British Journal of Pharmacology

British Journal of Pharmacology (2017) **174** 454–467 454

RESEARCH PAPER

# Genistein and tyrphostin AG556 decrease ultra-rapidly activating delayed rectifier $K^+$ current of human atria by inhibiting EGF receptor tyrosine kinase

**Correspondence** Dr Gui-Rong Li, Xiamen Cardiovascular Hospital, Xiamen University, Xiamen, Fujian 361004, China.
E-mail: grli8@outlook.com

**Received** 27 January 2016; **Revised** 16 December 2016; **Accepted** 5 January 2017

Guo-Sheng Xiao[1], Yan-Hui Zhang[1,2], Wei Wu[2], Hai-Ying Sun[2], Yan Wang[1] and Gui-Rong Li[1,2]

*[1]Xiamen Cardiovascular Hospital, Xiamen University, Xiamen, Fujian, China, and [2]Department of Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong, China*

## BACKGROUND AND PURPOSE

The ultra-rapidly activating delayed rectifier $K^+$ current $I_{Kur}$ (encoded by $K_v1.5$ or *KCNA5*) plays an important role in human atrial repolarization. The present study investigates the regulation of this current by protein tyrosine kinases (PTKs).

## EXPERIMENTAL APPROACH

Whole-cell patch voltage clamp technique and immunoprecipitation and Western blotting analysis were used to investigate whether the PTK inhibitors genistein, tyrphostin AG556 (AG556) and PP2 regulate human atrial $I_{Kur}$ and hKv1.5 channels stably expressed in HEK 293 cells.

## KEY RESULTS

Human atrial $I_{Kur}$ was decreased by genistein (a broad-spectrum PTK inhibitor) and AG556 (a highly selective EGFR TK inhibitor) in a concentration-dependent manner. Inhibition of $I_{Kur}$ induced by 30 μM genistein or 10 μM AG556 was significantly reversed by 1 mM orthovanadate (a protein tyrosine phosphatase inhibitor). Similar results were observed in HEK 293 cells stably expressing $hK_v1.5$ channels. On the other hand, the Src family kinase inhibitor PP2 (1 μM) slightly enhanced $I_{Kur}$ and $hK_v1.5$ current, and the current increase was also reversed by orthovanadate. Immunoprecipitation and Western blotting analysis showed that genistein, AG556, and PP2 decreased tyrosine phosphorylation of $hK_v1.5$ channels and that the decrease was countered by orthovanadate.

## CONCLUSION AND IMPLICATIONS

The PTK inhibitors genistein and AG556 decrease human atrial $I_{Kur}$ and cloned $hK_v1.5$ channels by inhibiting EGFR TK, whereas the Src kinase inhibitor PP2 increases $I_{Kur}$ and $hK_v1.5$ current. These results imply that EGFR TK and the soluble Src kinases may have opposite effects on human atrial $I_{Kur}$.

## Abbreviations

EGFR, EGF receptor; $I_{Kur}$, ultra-rapidly activating delayed rectifier potassium current; PP2, 3-(4-chlorophenyl) 1-(1,1-dimethylethyl)-1*H*–pyrazolo[3,4-d] pyrimidin-4-amine; PTK, protein tyrosine kinase; TK, protein kinase; tyrphostin AG556, AG556

DOI:10.1111/bph.13710

## Tables of Links

| TARGETS | |
|---|---|
| **Voltage-gated ion channels**[a] | **Catalytic receptors**[c] |
| $K_v$1.5 ($I_{Kur}$) channels | EGFR tyrosine kinases |
| **Enzymes**[b] | Protein tyrosine phosphatases |
| Src tyrosine kinases | |

| LIGANDS |
|---|
| Daidzein |
| Genistein |

These Tables list key protein targets and the ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a,b,c]Alexander *et al.*, 2015a,b,c)

## Introduction

The ultra-rapidly activating delayed rectifier current $I_{Kur}$ was initially reported in human atrial myocytes (Wang *et al.*, 1993) and later found in atria, but not in the ventricles of the human heart (Li *et al.*, 1996b). This current contributes to the repolarization of human atrial action potentials (Feng *et al.*, 1997; Wettwer *et al.*, 2004; Li *et al.*, 2008). $I_{Kur}$ is carried by $K_v$1.5 (*KCNA5*) channels (Fedida *et al.*, 1993; Snyders *et al.*, 1993; Feng *et al.*, 1997). Loss-of-function and/or gain-of-function $K_v$1.5 mutations may increase atrial fibrillation susceptibility (Olson *et al.*, 2006; Christophersen *et al.*, 2013) and so $K_v$1.5 channels are attractive targets for the treatment of atrial arrhythmias (Li *et al.*, 2008; Schumacher *et al.*, 2009; Tamargo *et al.*, 2009; Loose *et al.*, 2014). Our earlier study demonstrated that stimulation of β-adrenoceptors increases, whereas α-adrenoceptor stimulation decreases, human atrial $I_{Kur}$ via activating protein kinase A and protein kinase C respectively (Li *et al.*, 1996a). The up-regulation by protein kinase A and the down-regulation by protein kinase C have been confirmed in $K_v$1.5 channels expressed in *Xenopus laevis* oocytes with $K_v\beta$1.3 (Kwak *et al.*, 1999) or $K_v\beta$1.2 subunits (Williams *et al.*, 2002). A recent study showed that AMP-activated protein kinase down-regulated $K_v$1.5 channels via activating the ubiqutin ligase Nedd4-2 with subsequent clearance of channel protein from the cell membrane (Mia *et al.*, 2012).

Receptor protein tyrosine kinases (PTKs) such as the EGF receptor (EGFR) kinase, and non-receptor PTKs (e.g. the Src family kinases) (Hubbard and Till, 2000) play crucial roles in mediating cell growth, embryonic development, differentiation, metabolism, immune system function and oncogenesis. In addition, PTKs regulate transmembrane ion channels (Davis *et al.*, 2001; Levitan, 1994). EGFR PTKs regulate several ion channels, including those carrying the cardiac voltage-gated sodium current ($I_{Na}$) (Liu *et al.*, 2007), $K_{ir}$2.3 and $K_{ir}$2.1 (Zhang *et al.*, 2011a,b), recombinant human cardiac $I_{Ks}$ (hKCNQ1/hKCNE1) (Dong *et al.*, 2010) and the human EAG1 ($K_v$10.1) channels (Wu *et al.*, 2012). Src family kinases also participate in regulation of hERG channels (Zhang *et al.*, 2008) and cardiac transient outward potassium current ($I_{to}$, carried by h$K_v$4.3 channels) (Zhang *et al.*, 2012). An earlier report demonstrated that Src TK was associated with native h$K_v$1.5 channels in human myocardium and cloned h$K_v$1.5 channels. Tyrosine phosphorylation of h$K_v$1.5 channels suppressed the channel current in cells coexpressing v-Src (Holmes *et al.*, 1996).

The present study investigated effects of EGFR TK and Src family kinases on $I_{Kur}$/h$K_v$1.5 channels using pharmacological tools. Our results demonstrated that genistein (a broad spectrum PTK inhibitor) and AG556 (a highly selective EGFR kinase inhibitor) inhibited $I_{Kur}$/h$K_v$1.5 current, whereas the inhibitor of Src family kinases, PP2, increased the current, by decreasing phosphorylation of h$K_v$1.5 channels.

## Methods

### Human atrial myocyte isolation

The protocols for obtaining human atrial tissues was approved by the Ethics Committee of the University of Hong Kong (UW-10-174) with patients' consent. The investigation follows the principles outlined in the Declaration of Helsinki (see *Cardiovascular Research* 1997;35:2–4) for using human tissue. Human right atrial tissues were collected from patients undergoing coronary artery bypass grafting. Human atrial myocytes were enzymatically dissociated using the procedure as previously described (Li *et al.*, 1996a, 2008). The isolated myocytes were kept at room temperature in a high-potassium medium for at least 2 h before the electrophysiological recordings and randomly used for testing the effects of genistein, AG556, PP2 and/or orthovanadate on human atrial $I_{Kur}$.

### Cell culture, mutagenesis and gene transfection

The HEK 293 cell line (Tang *et al.*, 2007; Wu *et al.*, 2011) stably expressing hKv1.5/pBK$_{CMV}$ vector provided by Dr M. Tamkun (Colorado State University, CO, USA) was cultured with DMEM (Invitrogen, Hong Kong) containing 400 $\mu g{\cdot}mL^{-1}$ G418 (Sigma-Aldrich, St Louis, MO, USA) and 10% fetal bovine serum. Cells for electrophysiological recording were seeded on a glass cover slip.

The high-potential tyrosine phosphorylation sites of h$K_v$1.5 channels were predicted with NetPhos 2.0 software (www.cbs.dtu.dk/cgi-bin). Mutants of h$K_v$1.5 channels were generated using the site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), and each mutant was confirmed via full

DNA sequencing analysis (Gene Centre, University of Hong Kong) as described previously (Zhang *et al.*, 2011b; Wu *et al.*, 2013). The mutants Y155F, Y521F, Y601F or Y155F–Y521F–Y601F were transiently transfected (10 μL of Lipofectamine 2000 with 4 μg of the plasmid) into HEK 293 cells for electrophysiological recording.

### *Electrophysiology*

$I_{\mathrm{Kur}}$ and $\mathrm{hK_v1.5}$ current were recorded using the experimental conditions and procedures as described previously (Li *et al.*, 1996a; Wu *et al.*, 2011). Briefly, atrial myocytes or HEK 293 cells were transferred into the cell chamber and superfused with Tyrode's solution. Whole-cell configuration was established by a gentle suction after obtaining a gigaohm seal. Series resistance (3–5 MΩ) was compensated by 50–80% to reduce voltage errors. The data of membrane electrical signals were acquired using an EPC-10 amplifier and Pulse software (Heka Elektronik, Lambrecht, Germany). All the experiments were conducted at room temperature (22–24°C). The data obtained in cells with unstable $R_s$ and/or leakage current increased during experiments were discarded for analysis.

### *Immunoprecipitation and Western blots*

The immunoprecipitation and Western blots were performed following the procedure described previously (Liu *et al.*, 2007; Wu *et al.*, 2012). Briefly, samples of HEK 293 cells stably expressing $\mathrm{hK_v1.5}$ channels and grown to 70–80% confluence, were treated with the different compounds (30 min at room temperature) and then detached and centrifuged (4°C). After the cell pellet was lysed (Liu *et al.*, 2007; Wu *et al.*, 2012), the protein lysate was quantified with a protein assay reader (Bio-Rad Laboratories, Hercules, CA, USA) and diluted to equal concentrations. Proteins were immunoprecipitated with 2 μg anti-$\mathrm{hK_v1.5}$ channel antibody (NeuroMab, Davis, CA, USA) and protein A/G beads (100 μL, Upstate) overnight at 4°C. Immunoprecipitated proteins bound to pelleted protein A/G beads were washed with PBS, denatured in Laemmli sample buffer, separated with SDS-PAGE and electroblotted onto nitrocellulose membranes. The immunoblots were probed with anti-phosphotyrosine antibody (1:2000; Cell Signaling Technology Inc., Danvers, MA, USA) overnight at 4°C in the blocking medium containing 5% BSA in Tris buffered saline (TBS) and Tween 20 and subsequently treated with goat anti-mouse IgG-HRP antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. Blots were developed with enhanced chemiluminescence (GE Healthcare, Hong Kong) and exposed on X-ray film (Fuji Photo Film GmbH). The blots were then stripped and reprobed with the anti-$\mathrm{hK_v1.5}$ antibody to determine total $\mathrm{hK_v1.5}$ channel proteins. The film was scanned, imaged by a Bio-Imaging System (Syngene, Cambridge, UK) and analysed via Gene Tools software (Syngene).

### *Data and statistical analysis*

The data collection and statistical analysis in this study comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Data are presented as mean ± SEM. A group size of number of 5 or more was determined based on previous experience (Li *et al.*, 2008; Zhang *et al.*, 2012) with SigmaPlot 12.5 (SPSS Science, Chicago, IL, USA). Paired and/or unpaired Student's two-tailed *t*-test was used as appropriate to determine the statistical significance of differences between two group means. One-way ANOVA for multiple groups was followed by Tukey's test. A value of $P < 0.05$ was considered to indicate statistical significance.

### *Materials*

3-(4-Chlorophenyl) 1-(1,1-dimethylethyl)-1*H*-pyrazolo[3,4-d] pyrimidin-4-amine (PP2) was obtained from Tocris Bioscience (Bristol, UK). All other compounds were obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of genistein (100 mM), daidzein (100 mM), AG556 (100 mM) and PP2 (10 mM) were prepared in DMSO and then aliquoted and stored at −20°C. Aqueous stock solutions of sodium orthovanadate (100 mM) was prepared and pH was adjusted to 9.0 with HCl.

Tyrode's solution contained the following: (mM) NaCl 140, KCl 5.4, $MgCl_2$ 1.0, $CaCl_2$ 1.8, HEPES 5.0 and glucose 10 (pH adjusted to 7.3 with NaOH). The pipette solution contained the following: (mM) KCl 20, K-aspartate 110, $MgCl_2$ 1.0, HEPES 10, EGTA 5, GTP 0.1, $Na_2$-phosphocreatine 5 and Mg-ATP 5 (pH adjusted to 7.2 with KOH).

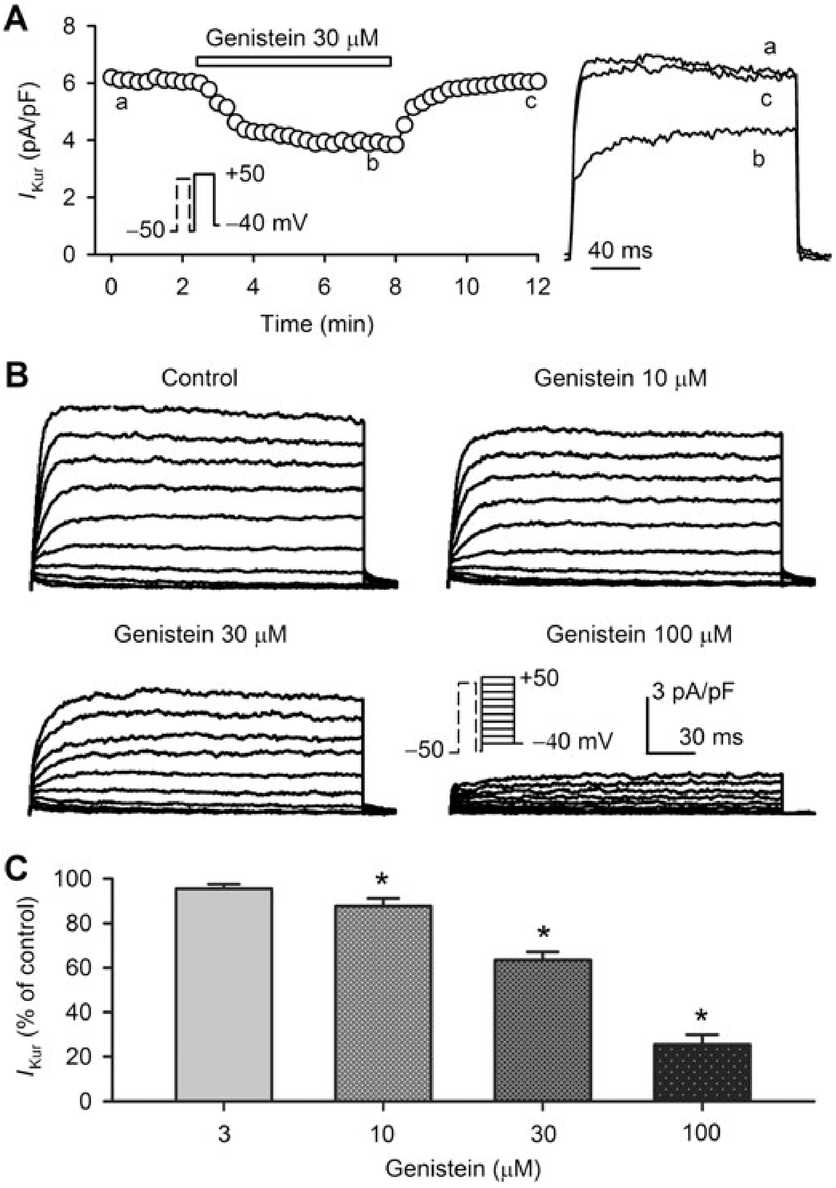
## Results

### *Effect of genistein on $I_{Kur}$*

The effect of genistein (a broad spectrum PTK inhibitor) on $I_{\mathrm{Kur}}$ was investigated in human atrial myocytes. The time course of $I_{\mathrm{Kur}}$ at +50 mV (Figure 1A) was determined in a representative human atrial myocyte with the voltage protocol (inset) in the absence and presence of 30 μM genistein. The current was gradually decreased by genistein, and the inhibition reached a steady-state level in 5 min and almost fully recovered on washout. Figure 1B displays the family of voltage-dependent $I_{\mathrm{Kur}}$ recorded in a representative cell with the voltage steps as shown in the inset in the absence (control) and presence of 10, 30 and 100 μM genistein. The current was inhibited by genistein in a concentration-dependent manner. The percentage values of current inhibition, measured (+50 mV, $n = 6$) at the steady-state current of $I_{\mathrm{Kur}}$ at end of voltage step in cells treated with 3, 10, 30 and 100 μM genistein, are illustrated in Figure 1C. Significant inhibition was observed at 10–100 μM ($n = 6$, $P < 0.05$ vs. control).

To determine whether the $I_{\mathrm{Kur}}$ reduction by genistein, as previously observed in rat cardiac $K_v$ currents (Gao *et al.*, 2004), is related to PTK inhibition, we tested daidzein (a PTK-inactive analogue of genistein) in human atrial myocytes. Daidzein at 30 μM had no inhibitory effect on $I_{\mathrm{Kur}}$, whereas genistein at 30 μM markedly suppressed this current in the same myocyte (Figure 2A), suggesting that PTK inhibition is likely to be involved in the decreased $I_{\mathrm{Kur}}$ induced by genistein.

Orthovanadate (a protein tyrosine phosphatase inhibitor) was then used to determine whether $I_{\mathrm{Kur}}$ inhibition induced by genistein could be reversed by this compound. Orthovanadate at 1 mM significantly reversed the $I_{\mathrm{Kur}}$ reduction induced by 30 μM genistein (Figure 2B). Figure 2C illustrates the percentage values of current amplitude (+50 mV) with daidzein, genistein and genistein plus orthovanadate or orthovanadate alone. No significant inhibition of $I_{\mathrm{Kur}}$ was



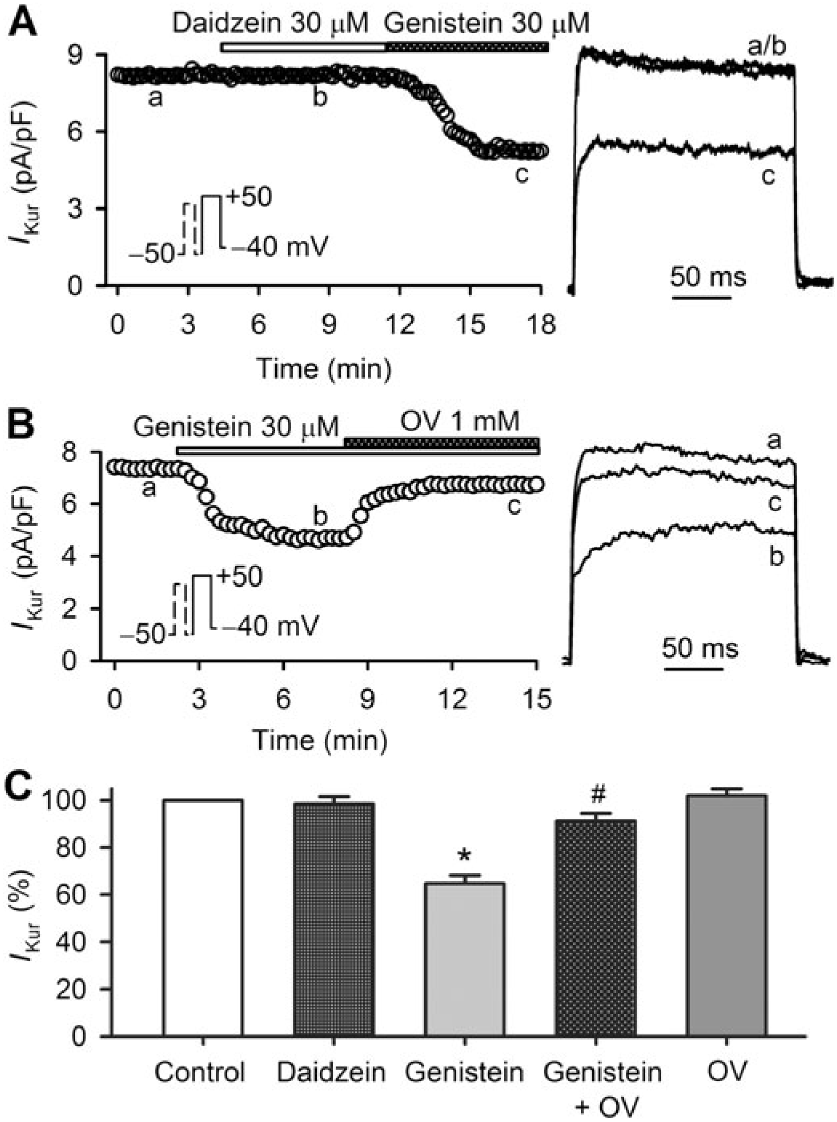
## Figure 1

Inhibition of human atrial $I_{\mathrm{Kur}}$ by genistein. (A) Time course of $I_{\mathrm{Kur}}$ recorded in a typical experiment with a 100 ms prepulse to +40 mV from −50 mV to inactivate $I_{\mathrm{to}}$, followed by 200 ms test pulse to +50 mV (inset) after a 10 ms interval every 15 s. The cell was treated with 30 μM genistein. $I_{\mathrm{Kur}}$ traces at corresponding time points are shown in right side of the panel. (B) Family of $I_{\mathrm{Kur}}$ (capacitance compensated) at various depolarization voltages (a 100 ms prepulse to +40 mV to inactivate $I_{\mathrm{to}}$, followed by 200 ms test pulses to between −40 and +50 from −50 mV after a 10 ms interval and then to −40 mV in a typical experiment) in the absence and presence of 10, 30 and 100 μM genistein. (C) Percent values of $I_{\mathrm{Kur}}$ at +50 mV in cells treated with 3, 10, 30 and 100 μM genistein ($n = 6$). *$P < 0.05$, significantly different from control.

observed with 30 μM daidzein ($n = 5$, $P > 0.05$). Orthovanadate (1 mM) alone did not affect $I_{\mathrm{Kur}}$ significantly ($n = 6$, $P > 0.05$ vs. control), while it reversed $I_{\mathrm{Kur}}$ inhibition by 30 μM genistein ($n = 7$, $P < 0.05$ vs. genistein alone). These results suggest that the $I_{\mathrm{Kur}}$ reduction by 30 μM genistein is a result of both PTK-dependent and -independent inhibition.

## *Effect of AG556 on human atrial $I_{Kur}$*

The human atrial $I_{\mathrm{to}}$ (Zhang *et al.*, 2012) is inhibited by the highly selective EGFR kinase inhibitor AG556. Here we determined the effects of AG566 on $I_{\mathrm{Kur}}$ in human atrial myocytes. Figure 3A shows the time course of $I_{\mathrm{Kur}}$ recorded in a representative cell with the voltage protocol shown in the *inset* before and after application of 10 μM AG556. The current was gradually decreased by AG556, and the inhibition was fully reversed on washout. Figure 3B displays the family of voltage-dependent $I_{\mathrm{Kur}}$ recorded in a typical experiment with the voltage protocol shown in the inset in the absence (control) and presence of 3, 10 and 30 μM AG556. AG556 decreased human atrial $I_{\mathrm{Kur}}$ in a concentration-dependent manner. Figure 3C illustrates the current inhibition (+50 mV) in cells treated with 1, 3, 10 and 30 μM AG556.



## Figure 2

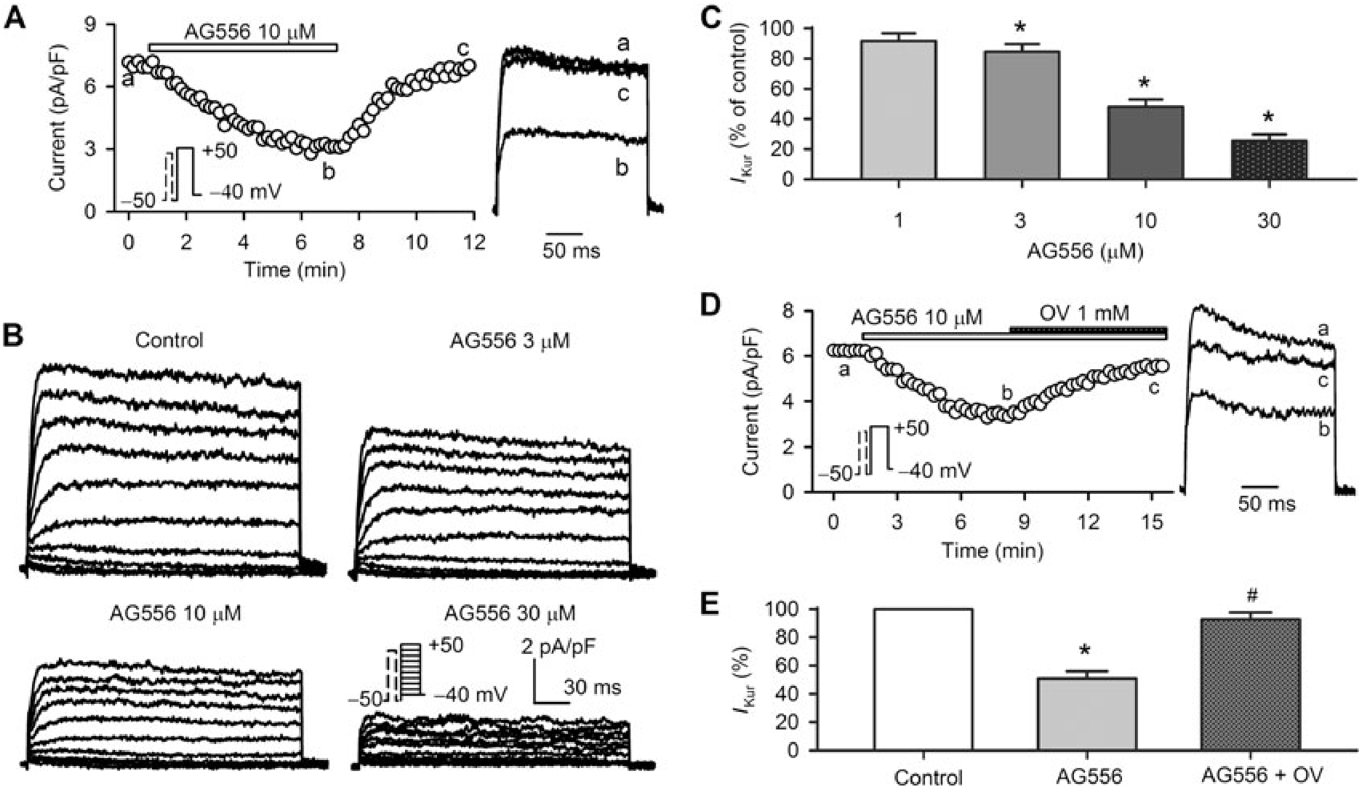
Effect of orthovanadate (OV) on genistein in human atrial myocytes. (A) Time course of $I_{Kur}$ recorded with the voltage protocol as shown in the inset in a typical experiment in the absence and presence of 30 μM daidzein or 30 μM genistein. $I_{Kur}$ traces at corresponding time points are shown in right side of the panel. (B) Time course of $I_{Kur}$ recorded in an atrial myocyte in the absence and presence of 30 μM genistein, and genistein plus 1 mM OV. $I_{Kur}$ traces at corresponding time points are shown in right side of the panel. (C) Histogram showing the mean percent values of $I_{Kur}$ during control, in the presence of 30 μM daidzein ($n = 5$), 30 μM genistein, genistein plus 1 mM OV ($n = 7$). $^*P < 0.05$, significantly different from control; #$P < 0.05$, significantly different from genistein alone or 1 mM OV alone ($n = 5$).

Significant current reduction was observed with 3–30 μM AG556 ($n = 6$, $P < 0.05$ vs. control).

Figure 3D shows the time course of $I_{Kur}$ recorded in another typical experiment before and after application of 10 μM AG556, and AG556 plus 1 mM orthovanadate. AG556 gradually reduced $I_{Kur}$, and the current reduction was significantly reversed by 1 mM orthovanadate. Figure 3E illustrates the $I_{Kur}$ at +50 mV (as % control) before and after application of 10 μM AG556 and AG556 plus 1 mM orthovanadate. AG556 decreased the current ($n = 6$, $P < 0.05$ vs. control), and this inhibition was reversed by orthovanadate ($n = 6$, $P < 0.05$ vs. AG556 alone). These results reveal that the decrease in $I_{Kur}$ caused by 10 μM AG556 is mostly a result of EGFR kinase inhibition with a much smaller PTK-independent inhibition.

### *Inhibition of hK$_v$1.5 current by genistein and AG556*

The human cardiac $I_{Kur}$ is carried by K$_v$1.5 channels, encoded by the *KCNA5* gene (Fedida *et al.*, 1993; Feng *et al.*, 1997). Thus, we determined whether hK$_v$1.5 channel current is affected by genistein or AG556 and whether their effects can be reversed by orthovanadate in HEK 293 cells stably expressing hK$_v$1.5 channels. Figure 4A shows that the family of voltage-dependent hK$_v$1.5 current was reversibly inhibited



## Figure 3

Effect of AG556 on human atrial $I_{Kur}$. (A) Time course of $I_{Kur}$ recorded in a typical human atrial myocyte in the absence and presence of 10 μM AG556. $I_{Kur}$ traces at corresponding time points are shown in right of the panel. (B) Family of $I_{Kur}$ recorded in a representative cell using the voltage protocol shown in the inset during control and application of 3, 10 and 30 μM AG556. (C) Percent values of $I_{Kur}$ at +50 mV in cells treated with 1, 3, 10 and 30 μM AG556 ($n = 6$). *$P < 0.05$ , significantly different from control. (D) Time course of $I_{Kur}$ recorded in an atrial myocyte in the absence and presence of 10 μM AG556, and AG556 plus 1 mM orthovanadate (OV). $I_{Kur}$ traces at corresponding time points are shown in right side of the panel. (E) Percent values of $I_{Kur}$ (at +50 mV) in the absence and presence of 10 μM AG556 or AG556 plus OV ($n = 7$). *$P < 0.05$, significantly different from control; #$P < 0.05$, significantly different from AG556 alone.

by 30 μM genistein and the inhibition was reversed by 1 mM orthovanadate (Figure 4B).

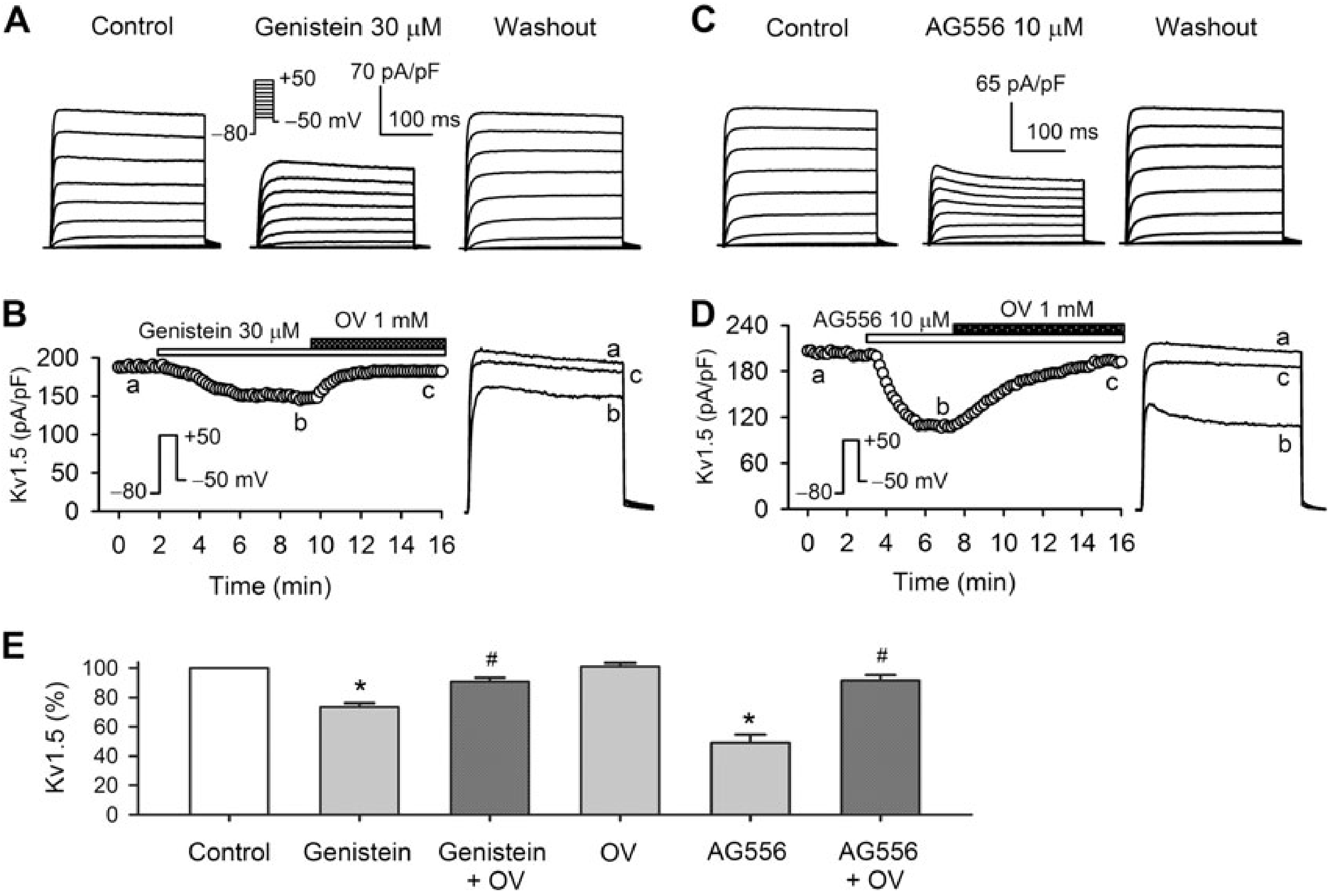
Voltage-dependent $hK_v1.5$ current was also reversibly inhibited by 10 μM AG556 (Figure 4C). Figure 4D displays the time course of $hK_v1.5$ current in a representative cell. AG556 at 10 μM gradually inhibited $hK_v1.5$ current, and the inhibition was reversed by orthovanadate (1 mM). The result was similar to $I_{Kur}$ in human atrial myocytes (Figure 3D).

Figure 4E shows the $hK_v1.5$ current (+50 mV), as %control, in cells treated with 30 μM genistein, genistein plus 1 mM orthovanadate, orthovanadate alone, 10 μM AG556 or AG556 plus orthovanadate. Orthovanadate, as in human atrial $I_{Kur}$, had no effect on $hK_v1.5$ channel current. Genistein inhibited $hK_v1.5$ current at +50 mV ($n = 9$, $P < 0.05$ vs. control), and this inhibition was reversed by orthovanadate ($n = 9$, $P < 0.05$ vs. genistein alone). The orthovanadate-insensitive fraction of genistein-induced current reduction indicates a PTK-independent inhibition of $hK_v1.5$ channels by 30 μM genistein, as observed in human atrial $I_{Kur}$.

AG556 at 10 μM decreased the current ($n = 8$, $P < 0.05$ vs. control), and the inhibitory effect was reversed by 1 mM orthovanadate ($n = 8$, $P < 0.05$ vs. AG556 alone), showing a small PTK-independent reduction. These results indicate that $hK_v1.5$ channels expressed in HEK 293 cells, similar to $I_{Kur}$, are inhibited by genistein or AG556 via PTK-dependent and PTK-independent mechanisms. The PTK-independent effect of genistein on $I_{Kur}$ and $hK_v1.5$ channels is also reflected by a delayed time of the current to peak (closed channel blocking, Figures 1A and B and 4A and B), while the direct inhibition of $hK_v1.5$ channels by AG556 is reflected by an increased current inactivation (open channel blocking, Figure 4C and D).

### *Effects of PP2 on $I_{Kur}$ and $hK_v1.5$ channels*

Whether PP2 (a Src family kinase inhibitor) could also affect $I_{Kur}$/$hK_v1.5$ current was determined in human atrial myocytes and HEK 293 cells expressing $hK_v1.5$channels. Figure 5A shows the family of voltage-dependent $I_{Kur}$ recorded in a typical experiment in the absence and presence of 1 μM PP2 [a concentration 200 times higher (Hanke *et al.*, 1996) than the $EC_{50}$ for inhibiting Src-related kinases]. A high concentration of PP2 is usually required for Src family kinase inhibition in experiments at cellular level (Du *et al.*, 2004; Zhang *et al.*, 2008; Zhang *et al.*, 2012), perhaps because PP2 does not easily cross the cell membrane. It is interesting to note that $I_{Kur}$ was slightly increased by PP2 (7 min superfusion), and the enhancement was also reversed by co-application of 1 mM orthovanadate. Similar results were



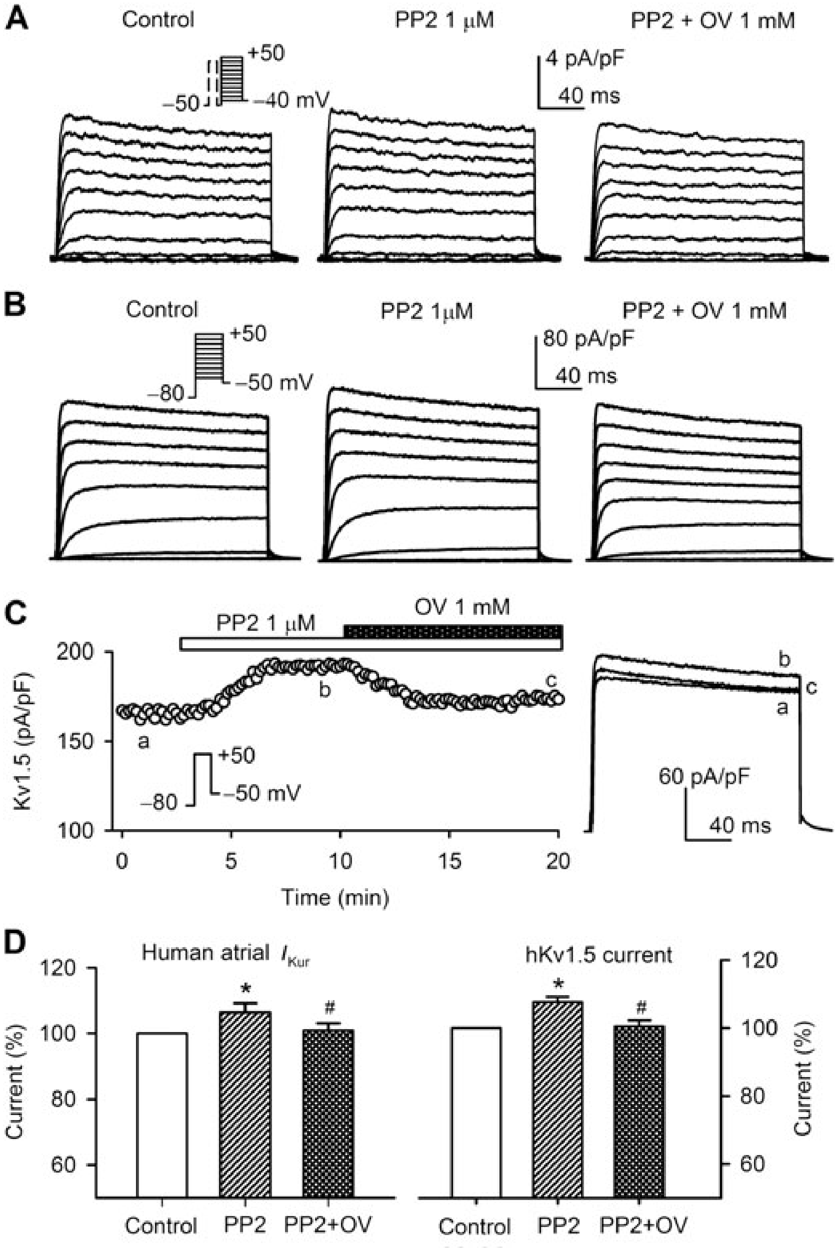
## Figure 4

Effects of PTK inhibitors on $hK_v1.5$ channels stably expressed in HEK 293 cells. (A) Voltage-dependent $hK_v1.5$ current was recorded with voltage protocol as shown in the inset in the absence (control) and presence of 30 μM genistein, and on washout. (B) Time course of $hK_v1.5$ current (left) and original current traces (right) in the absence and presence of 30 μM genistein, and genistein plus 1 mM orthovanadate (OV). The $hK_v1.5$ current traces at corresponding time points are shown in right side of the panel. (C) Voltage-dependent $hK_v1.5$ current was recorded in the absence and presence of 10 μM AG556, and on washout. (D) Time course of $hK_v1.5$ current (left) and original current traces (right) in the absence and presence of 10 μM AG556, and AG556 plus 1 mM OV. The $hK_v1.5$ current traces at corresponding time points are shown in right side of the panel. (E) Percentage values of hKv1.5 current (at +50 mV) in the absence (control) and presence of 30 μM genistein or genistein plus OV ($n = 9$). *$P < 0.05$, significantly different from control; #$P < 0.05$, significantly different from genistein alone, OV alone ($n = 5$), 10 μM AG556 and AG556 plus 1 mM OV ($n = 8$). *$P < 0.05$, significantly different from control; #$P < 0.05$, significantly different from AG556 alone).

observed in $hK_v1.5$ channels in HEK 293 cells (Figure 5B). Figure 5C shows the time course of $hK_v1.5$ current recorded in an HEK 293 cell expressing $hK_v1.5$ channels with the voltage step shown in the *inset* before and after application of 1 μM PP2, and PP2 plus 1 mM orthovanadate. PP2 gradually increased $hK_v1.5$ current, and the effect was gradually reversed by orthovanadate. The inhibition of $I_{Kur}$ and $hK_v1.5$ current at +50 mV are shown in Figure 5D. Human atrial $I_{Kur}$ was increased by PP2 ($n = 6$, $P < 0.05$), and the effect was reversed by orthovanadate, while $hK_v1.5$ current was similarly increased by PP2 ($n = 7$, $P < 0.05$) and was reversed by orthovanadate. These results indicate that PP2 slightly, but significantly, increased $I_{Kur}$/$hK_v1.5$ current in a PTK-dependent manner.

### *Tyrosine phosphorylation of $hK_v1.5$ channels*

If the suppression of $I_{Kur}$/$hK_v1.5$ channels by genistein and AG556 or the increase of $I_{Kur}$/$hK_v1.5$ channels by PP2 is mediated by EGFR kinase inhibition or Src family kinases reduction, tyrosine phosphorylation of the channel would be reduced by these PTK inhibitors. The tyrosine phosphorylation of $hK_v1.5$ protein was therefore determined in HEK 293 cells stably expressing $hK_v1.5$ channels, but not in human atrial myocytes due to the limited cells isolated from human atrial specimens. Figure 6A displays the tyrosine phosphorylation images of $hK_v1.5$ channels in the HEK 293 cells treated with 1 mM orthovanadate, 30 μM genistein, genistein plus orthovanadate, 10 μM AG556, AG556 plus orthovanadate, 1 μM PP2 or PP2 plus orthovanadate (30 min). Genistein, AG556 and PP2 significantly decreased the phosphorylation level of $hK_v1.5$ channel protein, and the reduction in phosphorylation was reversed by pretreatment (30 min) with 1 mM orthovanadate. Orthovanadate itself had no effect on phosphorylation levels of the $hK_v1.5$ protein. This indicates that the phosphorylation level of $hK_v1.5$ channels, like hERG channels (Zhang *et al.*, 2008), $K_{ir}2.1$ channels (Zhang *et al.*,

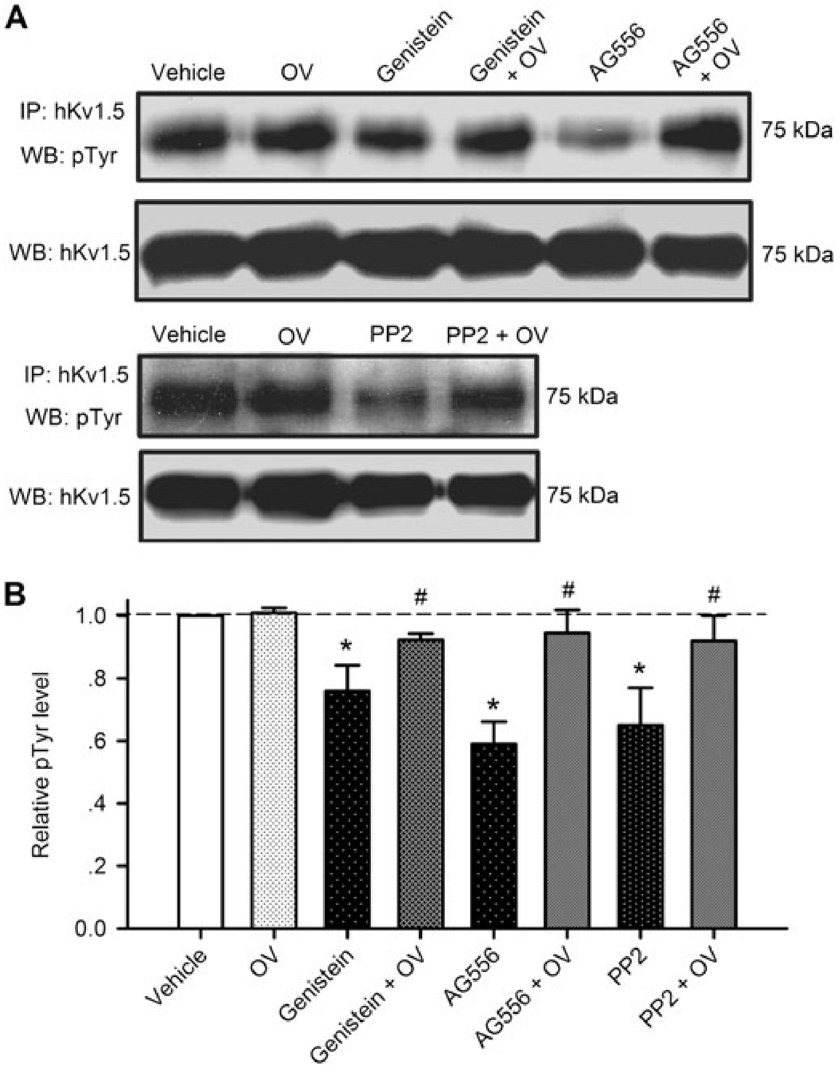


## Figure 5

Effects of PP2 on $I_{Kur}$/hK$_v$1.5current. (A) Voltage-dependent $I_{Kur}$ recorded in a human atrial myocyte with the voltage protocol as shown in the inset in the absence and presence of 1 μM PP2, and PP2 plus 1 mM orthovanadate (OV). (B) Voltage-dependent hK$_v$1.5current recorded in HEK 293 cells expressing *KCNA5* with the voltage protocol as shown in the inset in the absence and presence of 1 μM PP2, and PP2 plus 1 mM OV. (C) Time course of hK$_v$1.5 current recorded in a typical experiment in the absence and presence of 1 μM PP2 and PP2 plus 1 mM OV. The hK$_v$1.5 current traces at corresponding time points are shown in right side of the panel with expanded Y-axis. (D) Percentage values of $I_{Kur}$ (left panel, $n = 7$) and hK$_v$1.5 current (right panel, $n = 7$) during control, in the presence of 1 μM PP2, and PP2 plus 1 mM OV ($n = 7$). $*P < 0.05$, significantly different from control; $\#P < 0.05$, significantly different from PP2 alone.

2011a) and hK$_v$4.3 channels (Zhang *et al.*, 2012), is saturated under basal physiological conditions.

Figure 6B summarises the mean levels of hK$_v$1.5 tyrosine phosphorylation. Orthovanadate itself had no effect on the saturated tyrosine phosphorylation of hK$_v$1.5 channels. Genistein (30 μM) decreased the tyrosine phosphorylation of hK$_v$1.5 channel protein ($n = 5$, $P < 0.05$ vs. vehicle control), and the reduction was countered by 1 mM orthovanadate ($P < 0.05$ vs. genistein alone). AG556 (10 μM) decreased the tyrosine phosphorylation ($n = 5$, $P < 0.05$ vs. control) and this effect was reversed by 1 mM orthovanadate ($P < 0.05$ vs. AG556 alone). PP2 (1 μM) decreased the tyrosine phosphorylation level ($n = 5$, $P < 0.05$ vs. control) and the inhibition was reversed by co-application of orthovanadate ($P < 0.05$ vs. PP2 alone). These results indicate that the inhibition of hK$_v$1.5



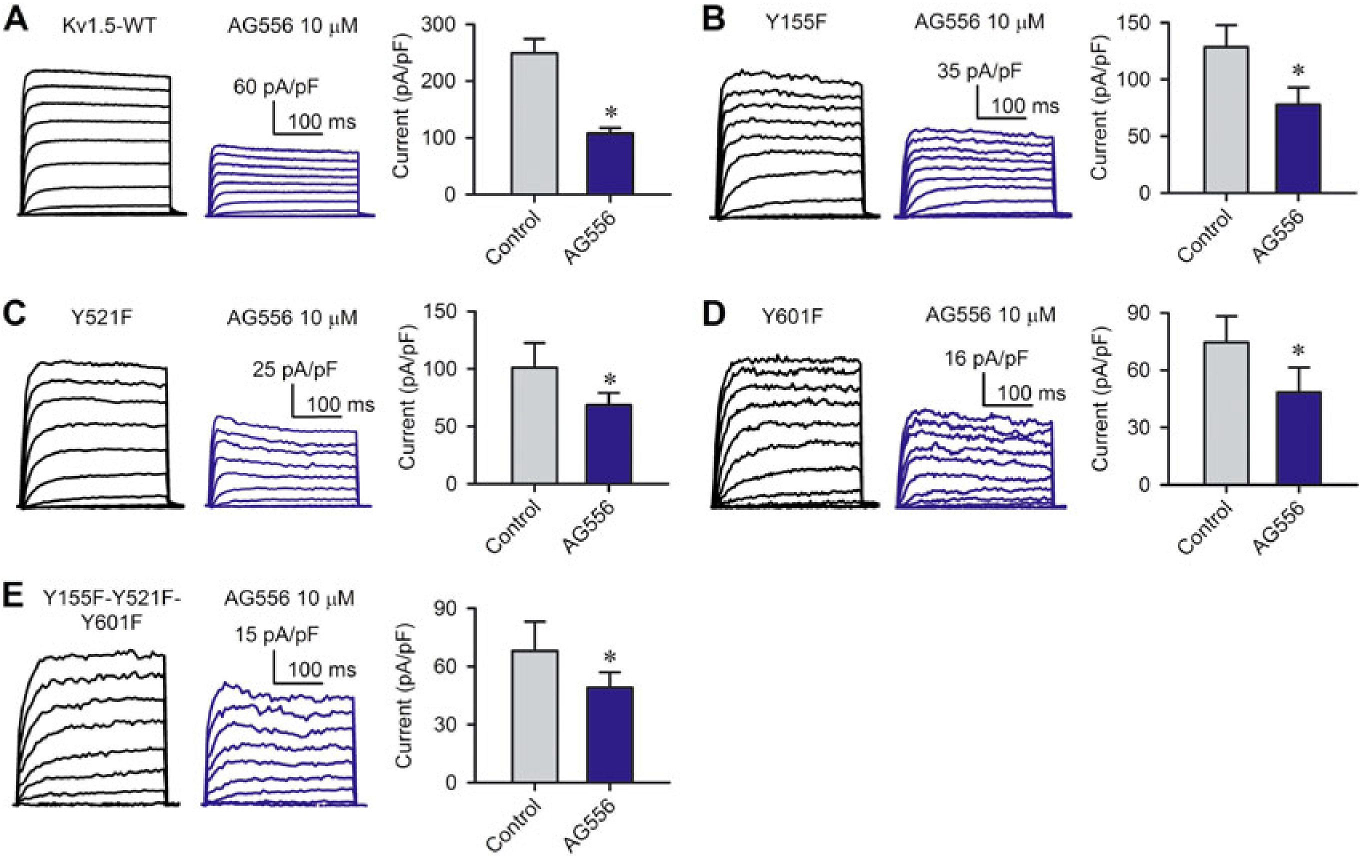
## Figure 6

Tyrosine phosphorylation levels of hK$_v$1.5 channels. (A) Images of immunoprecipitation (IP) and western blot (WB) in cells treated with vehicle (control), 1 mM orthovanadate (OV), 30 μM genistein, genistein plus 1 mM OV, 10 μM AG556, AG556 plus 1 mM OV, 1 μM PP2 and PP2 plus OV. (B) Relative phosphorylated hK$_v$1.5 levels were determined by dividing pTyr- K$_v$1.5 density by total hK$_v$1.5 protein density in cells treated with OV, genistein, AG556 or PP2 as described in (A) and then normalizing to vehicle control ($n = 5$). *$P < 0.05$, significantly different from vehicle control; #$P < 0.05$, significantly different from genistein, AG556 or PP2 alone.

current by genistein or AG556 and the increase of hK$_v$1.5current by PP2 are mediated by reducing the tyrosine phosphorylation of the channel by EGFR TK or Src family kinases.

## *Potential tyrosine phosphorylation sites of hK$_v$1.5channels*

To determine the potential EGFR tyrosine phosphorylation sites of hK$_v$1.5 channels, we initially generated three mutants (Y155F, Y521F and Y601F) of predicted tyrosine phosphorylation sites and tested the inhibitory response of these mutants to the selective EGFR kinase inhibitor AG556. The wild-type (WT) hK$_v$1.5 and the mutant currents recorded in HEK 293 cells transiently expressing the corresponding hK$_v$1.5 channel mutants are displayed in Figure 7A–D in the absence and presence of 10 μM AG556. It appears that current density is greater in WT hK$_v$1.5 channels than in hK$_v$1.5 mutants (Table 1, $n = 7–12$, $P < 0.05$). The sensitivity of Y155F, Y521F and Y601F to AG556 was reduced, which suggests that Y155, Y521 and Y601 may be the EGFR kinase phosphorylation sites. However, the triple mutant Y155F–Y521F–Y601F of hK$_v$1.5 channels still showed a significant inhibitory response to 10 μM AG556, though it was more sensitive to AG556 ($P < 0.05$ vs. other mutants). This differs from the hK$_v$10.1 channels, in which triple tyrosine phosphorylation site mutation abolishes the inhibitory response to AG556 (Wu *et al.*, 2012). These results suggest that the tyrosine phosphorylation sites of hK$_v$1.5 channels are not limited to Y155, Y521 and Y601.



## Figure 7

Effects of AG556 on mutant $hK_v1.5$ channels. (A) WT $hK_v1.5$ current recorded with the voltage protocol as shown in Figure 4A in a representative cell treated with 10 µM AG556; bar graph shows WT $hK_v1.5$ current densities at +50 mV ($n = 12$). $^*P < 0.05$, significant effect of AG556). (B) Y155F current recorded in a representative cell treated with 10 µM AG556; bar graph shows Y155F current densities at +50 mV ($n = 9$). $^*P < 0.05$, significant effect of AG556). (C) Y521F current recorded in a representative cell treated with 10 µM AG556; bar graph shows Y521F current densities at +50 mV ($n = 8$). $^*P < 0.05$, significant effect of AG556). (D) F601F current recorded in a representative cell treated with 10 µM AG556; bar graph shows Y601F current densities at +50 mV ($n = 8$). $^*P < 0.05$, significant effect of AG556). (E) Y155F–Y521F–Y601F current recorded in a representative cell treated with 10 µM AG556; bar graph shows the triple mutant current densities at +50 mV ($n = 7$). $^*P < 0.05$, significant effect of AG556).

## Table 1

Current inhibition by AG556 (10 µM) in HEK 293 cells expressing WT or various mutants of $hK_v1.5$ channels

| $hK_v1.5$ | *n* | Control | AG556 | Inhibition % |
|---|---|---|---|---|
| WT | 12 | 249.1 ± 25.2* | 107.5 ± 9.9[#] | 54.8[†] |
| Y155F | 9 | 128.5 ± 19.2 | 77.9 ± 14.8[#] | 39.3 |
| Y521F | 8 | 101.1 ± 21.5 | 60.3 ± 10.6[#] | 38.5 |
| Y601F | 8 | 76.4 ± 13.7 | 48.4 ± 13.1[#] | 37.7 |
| Triple mutant | 7 | 68.1 ± 14.9 | 49.1 ± 7.8[#] | 29.4[Ψ] |

*$P < 0.05$, significantly different from mutants.

[#] $P < 0.05$, significantly different from control (before AG556).

[†] $P < 0.05$, significantly different from $hK_v1.5$ mutants.

[Ψ] $P < 0.05$, significantly different from Y155F, Y521F or Y601F.

## Discussion

It is well known that PTKs not only modulate cell growth and differentiation (Hubbard and Till, 2000) but also regulate ion channels (Davis *et al.*, 2001). Earlier studies have used the inhibitors of receptor TKs, for example, the isoflavone genistein and the tyrphostin compounds (Levitzki and Mishani, 2006), or the non-receptor TK (e.g. Src family kinases) inhibitor PP2, and/or a protein tyrosine phosphatase inhibitor (e.g. orthovanadate) in different types of cells and investigated the regulation of a number of ion channels and currents by TKs, including L-type $Ca^{2+}$ current ($I_{Ca.L}$) in cardiac myocytes (Ogura *et al.*, 1999), volume-sensitive chloride current ($I_{Cl.vol}$) in dog and human atrial cardiac myocytes (Sorota, 1995; Du *et al.*, 2004), cardiac voltage-gated $Na^+$ current ($I_{Na}$) (Liu *et al.*, 2007) and several types of $K^+$ channels in different types of cells (Gao *et al.*, 2004; Zhang *et al.*, 2008, 2011a,b, 2012; Dong *et al.*, 2010; Wu *et al.*, 2012, 2013).

In the present study, the Src family kinase inhibitor PP2 slightly increased human atrial $I_{Kur}$ and hK$_v$1.5 current expressed in HEK 293 cells and significantly inhibited tyrosine phosphorylation of hK$_v$1.5 channels and the effects were countered by the protein tyrosine phosphatase inhibitor orthovanadate, suggesting that endogenous tyrosine phosphorylation of hK$_v$1.5 channels by Src family kinases would inhibit $I_{Kur}$/hK$_v$1.5 current. This notion is supported by the early report of a direct association of Src TK with native hK$_v$1.5 channels in human myocardium and cloned hK$_v$1.5 channels. This occurred at a proline-rich motif of the channel and the SH3 domain of Src, and tyrosine phosphorylation of hK$_v$1.5 channels suppressed the channel current in cells coexpressing v-Src (Holmes *et al.*, 1996). A recent study found that removing this proline-rich region resulted a mutant hK$_v$1.5 channel with reduced current and lack of response to v-Src-induced current reduction, which suggests the abnormal atrial repolarization control due to variable Src family TK signalling as a mechanism in familial atrial fibrillation (Yang *et al.*, 2010). A more recent report demonstrated that activation of the TK JAK3 down-regulated K$_v$1.5 channels with an effect similar to Src family kinases (Warsi *et al.*, 2015).

The present study provided new evidence that $I_{Kur}$/hK$_v$1.5 current may also be regulated by EGFR kinase. Genistein is a broad-spectrum PTK inhibitor. It strongly inhibits EGFR kinase and also the Src family kinases (Akiyama and Ogawara, 1991), and is widely utilized for investigating ion channel regulation by PTKs (Ogura *et al.*, 1999; Gao *et al.*, 2004). The present study showed that genistein did not increase but inhibited $I_{Kur}$/hK$_v$1.5 current. PTK-independent suppression by genistein has been previously observed in cardiac $I_{Ks}$ (Washizuka *et al.*, 1998), $I_{Kr}$ (Missan *et al.*, 2006), neuronal $I_{Na}$ (Liu *et al.*, 2004), K$_{ir}$2.1 and K$_{ir}$2.3 channels (Zhao *et al.*, 2008) and K$_v$4.3 channels expressed in CHO cells (Kim *et al.*, 2011). However, PTK-dependent inhibition of human cardiac $I_{Ks}$ and hERG channels and hK$_{ir}$2.1, hK$_{ir}$2.3 and hK$_v$4.3 channels by genistein was revealed by the application of the protein tyrosine phosphatase inhibitor orthovanadate and the determination of tyrosine phosphorylation levels of these channel in our earlier reports (Zhang *et al.*, 2008; Dong *et al.*, 2010; Zhang *et al.*, 2011a,b, 2012). In this study, we found that genistein (3–100 μM) inhibited human atrial $I_{Kur}$ in a concentration-dependent manner. The reduction of $I_{Kur}$/hK$_v$1.5 current by 30 μM genistein was significantly reversed by orthovanadate. A small fraction of PTK-independent inhibitory action of genistein is also involved in the overall inhibition of $I_{Kur}$/hK$_v$1.5 current. This suggests that the effects of 30 μM genistein on $I_{Kur}$/hK$_v$1.5 current is mainly due to inhibition of EGFR kinase, which was confirmed with the selective EGFR kinase inhibitor AG556.

AG556 is a highly selective inhibitor of EGFR TK (Levitzki and Mishani, 2006). Earlier studies demonstrated that AG556 can inhibit EGFR kinase activation and thereby improve rat spinal cord injury (Usul *et al.*, 2004) and cardiac arrhythmias (Feng *et al.*, 2012) induced by ischaemia/reperfusion injury accompanied with EGFR activation. Also, AG556 reversed the EGF-induced enhancement of cardiac $I_{Na}$ (Liu *et al.*, 2007) and K$_{ir}$2.3 current (Zhang *et al.*, 2011b). In the present study, we demonstrated that AG556 inhibited human atrial $I_{Kur}$ in a concentration-dependent manner. The decrease of $I_{Kur}$/hK$_v$1.5 current by 10 μM AG556 is mainly due to inhibiting EGFR TK, though a small fraction of PTK-independent effect cannot be excluded. The EGFR TK inhibition is supported by the evidence that the reduced current and tyrosine phosphorylation level by AG556 are reversed by the protein tyrosine phosphatase inhibitor orthovanadate. Tyrosine phosphorylation of hK$_v$1.5 channels by EGFR kinase activates the channel and may thereby enhance the current. However, orthovanadate did not affect $I_{Kur}$/hK$_v$1.5 current or the channel phosphorylation level, which suggests the basal tyrosine phosphorylation of hK$_v$1.5 channels is saturated, as observed in rat cardiac K$_v$ currentS (Gao *et al.*, 2004), human cardiac $I_{to}$ (Zhang *et al.*, 2012), hERG (Zhang *et al.*, 2008), $I_{Ks}$ (Dong *et al.*, 2010), K$_{ir}$2.1 (Zhang *et al.*, 2011a) and also hEAG1 (Wu *et al.*, 2012) and hSKCa1(Wu *et al.*, 2013).

$I_{Kur}$ is a rapidly activating, delayed rectifier current in response to depolarization, is predominantly present in human atria (Li *et al.*, 1996b) and is responsible for human atrial repolarization (Feng *et al.*, 1997; Wettwer *et al.*, 2004; Li *et al.*, 2008). Previous studies demonstrate that hK$_v$1.5 channels can be down-regulated by Src family kinases (Holmes *et al.*, 1996; Yang *et al.*, 2010). In this study, we showed that in addition to Src family kinase regulation, EGFR TK also regulates $I_{Kur}$/hK$_v$1.5 current. EGFR TK and Src kinases regulate $I_{Kur}$/hK$_v$1.5 current with opposite actions. Under physiological conditions, control by EGFR kinase is clearly dominant (increasing the current). The basal full-stoichiometric phosphorylation of hK$_v$1.5 channels with other $K^+$ channels (K$_{ir}$2.1, K$_{ir}$2.3, $I_{Kr}$, $I_{Ks}$) is responsible for maintaining normal human atrial repolarization. So inhibition of EGFR tyrosine phosphorylation of these $K^+$ channels by genistein and/or AG556 would decrease the current amplitude and therefore delay human atrial repolarization. On the other hand, under pathophysiological conditions, control by Src kinases may be important. Recent studies have demonstrated that loss-of-function and/or gain-of function mutations of hK$_v$1.5 channels increase susceptibility to atrial fibrillation (Olson *et al.*, 2006; Christophersen *et al.*, 2013). Abnormal Src kinase phosphorylation of hK$_v$1.5 channels is implicated in familial atrial fibrillation (Yang *et al.*, 2010).

In addition, K$_v$1.5 current plays a role in the maintenance of the cell membrane potential in a wide variety of cells/tissues, such as pancreatic beta cells (MacDonald and Wheeler, 2003), brain tissue (Tipparaju *et al.*, 2012), macrophages (Vicente *et al.*, 2006) and skeletal muscle (Kang *et al.*, 2009), as well as smooth muscles in vessels (Overturf *et al.*, 1994) and airways (Adda *et al.*, 1996). Therefore, tyrosine phosphorylation of $hK_v1.5$ channels could also be important in maintaining normal cellular function of these types of cells. Moreover, $K_v1.5$ channels are also expressed in several tumour cells and participate in the modulation of cell adhesion, proliferation and apoptosis (Bonnet *et al.*, 2007; Ousingsawat *et al.*, 2007; Arvind *et al.*, 2012). Thus, $K_v1.5$ channels has been considered to be a potential target to regulate tumour growth (Felipe *et al.*, 2012). Inhibition of $K_v1.5$ channel activity via EGFR inhibitors is a possible pathway for the clinical treatment of cancers (Tan *et al.*, 2015).

A major limitation of this study was that we did not identify all of the tyrosine phosphorylation sites involved in the EGFR kinase phosphorylation of $hK_v1.5$ channels. The mutants Y155F, Y521F and Y601F showed a reduced response to AG556 inhibition. However, the triple mutant Y155F–Y521F–Y601F did not completely eliminate the inhibitory response to 10 μM AG556 (−29.4%). The small fraction (8.5%) of PTK-independent inhibition of $hK_v1.5$ channels by 10 μM AG556 cannot account for the significant inhibition of the triple mutant, which suggests that in addition to Y155, Y521 and Y601, other tyrosine sites may also be involved in EGFR kinase phosphorylation.

The tyrosine phosphorylation of $hK_v1.5$ channels by EGFR TKs is clearly not simple as EGFR kinase phosphorylation of cardiac $K_{ir}2.1$, $K_{ir}2.3$ and SKCa1, in which only one tyrosine site is involved in EGFR kinase phosphorylation (Zhang *et al.*, 2011a,b; Wu *et al.*, 2013). A similar phenomenon was also observed in cardiac $I_{Ks}$ in which several tyrosine sites are involved in EGFR kinase phosphorylation of KCNQ1 (Missan *et al.*, 2009). Another limitation was that we were unable to obtain data on the changes in tyrosine phosphorylation in mutant $hK_v1.5$ channels because our very low transfection rate (typically <10%) precluded biochemical analysis by immunoprecipitation and Western blotting. Although mass spectrometry can be used to accurately determine the specific levels of protein phosphorylation in tiny amounts of sample (Mann *et al.*, 2002), we were not able to employ such approaches in the present study. The levels of tyrosine phosphorylation associated with the data reported in this paper remain to be determined.

Collectively, the present study provides the first indication that PTKs show dual regulating effects on $I_{Kur}/hK_v1.5$ current. Src family kinases decrease but EGFR kinase increases the activity of $I_{Kur}/hK_v1.5$ channels. This information is important not only for understanding cardiac electrophysiological regulation by PTKs but also for interpreting the therapeutic potential of PTK inhibitors in humans, especially EGFR inhibitors.

## Acknowledgements

The work was supported in part by a grant from Sun Chieh Yeh Heart Foundation of Hong Kong, Hong Kong, China, a Collaborative Grant for Study on Prevention and Control of Major Chronic Non-infectious Diseases of the 13th Five-Year National Research Plan from Ministry of Science and Technology, China, and a Key Cardiovascular Laboratory Fund (3502Z20150050) from Department of Xiamen Science and Technology, Xiamen, China. Zhang Y.H. and Wu W. were supported by a postgraduate studentship from the University of Hong Kong, Hong Kong, China.

## Author contributions

G.S.X., Y.W. and G.R.L. conceived and designed the experiments; G.S.X., Y.H.Z., W.W. and H.Y.S. performed the experiments; G.S.X., H.Y.S. and G.R.L. analysed the data; G.S.X. and G.R.L. wrote the paper; G.S.X., Y.H.Z., W.W., H.Y.S., Y. W. and G.R.L. approved the submission.

## Conflict of interest

The authors declare no conflicts of interest.

## Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

---

## References

Adda S, Fleischmann BK, Freedman BD, Yu M, Hay DW, Kotlikoff MI (1996). Expression and function of voltage-dependent potassium channel genes in human airway smooth muscle. J Biol Chem 271: 13239–13243.

Akiyama T, Ogawara H (1991). Use and specificity of genistein as inhibitor of protein-tyrosine kinases. Methods Enzymol 201: 362–370.

Alexander SPH, Catterall WA, Kelly E, Marrion N, Peters JA, Benson HE *et al.* (2015a). The Concise Guide to PHARMACOLOGY 2015/16: Voltage-gated ion channels. Br J Pharmacol 172: 5904–5941.

Alexander SPH, Fabbro D, Kelly E, Marrion N, Peters JA, Benson HE *et al.* (2015b). The Concise Guide to PHARMACOLOGY 2015/16: Enzymes. Br J Pharmacol 172: 6024–6109.

Alexander SPH, Fabbro D, Kelly E, Marrion N, Peters JA, Benson HE *et al.* (2015c). The concise guide to PHARMACOLOGY 2015/16: Catalytic receptors. Br J Pharmacol 172: 5979–6023.

Arvind S, Arivazhagan A, Santosh V, Chandramouli BA (2012). Differential expression of a novel voltage gated potassium channel–Kv1.5 in astrocytomas and its impact on prognosis in glioblastoma. Br J Neurosurg 26: 16–20.

Bonnet S, Archer SL, Allalunis-Turner J, Haromy A, Beaulieu C, Thompson R *et al.* (2007). A mitochondria-$K^+$ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth. Cancer Cell 11: 37–51.

Christophersen IE, Olesen MS, Liang B, Andersen MN, Larsen AP, Nielsen JB *et al.* (2013). Genetic variation in KCNA5: impact on the atrial-specific potassium current IKur in patients with lone atrial fibrillation. Eur Heart J 34: 1517–1525.

Curtis MJ, Bond RA, Spina D, Ahluwalia A, Alexander SP, Giembycz MA *et al.* (2015). Experimental design and analysis and their reporting: new guidance for publication in BJP. Br J Pharmacol 172: 3461–3471.

Davis MJ, Wu X, Nurkiewicz TR, Kawasaki J, Gui P, Hill MA *et al.* (2001). Regulation of ion channels by protein tyrosine phosphorylation. Am J Physiol Heart Circ Physiol 281: H1835–H1862.

Dong MQ, Sun HY, Tang Q, Tse HF, Lau CP, Li GR (2010). Regulation of human cardiac KCNQ1/KCNE1 channel by epidermal growth factor receptor kinase. Biochim Biophys Acta 1798: 995–1001.

Du XL, Gao Z, Lau CP, Chiu SW, Tse HF, Baumgarten CM *et al.* (2004). Differential effects of tyrosine kinase inhibitors on volume-sensitive chloride current in human atrial myocytes: evidence for dual regulation by Src and EGFR kinases. J Gen Physiol 123: 427–439.

Fedida D, Wible B, Wang Z, Fermini B, Faust F, Nattel S *et al.* (1993). Identity of a novel delayed rectifier current from human heart with a cloned $K^+$ channel current. Circ Res 73: 210–216.

Felipe A, Bielanska J, Comes N, Vallejo A, Roig S, Ramon YCS *et al.* (2012). Targeting the voltage-dependent K(+) channels Kv1.3 and Kv1.5 as tumor biomarkers for cancer detection and prevention. Curr Med Chem 19: 661–674.

Feng J, Wible B, Li GR, Wang Z, Nattel S (1997). Antisense oligodeoxynucleotides directed against Kv1.5 mRNA specifically inhibit ultrarapid delayed rectifier $K^+$ current in cultured adult human atrial myocytes. Circ Res 80: 572–579.

Feng M, Xiang JZ, Ming ZY, Fu Q, Ma R, Zhang QF *et al.* (2012). Activation of epidermal growth factor receptor mediates reperfusion arrhythmias in anaesthetized rats. Cardiovasc Res 93: 60–68.

Gao Z, Lau CP, Wong TM, Li GR (2004). Protein tyrosine kinase-dependent modulation of voltage-dependent potassium channels by genistein in rat cardiac ventricular myocytes. Cell Signal 16: 333–341.

Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ *et al.* (1996). Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. J Biol Chem 271: 695–701.

Holmes TC, Fadool DA, Ren R, Levitan IB (1996). Association of Src tyrosine kinase with a human potassium channel mediated by SH3 domain. Science 274: 2089–2091.

Hubbard SR, Till JH (2000). Protein tyrosine kinase structure and function. Annu Rev Biochem 69: 373–398.

Kang LS, Kim S, Dominguez JM 2nd, Sindler AL, Dick GM, Muller-Delp JM (2009). Aging and muscle fiber type alter $K^+$ channel contributions to the myogenic response in skeletal muscle arterioles. J Appl Physiol 107: 389–398.

Kim HJ, Ahn HS, Choi BH, Hahn SJ (2011). Inhibition of Kv4.3 by genistein via a tyrosine phosphorylation-independent mechanism. Am J Physiol Cell Physiol 300: C567–C575.

Kwak YG, Hu N, Wei J, George AL Jr, Grobaski TD, Tamkun MM *et al.* (1999). Protein kinase A phosphorylation alters Kvbeta1.3 subunit-mediated inactivation of the Kv1.5 potassium channel. J Biol Chem 274: 13928–13932.

Levitan IB (1994). Modulation of ion channels by protein phosphorylation and dephosphorylation. Annu Rev Physiol 56: 193–212.

Levitzki A, Mishani E (2006). Tyrphostins and other tyrosine kinase inhibitors. Annu Rev Biochem 75: 93–109.

Li GR, Feng J, Wang Z, Fermini B, Nattel S (1996a). Adrenergic modulation of ultrarapid delayed rectifier $K^+$ current in human atrial myocytes. Circ Res 78: 903–915.

Li GR, Feng J, Yue L, Carrier M, Nattel S (1996b). Evidence for two components of delayed rectifier $K^+$ current in human ventricular myocytes. Circ Res 78: 689–696.

Li GR, Wang HB, Qin GW, Jin MW, Tang Q, Sun HY*et al.* (2008). Acacetin, a natural flavone, selectively inhibits human atrial repolarization potassium currents and prevents atrial fibrillation in dogs. Circulation 117: 2449–2457.

Liu H, Sun HY, Lau CP, Li GR (2007). Regulation of voltage-gated cardiac sodium current by epidermal growth factor receptor kinase in guinea pig ventricular myocytes. J Mol Cell Cardiol 42: 760–768.

Liu L, Yang T, Simon SA (2004). The protein tyrosine kinase inhibitor, genistein, decreases excitability of nociceptive neurons. Pain 112: 131–141.

Loose S, Mueller J, Wettwer E, Knaut M, Ford J, Milnes J *et al.* (2014). Effects of $I_{Kur}$ blocker MK-0448 on human right atrial action potentials from patients in sinus rhythm and in permanent atrial fibrillation. Front Pharmacol 5: 26.

MacDonald PE, Wheeler MB (2003). Voltage-dependent $K^+$ channels in pancreatic beta cells: role, regulation and potential as therapeutic targets. Diabetologia 46: 1046–1062.

Mann M, Ong SE, Grønborg M, Steen H, Jensen ON, Pandey A (2002). Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome. Trends Biotechnol 20: 261–268.

Mia S, Munoz C, Pakladok T, Siraskar G, Voelkl J, Alesutan I *et al.* (2012). Downregulation of Kv1.5 K channels by the AMP-activated protein kinase. Cell Physiol Biochem 30: 1039–1050.

Missan S, Qi J, Crack J, McDonald TF, Linsdell P (2009). Regulation of wild-type and mutant KCNQ1/KCNE1 channels by tyrosine kinase. Pflugers Arch 458: 471–480.

Missan S, Zhabyeyev P, Linsdell P, McDonald TF (2006). Insensitivity of cardiac delayed-rectifier $I_{Kr}$ to tyrosine phosphorylation inhibitors and stimulators. Br J Pharmacol 148: 724–731.

Ogura T, Shuba LM, McDonald TF (1999). L-type $Ca^{2+}$ current in guinea pig ventricular myocytes treated with modulators of tyrosine phosphorylation. Am J Physiol Heart Circ Physiol 276: H1724–H1733.

Olson TM, Alekseev AE, Liu XK, Park S, Zingman LV, Bienengraeber M *et al.* (2006). Kv1.5 channelopathy due to KCNA5 loss-of-function mutation causes human atrial fibrillation. Hum Mol Genet 15: 2185–2191.

Ousingsawat J, Spitzner M, Puntheeranurak S, Terracciano L, Tornillo L, Bubendorf L *et al.* (2007). Expression of voltage-gated potassium channels in human and mouse colonic carcinoma. Clin Cancer Res 13: 824–831.

Overturf KE, Russell SN, Carl A, Vogalis F, Hart PJ, Hume JR *et al.* (1994). Cloning and characterization of a Kv1.5 delayed rectifier $K^+$ channel from vascular and visceral smooth muscles. Am J Physiol Cell Physiol 267: C1231–C1238.

Schumacher SM, McEwen DP, Zhang L, Arendt KL, Van Genderen KM, Martens JR (2009). Antiarrhythmic drug-induced internalization of the atrial-specific $K^+$ channel Kv1.5. Circ Res 104: 1390–1398.

Snyders DJ, Tamkun MM, Bennett PB (1993). A rapidly activating and slowly inactivating potassium channel cloned from human heart.

Functional analysis after stable mammalian cell culture expression. J Gen Physiol 101: 513–543.

Sorota S (1995). Tyrosine protein kinase inhibitors prevent activation of cardiac swelling-induced chloride current. Pflugers Arch 431: 178–185.

Southan C, Sharman JL, Benson HE, Faccenda E, Pawson AJ, Alexander SPH *et al.* (2016). The IUPHAR/BPS guide to PHARMACOLOGY in 2016: towards curated quantitative interactions between 1300 protein targets and 6000 ligands. Nucl Acids Res 44 (Database Issue): D1054–D1068.

Tamargo J, Caballero R, Gomez R, Delpon E (2009). $I_{\mathrm{Kur}}$/Kv1.5 channel blockers for the treatment of atrial fibrillation. Expert Opin Investig Drugs 18: 399–416.

Tan CS, Gilligan D, Pacey S (2015). Treatment approaches for EGFR-inhibitor-resistant patients with non-small-cell lung cancer. Lancet Oncol 16: e447–e459.

Tang Q, Jin MW, Xiang JZ, Dong MQ, Sun HY, Lau CP *et al.* (2007). The membrane permeable calcium chelator BAPTA-AM directly blocks human ether a-go-go-related gene potassium channels stably expressed in HEK 293 cells. Biochem Pharmacol 74: 1596–1607.

Tipparaju SM, Li XP, Kilfoil PJ, Xue B, Uversky VN, Bhatnagar A *et al.* (2012). Interactions between the C-terminus of Kv1.5 and Kvbeta regulate pyridine nucleotide-dependent changes in channel gating. Pflugers Arch 463: 799–818.

Usul H, Cakir E, Cobanoglu U, Alver A, Peksoylu B, Topbas M *et al.* (2004). The effects of tyrphostine Ag 556 on experimental spinal cord ischemia reperfusion injury. Surg Neurol 61: 45–54.

Vicente R, Escalada A, Villalonga N, Texido L, Roura-Ferrer M, Martin-Satue M *et al.* (2006). Association of Kv1.5 and Kv1.3 contributes to the major voltage-dependent $K^+$ channel in macrophages. J Biol Chem 281: 37675–37685.

Wang Z, Fermini B, Nattel S (1993). Sustained depolarization-induced outward current in human atrial myocytes. Evidence for a novel delayed rectifier $K^+$ current similar to Kv1.5 cloned channel currents. Circ Res 73: 1061–1076.

Warsi J, Elvira B, Bissinger R, Hosseinzadeh Z, Lang F (2015). Regulation of voltage-gated $K^+$ channel Kv1.5 by the Janus kinase JAK3. J Membr Biol 248: 1061–1070.

Washizuka T, Horie M, Obayashi K, Sasayama S (1998). Genistein inhibits slow component delayed-rectifier K currents via a tyrosine kinase-independent pathway. J Mol Cell Cardiol 30: 2577–2590.

Wettwer E, Hala O, Christ T, Heubach JF, Dobrev D, Knaut M *et al.* (2004). Role of IKur in controlling action potential shape and contractility in the human atrium: influence of chronic atrial fibrillation. Circulation 110: 2299–2306.

Williams CP, Hu N, Shen W, Mashburn AB, Murray KT (2002). Modulation of the human Kv1.5 channel by protein kinase C activation: role of the Kvbeta1.2 subunit. J Pharmacol Exp Ther 302: 545–550.

Wu HJ, Wu W, Sun HY, Qin GW, Wang HB, Wang P *et al.* (2011). Acacetin causes a frequency- and use-dependent blockade of hKv1.5 channels by binding to the S6 domain. J Mol Cell Cardiol 51: 966–973.

Wu W, Dong MQ, Wu XG, Sun HY, Tse HF, Lau CP *et al.* (2012). Human ether-a-go-go gene potassium channels are regulated by EGFR tyrosine kinase. Biochim Biophys Acta 1823: 282–289.

Wu W, Sun HY, Deng XL, Li GR (2013). EGFR tyrosine kinase regulates human small-conductance $Ca^{2+}$-activated $K^+$ (hSKCa1) channels expressed in HEK-293 cells. Biochem J 452: 121–129.

Yang T, Yang P, Roden DM, Darbar D (2010). Novel KCNA5 mutation implicates tyrosine kinase signaling in human atrial fibrillation. Heart Rhythm 7: 1246–1252.

Zhang DY, Wang Y, Lau CP, Tse HF, Li GR (2008). Both EGFR kinase and Src-related tyrosine kinases regulate human ether-a-go-go-related gene potassium channels. Cell Signal 20: 1815–1821.

Zhang DY, Wu W, Deng XL, Lau CP, Li GR (2011a). Genistein and tyrphostin AG556 inhibit inwardly-rectifying Kir2.1 channels expressed in HEK 293 cells via protein tyrosine kinase inhibition. Biochim Biophys Acta 1808: 1993–1999.

Zhang DY, Zhang YH, Sun HY, Lau CP, Li GR (2011b). Epidermal growth factor receptor tyrosine kinase regulates the human inward rectifier potassium $K_{IR}2.3$ channel, stably expressed in HEK 293 cells. Br J Pharmacol 164: 1469–1478.

Zhang YH, Wu W, Sun HY, Deng XL, Cheng LC, Li X *et al.* (2012). Modulation of human cardiac transient outward potassium current by EGFR tyrosine kinase and Src-family kinases. Cardiovasc Res 93: 424–433.

Zhao Z, Liu B, Zhang G, Jia Z, Jia Q, Geng X *et al.* (2008). Molecular basis for genistein-induced inhibition of Kir2.3 currents. Pflugers Arch 456: 413–423.