Effects of a Highly Selective Acetylcholine-Activated K⁺ Channel Blocker on Experimental Atrial Fibrillation

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Background—The acetylcholine-activated K⁺ current (I\textsubscript{\text{K,ACH}}) is a novel candidate for atrial-specific antiarrhythmic therapy. The present study investigates the involvement of I\textsubscript{\text{K,ACH}} in atrial fibrillation (AF) using NTC-801, a novel potent and selective I\textsubscript{\text{K,ACH}} blocker.

Methods and Results—The effects of NTC-801, substituted 4-(aralkylamino)-2,2-dimethyl-3,4-dihydro-2H-benzopyran-3-ol, on I\textsubscript{\text{K,ACH}} and other cardiac ionic currents (I\textsubscript{Na}, I\textsubscript{CaL}, I\textsubscript{to}, I\textsubscript{Kur}, I\textsubscript{Kr}, I\textsubscript{KATP}, and I\textsubscript{l}) and on atrial and ventricular action potentials were examined in vitro. NTC-801 potently inhibited carbachol-induced I\textsubscript{\text{K,ACH}} in guinea pig atrial cells and the GIRK1/4 current in Xenopus oocytes with IC\textsubscript{50} values of 5.7 and 0.70 nmol/L, respectively. NTC-801 selectively inhibited I\textsubscript{\text{K,ACH}} >1000-fold over other cardiac ionic currents. NTC-801 (10 to 100 nmol/L) reversed the action potential duration (APD\textsubscript{90}) shortened by carbachol or adenosine in atrial cells, whereas it did not affect APD\textsubscript{90} at 100 nmol/L in ventricular cells. Antiarrhythmic effects of NTC-801 were evaluated in 3 AF models in vivo. NTC-801 significantly prolonged atrial effective refractory period without affecting ventricular effective refractory period under vagal nerve stimulation. NTC-801 dose-dependently converted AF to normal sinus rhythm in both vagal nerve stimulation–induced (0.3 to 3 μg · kg\textsuperscript{-1} · min\textsuperscript{-1} IV) and aconitine-induced (0.01 to 0.1 mg/kg IV) models. In a rapid atrial pacing model, NTC-801 (3 μg · kg\textsuperscript{-1} · min\textsuperscript{-1} IV) significantly decreased AF inducibility with a prolonged atrial effective refractory period that was frequency-independent.

Conclusions—A selective I\textsubscript{\text{K,ACH}} blockade induced by NTC-801 exerted anti-AF effects mediated by atrial-selective effective refractory period prolongation. These findings suggest that I\textsubscript{\text{K,ACH}} may be important in the development and maintenance of AF. (Circ Arrhythm Electrophysiol. 2011;4:94-102.)

Key Words: acetylcholine ▪ antiarrhythmia agents ▪ ion channels ▪ potassium ▪ remodeling

Atrial fibrillation (AF) is the most common arrhythmia in clinical practice, and its occurrence increases with age. It is associated with increased cardiovascular morbidity and mortality and adversely affects the quality of life.\textsuperscript{1-3} Many randomized clinical trials have examined whether reestablishment and maintenance of sinus rhythm or control of heart rate alone is more effective in the management of AF. However, clinical trials have not yet shown that rhythm control is any better than rate control in decreasing the mortality and morbidity rates of patients with AF.\textsuperscript{4,5} One possible explanation for the ineffectiveness of rhythm control is that the proarrhythmic risk and toxic effects associated with currently available antiarrhythmic drugs adversely affect outcomes. Innovative approaches have been applied to discover an ideal antiarrhythmic drug that can effectively and safely terminate and prevent AF recurrence. An antiarrhythmic drug with selective affinity for the ion channels that are specifically involved in atrial repolarization would be an ideal candidate.\textsuperscript{6,7}

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Considerable attention has recently focused on acetylcholine (ACh)-activated K⁺ (K\textsubscript{ACH}) channels that are important for atrial, but not ventricular repolarization and in AF susceptibility.\textsuperscript{8,9} Atrial tachypacing in dogs produces electric remodeling in which the density of the ACh-activated K⁺ current (I\textsubscript{\text{K,ACH}}) in atrial cells increases, even in the absence of a muscarinic agonist.\textsuperscript{10} In addition, I\textsubscript{\text{K,ACH}} is constitutively active in atrial cardiomyocytes from patients with long-term...
AF.\textsuperscript{11} Paroxysmal AF occurring at night, at rest, and/or after consuming meals or alcohol is caused at least partly by $K_{ACh}$ channel activation.\textsuperscript{12} However, the contribution of $K_{ACh}$ channel activation to the development and maintenance of AF in patients remains unresolved because a highly selective $K_{ACh}$ channel blocker is not clinically available. Hence, we synthesized the benzopyrane derivative NTC-801 for application as a clinically oriented specific $K_{ACh}$ channel blocker. We show that NTC-801 is a highly selective $K_{ACh}$ channel blocker that effectively treated experimental AF in vitro and in vivo.

Methods
All experiments were performed under the regulations of the Animal Research Committee of Chiba University Graduate School of Medicine or those of the Animal Care and Use Committee of Teijin Institute for Bio-Medical Research.

Effects of NTC-801 on $I_{K,ACh}$ in Guinea Pig Atrial Cells
Whole-cell membrane currents were recorded from single atrial/ventricular cells of the guinea pig heart isolated by enzymatic dissociation,\textsuperscript{13} using the patch-clamp method.\textsuperscript{14} Detailed methods for atrial cell isolation and patch-clamp are described in the online-only Data Supplement.

Effects of NTC-801 on GIRK1/4 and GIRK1/2 Channels Expressed in Xenopus Oocytes
Oocytes were surgically removed from Xenopus laevis under anesthesia in iced water. Stage V and VI oocytes were injected with 5 ng each of GIRK1 and GIRK4 or GIRK1 and GIRK2 cRNA. Membrane currents were then recorded from the oocytes using the conventional 2-microelectrode voltage-clamp technique. Detailed methods are described in the online-only Data Supplement.

Effects of NTC-801 on 9 Cardiac Ionic Currents
Using human embryonic kidney (HEK) cell lines, Chinese hamster ovary (CHO) cell lines that expressed 1 of the human channels, or isolated ventricular myocytes of guinea pig or rat, effects of NTC-801 on 9 ionic currents of heart cells ($I_{Na}$, $I_{Cat}$, $I_{K1}$, $I_{K2}$, $I_{K3}$, $I_{K4}$, $I_{KATP}$, and $I_{Kr}$) were examined by the whole-cell patch-clamp technique. Detailed methods are described in the online-only Data Supplement.

Effects of NTC-801 on Action Potential in Guinea Pig Atrial/Ventricular Cells
Action potentials were recorded in the current clamp mode of patch-clamp in single atrial/ventricular cells isolated from guinea pig hearts.\textsuperscript{15} Detailed methods are described in the online-only Data Supplement.

Effects of NTC-801 on Vagal Nerve Stimulation–Induced AF in Dogs
Sixty-two male mongrel dogs weighing 19 to 25 kg were used. Vagal nerve stimulation (VNS)-induced AF models were prepared according to the procedure described previously.\textsuperscript{16} Under bilateral VNS, AF was induced by burst atrial stimulation with 4-fold diastolic threshold current. The incidence of AF termination within 15 minutes after the start of NTC-801 (0.3, 1, and 3 $\mu$g $\cdot$ kg$^{-1}$ $\cdot$ min$^{-1}$) was also measured. Detailed methods are described in the online-only Data Supplement.

Effects of NTC-801 on Atrial and Ventricular Effective Refractory Period and Intra-Atrial Conduction Time Under VNS
Twenty-six female beagle dogs weighing 10 to 12 kg were used. The surgical procedure was essentially the same as that of VNS-induced AF. Under bilateral VNS, effective refractory period (ERP) was determined using train 10 basic (S1) stimuli followed by a premature (S2) stimulus at a basic cycle length of 300 ms. The ERP was defined as the longest S1-S2 interval failing a propagated response. The conduction time from the low right atrium to the atrial appendage was also determined. The ERP and conduction time were measured before and after NTC-801 (0.3, 1, and 3 $\mu$g $\cdot$ kg$^{-1}$ $\cdot$ min$^{-1}$) or vehicle (saline) infusion for 15 minutes. The change of ERP before and after the infusion was analyzed. Detailed methods are described in the online-only Data Supplement.

Effects of NTC-801 on Aconitine-Induced AF in Dogs
Sixteen male beagle dogs weighing 9 to 13 kg were used. Aconitine-induced AF models were prepared according to the procedure described previously.\textsuperscript{15} AF was induced by a topical application of aconitine (0.1 mg/body) on the right appendage. NTC-801 (0.01, 0.03, and 0.1 mg/kg) or vehicle (10% DMSO-PEG200) was intravenously administered as a bolus injection and then the incidence of AF termination was evaluated within 10 minutes. Detailed methods are described in the online-only Data Supplement.

Blood Pressure, Heart Rate, ECG Parameters, and Arrhythmia Assessment in Conscious Dogs
These protocols are provided in the online-only Data Supplement.

Drugs
The following drugs were used: NTC-801 (substituted 4-(arylalkylamino)-2,2-dimethyl-3,4-dihydro-benzopyran-3-ol, Nissan Chemical Industries, Ltd, Tokyo, Japan), carbachol chloride (Tokyo Chemical Industry Co, Ltd, Tokyo, Japan), teratipin (Peptide Institute, Inc, Osaka, Japan), atenolol (Wako Pure Chemical Industries, Ltd, Osaka, Japan), and atropine sulfate monohydrate and aconitine (Sigma Chemical Co, Ltd, St Louis, MO). The compositions of the various buffer solutions and pipette solutions are described in the online-only Data Supplement.

Statistics
All data are expressed as mean±SEM, and nonparametric tests were used throughout the study except for the incidence of AF termination. For 2-group comparisons, the Wilcoxon test or Wilcoxon signed-rank test was used. For multiple comparisons, the Kruskal-Wallis test followed by a nonparametric-type Dunnott (joint ranking) test was used to compare each dose group versus a vehicle-treated group. For evaluation of the differences in the incidence of AF, the Fisher exact test was used. All data were statistically analyzed using SAS software Windows version, Release 8.2 (SAS Institute Inc, Cary, NC). Differences with a probability value of $<0.05$ were considered significant. The concentration-response data were fitted, and $IC_{50}$ values were obtained using GraphPad PRISM software version 4.01 (GraphPad, La Jolla, CA).

Results
Effects of NTC-801 on $I_{K,ACh}$ in Guinea Pig Atrial Cells
NTC-801 concentration-dependently inhibited carbachol-induced $I_{K,ACh}$ in isolated guinea pig atrial cells (Figure 1A, 1B, and 1G). Because adenosine can also induce $I_{K,ACh}$...
through activating pertussis toxin–sensitive GTP-binding protein in atrial cells,17 we evaluated the effects of NTC-801 on adenosine-induced I
\textsubscript{K,ACh}. NTC-801 also inhibited the adenosine-induced current to the same extent as it inhibited the carbachol-induced current (Figure 1C, 1D, and 1G). We then examined the effects of NTC-801 on I
\textsubscript{K,ACh} induced by intracellular loading with the nonhydrolysable GTP analog GTP\textsubscript{\gamma}S. NTC-801 also concentration-dependently inhibited the current induced by GTP\textsubscript{\gamma}S (Figure 1E, 1F, and 1G), and the concentration-response curve was superimposable on those of carbachol- and adenosine-induced I
\textsubscript{K,ACh}. The IC\textsubscript{50} values of NTC-801 for the inhibition of carbachol-, adenosine-, and GTP\textsubscript{\gamma}S-induced currents were 5.7, 6.2, and 5.7 nmol/L, respectively (Figure 1G). NTC-801 inhibited I
\textsubscript{K,ACh} with equal potency irrespective of the activation method.

Effects of NTC-801 on GIRK1/4 and GIRK1/2 Channels Expressed in Xenopus Oocytes
We examined the effects of NTC-801 on human K\textsubscript{ACh} channels and clarified channel selectivity among GIRK subtypes by comparing the effects of NTC-801 with those of tertiapin on GIRK1/4 and GIRK1/2 channel currents in the Xenopus oocyte expression system. NTC-801 concentration-dependently inhibited the GIRK1/4 current with an IC\textsubscript{50} of 0.70 nmol/L (Figure 2A, 2B, 2C, and Figure 3A). Tertiapin likewise inhibited the GIRK1/4 current with an IC\textsubscript{50} of 0.41 nmol/L (Figure 2G, 2H, 2I, and 3B). NTC-801 inhibited the GIRK1/2 current less potently with an IC\textsubscript{50} of 24 nmol/L (Figure 2D, 2E, 2F, and 3A), whereas the potency of tertiapin inhibition (IC\textsubscript{50}=0.45 nmol/L) was similar to that on the GIRK1/4 current (Figure 2J, 2K, 2L, and 3B). Thus, NTC-801 inhibition was 34-fold more selective for the GIRK1/4.
than the GIRK1/2 current, whereas tertiapin inhibition was almost equivalent between the two.

**Effects of NTC-801 on 9 Ionic Currents of Heart Cells**

NTC-801 inhibited $I_{\text{Na}}, I_{\text{to}},$ and $I_{\text{KATP}}$ with IC$_{50}$ values of 8.3, 11.6, and 7.8 mmol/L, respectively, and all the other ionic currents <50% with IC$_{50}$ values of >30 mmol/L (Table and online-only Data Supplement Figure 1). Thus, the IC$_{50}$ ratios for all other currents to that of $I_{\text{K,ACh}}$ were >1000-fold.

**Effects of NTC-801 on Action Potentials in Guinea Pig Atrial/Ventricular Cells**

We examined the effects of NTC-801 on atrial and ventricular action potentials as well as on carbachol- or adenosine-induced action potential shortening in atrial cells. Figure 4A through 4F shows that NTC-801 at 100 nmol/L did not affect action potential duration (APD$_{90}$) in atrial cells in the absence of agonists (Figure 4A and 4D) and concentration-dependently reversed the action potential shortening induced by 1 μmol/L of carbachol or 10 μmol/L of adenosine (Figure 4B, 4C, 4E, and 4F). Figure 4G and 4H shows that NTC-801 at 100 nmol/L did not affect APD$_{90}$ in ventricular cells. In addition, NTC-801 up to 30 μmol/L did not affect $dV/dt$ max or action potential duration in guinea pig papillary muscles (online-only Data Supplement Table 1). NTC-801 did not affect resting membrane potentials in atrial and ventricular cells or in papillary muscles (online-only Data Supplement Table 1).

**Effects of NTC-801 on VNS- and Aconitine-Induced AF in Dogs**

We evaluated the effects of NTC-801 on AF induced by VNS and aconitine. NTC-801 (0.3, 1, and 3 μg · kg$^{-1}$ · min$^{-1}$)
dose-dependently converted AF to sinus rhythm in the VNS-induced AF model (Figure 5A and 5C). The highest dose of NTC-801 restored sinus rhythm in all of 8 dogs, and the effect was statistically significant ($P=0.0014$) compared with the vehicle-treated group (1 of 8 dogs). The mean plasma concentration at the time of AF termination by NTC-801 at 3 μg·kg$^{-1}$·min$^{-1}$ was about 50 nmol/L.

NTC-801 (0.01, 0.03, and 0.1 mg/kg) also dose-dependently converted AF to sinus rhythm in the aconitine-induced AF model (Figure 5B and 5D). The rates of AF inducibility under VNS to determine the mechanism of its anti-AF properties. NTC-801 (0.3, 1, and 3 μg·kg$^{-1}$·min$^{-1}$) dose-dependently converted AF to sinus rhythm in the VNS-administered dogs at doses of 1 μg·kg$^{-1}$·min$^{-1}$ ($P=0.0204$) and 3 μg·kg$^{-1}$·min$^{-1}$ ($P=0.0006$). The intra-atrial conduction time after NTC-801 administration did not change in any group (data not shown).

Effects of NTC-801 on ECg Parameters in Conscious Dogs

We recorded ECG traces from conscious dogs. Oral administration of NTC-801 (0.1, 1, and 3 mg/kg) did not appreciably affect ECG parameters including QT interval (online-only Data Supplement Figure 3 and online-only Data Supplement Table 2).

Discussion

Main Findings

NTC-801 potently inhibited carbachol-induced $I_{K_{ACh}}$ in guinea pig atrial cells and the GIRK1/4 current in Xenopus oocytes expressing human GIRK1/4 channels. The inhibitory activity of NTC-801 on $I_{K_{ACh}}$ and the GIRK1/4 current was essentially equivalent to that of tertiapin. NTC-801 also selectively inhibited $I_{K_{ACh}}$ over other cardiomyocyte currents ($I_{Na}$, $I_{CaL}$, $I_{Ito}$, $I_{Ks}$, $I_{KvLQT1}$, $I_{Kr}$, $I_{KATP}$, and $I_{f}$), with a margin of >1000-fold. To the best of our knowledge, this is the first synthetic compound to selectively inhibit $I_{K_{ACh}}$. These selectivity data are consistent with the findings that NTC-801 hardly affected $I_{K_{f}}$ and $I_{K_{s}}$ in guinea pig atrial cells as well as dV/dt max and APD$_{90}$ in guinea pig papillary muscle (online-only Data Supplement Figure 2 and online-only Data Supplement Table 1). We also confirmed that NTC-801 did not affect the QRS and QTc in a conscious dog (online-only Data Supplement Table 2), which reflected the outcomes in vitro.

NTC-801 similarly inhibited $I_{K_{ACh}}$ induced by carbachol, adenosine, and GTPγS in guinea pig atrial cells. The electro-
physiological experiments using guinea pig atrial cells showed that NTC-801 also reversed the action potential shortening induced by carbachol or adenosine within a similar concentration-response range. These results indicate that the site of action by this compound is the K_{ACh} channel itself and/or G-protein. This concept is supported by the finding that NTC-801 at 10 \mu mol/L did not bind to M_{2} or A_{1} receptors in pharmacological profiling assays (online-only Data Supplement). Furthermore, the effects of NTC-801 on GIRK1/4 and GIRK1/2 currents differed in Xenopus oocytes. Because the GIRK overexpression systems were essentially identical between the GIRK1/4 and GIRK1/2 experiments, the effects would have been similar had NTC-801 acted on G-protein. Thus, NTC-801 probably acts directly on GIRK1/4 channels rather than on G-protein. Tertiapin inhibited both GIRK currents with equivalent potency, whereas NTC-801 inhibited GIRK1/4 current 34-fold more potently than the GIRK1/2 current. This GIRK channel selectivity indicates that NTC-801 should have high therapeutic value as an antiarrhythmic drug because GIRK1/2 channels are mainly expressed in the central nervous system.\textsuperscript{18,19} Hence, NTC-801 at therapeutic concentrations is less likely to exert neurological disturbances such as seizures that are mediated by GIRK1/2 current inhibition in the central nervous system.\textsuperscript{20}

We investigated the antiarrhythmic potential of a selective I_{K,ACh} blockade induced by NTC-801 in vivo using VNS- and aconitine-induced models as well as the RAP-induced model that simulates AF associated with electrical remodeling. Our findings in the VNS-induced model are consistent with a previous report indicating that a selective I_{K,ACh} blockade produces AF termination and atrial-selective ERP prolongation without affecting ventricular ERP or intra-atrial conduction time under VNS.\textsuperscript{21} These results demonstrated the crucial concept of the "atrial-selectivity" of I_{K,ACh} blocker.

We generated an ectopic automaticity–induced AF model using aconitine. Whereas class I antiarrhythmic drugs terminate AF in this model, the present study demonstrated that a selective blocker of I_{K,ACh} can also terminate AF. These results are consistent with the finding of a previous report showing the effectiveness of an antiarrhythmic drug with
I_{K_{ACH}}-blocking action against aconitine-induced AF.\textsuperscript{21} Aconitine is considered to inhibit Na\textsuperscript{+}/H\textsuperscript{+} channel inactivation,\textsuperscript{22} and resultant intracellular Na\textsuperscript{+} accumulation might increase K_{ACH} channel activity.\textsuperscript{23,24} Hence, the inhibitory effects of NTC-801 on I_{K_{ACH}}, activated by intracellular Na\textsuperscript{+} accumulation due to aconitine, might contribute to the AF termination. It is commonly assumed that AF induced by K_{ACH} channel activation resulting from muscarinic stimulation is an exclusive model of reentry, whereas aconitine-induced AF represents a model of atrial ectopy/automaticity. However, the results of this study that aconitine-induced AF can be inhibited by a highly-selective K_{ACH} channel blocker clearly indicate that K_{ACH} channel activation is important for atrial arrhythmias that are maintained by both reentry and ectopic mechanisms.

A constitutively active (independent of vagal influence) form of K_{ACH} channels has been identified in atrial cardiomyocytes from patients with chronic AF\textsuperscript{11} and dog atrial tissues after atrial tachypacing.\textsuperscript{10} Rapid atrial pacing causes electrical remodeling in the atrium, generates an arrhythmogenic substrate, and induces AF with a burst of atrial stimulation.\textsuperscript{25,26} Tertiapin suppresses AF in atrial preparations after atrial tachypacing,\textsuperscript{10} but whether a selective I_{K_{ACH}} blockade can result in antiarrhythmic effects in vivo has not been determined. We evaluated the effects of NTC-801 on AF inducibility in the RAP-induced AF model to address this issue. NTC-801 significantly decreased AF inducibility with atrial ERP prolongation, irrespective of stimulation frequency. This is the first report to show that a selective I_{K_{ACH}} blocker can exert antiarrhythmic effects on AF associated with electrical remodeling in vivo. The ERP prolongation was notably enhanced in the RAP (about 40 ms) compared with the VNS (about 30 ms) model after NTC-801 (3 μg · kg\textsuperscript{-1} · min\textsuperscript{-1})

Figure 5. Effects of NTC-801 on VNS-induced AF (A) and aconitine-induced AF (B). Data are expressed as means. Values in parentheses indicate numbers of animals per group. *P<0.05, **P<0.01 versus vehicle-treated group (Fisher exact test). Representative examples of AF termination by NTC-801 in VNS-induced (C) and aconitine-induced (D) AF are shown.

Figure 6. Effects of NTC-801 on AF inducibility (A) and atrial ERP (B) in a RAP model of AF. For measurement of AF inducibility, a burst of atrial stimulation was applied 10 times and AF inducibility was calculated. Atrial ERP was measured at different cycle lengths (basic cycle lengths of 300, 250, and 200 ms). Rapid atrial pacing for 3 to 4 weeks increased AF inducibility (C) and decreased ERP (D), suggesting electric remodeling. Data are expressed as mean±SEM. Values in parentheses indicate numbers of animals per group. *P<0.05, ***P<0.001 versus before treatment/pacing (A, C, and D, Wilcoxon signed-rank test). **P<0.01 versus vehicle-treated group (B, Wilcoxon test).
administration, suggesting that $I_{K,ACH}$ channels play a pathophysiological role in AF associated with electrical remodeling.

The present results differ from those of other class III antiarrhythmics ($I_{Kr}$ blockers), of which the antiarrhythmic effects are diminished in AF with electrical remodeling.16,27 Constitutively active $I_{K,ACH}$ is increased, whereas $I_{Kr}$ density is decreased, leading to a triangulated and shortened action potential in the electrically remodeled atrium.16 NTC-801 prolonged ERP independent of frequency, whereas $I_{Kr}$ blockers do so dependently on reverse frequency, which blunts the effects of $I_{Kr}$ blockers under tachyarrhythmias. Thus, AF might be more effectively treated using blockers of $I_{K,ACH}$ than of $I_{Kr}$.

Potential Clinical Implications and Study Limitations

Considerable clinical attention has recently focused on $I_{K,ACH}$ because of the involvement in atrial but not ventricular repolarization and in AF susceptibility.9 Atrial tachypacing in dogs results in electrical remodeling with an increased density of $I_{K,ACH}$ that is active in atrial cells even in the absence of muscarinic agonists.10 In addition, atrial cardiomyocytes from patients with chronic AF show constitutively active $I_{K,ACH}$ channels,11 although patients with paroxysmal AF probably do not have constitutively active $I_{K,ACH}$ channels.28 It is assumed that paroxysmal AF occurring at night, at rest, and/or after consuming meals or alcohol is caused at least partly by the activation of ligand-operated $I_{K,ACH}$ channels.12 However, the contribution of $I_{K,ACH}$ activation to the development and maintenance of AF in patients remains unknown because a highly selective $I_{K,ACH}$ blocker has not been clinically available. Here, we demonstrated that NTC-801 should be a useful pharmacological tool with which to investigate the $I_{K,ACH}$ contribution to AF and that it shows promise as a new treatment for AF.

The most recent data demonstrated the unequal distribution of inward rectifier $K^+$ currents in atria of patients with paroxysmal AF.29 The left-to-right gradient in inward rectifier background current is suggested to contribute to the left-to-right atrium dominant frequency gradients in paroxysmal AF patients, accelerating frequency and stability of reentry-promoting rotors. The NTC-801–mediated inhibition of $I_{K,ACH}$ might modify not only atrial refractoriness but also AF-promoting reentrant sources in the experimental models described.

The disadvantage of attempts at rhythm control using existing antiarrhythmic drugs comprise limited efficacy and a significant risk of adverse effects, such as proarrhythmia and reduced cardiac function.6,7 The present study showed that a selective $I_{K,ACH}$ blockade with NTC-801 has little effect on membrane currents and action potentials in ventricular cells, which is reflected by the absence of QRS and QTc changes in vivo. These results indicate that a selective $I_{K,ACH}$ blocker is unlikely to produce adverse ventricular effects.

We do not fully understand either the pathophysiological role of $I_{K,ACH}$ in AF or its relevance between humans and dogs, although $I_{K,ACH}$ plays key roles in the human and canine atrium. As with all animal experiments, whether the efficacy of NTC-801 in dogs can be directly extrapolated to humans remains unknown. From this perspective, NTC-801 will require clinical assessment to verify the importance of $I_{K,ACH}$ channels and to ascertain the therapeutic utility of a selective $I_{K,ACH}$ blocker. Since the phase I study has been completed, clinical proof of concept for this compound will be clarified soon.

Conclusions

The present study demonstrated that a highly selective $I_{K,ACH}$ blockade with NTC-801 prolongs atrial-selective ERP, decreases AF inducibility in an AF model with electrical remodeling, and converts AF to normal sinus rhythm in vagally induced and ectopic automaticity–induced AF models. These findings suggest that $I_{K,ACH}$ may be important in the development and maintenance of AF.

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Disclosures

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References


**CLINICAL PERSPECTIVE**

Atrial fibrillation (AF) is the most common arrhythmia in clinical practice. Innovative approaches have been applied to discover antiarrhythmic drugs that can effectively and safely terminate and prevent AF recurrence. An antiarrhythmic drug with selective affinity for the ion channels that are specifically involved in atrial repolarization would be an ideal candidate. Considerable attention has recently focused on acetylcholine (ACh)-activated K+ (IK_ACh) channels because of the involvement in atrial but not ventricular repolarization and in AF susceptibility. Atrial cardiomyocytes from patients with chronic AF have constitutively active IK_ACh channel current (I_K_ACh). Moreover, paroxysmal AF occurring at night, at rest, and/or after consuming meals or alcohol is caused at least partly by activation of ligand-activated IK_ACh channels. However, the contribution of IK_ACh channel activation to the development and maintenance of AF in patients remains unknown because a selective IK_ACh channel blocker has not been available. We have developed a highly selective I_K_ACh blocker, NTC-801, and demonstrate that selective I_K_ACh blockade with NTC-801 prolongs atrial-selective effective refractory period, decreases AF inducibility in an AF model with electrical remodeling, and converts AF to normal sinus rhythm in vagally induced and ectopic automaticity–induced AF models. These findings suggest that I_K_ACh may be important in the development and maintenance of AF. Furthermore, I_K_ACh blockade does not affect membrane currents or action potentials in ventricular cells, which is reflected by the absence of QRS and QTC changes in vivo. These results indicate that a selective I_K_ACh blocker is unlikely to produce adverse ventricular effects.
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SUPPLEMENTAL MATERIAL

I. Supplementary Methods

Isolation of single atrial/ventricular cells

Hearts were removed from guinea pigs (250 - 450g) anesthetized with sodium pentobarbital and mounted on a modified Langendorff system to perfuse the coronary arteries with HEPES-Tyrode’s solution (mmol/L: NaCl 143, KCl 5.4, CaCl₂ 1.8, MgCl₂ 0.5, NaH₂PO₄ 0.33, glucose 5.5, and HEPES-NaOH 5; pH 7.4). The perfusion medium was replaced with Ca²⁺-free HEPES-Tyrode’s solution, followed by the same solution containing 0.02% wt/vol collagenase (Wako Pure Chemical Industry Co. Ltd., Osaka, Japan). Thereafter, the hearts were perfused with a high K⁺, low Cl⁻ modified KB solution. Atrial/ventricular tissue was cut into small pieces in modified KB solution (mmol/L: KOH 70, l-glutamic acid 50, KCl 40, taurine 20, KH₂PO₄ 20, MgCl₂ 3, glucose 10, EGTA 1, and HEPES-KOH 10; pH 7.4) and gently shaken to isolate cells that were then stored at 4°C.

Patch-clamp study

Whole-cell membrane currents were recorded using the patch-clamp method. Single atrial cells were placed in a recording chamber (1 mL volume) attached to an inverted microscope (model IMT-2, Olympus, Tokyo, Japan) and superfused with HEPES-Tyrode’s solution. The temperature of the external solution was maintained at 36 ± 1.0°C. Patch pipettes made from glass capillaries with a diameter of 1.5 µm using a vertical microelectrode puller (model PB-7, Narishige, Tokyo, Japan) were filled with an internal solution (mmol/L: potassium aspartate 110, KCl 20, MgCl₂ 1, potassium
ATP 5, potassium phosphocreatine 5, EGTA 1, and HEPES-KOH 5; pH 7.4) without or with GTP (100 μmol/L) or GTPγS (100 μmol/L) at 2 - 4 MΩ of resistance. The free Ca\(^{2+}\) concentration in the pipette solution was adjusted to pCa 8 according to the calculation of Fabiato and Fabiato\(^3\) with the correction of Tsien and Rink.\(^4\) A gigaohm seal was established between the tip of the electrode and the cell membrane, and then the membrane patch was disrupted by more negative pressure to create the whole-cell voltage-clamp mode. The electrode was connected to a patch-clamp amplifier (model CEZ-2300, Nihon Kohden, Tokyo, Japan). Recording signals were filtered at a 1 kHz bandwidth, and series resistance was compensated. Command pulse signals were generated by a 12-bit digital-to-analog converter controlled by pClamp software version 9.0.2.06 (Axon Instruments, Inc., Foster City, CA). Current signals were digitized and stored on the hard disc of a personal computer. A liquid junction potential between the internal and bath solution was corrected.

Current-clamp experiments were performed in whole-cell recording mode at 36 ± 1.0ºC. The external and pipette solution were the same as those used to record whole-cell membrane currents. The cells were stimulated by passing 2-ms currents through the pipette at a rate of 0.2 Hz. After a stabilization of action potential configuration, effects of NTC-801 on action potentials in the presence or absence of carbachol (1 μmol/L) or adenosine (10 μmol/L) were evaluated.

The \(I_{K,ACH}\) was activated by the extracellular application of carbachol (1 μmol/L) or adenosine (10 μmol/L) to GTP-loaded atrial cells, or by intracellular loading with GTPγS (100 μmol/L), a nonhydrolyzable GTP analog, in atrial cells held at -50 mV. Effects of various concentrations of NTC-801 on the \(I_{K,ACH}\) activated in three different ways were examined. The difference between the persistent outward current in the
GTPγS-loaded cells in the absence of NTC-801 and the initial current immediately after breaking the patch membrane in the pipette was taken as 100%. Assuming that the current decay was a linear function of time, the % inhibition of $I_{K,ACh}$ was calculated from the difference between the current extrapolated from the current decay before applying NTC-801 and that altered by various concentrations of NTC-801. Concentration-response data were fit to the following equation:

$$\% \text{ Block} = \left\{1-1/\left[1+([\text{Test}]/IC_{50})^N\right]\right\} \times 100$$

where [Test] is the test article concentration, $IC_{50}$ is the test article concentration at half maximal inhibition, $N$ is the Hill coefficient, and % Block is the ratio of the current inhibited at each test article concentration.

**Effects of NTC-801 on GIRK1/4 and GIRK1/2 current expressed in Xenopus oocytes**

**Oocyte preparation** Oocytes were surgically removed from *Xenopus laevis* (Hamamatsu Seibutsu, Hamamatsu, Japan) under anesthesia in ice water. Stage V and VI oocytes were defolliculated using forceps in Ca$^{2+}$-free OR2 solution (mmol/L: NaCl 82.5, KCl 1, MgCl$_2$ 1, and HEPES-NaOH 5; pH 7.6). Human GIRK1, GIRK2, and GIRK4 cDNA (Dragon Genomics Center at Takara Bio Inc., Shiga, Japan) were subcloned into the pT7Blue T vector (Takara Bio Inc.). cRNAs were prepared with the mMessage mMachine Kit (Ambion, Austin, TX), using T7 RNA polymerase, after linearization of the plasmid with BamHI, according to the manufacturer’s protocols. The oocytes were injected with 5 ng each of GIRK1 plus GIRK4 or GIRK1 plus GIRK2 cRNA using a Drummond Nanoject microdispenser (Drummond Scientific, Broomhall, PA) and incubated for 3 days at 18°C in PS solution (mmol/L: NaCl 96, KCl 2, CaCl$_2$ 3).
1.8, MgCl\textsubscript{2} 1, HEPES-NaOH 5; pH 7.5 and sodium pyruvate 2.5, supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin). High K\textsuperscript{+} solution comprised (mmol/L) KCl 96, NaCl 2, MgCl\textsubscript{2} 1, CaCl\textsubscript{2} 1.5, and HEPES-NaOH 5 (pH 7.4).

**Electrophysiological experiments using Xenopus oocytes** Membrane currents were recorded from oocytes using the two-microelectrode voltage-clamping and an oocyte clamp amplifier model OC-725C (Warner Instrument Corp., Hamden, CT). The electrodes contained 3 μmol/L KCl and had 0.5-1.0 MΩ of resistance. Data were obtained using pClamp software version 5.7.1(Axon Instruments, Inc.) and Digidata 1200B (Axon Instruments, Inc.). Currents were recorded in high K\textsuperscript{+} solution at room temperature. Currents through the GIRK1/4 or GIRK1/2 channels were elicited via 20 mV voltage steps of 450 ms duration from -100 to +40 mV with 0 mV holding. The % inhibition of the GIRK1/4 and GIRK1/2 currents was calculated from the current at the end point of voltage pulse to -80 mV.

**Effects of NTC-801 on nine cardiac ion channel currents**

**Effects on I\textsubscript{Na}** HEK293 cells were transfected with hNav1.5 cDNA and then stable transfectants were selected by coexpression of the hNav1.5 cDNA and the G418-resistance gene incorporated into the expression plasmid. Selection pressure was maintained in culture medium containing G418. The cells were cultured in Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (D-MEM/F-12) supplemented with 10% fetal bovine serum, 100 U/mL penicillin G sodium, 100 μg/mL streptomycin sulfate and 500 μg/mL G418.

Cells stably expressing hNav1.5 were held at -80 mV. Onset and steady-state block of the hNav1.5 current due to the test article or positive control (2 mmol/L lidocaine)
was measured using a depolarizing pulse (-15 mV, 10-ms duration) immediately followed by a repolarizing pulse (-65 mV, 5-ms duration). This pulse profile was repeated at 3 Hz (0.33-s interval between pulses). Peak current was measured from the test pulse and monitored during repetitive pulsing to reach a steady state. The pipette (intracellular) solution for whole cell recording comprised (mmol/L) cesium-aspartate 130, MgCl₂ 5, EGTA 5, Na₂ATP 4, GTP 0.1 and HEPES-CsOH 10 (pH 7.2).

**Effects on I_{CaL}** Adult ventricular myocytes isolated from male and female Hartley guinea pigs were transferred to a recording chamber to settle. Current was recorded from cylindrical calcium-tolerant myocytes with a smooth surface, a well-defined border and clear striations. The cells were maintained at room temperature if used on the day of isolation or refrigerated if intended for use within 48 h of isolation.

The onset and steady state block of I_{CaL} due to the test article or positive control (1 µmol/L nifedipine) were measured using a pulse profile comprising fixed amplitudes (prepulse: -40 mV amplitude, 1000-ms duration; test pulse: 10 mV amplitude, 300-ms duration; postpulse: -80 mV amplitude, 730-ms duration) repeated at 3-s intervals from a holding potential of -60 mV. Peak current was measured during the step to 10 mV. Peak current was monitored during repetitive stimulation until a new steady state was achieved. A steady state was maintained for at least 30 s before applying the test article or positive control. The pipette (intracellular) solution for whole cell recording comprised (mmol/L) Cs-methanesulfonate 130, tetraethylammonium chloride 20, MgCl₂ 1, EGTA 10 and HEPES-methanesulfonic acid 10 (pH 7.2), and were supplemented with ATP, GTP, phosphocreatine and creatine phosphokinase to minimize current rundown.

**Effects on I_{to}** HEK293 cells were transfected with hKv4.3 cDNA and then stable
transfectants were selected by coexpression with the G418-resistance gene incorporated into an expression plasmid. Selection pressure was maintained in medium containing G418. The cells were cultured in D-MEM/F-12 supplemented with 10% fetal bovine serum, 100 U/mL penicillin G sodium, 100 µg/mL streptomycin sulfate and 500 µg/mL G418.

The onset and steady state block of $I_{K_{ur}}$ due to the test article or positive control (1 mmol/L flecainide) were measured using a pulse profile of fixed amplitudes (depolarization: 0 mV for 300 ms) repeated at 5-s intervals from a holding potential of −80 mV. Peak current was monitored during the test step to 0 mV. The absolute change in total charge (current time integral, AUC) was also monitored. Peak current and total charge were monitored during repetitive stimulation until a new steady state was achieved. A steady state was maintained for at least 30 s before applying the test article or positive control. The pipette (intracellular) solution for whole cell recordings comprised (mmol/L) potassium aspartate 130, MgCl$_2$ 5, EGTA 5, ATP 4 and HEPES-KOH 10 (pH 7.2).

**Effects on $I_{K_{ur}}$** HEK293 cells were transfected with hKv1.5 cDNA and then stable transfectants were selected by coexpression with the G418-resistance gene incorporated into an expression plasmid. Selection pressure was maintained in culture medium containing G418. Cells were cultured in D-MEM/F-12 supplemented with 10% fetal bovine serum, 100 U/mL penicillin G sodium, 100 µg/mL streptomycin sulfate, and 500 µg/mL G418.

The onset and steady state block of $I_{K_{ur}}$ due to the test article or positive control (2 mmol/L 4-aminopyridine) were measured using a pulse profile of fixed amplitudes (depolarization: +20 mV amplitude, 300 ms-duration) repeated at 10-s intervals from a
holding potential of –80 mV. Current amplitude was measured at the end of the step to +20 mV. Currents were monitored until a new steady state was achieved. A steady state was maintained for at least 30 s before applying the test article or positive control. The pipette (intracellular) solution for whole cell recordings comprised (mmol/L) potassium aspartate 130, MgCl$_2$ 5, EGTA 5, ATP 4, and HEPES-KOH 10 (pH 7.2).

**Effects on I$_{Kr}$** CHO-K1 cells transfected with hERG (Cytomyx Ltd., Cambridge, UK) were cultured once upon receipt and stored in liquid nitrogen for later culture in Ham’s F-12 medium (Invitrogen Corp., Carlsbad, CA) containing 10% inactivated fetal bovine serum (Invitrogen Corp.) and 200 µg/mL of geneticin (Invitrogen Corp.).

Pulses were applied at 15-s intervals as follows: holding voltage, –80 mV; depolarization pulse, 4 steps of 1.5 s each with 20 mV increases per step from −40 to +20 mV, and repolarization pulses for 1.5 s at −50 mV. The pipette (intracellular) solution for whole cell recordings was (mmol/L) potassium gluconate 100, KCl 20, CaCl$_2$ 1, MgCl$_2$ 1, HEPES 10, EGTA 11, ATP-Na 5, and GSH 2 (pH 7.2).

**Effects on I$_{Ks}$** HEK293 cells were stably co-transfected with plasmids containing hKvLQT1 cDNA and hminK cDNA. Stable transfectants were selected based on coexpression of the cDNAs, neomycin (incorporated into the hKvLQT1 plasmid) and the Zeocin (incorporated into the hminK plasmid) resistance genes. Selection pressure was maintained in culture medium containing G418 and Zeocin. The cells were cultured in D-MEM/F-12 supplemented with 10% fetal bovine serum, 100 U/mL penicillin G sodium, 100 µg/mL streptomycin sulfate, 500 µg/mL G418 and 750 µg/mL Zeocin.

Cells stably expressing hKvLQT1/hminK were held at -80 mV. The onset and steady state block of hKvLQT1/hminK current due to the test article or positive control
(30 µmol/L Chromanol 293B) were measured using a pulse profile of fixed amplitudes (depolarization: +20 mV for 2 s; repolarization: -40 mV for 0.5 s) repeated at 15-s intervals. Current was measured at the end of the step to +20 mV. A steady state was maintained for at least 45 s before applying the test article or positive control. Current was monitored until a steady state of current blockade was achieved. The pipette (intracellular) solution for whole cell recording comprised (mmol/L) potassium aspartate 120, MgCl$_2$ 5, EGTA 5, ATP 4, and HEPES-KOH 10 (pH 7.2), supplemented with GTP 0.3, phosphocreatine 14, and 50 units/mL creatine phosphokinase to prevent rundown of the hKvLQT1/minK currents.

**Effects on $I_{K1}$** Ventricular myocytes isolated from adult male and female Hartley guinea pigs were transferred to the recording chamber and allowed to settle. Current recordings were obtained from calcium-tolerant, cylindrical myocytes with a smooth surface, a well-defined border and clear striations. Cells were maintained at room temperature or at 4°C if used on the day of isolation or within 48 h, respectively.

Membrane current was elicited by voltage steps (-120 mV amplitude, 400-ms duration) repeated at 10-s intervals from a holding potential of -70 mV. Current was monitored for at least 4 min under control conditions to allow stabilization. Recordings were terminated with a final application of a supramaximal concentration of the positive control (100 µmol/L BaCl$_2$), to assess the contribution of endogenous currents and verify sensitivity to blockers. The remaining unblocked current was digitally subtracted off-line from the data to determine the $I_{K1}$ inhibition potency of the test substance. The pipette (intracellular) solution for whole cell recordings comprised (mmol/L) potassium aspartate 130, MgCl$_2$ 5, EGTA 5, ATP 10, and HEPES-KOH 10 (pH 7.2).
**Effects on $I_{\text{KATP}}$** Primary cell cultures of neonatal myocytes were prepared from rat heart ventricles. Briefly, hearts from a litter of 8-10 pups were removed under sterile conditions, the atria and ventricles were separated and then the atria were discarded. Ventricular tissue was minced, incubated at 37°C in nominally Ca$^{2+}$-free Hanks solution containing 0.05% trypsin and 0.01% collagenase, and then individual cells were dissociated by trituration through Pasteur pipettes. The supernatant was removed and the pelleted cells were added to culture medium (DMEM and 10% fetal calf serum) to stop enzyme action. Resuspended cells were seeded at low density ($2 \times 10^5$ cells/mL) onto glass coverslips and incubated in culture medium at 37°C in a 5% CO$_2$ - 95% air atmosphere. After incubation for 6-18 h, the cells were washed twice with serum-free D-MEM/F-12 medium. The antibiotic Ara-c (1 mmol/L) was added to the culture medium to prevent the growth of non-myocyte cells. Electrophysiological recordings were obtained within 24 - 72 h.

Membrane currents were elicited by voltage ramps (-120 to +40 mV at 80 mV/s) repeated at 10-s intervals from a holding potential of -70 mV. Currents were monitored for at least 4 min under control conditions or until steady-state activation of the $I_{\text{KATP}}$ current. Peak outward current in the absence and presence of the test article was measured until a new steady state was achieved. At the end of each recording, glibenclamide (1 µmol/L) was applied to block $I_{\text{KATP}}$ and the glibenclamide-sensitive component of the ionic current was obtained by digital subtraction.

One test article concentration was applied to each cell and peak current was measured at the end of the test ramp. A steady state was maintained for at least 30 s before applying the test article or positive control. Peak current was measured until a new steady state was achieved. The pipette (intracellular) solution for whole cell recording...
comprised (mmol/L) potassium aspartate 130, MgCl$_2$ 5, EGTA 5, ATP 0.01, Na$_3$VO$_4$ 2, NaF 10, Na$_4$P$_2$O$_7$ 1 and HEPES-KOH 10 (pH 7.2).

**Effects on I$_f$** hHCN4 cDNA was subcloned into the tetracycline inducible expression vector pcDNA4/TO and transfected into the TRex-293 cell line. Cells were cultured in D-MEM/F-12 supplemented with 10% fetal bovine serum, 100 U/mL penicillin G sodium, 100 µg/mL streptomycin sulfate, and 200 µg/mL Zeocin. Expression was induced by incubation with tetracycline (1 µg/mL) for 24 h.

The onset and steady state block of the hHCN4 channel current due to the test article or positive control (10 µmol/L Zatebradine) was measured using a stimulus voltage profile comprising a hyperpolarizing test pulse (−120 mV, 1-s duration) repeated every 10-s from a holding potential of −30 mV. Peak current was monitored during the test step to −120 mV until a new steady state was achieved or 12 min had elapsed. A steady state was maintained for at least 30 s before applying the test article or positive control. The pipette (intracellular) solution for whole cell recordings comprised (mmol/L) potassium aspartate 130, MgCl$_2$ 5, EGTA 5, ATP 4, and HEPES-KOH 10 (pH 7.2).

**Concentration Response** NTC-801 at 30 µmol/L was applied to at least three cells and the effects on current amplitude were monitored. If the steady state inhibitory response was ≥50%, the IC$_{50}$ was estimated and concentrations were selected to span the range of approximately 10 - 90% inhibition in half-log increments to evaluate the concentration-response relationship. If the inhibitory response was <50% at 30 µmol/L, then the study was deemed complete.

**Data Analysis** Data acquisition and analysis was performed using the pCLAMP version 8.2 suite of programs (Molecular Devices, Union City, CA). We defined
steady state as the limiting constant rate of change with time (linear time dependence). The steady states before and after test article application were used to calculate its inhibitory effect on peak current amplitude. Concentration-response data were fit to the following equation:

\[
\%\text{ Block} = \{1-1/[1+(\text{[Test]}/\text{IC}_{50})^N]\}*100
\]

where \([\text{Test}]\) is the test article concentration, \(\text{IC}_{50}\) is the test article concentration at half maximal inhibition, \(N\) is the Hill coefficient, and \(\%\text{Block}\) is the ratio of the current inhibited at each test article concentration. Nonlinear least squares fit was solved with the Solver add-in for Excel 2000, or a later version (Microsoft, Bellevue, WA), and the \(\text{IC}_{50}\) was calculated if the test article produced >50% block at the highest concentration. If the inhibitory response was <50%, the \(\text{IC}_{50}\) was not determined.

**Effects of NTC-801 on \(I_{Kr}\) and \(I_{Ks}\) in guinea pig atrial cells.**

The delayed rectifier \(K^+\) current \((I_K)\) was elicited by 10 mV voltage steps between -30 and +40 mV from a holding potential of -40 mV after inhibiting the L-type \(Ca^{2+}\) current using nisoldipine (1 µmol/L), and the effects of NTC-801 on \(I_{Kr}\) and \(I_{Ks}\) were examined. The amplitude of the deactivating current was measured as the difference between the holding current and the peak current recorded upon the clamp back to the holding potential, as described previously. The positive controls comprised the selective \(I_{Ks}\) and \(I_{Kr}\) blockers, chromanol 293B (100 µmol/L) and E-4031 (10 µmol/L), respectively.

**Effects of NTC-801 on muscarinic \(M_2\) and adenosine \(A_1\) receptor binding.**

The effects of NTC-801 at 10 µmol/L (n=2) on the specific binding of \(^3\text{H-N-methylscopolamine (0.8 nmol/L) or } ^3\text{H-DPCPX (1 nmol/L) to M_2 receptor or A_1 receptor.}\)
receptors, respectively, were examined in Chinese hamster ovary (CHO) cells expressing human muscarinic M₂ or adenosine A₁ receptors.

**Effects of NTC-801 on action potential in guinea pig papillary muscles**

*Isolation of Papillary Muscles*  Hearts removed from animals anesthetized with diethylether were placed in Tyrode’s solution (mmol/L: NaCl 125, KCl 4, NaHPO₄ 1.8, MgCl₂ 0.5, CaCl₂ 2.7, glucose 11.1 and NaHCO₃ 25) and bubbled with a 95% O₂ - 5% CO₂ mixture. One specimen of right ventricular papillary muscles from each heart was stabilized by perfusion at a rate of 300 mL/h using a DynamaxR, Model RP-1 peristaltic pump (Rainin Instrument Co., Inc., Woburn, MA) for 30 min at 35.7 - 36.2°C maintained by a TC-324B automatic temperature controller (Warner Instruments LLC., Hamden, CT).

*Action Potential Recording from Papillary Muscles*  Stabilized papillary muscle was electrically stimulated (frequency, 0.5 Hz, pulse amplitude, 1 ms; threshold voltage, 150%) using a stimulating electrode, an isolator (Constant Current Stimulus Isolator A360D; World Precision Instruments, Inc., Sarasota, FL) and an electric stimulator (AccupulsereTM Pulse Generator A310; World Precision Instruments, Inc.) under stereoscopic guidance. A glass microelectrode prepared using a puller (PP-830; Narishige Co., Ltd.) was brought to 16 - 20 MΩ using 3 mol/L KCl, connected to an electrode holder and a micromanipulator, and inserted into the papillary muscle. Resting membrane potential (RMP), action potential amplitude (APA), maximum rate of depolarization (dV/dt max), and action potential duration at 30, 60, and 90% repolarization (APD₃₀, APD₆₀, APD₉₀) were monitored using a Duo 773 Dual Microprobe System microelectrode amplifier (World Precision Instruments, Inc.), a
DCS-7020 memory oscilloscope (Kenwood TMI Corp., Tokyo, Japan), and the WinCAPA Ver 1.4.3 data analysis system (Physio-Tech Co., Ltd., Tokyo, Japan).

**Vagal nerve stimulation (VNS)-induced AF model**

Sixty-two male mongrel dogs (HBD dogs, Kitayama Labes Co., Ltd., Gifu, Japan) weighing 19-25 kg were anesthetized with pentobarbital (40 mg/kg, i.v.), supplemented as necessary during the experiment. Each dog was artificially ventilated with room air (SN-480-3, Shinano, Tokyo, Japan). The forepaw and femoral veins were cannulated to administer test articles and to maintain fluid balance, respectively. The chest was opened via a midsternal thoracotomy, and a bipolar electrode was placed on the low right atrium and right atrial appendix for pacing and recording, respectively. Two Teflon®-coated electrodes were inserted into the middle of the bilateral cervical vagal nerve to stimulate efferent vagi. Atenolol (0.03 μmol/kg/h over 1 h) was administered intravenously to block beta-adrenergic effects on the heart.

Bilateral VNS was delivered from a SEN3301 stimulator (Nihon Kohden, Tokyo, Japan) with a pulse width of 3 ms and an applied voltage of 1 V. Thirty seconds later, a 5-s burst of atrial stimulation with a 4-fold diastolic threshold current (60 ms interval) induced AF defined as a rapid and irregular rhythm with varying atrial electrocardiographic morphology. If AF persisted for more than 15 minutes, AF was terminated by stopping VNS. Ten minutes after the termination of AF, AF was induced again by VNS and a burst of stimulation. Thirty seconds after the onset of AF, NTC-801 (0.3, 1, and 3 μg/kg/min) or vehicle (saline) was intravenously infused for a maximum of 15 min. The incidence of AF termination within 15 min after the start of test article infusion was evaluated. Blood samples were collected immediately after
AF was terminated to measure plasma NTC-801 concentrations.

**Measurement of effective refractory period (ERP) and intra-atrial conduction time under VNS**

Twenty-six female beagle dogs (Kitayama Labes Co., Ltd.) weighing 10-12 kg were used. The surgical procedure was essentially the same as that of VNS-induced AF. Three bipolar electrodes were placed in the right atrial appendix, low right atrium and right ventricular free wall for pacing and recording. Bilateral VNS was delivered with a pulse width of 0.1 ms and an applied voltage of 0.05-0.1 V. The ERP was determined using train 10 basic (S1) stimuli followed by a premature (S2) stimulus. The basic cycle length was 300 ms. S2 was initially delivered late in diastole and applied in 10-ms decrements until no response was elicited. The procedure was then repeated for the final 10-ms window in 2-ms decrements. The ERP was defined as the longest S1-S2 interval failing a propagated response. The conduction time from the low right atrium to the atrial appendix was determined at a cycle length of 300 ms. After baseline measurement, NTC-801 (0.3, 1, and 3 µg/kg/min) or vehicle (saline) was intravenously infused for 15 min and ERP was measured again 30 s after the end of test article administration. Differences in ERP before and after administration were assessed.

**Aconitine-induced AF model**

Sixteen male beagle dogs (Kitayama Labes Co., Ltd.) weighing 9-13 kg were used. Two recording bipolar electrodes were placed in the low right atrium and right ventricular free wall. Atrial fibrillation was induced by the topical application of
aconitine-impregnated (0.1 mg/body) absorbent cotton on the right appendage. Two minutes after the onset of AF, the NTC-801 (0.01, 0.03, and 0.1 mg/kg) or vehicle (10% DMSO-PEG200) was intravenously injected as a bolus and the incidence of AF termination within 10 min after administration of the test article was evaluated.

**Rapid atrial pacing (RAP)-induced AF model**

Twenty-two male mongrel dogs (HBD dogs, Kitayama Labes Co., Ltd.) weighing 17-23 kg each were anesthetized with pentobarbital (about 30 mg/kg i.v.), which was also supplemented as necessary during the experiment. Each animal was intubated and ventilated with room air, delivered via an SN-480-3 respirator (Shinano Manufacturing Co., Ltd, Tokyo, Japan). The chest was opened via right thoracotomy, and the heart was exposed by incising the pericardium. A bipolar electrode (Physio-Tech Co., Ltd.) was sutured with 3-0 nylon thread on the low right atrium and the right atrial appendix for pacing and recording, respectively, and the chest was closed. Infection was prevented by the intramuscular administration of antibiotics (Viccillin S1000 and streptomycin). After a recovery period of at least one week, a cardiac pacemaker (APC Medical Ltd., Waltham, MA) was connected via the stimulation electrode. Rapid atrial pacing at a rate of about 400 bpm was delivered for 3 to 4 weeks at an output of 12V.

Thereafter, 5-s bursts of atrial stimulation were applied 10 times at a voltage of 2-fold the diastolic threshold and a stimulus interval of 20 ms, and then the number of AF inductions was counted. NTC-801 (3 µg/kg/min) or vehicle (saline) was intravenously infused for 15 min, followed by repeated AF challenges and the number of AF inductions was evaluated. In addition, atrial ERP with basic cycle lengths (BCL) of
200, 250 and 300 ms was measured before and after test article infusion. Differences in atrial ERP before and after the infusion were assessed as described above.

**Blood pressure, heart rate, electrocardiogram (ECG) parameters, and arrhythmia assessment in conscious dogs**

Four conscious and unstrained, fasted male beagle dogs (Japan Laboratory Animals, Inc., Tokyo, Japan) weighing 11-12 kg each were orally administered with an empty gelatin capsule (control), or a capsule containing NTC-801 (0.1, 1, 3 mg/kg) at intervals of >3 days. Blood pressure, heart rate, and ECG parameters (PR-interval, QRS-width, QT-interval and QTcF corrected using the Fridericia formula), were measured before and 1, 2, 3, 4, 8 and 24 h after dosing. Arrhythmia was continuously monitored from before to 24 h after dosing using an automatic telemetry measurement system (blood pressure and heart rate) and Holter electrocardiography (ECG parameters and arrhythmia).
II. Supplementary Results

Effects of NTC-801 on $I_{Kr}$ and $I_{Ks}$ in guinea pig atrial cells.
We examined the effects of NTC-801 at 100 nmol/L in guinea pig atrial cells after blocking the L-type Ca$^{2+}$ current with 1 µmol/L nisoldipine to confirm whether NTC-801 affects $I_{Kr}$ and $I_{Ks}$. The results were compared with the effects of the $I_{Kr}$ and $I_{Ks}$ blockers E-4031 (10 µmol/L), and chromanol 293B (100 µmol/L), respectively. NTC-801 (100 nmol/L) did not affect $I_{Kr}$ or $I_{Ks}$, whereas E-4031 and chromanol 293B respectively inhibited $I_{Kr}$ and $I_{Ks}$. Co-administration of E-4031 and chromanol 293B completely blocked $I_{Kr}$ and $I_{Ks}$ in guinea pig atrial cells (Supplementary Figure 2).

Effects of NTC-801 on muscarinic M$_2$ and adenosine A$_1$ receptor binding.
The mean values for the inhibition of M$_2$ and A$_1$ receptor binding by NTC-801 (10 µmol/L) were 6 and 47%, respectively. Hence, the IC$_{50}$ values of NTC-801 for the binding of these two receptors were both >10 µmol/L.

Effects of NTC-801 on action potential in guinea pig papillary muscles.
The effects of NTC-801 at 3-30 µmol/L were examined using guinea pig papillary muscles to confirm whether NTC-801 at higher concentrations affects action potential parameters in ventricular tissue, and the results were compared with those of the $I_{Kr}$ blocker E-4031 at 0.1 µmol/L (positive control). NTC-801 at 3, 10, and 30 µmol/L did not affect any of RMP, APA, dV/dt max, APD$_{30}$, APD$_{60}$, or APD$_{90}$, whereas APD$_{30}$, APD$_{60}$ and APD$_{90}$ were significantly prolonged in the group given E-4031 (Supplementary Table 1). NTC-801 up to 30 µmol/L did not affect the action potential of the guinea pig papillary muscles.
Effects on blood pressure, heart rate, ECG parameters and arrhythmia in conscious dogs

NTC-801 up to 3 mg/kg hardly affected blood pressure, heart rate and ECG parameters (PR-interval, QRS-width or QTcF) (Supplementary Table 2). Analysis of continuous ECG data before and after dosing revealed no induction of arrhythmia, including premature ventricular contraction or atrioventricular block (Supplementary Figure 3).
References


Supplementary Table 1.
Effects of NTC-801 on Action Potential Parameters in Guinea Pig Papillary Muscles.

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<th>Test and control article</th>
<th>Application concentration (µmol/L)</th>
<th>Number</th>
<th>RMP (mV)</th>
<th>APA (mV)</th>
<th>dV/dt max (V/second)</th>
<th>APD₃₀ (millisecond)</th>
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RMP: Resting membrane potential.
APA: Action potential amplitude.
dV/dt max: Maximum rate of depolarization.
APD₃₀, APD₆₀, and APD₉₀: Action potential duration at 30%, 60% and 90% repolarization.
Pre: Pre-application of test or control article.
Post: 30 minutes after the application of test or control article.

No significant differences were found between the control and the NTC-801-treated groups.

* p<0.05, ** p<0.01: Significantly different from the control (Wilcoxon test).
Supplementary Table 2.
Effects of NTC-801 on blood pressure, heart rate, and ECG parameters in conscious dogs.

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<th>MBP (mmHg)</th>
<th>HR (bpm)</th>
<th>PR (ms)</th>
<th>QRS (ms)</th>
<th>QT (ms)</th>
<th>QTc (ms)</th>
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<td>95 ± 7</td>
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<td>75 ± 3</td>
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<tr>
<td>24</td>
<td>98 ± 7</td>
<td>98 ± 7</td>
<td>99 ± 9</td>
<td>72 ± 2</td>
<td>245 ± 9</td>
<td>294 ± 7</td>
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</table>

| NTC-801 0.1mg/kg | Baseline | 102 ± 5 | 87 ± 4 | 104 ± 6 | 76 ± 1 | 253 ± 7 | 274 ± 5 |
| NTC-801 1mg/kg   | Baseline | 107 ± 6 | 94 ± 15| 105 ± 6 | 75 ± 1 | 256 ± 2 | 278 ± 7 |
| NTC-801 3mg/kg   | Baseline | 120 ± 3 | 99 ± 7 | 103 ± 10| 71 ± 2 | 253 ± 10| 264 ± 12|

| NTC-801 0.1mg/kg | 1        | 109 ± 5 | 102 ± 10| 106 ± 9 | 73 ± 4 | 250 ± 12| 270 ± 7 |
| NTC-801 1mg/kg   | 2        | 101 ± 5 | 76 ± 5  | 105 ± 10| 76 ± 1 | 258 ± 5 | 274 ± 11|
| NTC-801 3mg/kg   | 3        | 104 ± 6 | 80 ± 3  | 109 ± 10| 72 ± 2 | 263 ± 6 | 273 ± 7 |
| NTC-801 0.1mg/kg | 4        | 110 ± 4 | 89 ± 3  | 98 ± 7  | 74 ± 2 | 244 ± 7 | 283 ± 3 |
| NTC-801 1mg/kg   | 5        | 104 ± 8 | 88 ± 5  | 104 ± 7 | 77 ± 1 | 259 ± 7 | 271 ± 3 |
| NTC-801 3mg/kg   | 6        | 102 ± 6 | 85 ± 7  | 102 ± 9 | 75 ± 1 | 254 ± 7 | 275 ± 2 |
Supplementary Figure legends

Supplementary Figure 1.
Current traces for nine cardiac ion channels for $I_{\text{Na}}$ (A), $I_{\text{CaL}}$ (B), $I_{\text{to}}$ (C), $I_{\text{Kur}}$ (D), $I_{\text{Kr}}$ (E), $I_{\text{Ks}}$ (F), $I_{\text{KI}}$ (G), $I_{\text{KATP}}$ (H), and $I_{\text{f}}$ (I).

Supplementary Figure 2.
Effects of NTC-801 on delayed rectifier K$^+$ current ($I_{\text{K}}$) in guinea pig atrial cells. Current traces elicited by 300 ms depolarizing pulses from a holding potential of $-40$ mV in the control (A), in presence of 100 nmol/L NTC-801 (B), 10 µmol/L E-4031 (C), 100 µmol/L Chromanol 293B (D), or 10 µmol/L E-4031 plus 100 µmol/L Chromanol (E). Panel F summarizes data from $I_{\text{K}}$ measured after clamping back to $-40$ mV from indicated potential ($I_{\text{K, tail}}$). Data are expressed as mean ± SEM of 5 cells.

Supplementary Figure 3.
Representative examples of ambulatory electrocardiography (ECG) before (A) and after (B) NTC-801 (3 mg/kg) administration. Electrodes were fixed at episternum (-), xiphisternum (+) (M-X lead) and right thorax (-), left thorax (+) (R-L lead), respectively. Ambulatory ECG was continuously recorded from the day before dosing to the day after dosing using Holter’s electrocardiograph. Data were obtained at 22h before (A; a time match control for B in the circadian rhythm) and 2h after (B) the administration.
Supplementary Figure 3