Supplemental Information for manuscript #JPET/2012/198887

Title: A Novel, Potent, and Selective Inhibitor of Cardiac Late Sodium Current Suppresses Experimental Arrhythmias

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Journal: *The Journal of Pharmacology and Experimental Therapeutics*

This Supplement summarizes methods and results of studies of GS967 that are not described in the printed manuscript, including: (1) ion channel currents recorded from rabbit isolated myocytes; (2) action potentials (APs) recorded from rabbit isolated myocytes; (3) relative concentrations of intracellular Na^+ and Ca^{2+} in rabbit isolated myocytes; (4) currents recorded from human embryonic kidney 293 or Chinese hamster ovary cells expressing individual human cardiac ion channels, and (5) receptor and enzyme radioligand binding, and kinase active site binding.

(1) Effects of GS967 on Additional Rabbit Isolated Myocyte Ion Channel Currents

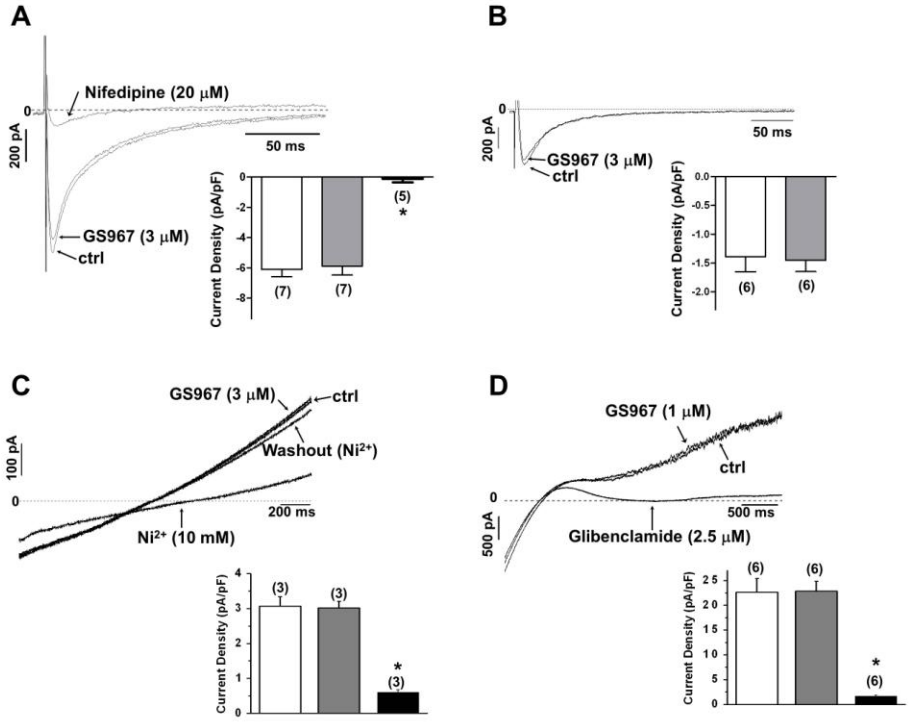
Methods: L-type calcium current (I_{CaL}) was measured using the amphotericin-B perforated patch technique. I_{CaL} was activated by applying 360-ms pulses to +10 mV every 15 s from a holding potential of -80 mV. A preconditioning pulse to -40 mV for 300 ms was used to inactivate I_{Na} and I_{CaT}. Nifedipine (5-20 µM) was added at the end of an experiment to confirm the identity of I_{CaL}. The contents of the pipette solution were (in mM): 200–300 µg/mL amphotericin B, 90 aspartic acid, 10 NaCl, 100 CsOH, 30 CsCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 5 EGTA, 2 $ATPNa_2$, and 0.1 $GTPNa_2$, pH 7.2 with CsOH. Pipette resistance was 1-2 MΩ. Myocytes were continuously superfused with a 22±1 ºC bath solution containing (in mM): 135 NaCl, 10 CsCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES, 10 glucose, and 0.01 tetrodotoxin, pH 7.4 with NaOH.

T-type calcium current (I_{CaT}) was calculated as the difference current between peak total Ca^{2+} current and I_{CaL} in whole cell-attached patches. Total I_{Ca} was evoked using depolarizing pulses of 360-ms duration from a holding potential of -80 mV to +10 mV every 15 s. Peak I_{CaL} was measured using the same depolarizing step, but from a holding potential of -50 mV (to inactivate I_{CaT}). After a seal was formed, the membrane was ruptured, and capacitance currents were compensated; the cell was continuously superfused with a 22±1 ºC bath solution containing (in mM): 135 CsCl, 1 $MgCl_2$, 10 $CaCl_2$, 10 HEPES, 10 glucose, and 0.02 TTX, pH 7.3 with CsOH. The pipette resistance was 2-3 MΩ when filled with the following internal solution (in mM): 96 CsCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 MgATP, 20 HEPES, and 14 EGTA, pH 7.1 with CsOH.

For measurement of Na^+/Ca^{2+} exchange current (I_{NCX}), a 2-s descending ramp from 60 mV to -120 mV at a frequency of 0.1 Hz was used. I_{NCX} was recorded at 36±1ºC and myocytes were continuously superfused with bath solution containing (in mmol/L): 140 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 7.5 glucose, 5 HEPES, pH 7.2 with CsOH. Niflumic acid (100 μmol/L), Ouabain (10 μmol/L), nifedipine (10 μmol/L) and ryonadine (10 μmol/L) were used to block Ca^{2+} -activated Cl^- channels, Na^+-K^+ ATPase, I_{CaL} and ryonadine receptor, respectively. The contents of the pipette solution were (in mmol/L): 20 NaCl, 10 CsCl, 0.8 $MgCl_2$, 2.0 $CaCl_2$, 5 BAPTA, 5 MgATP, 10 TEA, 5 glucose, 10 HEPES pH 7.3 CsOH. The Ni^{2+}-sensitive (10 mmol/L) current was taken as I_{NCX}.

For measurement of the ATP-sensitive potassium current (I_{KATP}), a 4-s ramp from -120 mV to +60 mV was used and the protocol was repeated every 6 s. Cromakalim (100 μM) was added to evoke I_{KATP}. The bath solution contained (in mM): 140 NaCl, 4.0 KCl, 0.33 NaH_2PO_4, 1 $MgCl_2$, 1 $CaCl_2$, 5 HEPES, 0.25 $CdCl_2$, pH 7.4 with NaOH. The contents of the pipette solution were (in mM): 120 KCl, 1 $MgCl_2$, 10 EGTA, 10 HEPES, pH 7.3 with KOH.

Results: GS967 effects on sodium currents and I_{Kr} are presented in the print version. GS967 (1-3 μM) had no significant effects on I_{CaL}, I_{CaT}, or I_{KATP} in rabbit ventricular myocytes (Supplemental Figure 1).



Supplemental Figure 1. Effects of GS967 (1-3 μM) on I_{CaL} (panel **A**), I_{CaT} (panel **B**), I_{NCX} (panel **C**), and I_{KATP} (panel **D**) in rabbit ventricular myocytes. Representative experiments are shown on the left and summary data on the right side in each panel. Open bars represent no drug control (ctrl), gray bars GS967, and black bars either nifedipine or glibenclamide data as appropriate. The number of myocytes is indicated in parentheses. **, $p<0.01$ compared to control or GS967. Data are mean±S.E.M.

(2) Effects of GS967 on the Rabbit Ventricular Myocyte Action Potential (AP)

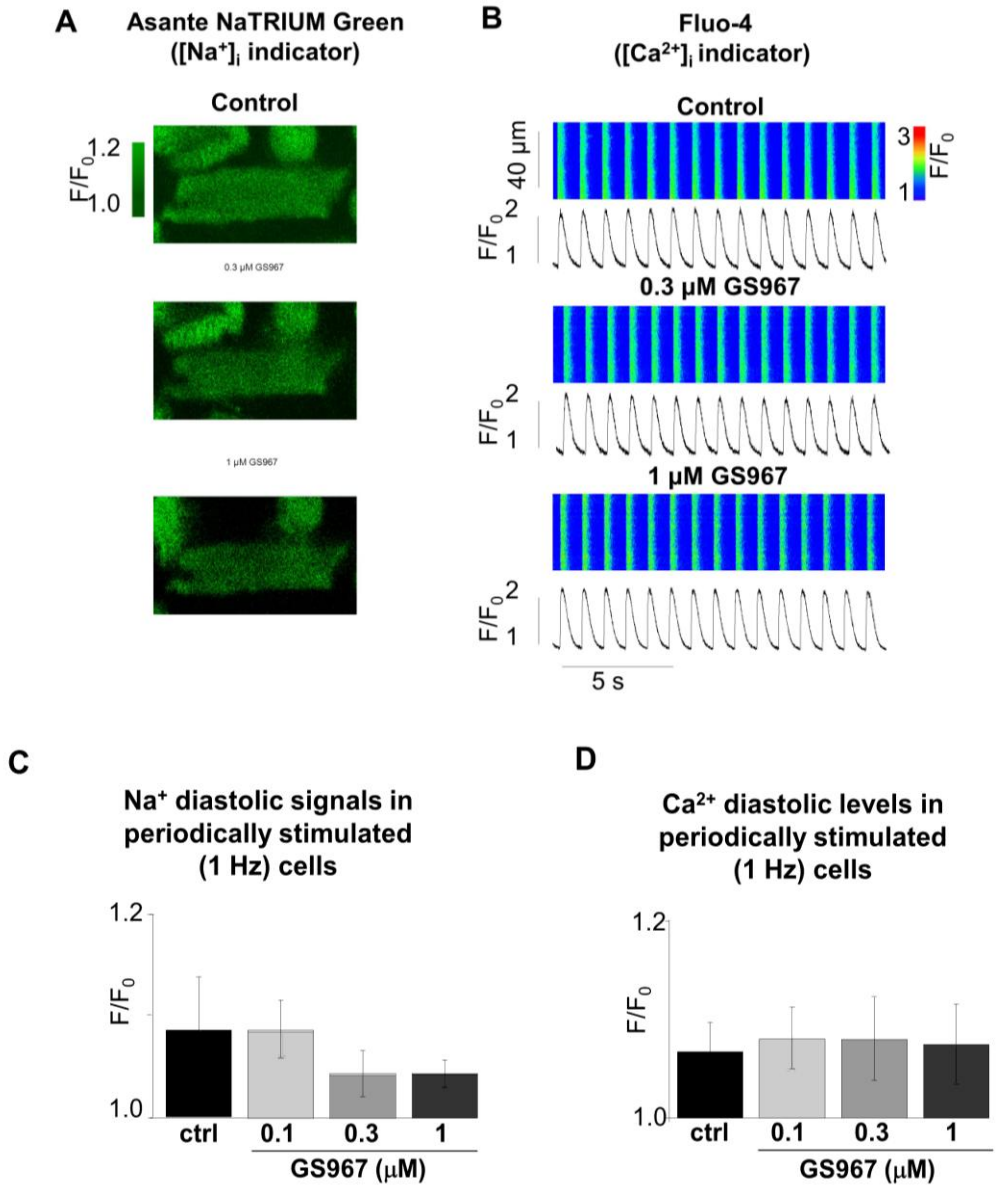
Methods: New Zealand White adult female rabbits were anesthetized and the heart was excised. Left ventricular tissue samples were quickly immersed in a cardioplegia solution consisting of (mM): 50 KH_2PO_4, 8 $MgSO_4$, 10 $NaHCO_3$, 5 adenosine, 25 taurine, 140 glucose, and 100 mannitol, pH 7.4, and bubbled with 100% O_2 at 0-4°C. Tissue was minced into 0.5-1 mm cubes and transferred to a 50 mL conical tube containing a low-calcium wash solution with (in mM): 137 NaCl, 5 KH_2PO_4, 1 $MgSO_4$, 10 taurine, 10 glucose, 5 HEPES, and 0.1 EGTA, pH 7.4 (22-24°C). The solution was continuously bubbled with 100% O_2 for 5 min. The tissue was next incubated in a 5 mL solution containing (mM): 137 NaCl, 5 KH_2PO_4, 1 $MgSO_4$, 10 taurine, 10 glucose, 5 HEPES, supplemented with 0.1% bovine albumin, 2.2 mg/ml collagenase type V and 1.0 mg/ml protease type XXIV (Sigma, St Louis, MO), pH 7.4 (37°C) and bubbled continuously with 100% O_2. The supernatant was removed after 20 min and discarded. Tissue chunks were then incubated in a solution of the same ionic composition but supplemented with only collagenase and 100 μM $CaCl_2$. Microscopic examination of the medium was performed every 5-10 min to determine the number and quality of the isolated cells. When the yield appeared to be maximal, the cell suspension was centrifuged for 2 min and the resulting pellet was resuspended in a modified Kraftbruhe solution containing (mM): 25 KCl, 10 KH_2PO_4, 25 taurine, 0.5 EGTA, 22 glucose, 55 glutamic acid, and 0.1% bovine albumin, pH 7.3 (22-24°C). The isolation procedure produced an initial yield of approximately 40-60% rod-shaped, calcium-tolerant cells. Cells were used within 24 h after isolation. APs were recorded at 36.0±0.5 ^{o}C using intracellular microelectrodes with resistances ranging from 25 to 40 MΩ when filled with 3 M KCl. Cells were superfused (~2 mL/min) with a bath solution containing (mM): 137 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES, pH 7.4. APs were recorded from each cell at stimulus frequencies of 1, 2, and 3.3 Hz in the absence of drug (control). Each cell was then superfused with drug for 4 min and APs were again recorded at each stimulation frequency in the presence of drug. Only one concentration of drug was studied with each cell.

Results: See Table 2 and text in the print version of this manuscript.

(3) Effect of GS967 on Rabbit Myocyte Intracellular Na^+ and Ca^{2+} Concentrations

Methods: Myocytes were enzymatically isolated from hearts of 2-3 kg adult New Zealand white female rabbits and studied using confocal microscopy of intracellular Na^+ and Ca^{2+} concentrations as previously described (Yao et al., 2011). Cells were bathed in a solution containing (in mM): 140 NaCl, 5.4 KCl, 2.0 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 5.6 glucose, pH 7.3. Isolated myocytes were placed on laminin-coated coverslips, stored in Ca^{2+}- free Tyrode solution at room temperature, and used within 4 hr of isolation. Myocytes were imaged with a LSM 5 PASCAL (Carl Zeiss, Oberkochen, Germany) Laser Scanning Confocal System, equipped with a Zeiss Plan-Apochromat 63 X 1.4 numerical aperture oil immersion objective. For dye loading, cells were incubated for 25 min in bath solution with reduced Ca^{2+} (0.25 mM) concentration and either 5 μM of the

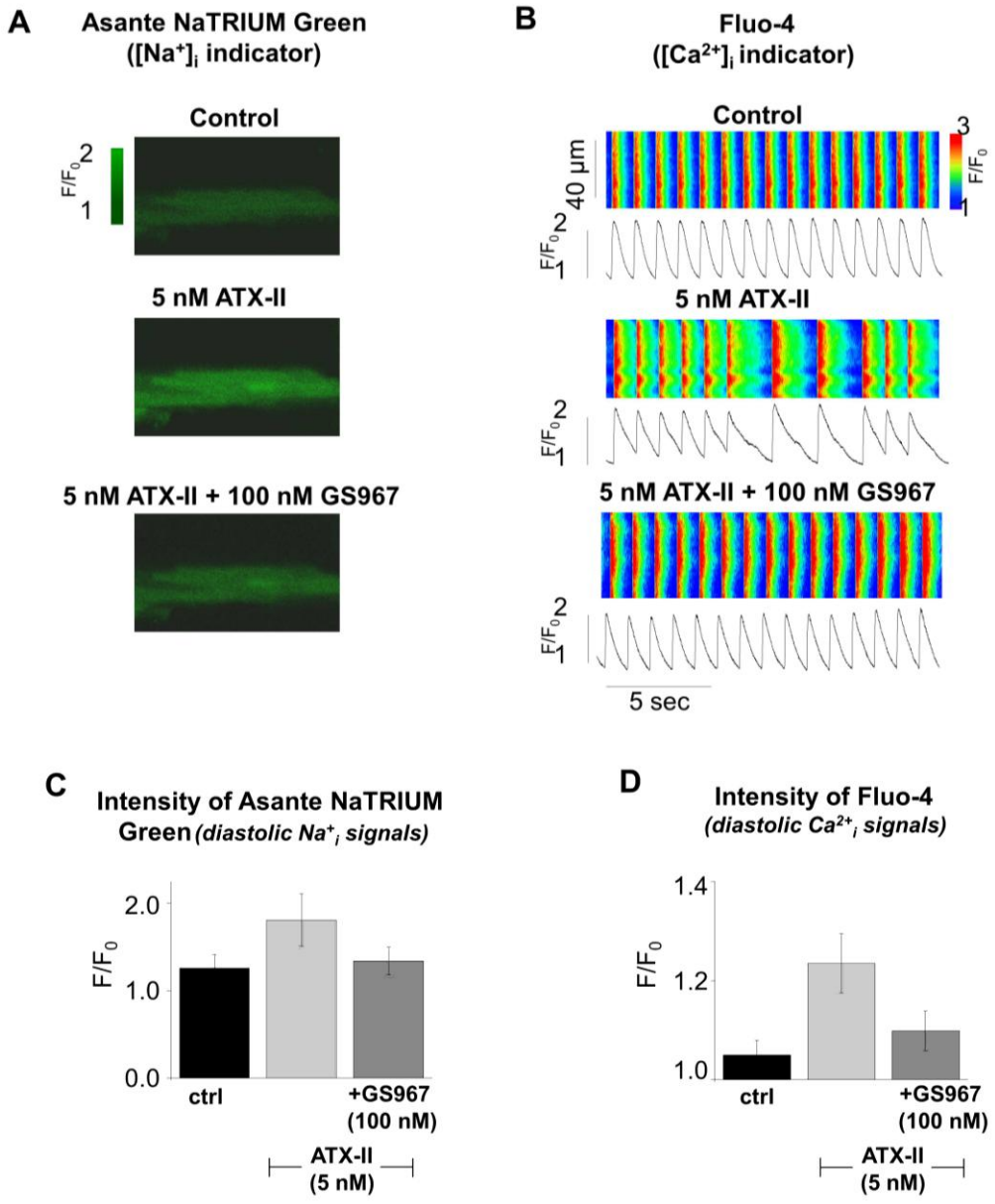
Ca^{2+} indicator Fluo-4AM (Invitrogen, Carlsbad, CA) or 5 μM of the Na^+ indicator Asante NaTRIUM Green AM (TEFLabs, Austin, TX). Fluorescence intensity was measured as F/F_0, were F is the dynamically-changing intensity of the fluorescent dye signal and F_0 is the fluorescence intensity in non-stimulated cells at the beginning of each experiment.



Supplemental Figure 2. Effect of GS967 on rabbit isolated myocyte intracellular Na^+ and Ca^{2+} concentrations. Results of representative experiments are shown in panels **A** and **B** for measurements of intracellular levels of Na^+ and Ca^{2+} using Asante NaTRIUM Green and Fluo-4, respectively. Summary data for all experiments are shown in panels **C** (Na^+) and **D** (diastolic and systolic Ca^{2+}), respectively. Bars represent the values of mean±S.E.M. of data from the number of myocytes indicated in parentheses.

Results: GS967 (0.3 and 1 μM) did not significantly affect the concentration of intracellular Na^+ or the Ca^{2+} transient in rabbit isolated myocytes stimulated at a rate of 1

Hz (Supplemental Figure 2). Neither the peak systolic nor the diastolic level of intracellular Ca^{2+} was altered from control (no drug) by GS967 (0.3-1 μM) (Supplemental Figure 2). ATX-II caused increases of intracellular Na^{+} and Ca^{2+} and dysrhythmic Ca^{2+} transients, which were attenuated by GS967 (0.1 μM) (see main manuscript and Supplemental Figure 3).



Supplemental Figure 3. GS967 (100 nM) attenuated anemone toxin-II (ATX-II)-induced increases of intracellular Na^{+} and Ca^{2+} concentrations in rabbit ventricular myocytes. ATX-II (5 nM) increased the intensity of Asante NaTRIUM Green (relative Na^{+} concentration; panels **A**, **C**) and Fluo-4 (relative Ca^{2+} concentration; panels **B**, **D**) fluorescence signals, and caused dysrhythmic Ca^{2+} transients (panel **B**). GS967 (100 nM) attenuated each of these effects of ATX-II (all panels). Results of representative experiments are shown in panels A and B; summary data in panels C and D for the number of myocytes indicated in

parentheses. Myocytes were paced at a rate of 1 Hz. *,** significantly different ($p<0.05$) from control (ctrl) or ATX-II, respectively.

(4) Effects of GS967 on currents recorded from HEK293 or CHO cells expressing individual human cardiac ion channels

Methods: Assays of the effects of GS967 on cardiac ion channels were performed at ChanTest (Cleveland, OH) or Gilead Sciences (peak and late I_{Na}) using PatchXpress 7000A or QPatch16 automated patch clamp technology and CHO or HEK293 cell lines stably expressing human channel genes and channel subunits, as indicated in Supplemental Table 2. All experiments were carried out at room temperature (22-24 ^{o}C). Cells were stimulated at a frequency of 0.1 Hz in all protocols except that for recording of I_{Ks}, wherein the frequency was 0.067 Hz. Peak I_{Na} was measured as the peak inward current during a 20-ms voltage step to -20 mV from a holding potential (HP) of -120 mV. Late I_{Na} was measured in the presence of 10 nM ATX-II as the mean inward current at the end of a 250-ms voltage step to -20 mV from a HP of -120 mV, after subtraction of basal current recorded in Na^+-free bathing solution. Peak I_{CaL} was measured as the peak inward current during a 200-ms step to +10 mV from a HP of -80 mV. Peak I_{CaT} was measured as inward current during a 50-ms test pulse to -30 mV that immediately followed a 250-ms hyperpolarizing conditioning pulse to -120 mV, from a HP of -80 mV. Funny current I_f (HCN2/HCN4) was measured as the steady-state inward current at the end of a 1-s hyperpolarizing step to -120 mV from a HP of -30 mV. The hERG current voltage protocol consisted of a 500-ms prepulse to -40 mV from an HP of -80 mV (used for leak correction) followed by a 2-s pulse to +40 mV to activate the channel and then a 2-s test pulse to -40 mV. Block of hERG current was measured as a decrease in the outward tail current during the test pulse to -40 mV. Inwardly-rectifying background K^+ current I_{K1} ($K_{ir}2.1$) was measured as the steady-state current at the end of a 300-ms hyperpolarizing step to -110 mV from a HP of -70 mV. The acetylcholine-activated K^+ current I_{KAch} ($K_{ir}3.1/3.4$) current protocol consisted of a 400-ms hyperpolarizing step to -100 mV from an HP of -70 mV, followed by a 1-s ramp from -100 mV to +40 mV. I_{KAch} was measured as the steady-state current at the end of the 400-ms step to -100 mV. The voltage protocol for measurement of I_{KATP} ($K_{ir}6.2$/SUR2A) consisted of a 100-ms hyperpolarizing step to -110 mV from an HP of -60 mV, followed by a 1-s ramp from -110 mV to +10 mV, holding at +10 mV for 100 ms, then returning to the HP. I_{KATP} was measured as the current at the end of the step to +10 mV. I_{KATP} was activated using a 5-min exposure of cells to 100 µmol/L pinacidil. The ultra-rapidly-activating K^+ current I_{Kur} ($K_V1.5$) was measured as outward current at the end of a 300-ms test pulse to +20 mV from a HP of -80 mV. The transient outward K^+ current I_{to} ($K_V4.3$) was measured as peak outward current during a 300-ms test pulse to 0 mV from a HP of -80 mV. The slowly-activating delayed-rectifier K^+ current I_{Ks} (K_VLQT/minK) protocol consisted of a 2-s step to +40 mV from an HP of -80 mV, followed by a 0.5-s step to -40 mV. I_{Ks} was measured at the end of the step to +40 mV. At the end of each experiment, leak current was measured in the presence of 300 µM chromanol 293B and subtracted from total membrane current to obtain I_{Ks}. The contents of all extracellular and intracellular solutions used in assays of the various currents are presented in Supplemental Tables 2 and 3.

Results: GS967 (1 μM) caused significant inhibition of $Na_V1.5$ late I_{Na} (90%), modest inhibition of hERG (22.7%), minor inhibitions of $Na_V1.5$ peak I_{Na} (16.3%) and $Ca_V3.2$ I_{CaT} (14.6%), and insignificant effects on other currents (Supplemental Table 1). GS967 did not inhibit hERG in rabbit ventricular myocytes (see manuscript Figure 1).

Supplemental Table 1. Inhibition by GS967 (1 μM) of human ion channels heterologously-expressed in human embryonic kidney (HEK) 293 or Chinese hamster ovary (CHO) cells.

Cardiac Ion Channels	Cardiac Currents	1 μM % Block	n	Cell Line	Subunits	
					Type	Gene(s)
$Na_V1.5$ late	**Late I_{Na}**	90.0 ± 1.6	5	HEK293	α	SCN5A
$Na_V1.5$ peak	**Peak I_{Na}**	16.3 ± 3.5	10	HEK293	α	SCN5A
$Ca_V1.2$	**I_{CaL}**	9.7 ± 7.7	4	CHO	α, β2, α2δ	hCACNA1C, hCACNB2, hCACNA2D1
$Ca_V3.2$	**I_{CaT}**	14.6 ± 2.7	4	HEK293	α	hCACNA1H
HCN2	**I_f**	-0.5 ± 3.0	4	CHO	α	hHCN2
HCN4	**I_f**	-1.1 ± 3.9	4	HEK293	α	hHCN4
$K_{ir}2.1$	**I_{K1}**	2.6 ± 2.7	4	HEK293	α	hKCNJ2
$K_{ir}3.1/3.4$	**I_{KAch}**	2.9 ± 4.4	4	HEK293	α, α	hKCNJ3, hKCNJ5
$K_{ir}6.2/SUR2A$	**I_{KATP}**	0.5 ± 3.8	4	HEK293	α, β	hKCNJ11, SUR2A
$K_V1.5$	**I_{kur}**	2.0 ± 2.4	4	CHO	α	hKCNA5
$K_V4.3$	**I_{to}**	6.1 ± 5.4	4	HEK293	α	hKCND3
K_VLQT/minK	**I_{Ks}**	4.0 ± 2.3	4	CHO	α, β	hKCNQ1, hKCNE1
hERG	**I_{Kr}**	22.7 ± 3.4	4	HEK293	α	hKCNH2

Values of % block are mean±S.D. of current recorded from "n" cells in the presence of 1 μM GS967 after normalization to control current (absence of drug) recorded from the same cell.

Supplemental Table 2. Extracellular Saline Solutions Used to Record Currents from Human Cardiac Ion Channels.

Channel Test System	Solution ID	Extracellular Saline - Concentrations (mM)						
		NaCl	$BaCl_2$	KCl	$CaCl_2$	$MgCl_2$	HEPES	Glucose
$Ca_V1.2$	HB-PS	137	0	4	1.8	1	10	10
$Ca_V3.2$	HB-PS	137	0	4	1.8	1	10	10
HCN2, HCN4	HCN4-HB-PS	100	1	40	1.8	0.5	10	10
hERG	HB-PS	137	0	4	1.8	1	10	10
$K_{ir}2.1$	HB-PS	137	0	4	1.8	1	10	10
$K_{ir}6.2/SUR2A$	HB-PS	137	0	4	1.8	1	10	10
$K_{ir}3.1/3.4$	40 K^+ HB-PS	100	0	40	1.8	1	10	10
$K_V1.5$	HB-PS	137	0	4	1.8	1	10	10
$K_V4.3$	HB-PS	137	0	4	1.8	1	10	10
K_VLQT1/MinK	HB-PS	137	0	4	1.8	1	10	10
$Na_V1.5$	CJL-PS	140/20*	0	4	1.8	0.75	5	0

*140 mM NaCl was used for $Na_V1.5$ late I_{Na} and 20 mM NaCl + 120 mM N-methyl-D-glucamine were used for peak I_{Na} assays.

Supplemental Table 3. Intracellular Saline Solutions Used to Record Currents from Human Cardiac Ion Channels.

Ion Channel Test System	Intracellular Saline - Concentrations (mM)												
	Cesium Aspartate	Cesium Fluoride	Potassium Aspartate	$MgCl_2$	EDTA	EGTA	ATP	CsCl	NaF	Na_3VO_4	$Na_4P_7O_2$	GTP	HEPES
$Ca_V1.2$	130	0	0	5	0	10	4	0	0	0	0	0.5	10
$Ca_V3.2$	130	0	0	5	0	5	4	0	0	0	0	0	10
HCN2, HCN4	0	0	130	5	0	5	4	0	0	0	0	0	10
hERG	0	0	130	5	0	5	4	0	0	0	0	0	10
$hK_{ir}2.1$	0	0	130	5	0	5	4	0	0	0	0	0	10
$hK_{ir}6.2$/ SUR2A	0	0	130	5	0	5	4	0	0	0	0	0	10
$hK_{ir}3.1/3.4$	0	0	130	5	0	5	4	0	5	1	1	0.1	10
$hK_V1.5$	0	0	130	5	0	5	4	0	0	0	0	0	10
$hK_V4.3$	0	0	130	5	0	5	4	0	0	0	0	0	10
K_VLQT1/ MinK	0	0	130	5	1	5	4	0	0	0	0	0	10
$Na_V1.5$	0	120	0	0	0	5	0	20	0	0	0	0	5

(5) Effects of GS967 on Receptor and Enzyme Radioligand Binding, and Kinase Activity

GS967 was tested in receptor and enzyme binding and kinase active site binding assays conducted at CEREP (Redmond, WA) and DiscoveRx (Fremont, CA), respectively. In assays of displacement of radioligand binding to 162 receptors, ion channels, transporters, and enzymes at CEREP, GS967 (10 µM) displaced veratridine binding to site 2 (close to or overlapping the local anesthetic binding site) of the voltage-gated Na^+ channel by 70%. GS967 (10 µM) also inhibited picrotoxin binding to the GABA-gated chloride channel by 46%, and nitrendipine binding to the L-type Ca^{2+} channel by 41%. Other effects of GS967 (increased/decreased binding) were considered to be low or minimal. In the DiscoveRx KinomeScreen of effects of 3 µM GS967 on 442 kinases, a single hit – MARK4 – was identified as more than "minimal". In a subsequent follow-up study the IC_{50} value for GS967 inhibition of MARK4 was found to be >30 µM (i.e., very much higher than is needed for inhibition of late I_{Na}).

REFERENCES

Wu L, Ma J, Li H, Wang C, Grandi E, Zhang P, Luo A, Bers DM, Shryock JC, Belardinelli L (2011) Late sodium current contributes to the reverse rate-dependent effect of IKr inhibition on ventricular repolarization. *Circulation* **123**:1713-1720.