The strength of hERG inhibition by Erythromycin at different temperature might be due to its different critical binding sites on the channels

yuan chen¹, Qian Zhang², Dongrong Cheng², Yanting Zhang¹, Jianwei Xu², Dian Zhong¹, Xiaofeng Wei², Jingtao Wang², Huan Liu², Junjie Yu², Jiaxin Yang², Lanying Pan², and Wei Zhao¹

¹Zhejiang Agriculture and Forest University
²Zhejiang A and F University

March 07, 2024

Abstract

The human ether-a-go-go-related gene (hERG) inhibition is a serious cardiac safety issue. Although the inhibition of hERG by majority of compounds are stable, the strength of the inhibition of a few compounds, including Erythromycin, is remarkably rising from room temperature (RT) to physiological temperature (PT). Understanding the features of erythromycin against hERG could help us to decide which compounds are needed for further study. The whole cell patch clamp technique was used to investigate the effects of erythromycin on hERG channels in different temperatures. We found that erythromycin caused a concentration dependent inhibition of cardiac hERG potassium channels. The half maximal inhibitory concentration (IC50) value was 1671 ± 593 μM (n = 5-10) at RT, about 11 folds higher than its IC50 (150 ± 26 μM, n = 7) at PT. Although temperature does have a profound change of hERG channel dynamic, the erythromycin further left shifted channel’s steady state activation, steady state inactivation, and make onset of inactivation significantly faster at both temperatures. It is interesting that our data suggests that there is critical binding site shifted from V625 at RT to Y652 at PT. By contrast, Cisapride, a well-known hERG blocker and its inhibition is not affected by temperature, does not change its critical binding sites after the temperature is raised to PT. The data suggests that increase strength of the inhibition could be due to the shift of its binding sites of hERG channels.

The strength of hERG inhibition by Erythromycin at different temperature might be due to its different critical binding sites on the channels


aChinese Herb Medicine Division, Zhejiang Agriculture and Forestry University, 666 Wusu street, Lin’an 311300, P. R., China
bThe State Key Laboratory of Subtropical Silviculture, Zhejiang Agriculture and Forestry University, 666 Wusu St, Lin’an 311300, P. R., China;
* Equal contributors
**Corresponding author

Correspondence should be addressed to ychen@zafu.edu.cn or wzhao@zafu.edu.cn or lanyingpan@126.com

ABSTRACT
The human ether-a-go-go-related gene (hERG) inhibition is a serious cardiac safety issue. Although the inhibition of hERG by majority of compounds are stable, the strength of the inhibition of a few compounds, including Erythromycin, is remarkably rising from room temperature (RT) to physiological temperature (PT). Understanding the features of erythromycin against hERG could help us to decide which compounds are needed for further study. The whole cell patch clamp technique was used to investigate the effects of erythromycin on hERG channels in different temperatures. We found that erythromycin caused a concentration dependent inhibition of cardiac hERG potassium channels. The half maximal inhibitory concentration ($IC_{50}$) value was $1671 \pm 593 \, \mu \text{M} \, (n = 5-10)$ at RT, about 11 folds higher than its $IC_{50}$ ($150 \pm 26 \, \mu \text{M}, n = 7$) at PT. Although temperature does have a profound change of hERG channel dynamic, the erythromycin further left shifted channel’s steady state activation, steady state inactivation, and make onset of inactivation significantly faster at both temperatures. It is interesting that our data suggests that there is critical binding site shifted from V625 at RT to Y652 at PT. By contrast, Cisapride, a well-known hERG blocker and its inhibition is not affected by temperature, does not change its critical binding sites after the temperature is raised to PT. The data suggests that increase strength of the inhibition could be due to the shift of its binding sites of hERG channels.

**Key words**: erythromycin, temperature, hERG, steady state activation, Steady state inactivation

**Introduction**

The human ether-a-go-go-related gene (hERG) is important for cardiac action potential (AP). It is one of critical components to form plateau of cardiac AP and determines the duration of QT interval in the electrocardiogram (ECG) since it plays essential roles of AP repolarization (Sanguinetti et al., 1991; Virag et al., 2001; Helbing et al., 2002; Jouven et al., 2002; Schram et al., 2004; Tamargo et al., 2004). Thus, inhibition of hERG channel will lead to the prolongation of the QT interval of the ECG, which is associated with a risk of the potentially fatal arrhythmia Torsade de Pointes (TdP) (Vandenberg et al., 2001; Hancox et al., 2008).

hERG channel is a tetramer consisting of four pore-forming $\alpha$ subunits (Sanguinetti et al., 1995). It has a unique pore structure, which has a small center cavity with extended pockets, which is favor for catonic drugs to bind (Wang et al., 2017). The extended pockets have features also favor drug binding as it is elongated and relative hydrophobic. In addition, pore region also contains two aromatic amino acid residues, Y652 and F656, in the S6 helices. Those unique features are believed to contribute to hERG’s sensitivity to variety pharmacological blockades (Mitcheson et al., 2000; Vandenberg et al., 2001; Chen et al., 2002; Perry et al., 2004; Sanguinetti et al., 2006).

Since it is clear relationship between hERG channel inhibition and many non-antiarrhythmic drugs that produce torsades de pointes associated with QT prolongation, early evaluation of hERG toxicity has been strongly recommended by the regulatory agencies such as U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA). Such evaluations are typically carried out at room temperature (RT), which is different with 37°C physiological temperature (PT). A few compounds have much stronger inhibition at physiological temperature (Kirsch et al., 2004; Guo et al., 2005). Although there is the concept of the comprehensive in vitro proarrhythmic assay (CiPA) having been developed to ensure cardia safety (Fermini et al., 2016), hERG screen is still major cardiac safety concern. It will be worrisome if the compounds have been studied against hERG only at RT; it is also very time consuming and money waste if all of compounds are done at PT since it is much more difficult to be investigated at PT. Thus, it is important to understand when compounds need to be further exanimated at physiological temperature.

Erythromycin, a macrolide antibiotic drug, is interesting because it is one of few compounds having different strength in different temperature (Kirsch et al., 2004; Duncan et al., 2006). Erythromycin has also been suggested that it has protection effect Erythromycin protective effect against hERG block (Meng et al., 2013; Crumb, 2014). By characterizing the behavior of erythromycin against hERG at RT and PT, it will help us to understand the potential features of a compound having different strength of hERG inhibition in different temperature.
Materials and Methods

Preparation of Erythromycin and Cisapride

The 100% purity product of erythromycin (Prefa, Chengdu) was dissolved in DMSO (Aladdin, China) as a storage solution and stored in refrigerator at 4°C before the experiment. In the day of the experiment, the erythromycin was added to the extracellular solution to be the required concentration for the test. Cisapride (Sigma, US) with concentration of 9 mM as stock solution was diluted to required concentration for patch clamp test.

Cell preparation

Human Embryonic Kidney (HEK-293) Cells were cultured in the solution with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) P/S (100U Penicillin and 0.1 mg/ml streptomycin) and 100 μg/ml G418. Cells were passaged and transiently transfected with 1 μg of the WT and its mutation of T623A, S624A, S625A, T652A and F656A by transient transfection reagents (Attractene Transfection Reagent, Qiagen) while CD8 cDNA was co-transfected. The successfully transfected cells were identified by labeling with CD8-specific antibody-coated microspheres (Dynal, Oslo, Norway).

Electrophysiological recordings

Different concentrations of erythromycin solution were diluted into the external solution from its stock solution, which were taken from -80 freezer, for the test and transferred to 50mL syringes while recording the hERG channel currents by a whole cell patch clamp. The current curves were obtained by PC 505B (Warner) clamp amplifier at room temperature (RT) and physiological temperature (PT). The PT in our study refers to 35 ± 2°C.

Data analysis

All test data was expressed in Mean +- S.E.M. Clampfit, Excel 2010 and Origin 8.0 software were used for statistical analysis and image processing. For the steady state activation and inactivation, Boltzmann equation was used; Hill equation was used to calculate the IC50; Other fitting functions will be mentioned in result section. Statistical processing was carried out by T-test. The difference, P < 0.05, was significant.

Results

Temperature-dependent inhibition to hERG by erythromycin.

In this set of experiment, different concentrations of erythromycin were used to investigate the response of the hERG channels at RT and at PT respectively. The protocol is shown in figure 1A (right panel). Current recordings were evoked with a depolarizing step to +40 mV for 2 s from a -80 mV holding voltage. A repolarizing step to -40 mV was used to record hERG tail currents before and after erythromycin application. The inhibitory effect of erythromycin on hERG potassium channel was different between at RT and that at PT. At RT, the percentage inhibition on hERG currents of 3, 10, 30, 100, 300, 1000, and 3000 μM erythromycin were -3.9 ± 1.3%, 1.8 ± 2.4%, 9.90 ± 2.9%, 15.0 ± 4.2%, 23.1 ± 6.1%, 37.0 ± 7.1%, 60.1 ± 4.3%(n=5-10) respectively (Fig. 1B); while the percentage inhibition were 10.9 ± 2.0%, 20.0 ± 2.0%, 30.1 ± 2.4%, 41.9 ± 3.8%, 58.1 ± 3.4%, 89.2 ± 4.2%, 97.1 ± 1.1%(n=7) at PT (Fig. 1C). Although the inhibition at RT far away from maximal, which is not ideal to estimate its IC50, the erythromycin at 3000 μM or above was not tested since it was insoluble at that concentration. The data was fitted by was fitted by Hill equation. The IC50 at RT is 1671 ± 593 μM at RT and is 150 ± 26 μM at PT respectively (Fig. 1B and 1C). The IC50 at RT is about 11 times higher than that at PT, which suggested that erythromycin inhibits hERG current not only in a concentration-dependent but also in a temperature-dependent manner. These results were similar to what were reported in the literature (Desai et al., 2004). The inhibitory effects of 3 mM erythromycin at RT (67.8 ± 4.3%, n=5) and 300 μM erythromycin at PT (58.4 ± 3.4%, n=7) were obtained and shown at Figure 1D, which was also similar to the literature reported (Duncan et al., 2006). These two concentrations were further used to study the effect of erythromycin on hERG channels influenced by the temperature.
Effect of erythromycin on the steady state activation at RT and PT

The steady state activation of ion channel is an important parameter of voltage gated ion channel, which reflects the process of channel opening. To further understand the mechanism, we investigated the steady state activation of hERG with erythromycin at different temperature and then explored the kinetic properties of its action. The concentrations of 3 mM erythromycin at RT and 300 μM at PT were chosen in the study since the similarity inhibitory effect.

Patch clamp test was carried out at RT and PT. After whole cell patch clamp configure was set up, the extracellular fluid was perfused until the current amplitudes were stable. Followed the protocol illustrated in the figure 2A, the outward currents at different voltage steps were recorded and the peak tail current amplitudes, which the voltage steps were repolarized to -40 mV, were measured before erythromycin application. To record currents treated with erythromycin, the erythromycin was perfused for 3-5 minutes (Figure 2B) before the pulse stimulation program in Figure 2A was given. After the current was stabilized, the current value was recorded under the same pulse stimulation program (Figure 2A). The test needs to turn on the heater to heat the drug superfusion. The initial data are processed by relevant software, and the maximum current value is set to 1 with each activation voltage as Abscissa, and the percentage of tail current to maximum current (current density) under other different voltages is used as ordinate to make I-V curve. Both steady state activation curves were shown in Figure 2B and Figure 2C at RT and PT. At RT, the ion channel opened at -40 mV and the current amplitudes were increased until about 20 - 30 mV following the increase of voltage. However, the peak amplitudes were shifted to 0 mV (Fig. 2B), similar shift of V_{1/2}, which were the V_{1/2} was -7.8 ± 0.4 mV before and shifted to -22.4 ± 0.4 mV (n=6) after adding erythromycin, suggesting that erythromycin changes the voltage sensitivity of the channels. At PT, the V_{1/2} of steady state activation was left shifted to -17.1 ± 0.5 mV at PT comparing that at RT. At the same time, the V_{1/2} was further left shifted to -33.0 ± 1.6 mV (n=5) after erythromycin treatment. It was found that the slope (K) was 11.7 ± 0.5 and 8.2 ± 0.4 (n=6) (Figure 2B) before and after adding medicine at RT respectively, while which was 9.5 ± 0.6 and 6.3 ± 2.4 (n=5) at PT respectively (Figure 2C). The curve was positively left shifted after the addition of erythromycin (Figure 2B and Figure 2C), which indicating that erythromycin influences the I-V curve of hERG at RT and PT. But the difference of effect of erythromycin between RT and PT are small and insignificant. The inactivation kinetics of currents were measured (figure 2D). There were only few measurable traces at RT. Their time constancies were 2914.6 ms (n=1) at 30 mV, 3879.5 (n=1) at 40 mV and 3766.7 ± 192.7 ms (n=3) at 50 mV respectively. After 3 mM erythromycin, the time constancies were 413.3 ± 45.6 ms (n=6) 10 mV, 345.6 ± 57.7 (n=6) at 20 mV, 222.0 ± 45.9 (n=6) at 30 mV, 155.6 ± 25.0 (n=6) at 40 mV and 114.0 ± 26.2 (n=6) at 50 mV. It is clearly much faster and at least 10 folds faster after erythromycin application. At PT, the time constancies were 728.8 ms (n=1) 10 mV, 702.0 (n=1) at 20 mV, 1068.9 ± 122.6 (n=5) at 30 mV, 867.8 ± 32.3 (n=5) at 40 mV and 825.5 ± 36.8 (n=5) at 50 mV. The time constancies were 139.7 ms ± 32.4 (n=5) at 10 mV, 129.0 ± 32.8 (n=5) at 20 mV, 88.4 ± 21.8 (n=5) at 30 mV, 48.9 ± 15.7 (n=5) at 40 mV and 40.1 ± 14.4 (n=5) at 50 mV. Comparing the time constancies from RT, Time constancies at PT is significant faster that that at the same voltage at RT. The time constancies are further accelerated significantly after erythromycin application. Effect of erythromycin on steady-state inactivation of hERG channels at RT and PT To further understand erythromycin on hERG potassium channels at different temperature, we have investigated the effect of erythromycin on the steady-state inactivation of hERG following the pulse stimulation protocol shown in figure 3A. When the whole cell configuration was established, the extracellular fluid was perfused until the current amplitude was stable; it takes for 3-5 minutes typically. The example current traces are shown in figure 3A middle panel. When the recording was completed, 300 μM of erythromycin was added and the current data were recorded in figure 3A bottom panel. For easy comparison, the data was normalized with their maximum current amplitudes, the average data was shown in figure 3B and 3C at RT or PT respectively. The figure 3B shows the data at RT (n=10). Before erythromycin, it reaches peak at 20 mV (V_{1/2} = 39.2 ± 0.6, n=10) and the peak shift to left at -40 mV (V_{1/2} = -7.3 ± 1.3, n=10) after adding erythromycin. At PT, figure 3C shows that the peak tail current reaches its peak at around 0 mV (V_{1/2} = 25.9 ± 0.3, n=5) before erythromycin, and the peak shift to left at -20 mV (V_{1/2} = 8.5 ± 1.0, n=5) after adding erythromycin, which is less shifted than
that at RT (n=5). The data shows that the higher temperature at PT left shifts steady state inactivation. Since the concentrations at RT and PT have similar inhibitory effect, data also suggests less influence of erythromycin at PT. However, it can also be explained that shift caused by erythromycin is concentration dependent since 300 μM at PT is 10 times less than 3 nM erythromycin at RT. Effect of erythromycin on onset of inactivation of hERG channel at RT and PT By increase the length of pre-pulses, onset protocol is to measure how fast the channel turns into inactivation state. The protocol shown in figure 4A is used to investigate the effect of erythromycin on hERG channels. The peak tail currents, which are evoked by test pulses at -40 mV, are used to measure the inactivation since the tail currents reflect the fully recovery from inactivation of hERG channels. The individual peak currents are normalized to their maximal peak tail current amplitudes and then plotted against their pre-pulse lengths. At RT, the peak tail current reaches its maximum at 320 ms and tended to be stable afterwards (figure 4B, n=7). Its time constancy is 144.2 ± 0.3 ms. After adding the compound, the maximal peak current is shifted to 80 ms length and time constancy is 48.8 ± 0.1 ms, significantly faster than before (n=5, P<0.01). Meanwhile the maximal peak current is also shifted from 80 ms pre-pulse length (τ=12.6 ± 3.0 ms) to 40 ms pre-pulse length (time constancy cannot be fit due to only two data points before the peak current, including the peak current) at PT before and after addition of erythromycin (figure 4C, n=8). Besides increase the rate of inactivation, erythromycin enhances its inhibition of tail currents followed the pre-pulse length increase at both temperatures. The rate of inhibition is much fast at PT than that at RT. Inhibitory effect of erythromycin on mutations of hERG channel at RT and PT As we mention before, the concentration 3 mM erythromycin inhibits 67.8 ± 4.3% hERG tail currents at RT and 300 μM erythromycin inhibits 58.4 ± 3.4% hERG tail currents at PT (Fig. 1D). Further experiment found that the percentage inhibitions of hERG mutations, T623A, S624A, V625A, Y652A and F656A were 52.1 ± 6.0% (n=5), 67.2 ± 4.1% (n=7), 46.9 ± 4.2% (n=13), 67.1 ± 5.0% (n=8), 61 ± 2.7% (n=5) at RT, respectively (Fig. 5A). Among them, mutations T623A and S625A have significantly reduced inhibition of erythromycin and the V625A mutation reduces more. The percentage inhibition of 300 μM erythromycin to T623A, S624A, V625A, Y652A and F656A were 46.1 ± 2.8% (n=5), 45.0 ± 2.8% (n=11), 60.2 ± 4.5% (n=5), 36.9 ± 5.15% (n=16), 55.2 ± 3.3% (n=6) respectively at PT (Figure 5B). Among them, mutations T623A, S624A and F656A have significantly reduced the inhibition and F656A has the most. It is interesting that erythromycin binding sites are different in the different temperature except of T623A. Opposite to RT, which V625A is a stronger influence site at RT, V625A has no significant effect at PT; While Y656A has little effect at RT, it is the strongest impact site at PT (Figure 5C). Thus, our data suggests that the hERG critical binding sites of erythromycin have remarkable shift between RT and PT. Inhibitory effect of Cisapride on hERG potassium channels and mutants at RT and PT Erythromycin is one of few compounds that enhance their inhibitory effects on hERG following the increase of temperature (Kirsch et al, 2004). It is surprising to know it has preferent binding sites on hERG at RT and PT. Further investigation was persuaded to understand whether other compounds, which have stable inhibition on hERG in different temperature, have the same phenomenon. Cisapride is a typical hERG channel blocker. It is not a surprise that there is no significant difference of inhibition of Cisapride between at RT and PT (Figure 6A) since the percentage inhibition of 100 nM Cisapride on hERG was 75.9 ±2.2% (n=6) at RT and 75.1 ±2.0% (n=6) at PT respectively, there is no significant different (p > 0.05). Further experiments found that the percentage inhibition of 100 nM erythromycin to T623A, S624A, V625A, Y652A and F656A mutations was 7.1 ±1.9% (n=8), 44.0 ±2.8% (n=5), 10.0 ±0.9% (n=7), 33.9 ±3.6% (n=6) and 8.0 ±3.2% (n=7) respectively at RT (Figure 6B). All of them significantly reduced the inhibition of Cisapride (p < 0.05). Among them, T623A, V625A, and F656A have shown similar strength of inhibition. In the meanwhile, the inhibition of these mutation was 15.8 ±3.1% (n=4), 42.2 ±1.8% (n=6), 6.1 ±1.7% (n=5), 22.1 ±2.1% (n=5), 12.9 ±3.5% (n=6) at PT respectively (Figure 6C). Although the strength of inhibition slightly changed, T623A, V625A, and F656A were still the mutation having the most reduction of the inhibition of Cisapride. The current percentage inhibition of Cisapride of 100 nM on five mutants at RT and PT were all significantly different from their WT. The data of each mutant at RT and PT were compared (Figure B vs Figure C), which showed that the pattern of the inhibition of Cisapride on hERG are similar at RT and PT. Mutations S624A and Y652A are less effective on the inhibition while mutations T623A, V625A and F656A remain robust inhibition at both temperatures. DiscussionhERG is such an important channel that is listed as a biomark
for cardiac safety. hERG channel participates in cardiac action potential plateau phase and precisely controls the length of the action potential. Any drug blocks the hERG channel will prolong the action potential thereby prolong the QT-period in electro-cardiac graph, which could cause sudden death. Many compounds have been examined to see whether they block hERG channels before being selected as drug candidates. Those blocking experiments are overwhelmingly done at room temperature, which has a distinguish distance with physiological temperature (36-37°C). Thus, whether the compounds should be tested in physiological temperature becomes a billion-dollar question. Our experiments showed that there are many distinct features of hERG channel at room temperature or at physiological temperature. Overall, hERG is faster and left shift at PT, comparing with the channel at RT. For example, V_{1/2} of the steady state activation is left shifted to -33 mV at PT from -17 mV at RT. Although the slope of the steady state activation is not significantly changed by the temperature. In the steady state inactivation protocol, the voltage to activate the maximal currents is also shifted to 0 mV at PT from 20 mV at RT. A similar left shift is also found from onset of inactivation protocol (Mauerhofer et al., 2016). These features are similar to what is already found in the literature ( ). Left shift of steady state activation is expected in voltage gated ion channels in rising temperature since the same changes have been reported at least in voltage gated bacterial sodium channels (Chen et al., 2021) and hERG channel itself (Zhou et al., 1998; Windley et al., 2018; Lei et al., 2019). It is known that the inhibitory of erythromycin on hERG is temperature dependent (Kirsch et al., 2004). As expected, we found that the IC_{50} were 1684 μM at RT and 150 μM at PT respectively, which suggests the inhibition is temperature dependent. The result is very similar to the literature. Using different concentration but similar inhibitory strength, we found that the effect of erythromycin on the gating of hERG potassium channel. Although both steady state activation and inactivation are left shifted at rising temperature, we notice that erythromycin seems less effective on steady state activation than inactivation. After erythromycin, the V_{1/2} of steady state is shifted to the left by 18 mV at RT vs 16 mV at PT, the difference is 2 mV and not significant statistically; in contrast, the maximal currents at the steady state inactivation are shifted to left by 60 mV at RT vs 20 mV at PT, the difference is 40 mV. Although there are more data needed to support, we speculate that the shift is more likely erythromycin concentration dependent rather than the strength of inhibition of erythromycin. The 16-18 mV shift of steady state activation could be a maximal shift at concentration near 300 μM. If this assumption is correct, then the steady state activation is very sensitive to erythromycin because 300 μM of erythromycin already maximizes the effect. The effect on steady state activation seems separated event from steady state inactivation since such a huge different shift. Although the activation and inactivation are linked, it should not be too surprising because the steady state activation is more determined by S4 transmembrane helix, and the steady state inactivation is more determined by C type inactivation at filter region. S4 and filter, which is constructed by L5-6 helix, have a quite distance between each other in hERG crystal structure (Wang et al., 2017). Therefore, the erythromycin could have two separated binding sites close to S4 and the filter. Onset of inactivation is using the length of voltage pulses to push the channels into inactivation state. Both recoveries of peak tail currents are faster after the cells treated with erythromycin. The recovery can be fit by double exponential in general, reflexing fast and slow inactivation states respectively. It is interesting that the second part of recovery decays following the increase of pulse length at PT. The same pattern is also found after erythromycin treatment at RT. It could be that higher temperature or erythromycin disrupts slow inactivation or the process of decay since the large tail current is due to slow decay and super-fast recovery of inactivation. The disruption follows the increase of prepulse length and must overcome the recovery of inactivation and suppress the peak tail currents. In addition to that, erythromycin could affect S6 water cavity domain, similar to mutations in S6 of sodium channels, which alter the slow inactivation (Chen et al., 2006). It is surprising that the keys amino acids have flipped upon the different temperature (RT vs PT). According to our data, V625 is the most important amino acid for erythromycin to bind at RT. However, V625A mutation has no effect at PT. The key amino acid is shifted to Y652, which has no effect at RT. S624 has no effect at RT but significantly reduced the inhibition at PT. Only the inhibition of T623A remains stable. Contrast to erythromycin, although the strength of inhibition of Cisapride from the same mutation does change significantly at between RT and PT, the pattern remains the same and there is no flip of inhibition. From T623A, S624A, V625A, Y652A, and F656A, the disruption of Cisapride inhibition remains strong, weak, strong, weak, and strong pattern at
both temperatures (figure 7B and C). The inhibition of these three mutations is about to or less than 20% inhibition of their wild type at both RT and PT. The difference of inhibition could suggest that there is a change of hERG inhibition of most compounds (Kirsch et al., 2004; Windley et al., 2018) but the change is in acceptable range in majority cases. The flip of hERG inhibition of erythromycin could be unique. First, erythromycin is quite large as a small molecule since its molecule weight (MW) is 734 Dalton and the MW of most small molecule of clinical medicine is less than 500 Dalton according to Lipinski’s rule of five. The larger size of erythromycin could be more sensitive to the change of structure of hERG protein with unique pore domain (Wang et al., 2017) causing by temperature difference. Another unique feature of erythromycin is that erythromycin has double structures, fold-out and fold-in structures. Majority is “foldout” structure but the percentage of “fold-in” structure will rise as the temperature rises (Koltun et al., 2016). It might explain the phenomenon of the dramatic change since different structure binds different sites. In conclusion, erythromycin is a unique molecule. It inhibits hERG channels with left shift of steady state activation and inactivation. Moreover, Key binding sites in pore are domain dramatically changes at different temperature (RT and PT).

Acknowledgements
This work is supported by Grants from Scientific Research & Development Fund of Zhejiang A & F University 2013FR032 to Dr Yuan Chen, 2016FR027 to Dr Lanying Pan and fund of Zhejiang provincial Natural Science Foundation LBY22H270002 to Dr Wei Zhao and LY20C040001 to Lanying Pan.

Competing Interests’ statement
none

Data Availability Statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Reference


Guo J, Zhan S, Lees-Miller JP, Teng G, Duff HJ (2005). Exaggerated block of hERG (KCNH2) and prolongation of action potential duration by erythromycin at temperatures between 37 degrees C and 42 degrees
C. Heart rhythm 2 (8): 860-866.


Koltun DO, Parkhill EQ, Elzein E, Kobayashi T, Notte GT, Kalla R, et al. (2016). Discovery of triazolopyridine GS-458967, a late sodium current inhibitor (Late INaI) of the cardiac NaV 1.5 channel with improved efficacy and potency relative to ranolazine. Bioorganic & medicinal chemistry letters 26 (13): 3202-3206.


**Figure Legend**

**FIGURE 1**. The percentage inhibitions of erythromycin on hERG currents at room temperature (RT) and physiological temperature (PT). (A) Left panel is the command voltage protocol. Middle and right panels are the example current traces at RT and at PT. (B) Concentration response curve shows the inhibition of erythromycin on hERG currents at different concentration at RT (n=5-10). Data was fitted by Boltzmann function. (C) Different concentrations of erythromycin on hERG currents at PT (n=7). (D) The average percentage inhibition rates of 3 mM erythromycin at RT(n=5) and 300 μM at PT (n=7) on hERG channels.

**FIGURE 2**. The effect of erythromycin on hERG steady state activation. (A) upper panel is the steady state protocol. Middle two panels are the example steady state current traces before and after erythromycin at RT and the bottom two panels are the currents traces before and after erythromycin at RT. (B) and (C) represents the data before erythromycin. * represents the data after erythromycin. The steady state current traces at RT and PT are shown from the middle panels and the bottom panel is the example current traces after erythromycin application at RT and PT respectively. * represents the data before erythromycin. * represents the data after erythromycin. 

**FIGURE 3**. Steady state inactivation before and after erythromycin application at RT and PT. (A) Upper panel is the voltage command protocol. A 4 s prepulse to +40 mV was applied to inactivate the channels from a holding potential at -80 mV and following test pulses from -60 to +50 mV in 10 mV increments to elicit the currents. Middle panel is the example current traces before erythromycin application at RT and PT. Bottom panel is the example current traces after erythromycin application at RT and PT respectively. * represents the data before erythromycin. * represents the normalized data after erythromycin. (B) and (D) show the tail peak currents follow the change of voltages before and after erythromycin. (C) and (E) show that the steady state inactivation is left shift by erythromycin. Normalized data fit by Boltzmann equation.

**FIGURE 4**. Shows the onset of inactivation. (A) The command voltage protocol. The holding potential was -80 mV, pulsed to +40 mV depolarizing voltage for variable durations from 20 to 2560 ms in double duration increments. The example current traces are shown from the middle panels and the bottom panels. Currents were recorded upon repolarization to -40 mV before and after erythromycin application. (B) and (C) represents the data before erythromycin. * represents the data after erythromycin.

**FIGURE 5**. Effects of erythromycin on hERG channel mutations at RT and PT. At PT, T623A (n=5), S624A (n=11), S625A (n=5), T652A (n=16), F656A (n=6)). The holding potential was -80 mV, +40 mV voltage was given, pulse stimulation lasted for 2 seconds, and then repolarized to-40 mV. (A) Effects of erythromycin on hERG channel mutations at RT. * P<0.05. (B) Effects of erythromycin on hERG channel mutations at PT. * P<0.05. (C) Example tail current traces before and after erythromycin.
FIGURE 6. Effects of Cisapride on mutations of hERG channel. (A) The average percentage inhibition of 100 nM Cisapride on hERG channels at RT (n=6) and PT (n=6). The holding potential is -80 mV, test pulse is +40 mV voltage, pulse duration is 2 s, then repolarization to -40 mV. Bar graphs show the effects of 100 nM Cisapride on mutations of hERG channel at RT (B) and PT (C). N numbers are on top of the bars. (D) Example tail current traces before and after erythromycin.