determined the protein abundance of DPP6 in ventricular myocardium of dog and mouse, as well as in human hiPSC-CMs. An antibody that binds with both DPP6-L and DPP6-S was used. The brain tissue was used as a positive control for the western blots since both DPP6-L and DPP6-S are present in the brain.11,27 As shown in Figure 5A1, two protein bands were detected in the brain, the smaller molecule was more abundant than the larger one. This finding is consistent with higher abundance of DPP6-S, as compared to DPP6-L, in the brain.29 Thus, we assumed that two bands seen in our western blot experiments correspond to DPP6-S and DPP6-L, respectively. Intriguingly, a different expression pattern of DPP6 isoforms was found among the species tested. Thus, DPP6-L was dominantly expressed in both mouse ventricular myocardium and hiPSC-CMs, while it was almost undetectable in canine ventricular myocardium (Figure 5A1). In addition, DPP6-S was virtually undetectable in canine heart preparation (Figure 5A1). Specificity of the DPP6 antibody was tested by western blot after pre-incubation with the blocking antigen, which eliminated both bands in brain and heart preparations (Figure 5A2). Notably, the expression level of KChIP2 protein was comparable among these species (Figure S6). Based on the above results we hypothesized that the dominant expression of DPP6-L might be responsible for the inhibitory effect of NS5806 on native \(I_{Io}\) in mouse cardiomyocytes and hiPSC-CMs.

3.5 | Analysis on the association between KChIP2 and DPP6

Although our findings revealed the key role of DPP6 subunits in defining pharmacological profile of \(I_{Io}\), the fact that NS5806 failed to affect the current amplitude and inactivation kinetics in Kv4 and Kv4/DPP6 channels suggests that KChIP2 is also required for the modulatory effects of NS5806. Specifically, both DPP6 and KChIP2 are required for NS5806-mediated channel inhibition, while KChIP2 alone confers channel potentiation by NS5806. We further hypothesized that the inhibitory effect of NS5806 on the Kv4 channel may depend on the association between DPP6-L and KChIP2.

To identify the possible sites involved in association between two proteins, the structural models of KChIP2 and DPP6-L were generated. Based on a known structure of KChIP4a,23 a homology model of KChIP2 was generated (Figure 6A1). The structure of intracellular domain of DPP6-L (DPP6-Lin) was predicted using \textit{ab initio} protein prediction approach (Figure 6A2). Top-ranked models of both proteins are shown in Figure 6A3. Then, molecular docking simulations were performed to identify possible binding sites between these two proteins. Ten models were generated in total and the model with the most favorable binding energy (Figure 6A4) was used as a template for site-directed mutagenesis study. The detailed predicted results of interactions between DPP6-Lin and KChIP2 in the model are shown in Table S4. Residues R7, P33, D36, G38, and L44 within the putative KChIP2-associating site of DPP6-L (Figure 6B1) and were mutated to alanines with an aim to generate a DPP6-L mutant with reduced
KChIP2-binding affinity (DPP6-L-Mut). The current for Kv4.3/KChIP2/DPP6-L-WT and Kv4.3/KChIP2/DPP6-L-Mut (1:1:1) channels were then recorded and compared (Figure 6B2). The current inactivation kinetics of Kv4.3/KChIP2/DPP6-L-Mut was significantly slower, as compared to Kv4.3/KChIP2/DPP6-L-WT (Figure 6B3). The representative current traces of Kv4.3/KChIP2/DPP6-L-WT channels and Kv4.3/KChIP2/DPP6-L-Mut channels in the absence and presence of NS5806 are shown in Figure 6C1. Strikingly, the mutations conferred a reversal of the effect of NS5806 on the current amplitude: from inhibition of \( I_{Kv4.3/KChIP2/DPP6-L-WT} \) to modest (but significant) enhancement of \( I_{Kv4.3/KChIP2/DPP6-L-Mut} \) (Figure 6C2). In addition, mutations within the putative KChIP2 interaction sites also reversed the effect of NS5806 on the current inactivation kinetics (Figure 6C3). These experiments add further support to the hypothesis that an association between DPP6 and KChIP2 confers the inhibitory effect of NS5806 on Kv4 channel.

4 | DISCUSSION

4.1 | NS5806 suppressed Kv4-generated \( I_{to} \) in mouse cardiomyocytes and hiPSC-CMs

NS5806 has been put forward as a prototypic activator of \( I_{to} \)[12,13] Yet, the efficacy of the \( I_{to} \) enhancement by NS5806...
varies significantly between the tissue types and even within different regions across the ventricular wall (i.e., between epicardial, midmyocardial, or endocardial cells\textsuperscript{15,30}). The potentiating effects have also been demonstrated on heterologously expressed Kv4.3/Kv4.2 channels in the presence of KChIP2 with or without DPP6.\textsuperscript{12} A recent study has revealed that NS5806 markedly increased $I_{\text{to}}$ amplitude in rabbit ventricular myocytes, but it inhibited $I_{\text{to}}$ in rabbit atrial cells.\textsuperscript{31} These different responses are difficult to explain solely by contribution of Kv1.4 since Kv4.3, 4.2, and 1.4 are all expressed across the rabbit atri

Consistent with the previous finding, we observed a significant increase in $I_{\text{to}}$ amplitude in the presence of NS5806 in canine ventricular myocytes. However, the effect of NS5806 on native $I_{\text{to}}$ in mouse ventricular cardiomyocytes as well as in hiPSC-CMs was strikingly different as current inhibition was seen instead. In mouse ventricles, Kv4.2/Kv4.3 subunits conduct $I_{\text{to}}$, whereas Kv1.4 forms $I_{\text{to}}$, channels.\textsuperscript{32} Using specific Kv4 channel blocker combined with kinetic differentiation, our experiments further confirmed that $I_{\text{to}}$ was specifically inhibited by NS5806 in mouse ventricular myocytes and hiPSC-CMs. In both these cell types, NS5806 significantly accelerated current inactivation and shifted voltage dependence of steady-state
inactivation to more negative potentials. These effects on channel gating could account for the decrease in current amplitude.

Consistent with these results, a previous report has shown that NS5806 at 10 μM fails to enhance, but rather produced a small reduction of \( I_{\text{to}} \) amplitude in hiPSC-CMs. Our western blot analysis provided further evidence for a dominant expression of Kv4 in hiPSC-CMs, while only a trace amount of Kv1.4 was found in these cells. This observation is seemingly at odds with the previous study showing presence of Kv1.4 mRNA in hiPSC-CM; however, the abundance of Kv1.4 protein in hiPSC-CM was not investigated in that study. The different expression pattern of ion channels may depend on different maturation state of hiPSC-CMs. Yet, variable presence of Kv1.4 brings further complexity to \( I_{\text{to}} \) modulation by NS5806 since an inhibitory effect of NS5806 on cloned Kv1.4 channel has been demonstrated. Nevertheless, the fact that there were comparable levels of Kv1.4 protein abundance in mouse and canine ventricular tissue (while the effects of NS5806 on \( I_{\text{to}} \) were opposite) suggests that the effect of NS5806 on the Kv4 channel complex is a dominant mechanism of the \( I_{\text{to}} \) modulation in the heart.

### 4.2 | DPP6 and KChIP2 subunits confer the modality of \( I_{\text{to}} \) channel response to NS5806

We think that involvement of different auxiliary subunits could offer an explanation for the difference in NS5806 action in cardiac cells from different species. The KChIP2 and DPP6 are most likely candidates for auxiliary subunits of Kv4-containing \( I_{\text{to}} \) channel complex. We thus tested the effect of NS5806 on cloned Kv4 with KChIP2 and DPP6 subunits in HEK293 cells. In agreement with the previous reports, our results indicate that NS5806 significantly enhanced the current amplitude of Kv4.3/KChIP2 and Kv4.3/KChIP2/DPP6-S channels, and concomitantly slowed the inactivation kinetics of these channel complexes. On the contrary, NS5806 markedly decreased the peak amplitudes of currents mediated by Kv4.3/KChIP2/DPP6-L or Kv4.2/KChIP2/DPP6-L channel complexes and significantly sped the inactivation of these channels. The above effect of NS5806 was further potentiated when DPP6-L expression level was elevated. These changes recapitulated the effect of NS5806 on native \( I_{\text{to}} \) in the mouse ventricular cells. The results suggest that auxiliary subunits DPP6-L and KChIP2 confer the opposite responses to NS5806. Yet, KChIP2 was also necessary for the inhibitory action of NS5806 on \( I_{\text{to}} \) as Kv4/DPP6-L complex was insensitive to the compound (Figure 4A,B).

Consistent with the findings from heterologous expression system, our western blot analysis demonstrated that DPP6-L was dominantly expressed in both mouse ventricular myocardium and iPSC-CMs. In contrast, in canine ventricular myocardium, DPP6-L presence was undetectable while low levels of DPP6-S expression were seen. Levels of KChIP2 expression were comparable between all three species (Figure 5A). Furthermore, siRNA knockdown of DPP6 significantly antagonized the NS5806-induced reduction of current amplitude and acceleration of inactivation of native \( I_{\text{to}} \) in hiPSC-CMs. Thus, our data clearly demonstrate that the inhibitory response to NS5806 in cardiomyocytes depends on the DPP6-L subunit. The effects of NS5806 on cloned Kv4 channel complexes and native \( I_{\text{to}} \) reported here and in previous reports are summarized in Table 1.

| Cloned Kv4 channel complexes | K\text{v}4.2/\text{Kv}4.3 | K\text{v}4.2/\text{Kv}4.3 | K\text{v}4.2/\text{Kv}4.3 | K\text{v}4.2/\text{Kv}4.3 |
|-------------------------------|-----------------|-----------------|-----------------|-----------------|
| Subunits                      | \(+\) KChIP2    | \(+\) KChIP2    | \(+\) DPP6-S    | \(+\) KChIP2    |
| Peak current\(^a\)            | ↑               | ↑               | ↓               | ↓               |
| Inactivation\(^b\)            | ↑               | ↑               | ↑               | ↑               |

Note: Highlighted in blue color are data from the present study; others from previous reports (which have been cited in text).

\(^a\) No significant effect; \(^↑\) Decrease peak current, \(^↑↑\) increase peak current.
\(^b\) Accelerate inactivation, slow inactivation.

### 4.3 | Possible molecular mechanism underlying the inhibitory response to NS5806

Previous study suggested that the potentiating effect of NS5806 on Kv4 channels depends on the accessory protein KChIP2. KChIP2 is a cytosolic protein that interacts with the intracellular N-termini of Kv4 subunits and results in a slower inactivation kinetics and acceleration of recovery from inactivation. A recent study has shown that NS5806 binds to hydrophobic residues on the C-terminus of KChIP3 and increases the affinity between KChIP3 and the N-terminus of Kv4.3. It is likely that the similar mechanism underlies the effect of NS5806 on Kv4/KChIP2 complex channel since KChIP2 and KChIP3 have nearly identical NS5806-binding sites. However, how does DPP6-L confer the inhibitory effect of NS5806 on Kv4/KChIP2/DPP6-L channel complex? The DPP6 is a single
transmembrane subunit consisting of a short intracellular N-terminal domain and a long extracellular C-terminal domain.\(^8\) When co-expressed with Kv4 subunits, DPP6 (either long or short isoform) increases the rate of inactivation of a resulting channel complex, negatively shifts the voltage dependence of steady-state inactivation, and increases the rate of recovery from inactivation.\(^11\) Interestingly, the present study revealed that NS5806 produces biophysically very similar effects on both native \(I_{to}\) channels in tissues expressing DPP6-L (murine and human but not canine) and on recombinant Kv4/KChIP2/DPP6-L channel complexes. It is thought that the discrete and specific interactions mediate the effects of KChIP and DPP subunits on the gating of Kv4 channels.\(^34\) Thus, we speculate that the inhibitory effect of NS5806 on the \(I_{to}\) current might result from the increased binding affinity between DPP6-L and the Kv4/KChIP2 complex. One explanation for the observed effects might be in that (a) intracellular N-terminal domain of DPP6-L interacts with KChIP2, (b) the association between two subunits exposes NS5806-binding site in DPP6-L while hindering the binding of NS5806 to KChIP2 subunits. In support of this scenario, the molecular docking simulations indicated that several putative residues, including R7, P33, D36, G38, and L44 in the N-terminus of DPP6-L, are likely to associate with the C-terminus of KChIP2. Mutation of these residues reversed the inhibitory effect of NS5806 into potentiation of the \(I_{to}\) current. In addition, the acceleration of inactivation was turned into slowing down. Interestingly, according to the docking simulation, the putative KChIP2-DPP6-L association site is predominantly localized in the C-terminus of KChIP2, which has been reported to contain also the binding site for NS5806.\(^13\) This may be the reason why the association between two subunits could hamper binding of NS5806 to KChIP2 and promote binding to DPP6-L instead. Binding of NS5806 to DPP6-L might facilitate its association with the pore-forming Kv4 subunit and “switch” the potentiation of \(I_{to}\) into inhibition. Interestingly, previous studies reported that the peak amplitudes of currents mediated by ternary Kv4/KChIP2/DPP6-S channel complexes were potentiated and the current inactivation significantly slowed by NS5806.\(^12,28\) DPP6-S isoform lacks 62 N-terminal residues that form putative KChIP-interacting site. Hence, the ability of DPP6-S to associate with KChIP2 is likely to be compromised. This is a likely explanation for the divergent results observed with the DPP6-S and DPP6-L isoforms. Indeed, our data predict that similarly to the DPP6-L-Mut (Figure 6), the DPP6-S isoform would not be able to bind to KChIP2 and, thus, to switch the modality of NS5806 response of the \(I_{to}\) channel complex. However, further structural insights are needed to confirm the direct interaction between KChIP2 and DPP6-L. In addition, KCNE \(\beta\)-subunits also regulate Kv4 channels in the hearts of some species and may influence the response to channel modulator.\(^35\) Further studies are needed to test the possibility of the involvement of KCNE subunits in the NS5806 response.

## 5 CONCLUSION

This study discovers molecular mechanism for the opposing response of cardiac Kv4 channel complex to its modulator NS5806. We show that the alternative assembly of the complex with different auxiliary subunits in different species results in channels with strikingly different biophysical and pharmacological properties. These findings provide novel insight for the development of new ion channel modulators for treatment of cardiovascular diseases.

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### CONFLICT OF INTEREST

The authors report no conflict of interest.

### AUTHOR CONTRIBUTIONS

Hongxue Zhang, Hua Zhang, C. Wang, Y. Wang, R. Zou, C. Shi, and B. Guan performed the research. Y. Xu and N. Gamper designed the research strategy. Hongxue Zhang, C. Wang, R. Zou, and C. Shi analyzed the data. Hongxue Zhang, N. Gamper, and Y. Xu wrote the paper.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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