Inhibition of Small Conductance Ca\textsuperscript{2+}-Activated K\textsuperscript{+} Channels Terminates and Protects against Atrial Fibrillation

Running title: Diness et al.; SK inhibition terminates and protects against AF

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Abstract:

**Background** - Recently, evidence has emerged that small conductance Ca\(^{2+}\)-activated K\(^+\) (SK) channels are predominantly expressed in the atria in a number of species including human. In rat, guinea pig, and rabbit *ex vivo* and *in vivo* models of atrial fibrillation (AF) we utilized three different SK channel inhibitors UCL1684, N-(pyridin-2-yl)-4-(pyridin-2-yl)thiazol-2-amine (ICA), and NS8593 to assess the hypothesis that pharmacological inhibition of SK channels is antiarrhythmic.

**Methods and Results** - In isolated, perfused guinea pig hearts, AF could be induced in all control hearts (n=7) with a combination of 1\(\mu\)M acetylcholine (ACh) combined with electrical stimulation. Pre-treatment with 3\(\mu\)M NS8593, which had no effect on QT-interval, prolonged the atrial effective refractory period (aERP) by 37.1±7.7% (p<0.001) and prevented ACh-induced AF (p<0.001, n=7). After AF-induction, perfusion with NS8593 (10\(\mu\)M), UCL1684 (1\(\mu\)M), or ICA (1\(\mu\)M) terminated AF in all hearts, comparable to 10\(\mu\)M amiodarone.

In isolated, perfused rat hearts, AF was induced with electrical stimulation. 10\(\mu\)M NS8593 terminated AF and prevented re-induction of AF in all hearts (n=6, p<0.001). In all hearts, AF could be re-induced after washing.

In isolated, perfused rabbit hearts, AF was induced with 10 \(\mu\)M ACh and burst pacing. 10\(\mu\)M NS8593 terminated AF and prevented re-induction of AF in all hearts (n=6, p<0.001). After washing, AF could be re-induced in 75% of the hearts (n=4, p=0.03).

In an *in vivo* rat model of acute AF induced by burst-pacing, injection of 5mg/kg of either NS8593 or amiodarone shortened AF-duration significantly (23.2±20.0%, p<0.001, n=5, and 26.2±17.9%, p<0.001, n=5, respectively) as compared to injection of vehicle (96.3±33.2%, n=5).

**Conclusions** - Inhibition of SK channels prolongs aERP, without affecting QT-interval, and prevents and terminates AF *ex vivo* and *in vivo*, thus offering a promising new therapeutic opportunity in the treatment of AF.

**Key words:** antiarrhythmia agents, drugs, atrial fibrillation, ion channels, NS8593
Introduction

The use of conventional class III agents that decrease K⁺ currents has been limited because they can induce potentially fatal ventricular arrhythmias[1, 2]. Current antiarrhythmic drugs approved for the treatment of AF are limited in their efficacy and have side-effect profiles that often result in poor patient tolerance[3]. E.g. the most efficacious drug currently used to maintain sinus rhythm in patients, amiodarone, has a recurrence rate of 30% after 1 year of treatment and has prominent extra-cardiac adverse effects[3, 4]. With sotalol or propafenone, the 1-year recurrence rate after treatment is as high as 60%[3]. Thus, a considerable unmet medical need for safe and effective agents against AF exists and ongoing drug development has focused on increased safety by targeting ion channels specifically expressed in the atria.

Traditionally, SK channels have not been considered important cardiac ion channels. Recently, a small number of articles have linked SK channels to the hearts of mouse, rat, rabbit, and human[5-8]. Three subtypes of SK channels (SK1-3) exist, and biochemical evidence indicates that SK2 channels are predominantly expressed in the atria of human and mouse hearts as compared to the ventricles. Also, SK1 channels have been reported to be predominantly expressed in the atria of mouse hearts[5, 6]. Finally, functional evidence supports the notion that SK channels are more important in atria than in ventricles[5]. The exact role of SK channels in the heart remains uncertain, but they have been suggested to play an important role in atrial repolarization and fibrillation making them an interesting target in AF [7, 9]. However, no systematic investigations have yet been made into the possible pro- or antiarrhythmic effects of these channels.

SK channels can be blocked by the highly selective 18-amino-acid bee-venom toxin apamin as well as several scorpion toxins, such as scyllatoxin and and tamapin[10]. Compounds such as tubocurarine and UCL1684 mimick the structural elements of the binding residues on these selective SK neurotoxins whereas ICA has been suggested to act by blocking the channels through
its chelation to a cation[11, 12]. All the above compounds displace $[^{125}\text{I}]$apamin binding and are considered as pore blockers acting at the apamin binding site[13].

A novel class of selective SK channel inhibitors that do not block the channel pore has recently been described[13, 14]. $[^{125}\text{I}]$Apamin is not displaced by these compounds in binding studies, and they still inhibit SK channels in which point mutations of essential amino acids have disrupted the apamin binding site[13]. NS8593 is an example of a compound from this structural class which indiscriminately modulates all SK1-3 subtypes negatively by decreasing the sensitivity towards Ca$^{2+}$[14, 15].

In this study, we assessed the effects of a negative modulator of SK channels, NS8593, on the aERP and the capacity to prevent the induction of AF in isolated guinea pig hearts. We furthermore examined the ability of SK channel inhibition to revert AF in a model of paroxysmal AF in isolated guinea pig hearts. We used two structurally unrelated SK channel pore blockers, UCL1684 and ICA, as well as the negative modulator of SK channels NS8593 (Fig. 1). The finding of the ability of SK inhibition to revert AF in isolated guinea pig heart was subsequently confirmed in an isolated rat and rabbit heart models of paroxysmal AF. Finally, the anti-AF effects of negative modulation of SK channels was tested in vivo in a rat model of paroxysmal AF.

The findings in this study reveal SK channels as a promising new therapeutic target in the treatment of AF with a decreased risk of ventricular proarrhythmia.

**Materials and methods**

All studies were performed in accordance with the Danish guidelines for animal experiments according to the Helsinki declaration. Methods for isolation of single cardiac myocytes, recordings of action potentials, oocyte isolation and injection, in vitro transcription, and two-electrode voltage clamp recordings can be found in the supplemental material.
Ex vivo experiments

The isolated hearts were prepared as earlier described[16]. Four monophasic action potential (MAP) electrodes were placed on the heart surface, one on each ventricle and two on the left atrium (LA). An electrocardiogram (ECG) was obtained with 9 electrodes placed in the vicinity of the heart. All data were acquired using the 16 channel PowerLab system (ADInstruments, Oxfordshire, UK). Hearts were electrically stimulated on the right atrium (RA) with 2 ms double threshold stimuli.

AF prevention, guinea pig: Isolated, perfused female guinea pig hearts from Dunkin Hartley strain (HsdPoc:DH) continuously paced on the right atrium at a rate of 300 beats per minute (BPM) were allowed to stabilize and perfused for 30 minutes with either no drug or NS8593 (0.3 μM, 1 μM, or 3 μM). The aERP was measured (10 basic stimuli (S1) followed by premature S2 stimuli applied with 2 ms decrements) immediately before and at the end of the 30 minutes period. Following the 30 minutes of perfusion with either no drug or NS8593, 1 μM acetylcholine (ACh) was added to the perfusate and induction of AF was attempted with S2 stimuli 1-3 ms after the end of atrial refractoriness. If S2 stimuli did not elicit AF, the hearts were burst paced (50 Hz) 10 times 10 s with intervals of 10 s. AF was considered prevented if no episodes of AF>1 min could be induced by any of these interventions.

AF reversion, guinea pig: Isolated, perfused female guinea pig hearts from Dunkin Hartley strain (HsdPoc:DH) continuously paced on the right atrium (300 BPM) were allowed to stabilize, and aERP was measured. AF was induced by 1-3 μM ACh combined with S2 stimuli 1-3 ms after the end of atrial refractoriness. When sustained AF was obtained (>2 min), either no drug, amiodarone (10 μM), ICA (1 μM), UCL1684 (1 μM), or NS8593 (1 μM, 3 μM, or 10 μM) was added to the
perfusate. AF was considered reverted if sinus rhythm was obtained within 20 min of drug application and if AF could not be re-induced with S2 stimulation or burst pacing after this.

**AF reversion, rabbit:** Isolated, perfused, female rabbit hearts continuously paced on the right atrium (240 BPM) were allowed to stabilize, and aERP was measured. AF was induced by 10 μM ACh combined with burst pacing (50 Hz). When sustained AF was obtained (>2 min), 10 μM NS8593 was added to the perfusate. AF was considered reverted if sinus rhythm was obtained within 20 min of drug application and if AF could not be re-induced with burst pacing after this. Following NS8593 application, the drug was allowed to wash out until aERP reached steady state, usually within 20-25 minutes. After washing, induction of AF was attempted in 4 out of 6 rabbits by burst pacing (50 Hz).

**AF reversion, rat:** Isolated, perfused male rat hearts from the Sprague Dawley strain (Crl:SD) continuously paced on the right atrium (500 BPM) were allowed to stabilize, and aERP was measured. AF was induced by S2 stimuli 1-3 ms after the end of atrial refractoriness. When sustained AF was obtained (>2 min), NS8593 (10 μM) was added to the perfusate. AF was considered reverted if sinus rhythm was obtained within 20 min of drug application and if AF could not be re-introduced with S2 stimulation or burst pacing after this. Following NS8593 application, the compound was allowed to wash out until aERP reached steady state, usually within 20-25 minutes. After washing, induction of AF was attempted by S2 stimulation 1-3 ms after the end of atrial refractoriness for 5-10 minutes. If S2 stimulation did not elicit AF, burst pacing (83 Hz) 10 times 10 s with intervals of 10 s was used. AF was considered reintroduced if periods of AF>5s could be induced by any of these interventions.
**In vivo experiments**

*In vivo* experiments were conducted in a novel AF model inspired by Haugan et al. and Sugiyama et al.[17, 18]. Male Sprague Dawley rats (Crl:SD) rats weighing 350-450 g were mebumal-anaesthetized (50mg/kg, i.p.), artificially respirated and kept at 37°C. Following thoracotomy and a period of stabilization, short episodes of AF were induced every 2 minutes by open-chest burst pacing (83 Hz) of the RA for 30 s with concomitant asphyxia followed by 90 seconds of intrinsic heart rhythm and normal ventilation. MAP recordings were obtained with a MAP electrode placed on the RA and ECG recordings were obtained with subcutaneous needle electrodes, two near the forelimbs and one near the left hind limb. Animals were divided into three groups receiving i.v. injections of either NS8593 (5 mg/kg), amiodarone (5 mg/kg), or vehicle subsequent to 30 minutes of baseline recordings. AF durations were measured in a blinded fashion. The total duration of AF at all 90 s interburst intervals was measured over a period 30 minutes before injection to establish a baseline AF duration. After injection, the total duration of AF was measured over another period of 30 minutes.

**Data analysis**

Data analysis and drawings were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA), SAS 9.2 software (SAS Institute Inc., Cary, NC, USA), Chart 5 software (ADinstruments), or Igor software (WaveMetrics, Lake Oswego, OR, USA). Continuous data are summarized using the mean and standard deviation (SD). Fisher’s exact test was used to compare proportions of animals with with protection against AF by treatment with p-values adjusted for multiple comparisons with the Bonferroni method (p-values multiplied with the number of comparisons). 1-way ANOVA with Tukey’s *post hoc* test was used to compare mean values of aERP and QT-interval by concentration of NS8593 and for comparing AF-durations normalized to
baseline values for the three different treatment regimens in vivo, and paired Student’s t-test was applied for the selectivity data. Values of $P<0.05$ were considered significant and in figures denoted by *. In figures, $P<0.01$ and $P<0.001$ are denoted with ** and ***, respectively. The small sample sizes used in this study are a limitation with regards to the statistical analysis.

**Drugs and solutions**

Unless otherwise mentioned, all the chemicals used were of analytical grade and were obtained from Sigma-Aldrich. NS8593, UCL1684, and ICA were synthesized at NeuroSearch A/S, Ballerup, Denmark. Except from amiodarone, drugs were dissolved in dimethyl sulfoxide as concentrated stock solutions of 10 mM and diluted directly into the perfusion solution to yield the final concentration for the ex vivo experiments. Amiodarone liquid for injection 50 mg/ml produced by Sanofi-Aventis A/S and glucose liquid for infusion 50 mg/ml produced by B. Braun Melsungen AG was obtained from a Danish pharmacy. The vehicle solution for amiodarone liquid for injection 50 mg/ml consisted of polysorbate 80, benzilic alcohol and water (10:10:80, v/v/v), which was diluted in glucose liquid for infusion to a final concentration of 5 mg/ml. Glucose liquid for infusion with 5 % cremophor was used as vehicle for the in vivo experiments.

**Results**

If aERP is shortened the likelihood that circulating impulses perpetuate is dramatically increased[19]. In the ex vivo guinea pig model of AF that we use in this study, ACh is added to the perfusate in order to shorten aERP, thus providing an arrhythmic substrate. With aERP thus shortened, sustained AF can be elicited by electrical stimulation unless measures are taken to
terminate it. In Fig. 2, an example of sustained AF is shown along with an example of how NS8593 terminates AF and prevents its re-induction.

SK channel inhibition prevents AF induction in ex vivo guinea pig heart

To examine the effect of NS8593 on aERP and the capacity to prevent AF, isolated guinea pig hearts were perfused with different concentrations of the negative modulator of SK channels NS8593 (Fig. 3). Increasing concentrations of NS8593 (0.3 μM, 1 μM, and 3 μM) prolonged aERP (overall p-value<0.001) by 13.1±7.8% (p<0.05, n=4), 18.9±3.4% (p<0.01, n=3), and 37.1±7.7% (p<0.001, n=4), respectively, compared to controls (0.7±4.4%, n=4)(Fig. 3b). These effects are believed to be atrial-selective as no effect on QT-interval was observed in any of the concentrations tested (Fig. 3A and 3B). The increased aERP was associated with a protection against the induction of AF; in all of the time matched control experiments sustained AF was inducible (n=7) (Fig. 2), whereas increasing concentrations of NS8593 (0.3 μM, 1 μM, and 3 μM) protected against induction of AF (overall p-value=0.03) in 43% (p=0.19, n=7), 80% (p<0.05, n=5), and 100% (p<0.01, n=4) of preparations (Fig. 3A and 3C). Of the drug treated hearts, only one from the 0.3 μM group developed AF without burst pacing. In the rest of the drug treated hearts in which AF could be induced, burst pacing was necessary to elicit the AF. In comparison, the gentler AF induction protocol of S2 stimulation was sufficient to elicit AF in controls, suggesting a decreased vulnerability to AF even at the lowest dose of NS8593, although complete protection was not provided.

SK channel inhibition reverts acutely induced AF in ex vivo guinea pig heart

Drugs such as sotalol that are effective in preventing AF are not necessarily effective for conversion of AF to sinus rhythm[20]. Therefore, we next examined the capacity of SK channel inhibition to
revert sustained AF. When sustained AF (>2 min) had been induced in the time matched controls it lasted for the duration of the experiment (>45 minutes, n=7) (Fig. 2 and 4). In 1/7 controls AF reverted spontaneously after 3 minutes, but could be easily reintroduced with S2 stimulation. In hearts perfused with increasing concentrations of NS8593 (1 μM, 3μM, and 10μM) AF was reverted and non-reinducible (overall p-value<0.001) in 33% (p=0.58, n=6), 83% (p<0.01, n=6), and 100% (p<0.001, n=6), respectively, of the hearts (Fig. 4B).

Two other SK channel inhibitors, UCL1684 and ICA, with pore-blocking properties, as opposed to the inhibition on Ca\(^{2+}\)-sensitivity exerted by NS8593, also reverted and protected against AF (overall p-value<0.001)(Fig. 4C, Fig 1). In 100% of hearts perfused with 1 μM ICA or 1 μM UCL1684, AF was reverted and could not be re-induced (p<0.01, n=5 in both cases).

The effects of SK channel inhibition were comparable to those seen in hearts perfused with 10 μM amiodarone (reversion of and protection against AF in 100% of the hearts) (Fig. 4C). Amiodarone is normally used as a last-resort agent that dampens electrical activity in the heart by an unspecific block of multiple ion channels (I_{Na}, I_{Ks}, I_{Kf}, and L-type Ca\(^{2+}\) channels), and is the most efficient presently available agent for maintenance of sinus rhythm, but the compound has prominent extra-cardiac adverse effects and complicated pharmacokinetics that limit widespread application[20].

**SK channel inhibition reverts acutely induced AF in *ex vivo* rat heart**

In order to confirm the antiarrhythmic effects of SK inhibition seen in guinea pig, we examined the capability of 10 μM NS8593 to revert AF in a rat *ex vivo* model. Sustained AF was induced with S2 stimulation in all hearts before drug application (n=6) (Fig 5A and 5C). Following application of 10 μM NS8593, AF was reverted (overall p-value<0.001), and could not be re-induced in any (p<0.001, n=6) of the hearts. After a wash-out period of 20-25 minutes, during which aERP progressively shortened until reaching a steady state, AF could be re-induced in all hearts (n=6)(Fig. 5A and 4C). Application of 10 μM NS8593 prolonged aERP (overall p-value<0.001) in
the rat hearts from 15.5±3.8 ms in the control situation to 43.5±7.0 ms (p<0.001, n=6) (Fig. 5B).
After wash-out aERP was shortened to 23.0±5.2 ms, which was not significantly different from the control situation (Fig. 5B).

**SK channel inhibition reverts acutely induced AF in *ex vivo* rabbit heart**

The capability of 10 μM NS8593 to revert AF was further examined in a rabbit *ex vivo* model. Sustained AF was induced with ACh and burst pacing in all hearts (n=6)(Fig. 6). Following application of 10 μM NS8593, AF was reverted (overall p-value<0.001), and could not be re-induced in any (p<0.01, n=6) of the hearts. After a wash-out period of 20-25 minutes, AF could be re-induced in 3 of 4 hearts(p=0.06), which was not significantly different from the control situation (p=0.8). In two hearts (initial experiments), a wash-out was not conducted. The effects on aERP were as follows (overall p-value<0.001): Before drug application aERP was 75±9 ms, 10 μM ACh shortened aERP to 39±7 ms (p<0.01), co-application of 10 μM NS8593 increased aERP to 110±8 ms (p<0.001), and washout with 10 μM ACh could only partly bring down aERP to 72±15 ms which was significantly shorter than when SK inhibitor was present (p<0.01), but not significantly shorter than when no drug was present. However, the wash-out of NS8593 was sufficient to elicit AF in 3 of 4 hearts.

**SK channel inhibition protects against acutely induced AF in *rat in vivo***

The *ex vivo* data clearly show antiarrhythmic potential with SK channel inhibition and to further substantiate these findings, we employed a rat *in vivo* model of AF. The effects of SK channel inhibition in this model were compared to the effects of amiodarone. The average duration of AF episodes elicited by burst pacing at baseline was 2.6 s. The effects on the duration of AF were as follows (overall p-value<0.001): Injection of vehicle did not change AF durations significantly
compared to baseline recordings (96.3%±33.2%, n=5, p=0.80) (Fig. 7). The overall duration of AF normalized to baseline recordings was significantly shorter in the NS8593 treated group and in the group treated with amiodarone (23.2%±20.0%, p<0.001, n=5, and 26.2%±17.9%, p<0.001, n=5, respectively) compared to the vehicle treated group (96.3%±33.2%, n=5). There were no significant differences in the effects on AF duration in the groups treated with NS8593 or amiodarone.

**NS8593 is a selective inhibitor of SK channels**

Selectivity data on NS8593 have been published on recombinant big- and intermediate conductance Ca**2+**-activated K**+** channels (BK and IK channels, respectively), K<sub>v</sub>7.2, K<sub>v</sub>7.3, K<sub>v</sub>11.1, rNa,1.2 as well as native voltage-dependent Na**+, Ca**2+, and K**+** channels in rat embryonic dorsal root ganglion neurons[14]. To obtain further evidence for a cardiac specific SK channel inhibition of NS8593, additional selectivity data towards K<sub>v</sub>1.4, K<sub>v</sub>1.5, K<sub>v</sub>4.3, K<sub>v</sub>7.1, K<sub>i</sub>2.1, and GIRK3.1/3.4 co-expressed with M2-type ACh receptors were obtained(Fig. 8). NS8593 show no effect in any of these cardiac relevant channels in concentrations of 10 μM.

**Discussion**

**Antiarrhythmic properties of SK channel inhibition**

We here provide evidence for the first time that pharmacological inhibition of SK channels can prevent and revert AF in different models of AF in guinea pig, rat, and rabbit.

Theories of the mechanism of AF involve two main processes: one or more rapidly depolarizing foci that function as triggers of arrhythmia, and a fibrillation-prone atrium that allows re-entry involving one or more circuits[20]. Typically, the triggers of AF originate in the pulmonary vein-atrial junctions[21, 22]. Single- and multiple-circuit re-entry play key roles in the mechanism of AF[19, 20, 22, 23]. The aERP plays a crucial role in re-entry – a long aERP increases the chances that a circulating impulse encounters refractory tissue and subsequently terminates. Both single-
and multiple-circuit re-entry should be amenable to drugs that prolong the aERP and the primary
approach to treating AF is therefore to increase aERP. Episodes of AF leads to electrical
remodeling of the atria in a manner that shortens atrial action potential duration (APD) and aERP,
and promotes AF initiation and maintenance[24].
Our data demonstrate that SK inhibition prevents and reverts AF in acute models of AF, probably
by prolonging aERP, thereby inhibiting re-entry. In neither the *ex vivo* nor the *in vivo* experiments
did we observe any proarrhythmic effects by prolonging aERP. Pharmacological inhibition of SK
channels combined with S2-stimulation did not elicit any episodes of AF *ex vivo*. Similarly,
pharmacological inhibition of SK channels *in vivo* decreased AF-susceptibility to burst pacing
rather than increasing it. Importantly, it should be noted, that from a functional point of view SK
channel inhibition appeared atria-selective since no effect in QT intervals was observed in the tested
concentrations of NS8593.
The involvement of SK channels in AF genesis is substantiated by data from Özgen et al. which
suggest that SK2 channels are involved in atrial remodeling and that upregulation of these channels
in the pulmonary vein-atrial junctions provides a basis for evolution of an arrhythmogenic substrate
by shortening APD[7]. Furthermore, very recently a link between SNP polymorphisms in the gene
encoding SK3 and AF in man has been found[25].
A different piece of evidence for a role of SK channels in AF has been provided by Li et al. who
have demonstrated that SK2 knock-out mice have prolonged atrial APD, show S2-inducible AF,
and atrial EADs under appropriate conditions[9]. Prolongation of atrial APD would normally not be
expected to promote AF, and the electrophysiological remodeling in patients with AF involves
shortening of APD, and thereby the aERP. Thus, Li et al. show that loss of function of SK2
promotes AF in mice, while Özgen et al. suggest that increased SK2 function provides an
arrhythmogenic substrate in rabbits, and we demonstrate that inhibition of SK channels prolongs aERP and prevents and reverts AF in rabbit s, guinea pigs and rats.

This apparent discrepancy may be insignificant due to the fact that both augmentation and reduction of repolarisation in principle can lead to arrhythmogenesis[26, 27]. Also, in the study of Li et al., SK2 channels were completely absent in the mice, why indirect mechanisms of AF cannot be excluded. Furthermore, there is a difference in the species used, as Li et al. used mice, Özgen et al. used rabbits, and we have used rabbit, guinea pig and rat. Another possible explanation could be the fact that mice tend to develop AF episodes of focal origin maintained primarily by early and/or late afterdepolarizations, whereas it is still controversial and unclear whether triggered activity contributes to AF maintenance in large-animal models and patients. This may explain why SK2 knock-out mice develop AF due to APD prolongation and EAD evolution, whereas in rabbits with electrical remodeling the higher SK2 membrane expression likely promotes AF by providing a substrate for AF. Variable expression of ion channels, as well as differences in heart rate and action potential morphology in mice compared to other species might also explain the dissimilar effects of enhancing and reducing SK current observed in different species.

**Apamin and SK channel inhibition**

Apamin is considered the state-of-the art blocker of SK channels. However, evidence for the effects of apamin in cardiac preparations are conflicting; in 2003 Xu et al. reported a significant prolongation of APD from human and mouse cardiac myocytes, that was significantly greater in atrial myocytes as compared to ventricular myocytes, whereas Nagy et al. in 2009 saw no effects of apamin in rat and dog atrial and ventricular multicellular preparations, and in isolated cardiomyocytes.[5, 28] This led Nagy et al. to conclude that SK channels, though present, are not active and do not contribute to cardiac repolarization under normal physiological conditions. In
preliminary experiments we also tested the effects of apamin in atrial myocytes from guinea pigs and in guinea pig Langendorff hearts with induced AF. In atrial myocytes apamin only showed a slight increase in APD at 2 Hz pacing rate and no significant effect at 1 Hz. Concentrations of apamin as high as 1 μM did not revert sustained AF in guinea pig Langendorff hearts (n = 2). This lack of effect of apamin could have a number of explanations. At supramaximal concentrations (1 μM) apamin has previously been found to leave almost half of the SK currents unblocked in chinese hamster ovary cells stably expressing SK1-3.[29] There can be several possible explanations to this; e.g. auxiliary subunits exist that can prevent apamin binding, or apamin block can be modulated by phosphorylation, or apamin has separate mechanisms for binding and blocking that allow the channel to be in a conducting state even when apamin is bound, as opposed to other blockers of SK channels[29, 30]. Thus, apamin may constitute its own third class of SK channel inhibitors different from both the negative modulators and the other pore blockers. Also the existence of an apamin- and scyllatoxin-insensitive isoform of the human SK3 channel has been reported[31]. It seems possible that SK channels in the heart have properties that decrease the blocking effects of apamin, making apamin unsuited for addressing SK currents in the heart.

Potential for SK inhibition in the treatment of AF

The use of conventional antiarrhythmic compounds has been limited by potentially fatal ventricular arrhythmias. Consequently, ongoing drug development has focused on increasing safety by developing atrial-specific agents. Atrial selectivity can be achieved by targeting ion channels that are selectively expressed in the atria or by blocking Na⁺ channels in a state dependent manner that favours blocking of atrial rather than ventricular action potentials, or that are effective at rapid rhythms as AF. The number of known atrial-specific ion channels has until recently been limited to I_{Kur} and I_{KACl}. Several compounds have been developed that show atrial selectivity. Vernakalant
and AZD7009 affects cardiac Na\(^+\) channels and a number of K\(^+\) channels including I\(_{Kur}\) and I\(_{KAC}\), and have proven effective in terminating AF in patients\([32-37]\). Other atrial selective compounds effective in treating AF in pre-clinical models are AVE0118 and NIP-142. Both AVE0118 and NIP-142 block several K\(^+\) channels and NIP-142 blocks Ca\(^{2+}\) and Na\(^+\) channels as well\([38-47]\). Since all of these atrial selective compounds exert their effect by a multiple-channel block, it would be reasonable to think that the SK inhibitors used in this study are not solely affecting SK channels. However, selectivity data on NS8593 show no effect of the compound on other ion channels than SK channels. In addition, two potent SK channel pore blockers, UCL1684 and ICA, structurally unrelated to NS8593 and with a different mode of action than NS8593, exert antiarrhythmic effects comparable to those of NS8593. In combination with the selectivity data, this suggests that the antiarrhythmic effect of NS8593 is primarily a result of SK channel inhibition.

Within the last few years, biochemical evidence has emerged that SK channels are predominantly expressed in the atria compared to the ventricles\([5, 6]\). In the present study, we show that a selective negative modulator of SK channels prolongs aERP concentration-dependently and is able to revert and prevent the induction of AF without affecting the QT-interval.

Thus, the use of specific inhibitors of SK channels may offer a new therapeutic opportunity in the treatment of AF with a decreased risk of ventricular proarrhythmia.

**Conclusion**

We here show that three mechanistically different SK channel inhibitors possess antiarrhythmic properties in three different species both *ex vivo* and *in vivo*. From a mechanistical point of view, the negative modulation of SK channels prolongs aERP concentration-dependently and is able to revert and prevent against the induction of AF without affecting the QT-interval.
Hence, although further studies are needed to address the clinical relevance of these findings, SK channels offer a promising new therapeutic target in the treatment of AF with a decreased risk of ventricular proarrhythmia.

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Figure Legends:

**Figure 1:** Structures and modes of action of the SK channel inhibitors used in this study. Large structural differences can be observed between all three compounds, the negative modulator NS8593 and the two pore-blockers ICA and UCL1684. The difference in mode of action of these compounds is emphasized by their ability to displace [125I]apamin binding. Data on NS8593 and UCL1684 are from Sørensen et al. 2008 and data on ICA is from Gentles et al. 2008.

**Figure 2:** Representative MAP recordings from the left atria of two guinea pig ex vivo hearts. Left column shows a time matched control heart perfused with 1 μM ACh, right column shows a heart perfused with 1 μM ACh followed by co-perfusion of 1 μM ACh and 3 μM NS8593 after the induction of sustained AF. In the time matched control, sustained AF with no spontaneous reversion is induced by S2 stimulation. SK channel inhibition with 3 μM NS8593 reverts sustained AF, and no new episodes of AF can be induced. Time is given in minutes.

**Figure 3:** AF prevention in isolated, perfused guinea pig heart. A) Representative recordings from isolated, perfused guinea pig heart of ECG and MAPs from left ventricle (LV) and left atrium (LA) in the presence of no drug, 3 μM NS8593, and 3μM NS8593 + 1 μM ACh combined with S2 stimulation and with 3μM NS8593 + 1 μM ACh combined with burst pacing. aERP is prolonged in the presence of 3 μM NS8593 with no effect on QT interval or ventricular APD. Addition of 1 μM ACh blocks AV node conduction and shortens aERP, but this heart is protected against AF induction by S2 stimulation and burst pacing. B) NS8593 concentration-dependently increases aERP with no effect on QT interval in concentrations from 0.3 – 3 μM. C) NS8593 concentration-dependently protects against the induction of AF.
**Figure 4:** AF reversion in isolated, perfused guinea pig heart. A) Representative recordings from isolated, perfused guinea pig heart of ECG and MAPs from left ventricle and left atrium in the presence of no drug, 1 μM ACh, and 1 μM ACh + 3μM NS8593 combined with S2 stimulation and with μM ACh + 3μM NS8593 combined with burst pacing. 1 μM ACh combined with S2 stimulation elicits sustained AF. Co-perfusion of 3 μM NS8593 terminates AF and protects against the re-induction of AF by S2 stimulation and by burst pacing. B) NS8593 concentration-dependently reverts and protects against AF. C) UCL1684 and ICA, two SK channel inhibitors with modes of action different from NS8593, reverts and protects against AF in a concentration of 1 μM in the same manner as 10 μM NS8593. The effects of the SK channel inhibitors are comparable to the effects of 10 μM amiodarone.

**Figure 5:** AF reversion in isolated, perfused rat heart. A) Representative recordings from isolated, perfused rat heart of ECG and MAPs from left ventricle and left atrium in the presence of no drug, 10μM NS8593, and after washing combined with S2 stimulation and with 10μM NS8593 combined with burst pacing. S2 stimulation elicits sustained AF in the presence of no compound. Perfusion with 10 μM NS8593 terminates AF and protects against the re-induction of AF by S2 stimulation and by burst pacing. After washing, AF can be re-induced by S2 stimulation. B) Application of 10 μM NS8593 prolongs aERP in the rat hearts from 15.5±3.8 ms in the control situation to 43.5±7.0 ms (p<0.001, n=6). After wash-out aERP is shortened to 23.0±5.2 ms, which is not significantly different from the control situation. C) S2 stimulation elicits sustained AF in the presence of no compound in all hearts. Perfusion with 10 μM NS8593 terminates AF and protects against the re-induction of AF by S2 stimulation and by burst pacing in all hearts. After washing, AF can be re-induced by S2 stimulation in all hearts.
**Figure 6:** A) Application of 10 μM ACh shortens aERP in the rabbit hearts from 75±9.ms to 39±7ms (p<0.01, n=6) co-application of 10 μM NS8593 with ACh increases aERP to 110±8ms (p<0.001), and washout with 10 μM ACh decreases aERP to 72±15ms which is significantly shorter than with SK inhibitor present (p<0.01), but not significantly shorter than when no drug was present. However, the wash-out of NS8593 was sufficient to elicit AF in 3 of 4 hearts as shown in B) Burst pacing elicits sustained AF in the presence of 10 μM ACh in all hearts. Co-perfusion with 10 μM NS8593 terminates AF and protects against the re-induction of AF by burst pacing in all hearts (p=0.002, n=6). After washing with ACh, AF can be re-induced by burst pacing in 3 of 4 hearts, which is a significant increase (p=0.03), and not significantly different from the control situation (p=0.4).

**Figure 7:** AF protection in rat heart *in vivo*. A) Representative recordings of MAPs from the right atrium of an *in vivo* rat heart in the presence of no drug and after injection of 5 mg/kg NS8593. Burst pacing of the right atrium for 30 s with concomitant asphyxia elicits an episode of AF which reverts spontaneously within seconds. Injection of 5 mg/kg NS8593 shortens the duration of the episodes of AF. B) In the vehicle-injected group the duration of AF does not change significantly compared to baseline recordings. Injection of 5 mg/kg of either NS8593 or amiodarone shortens AF duration significantly compared to the vehicle-injected group.

**Figure 8:** Selectivity data on NS8593. A) Representative traces from *Xenopus laevis* oocytes expressing either Kv1.4, Kv1.5, Kv4.3, Kv7.1, Kᵦ.2.1, and GIRK3.1/3.4, thereby mimicking the pore forming subunits of Iᵦ, Iᵦᵦ, Iᵦᵦ, Iᵦ, and Iᵦᵦ, respectively. No significant effect of 10 μM NS8593 was observed on any of these currents (n=4-6).
<table>
<thead>
<tr>
<th>Mechanism of action</th>
<th>$[^{125}]$-Apamin binding (µM)</th>
<th>SK3 whole-cell (µM)</th>
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<td>Pore blocker</td>
<td>0.0018 ± 0.0003 ($K_i$)</td>
<td>0.0027 ± 0.0004 ($K_d$)</td>
</tr>
<tr>
<td>UCL1684</td>
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<td></td>
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<td>Pore blocker</td>
<td>0.025 ± 0.008 ($IC_{50}$)</td>
<td>0.29 ± 0.05 ($IC_{50}$)</td>
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<tr>
<td>Modulator</td>
<td>&gt;50</td>
<td>0.077 ± 0.007 ($K_d$)</td>
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<tr>
<td>NS8593</td>
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<td></td>
</tr>
<tr>
<td>T = 0</td>
<td>Control</td>
<td>3 μM NS8593 at T = 2</td>
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<td>T = 40</td>
<td><img src="T=40" alt="Control" /></td>
<td><img src="T=40" alt="3 μM NS8593 at T = 2" /></td>
</tr>
</tbody>
</table>
A

Control + S2 stimulation

ECG
LV
LA

10 μM NS8593 + S2 stimulation

ECG
LV
LA

10 μM NS8593 + burst pacing

ECG
LV
LA

Wash + S2 stimulation

ECG
LV
LA

B

\( \text{aERP (ms)} \)

\[ \begin{align*}
\text{Control} & \quad 30.0 \pm 2.5 \\
10 \mu M \text{NS8593} & \quad 45.0 \pm 3.0 \\
\text{Wash} & \quad 35.0 \pm 1.5 \\
\end{align*} \]

C

Reversion and protection against AF (%)

\[ \begin{align*}
\text{Control} & \quad 0/6 \\
10 \mu M \text{NS8593} & \quad 6/6 \\
\text{Wash} & \quad 0/6 \\
\end{align*} \]
Initiation of burst pacing, baseline recording

Termination of burst pacing, baseline recording

Reversion to sinus rhythm, baseline recording

Termination of burst pacing after 5 mg/kg NS8593

AF-duration compared to baseline

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>NS8593</th>
<th>Amiodarone</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*** p < 0.001
Inhibition of Small Conductance Ca$^{2+}$-Activated K$^+$ Channels Terminates and Protects against Atrial Fibrillation

Jonas Goldin Diness, Ulrik Svane Sørensen, Jakob Dahl Nissen, Baha Al-Shahib, Thomas Jespersen, Morten Grunnet and Rie Schultz Hansen

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Supplemental Material

Supplemental Methods

Isolation of Single Cardiac Myocytes and Recordings of Action Potentials

Atrial myocytes were isolated from 9-16 weeks old female guinea pigs of the Dunkin Hartley strain (HsdPoc:DH) weighing 500 g to 800 g using the procedure described in 1. Effects of 10 µM NS8593 and 100 nM apamin on action potentials from atrial myocytes paced at 1 Hz or 2 Hz were recorded as previously described 2.

Cell Culture and Electrophysiology on Recombinant Channels

Patch-clamp studies in the whole-cell configuration using recombinant channels were performed with 10 µM amiodarone on HEK293 cells stably expressing SK2 channels as described in3. A holding potential of 0 mV was used in combination with a voltage-ramp protocol from -100 mV to 100 mV over 500 ms. Mean current from -80mv to -60mv and from 20 mV to 40 mV was analyzed.

Oocyte Isolation and Injection

Isolation, maintenance and cRNA injection of Xenopus laevis oocytes was performed as previously described⁴.
**In Vitro Transcription**

cDNAs encoding human Kv1.4, Kv1.5, Kv4.3, Kv7.1, Kv2.1, or GIRK3.1/3.4 with M2-type ACh receptors (conducting $I_{KACH}$) were subcloned into the dual-function expression vector pXOOM, containing the 5’- and 3’-untranslated regions for *Xenopus laevis* β-globine as well as a poly-A segment, as described previously\textsuperscript{5}. cRNA for injection was prepared from linearized cDNA using the T7 m-Message Machine kit (Ambion, Austin, TX, USA) according to the manufacturer’s instructions. RNA concentrations were quantified by UV spectroscopy and RNA quality was checked by gel electrophoresis.

**Two-electrode Voltage Clamp Recordings**

Currents were recorded at room temperature 48-96 hours after injection using a two-electrode voltage-clamp amplifier (CA-1B, Dagan, Minneapolis, MN) and PULSE acquisition software (HEKA, Lambrecht, Germany). Oocytes were immersed in Kulori medium (90 mM NaCl, 4 mM KCl, 1 mM MgCl\textsubscript{2}, 1 mM CaCl\textsubscript{2}, 5 mM HEPES, pH 7.4) and subject to a constant flow of Kulori medium during recordings. Electrodes were pulled from borosilicate glass capillaries on a horizontal patch electrode puller and had a tip resistance between 0.3 and 2.0 MΩ when filled with 2 M KCl. Step protocols from -100 mV to + 60 mV in 10 mV increments lasting for 1 s were applied to activate $K_v$ channels. $K_v$2.1 and GIRK channels were activated by application of continuous 0.5 s ramps from -120 mV to + 60 mV. The effect on GIRK channels with the M2-type ACh receptor co-expressed was measured in the presence of 10 µM ACh.
Supplemental Results

Effects of SK Inhibitors on Atrial APD

Application of 10 µM of the negative SK channel modulator NS8593 caused a significant increase in APD$_{90}$ at both 1 Hz and 2 Hz pacing frequency (37.0 ± 15.3%, $p = 0.02$, $n = 5$ and 39.7 ± 16.5%, $p < 0.0001$, $n = 7$, respectively)(Suppl. Fig. 1).

The application of 100 nM of the SK channel blocker apamin caused no significant increase in APD$_{90}$ at 1 Hz and a slight, but significant increase in APD$_{90}$ at 2 Hz pacing frequency (5.3 ± 8.0%, $p = 0.24$, $n = 4$ and 10.7 ± 12.5 %, $p = 0.004$, $n = 10$, respectively)(Suppl. Fig. 1).

Effects of Amiodarone on SK2 Channels

Application of 10 µM on HEK293 cells stably expressing SK2 had no effects on current levels compared to the control situation (Suppl. Fig. 2).
Supplemental Figure 1

Effects of SK channel inhibitors on APD$_{90}$ in isolated atrial myocytes at room temperature.

Top: Apamin (100 nM) caused no significant increase in APD$_{90}$ at 1 Hz and a very slight, but significant increase in APD$_{90}$ at 2 Hz pacing frequency (5.3 ± 8.0%, $p = 0.24$, $n = 4$ and 10.7 ± 12.5 %, $p = 0.004$, $n = 10$, respectively). Bottom: NS8593 (10 µM) caused a significant increase in APD$_{90}$ at both 1 Hz and 2 Hz pacing frequency (37.0 ± 15.3%, $p = 0.02$, $n = 5$ and 39.7 ± 16.5%, $p<0.0001$, $n = 7$, respectively).
Supplemental Figure 2

Effects of amiodarone on HEK293 cells stably expressing SK2 Channels. Top:
Representative current traces from a cell in the presence of either no compound or 10 µM amiodarone. Bottom: Analysis of mean currents from -80mv to -60mv and from 20 mV to 40 mV revealed no effects of amiodarone.


