

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

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ABBREVIATIONS: AP, action potential; APD, action potential duration; ATX, *Anemonia sulcata* toxin; ECG, electrocardiogram; GS967, 6-(4-(trifluoromethoxy)phenyl)-3-(trifluoromethyl)-[1,2,4]triazolo[4,3-a]pyridine; I_{Na} , sodium current; I_{Kr} , rapidly-activated delayed rectifier potassium current; IC_{50} , 50% effective inhibitory concentration; MAP, monophasic action potential; MAPD, monophasic action potential duration; PVC, premature ventricular contraction; TdP, *torsades de pointes*; TTX, tetrodotoxin; V_{max} , upstroke velocity of the action potential; VF, ventricular fibrillation; VT, ventricular tachycardia.

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ABSTRACT

Inhibition of cardiac late Na⁺ current (late I_{Na}) is a strategy to suppress arrhythmias and sodium-dependent calcium overload associated with myocardial ischemia and heart failure. Current inhibitors of this current are unselective and can be proarrhythmic. This study introduces GS967 (6-(4-(trifluoromethoxy)phenyl)-3-(trifluoromethyl)-[1,2,4]triazolo[4,3-a]pyridine), a potent and selective inhibitor of late I_{Na}, and demonstrates its effectiveness to suppress ventricular arrhythmias. The effects of GS967 on rabbit ventricular myocyte ion channel currents and action potentials were determined. Anti-arrhythmic actions of GS967 were characterized in *ex vivo* and *in vivo* rabbit models of reduced repolarization reserve and ischemia. GS967 inhibited *Anemonia sulcata* toxin II (ATX-II)-induced late I_{Na} in ventricular myocytes and isolated hearts with IC₅₀ values of 0.13 and 0.21 micromolar, respectively. Reduction of peak I_{Na} by GS967 was minimal at a holding potential of -120 mV but increased at -80 mV. GS967 did not prolong action potential duration or the QRS interval. GS967 prevented and reversed proarrhythmic effects (afterdepolarizations, torsades de pointes) of the late I_{Na} enhancer ATX-II and the I_{Kr} inhibitor E-4031 in isolated ventricular myocytes and hearts. GS967 significantly attenuated the proarrhythmic effects of methoxamine+clofilium, and suppressed ischemia-induced arrhythmias. GS967 was more potent and effective to reduce late I_{Na} and arrhythmias than either flecainide or ranolazine. Results of all studies and assays of binding and activity of GS967 at numerous receptors, transporters and enzymes indicated that GS967 selectively inhibited late I_{Na}. In summary, GS967 selectively suppressed late I_{Na} and prevented and/or reduced the incidence of experimentally-induced arrhythmias in rabbit myocytes and hearts.

INTRODUCTION

Sodium (Na^+) channel opening and influx of Na^+ are responsible for the upstroke of the cardiac action potential (AP). When Na^+ channels in myocytes fail to inactivate after opening, Na^+ influx continues throughout the AP plateau. The resulting Na^+ current (I_{Na}) is referred to as late I_{Na} to distinguish it from the larger and transient peak I_{Na} . Late I_{Na} in the normal heart is small but its magnitude is increased in many pathological conditions, as in the failing and/or ischemic heart, in the heart exposed to oxidative stress, and in hearts of patients with congenital long QT3 syndromes (Ver Donck et al., 1993; Le Grand et al., 1995; Bennett et al., 1995; Wang et al., 1995; Ju et al., 1996; Maltsev et al., 1998; Maltsev and Undrovinas, 2006; Song et al., 2006; Sossalla et al., 2010).

Regardless of cause, an enhanced cardiac late I_{Na} is pro-arrhythmic (Boutjdir and El-Sherif, 1991; Sicouri et al., 1997; Undrovinas and Maltsev, 2008; Zaza et al., 2008). Late I_{Na} during the plateau of the AP reduces repolarization reserve (i.e., net outward current) and may prolong AP duration (APD). Prolongation of APD can result in early afterdepolarizations as a result of L-type Ca^{2+} channel re-opening (Ca^{2+} window current) (January and Riddle, 1989). Enhancement of late I_{Na} has been shown to elicit afterdepolarizations, triggered arrhythmic activity, and torsades de pointes (TdP) tachycardia in studies of Purkinje fibers, isolated atrial and ventricular myocytes, isolated wedges of cardiac tissue, and intact hearts (Boutjdir and El-Sherif, 1991; Sicouri et al., 1997; Song et al., 2004, 2008). Conversely, drug-induced reduction of late I_{Na} has been associated with improvement of electrical function in myocytes isolated from failing hearts, and in hearts made ischemic or that have been exposed to cardiac glycosides, H_2O_2 , enhancers of late I_{Na} , or drugs that block the rapidly-activating delayed-rectifier K^+ current (I_{Kr}) and reduce repolarization reserve (Ver Donck et al., 1993; Haigney et al., 1994; Le Grand et al., 1995; Sicouri et al., 1997; Song et al., 2004, 2006, 2008; Sossalla et al., 2010; Undrovinas and Maltsev 2008; Wu et al., 2011).

Selective inhibition of late I_{Na} is a therapeutic target for treatment of electrical and contractile dysfunction in cardiac ischemia and heart failure (Ver Donck et al., 1993; Undrovinas and Maltsev, 2008; Saint 2006). However, no potent and selective inhibitor of late I_{Na} is currently available. The Na^+ channel blocker tetrodotoxin (TTX) inhibits late with greater potency than peak I_{Na} (Wu et al., 2009). Nevertheless, TTX blocks neuronal and skeletal muscle isoforms of Na^+ channels with much greater potency than it blocks the cardiac isoform $Na_v1.5$ (Heinemann et al., 1992), and is therefore not suitable for *in vivo* block of cardiac late I_{Na} . Compounds R56865 and F15845 are potent inhibitors of cardiac late I_{Na} (Le Grand et al., 2008), but their efficacies for block of late versus peak I_{Na} and specificity for binding to Na^+ channels relative to other proteins are unknown. Drugs such as lidocaine, mexiletine, and flecainide are prototypical Na^+ channel blockers that inhibit both late and peak I_{Na} . The anti-ischemic, antianginal drug ranolazine is reported to inhibit late I_{Na} with greater potency (IC_{50} value of 6 μM) than it inhibits other ion currents (Antzelevitch et al., 2004). Ranolazine is more selective for inhibition of late I_{Na} relative to peak I_{Na} than either lidocaine or amiodarone (Undrovinas et al., 2006). However, like flecainide, ranolazine reduces I_{Kr} (IC_{50} value of 12-14 μM) (Antzelevitch et al., 2004). Ranolazine also blocks both α - and β -adrenergic receptors (Zhao et al., 2011), and in one (Parikh et al., 2012) but not in another (Galimberti and Knollmann, 2011) study was reported to regulate sarcoplasmic reticulum Ca^{2+} release. Thus, the interpretation of results of experimental studies with ranolazine and other nonselective inhibitors of late I_{Na} may not be straightforward, and the effect of selective inhibition of late I_{Na} on cardiac electrical function has yet to be clearly demonstrated.

Here we describe a novel late I_{Na} inhibitor, GS-458967 (GS967), and use this agent to demonstrate that selective block of late I_{Na} has anti-arrhythmic actions. In some experiments, the sodium channel toxin *Anemonia sulcata* toxin II (ATX-II) was used to selectively

enhance the Na⁺ channel late current. ATX-II alters Na⁺ channel gating to delay and increase recovery from Na⁺ channel inactivation (Chahine et al., 1996), thereby mimicking the effects of long-QT type 3 mutations (Bennett et al., 1995; Zimmer and Surber, 2008). For comparison with GS967, the drugs flecainide and ranolazine (nonselective inhibitors of both late I_{Na} and I_{Kr}) were used.

MATERIALS AND METHODS

The use of rabbits (New Zealand White adult females, 2-4 kg; Western Oregon Rabbit Company, Philomath, OR) in this investigation conformed to the “*Guide for the Care and Use of Laboratory Animals*” (NIH publication No. 85-23, revised 1996) and was approved by the Institutional Animal Care and Use Committees of Gilead Sciences and Zenas Technologies. The use of the female rabbit is based on previous reports that this is a sensitive model for detection of drug-induced proarrhythmia (Hondeghem et al., 2003), and for preclinical evaluation of new drugs believed to affect cardiac AP repolarization (Lengyel et al, 2001). Late I_{Na} is reported to be greater in myocytes isolated from female than from male mice (Lowe et al., 2012).

Chemicals

GS967 and ranolazine were synthesized at Gilead Sciences. GS967 (mol wt 347) is a triazolopyridine derivative, 6-(4-(trifluoromethoxy)phenyl)-3-(trifluoromethyl)-[1,2,4]triazolo[4,3-a]pyridine (Figure 1). ATX-II, E-4031, and flecainide were purchased from Alomone Labs (Jerusalem, Israel), Tocris (Bristol, UK), and Sigma (St. Louis, MO), respectively. Stock solutions of GS967, flecainide, and ranolazine were prepared and stored in dimethylsulfoxide in glass vials.

Voltage/Current-Clamp Recording of Ion Currents in Rabbit Isolated Ventricular Myocytes

Ventricular myocytes were isolated from the septal portions of hearts of 2-4 kg New Zealand White female rabbits as previously described (Liu et al., 2012). For peak and late I_{Na} , myocytes were depolarized from a holding potential of -120 mV to a test potential of -20 mV for 20 or 220 ms, respectively, at a frequency of 0.1 Hz. ATX-II (10 nM) was used to increase late I_{Na} . For I_{Kr} measurement, myocytes were depolarized from -40 to +20 mV for 3

s, followed by a 2.5-s step to -40 mV to record tail current, at a frequency of 0.1 Hz. I_{Kr} was measured at $36 \pm 1^\circ\text{C}$. All other currents were measured at $22 \pm 1^\circ\text{C}$ (see Supplemental Data).

Rabbit Myocyte Intracellular Na^+ and Ca^{2+} Concentrations

Myocytes were enzymatically isolated from hearts of 2-3 kg adult New Zealand white female rabbits and intracellular Na^+ and Ca^{2+} concentrations were measured using confocal microscopy as previously described (Yao et al., 2011; see Supplemental Data).

Rabbit Isolated Heart Experiments

Hearts were isolated and perfused by the method of Langendorff as previously described (Wu et al., 2009). The atrioventricular nodal area was thermally ablated to produce heart block, and hearts were paced at a rate of 1 Hz. Monophasic APs (MAPs) from the left ventricular epicardium and pseudo 12-lead electrocardiograms (ECGs) were recorded. Following a 10-20 min period of equilibration, hearts were exposed to vehicle (modified Krebs-Henseleit buffer), ATX-II or E-4031, and then to increasing concentrations of either GS967 or ranolazine until a steady-state effect was reached. The duration of the MAP at the level at which repolarization is 90% complete (MAPD_{90}) was measured. The QRS interval was determined from the ECG record.

Rabbit In Vivo Experiments

The effect of GS967 on MAPD_{90} was determined as previously described (Wang et al., 2008). Hearts were atrial-paced beginning at a cycle length of 320 ms. Either vehicle (15% N-methyl-2-pyrrolidone, 10% solutol, and 75% water) or GS967 was then given intravenously as a bolus followed by an infusion at each of 4 dose levels in ascending order: $30 \mu\text{g}/\text{kg} + 8 \mu\text{g}/\text{kg}/\text{min}$, $30 \mu\text{g}/\text{kg} + 16 \mu\text{g}/\text{kg}/\text{min}$, $60 \mu\text{g}/\text{kg} + 32 \mu\text{g}/\text{kg}/\text{min}$ and $120 \mu\text{g}/\text{kg} + 64 \mu\text{g}/\text{kg}/\text{min}$. Each dose was administered for 5 min. After recording the effects of the highest dose of GS967 or vehicle, the cardiac pacing cycle length was decreased in steps,

from 320 to 200 ms, during continued infusion of the highest dose of GS967 or vehicle.

Blood samples were taken at 1 and 5 min after the onset of infusion of each dose of GS967 to determine the plasma drug level.

To determine the effect of GS967 on the inducibility of TdP by clofilium in the presence of methoxamine, rabbits were first treated with either vehicle or GS967 (in randomized manner) given as a 60 $\mu\text{g}/\text{kg}$ bolus followed by a 16 $\mu\text{g}/\text{kg}/\text{min}$ infusion that was maintained for the duration of an experiment. After 10 min, methoxamine was infused intravenously at 15 $\mu\text{g}/\text{kg}/\text{min}$, followed 10 min later by clofilium at 100 $\text{nmol}/\text{kg}/\text{min}$. The incidences of premature ventricular contractions (PVCs), ventricular tachycardia (VT; defined as three or more consecutive abnormal beats), and TdP were determined from the ECG recordings.

Rabbit Heart Ischemia-Induced Arrhythmia Model

Rabbits were anesthetized using pentobarbital (30 mg/kg intravenous bolus followed by infusion at a rate of 15 $\text{mg}/\text{kg}/\text{hr}$). A coronary artery occluder was made by placing a 6-0 Prolene suture around the origin of the left circumflex artery and pulling both ends through 5 cm of PE-10 tubing. Animals were randomly assigned to vehicle, flecainide, ranolazine and GS967 treatment groups. Vehicle or drugs were administered as an intravenous bolus injection followed by an infusion as follows: flecainide, 1 mg/kg + 100 $\mu\text{g}/\text{kg}/\text{min}$; ranolazine, 0.75 mg/kg + 60 $\mu\text{g}/\text{kg}/\text{min}$; GS967, 15 $\mu\text{g}/\text{kg}$ + 4 $\mu\text{g}/\text{kg}/\text{min}$. After 30 min of drug infusion the heart was subjected to 30 min of occlusion of the left circumflex artery. Electrical activity of the heart was monitored continuously for PVCs, VT, and ventricular fibrillation (VF). Blood samples were collected at 1, 5, 10, and 30 min after the onset of drug administration. At the end of an experiment, the heart was removed and the ischemic area at risk was visualized by perfusion with 0.03% Evans Blue dye in saline after ligation of the left circumflex artery. Concentrations of drugs in blood plasma were determined after removal of

protein by use of a high performance liquid chromatograph-tandem mass spectrometric system.

Data Analysis

Data are expressed as mean \pm S.E.M. To determine the 50% effective inhibitory concentration (IC_{50}) for a drug to inhibit an ion channel current, concentration-response data were fitted with the Hill equation. The statistical significance of differences in values before and after interventions in the same hearts was evaluated using repeated-measure one-way analysis of variance followed by a Student-Newman-Keuls test. An unpaired Student *t*-test was used to determine the difference between values of two means obtained from different groups of cells or hearts. One-way analysis of variance with repeated measures, followed by Dunnett's multiple comparison procedure, was used to determine the significance of drug and vehicle effects (at several rates of infusion in each animal) on values of $MAPD_{90}$ recorded from hearts of anesthetized rabbits. Fisher's exact test was used to analyze differences in categorical variables (i.e., the presence or absence of various arrhythmias and mortality). A difference with a *P* value < 0.05 was considered statistically significant.

RESULTS

Effects of GS967, Flecainide, and Ranolazine on Ion Channel Currents

The effects of GS967, flecainide, and ranolazine to reduce peak and late I_{Na} and I_{Kr} in rabbit isolated ventricular myocytes are shown in Figure 2 (panels A-C) and Table 1. The order of potency of the compounds to inhibit ATX-II-stimulated late I_{Na} was GS967 >> flecainide > ranolazine (Table 1). All three compounds selectively inhibited late relative to peak I_{Na} . The ratios of values of IC_{50} for inhibition of peak and late I_{Na} were 78 (i.e., 1329/17 μ M) and 25 (84/3.4 μ M) for ranolazine and flecainide, respectively (Table 1). GS967 caused minimal inhibition ($\leq 7.5\%$) of peak I_{Na} (Figure 2, Table 1) at concentrations up to 10 μ M (the limit of solubility of the agent in aqueous buffer). The ratio of IC_{50} values for inhibition of peak and late I_{Na} by GS967 therefore exceeded 76-fold ($>10/0.13 \mu$ M). To further clarify the effect of GS967 on peak I_{Na} , rabbit ventricular myocytes were paced at rates of 0.1, 1, and 3 Hz and I_{Na} was elicited by depolarization to -20 mV from holding potentials of either -120 mV or -80 mV. GS967 (0.1, 1, and 5 μ M) did not reduce peak I_{Na} in myocytes held at -120 mV and paced at rates of 0.1, 1, or 3 Hz. When myocytes were held at a diastolic potential of -80 mV, however, GS967 concentration-dependently reduced peak I_{Na} at each pacing rate. GS967 at 0.1, 1, and 5 μ M reduced peak I_{Na} by 17 ± 3 , 48 ± 7 , and $65 \pm 4\%$ at a pacing rate of 0.1 Hz, 18 ± 4 , 50 ± 7 , and $67 \pm 4\%$ at a rate of 1 Hz, and 25 ± 5 , 56 ± 8 , and $70 \pm 4\%$ at a rate of 3 Hz, respectively (n=4 for all values). The results suggest that inhibition of peak I_{Na} by GS967 was significantly concentration- and voltage-dependent, but minimally use-dependent. We have previously reported that block by ranolazine of $Nav1.5$ peak I_{Na} is use-dependent (Rajamani et al., 2009).

Ranolazine and flecainide were not selective for inhibition of late I_{Na} relative to I_{Kr} , whereas GS967 caused minimal inhibition of I_{Kr} (Figure 2, panels C, F). The ratios of values of IC_{50} for inhibition of I_{Kr} and late I_{Na} were ≤ 1 , < 1 , and > 76 -fold ($> 10/0.13 \mu$ M) for ranolazine,

flecainide, and GS967, respectively. Representative recordings of effects of GS967 on peak I_{Na} , late I_{Na} , and I_{Kr} are shown in Figure 2, panels D-F. GS967 (3 μ M) had no significant effect on L- or T-type calcium channel currents or Na^+ - Ca^{2+} exchanger current (I_{NCX}), and 1 μ M GS967 had minimal or no effect on the ATP-inhibited K^+ current or on human cardiac ion channels expressed in human embryonic kidney 293 or Chinese hamster ovary cells (Supplemental Figure 1). In addition, assays of effects of GS967 on 162 receptors, ion channels, transporters, and enzymes, and on 442 kinases did not identify any target of the compound at concentrations that are likely to be used for inhibition of cardiac late I_{Na} (i.e., ≤ 1 μ M) (Supplemental Tables 1-3).

Effects of GS967 on the Action Potential of Rabbit Isolated Ventricular Myocytes

GS967 (10, 100, 300 nM; Figure 3) completely attenuated the effect of ATX-II (10 nM) to increase APD and APD variability in ventricular myocytes, with an apparent IC_{50} value of approximately 10 nM (Figure 3F) and decreased the beat-to-beat variability of APD (Figure 3). The effects of 100 nM GS967 mimic the reported effects of 10 μ M ranolazine (Figure 5 in Song et al., 2004).

GS967 (0.1, 1, and 3 μ M) had no significant effect on the resting (diastolic) membrane potential, APD_{50} or APD_{90} of ventricular myocytes stimulated at a rate of 1 Hz, although there was a trend for GS967 to decrease APD (Table 2). GS967 (3 μ M) significantly decreased the upstroke velocity (V_{max}) of the AP whereas lower concentrations of GS967 did not (Table 2). The effect of GS967 (3 μ M) to decrease V_{max} of the AP upstroke was not significantly increased when the frequency of myocyte stimulation was increased from 1 to 2 and 3.3 Hz (data not shown). Flecainide was reported to reduce V_{max} of the AP upstroke in rabbit ventricular myocytes by 52.5% at a concentration of 2.5 μ M in a frequency (use)-dependent manner (Ikeda et al., 1985). Ranolazine was reported to reduce V_{max} of the AP

upstroke in dog Purkinje fibers at concentrations exceeding 10 μM (Antzelevitch et al., 2004).

GS967 Reduced Na^+ and Ca^{2+} Overload in Rabbit Isolated Ventricular Myocytes

GS967 alone (0.1, 0.3 or 1 μM) had no significant effect on either systolic or diastolic Asante NaTRIUM Green or Fluo-4 fluorescence intensity (i.e., Na^+ or Ca^{2+} concentration) in myocytes stimulated at a frequency of 1 Hz (Supplemental Figure 2). ATX-II (5 nM) increased cytosolic concentrations of Na^+ and Ca^{2+} during diastole, increased the duration of the Ca^{2+} transient, and caused loss of rhythmicity (Supplemental Figure 3), consistent with previous findings (Yao et al., 2011). GS967 (0.1 μM) attenuated the ATX-II-induced increases of diastolic Na^+ and Ca^{2+} concentrations by 85 and 82%, respectively, and restored normal rhythmic Ca^{2+} transients (Supplemental Figure 3). Ranolazine similarly attenuated the effects of ATX-II to increase cytosolic Na^+ and Ca^{2+} concentrations and arrhythmic activity (Yao et al., 2011).

Effects of GS967, Flecainide, and Ranolazine on the Rabbit Isolated Heart

Three assays were used to assess block of I_{Kr} , late I_{Na} , and peak I_{Na} by GS967 in the intact heart: the effect of drug to (1) prolong the duration of the left ventricular epicardial MAPD_{90} , (2) shorten the duration of the left ventricular epicardial MAPD_{90} in the presence of the late I_{Na} enhancer ATX-II, and (3) prolong the QRS interval in the ECG, respectively. GS967 at concentrations of 0.01-3 μM did not prolong, but rather shortened MAPD_{90} by $4 \pm 2\%$ from 184 ± 4 to 174 ± 4 ms ($n=6$, $P < 0.05$, Figure 4A). This result is consistent with the finding that GS967 blocked I_{Kr} in rabbit isolated myocytes by only 17% at a concentration of 10 μM and tended to shorten the APD_{90} of the rabbit isolated myocyte. In contrast, both flecainide and ranolazine prolonged MAPD_{90} in a concentration-dependent manner. Flecainide (0.01-30 μM) prolonged MAPD_{90} by $29 \pm 4\%$ from 185 ± 5 to 235 ± 7 ms ($n=7$, $P < 0.01$), whereas

ranolazine (0.1-100 μM) similarly prolonged MAPD_{90} by $28\pm 3\%$ from 185.1 ± 5.7 to 236.9 ± 6.9 ms ($n=11$, $P<0.05$, Figure 4A). These results are consistent with previous reports that flecainide and ranolazine blocked I_{Kr} with IC_{50} values of 2.1 and 12-14 μM , respectively, and increased APD of ventricular myocytes (Follmer et al., 1992; Antzelevitch et al., 2004).

GS967 shortened the duration of the left ventricular epicardial MAPD_{90} in the presence of the late I_{Na} enhancer ATX-II (Figure 4B). ATX-II (3 nM) prolonged MAPD_{90} by $68\pm 11\%$, from 181 ± 7 (control) to 306 ± 28 ms ($n=6$, $P<0.01$) (not shown). GS967 (0.01-3 μM , $n=6$) completely reversed the ATX-II-induced increase in MAPD_{90} with an IC_{50} value of 0.21 μM (Figure 4B), which is consistent with the IC_{50} value of 0.13 μM for GS967 to block late I_{Na} in ventricular myocytes. Flecainide reversed the effect of ATX-II by up to $71\pm 4\%$, with an IC_{50} value of 1.48 μM ($n=5$, Figure 4B). Ranolazine (0.1-100 μM) also shortened MAPD_{90} in the presence of ATX-II by a maximum of $55\pm 9\%$ at 100 μM ($n=6$, Figure 4B), with an IC_{50} value of 15 ± 4 μM ($P<0.001$ compared to GS967). Whereas the antagonism of an ATX-II-induced prolongation of MAPD_{90} by GS967 was complete, those by flecainide and ranolazine were only partial. The efficacies of flecainide and ranolazine to shorten the rabbit epicardial MAPD_{90} in the presence of ATX-II by blocking late I_{Na} may be limited (Figure 4B) by their concomitant effects to block I_{Kr} (which causes an increase of MAPD_{90}).

Neither GS967 (0.01 to 3 μM , $n=7$) nor ranolazine (up to 100 μM , $n=9$) caused a significant change in the QRS interval in hearts paced at a rate of 1 Hz (Figure 4C). In contrast, flecainide ($n=7$) at concentrations of 10 and 30 μM significantly prolonged the QRS interval (Figure 4C), consistent with its effects to block peak I_{Na} in myocytes with an IC_{50} value of 84 μM (Table 1). An increase of the rate of pacing from 1 to 3 and 4 Hz increased the effect of flecainide (2 μM ; $n=4$) but not the effects of ranolazine (10 μM ; $n=4$) or GS967 (3 μM ; $n=4-7$) on the QRS interval (Figure 4D).

GS967 Terminated TdP Induced by ATX-II or E-4031 in the Rabbit Isolated Heart

Enhancement of late I_{Na} with ATX-II or reduction of I_{Kr} with E-4031 prolonged APD and caused TdP-type VT in rabbit isolated hearts (Figure 5). GS967 (0.01-3 μ M) concentration-dependently decreased MAPD₉₀ in the presence of either 3 nM ATX-II (Figure 5A, n=11) or 60 nM E-4031 (Figure 5B, n=6) with IC₅₀ values of 147±22 and 460±56 nM, respectively, and terminated VT (both panels). We have previously demonstrated that ranolazine (5, 10 and 30 μ M) reduces MAPD₉₀ and the incidence of TdP in the rabbit isolated heart treated with 60 nM E-4031 (Wu et al., 2009).

GS967 Decreased MAPD₉₀ but Did Not Alter Cardiac Conduction Time in the Anesthetized Rabbit

GS967 significantly decreased the left ventricular epicardial MAPD₉₀ (relative to vehicle) in hearts paced at a cycle length of 320 ms (Figure 6A). There were no significant changes in either PR or QRS intervals in the ECG at these concentrations of GS967, relative to vehicle (not shown). The PR intervals were 75±5 ms before and during drug administration (n=5 each), the QRS intervals varied from 34±1 to 38±2 ms in both vehicle and GS967-treated hearts (n=4-5), and the duration of the QT interval was 169±9 ms before drug administration and 159±5 ms during administration of 1.46 μ M GS967 (n=5; $P>0.05$). The administration of increasing doses of vehicle alone (n=4 rabbits) caused no significant effect on MAPD₉₀ (not shown). A decrease of the pacing cycle length from 320 to 200 ms in the presence of the highest tested dose of GS967 or vehicle (120 μ g/kg bolus + 64 μ g/kg/min infusion) was associated with a similar trend ($P>0.05$) toward a decrease of MAPD₉₀ in both vehicle and GS967 (1.46 μ M plasma concentration)-treated animals (Figure 6B), suggesting that the magnitude of the effect of GS967 on MAPD₉₀ is not altered by a change in heart rate. We have previously observed that ranolazine (\leq 28 μ M) did not alter the PR or QRS intervals but

prolonged the QT interval in the anesthetized rabbit (Wang et al., 2008). Flecainide is known to increase QRS duration and slow conduction of electrical activity (Hellestrand et al., 1982).

GS967 Prevented the Induction of Arrhythmic Activity and TdP by Clofilium in Anesthetized Rabbits

The induction of TdP in rabbits by administration of the I_{Kr} blocker clofilium in the presence of the α_1 -agonist methoxamine was inhibited by GS967 (Figure 7A). Baseline values of mean arterial blood pressure, heart rate, and ECG intervals (PR, QRS and QT) were 66 ± 1 mmHg, 172 ± 5 beats per min, 76 ± 2 ms, 34 ± 2 ms and 164 ± 4 ms (all $n=12$), respectively, before drug administration. Either vehicle ($n=6$ rabbits) or GS967 ($n=6$ rabbits) was administered as a 60 $\mu\text{g}/\text{kg}$ bolus followed by a 16 $\mu\text{g}/\text{kg}/\text{min}$ infusion. The plasma concentration of GS967 was 0.3 $\mu\text{mol}/\text{L}$ at 5 min after onset of infusion. Neither vehicle nor GS967 alone had any significant effect on measured cardiovascular parameters. Methoxamine was then infused intravenously at a rate of 15 $\mu\text{g}/\text{kg}/\text{min}$. After the administration of methoxamine, no arrhythmias were observed in either vehicle- or GS967-treated animals, suggesting that GS967 does not have a pro-arrhythmic effect. The subsequent administration of clofilium in the continued presence of methoxamine prolonged the QT interval by 63 ± 4 ms from 170 ± 5 to 233 ± 6 ms in vehicle-pretreated rabbits. In the rabbits pretreated with GS967, clofilium-induced QT prolongation was significantly ($P<0.01$) reduced from 63 ± 4 to 46 ± 5 ms (i.e., from 158 ± 4 to 204 ± 6 ms). Clofilium and methoxamine caused PVCs, VT, and TdP in 5 of 6 (83%) rabbits pretreated with vehicle (Figure 7A). Treatment of rabbits with GS967 before the administration of methoxamine and clofilium significantly reduced the incidences of PVCs, VT, and TdP to 2/6, 1/6, and 0/6 rabbits, respectively (Figure 7A). Pretreatment of rabbits with ranolazine (14 and 28 μM) similarly decreased the incidence of TdP caused by methoxamine and clofilium in the anesthetized rabbit (Wang et al., 2008).

GS967 Decreased the Incidence of Ischemia-Induced Arrhythmias in Anesthetized Rabbits

GS967 (0.1-0.2 μM , the approximate IC_{50} value for inhibition of late I_{Na}) suppressed cardiac arrhythmias caused by acute ischemia in the anesthetized rabbit (Figure 7B). Ligation of the left circumflex coronary artery for 30 min resulted in occurrences of VT, VF, and mortality in 5/10, 6/10, and 4/10 vehicle-treated rabbits, respectively (Figure 7B). Treatment of rabbits with flecainide at a therapeutically-relevant plasma concentration of 0.5 μM before and during ischemia caused VT and VF in 7/7 and mortality in 6/7 rabbits (Figure 7B). In rabbits treated with a therapeutically-relevant concentration of ranolazine (4 μM), the incidence of VT (4/9), VF (6/9), and mortality (4/9), was similar to that in vehicle-treated rabbits (Figure 7B). In contrast, in rabbits treated with GS967 the incidences of ischemia-induced VT, VF, and mortality were 2/8, 2/8, and 2/8 rabbits, respectively (Figure 7B). The mean time to occurrence of fatal arrhythmia after ligation of the circumflex coronary artery in vehicle, flecainide, ranolazine and GS967 treatment groups was 16 ± 3 (n=4 deaths), 7 ± 1 (n=6), 11 ± 2 (n=4) and 8 (n=2) min, respectively. The mean size of the ischemic area at risk (30-34% of the left ventricular mass) was not significantly different among treatment groups.

DISCUSSION

The results of this study demonstrate that GS967 is a potent and highly selective blocker of late I_{Na} in female rabbit hearts and isolated ventricular myocytes. Moreover, GS967 was not proarrhythmic, but rather reduced the induction of arrhythmias by an enhancer of late I_{Na} , or by inhibitors of I_{Kr} , or by myocardial ischemia in rabbit hearts *ex vivo* and *in vivo* (Figures 5, 7). The results strongly implicate late I_{Na} as a cause of arrhythmogenesis. Because of its selectivity, GS967 should be a useful “tool” for determination of the roles of late I_{Na} in physiological and pathological processes in cardiac and other excitable cells both *in vitro* and *in vivo*.

The potency of GS967 to inhibit late I_{Na} induced by 10 nM ATX-II in isolated ventricular myocytes was more than 10-fold greater than that of either flecainide or ranolazine: 0.13 ± 0.01 versus 3.4 ± 0.4 and 17.1 ± 1.2 μM , respectively. In intact rabbits, GS967 plasma concentrations of 0.1-0.3 μM were effective to suppress arrhythmic activity induced by clofilium/methoxamine or ischemia (Figure 7). In comparison, ranolazine plasma concentrations of 14-28 μM are required to reduce the incidence of TdP in clofilium/methoxamine-treated rabbits (Wang et al., 2008). GS967 (10 μM) inhibited I_{Kr} and peak I_{Na} by only 17 and 7%, respectively. Consistent with the absence of an effect to block I_{Kr} or peak I_{Na} in myocytes, GS967 did not prolong APD or increase the duration of the QRS interval in either isolated hearts or anesthetized rabbits. In contrast, ranolazine and flecainide increased APD in isolated perfused hearts (Figure 4), and flecainide increased the QRS interval (Figure 4) and mortality during acute myocardial ischemia (Figure 7). Thus, GS967 is a more selective inhibitor of late I_{Na} than either flecainide or the current standard ranolazine, which is reported to inhibit I_{Kr} and blocks α - and β -adrenergic receptors at

concentrations only 2-fold higher than the 2-8 μM used to inhibit late I_{Na} (Antzelevitch et al., 2004; Zhao et al., 2011).

Physiological Importance of Late I_{Na}

The magnitude of endogenous late I_{Na} is small (≤ 60 pA) in the normal heart but its contribution of inward current during the AP plateau is proarrhythmic in the presence of blockers of I_{Kr} (Wu et al., 2009, 2011) (Figure 5B). Due to the high input resistance during the AP plateau even a small inward current, such as late I_{Na} , can contribute significantly to the AP waveform and duration. Although the effect of GS967 caused small but consistent decreases in APD_{90} , MAPD_{90} and the QT interval in isolated myocytes (Table 2) and hearts (Figure 4A), and anesthetized rabbits (Figure 6). The result suggests that endogenous late I_{Na} contributes to action potential duration. In hearts treated with either E-4031 (Figure 5) or clofilium/methoxamine (Figure 7) to reduce I_{Kr} , GS967 shortened MAPD_{90} and prevented (Figure 7) or terminated (Figure 5) the induction of arrhythmic activity. This finding confirms earlier data demonstrating the proarrhythmic role of endogenous late I_{Na} when repolarization reserve is reduced (Wu et al., 2009, 2011). GS967 was more potent and efficacious in reversing the effects of ATX-II than that of E-4031 (Figure 5). An explanation is that in the presence of ATX-II, GS967 inhibits both the endogenous and enhanced late I_{Na} , whereas in the presence of E-4031, GS967 reduces only the endogenous late I_{Na} . Taken together, these findings provide evidence that reduction of endogenous late I_{Na} is beneficial in preventing ventricular arrhythmias when repolarization reserve is reduced.

Pathological Role for late I_{Na}

An enhanced late I_{Na} is pathological because it destabilizes AP repolarization, causes Na^+ -dependent Ca^{2+} overload and intracellular acidosis, and promotes phosphorylation and activation of CaMKII (Yao et al., 2011). The late I_{Na} enhancer ATX-II caused Na^+ and Ca^{2+}

loading of rabbit isolated myocytes (Supplemental Figure 3), markedly prolonged the AP (Figure 3), increased the variability of APD (Figure 3), and caused TdP-like VT (Figure 5A). GS967 (0.1 $\mu\text{mol/L}$) reduced ATX-II-induced late I_{Na} (Figures 2 and 3) and reversed the Na^+ -dependent Ca^{2+} overload (Supplemental Figure 3) and the pro-arrhythmic effects of ATX-II. The present results are consistent with the increasingly recognized roles of an enhanced late I_{Na} to prolong repolarization, to cause Na^+ and Ca^{2+} loading of myocardial cells and consequently electrical dysfunction and arrhythmias (Ver Donck et al., 1993; Haigney et al., 1994; Le Grand et al., 1995; Sicouri et al., 1997; Saint, 2006; Undrovinas and Maltsev, 2008; Wu et al., 2009).

Blockers of Late I_{Na} as Therapeutic Agents: Opportunities and Challenges

The effects of GS967 on rabbit ventricular myocytes, rabbit isolated hearts, and anesthetized rabbits show that the compound reduces the pro-arrhythmic consequences of either enhancing late I_{Na} or inhibiting I_{Kr} . The finding suggests that selective inhibition of late I_{Na} will be effective to increase repolarization reserve and prevent the induction of arrhythmias in the face of various interventions that delay and/or increase the instability of ventricular repolarization in the heart. In failing hearts, late I_{Na} is enhanced, and non-selective inhibitors of the current, albeit not as potent, selective and efficacious as GS967, shorten the APD, improve diastolic function, and suppress arrhythmic activity (Undrovinas et al., 2006, 2008). Inhibition of late I_{Na} has not been shown to have adverse consequences in either normal or diseased hearts. The adverse effects of nonselective inhibitors of late I_{Na} such as flecainide and ranolazine can be attributed to effects to cause use-dependent reduction of peak I_{Na} and/or reduction of I_{Kr} , respectively. The challenges are therefore to develop a selective inhibitor of late I_{Na} with good pharmaceutical properties, and to confirm that inhibition of late I_{Na} is indeed safe and beneficial therapeutically. The potent effect of GS967 to block late I_{Na} ,

reduction by the compound of peak I_{Na} in a voltage- but not a use-dependent manner, and its effect to decrease arrhythmic activity in the ischemic rabbit heart suggest that the compound may be beneficial in the setting of myocardial ischemia.

Limitations

The effects of GS967, ranolazine and flecainide to reduce arrhythmic activity induced by ischemia in the anesthetized rabbit were determined using single doses of each agent. These doses produced therapeutically-relevant plasma concentrations of ranolazine and flecainide, and an IC_{50} concentration of GS967 to reduce late I_{Na} . However, it is possible that improved efficacy could be demonstrated using different dosing regimens. Although ranolazine (4 μ M) did not reduce the arrhythmic activity induced by ischemia in the present study, it was reported to do so in a study of anesthetized rats (Dhalla et al., 2009). The different results may reflect either a species or a methodological (i.e., 5 versus 30 min duration of ischemia) difference between the studies. Additional studies are also needed to confirm the high potency of GS967 that is reported in this study of rabbit heart tissues. Although we have tested for effects of GS967 on hundreds of potential targets, further testing is nonetheless needed (e.g., the effect of GS967 on ryanodine receptors). This testing should include preparations of “remodeled” as well as normal cardiac tissues.

Conclusion

In conclusion the results demonstrate GS967 is a selective inhibitor of late I_{Na} . GS967 shortened the duration of the ventricular action potential and reduced the proarrhythmic effects of ATX-II, ischemia, and I_{Kr} block. GS967 was not proarrhythmic. The results provide compelling evidence of the major contribution of late I_{Na} to repolarization reserve and genesis of cardiac arrhythmias and point to a potential important contribution of this current to Na^+ -dependent intracellular Ca^{2+} homeostasis.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Belardinelli, Shryock, Rajamani, Dhalla, Wu, Smith-Maxwell, Crumb

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Contributed new reagents or analytic tools: Koltun, Zablocki

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Wrote or contributed to the writing of the manuscript: Shryock, Belardinelli, Rajamani, Dhalla, Smith-Maxwell, Crumb, Yao

REFERENCES

- Antzelevitch C, Belardinelli L, Zygmunt AC, Burashnikov A, Di Diego JM, Fish JM, Cordero JM, Thomas G (2004) Electrophysiological effects of ranolazine, a novel antianginal agent with antiarrhythmic properties. *Circulation* **110**:904-910.
- Bennett PB, Yazawa K, Makita N, George Jr. AL (1995) Molecular mechanism for an inherited cardiac arrhythmia. *Nature* **376**:683-685.
- Boutjdir M, El-Sherif N (1991) Pharmacological evaluation of early afterdepolarizations induced by sea anemone toxin (ATX II) in dog heart. *Cardiovasc Res* **25**:815-819.
- Chahine M, Plante E, Kallen RG (1996) Sea anemone toxin (ATX II) modulation of heart and skeletal muscle sodium channel alpha-subunits expressed in tsA201 cells. *J Membr Biol* **152**:39-48.
- Dhalla AR, Wang WQ, Dow J, Shryock JC, Belardinelli L, Bhandari A, Kloner RA (2009). Ranolazine, an antianginal agent, markedly reduces ventricular arrhythmias induced by ischemia and ischemia-reperfusion. *Am J Physiol* **297**:H1923-1929.
- Follmer CH, Cullinan CA, Colatsky TJ (1992) Differential block of cardiac delayed rectifier current by class Ic antiarrhythmic drugs: evidence for open channel block and unblock. *Cardiovasc Res* **26**:1121-1130.

Galimberti ES, Knollmann BC (2011) Efficacy and potency of class I antiarrhythmic drugs for suppression of Ca²⁺ waves in permeabilized myocytes lacking calsequestrin. *J Mol Cell Cardiol* **51**: 760-768.

Haigney MCP, Lakatta EG, Stern MD, Silverman HS (1994) Sodium channel blockade reduces hypoxic sodium loading and sodium-dependent calcium loading. *Circulation* **90**:391-399.

Heinemann SH, Terlau H, Imoto K (1992) Molecular basis for pharmacological differences between brain and cardiac sodium channels. *Pflugers Arch* **422**:90-92.

Hellestrand KJ, Bexton RS, Nathan AW, Spurrell RA, Camm AJ (1982) Acute electrophysiological effects of flecainide acetate on cardiac conduction and refractoriness in man. *Br Heart J* **48**, 140-148.

Hondeghem LM, Lu HR, van Rossem K, De Clerck F (2003) Detection of proarrhythmia in the female rabbit heart: blinded validation. *J Cardiovasc Electrophysiol* **14**:287-294.

Ikeda N, Singh BN, Davis LD, Hauswirth O (1985) Effects of flecainide on the electrophysiological properties of isolated canine and rabbit myocardial fibers. *J Am Coll Cardiol* **5**:303-310.

January CT, Riddle JM (1989) Early afterdepolarizations: mechanism of induction and block. A role for L-type Ca²⁺ current. *Circ Res* **64**:977-990.

Ju YK, Saint DA, Gage PW (1996) Hypoxia increases persistent sodium current in rat ventricular myocytes. *J Physiol (Lond)* **497**:3337-347.

Le Grand B, Vie B, Talmant JM, Coraboeuf E, John GW (1995) Alleviation of contractile dysfunction in ischemic hearts by slowly inactivating Na⁺ current blockers. *Am J Physiol* **269**:H533-540.

Le Grand B, Pignier C, Letienne R, Cuisiat F, Rolland F, Mas A, Vacher B (2008) Sodium late current blockers in ischemia reperfusion: Is the bullet magic? *J Med Chem* **51**:3856-3866.

Lengyel C, Iost N, Virag L, Varro A, Lathrop DA, Papp JG (2001) Pharmacological block of the slow component of the outward delayed rectifier current (I_{Ks}) fails to lengthen rabbit ventricular muscle QTc and action potential duration. *Br J Pharmacol* **132**:101-110.

Liu GX, Choi BR, Ziv O, Li W, de Lange E, Qu Z, Koren G (2012) Differential conditions for early after-depolarizations and triggered activity in cardiomyocytes derived from LQT1 and LQT2 rabbits. *J Physiol* **590**:1171-80.

Lowe JS, Stroud DM, Yang T, Hall L, Atack TC, Roden DM (2012) Increased late sodium current contributes to long QT-related arrhythmia susceptibility in female mice. *Cardiovasc Res* **95**:300-307.

Maltsev VA, Sabbah HN, Higgins RSD, Silverman N, Lesch M, Undrovinas AI (1998)

Novel, ultraslow inactivating sodium current in human ventricular cardiomyocytes.

Circulation **98**:2545-2552.

Maltsev VA, Undrovinas AI (2006) A multi-modal composition of the late Na⁺ current in

human ventricular myocytes. *Cardiovasc Res* **69**:116-127.

Parikh A, Mantravadi R, Kozhevnikov D, Roche MA, Ye Y, Owen LJ, Puglisi JL, Abramson

JJ, Salama G (2012) Ranolazine stabilizes cardiac ryanodine receptors: a novel mechanism

for the suppression of early afterdepolarization and torsade de pointes in long QT type 2.

Heart Rhythm **9**:953-960.

Rajamani S, El-Bizri N, Shryock JC, Makielski JC, Belardinelli L (2009) Use-dependent

block of cardiac late Na⁺ current by ranolazine. *Heart Rhythm* **6**:1625-1631.

Saint DA (2006) The cardiac persistent sodium current: an appealing therapeutic target? *Br J*

Pharmacol **153**:1133-1142.

Sicouri S, Antzelevitch D, Heilmann C, Antzelevitch C (1997) Effects of sodium channel

block with mexiletine to reverse action potential prolongation in in vitro models of the long

term QT syndrome. *J Cardiovasc Electrophysiol* **8**:1280-1290.

Song Y, Shryock JC, Wu L, Belardinelli L (2004) Antagonism by ranolazine of the pro-

arrhythmic effects of increasing late I_{Na} in guinea pig ventricular myocytes. *J Cardiovasc*

Pharmacol **44**:192-199.

Song Y, Shryock JC, Belardinelli L (2008) An increase of late sodium current induces delayed afterdepolarizations and sustained triggered activity in atrial myocytes. *Am J Physiol Heart Circ Physiol* **294**:H2031-2039.

Song Y, Shryock JC, Wagner S, Maier, LS, Belardinelli L (2006) Blocking late sodium current reduces hydrogen peroxide-induced arrhythmogenic activity and contractile dysfunction. *J Pharmacol Exp Ther* **318**:214-222.

Sossalla S, Kallmeyer B, Wagner S, Mazur M, Maurer U, Toischer K, Schmitto JD, Seipelt R, Schondube FA, Hasenfuss G, Belardinelli L, Maier LS (2010) Altered Na⁺ currents in atrial fibrillation. Effects of ranolazine on arrhythmias and contractility in human atrial myocardium. *J Am Coll Cardiol* **55**:2330-2342.

Undrovinas AI, Belardinelli L, Undrovinas NA, Sabbah HN (2006) Ranolazine improves abnormal repolarization and contraction in left ventricular myocytes of dogs with heart failure by inhibiting late sodium current. *J Cardiovasc Electrophysiol* **17**: Suppl 1:S169-S177.

Undrovinas A, Maltsev VA (2008) Late sodium current is a new therapeutic target to improve contractility and rhythm in failing heart. *Cardiovasc Hematol Agents Med Chem* **6**:348-359.

Ver Donck L, Borgers M, Verdonck F (1993) Inhibition of sodium and calcium overload pathology in the myocardium: A new cytoprotective principle. *Cardiovasc Res* **27**:349-357.

Wang Q, Shen J, Splawski I, Atkinson D, Li Z, Robinson JL, Moss AJ, Towbin JA, Keating MT (1995) *SCN5A* mutations associated with an inherited cardiac arrhythmia, long QT syndrome. *Cell* **80**:805-811.

Wang W-Q, Robertson C, Dhalla AK, Belardinelli L (2008) Antitortadogenic effects of (\pm)-N-(2,6-dimethyl-phenyl)-(4[2-hydroxy-3-(2-methoxyphenoxy)propyl]-1-piperazine (Ranolazine) in anesthetized rabbits. *J Pharmacol Exp Ther* **325**:875-881.

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 I_{Kr} inhibition on ventricular repolarization. *Circulation* **123**:1713-1720.

Wu L, Rajamani S, Li H, January CT, Shryock JC, Belardinelli L (2009) Reduction of repolarization reserve unmasks the proarrhythmic role of endogenous late Na^+ current in the heart. *Am J Physiol Heart Circ Physiol* **297**:H1048-1057.

Yao L, Fan P, Jiang Z, Viatchenko-Karpinski S, Wu Y, Kornyejev D, Hirakawa R, Budas GR, Rajamani S, Shryock JC, Belardinelli L (2011) $Na_v1.5$ -dependent persistent Na^+ influx activates CAMKII in rat ventricular myocytes and $N_{1325}S$ mice. *Am J Physiol Cell Physiol* **301**:C577-C586.

Zaza A, Belardinelli L, Shryock JC (2008) Pathophysiology and pharmacology of the cardiac “late sodium current”. *Pharmacology & Therapeutics* **119**:326-339.

Zhao G, Walsh E, Shryock JC, Messina E, Wu Y, Zeng D, Xu X, Ochoa M, Baker SP, Hintze TH, Belardinelli L (2011) Antiadrenergic and hemodynamic effects of ranolazine in conscious dogs. *J Cardiovasc Pharmacol* **57**:639-647.

Zimmer T, Surber R (2008) SCN5A channelopathies—an update on mutations and mechanisms. *Prog Biophys Molec Biol* **98**:120-136.

FOOTNOTES

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FIGURE LEGENDS

Figure 1. Chemical structure of GS967

Figure 2. Concentration-response relationships for flecainide (**A**), ranolazine (**B**), and GS967 (**C**) to inhibit peak I_{Na} (filled squares), ATX-II-enhanced late I_{Na} (filled circles) and I_{Kr} (open triangles) in rabbit ventricular myocytes. Examples of effects of GS967 on peak I_{Na} , late I_{Na} , and I_{Kr} are shown in panels **D**, **E**, and **F**, respectively. Symbols represent mean \pm SEM of data from the number of myocytes indicated in Table 1.

Figure 3. GS967 (100 and 300 nM) attenuated the effects of ATX-II (10 nM) to increase action potential duration (APD, panels **A-D**) and beat-to-beat variability of APD (panel **E**) in rabbit ventricular myocytes at $36\pm 1^\circ\text{C}$. Results of a representative experiment showing APs recorded in the absence of drug (control), in the presence of 10 nM ATX-II, and after additions of 100 and 300 nM GS967 in the continued presence of ATX-II are shown in panels **A-D**. The durations of 10 consecutive APs recorded during each period shown in panels **A-D** are depicted in panel **E**. Data ($n=5$ myocytes) for GS967 (10, 100, 300 nM) to decrease APD from upstroke to 90% of repolarization (APD_{90}) in the presence of 10 nM ATX-II are summarized in panel **F**. ATX-II alone increased APD_{90} compared to ctrl (control; no drug) by $390\pm 30\%$. †, $P<0.05$ compared to control. *, $P<0.05$ compared to ATX-II.

Figure 4. Concentration-response relationships for GS967 (circles), flecainide (diamonds), and ranolazine (squares) to: **A**, increase duration of the left ventricular epicardial monophasic action potential (MAPD_{90}); **B**, shorten MAPD_{90} in the presence of ATX-II (3 nM); and **C**, increase the QRS interval, in rabbit isolated hearts. **Panel D.** Effects of ranolazine (10 μM), flecainide (2 μM), and GS967 (3 μM) on the duration of the QRS interval at pacing rates of

1, 3, and 4 Hz. The number of hearts used in each assay is stated in the text. *, $P < 0.05$ compared to no drug.

Figure 5. Effects of GS967 to shorten the prolonged monophasic action potential (MAP) and to suppress torsades de pointes (TdP) ventricular tachyarrhythmia induced by either 3 nM ATX-II (**A**) or 60 nM E-4031 (**B**) in the rabbit isolated heart. Top panels: concentration-response relationships for GS967 to shorten the MAPD₉₀ and to reduce the incidence of TdP during exposure of hearts to either ATX-II (**A**) or E-4031 (**B**). **Bottom traces (a-d) in both panels:** representative sequential recordings of the left ventricular epicardial MAP (top record in each panel) and the pseudo-electrocardiogram (bottom record in each panel) during exposure of a heart to perfusate alone (ctrl), ATX-II (**A**) or E-4031 (**B**), and GS967 in the continued presence of either ATX-II or E-4031. Hearts were paced at a rate of 1 Hz. *, $P < 0.05$ compared to either ATX-II or E-4031 alone. Numbers in parenthesis represent hearts with VT/total number of hearts studied.

Figure 6. Plasma concentration-response relationship for GS967 to decrease the duration of the left ventricular epicardial monophasic action potential (MAPD₉₀), and rate-dependence of effect of GS967, in the anesthetized, open-chest rabbit. **A**, summary of values of MAPD₉₀ recorded before (0 drug) and 5 min after intravenous administration of increasing doses of GS967. Mean (n=5 rabbits) plasma concentrations of GS967 at 5 min after the onset of infusion of each of the 4 doses were 0.14, 0.33, 0.72, and 1.46 μM . ***significantly different from the pre-drug value, $P < 0.001$. **B**, Effect of an increase of atrial pacing rate (reduced cycle length) on MAPD₉₀ in the presence of vehicle (n=4 rabbits, open circles) or 1.46 μM GS967 (n=5, filled circles).

Figure 7. Effects of GS967 to suppress ventricular arrhythmias in the anesthetized rabbit induced by inhibition of I_{Kr} (**A**) or acute ischemia (**B**). **A**, incidence of premature ventricular

contractions (PVCs), ventricular tachycardia (VT), and torsades de pointes (TdP) in rabbits exposed to methoxamine and clofilium in the presence of either vehicle (n=6; open bars) or GS967 (plasma concentration of 0.3 μ M; n=6, filled bars). * P <0.05 and ** P <0.01 versus vehicle treatment. **B**, incidence of VT, ventricular fibrillation (VF), and mortality in rabbits treated with vehicle (n=10, open bars), flecainide (0.5 μ M; n=7, black bars), ranolazine (4 μ M; n=9, striped bars), and GS967 (0.1-0.2 μ M; n=8, checkered bars) during occlusion of the left circumflex coronary artery for 30 min or until the occurrence of fatal arrhythmias.

TABLES

Table 1. Potencies (IC₅₀ values, mean±S.E.M.) of drugs to inhibit ion channel currents

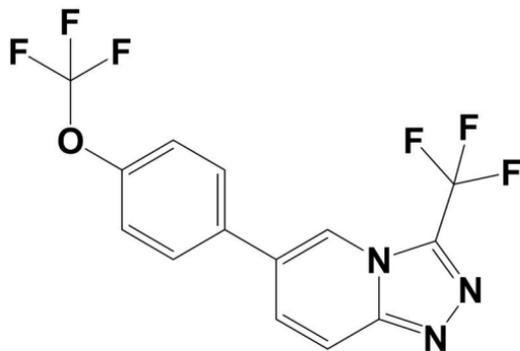
Drug/cmpd	Peak I_{Na}	Late I_{Na}	I_{Kr}
Flecainide	84±4 μM (n=20)	3.4±0.5 μM (n=23)	1.5±0.1 μM (n=21)
Ranolazine	1329±144 μM (n=23)	17±1 μM (n=23)	13±1 μM (n=21)
GS967	7.5% @10 μM (n=4)	0.13±0.01 μM (n=19)	16.7% @10 μM (n=6)

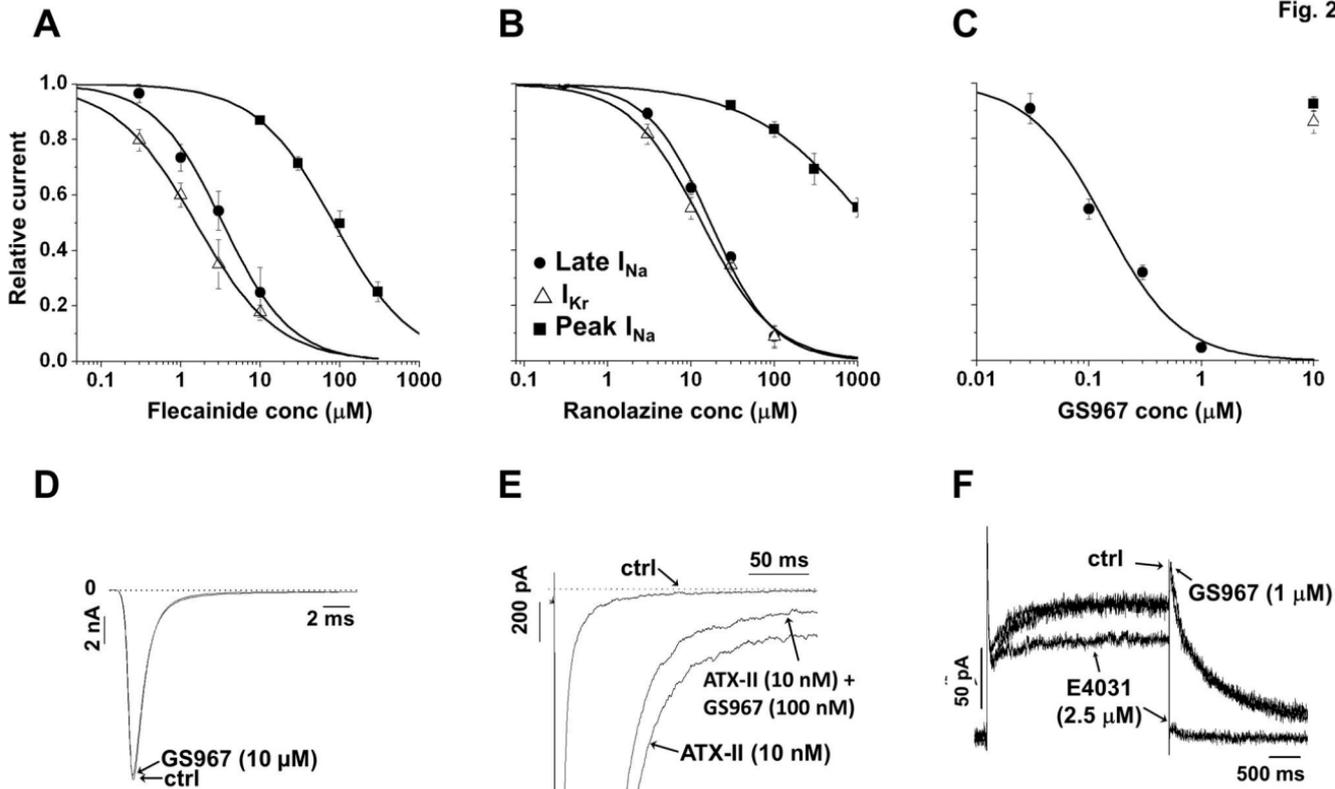
Table 2. Effect of GS967 on rabbit myocyte resting membrane potential (RMP), peak rate of voltage change during the AP upstroke (V_{max}), and action potential duration (APD).

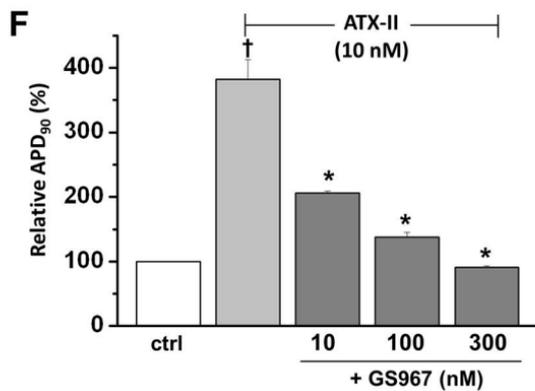
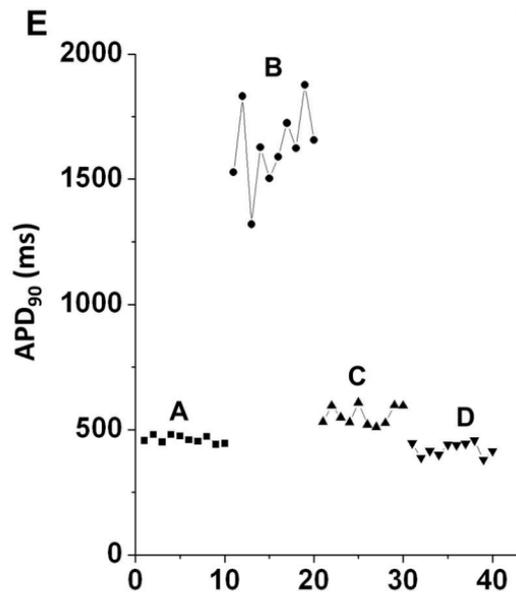
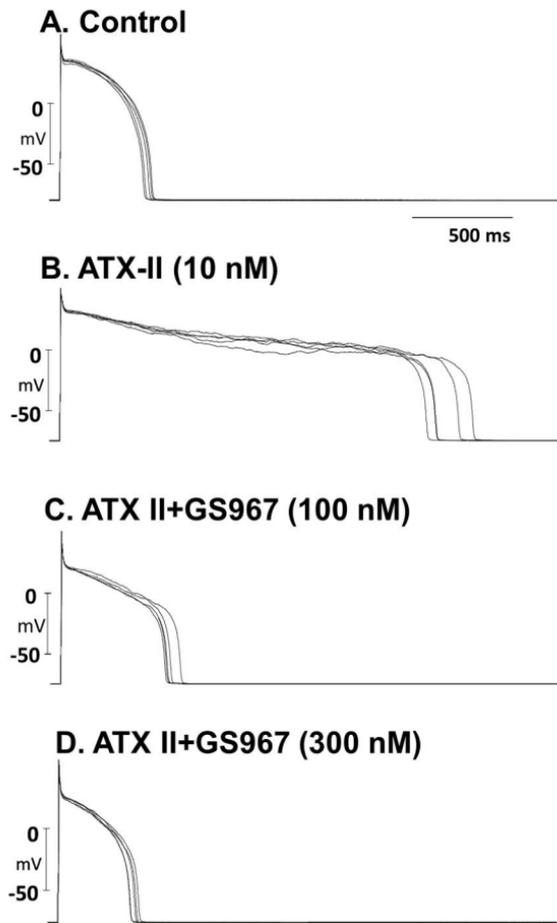
GS967 (μ M)	RMP		V_{max}		APD ₅₀		APD ₉₀	
	mV	(%)	V/s	(%)	ms	(%)	ms	(%)
0	-82.8 \pm 0.7		309.0 \pm 11.4		147.2 \pm 13.6		184.3 \pm 13.1	
0.1	-83.1 \pm 0.5	(0.3)	304.4 \pm 12.5	(-1.6)	143.1 \pm 13.1	(-2.7)	179.8 \pm 13.1	(-2.5)
1	-82.7 \pm 0.9	(-0.8)	293.9 \pm 11.2	(-4.9)	138.1 \pm 11.1	(-5.9)	172.3 \pm 13.1	(-6.6)
3	-83.2 \pm 0.3	(0.5)	272.0 \pm 10.7	(-12.0)	126.2 \pm 10.5	(-14.1)	162.8 \pm 12.5	(-11.8)

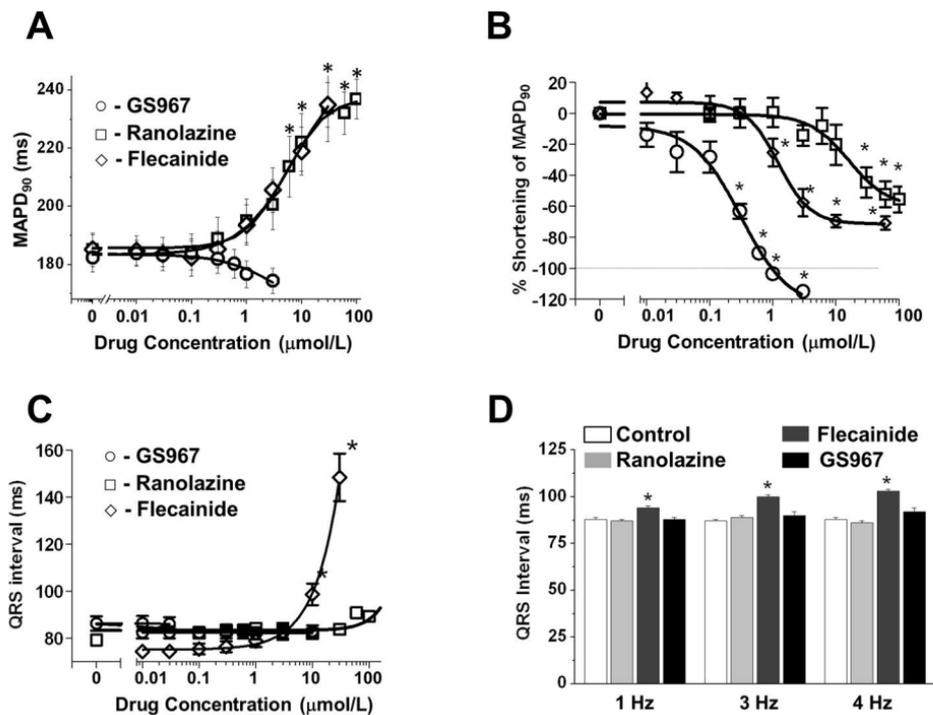
Values are mean \pm S.E.M., n=4. (%) is percentage change from control (0 drug).

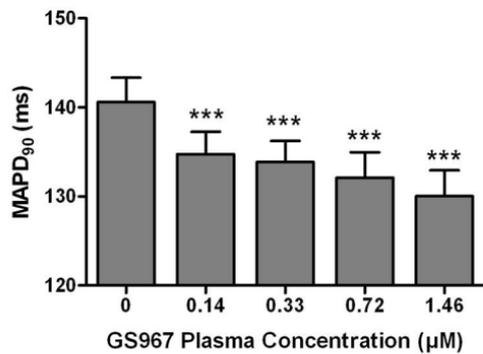
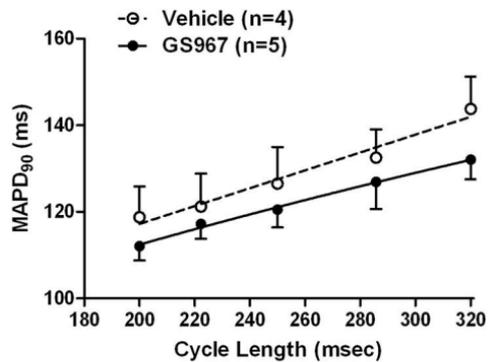
Experiments were done at 36 \pm 1°C.

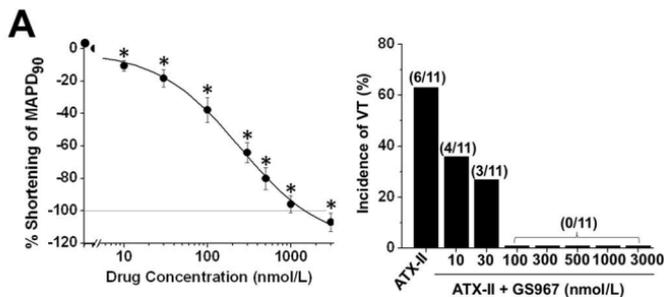




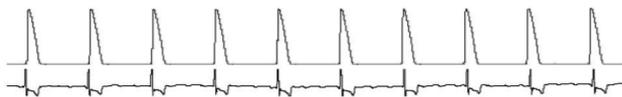




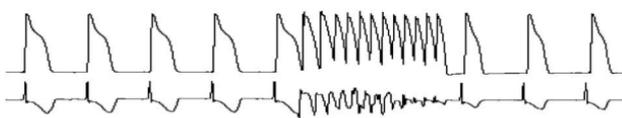
A**B**



a. ctrl



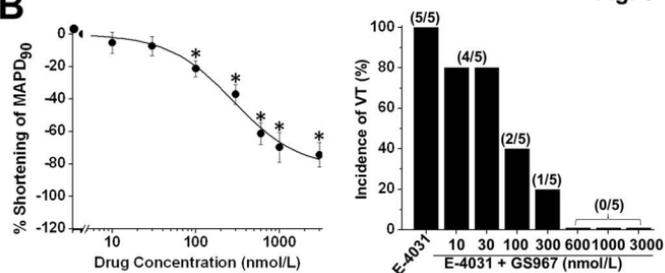
b. ATX-II (3 nM)



c. ATX-II+GS967 (30 nM)



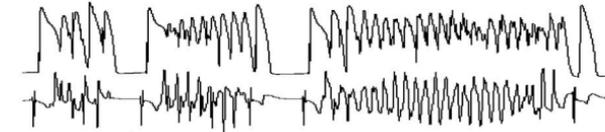
d. ATX-II+GS967 (100 nM)

**B**

a. ctrl



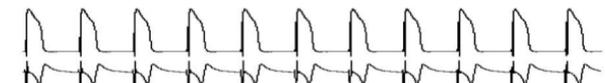
b. E-4031 (60 nM)

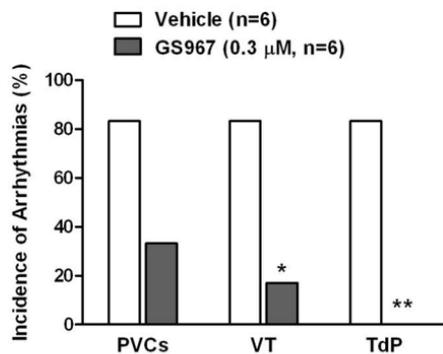
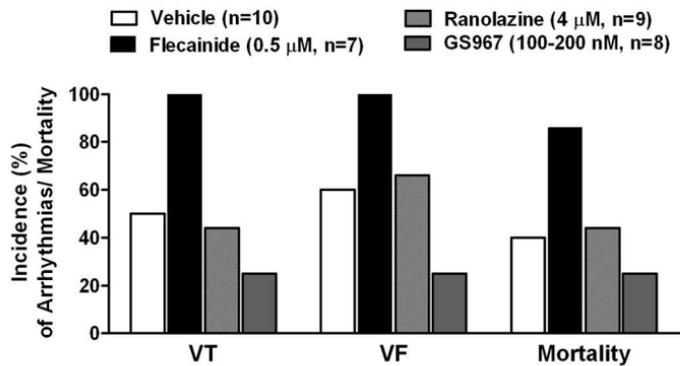


c. E-4031+GS967 (100 nM)



d. E-4031+GS967 (1 μM)



A**B**

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

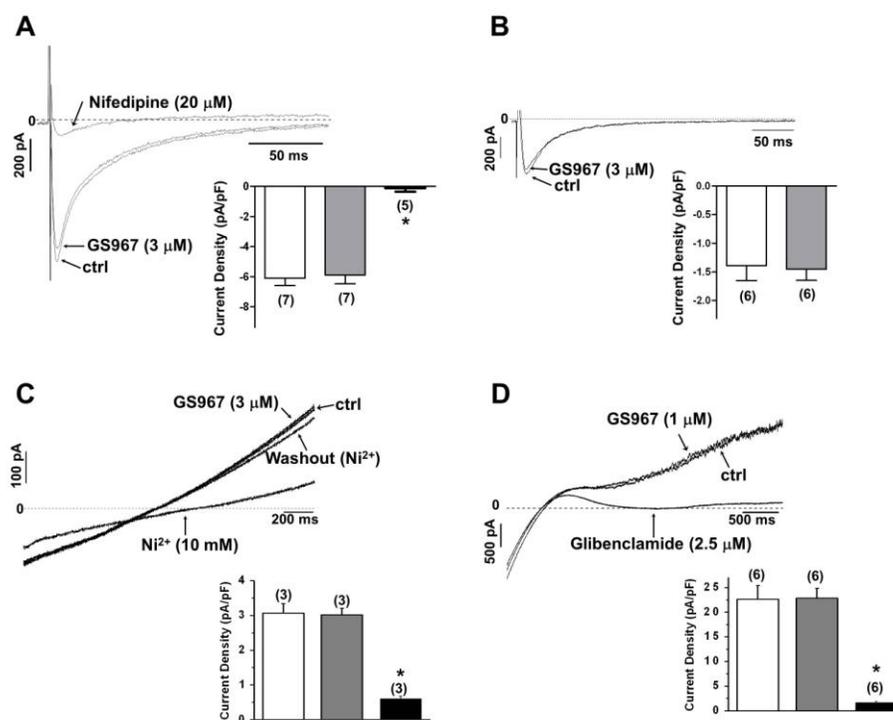
Methods: L-type calcium current (I_{CaL}) was measured using the amphotericin-B perforated patch technique. I_{CaL} was activated by applying 360-ms pulses to +10 mV every 15 s from a holding potential of -80 mV. A preconditioning pulse to -40 mV for 300 ms was used to inactivate I_{Na} and I_{CaT} . 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 $\mu\text{g/mL}$ 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 M Ω . Myocytes were continuously superfused with a 22 ± 1 °C 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 °C 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 M Ω 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 $\text{Na}^+/\text{Ca}^{2+}$ 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 $36 \pm 1^\circ\text{C}$ 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 $\mu\text{mol/L}$), Ouabain (10 $\mu\text{mol/L}$), nifedipine (10 $\mu\text{mol/L}$) and ryonadine (10 $\mu\text{mol/L}$) 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 \pm S.E.M.

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

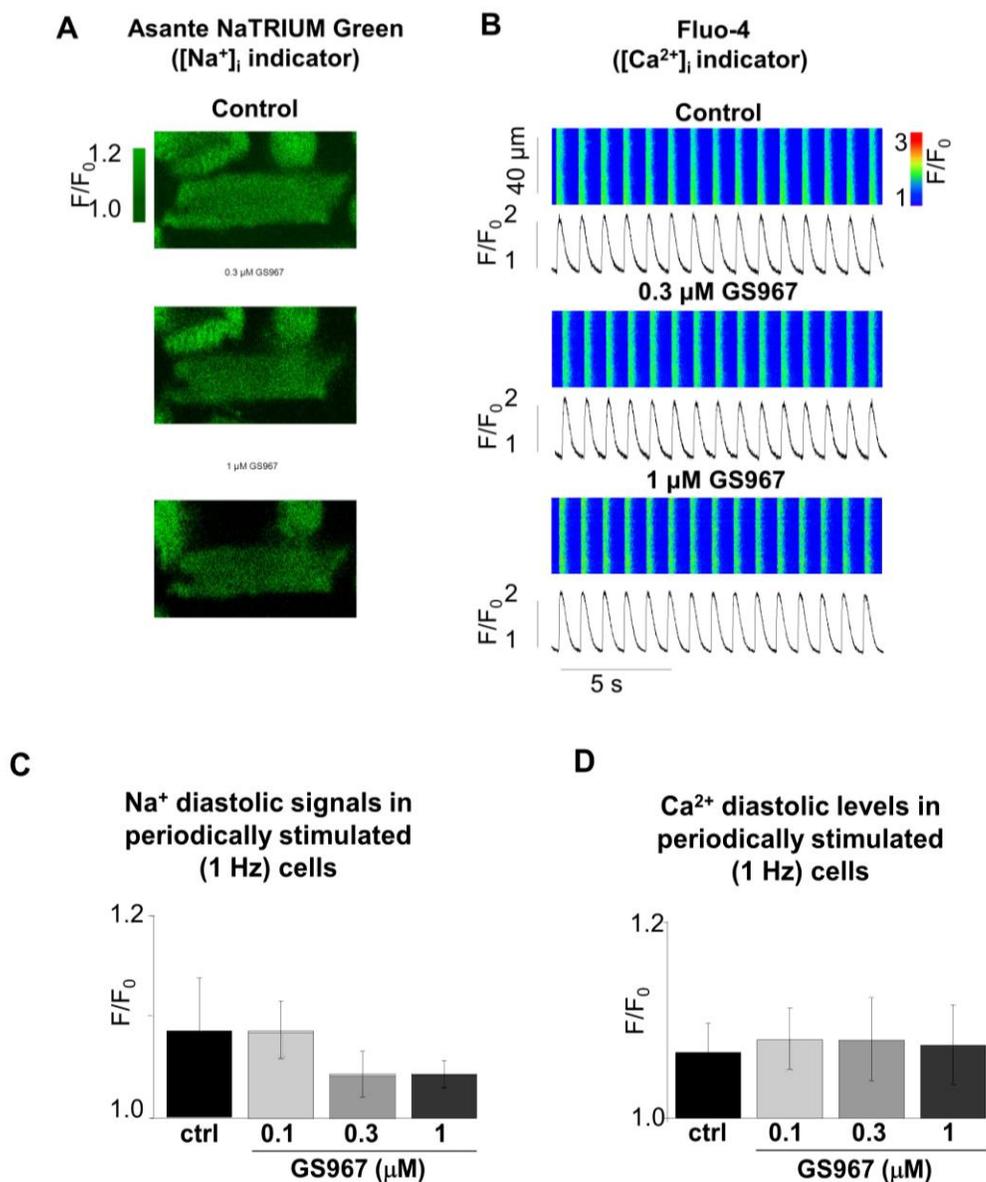
Methods: New Zealand White adult female rabbits were anesthetized and the heart was excised. Left ventricular tissue samples were quickly immersed in a cardioplegia solution consisting of (mM): 50 KH_2PO_4 , 8 MgSO_4 , 10 NaHCO_3 , 5 adenosine, 25 taurine, 140 glucose, and 100 mannitol, pH 7.4, and bubbled 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 Kraftbruehe 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 °C using intracellular microelectrodes with resistances ranging from 25 to 40 $\text{M}\Omega$ 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

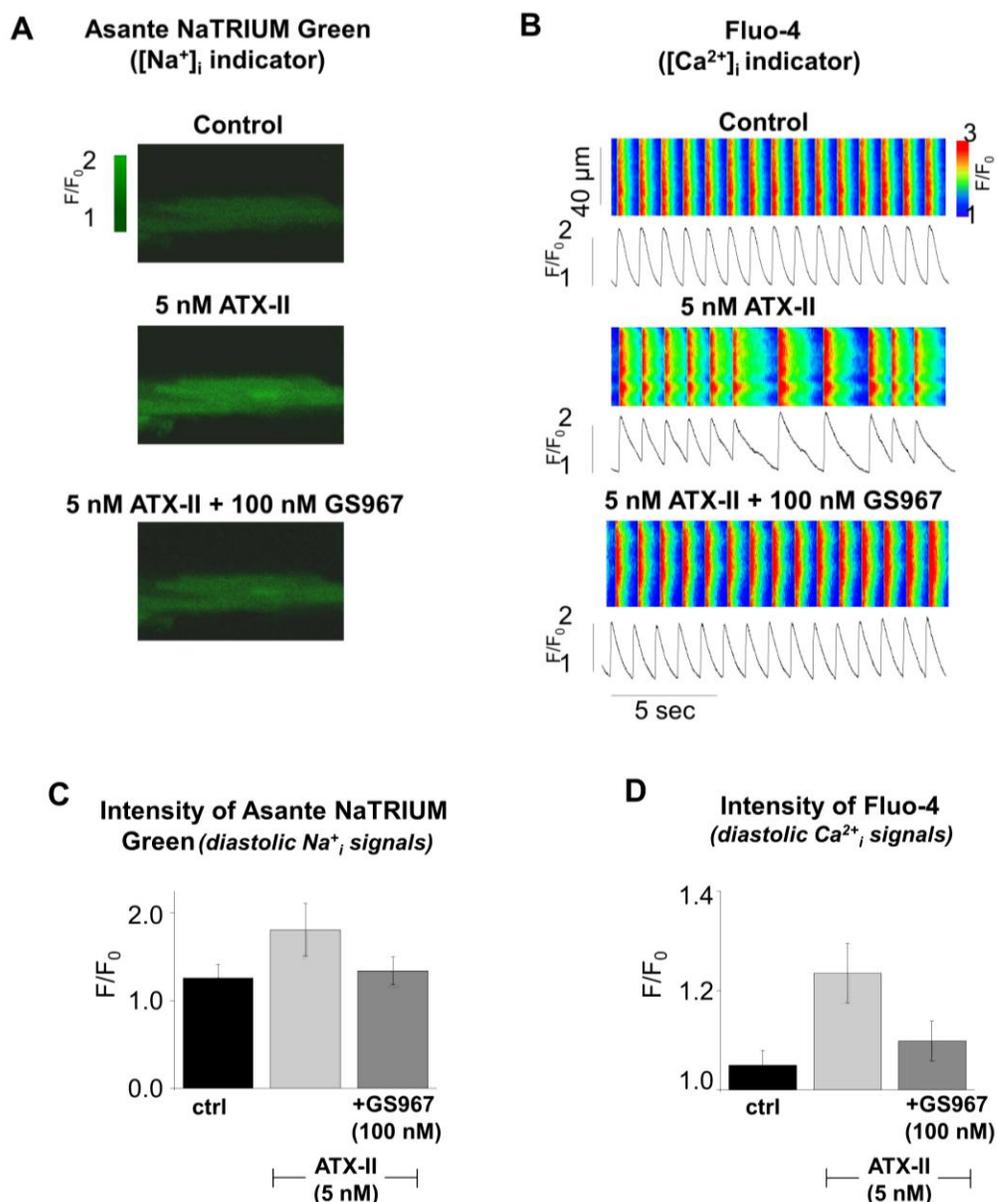
Ca^{2+} indicator Fluo-4AM (Invitrogen, Carlsbad, CA) or 5 μM of the Na^+ indicator Asante NaTRIUM Green AM (TEFLabs, Austin, TX). Fluorescence intensity was measured as F/F_0 , where F is the dynamically-changing intensity of the fluorescent dye signal and F_0 is the fluorescence intensity in non-stimulated 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 \pm 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 °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} ($K_{ir2.1}$) 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} ($K_{ir3.1/3.4}$) 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} ($K_{ir6.2/SUR2A}$) 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 μ M 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 μ M 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 μ M) 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 μ M) of human ion channels heterologously-expressed in human embryonic kidney (HEK) 293 or Chinese hamster ovary (CHO) cells.

Cardiac Ion Channels	Cardiac Currents	1 μ M % Block	n	Cell Line	Subunits	
					Type	Gene(s)
Na _v 1.5 late	Late I _{Na}	90.0 \pm 1.6	5	HEK293	α	SCN5A
Na _v 1.5 peak	Peak I _{Na}	16.3 \pm 3.5	10	HEK293	α	SCN5A
Ca _v 1.2	I _{CaL}	9.7 \pm 7.7	4	CHO	α , β 2, α 2 δ	hCACNA1C, hCACNB2, hCACNA2D1
Ca _v 3.2	I _{CaT}	14.6 \pm 2.7	4	HEK293	α	hCACNA1H
HCN2	I _f	-0.5 \pm 3.0	4	CHO	α	hHCN2
HCN4	I _f	-1.1 \pm 3.9	4	HEK293	α	hHCN4
K _{ir} 2.1	I _{K1}	2.6 \pm 2.7	4	HEK293	α	hKCNJ2
K _{ir} 3.1/3.4	I _{KAch}	2.9 \pm 4.4	4	HEK293	α , α	hKCNJ3, hKCNJ5
K _{ir} 6.2/SUR2A	I _{KATP}	0.5 \pm 3.8	4	HEK293	α , β	hKCNJ11, SUR2A
K _v 1.5	I _{kur}	2.0 \pm 2.4	4	CHO	α	hKCNA5
K _v 4.3	I _{to}	6.1 \pm 5.4	4	HEK293	α	hKCND3
K _v LQT/minK	I _{Ks}	4.0 \pm 2.3	4	CHO	α , β	hKCNQ1, hKCNE1
hERG	I _{Kr}	22.7 \pm 3.4	4	HEK293	α	hKCNH2

Values of % block are mean \pm S.D. of current recorded from “n” cells in the presence of 1 μ M 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 _v 1.2	HB-PS	137	0	4	1.8	1	10	10
Ca _v 3.2	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 _v 1.5	HB-PS	137	0	4	1.8	1	10	10
K _v 4.3	HB-PS	137	0	4	1.8	1	10	10
K _v LQT1/MinK	HB-PS	137	0	4	1.8	1	10	10
Na _v 1.5	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 ₃ VO ₄	Na ₄ P ₇ O ₂	GTP	HEPES
Ca _v 1.2	130	0	0	5	0	10	4	0	0	0	0	0.5	10
Ca _v 3.2	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 _v 2.1	0	0	130	5	0	5	4	0	0	0	0	0	10
hK _v 6.2/ SUR2A	0	0	130	5	0	5	4	0	0	0	0	0	10
hK _v 3.1/3.4	0	0	130	5	0	5	4	0	5	1	1	0.1	10
hK _v 1.5	0	0	130	5	0	5	4	0	0	0	0	0	10
hK _v 4.3	0	0	130	5	0	5	4	0	0	0	0	0	10
K _v LQT1/ MinK	0	0	130	5	1	5	4	0	0	0	0	0	10
Na _v 1.5	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 μ M) 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 μ M) also inhibited picrotoxin binding to the GABA-gated chloride channel by 46%, and nitrendipine binding to the L-type Ca²⁺ 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 μ M GS967 on 442 kinases, a single hit – MARK4 – was identified as more than “minimal”. In a subsequent follow-up study the IC₅₀ value for GS967 inhibition of MARK4 was found to be >30 μ M (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.