**The SK4 channel allosteric blocker, BA6b9, reduces atrial fibrillation substrate in rats with reduced ejection fraction following myocardial infarction**

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**Short title:** BA6b9 inhibits atrial fibrillation substrate

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**Summary**

**Background** Atrial fibrillation (AF), the most common cardiac arrhythmia, is strongly associated with several comorbidities including heart failure (HF). AF in general and specifically in the setting of HF has a progressive nature and is associated with poor clinical outcome. Current therapies for AF are limited in number and efficacy and do not target the underlying causes of atrial remodeling like inflammation and fibrosis. We previously identified a new druggable target against AF, namely the calcium-activated SK4 K+ channels, which are preferentially expressed in the atria vs. the ventricles in both rat and human hearts.

**Methods and Findings** We examined the ability of BA6b9, a novel allosteric blocker inhibiting SK4 channels at the specific calmodulin-PIP2 binding domain, to alter AF susceptibility and atrial remodeling in a systolic HF rat model of post-myocardial infarction (post-MI). Daily BA6b9 injection (20mg/kg/day) for 3 weeks starting 1-week post-MI prolonged the atrial effective refractory period, reduced AF induction and duration and dramatically prevented atrial structural remodeling. In the post-MI left atrium (LA) the level of SK4 K+ channel expression was markedly upregulated in association with increased collagen deposition, α-SMA and the NLRP3 inflammasome. Remarkably, the treatment with BA6b9 reversed these changes. Moreover, BA6b9 treatment significantly reduced the lateralization of atrial connexin Cx43 in the LA of post-MI rats.

**Interpretation** Our findings indicate that blockade of SK4 K+ channels by BA6b9 not only favors rhythm control, but remarkably reduces atrial structural remodeling, a property that is highly desired for novel AF therapies, particularly in patients with HF co-morbidity.

Keywords: potassium channel, SK4 channel, KCa3.1, atrial fibrillation, atrial remodeling, HFrEF

**Research in context**

**Evidence before this study**

Atrial fibrillation (AF) and heart failure (HF) are two cardiovascular challenges of major significance. Synergistically with HF, AF leads to poor clinical outcome and increased mortality. AF has a progressive nature and current therapies, especially in HF patients, are limited in number and efficacy and do not target AF promoting processes in the atrial tissue such as inflammation and fibrosis, leaving a substantial unmet need for therapeutic innovation. Recent data indicate that SK4 calcium-activated K+ channel, are expressed in cardiomyocytes predominantly in supraventricular tissues. In addition, these channels are overexpressed in activated fibroblasts and macrophages. Thus, blockage of these channels may serve as an attractive new therapeutic strategy to prevent AF progression.

**Added value of this study**

We found that BA6b9, a novel allosteric blocker inhibiting SK4 channels at the specific calmodulin-PIP2 binding domain, markedly inhibit AF susceptibility and atrial inflammation and fibrosis in rats with systolic HF following myocardial infarction.

**Implications of all the available evidence**

Blockade of SK4 K+ channels by BA6b9 can be an attractive new strategy to inhibit AF development and progression in patients suffering from systolic HF.

**Introduction**

Atrial fibrillation (AF) is the most common cardiac arrhythmia, affecting 1-2% of the population worldwide (1, 2). The prevalence of AF steeply increases with age and the lifetime risk is estimated to be as high as 22-26% (3). AF is characterized by rapid and disordered electrical activation of the atria leading to compromised contraction. It is associated with higher rates of stroke, heart failure, hospital admissions and mortality, thus representing an important medical challenge (4). Common conditions such as ischemic heart disease and heart failure (HF) with either reduce ejection fraction (EF) or preserved EF (HFrEF and HFpEF, respectively) are important etiological factors for AF (5, 6) and they converge together with additional etiologies such as hypertension, obesity, diabetes mellitus and sleep apnea to increase the risk of AF in ways that are still elusive (7). AF is a progressive condition resulting in greater risk of recurrence and persistence over time, challenging all treatment efforts (8). Importantly, when associated with HF, AF leads to poor clinical outcome and pharmacological treatment options for rhythm control are limited (9, 10).

The pathophysiology of AF is complex and multi-factorial. AF initiation involves sources of sustained rapid electrical activity (“drivers”) that can trigger arrhythmic episodes (11). In addition, various mechanisms may alter the electrical and structural properties of the atria and thus, promote tissue susceptibility to AF episodes (“AF substrate”) (1, 8). In addition, AF itself induces atrial electrical and structural remodeling increasing the AF substrate (8, 12). Antiarrhythmic drugs aimed to prevent AF recurrence and to sustain normal sinus rhythm are extensively used but are only partially effective and are not exempt of pro-arrhythmic and extra-cardiac side effects (8, 13-15). Number of novel atrial selective antiarrhythmic targets have been the focus of extensive research in the last two decades without success in reaching advanced clinical studies so far (15, 16 ). From a different point of view, therapeutic modalities intended to attenuate or revert underlying pathophysiological processes that promote AF substrate are attractive potential adjuncts to current management. Various insults such as inflammation, oxidative and ER stress, abnormal proteostasis, apoptotic signaling, autophagy and fibrosis are all considered as drug targets in this context (17-21).

We previously identified a new druggable target in the heart, namely the calcium-activated SK4 K+ channels, whose existence was overlooked in cardiac tissues (22-26). More recently, other groups identified SK4 channels in canine heart as well (27-29). SK4 K+ channels are well known to be expressed in the immune system such as T cells, B cells or macrophages as well as in endothelial cells, fibroblasts and proliferating smooth muscle cells of the vascular system (30-33). Recently, we showed that the SK4 channel protein is widely expressed in atria of rat and human hearts and to a lower extent in the ventricles (23). A novel allosteric blocker, BA6b9, was designed to act to the calmodulin-PIP2 binding domain, a previously untargeted region of SK4 channels, at the specific interface of the proximal C-terminus and the linker S4-S5 (23). It does not interact with SK1, SK2 and SK3 K+ channels as well as with other important cardiac channels. In rat heart *ex vivo*, BA6b9 as well as the generic SK4 channel blocker Tram-34, significantly prolonged atrial and atrioventricular effective refractory periods (AERP and AVERP, respectively) and inhibited the ability of carbachol to sustain AF following burst pacing. SK4 K+ channels are also expressed in mononuclear cells and fibroblasts (34) and their suppression by Tram-34 was recently shown to inhibit pro-inflammatory processes and tachypacing-related atrial remodeling in a canine model (27, 28). Thus, we hypothesized that apart from the direct effect of SK4 K+ channels blockage on the atrial electrophysiology, it may have profound effects on atrial remodeling by inhibiting inflammation and fibrosis, which appear to be specifically prominent in the context of HF (9, 35). Hence, we assumed that SK4 K+ channels are pro-arrhythmic and pro-inflammatory and blocking them may prevent atrial remodeling and AF progression in the context of HF.

Here, we examined the ability of the SK4 channel allosteric blocker, BA6b9, to alter AF susceptibility and atrial remodeling in a HFrEF rat model (EF<40%). Results indicate that daily BA6b9 injection (20mg/kg/day) for 3 weeks starting 1-week post-myocardial infarction (MI) did not affect ventricular function but markedly attenuated both AF induction and duration in this setting. In addition, we found that the atrial levels of SK4 K+ channels are markedly upregulated in the post-MI rats in conjunction with increased expression of NLRP3 inflammasome, lateralization of atrial connexin Cx43 and increased collagen deposition. Treatment with BA6b9 attenuated all the above detrimental changes indicating that blockade of SK4 K+ channels by BA6b9 not only favors rhythm control but can also inhibit structural remodeling, a property that is highly desired, notably in the setting of HF.

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**Materials and Methods**

**Patch clamp validation recordings**

*In vitro* expression of SK4 channels and performance of validation whole cell and inside out patch clamp recordings were performed as described in details in Burg et al. 2022 (23). In brief:

Human SK4 inserted into pEGFP-C1 vector plasmid (pEGFP-SK4) was used to transfect Chinese hamster ovary (CHO) cells that were grown in Dulbecco’s modified Eagle’s medium supplemented with 2 mM glutamine, 10% fetal calf serum, and antibiotics, The cells were seeded (20,000 / well) on poly-L-lysine-coated glass coverslips (13 mm in diameter) in a 24-multiwell plate and transfected with 0.5µg pEGFP-SK4 using TransIT-LT1 Transfection Reagent (Mirus Bio) according to the manufacturer’s protocol. For electrophysiology, transfected cells were visualized approximately 40h after transfection with a Zeiss Axiovert 35 inverted florescence microscope.

**BA6b9**

BA6b9 synthesis has been described in detail previously (23). For *in vitro* validation studies BA6b9 powder was dissolved in DMSO and applied to the cells at a concentration of 10 M. The final concentration of DMSO was 0.1% (23). For the rat studies BA6b9 was dissolved in sesame oil Sigma-Aldrich (St. Louis, MO, USA) to create a stock solution of 10mg/ml which was stored at 4oC and injected intra-peritoneally once a daily at a concentration of 20mg/Kg. Vehicle treatment contained sesame oil alone.

**Animals**

All animal studies in this study were approved by the Institutional Ethics Committee of Ben-Gurion University of the Negev, Israel (Protocol IL-24-05-2020D). Experiments were performed on adult (8 weeks, ~280gr) Sprague-Dawley male rats obtained from Envigo Laboratories LTD (Jerusalem, Israel). Rats were kept under standardized conditions throughout the study (36, 37) The rats had environmental enrichment and were monitored on a daily basis for signs of stress or inappropriate weight loss, according to guidance from the Ben-Gurion University veterinary services (assured by the Office of Laboratory Animal Welfare (OWLA) no. A5060-01 and fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

**Implantable electrophysiological device**

For atrial electrophysiological (EP) studies we used a chronically implanted EP device, which was previously described in details ((36-38) and includes peripheral ECG leads as well as an atrial quadripolar electrode adapted for advanced supraventricular EP studies in the freely moving state. Briefly, the device includes an 8-pin connector that is attached by highly flexible insulated electrical wires (AS155-36, Cooner wires, Chatsworth, CA) to the atrial quadripolar electrode and to three additional peripheral ECG leads. The atrial quadripolar electrode is composed of 4 Platinum-Iridium electrical poles that are embedded in medical grade silicon (MED-6219P, Nusil, CA) and fixed to the atria by miniature stainless steel hooking pins (26002-10, Fine Science Tools, Vancouver, Canada). Full details and photos of the design and fabrication of the quadripolar electrode are described in Murninkas et al. 2021 (37). All components of the instrumented device are biocompatible. Following preparation and before instrumentation each device was thoroughly cleaned with 70% ethanol and was sterilized by electron beam radiation.

**Surgical procedure for EP device implantation and induction of MI**

A procedure including EP device implantation and left coronary artery ligation was performed in each animal as previously described (36). Briefly, animals were anesthetized, (IM ketamine/xylazine 75/5 mg/kg) mechanically ventilated and placed on a heating pad. Under sterile conditions, left thoracotomy was performed and the left coronary artery was ligated followed by chest closure. Next, right thoracotomy was performed and the atrial quadripolar electrode was inserted on the right atrium followed by chest closure. ECG leads were inserted subcutaneously, and the device connector was exteriorized through the skin of the neck. A shielding ring that was sutured to the skin of the neck prevented extraction of the device over time (36). Postoperative recovery and analgesia were performed as described previously (36, 37).

**Echocardiography**

Echocardiography measurements were performed with Vevo 3100 Preclinical Imaging System (FUJIFILM VisualSonics, Canada) as previously described (36, 37). During the procedure, rats were lightly anesthetized by 1.5% isoflurane and positioned on a heating pad to maintain a rectal temperature of ~37 °C. 2D images of the left ventricle were taken in parasternal long and short axis, and apical four chamber views. Long and short axis M-mode images were obtained at the mid papillary muscles area. LV end-diastolic diameter (LVIDd) and LV end-systolic diameter (LVIDs) were obtained from the long-axis M-mode trace. Left ventricular EF was calculated using planimetry as follows: EF = 100 × (LVIDd − LVIDs/LVIDd). LV trace was obtained from parasternal long-axis B-Mode. Left atrial diameter was obtained from an apical four chamber view. All measurements were averaged for three consecutive cardiac cycles and performed by an experienced technician who was blinded to the pharmacological treatment of the animals.

**Experimental design and EP evaluation**

The experimental design and flow of the current study are illustrated schematically in Figure 1. Seven days following the initial surgery a first echocardiogram was performed. Rats having an LV trace EF ≤40% were considered as having significant LV dysfunction and were included in the study and connected to the EP setup for initial EP study on the next day (Post-operative day 7). Following initial EP study, animals were randomized for treatment with daily intraperitoneal (i.p.) injection of either BA6b9 (20mg./kg/day) dissolved in sesame oil or vehicle treatment (sesame oil alone). On post-operative day 28 the animals went through a final echocardiography and were connected to the EP setup for a final EP study on the next day. This EP study was performed 20-24 hours following the last i.p. injection of BA6b9 or vehicle treatments. Following the final EP, tissues were collected under deep pentobarbital anesthesia.

EP studies were performed as previously described (37), with minor modifications. In brief, for each EP evaluation, rats were placed in dedicated recording chambers where each instrumented device connector was attached to the pacing and recording apparatus through an elastic electrical cable. The proximal part of the elastic cable was connected to the electrophysiological system through a multi-channel commutator (PLA-SL12C/SB, PLASTICS One Inc., CA) that maintained stable electrical connections while the rats were freely rotating in the cages. In each animal two atrial poles were selected for pacing and were electrically connected to an optically isolated pacing unit (STG4002-16mA, Multichannels, Reutlingen, Germany). The remaining two atrial poles and the three peripheral ECG electrodes were connected to a voltage amplifier (Amplifier 1700, A-M systems, Carlsborg, WA). As previously described (37), the electrode side that was used for pacing was empirically determined based on a relatively low capture threshold and an ability to differentiate the atrial signal from the stimulus artifact in the recordings from the other side. Signals were filtered (1–1000 Hz) and sampled to the PC at a digital sample rate of 2 KHz. A self-made program created using Labview 7.1 (National Instruments, Austin, TX) controlled data acquisition and electrical stimulation. EP studies were performed in an unanesthetized state following overnight adaptation to the EP cages and during daylight hours of the circadian cycle when animals were inactive, as previously described (36, 37, 39). RR, PR and QT intervals were averaged from five cycles on the non-paced ECG. An atrial pacing capture threshold was obtained using bipolar square current pulses (total duration 4 ms; 2 ms in each direction), and stimulus intensity was raised to double threshold for the rest of the EP study. A programmed S1S2 stimulation protocol (S1=10) was used to determine the atrioventricular node refractory period (AVERP) and the atrial effective refractory period (AERP) in the millisecond range. AVERP was determined using a basic cycle length (BCL) of 120ms. AERP measurements were performed using two different basic BCL; 70ms and 120ms. AVERP and AERP were confirmed three consecutive times. Sinus node recovery time (SNRT) was obtained using 30s burst pacing protocols applied 3 times with 30s pause between the bursts. Arrhythmic substrate evaluation included 20 consecutive triggering bursts (1s duration, 10ms cycle length). Arrhythmic episodes lasting more than 4 minutes were aborted using short (1S) pacing bursts of increasing intensity until sinus rhythm was restored. The minimal time between pacing bursts was 1 minute from the end of an event. If an episode of more than 60s was detected, the delay from the end of this episode to the next pacing burst was equal to the duration of the AF episode. The cutoff for defining a positive arrhythmic event was defined as >1s following the burst pacing protocol.

To avoid bias of the AF analysis by regular stable arrhythmic episodes (36, 37) we separated between regular and irregular events in our analysis. We have previously noted that regular arrhythmic episodes are characterized stable cycle length > 60 ms (38). Thus, AF was defined in the current study as fast irregular atrial electrocardiograms or atrial waveforms in which the main repeating component had a duration < 55 ms. Regular arrhythmic waveforms were analyzed separately from the AF analysis.

**Power spectrum analysis of AF waveforms**

For power spectrum analysis of the AF waveforms, we cleaned the atrial signals from ventricular mixing based on QRS detection in the peripheral ECG, as described in detail in Murninkas et al. 2023 (38). Next, power spectrum analysis of the AF signals was performed using conventional Fast- Fourier-Transform (FFT) (38). Since recordings were all performed in the presence of a notch filter reducing the signals around 50 Hz, an artificial depression was detected around this frequency (38).

**Histology**

Hearts were excised and thoroughly washed with PBS solution. Left atrial (LA) tissues were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned in short-axis slices (4 μm thickness) and stained on positively charged glass slides (Histobond+,Marienfeld). LA tissue of heathy (naïve) adult male SD rats were used as reference for the baseline state in all stains.

Immunohistochemistry staining, an automated immunohistochemistry (IHC) system (BONDIII fully automated IHC staining system, Leica Biosystems, Buffalo Grove, Illinois, 60089, USA) was used. IHC antigen retrieval was performed using BOND Epitope Retrieval Solution 2 (Tris-EDTA, prediluted, pH 8.0) for 20 min at 100 °C. For left atrial fibrosis and ventricular infarct size assessment, the sections were stained with Masson’s trichrome. For detection and localization of SK4 channels in the working myocardium and endocardial tissue of rat hearts, we incubated the specimens with the primary antibody (IK1 D-5: sc-365265, SANTA CRUZ, mouse monoclonal antibody, 1:50) for 40min, stained with the secondary antibody for 30min, and stained with 3,3′-Diaminobenzidine (DAB) for 15min (Bond Polymer refine detection kit, LE-DS9800, Leica biosystems). For detection of α-SMA and NLRP3 we have incubated the specimens with the primary antibodies α-SMA (EPR5368, ab124964, abcam, rabbit monoclonal antibody against the N-terminus of human α-SMA, 1:1000) and NLRP3 (bs-10021R, Bioss Antibodies, rabbit polyclonal antibody against human NLRP3, 1:200), respectively, for 40min, stained with the secondary antibody for 30min and followed by labeling with RED-alkaline phosphatase for 15min (Bond Polymer Refine Red detection kit, LE-DS9390, Leica biosystems). The specimens were then counterstained with hematoxylin and cover-slipped. The stained sections were left to dry for 24h and scanned the following day via slide scanner Aperio (Aperio Versa 200 DM6000 B, Leica Biosystems) and analyzed using Qupath, a quantitative pathology and bioimage analysis software as described (23).

**Immunofluorescence**

The slides were visualized with a laser confocal microscope (Leica SP8. Leica Biosystems, Buffalo Grove, Illinois, 60089, USA) and the images were analyzed and processed using Qupath, ImageJ (National institute of health and University of Wisconsin, USA), and Adobe illustrator (Adobe Systems, Inc., Mountain View, CA). Antigen retrieval was performed using BOND Epitope Retrieval Solution 1 (Citrate Buffer, prediluted, pH 6.0) for 20 min at 100 °C, followed by peroxide for 10min. For membranal staining, we primarily incubated the sections with Wheat Germ Agglutinin (WGA) (29022, CF®488 WGA, Biotium, 1:500) conjugated with Alexa Fluor® 488 for 30min. WGA was diluted with PBS and only then, followed by blocking buffer (composed of normal goat serum 10%, Triton 0.1% and Bovine Serum Albumin 10%) for 30min. For SK4 expression and localization we incubated the specimens with the primary antibody (ALM-051, Alomone labs, mouse monoclonal antibody against the 3rd extracellular loop of human SK4, 1:50) for 90min and stained with the secondary antibody Cy3 (711-165-151, Jackson immunoresearch laboratories, CyTM3-conjugated AffiniPure Donkey Anti-Mouse, 1:100) for 90min. For connexin 43 expression and localization we incubated the specimens with the primary antibody (C6219, Sigma-Aldrich, rabbit polyclonal antibody against the C-terminus of human/rat conexin-43, 1:400) for 90min and stained with the secondary antibody Cy5 (711-175-152, Jackson immunoresearch laboratories, CyTM5-conjugated AffiniPure Donkey Anti-Rabbit, 1:200) for 90min. At the end of the staining, we incubated the specimens with Vector® TrueVIEW® Autofluorescence Quenching kit (SP-8400-15, Vector laboratories) for improvement of signal-to-noise ratio and reducing autofluorescence, for 10min. Lastly, the specimens were stained with DAPI (D9564, Sigma-Aldrich, 1:1000). The specimens were then cover-slipped with VECTASHIELD Vibrance® Antifade Mounting (SP-8400-15, Vector laboratories) and were left to dry for 24h and scanned the following day.

**Histological analyses**

Cardiac fibrosis and collagen deposition were measured by Masson’s trichrome staining. LA myocardial and epicardial fibrosis were analyzed in the myocardium and epicardium of the entire section for a total of 18 images per animal. A pre-determined threshold was selected, differentiating between normal and fibrotic tissue. Total fibrosis was calculated as the percentage of interstitial fibrosis in Qupath, a quantitative pathology and bioimage analysis software. SK4 channel, α-SMA and NLRP3 expression were measured by IHC by implementing the same analysis methods. Extra-cellular matrix (ECM) accumulation was measured by WGA staining in left atrial myocardial tissue 0.25µm snapshots. The entire image was selected with a total of 18 images per animal. A pre-determined threshold was selected, measuring the amount of ECM in healthy and post MI atrial tissue. For connexin-43 lateralization analysis in atrial sections, only longitudinal sections were selected. we performed immunofluorescent double staining, using WGA and Connexin-43. Lateralization was determined by Connexin-43 localization compared to WGA staining marking the membrane of longitudinal cardiomyocytes only. Lateralization results were presented and quantified as the ratio between Connexin-43 length (µm) when paralleled/colocalized with the cell’s membrane (+SEM) to the cell’s width+SEM (i.e. Connexin-43 length in the gap junction).

**Statistical Data analysis**

A total of 4 naïve, 7 vehicle-treated and 8 BA6b9-treated rats were used in the final analyses. Data analysis was performed using Prism 9.0 (GraphPad Software, Inc., San Diego, CA). All data were expressed as mean ± S.E.M. For analysis, normality assumptions were tested using the Shapiro-Wilk test. Accordingly, unpaired students t-test and one-way ANOVA with Tukey post hoc testing were used for normally distributed results whereas Mann-Whitney and Kruskal–Wallis with Dunn’s multiple comparisons post-test were used for non-normally distributed results. Analysis comparing the results of the same instrumented rats at 1 week vs. 4 weeks were performed using paired students t-test or Wilcoxon rank sum test for normal and non-normal distributed results, respectively. Details of the specific statistical analysis are indicated in each figure / table. The criterion for significance was set at p < 0.05. The p-values are displayed graphically as follows: \* p < 0.05, \*\* p < 0.01, and \*\*\* p < 0.001.

**Results**

**In vitro confirmation of BA6b9 potency**

Before performing the *in-vivo* experiments, we confirmed that the batch of our BA6b9 drug worked properly on recombinant human SK4 K+ channels expressed in transfected CHO cells. Like in our previous work, BA6b9 inhibited by 56 ± 2% (20 µM, n=34, p<0.0001) and 66 ± 5% (10 µM, n=6, p=0.0018) the SK4 K+ currents recorded using the whole-cell and the inside-out configurations of the patch-clamp, respectively (Figure 2A).

**Long-term BA6b9 treatment attenuates the AF substrate of post-MI rats with HFrEF**

Our instrumented EP device (36, 39) including the recently developed atrial-quadripolar electrode (37, 38), enabled detailed evaluation of the impact of BA6b9 treatment on the supraventricular EP and particularly the AF substrate of post-MI rats in the freely moving state. As we previously showed, the AF substrate that develops in this model inversely correlates with EF (36). Thus, we specifically focused our current study on rats with an EF≤40% at the end of the post-operative recovery week. In addition, since our study focused on atrial remodeling and AF substrate, we intentionally started the randomized treatment protocol only following the post-operative recovery week (Figure 1), so the acute recovery phase from the device implantation and the MI already passed. As expected following randomization, the baseline parameters in the two arms of the study were not different from each other in-terms of body weight, heart rate, echocardiographic parameters (including EF) and the various EP parameters (Table 1). The treatment with BA6b9 (20mg/kg/day) for 3 weeks had no effect on the body weight gain during the study. In addition, echocardiographic analysis revealed that the BA6b9 treatment did not significantly affect LVIDd and LVIDs, EF or LA diameter of the animals (Table 1). EP analysis indicated no effect of long-term BA6b9 treatment on the heart rate (HR), the PR and QT intervals as well as the corrected sinus node recovery time (CSNRT). Importantly, the BA6b9 treatment group did have significantly longer AERP at the end of the study as well as a tendency of increase in AVERP, which did not reach significance. Remarkably, BA6b9 treatment significantly reduced both AF induction and AF duration (Figure. 2B). In addition, in the BA6b9 group eyeball tracing of arrhythmic episodes typically indicated a less complex pattern (Figure. 2C). Accordingly, power spectrum analysis of the induced arrhythmic episodes indicated marked differences between the BA6b9 and the vehicle treated group (Figure. 2D), with attenuated power in the BA6b9 group over a wide range of frequencies, which was most prominent around 15Hz (the dominant frequency in both groups) (Figure 2E and Supplementary Figure S1). Overall, these findings demonstrate that while BA6b9 did not alter ventricular function in HFrEF rats, it markedly attenuated the AF substrate, mildly increased AERP and modified the properties of induced arrhythmic episodes (see discussion).

**BA6b9 inhibits post-MI structural remodeling of the LA**

As SK4 K+ channels are not only expressed in cardiomyocytes but also in fibroblasts and macrophages (23, 28-30, 32, 33), which all possess the inflammatory signaling machinery (17), we hypothesized that their pharmacological inhibition can reduce AF substrate by preventing atrial structural remodeling.

In order to properly evaluate the structural effects of BA6b9 treatment in the post -MI setting, we added a control group on naïve rats to the analysis. As expected, Figure 3 shows that collagen deposition in the LA as measured by Masson-trichrome staining, markedly increased in vehicle-treated rats subjected to MI as compared to control rats. In contrast, BA6b9 treatment for 3 weeks markedly attenuated LA collagen deposition in the post-MI setting. Along the same line, α-SMA a marker that is known to increase in cardiac stress conditions such as pathological hypertrophy (40) was also increased in the LA of vehicle-treated rats and was markedly inhibited by BA6b9 treatment (Supplementary Figure S2). In addition, WGA, which binds and labels glycoproteins of the cell membrane as well as extracellular fibrotic tissue (41) was markedly increased in the LA of post-MI rats relative to the controls and was markedly inhibited by BA6b9 treatment in the post-MI rats (Supplementary Figure S3). Overall, our findings show a remarkable effect of BA6b9 against the atrial structural remodeling induced by HFrEF in the post-MI setting.

**BA6b9 treatment reduces the upregulated expression of atrial SK4 K+ channels and NLRP3 inflammasome following MI**

In line with our hypothesis that SK4 K+ channels are involved in atrial remodeling in the context of HF, we found that SK4 K+ channel expression in the LA, as measured by immunohistochemistry, was noticeably upregulated in vehicle-injected rats subjected to MI when compared to control rats (Figure 4). Interestingly, post-MI blockage of SK4 K+ channels with BA6b9 markedly reduced the upregulated expression of atrial SK4 K+ channels in the LA, indicating that the overexpression of SK4 K+ channels is both a marker of stressed atrial myocardium, as well as an active player in the stress leading to atrial remodeling (see discussion). The NLRP3 inflammasome is known to be activated during atrial remodeling and AF progression in animal models and patients with AF (17, 42, 43). Figure 5 shows that in the LA, NLRP3 expression measured by immunohistochemistry was markedly increased in vehicle-treated rats subjected to MI as compared to control rats and that BA6b9 treatment virtually eliminated NLRP3 upregulation in this setting.

**BA6b9 reduces the lateralization of atrial Cx43 in post-MI rats**

Remodeling of Cx43 is commonly observed in patients with AF (44). The shift of connexins from the intercalated disc to the lateral membranes of cardiomyocytes is referred to as lateralization that can slow the conduction velocity and increase conduction heterogeneity(44). Thus, we examined by immunofluorescence experiments whether the AF substrate of post-MI rats is associated with altered atrial localization of Cx43 and if so, whether blockade of SK4 K+ channels could inhibit this lateralization process. Indeed, we found that following MI, the lateralization ratio of Cx43 markedly increased and BA6b9 treatment could potently inhibit this remodeling process (Figure 6).

**BA6b9 treatment inhibits structural changes of the LA epicardium post-MI**

Data from the recent years support the notion that the atrial epicardium have important roles in atrial electrical and structural remodeling. AF is accompanied by progressive epicardial fibrosis affecting transmural conduction. (45-48). In addition, the secretory epicardial adipose tissue enfolding the atria secretes various pro-inflammatory and pro-fibrotic mediators that promote atrial remodeling and increase the susceptibility to AF (49). While the atrial epicardium is far less studied in rodent models, we have noted marked changes in the LA epicardium of post-MI rats relative to the controls. Therefore, we aimed to systematically examine these changes as well as the effects of SK4 channel blockade by BA6b9 on the LA epicardial tissue. Indeed, LA epicardial fibrosis measured by Masson-trichrome staining was significantly increased in vehicle-injected rats subjected to MI as compared to control rats, while BA6b9 treatment inhibited the development of excess epicardial fibrosis (Figure 7A and 7A1-A3). Very similar results were obtained when α-SMA was quantified (Figure 7B and 7B1-B3). The LA epicardial remodeling post-MI was also characterized by upregulation of SK4 K+ channels that was potently blocked by BA6b9 (Figure 7C and 7C1-C3) and a similar pattern was noted in the expression of the NLRP3 inflammasome (Supplementary Figure S4)

**Discussion**

Initially assuming that SK4 K+ channels are pro-arrhythmic and pro-inflammatory, we examined in this study whether atrial SK4 channels are upregulated in the post-MI setting in rats and whether their blockage by our recently developed allosteric blocker BA6b9 could inhibit the AF substrate and atrial remodeling that develops in this HFrEF model mimicking important aspects of the co-morbidity between MI and AF in humans (36, 50, 51).

 As previously shown, post-MI rats develop a progressive increase of their AF substrate over time, which correlates with the level of systolic dysfunction. In addition, there is a clear association between the atrial fibrosis and the sustainability of arrhythmic events (36). In humans, HF occurs as frequently as AF and the co-existence of the two diseases is very common leading to poor clinical outcome (9, 10). HFrEF in particular, stimulates ion channel remodeling, inflammation and interstitial fibrosis, which makes the atria more prone to AF (1, 17). Thus, the HFrEF rat model of post-MI AF substrate development is clinically relevant. In addition, technical advances introduced by our group (37, 38), enabled us to chronically implant an atrial quadripolar electrode for simultaneous pacing and high resolution recordings in the unanesthetized rat, making this model even more potent and valuable for atrial EP and AF substrate analyses.

In this work, we showed that *in vivo* BA6b9 treatment for 3 weeks, starting 1 week following LAD ligation, prolongs the AERP, reduces AF induction and duration and markedly affects the power spectrum of induced AF episodes. Importantly, our final EP analysis was performed ~ 24 hours following the last injection of BA6b9. This design was aimed to let the animals accommodate overnight to the EP cage before EP testing in the resting state. However, it also helped us to focus on the sustained effects of the treatment rather than the acute effects of BA6b9. We recently demonstrated in the isolated rat heart *ex vivo* that both Tram-34 and BA6b9 significantly prolongs the RR and PR intervals and increases the AERP and the AVERP without affecting the ventricular effective refractory period (VERP) (23). Prolongation of RR interval (i.e. bradycardia) and PR interval were also noted in unanesthetized mice following acute applications of SK4 channel blockers (24). Thus, the fact that in our final EP study RR, PR and AVERP were not significantly different between the BA6b9-treated and the vehicle-treated rats clearly supports the notion that the main effects we detected here are indeed sustained. Another important aspect in our study design was the delayed initiation of treatment on day seven following MI induction. This was intentionally performed to avoid possible effects on the acute post-operative recovery. However, since our main focus was on atrial remodeling, it was also important to avoid possible effects of SK4 channels blockage on the acute inflammatory phase of post-MI healing (52, 53). Indeed, while our data indicate that the BA6b9 treatment was well tolerated and did not affect the weight gain of the rats, it also did not affect ventricular function using our current treatment protocol. This issue is critical since it strongly support the notion that the inhibition of AF substrate and atrial remodeling, we describe here are related to the effects of BA6b9 on the atria rather than to indirect effects on ventricular function. Of note, given the existing data regarding the possible role of SK4 channels in the acute inflammatory phase of post-MI healing (52, 53), it would be clearly interesting to test the effects of early post-MI treatment with BA6b9 on the ventricular healing and remodeling. However, this issue was clearly out of the scope of the present study.

 Our histological findings indicate that blocking SK4 K+ channels with BA6b9 not only attenuates AF induction and duration but also dramatically reduces post-MI overexpression of the SK4 channels themselves as well as multiple measures of atrial structural remodeling. Studies of the recent past years revealed a key role for inflammatory signaling pathways in AF development (17). Inflammation is also recognized as an important pathophysiological mediator in the post-MI HFrEF setting (54). Though inflammatory signaling crucially involves non-cardiomyocyte cells such as fibroblasts, adipocytes or macrophages, emerging evidence indicates that atrial cardiomyocytes have the same inflammatory signaling pathways, the activation of which contributes to AF substrate development (17, 55-57). Indeed, atrial cardiomyocytes were not only shown to secrete pro-inflammatory cytokines, but also to express their respective receptors that can stimulate via autocrine or paracrine mechanisms various important AF-related signaling cascades (17). Although our work did not explore which specific signaling pathway may be involved, we found that 3 weeks of SK4 K+ channel blockade by BA6b9 treatment drastically prevented atrial structural remodeling, as seen by the inhibition of increased collagen deposition (Masson-trichrome staining), ECM accumulation (conjugated-WGA fluorescence) and α-SMA expression in BA6b9- versus vehicle-treated post-MI animals. BA6b9 treatment also inhibited the upregulation of NLRP3 inflammasome in the LA of post-MI rats. Recent data demonstrate that NLRP3 inflammasome activation in atrial cardiomyocytes might be a sufficient and necessary condition for AF occurrence (17). In addition, NLRP3 inflammasome activity is increased in atrial cardiomyocytes of patients with paroxysmal AF or long-lasting persistent AF (17). Importantly, increases in cytosolic Ca2+ levels can lead to mitochondrial Ca2+ overload and the generation of mitochondria-derived reactive oxygen species (ROS), with subsequent NLRP3 inflammasome activation (58, 59). This latter feature might provide an additional deleterious link between abnormal diastolic Ca2+ handling, NLRP3 activation and increased SK4 channel activity. Reflecting their probable pro-inflammatory nature, the atrial SK4 K+ channels were upregulated by 3.5-fold in the setting of HFrEF post-MI. Remarkably, blockage of the SK4 K+ channels by BA6b9 treatment virtually suppressed this upregulation of atrial SK4 channels as well as that of the NLRP3 inflammasome.

The profound effects of BA6b9 treatment on collagen accumulation and the upregulation of α-SMA, SK4 channel and NLRP3 in the atrial epicardium also strengthens the clinical relevance of targeting AF by blocking SK4 K+ channel activity. Indeed, various studies support the view that endo–epicardial dissociation is an important driving force for breakthroughs to occur in AF. Moreover, in large mammals, epicardial adipose tissue properties correlate with AF severity, postoperative AF incidence and AF recurrence rates after cardioversion or ablation (60). Although rodents do not normally have notable epicardial adipose tissue, it will be important to further evaluate how the noted above epicardial changes can contribute to the post-MI AF substrate and whether BA6b9 can have similar effects on the epicardium of large mammalian models.

AF progression also includes connexin remodeling. Hence, a progressive reduction in Cx43 levels, often accompanied by enhanced lateralization, has been described in myocardial samples from patients with heart failure of different etiologies, including those secondary to AF, dilated and inflammatory cardiomyopathy (44, 61). The topological change of Cx43 from the intercalated disc to the lateral membranes of cardiomyocytes is often accompanied by changes in phosphorylation and ischemia was shown to induce a progressive dephosphorylation of Cx43. Mis-location of connexins outside intercalated discs and modifications in their phosphorylation state was found to result in important alterations of cardiomyocyte electrical coupling, thus disturbing action potential propagation and contributing to arrhythmia susceptibility (44). In this work, BA6b9 treatment virtually reversed the lateralization of atrial connexin Cx 43 in the setting of HF rat model of post-MI.

At this stage, it is hard to conclude which of the BA6b9- dependent effects (i.e. AERP prolongation, inhibition of atrial inflammation / fibrosis and attenuated Cx43 lateralization) contribute to the potent AF substrate inhibition by the treatment. However, all of these findings strongly support the notion that such treatment can have multiple long term beneficial effects that are independent of pure electrophysiological effects we have recently described (23). Current pharmacological therapies aiming to convert AF to sinus rhythm ('rhythm control' modalities) are very limited, especially in the setting of HF (1, 12-14, 16, 30, 62-66). Moreover, none of them has been shown to prevent the progressive structural changes such as inflammation and interstitial fibrosis that appear to be critical for AF progression. In this regard, our novel therapeutic strategy aimed to target the deleterious overexpression of atrial SK4 K+ channels is a novel approach that appears to target both the atrial electrical and l structural remodeling in a favorable manner.

**Limitations**

Our study does not provide information about which cell type is specifically affected by the SK4 channel block and it is likely that complex multidirectional autocrine and paracrine signaling pathways are involved in the process. However, precisely because of their unique expression in all cell types that are involved in AF induction and progression, SK4 K+ channels represent a suitable new drug target that has the combined qualities of rhythm control and anti-remodeling therapy. In addition, while our immunohistochemical analysis of SK4 K+ channels clearly indicates that the atrial overexpression is at least partially related to cardiomyocytes, we could not get reliable details on the exact cellular localization of the overexpressed channels. Nevertheless, our clear evidence regarding the beneficial effects of BA6b9 demonstrates that this SK4 K+ channel overexpression is a critical part of the pathophysiology rather than an epiphenomenon. Finally, our current proof-of-concept study was performed using male rats only. The logic for this design is that the AF substrate of freely moving female rats was found to be substantially lower compared to male rats of the same age, in an experiment we performed (Unpublished data). Thus, reaching conclusive results for female has yet to be demonstrated.

**Contributors**

O.L., S.EL., S.S., G.G., M.M. performed the *in vivo* experiments and analyzed physiological data. S.B. performed *ex vivo* patch-clamp studies. S.B., D.M., A.L., S.ET. performed the analyses of collected samples. S.B., O.L. generated figures and wrote the first draft of the manuscript. B.A., Y.E. conceived and designed the study, guided the analysis and wrote the final version of the MS. All authors read and approved the final version of the manuscript.

**Data sharing statement**

All data of the work are provided in the main figures, supplementary figures, and tables. Any additional information are openly available upon request to the corresponding authors (battali@tauex.tau.ac.il, tzion@bgu.ac.il)

**Declaration of interests**

B.A. and Y.E. declare a competing interest. There is a pending patent application filed for the new chemical entities including BA6b9. The other authors have no conflict of interest to declare.

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**Appendix A. Supplementary data**

Supplementary information is available for this paper at https:// \*\*\*\*\*

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