Tetramisole is a new $I_{K1}$ channel agonist and exerts $I_{K1}$-dependent cardioprotective effects in rats

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Abstract
Cardiac ischemia, hypoxia, arrhythmias, and heart failure share the common electrophysiological changes featured by the elevation of intracellular Ca$^{2+}$ (Ca$^{2+}$ overload) and inhibition of the inward rectifier potassium ($I_{K1}$) channel. $I_{K1}$ channel agonists have been considered a new type of anti-arrhythmia and cardioprotective agents. We predicted using a drug repurposing strategy that tetramisole (Tet), a known anthelmintic agent, was a new $I_{K1}$ channel agonist. The present study aimed to experimentally identify the above prediction and further demonstrate that Tet has cardioprotective effects. Results of the whole-cell patch clamp technique showed that Tet at 1–100 μmol/L enhanced $I_{K1}$ current, hyperpolarized resting potential (RP), and shortened action potential duration (APD) in isolated rat cardiomyocytes, while without effects on other ion channels or transporters. In adult Sprague–Dawley (SD) rats in vivo, Tet showed anti-arrhythmia and antiacardiac remodeling effects, respectively, in the coronary ligation-induced myocardial infarction model and isoproterenol (Iso, i.p., 3 mg/kg/day, 10 days) infusion-induced cardiac remodeling model. Tet also showed antiacardiomyocyte remodeling effect in Iso (1 μmol/L) infused adult rat ventricular myocytes or cultured H9c2 (2-1) cardiomyocytes. Tet at 0.54 mg/kg in vivo or 30 μmol/L in vitro

Abbreviations: AP, action potential; APD, action potential duration; ARVM, adult rat ventricular myocyte; AS, Andersen syndrome; CaMKII, calmodulin-dependent protein kinase II; ChIP, channel interacting proteins; DAD, delayed afterdepolarization; EF, ejection fraction; FS, fractional shortening; HBSS, Hank’s Balanced Salt Solution; HE, hematoxylin and eosin; HF, heart failure; HPF, high-powered field; $I_{K1}$, inward rectifier potassium channel; Iso, isoproterenol; LV, left ventricle; MAGUK, membrane-associated guanylate kinase; MI, myocardial infarction; p-PKA, phosphorylated protein kinase A; PVC, premature ventricular contraction; RP, resting potential; SD, Sprague–Dawley; Tet, tetramisole; VF, ventricular fibrillation; VT, ventricular tachycardia.

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1 | INTRODUCTION

Cardiac ischemia, hypoxia, malignant arrhythmias, and heart failure (HF) are the leading causes of morbidity and mortality in cardiovascular diseases.\(^1\) Convincing data have shown that these cardiac diseases share the common electrophysiological changes in ventricles, such as prolongation of the action potential duration (APD), elevation of intracellular \(Ca^{2+}\) overload, and decrease of inward rectifier potassium channel (\(\text{I}_{\text{K1}}\)).\(^3,\)\(^4\) Ventricular \(\text{I}_{\text{K1}}\) channels, primarily constituted by \(\text{Kir2.1}\) channel subunit (encoded by KCNJ2 gene), play key roles in maintaining the resting potential and cardiac Ca\(^{2+}\) homeostasis. Flecainide, a widely used \(\text{I}_{\text{K1}}\) channel blocker, exhibits effective control over ventricular arrhythmias in Andersen syndrome (AS) associated with loss of function mutations in KCNJ2.\(^7,\)\(^8\) Caballero et al unraveled that flecainide increases \(\text{Kir2.1}/\text{I}_{\text{K1}}\) channels, an effect underlies the pharmacological rescue of AS-associated ventricular arrhythmias.\(^1\) However, due to the multifaceted influence of flecainide on ionic channels, the mechanisms of flecainide therapy may be complex and require detailed investigation. We reported a selective \(\text{I}_{\text{K1}}/\text{Kir2.1}\) channel agonist, zacopride, and showed its striking anti-arrhythmic effect in anesthetic rats postcardiac myocyte infarction (MI) or in conscious rats with healing MI.\(^3,\)\(^1\) Zacopride also attenuated maladaptive cardiac repair following MI, Iso-, or L-thyroxine- toxicity.\(^1\) So far, zacopride is the only documented selective \(\text{I}_{\text{K1}}\) channel agonist. Developing more pharmacological tools is an issue to support the theoretical link between \(\text{I}_{\text{K1}}\) agonism and cardioprotection.

Drug repurposing (also known as drug repositioning, drug repurposing) is to rediscover new uses for existing drugs. Compared with traditional de novo drug development, it has been an alternative method in the advantage of reducing the costs and time-to-market of a medication.\(^1\)\(^6\)\(^-\)\(^8\) This strategy is largely driven by integrating, analyzing, and interpreting the data generated by high-throughput DNA and RNA sequencing, mass spectrometry, metabolomics and transcriptomic data, phenotyping, clinical data, etc. These data are often referred to as big data.\(^1\)\(^6\) In the present study, we took a drug repurposing strategy and found that tetramisole (Tet) might be a potential \(\text{I}_{\text{K1}}\) agonist, anti-arrhythmic, and antiventricular remodeling drug.

Tet is an anthelmintic agent used in veterinary applications to treat helminth or worm infections. It is a racemic mixture of (\(+\)) and (\(-\)) isomers. The (\(+\)) isomer (levamisole) accounts for most of the biological activity of tetramisole.\(^1\) The whole-cell patch clamp technique was applied to identify whether Tet is a selective \(\text{I}_{\text{K1}}\) agonist by comparing its potential influences on other channels or transporters, such as voltage-gated Na\(^+\) channel (\(\text{I}_{\text{Na}}\)), transient outward K\(^{+}\) channel (\(\text{I}_{\text{to}}\)), and Na\(^+\)-Ca\(^{2+}\) exchanger (\(\text{I}_{\text{NaCa}}\)). We further observed the effects of Tet on RP, AP, and intracellular calcium. Acute MI-induced arrhythmias and Iso-induced ventricular remodeling model were established to clarify whether increasing \(\text{I}_{\text{K1}}\) is a feasible anti-arrhythmic and antiremodeling strategy.

2 | MATERIALS AND METHODS

2.1 | Animal and ethical approval

Male Sprague–Dawley (SD) rats (2 months old) were provided by the Laboratory Animal Research Center of Shanxi Medical University (Taiyuan, China). The rats were housed under standard conditions, room temperature 20–24°C, humidity 40%–60%, 12:12 h light-dark cycles with light intensity up to 200 lux, and fed standard chow and water ad libitum. This study was carried out in accordance with the recommendations of the guidelines for the Care and Use of Laboratory Animals (NIH, revised 2011), and approved by the Ethics Committee of Shanxi Medical University (No. SYDL2021004).

2.2 | Isolation of adult rat ventricular myocytes

Single adult rat ventricular myocytes (ARVMs) were isolated using an enzymatic dissociation procedure. In brief, a rat heart was...
quickly removed and mounted via the aorta on an 80 cm H$_2$O high Langendorff retrograde perfusion apparatus. The heart was perfused first with oxygenated Ca$^{2+}$-free Tyrode’s solution (at 37°C) for approximately 10 min, then with collagenase P (0.1 g/L; Roche)-Tyrode solution for about 20 min. The composition of the Tyrode’s solution was (in mmol/L): NaCl 135, KCl 5.4, CaCl$_2$ 1.8, MgCl$_2$ 1.0, NaH$_2$PO$_4$ 0.33, HEPES 10, glucose 10, pH adjusted to 7.3–7.4 with NaOH. When the heart was well digested, the left ventricle (LV) was separated, minced, and filtrated in KB solution with the composition (in mmol/L): KOH 85, L-glutamic acid 50, KCl 30, MgCl$_2$ 1.0, KH$_2$PO$_4$ 30, glucose 10, taurine 20, HEPES 10, EGTA 0.5, pH adjusted to 7.4 with KOH. The isolated myocytes were stored at room temperature (23–25°C) at least 2 h before use.

### 2.3 | Patch clamp

Isolated ARVMs suspension was transferred to a chamber mounted on an inverted microscope (Nikon Diaphot; Nikon Co.) and superfused with Tyrode’s solution. The whole-cell recording was performed with an amplifier Axopatch 200B (Molecular Device) or Patchmaster EPC10 (HEKA Electronic). Filled with the pipette solution, the electrode resistance was maintained at 2–5 MΩ except for 1–1.5 MΩ for Na$^+$ current recording. The current signal was filtered at 2 kHz and sampled at 10–20 kHz. Current recordings were performed in the voltage clamp mode, and APs were recorded in the current clamp mode at a frequency of 1.0 Hz. Currents or APs were recorded in the presence of tetramisole (Sigma) at different concentrations (1, 10, 30, and 100 μmol/L) compared with baseline recording. The currents were expressed in terms of cell capacitance (pA/pF). All experiments were conducted at room temperature (23–25°C) except 36°C for APs recordings.

To measure AP, Tyrode’s solution was used as the extracellular solution. The pipette solution contained (in mmol/L) KCl 150.0, MgCl$_2$ 1.0, EGTA 5.0, HEPES 5.0, K$_2$-ATP 3.0, and the pH adjusted to 7.3 with KOH. The membrane potentials were corrected for the liquid junction potential (8 mV).

For $I_{K_1}$ recording, Tyrode’s solution was applied as the extracellular solution except for 0.2 mM CdCl$_2$ used to block $I_{Ca,L}$. The pipette solution contained (in mmol/L): KCl 150.0, MgCl$_2$ 1.0, EGTA 5.0, HEPES 5.0, K$_2$-ATP 3.0, and 4-aminoopyridine (4-AP) 5.0, pH 7.4 adjusted with KOH. BaCl$_2$ (0.2 mmol/L) was used to block $I_{K_1}$ channels. $I_{K_1}$ was determined as Ba$^{2+}$-sensitive current.

For $I_{Na}$ recording, the extracellular solution contained (in mmol/L) NaCl 60.0, CsCl 5.0, CdCl$_2$ 0.1, MgCl$_2$ 2.5, glucose 10.0, 4-AP 5.0, HEPES 5.0, saccharose 80.0, and pH 7.4 adjusted with NaOH. The pipette solution contained (in mM) EGTA 11.0, KCl 130.0, Na$_2$-ATP 5.0, HEPES 10.0, MgCl$_2$ 2.0, CaCl$_2$ 1.0, 4-AP 5.0, and pH 7.2 adjusted with CsOH.

For $I_{Ca,L}$ measurement, Tyrode’s solution was used as the extracellular solution. The pipette solution contained (in mM) EGTA 5.0, KCl 150.0, K$_2$-ATP 3.0, HEPES 5.0, 4-AP 5.0, MgCl$_2$ 1.0, Mg-ATP 1.0, and pH 7.3 adjusted with KOH.

The extracellular solution for $I_{Na}$ and $I_{K_1}$ recordings was Tyrode’s solution adding 0.1 mmol/L CdCl$_2$ and 0.2 mmol/L BaCl$_2$ used to block $I_{Ca,L}$ and $I_{K_1}$, respectively. The pipette solution contained (in mM) KCl 150.0, MgCl$_2$ 1.0, EGTA 5.0, HEPES 5.0, K$_2$-ATP 3.0, and pH 7.3 adjusted with KOH.

To measure $I_{NCX}$, the extracellular solution contained (in mmol/L) NaCl 140.0, CaCl$_2$ 2.0, MgCl$_2$ 2.0, glucose 10.0, HEPES 5.0, and pH 7.4 adjusted with CsOH. In addition, the Na$^+$-K$^+$ pump, K$^+$ channel, and Ca$^{2+}$ channel were blocked by 0.02 mmol/L ouabain, 1.0 mmol/L BaCl$_2$, 2.0 mmol/L CsCl, and 1.0 μmol/L nicardipine, respectively. The pipette solution contained (in mM) EGTA 42.0, CaCl$_2$ 29.0, MgCl$_2$ 13.0, potassium aspartate 42.0, K$_2$-ATP 10.0, Na$_2$-creatinephosphate 5.0, 4-AP 20.0, HEPES 5.0, and pH 7.4 with CsOH. $I_{NCX}$ could be blocked by a high concentration of Ni$^{2+}$ at 5.0 mmol/L.

### 2.4 | Induction of ischemic arrhythmias and preexposure with tetramisole

Arrhythmias were induced by ligating the left main coronary artery as previously described (Huang et al., 2001). After anesthetized with sodium pentobarbital (65 mg/kg, i.p.), rats were ventilated at 60 strokes/min and a stroke volume of 30 ml/kg by a small animal respirator (DH-1; Chengdu Instrument Factory) to maintain normal blood pO$_2$, pCO$_2$, and pH. The right femoral vein was cannulated for drug administration. Body temperature was maintained with an air conditioner. A left thoracotomy was performed in the fourth intercostal space. After opening the pericardium, a 6–0 suture was placed around the proximal portion of the left coronary artery, and the artery was ligated for 15 min. Myocardial ischemia was certified by elevation of the ST segment. Pharmacological treatments were as follows: Tet 0.18, 0.54, 1.8 mg/kg, Tet + chloroquine (CQ, $I_{K_1}$ antagonist, 7.5 μg/kg). The agent was dissolved in 0.2 ml of saline and administered intravenously 3 min before coronary artery occlusion. The control rat received 0.2 ml saline.

Prior to ischemia, a Lead II electrocardiogram (ECG) was continuously recorded with a waveform data analysis software (RM6240, BiopacSystem, Chengdu Instrument Factory). The rats exhibiting spontaneous arrhythmias were discarded. The ventricular ectopic activity was evaluated according to the diagnostic criteria advocated by Lambeth Convention. The ECGs were analyzed to determine the individual episode of arrhythmias, total episodes, and episode duration of ventricular tachyarrhythmias, including premature ventricular contraction (PVC), ventricular tachycardia (VT), and ventricular fibrillation (VF).

### 2.5 | Pretreatment with tetramisole and detection of Kir2.1 expression

Rats were randomly divided into MI, Tet pretreatment (0.54 mg/kg/day), and Tet + CQ (7.5 μg/kg/day) groups. The agents or saline was
administered by intraperitoneal injection once a day for 10 days. Then acute ischemic arrhythmia was established by ligating the left anterior descending artery. ECGs were recorded to observe the effects of Tet pretreatment on arrhythmias. The effect of Tet on Kir2.1 expression was observed by Western blotting.

2.6 | Induction of cardiac hypertrophy and remodeling

Cardiac hypertrophy was induced by daily injection of Iso (3 mg/kg/day; Sigma) for 10 days in rats in vivo. Pharmacological treatments were as follows: Iso, Tet (0.54 mg/kg/day, i.p.), Iso + Tet, and Iso + Tet + CQ (7.5 μg/kg/day, i.p.). Control rats were administered with the same volume of saline. The dose of Tet and CQ were applied according to our previous study and preliminary experiment. Ventricular remodeling was evaluated by echocardiography and histology.

2.7 | Echocardiography

The GE Vivid 7 Pro Ultrasound System (10 S probe, probe frequency 8.0 MHz, equipped with 2D strain imaging software, and EchoPAC workstation) was used in M-mode for rodent hearts. Approximate exploration angle was at 15°–30°, depth at 2–3 cm, frame rate > 250/s, and maximum frame rate up to 400/s. The positioning criterion was the LV long-axis section. The measured parameters included LV dimensions at end-diastole (LVIDd) and end-systole (LVIDs), interventricular septum thickness at end-diastole (IVSd) and end-systole (IVSs), LV posterior wall thickness at end-diastole (LVPWd) and end-systole (LVPWs), and LV ejection fraction (EF) and LV short-axis fractional shortening (FS).

2.8 | Histology

Samples of LV from all groups were fixed in 10% phosphate buffered formalin and subjected to routine histological processing. Transverse LV sections (5 μm thick) were cut using a cryostat microtome (Leica). After hematoxylin and eosin (HE) staining, the cross-sectional area of myofibers was measured using a microscope (Olympus) under a high-powered field (HPF) (×400 magnification). Fibrosis was evaluated by Masson’s trichrome staining, and the collagen content in the interstitial space was estimated by analyzing the images of each group. Total collagen area was calculated and expressed as the percent of the total ventricular area under HPF.

2.9 | Induction of cardiomyocyte remodeling

In vitro remodeling models were established by Iso infusion in native ARVMs or H9c2 (2-1) cells (National Collection of Authenticated Cell Cultures).

2.10 | [Ca2+], Imaging

The intracellular Ca2+ fluorescence in native ARVMs was indicated by the dual-wavelength Ca2+ indicator Fura-2 AM (Dojindo Laboratories). Post-24 h of pharmacological treatment, the cells were incubated with 3 μM Fura-2 AM in the dark at 37°C for 30 min then washed three times with Tyrode’s solution.

The intracellular Ca2+ fluorescence in cultured H9c2 (2-1) cells was indicated either by single-wavelength Ca2+ indicator Fluor-4 AM (Dojindo Laboratories) or by Fura-2 AM. H9c2 (2-1) cells were incubated with 5 μM Fluor-4 AM or 3 μM Fura-2 AM in the dark at 37°C for 30 min. The loaded cells were then washed three times with Hank’s Balanced Salt Solution (HBSS) and kept in HBSS for another 30 min to allow de-esterification of calcium indicators in cells.

The resting [Ca2+]i fluorescence indicated by Fluor-4 was measured by an FV1000 laser scanning confocal microscope (Olympus). The data were collected and analyzed with Fluoview 1.7a software (Olympus). The Ca2+ fluorescence indicated by Fura-2 was measured as fluorescence ratios (excitation at 340 and 380 nm; emission at 510 nm) from single cells using an Olympus IX71 inverted fluorescence microscope (Olympus) and a Luca EMCCD camera and collected at 2 s intervals. The data were recorded and analyzed by MetaFluor® Fluorescence Ratio Imaging System (Molecular Devices).

2.11 | Recombinant lentivirus

The Open Reading Frame of Kir2.1 was inserted into the MCS sites of pHBLV-CMV-MCS-3FLAG-EF1-ZsGreen-T2A-PURO to generate...
lentiviral vector overexpression of Kir2.1 (Hanbio Biotechnology). The lentiviral vectors mediated Kir2.1 knockdown were generated by inserting three shRNA sequences target for Kir2.1, respectively, to pHBLV-U6-MCS-CMV-ZsGreen-PGK-PURO (Hanbio Biotechnology). To generate lentiviruses, the lentiviral vectors and packaging vectors were cotransfected into 293T cells. Cells were lysed after good transfection, and extracts were subjected to Western blot analysis.

2.12 | Western blotting

Proteins from samples of LV tissue or H9c2 (2-1) cells were loaded on 12% acrylamide gels. After electrophoretic transfer, the nitrocellulose membranes were incubated overnight at 4°C with primary antibodies against Kir2.1 (dilution 1:1000, rabbit monoclonal; Abcam), Synapse-associated protein-97 (SAP97, DLG1, dilution 1:1000, rabbit polyclonal; ABclonal), phosphorylated protein kinase A (p-PKA, dilution 1:500, rabbit polyclonal; ABclonal), PKA (dilution 1:1000, mouse monoclonal; ABclonal), Kir6.1 (KCNJ8, dilution 1:1000, rabbit polyclonal; Proteintech), and AKAP5 (dilution 1:5000, rabbit polyclonal; ABclonal). GAPDH (dilution 1:1000, rabbit polyclonal; ABclonal) was used as the loading control in each case. The secondary antibody is goat antirabbit or goat antimouse IgG (ABclonal). Quantification was performed using Image J.

2.14 | Statistical analysis

Quantitative data were presented as the mean ± SEM and analyzed using the least significant difference or Games-Howell tests of ANOVA (analysis of variance). The statistical difference in the occurrence frequency for an individual type of arrhythmias between groups was assessed using the χ2 (chi-square) test of two variables. Statistical differences were considered significant when the p value was <.05.

2.15 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY,24 and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).25

3 | RESULTS

3.1 | Tetramisole selectively increases the $I_{K1}$ current in ARVMs

The Ba2+-sensitive currents at the end of each 500-ms step pulse were considered the steady-state $I_{K1}$ currents. As Figure 1 showed, tetramisole at 1–100 μmol/L enhanced both the inward and outward components of $I_{K1}$ in a concentration-dependent manner. The maximal efficacy appeared at 30 μmol/L, with a mean increase of 66.4% in the inward current (Figure 1C, at −120 mV, p < .05) and 60.4% in the outward current (Figure 1D, at −50 mV, p < .01). Tet at 100 μmol/L showed a minor weak trend on $I_{K1}$ enhancement than that at 30 μmol/L. The mean value of membrane capacitances is shown in Table S1.

3.2 | Tetramisole does not affect other ion channels and Na/Ca exchanger

3.2.1 | L-type Ca2+ current ($I_{Ca-L}$)

The typical trace recordings and I–V curves of $I_{Ca-L}$ are shown in Figure 2A. $I_{Ca-L}$ traces were recorded using 500-ms voltage steps from a holding potential of −40 mV to voltages between −40 and 50 mV by a 10-mV step. The I–V curves of $I_{Ca-L}$ in the absence and presence of Tet are almost overlapped. Tet at 1–30 μmol/L had no significant effect on $I_{Ca-L}$ (n = 6, p > .05).

3.2.2 | Voltage-gated Na+ channel

I–V curves of $I_{Na}$ were recorded by 200 ms depolarizing pulses from the holding potential of −110 mV to voltages between −100 mV and 30 mV by a 5-mV step. Tet at 1–30 μmol/L had no effect on $I_{Na}$ (Figure 2B, N = 6, p > .05).

3.2.3 | $I_{to}$ and $I_{KSUS}$ currents

The method used for resolving $I_{to}$ and $I_{KSUS}$ was modified to that described previously.26 $I_{to}$ and $I_{KSUS}$ were elicited by 500 ms voltage steps from a holding potential of −40 mV to voltages between −40 and 80 mV by a 10-mV step. Figure 2C showed that there was no...
significant difference between the absence and the presence of 1–30 μmol/L Tet (n = 6, p > .05).

3.2.4 | Na/Ca exchanger current

The I_{NCX} current was measured with a ramp voltage clamp pulse depolarized to a holding potential of −40 to 60 mV, then hyperpolarized to −120 mV at a rate of 90 mV/s (Figure 2D). After the application of NiCl\(_2\) (5.0 mmol/L), the current decreased in both the inward and the outward directions. I_{NCX} is defined as Ni\(^{2+}\)-sensitive current. Tet at 1–100 μmol/L had no effect on I_{NCX} (n = 6, p > .05).

3.3 | Tetramisole hyperpolarizes the RP and moderately shortens the APD

The effects of Tet on RP and APs in ARVMs are shown in Figure 3. At the current clamp mode, APs were triggered by injection currents (Figure 3A, 2-ms pulse width, twice of threshold at 1 Hz). In the presence of 1–100 μmol/L Tet, RP was hyperpolarized, and APD was moderately shortened in a dose-relative manner. At 30 μmol/L, Tet hyperpolarized the RP from −74.1 ± 2.1 mV (baseline) up to −79.8 ± 1.7 mV (Figure 3B, p < .05). Meanwhile, the APD\(_{50}\) was shortened from 37.4 ± 4.4 ms (baseline) to 25.2 ± 3.2 ms (Figure 3C, p < .05). Tet had no significant effect on APD\(_{50}\) and APA (Figure 3C,D). Considering the importance of I_{K1} in maintaining RP and terminal repolarization of AP, the effects of tetramisole on RP and APD highly fit the profile of a specific I_{K1} agonists.

3.4 | Tetramisole prevents acute ischemic arrhythmias either in 3-min preexposure setting or 10-day pretreatment

Acute MI-induced tachyarrhythmias usually occurred 5–6 min postcoronary occlusion and intensively appeared at 9–12 min (Figure 4Aa). So, 15 min MI was set as the model criteria. The cases with spontaneous arrhythmia were weeded out. In the setting of 3 min preexposure, Tet significantly reduced or even eliminated ventricular arrhythmias both in episode number and duration compared with the saline control. In detail, 92.3% (12 out of 13) of rats developed VT, and 84.6% (11/13) developed VF post-MI. The duration of VT and VF were 59.4 ± 17.7 s and 5.6 ± 1.6 s, respectively. Preexposure with 0.54 mg/kg Tet exhibited the most striking anti-arrhythmic effects as evidenced by reduction in the episodes of PVC (from 134 ± 23 to 16 ± 7, p < .01), the duration (8.1 ± 5.9 s, p < .01) and incidence (44.4%, p < .05) of VT, and the duration (0 s, p < .05) and incidence (0%, p < .01) of VF. The effects could be largely counteracted by 7.5 μg/kg chloroquine, a relatively specific I_{K1} blocker at low dosage (p < .05 or p < .01). All these data suggest that the anti-arrhythmic effects of Tet are mediated by I_{K1} activation (see the details in Figure 4A).

Tet 10-day pretreatment could inhibit ventricular arrhythmia induced by acute myocardial ischemia (Figure 4B). Tet at 0.54 mg/kg/day strikingly reduced the duration of VT (from 42.7 ± 13.7 to 6.5 ± 2.4, p < .01) and VF (8.2 ± 3.4 to 0, p < .01) and the incidence of VF (from 85.7% to 0, p < .01). The anti-arrhythmic effect could be largely abolished by chloroquine (p < .05). Native I_{K1} channels in the ventricles predominantly comprise Kir2.1 (KCNJ2) subunits.
Western blot data showed that pretreatment with Tet (0.54 mg/kg/day) for 10 days significantly increase the expression of Kir2.1 channel protein (p < .01), which could be reversed by chloroquine, an IK1 blocker ($p < .05$).

All these data suggested that the anti-arrhythmic effects of Tet are mediated by IK1 activation.

### 3.5 | Tetramisole improves Iso-induced cardiac remodeling in vivo

#### 3.5.1 | Echocardiography

Echocardiographic observation demonstrated the typical characteristics of concentric hypertrophy and enhanced pumping function (Figure 5A; Table 1). Post-10 days of Iso infusion, IVSd, IVSs, EF, and FS were increased ($p < .01$); LVIDs ($p < .05$) were reduced compared with control rats. Tet treatment prevented the thickening of interventricular septum, increased LV volume ($p < .05$), and normalized cardiac pumping function ($p < .01$). The effect could be largely reversed by the IK1 antagonist, chloroquine ($p < .01$ or $p < .05$). Tet (0.54 mg/kg/day) in present study or chloroquine (7.5 μg/kg/day) per se had no significant effects on cardiac structure or function.

#### 3.5.2 | Morphological features

Cardiac remodeling is a response to damages characterized by myocardial hypertrophy and interstitial fibrosis. Transverse sections of the LV were stained with HE. As shown in Figure 5B, compared with controls, the cardiac myofibers in Iso-infused rats are disorganized and hypertrophic, with a certain degree of cell necrosis and relatively light staining of the cytoplasm. In tetramisole-treated rats, cardiac myofibers are better arranged with normalized size. IK1 channel blocker chloroquine counteracted the protection, indicating that the antiremodeling effect of Tet is mediated by IK1 activation.
After 10 days of Iso infusion, rat hearts exhibited significant fibrosis, validated by increased collagen deposition (Figures 5C). Tet strikingly attenuated the fibrosis (p < .01), and this effect was largely abolished by chloroquine (p < .01). Tet (0.54 mg/kg/day) in present study or chloroquine (7.5 μg/kg/day) pabolished by chloroquine (p < .05 or < .01), and the effects were largely reversed by I* (0.54 mg/kg/day). Tet at 10 or 30 μmol/L normalized the expression of Kir2.1 (p < .01), and the effects were largely reversed by I* blocker BaCl₂. Although both Iso and BaCl₂ downregulated the expression of Kir2.1, Iso had no significant effect on SAP97 whereas the latter counteracted the effects of Tet on SAP97 (p < .05). Iso might downregulate Kir2.1 in a SAP97-independent pathway. We further observed the interactions involving SAP97 and Kir2.1 by lentivirus-driven knockdown or overexpression of Kir2.1 in H9c2 (2-1) cells. As shown in Figure S2, compared with negative control, neither Kir2.1 knockdown nor Kir2.1 overexpression had effects on the expression of SAP97.

ATP-sensitive K⁺ channel (Kᵦᵦ) and Iᵦᵦ channels are both members of the inward rectifier potassium (Kir) channel family and, respectively, constituted by Kir2.x and Kir6.x subunits. Kᵦᵦ is documented involved in ventricular remodeling, and Kᵦᵦ channel agonists improved cardiac structural remodeling and dysfunction. To rule out the possibility that the cardioprotective features of Tet are mediated by activation of Kᵦᵦ, the expression of Kir6.1, the predominant subunit of Kᵦᵦ, was observed in Iso-infused H9c2 (2-1) cells. The present results suggested that 1–100 μmol/L Tet had no significant effects on Kir6.1 in neither Iso stress nor control condition (Figure 7C).
β-adrenergic receptors (β-AR) stimulation in the heart could activate the cAMP/PKA pathway. Phosphorylation of PKA plays key roles in the progression of cardiac remodeling and HF. Figure 7D shows that 1 μmol/L Iso-induced hyperphosphorylation of PKA (p < .05), and the effects were reversed by 30 μmol/L Tet (p < .01). To further clarify how Tet inhibits phosphorylation of PKA, we detected the expression of A-kinase-anchoring protein 5 (AKAP5, also known as AKAP150 in rats) is a widely expressed...
LIU et al. | anchor protein that binds to the regulatory subunit of protein kinase A (PKA). Figure S3B shows that 10 and 30 μmol/L Tet downregulated the expression of AKAP5 (p < .01), and 1 μmol/L BaCl₂ counteracted the effect of Tet at 30 μmol/L (p < .05). Notably, in the absence of Iso stress, 1-100 μmol/L Tet had no significant effects on the expression of AKAP5 and the phosphorylation of PKA (Figure 7D; Figure S3A).

3.8 | Tetramisole selectively binds to Kir2.1

Molecular docking was performed to predict the possible interaction between Tet and the most important ion channel protein contributing to ventricular action potentials. Tet was found to be formed a special S…O bond at residue Glu224 and an alkyl–π interaction at residue Arg260 from Chain B of Kir2.1 (Figure 8). Further docking study showed that a Pi–Pi interaction was observed between the benzene ring of Tet and PHE 98 residue of Kv4.3 (Figure S4A), but there was no binding activity between Tet with Kv4.2, Nav1.5, or Cav1.2 (Figure S4B–D).

4 | DISCUSSION

The novel findings in the present study include (1) by drug repurposing, big data analysis, molecular docking, and experimental evidence,
Tet was identified as a specific \( l_{K1} \) agonist in rat, and \( l_{K1} \) channel is a promising target for the regulation of intracellular calcium overload that linking cardiac remodeling and arrhythmias.

### 4.1 Activation of \( l_{K1} \) is a new regulatory strategy on intracellular \( Ca^{2+} \) dyshomeostasis associated with cardiac remodeling and arrhythmogenesis

Cardiac remodeling generally encompasses two components, structural and electrical remodeling. Electrical remodeling, such as alterations in ion channels or \( Ca^{2+} \) cycling, may constitute the electrophysiological basis underlying arrhythmogenesis. Amelioration of electrical remodeling might be an effective strategy against HF and associated arrhythmia. Calcium dyshomeostasis, especially pathologic elevation of intracellular \( Ca^{2+} \) (\( Ca^{2+} \) overload), is a pivotal electrical event linking arrhythmogenesis and cardiac remodeling.\(^{14,33}\) \( \beta \)-AR stimulation increases calcium influx through LTCC,\(^{34} \) phosphorylates CaMKII and CAMP-dependent PKA that facilitates calcium leakage from RyRs, concurrently reducing \( Ca^{2+} \) uptake into the SR by downregulating SERCA2.\(^{2,4,35} \) All these changes ultimately lead to the intracellular \( Ca^{2+} \) overload. Furthermore, in cardiac hypertrophy and HF, prolongation of APD, downregulation of \( l_{K1} \) channel, enhanced depolarization drive, and reduced repolarization reserve are hallmarks of electrical remodeling.\(^{14,34,35} \) From our previous work, the RP depolarization, \( l_{K1} \) reduction, and APD prolongation were well recognized during the early stage of simulated ischemia in ARVMs in vitro.\(^{5} \) Downregulation of \( l_{K1} \) also contributes to APD prolongation and terminal repolarization of AP. Depolarized RP and prolonged APD facilitate the opening of LTCC thus promoting intracellular \( Ca^{2+} \) accumulation.\(^{38} \) We have proven that in conditions of ischemic membrane damage or adrenergic stimulation, \( Ca^{2+} \) overload, and APD prolongation may initiate delayed afterdepolarization (DAD), which underlies the genesis of triggered arrhythmias.\(^{3,12} \) Elevation of cardiomyocyte \([Ca^{2+}]_i\) elicits a series of biochemical signals through multifaceted \( Ca^{2+}\)-activated enzymes, such as \( Ca^{2+/-}\)-calmodulin-dependent protein kinase II (CaMKII). Activated CaMKII phosphorylates multiple ion channels and \( Ca^{2+} \) handling proteins, in turn, aggravating intracellular \( Ca^{2+} \) dyshomeostasis.\(^{14} \)

\( Ca^{2+} \) also produces voltage-dependent blockade of \( l_{K1} \). In Guinea pig ventricular myocytes, transient increase of \([Ca^{2+}]_i\) during the AP lead to inhibition of \( l_{K1} \) outward current by decreasing the open probability of the channel.\(^{39,40} \) In HF, \( l_{K1} \) is observed reduced by elevated diastolic \( Ca^{2+} \) in HF which provides a paradigm for \( Ca^{2+} \)-dependent modulation of RP.\(^{31} \)

By enhancing \( l_{K1} \), Tet reversed RP depolarization and APD prolongation, consequently inhibiting cardiac \( Ca^{2+} \) overload. It is benign to diminish the onset of DAD/EAD and \( Ca^{2+} \)-dependent transcriptional pathway for cardiac hypertrophy.

Similar to zacopride, Tet per se had no effect on LTCCs or \( l_{NCX} \). The regulation of calcium homeostasis is probably \( l_{K1} \)-dependent. The convincing data came from the coapplication of \( l_{K1} \) antagonist
**FIGURE 6** Tet attenuated Iso-induced [Ca\(^{2+}\)]i overload in H9c2 (2–1) cells. (A) Fluorescent images of Fluo-4/AM loaded H9c2 (2–1) cardiomyocytes. Magnification: 200×. Bars represent 100 μm. (B) In Fluo-4/AM loaded H9c2 (2–1) cardiomyocytes, Tet treatments attenuated Iso-induced intracellular calcium overload, and the effects could be reversed by I\(_{K1}\) blocker BaCl\(_2\). (C) In Fura-2/AM loaded H9c2 (2–1) cardiomyocytes, 30 μmol/L Tet attenuated Iso-induced intracellular calcium overload, and the effects could be reversed by I\(_{K1}\) blocker BaCl\(_2\). Ba, BaCl\(_2\); Iso, isoproterenol; N, numbers embedded in the columns; Tet, Tetramisole. Values are presented as the mean ± SEM. *p < .05; **p < .01.

**FIGURE 7** Effects of Tet on Kir2.1, SAP97, Kir6.1, and PKA signaling in H9c2 (2–1) cells. (A) Tet upregulated Kir2.1 channel expression. (B) Tet upregulated SAP97 expression. The representative blots in A&B derived from the same gel but different nitrocellulose membranes according to a certain molecular range. (C) Tet had no effects on Kir6.1 channel expression. (D) Tet inhibited the phosphorylation of PKA in Iso-infused H9c2 (2–1) cells. PKA activity is expressed as the ratio of phosphorylated PKA to total PKA. Ba, BaCl\(_2\); N, numbers embedded in the columns; Iso, isoproterenol; Tet, Tetramisole. Values are presented as the mean ± SEM. *p < .05; **p < .01.
BaCl₂ or chloroquine. BaCl₂ in vitro or chloroquine in vivo blunted the cardioprotection of Tet. Specifically, Tet only attenuated Ca²⁺ overload in cardiomyocytes upon Iso stress, compared with no effect in normal myocytes. The Iᵥ₁₉ channel might be a novel target for maintaining calcium homeostasis.

4.2 | Tetramisole showed high selective affinity with Kir2.1 and might upregulate Iᵥ₁₉ by facilitating Kir2.1 forward trafficking

In the present study, an in silico study molecular docking was performed to clarify the pharmacological selectivity of the ligand (Tet) with the receptor (Kir2.1). Tet showed binding activity with Kir2.1 and Kv4.3 but had no interaction with Kv4.2, Nav1.5, or Cav1.2 channel protein. Considering that Iᵥ₁₉ channels are constituted by both Kv4.2 and Kv4.3 in rodent ventricles and Tet had no significant on Iᵥ₁₉ current by patch clamp technique, Tet might be a selective pharmacological tool in regulation of Kir2.1 channel.

The trafficking of channel proteins might determine the channel density. Forward trafficking allows correctly folded nascent Kir2.1 protein trafficking toward and anchoring at the plasma membrane, where the channel plays biophysical roles. Upon removal from the plasma membrane, Kir2.1 channel proteins can enter the lysosome degradation pathway named backward trafficking.⁴² MAGUK proteins, serve as regulatory partners (channel interacting proteins, ChIPs) to the channel proteins and are characterized by sharing multiple protein–protein interaction domains, including PDZ (postsynaptic density protein-95), Drosophila disc large tumor suppressor (Dlg1), and SH3 domains (SRC Homology 3 Domain).²⁷ In hearts, SAP97 is the most well-characterized MAGUK protein which regulates Kir2.1 localization, forward trafficking, and formation of signaling complexes.²⁸,⁴³,⁴⁴ SAP97 knockdown in ventricular myocytes resulted in a downregulation of Kir2.1 and severe disruption of Kir2.1 localization. Vaidyanathan et al. demonstrated that silencing SAP97 decreased Iᵥ₁₉ density which was likely due to a decrease in the abundance of Kir2.1 channels on the membrane.²⁸ These alterations were associated with a prolongation of the APD which signifies arrhythmogenic propensity.⁴³ Conversely, in the present study, lentivirus-mediated Kir2.1 overexpression or knockdown had no effects on the expression of SAP97. Tet upregulated the expression of SAP97 in a dose-relative manner, which is in line with the effects of Tet on Kir2.1 current and expression. All these data support that Tetramisole increases cardiac Kir2.1 by positively regulating SAP97. Upregulation of SAP97 may facilitate the forward trafficking of Kir2.1 and increase the membrane conductance of Kir2.1 which well explain the hyperpolarization of RP and acceleration of terminal repolarization of AP. A notable observation indicated that Iso stimulation did not alter the expression of SAP97 although Kir2.1 was downregulated. So, Iso might inhibit Kir2.1 by a SAP97-independent pathway. A low dose of BaCl₂ (e.g., 1 μmol/L) and chloroquine (0.3 μmol/L or 7.5 μg/kg in vivo) are used as a relative specific blocker of the Iᵥ₁₉ channel. Our previous work showed that 1 μmol/L BaCl₂ or 0.3 μmol/L chloroquine...
downregulated the expression of SAP97 in cardiomyocytes, parallel to the inhibition of the Kir2.1 channel. It partly elucidated why BaCl₂ or chloroquine counteracted the cardioprotection of tetramisole.

4.3 The cardioprotection of tetramisole is associated with negative regulation of PKA-AKAP5 signaling in H9c2 (2-1) cardiomyocytes

Beta-adrenergic receptor (β-AR) stimulation (such as by acute MI or Iso) could activate the cAMP-dependent PKA. PKA is considered to be a key downstream effector in cAMP/PKA signaling. Activated PKA phosphorylates multiple substrates such as LTCC, RyR2, cardiac myosin binding protein (cMyBP), and PLN, resulting in increased intracellular Ca²⁺, the loss of excitation–contraction coupling, contraction, and relaxation dysfunction (reviewed by Saad et al., 2018). In HF, the activity and expression of PKA were significantly increased compared with nonfailing hearts. While PKA inhibition decreased cell death occurring in I/R and HF. Although it is still a contentious issue about the exact role of PKA in cardiomyopathy, most reports showed PKA inhibition as a potential target in the treatment of cardiac hypertrophy, cardiac dilation, I/R, MI, and HF.

The PKA holoenzyme is a heterotetramer consisting of two catalytic and two regulatory subunits. Phosphorylation of PKA in cardiomyocytes regulates multiple ion flux. Kir2.x channels including Kir2.1 are the substrates of PKA too. But the conditioning effect remains a matter of debate. In heterologous expression systems, activation of PKA via Iso, cAMP, or forskolin enhances the outward current of Kir2.1. While in native cardiomyocytes, Iₖ1 was suppressed by PKA-mediated phosphorylation in response to β-AR stimulation. Protein kinase A anchoring proteins (AKAPs) are a family of anchoring proteins, which are localized by binding to the regulatory subunits of the cyclic AMP-dependent PKA. It has been reported that overexpression of muscle AKAP in cardiomyocytes increased PKA-catalyzed phosphorylation of ryanodine Ca²⁺ release channels and inhibit adrenergic-induced hypertrophy. AKAP5 is a membrane-bound AKAP and was involved in tethering β-AR and spatiotemporal regulating phosphorylation of PKA. In the present study, Iso elicited phosphorylation of PKA and downregulation of Kir2.1 in H9c2 (2-1) cardiomyocytes, as was consistently observed in previous studies. In line with the activity of PKA, tetramisole inhibited the expression of AKAP5 in Iso-infused H9c2 (2-1) cells. The inhibition of PKA and AKAP5 by tetramisole may partially explain its activation on Kir2.1 upon Iso stimuli. All these effects could be reversed by Iₖ1 blocker BaCl₂.

4.4 Limitations and prospects

In the present study, we applied an in silico structure-based method to predict ligand–target interactions at molecular levels. The combination of drug repositioning and molecular docking might complement each other in more accurate target identification and drug discovery. But validation is necessary using in vitro binding assays, such as western blotting, immunofluorescence staining, and genetic modulation to confirm the key binding sites of the ligands to the receptor (Kir2.1).

Both loss and gain of function in cardiac Iₖ1 are associated with severe arrhythmias and even sudden cardiac death. Loss of ventricular Iₖ1 current is a hallmark of electrical alteration underlying arrhythmogenesis in human failing hearts. While a ventricular Iₖ1 increase may accelerate and stabilize the reentry rotors and ventricular fibrillation dynamics. Therefore, when judging an anti-arrhythmic strategy, a comprehensive understanding of mechanisms underlying certain arrhythmogenesis and drug therapy would be essential and beneficial. Based on previous and present research, we suggest that Iₖ1 channel agonists and related new anti-arrhythmic drugs might be applied under conditions of automaticity and triggered arrhythmias. Further, many clinical anti-arrhythmic drugs have been revealed of potential pro-arrhythmic risks. Compared with dramatic changes by genetic manipulation, Tet and zacopride are moderate Iₖ1 agonists, with limited efficacy on Iₖ1 and AP. Modulating Iₖ1 within the physiological range is important to minimize the pro-arrhythmic effects of anti-arrhythmic medications. The last concern is the risk of atrial arrhythmia. Gain of Iₖ1 function has been deemed a risk factor for atrial arrhythmogenesis. Because that Kir2.1 is the major isoform underlying human ventricular Iₖ1 and a minor constituent in atrial Iₖ1, an ideal and promising anti-arrhythmic agent targeting Iₖ1 should be Kir2.1-specific and of lower disturbance on atrial Iₖ1.

AUTHOR CONTRIBUTIONS

Participated in research design: Bowei Wu, Peifeng He, Qi Yu, Xuechun Lu, and Jimin Cao. Conducted experiments: Qinghua Liu, Jiaying Sun, Yangdong Pang, Lin Li, Jin Wang, Yulan Wang, and Yanwu Xu. Performed data analysis: Qinghua Liu and Xinrui Tian. Wrote or contributed to the writing of the manuscript: Qinghua Liu and Jimin Cao.

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