H<sub>1</sub> antihistamine drug promethazine directly blocks hERG K<sup>+</sup> channel

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Promethazine is a phenothiazine derivative with antihistaminic (H<sub>1</sub>), sedative, antiemetic, anticholinergic, and antinomotion sickness properties that can induce QT prolongation, which may lead to torsades de pointes. Since block of cardiac human ether-a-go-go-related gene (hERG) channels is one of the leading causes of acquired long QT syndrome, we investigated the acute effects of promethazine on hERG channels to determine the electrophysiological basis for its proarrhythmic potential. Promethazine increased the action potential duration at 90% of repolarization (APD<sub>90</sub>) in a concentration-dependent manner, with an IC<sub>50</sub> of 0.73 μM when action potentials were elicited under current clamp in guinea pig ventricular myocytes. We examined the effects of promethazine on the hERG channels expressed in Xenopus oocytes and HEK293 cells using two-microelectrode voltage-clamp and patch-clamp techniques. Promethazine induced a concentration-dependent decrease of the current amplitude at the end of the voltage steps and hERG tail currents. The IC<sub>50</sub> of promethazine dependent hERG block in Xenopus oocytes decreased progressively relative to the degree of depolarization. The IC<sub>50</sub> for the promethazine-induced block of the hERG currents in HEK293 cells at 36°C was 1.46 μM at +20 mV. Promethazine affected the channels in the activated and inactivated states but not in the closed states. The S6 domain mutations, Y652A and F656A partially attenuated (Y652A) or abolished (F656A) the hERG current block. These results suggest that promethazine is a blocker of the hERG channels, providing a molecular mechanism for the arrhythmogenic side effects during the clinical administration of promethazine.

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1. Introduction

Promethazine is a phenothiazine derivative with antihistaminic (H<sub>1</sub>), sedative, antiemetic, anticholinergic, and antinomotion sickness properties. Since its introduction in 1946, promethazine has been used for the prevention and treatment of nausea and vomiting caused by narcotic therapy [1], migraine episodes [2], and cancer chemotherapy [3], motion sickness [4], and during labor [5]. In addition, this agent has been shown to modify the electrical activity in cardiac tissues. For example, it has a negative chronotropic effect in atrial [8] and ventricular preparations [9] have also been reported under certain experimental conditions. The class III arrhythmogenic effect of this drug manifests as changes in the ECG of isolated perfused hearts from rats, such as a prolonged PR interval and increased QT intervals [10] and feline [11].

Almost all drugs causing significant QT prolongation interact with the K<sup>+</sup> channels, particularly the rapid component of the delayed rectifier K<sup>+</sup> channels (I<sub>Kr</sub>), which is important for the repolarization phase (phase 3) of the cardiac action potential [12]. The human ether-a-go-go-related gene (hERG) encodes the pore-forming subunit of the channel underlying I<sub>Kr</sub> [13]. Drug trapping and structure–function studies suggest that the inner cavity of the hERG channels is larger than that in the other voltage-gated K<sup>+</sup> channels, and can accommodate a wide range of chemical structures [14]. It has been reported that two aromatic residues in the S6 transmembrane domain, Tyr-652 and Phe-656, are particularly important in blocking the hERG channels through structurally diverse drugs [15]. However, there are hERG-inhibiting compounds that are not absolutely dependent on these two aromatic residues. Vesnarinone does not require Tyr-652 [16], and fluvoxamine can block hERG when there are mutations in Phe-656 or Tyr-652 [17]. In addition, the hERG-blocking scorpion toxins, including BeKm-1, have been reported to block from outside the cell [18]. This suggests that promethazine can prolong the action potential duration (APD) in vivo, and increase the QT interval by inhibiting the hERG channel.

This study examined the possible promethazine block of the hERG channels expressed in Xenopus oocytes and HEK cells as well as the inhibitory mechanisms and molecular determinants for the block using two mutant hERG channels (Y652A and F656A). In addition, a virtual docking simulation was carried out to understand the blocking mode of the hERG channel by the drug using the KvAP channel structure as a template.

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2. Materials and methods

2.1. Ventricular myocyte isolation

Single ventricular myocytes were isolated from each guinea pig heart using a method described previously [19]. Briefly, guinea pigs (300–500 g) were anesthetized with pentobarbital (~50 mg/kg, i.p.) and the heart was quickly excised. The heart was gotogradely perfused at 37 °C with a 750 μM Ca\(^{2+}\) solution and a Ca\(^{2+}\)-free solution followed by an enzyme solution. The enzyme solution contained 150 μM Ca\(^{2+}\), collagenase type I, and protease type XIV. The heart was then flushed with a 150 μM Ca\(^{2+}\) solution. The venticles were removed and chopped into small pieces, which were then shaken in a flask containing a 150 μM Ca\(^{2+}\) solution. The cell suspension was then left to sediment. The supernatant was replaced with a 500 μM Ca\(^{2+}\) solution. The cells were kept at room temperature. This study was performed according to the Research Guidelines of Kangwon National University IACUC.

2.2. Solutions and action potential recordings from myocytes

Myocytes in the experimental chamber were continuously superfused at room temperature (24–26 °C) with Tyrode solution containing 10 mM glucose, 5 mM HEPES, 140 mM NaCl, 4 mM KCl, 1 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), titrated to pH 7.4 with 4 M NaOH. The experimental chamber had a volume of 150 μl and the flow rate of the Tyrode solution was 2 ml/min. Miniature solenoid valves (LFA1201618H; Lee Products, Bucks, UK) selected the solution entering the chamber, and the superfusate within the chamber could be changed within 5 s. The solution level in the chamber was controlled with a suction system. The chamber and solenoid valves were mounted on the sliding stage of a microscope (Diaphot, Nikon, Japan) that sat on an antivibration table (Newport, USA).

Protease type XIV, dimethyl sulfoxide (DMSO), tetraethylammonium chloride (TEA-Cl) (Sigma, St. Louis, MO, USA), and Collagenase type 1 (Worthington Biochemical, Freehold, NJ, USA) were used in the form of stock solutions or test solutions. The antihistamine promethazine and the other reagents were purchased from Sigma (St. Louis, MO, USA). A stock solution of promethazine was prepared in distilled water and added to the external solutions at suitable concentrations shortly before each experiment.

Membrane potential was measured with conventional micro-electrodes pulled from filaments of thin wall glass tubing of 1.5-mm outer diameter and 1.2-mm inner diameter (World Precision Instruments, USA). They were filled with filtered 300 mM KCl and had a resistance of between 25 MΩ and 40 MΩ. Membrane potential was measured with an Axoclamp 900A amplifier (Axon Instruments, USA). Action potentials were elicited at 0.33 Hz by 2-ms depolarizing current pulses passed through the microelectrode. We selected recording electrodes and 0.6–1 M\(^{−}\) for voltage- clamp recordings from oocytes.

2.3. Expression of hERG in oocytes

hERG (accession no. U04270) cRNA was synthesized by in vitro transcription from 1 μg of linearized cDNA using T7 message machine kits (Ambion, Austin, TX, USA) and stored in 10 mM Tris–HCl (pH 7.4) at ~80 °C. The amino acid mutations where generated by polymerase chain reaction (PCR) with synthetic mutant oligonucleotide primers. The mutations Y652A and F656A were verified by sequencing (ABI3100). Stages V–VI oocytes were surgically removed from female Xenopus laevis (Nasco, Modesto, CA, USA) anesthetized with 0.17% tricane methanesulphonate (Sigma, St. Louis, MO, USA). Using fine forceps, the theca and follicle layers were manually removed from the oocytes, and then each oocyte was injected with 40nl of cRNA (0.1–0.5 μg/μl). The injected oocytes were maintained in a modified Barth’s Solution. The modified Barth’s Solution contained (mM): 88NaCl, 1KCl, 0.4CaCl\(_2\), 0.33Ca(NO\(_3\))\(_2\), 1MgSO\(_4\), 2.4NaHCO\(_3\), 10HEPES (pH 7.4), and 50 μg/ml gentamicin sulphonate. Currents were studied two to seven days after injection.

2.4. Solutions and voltage-clamp recordings from oocytes

Normal Ringer’s Solution contained 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl\(_2\), 1 mM MgCl\(_2\), and 10 mM HEPES (pH adjusted to 7.4 with NaOH). Solutions were applied to oocytes by continuous perfusion of the chamber while recording. Solution exchanges were completed within three min, and the hERG currents were recorded 5 min after the solution exchange. The effects of several concentrations of promethazine on the hERG currents were determined after the currents showed reversibility when washed with normal Ringer’s Solution. It took about 15 min to wash out ≤50 μM of the drug and about 30 min to wash out >100 μM of the drug. If the oocyte did not recover a current to its initial amplitude after 30 min of washing with a normal Ringer’s Solution, it was not used further. In general, each oocyte was treated with four to seven concentrations of promethazine. Currents were measured at room temperature (20–23 °C) with a two-microelectrode voltage-clamp amplifier (Warner Instruments, Hamden, CT, USA). Electrodes were filled with 3 M KCl and had a resistance of 2–4 MΩ for voltage- recording electrodes and 0.6–1 MΩ for current-passing electrodes. Stimulation and data acquisition were controlled with an AD–DA converter (Digidata 1200, Axon Instruments) and pCLAMP software (v5.1, Axon Instruments).

2.5. HEK cell culture and whole-cell patch recording

HEK293 cells stably expressing the hERG channel, a kind gift from Dr. C. January [20], were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM non-essential amino acid solution, 100 units/ml penicillin–streptomycin, 100 g/ml streptomycin sulfate, and 100 g/ml zeocin in an atmosphere of 95% air and 5% CO\(_2\). At 60–80% confluence, cells were treated with media containing 0.25% trypsin and 0.02% EDTA for 3 min, washed with fresh media, and dispersed to new plastic culture dishes. For electrophysiological recording, cells were seeded on 5 mm diameter glass cover slips, incubated for 5–24 h in 24-well plates, and then transferred to the recording chamber. Whole cell currents were recorded using patch-clamp techniques. The external bath solution contained 136 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM glucose and 10 mM HEPES, adjusted to pH 7.4 with NaOH. The intracellular pipette solution contained 130 mM KCl, 1 mM MgCl\(_2\), 10 mM EGTA, 5 mM Mg-ATP and 10 mM HEPES, adjusted to pH 7.2 with KOH. The patch pipettes had resistances of 2–4 MΩ in the external bath solutions. All ionic currents were recorded using EPC10 amplifiers (HEKA electronic, Lambrecht–Pfalz, Germany). The Pulse + PulseFit computer program (HEKA electronic) was used for voltage-clamp amplifier control, data acquisition, and analysis. Solutions were applied to the HEK 293 cells by continuous perfusion of the chamber during recording. All experiments were performed at 36 ± 1 °C.

2.6. Pulse protocols and analysis

To obtain concentration–response curves in the presence of promethazine, dose-dependent inhibition was fitted with the
concentrations of promethazine. The APD90 increased with increasing promethazine concentration from 1 to 10 μM in steps from 0.01 to 100 μM in steps of 0.1, and then to 100 μM. The IC50 for the promethazine-induced prolongation of the APD90 in the guinea pig ventricular myocytes was 0.73 ± 0.58 μM, as shown in Fig. 1B.

2.7. Virtual docking

A homology model of the hERG potassium channel was built on the basis of the 1.7 Å crystal structure (PDB ID code: 1R3J) of the KvAP channel [21], using the homology modeling program, MODELLER v8.0 [22]. A long stretch of amino acids (M579 to G603), located at the third extracellular loop of the hERG channel, was not included for the modeling because this region was not present in the template structure and does not appear to be involved in the drug-induced inhibition of the hERG channel. The 3D energy-minimized conformation of promethazine was generated using LigPrep version 2.0 (Schrodinger LLC, New York, NY). In the LigPrep operation, the ionization state was calculated at pH 7.4 without additional generation of the tautomeric forms. The virtual docking of promethazine was performed using GLIDE version 4.0 (Schrodinger LLC, New York, NY) [23]. Extra-precision (XP) mode of GLIDE was used for accurate pose prediction. The GLIDE XP mode utilizes an improved scoring function, including water desolvation energy term and protein–ligand structural motifs resulting in enhanced binding affinity [24]. For docking, receptor structures were preprocessed using protein refinement components in GLIDE docking package. A restrained minimization using the OPLS-AA force field was performed for the refinement of the hERG channel structure. This minimization continued until the average RMS deviation of the non-hydrogen atoms reached the specified limit of 0.3 Å. To assign the candidate docking site, a grid center was defined as a centroid of Y652 and F656 residues in four monomers of 0.3 Å. To assign the candidate docking site, a grid center was set to 15 Å from the grid center.

2.8. Statistical evaluations

All data are expressed as mean ± S.E.M. Unpaired or paired Student t-tests, or ANOVA were used for statistical comparisons when appropriate, and differences were considered significant at P < 0.05.

3. Results

3.1. Effects of the promethazine on action potentials in guinea pig ventricular myocytes

This study examined the effects of promethazine on the action potentials in guinea pig ventricular myocytes. The mean control action potential duration at 90% (APD90) of myocytes was 460 ± 13 ms (n = 18). Fig. 1A shows superimposed traces of the action potentials recorded before and after exposure to different concentrations of promethazine. The APD90 increased with increasing promethazine concentration from 1 to 10 μM in steps from 0.01 to 0.1, and then to 100 μM. The IC50 for the promethazine-induced prolongation of the APD90 in the guinea pig ventricular myocytes was 0.73 ± 0.58 μM, as shown in Fig. 1B.

3.2. Concentration-dependence of WT hERG channel block by promethazine in Xenopus oocytes

Next, to examine whether the promethazine-induced prolongation effect of APD90 could be due to inhibition of IKr, we examined the effect of promethazine on the hERG currents using a Xenopus oocyte expression system. Throughout these experiments, the holding potential was maintained at −70 mV, and tail currents (Itail) were recorded at −60 mV after depolarizing pulses from −50 mV to +40 mV. Fig. 2A gives an example of a voltage-clamp recording from a Xenopus oocyte and the representative current traces both under the control conditions and after exposure to 50 μM promethazine. Under the control conditions, the depolarizing steps activated the time-dependent outward currents. The amplitude of the outward currents measured at the end of the pulse (IHERG) increased with increasing positive voltage steps, reaching a maximum at −10 mV. The amplitude of IHERG was normalized to the maximum amplitude of the IHERG obtained under the control conditions, and was plotted against the potential of the step depolarization (IHERG, nor). Fig. 2B shows the current–voltage relationships for IHERG, nor obtained at various promethazine concentrations. The amplitude of IHERG, nor showed a concentration-dependent decrease with increasing promethazine concentration.

After the depolarizing steps, repolarization to −60 mV induced an outward Itail, which had an amplitude even greater than that of IHERG during depolarization. This is a characteristic property of the hERG currents, and is due to rapid recovery from inactivation and a slow deactivation mechanism [20]. The amplitude of Itail increased with the depolarizing steps from −50 mV to +20 mV, and was superimposed with the further depolarizing steps to +40 mV. When 50 μM promethazine was added to the perfusate, both the IHERG and Itail were reduced (Fig. 2A, bottom panel). The amplitude of Itail
was normalized to the peak amplitude obtained under the control conditions at the maximum depolarization, and was plotted against the potential of the step depolarization (Fig. 2C). The normalized $I_{\text{tail}}$ reflects the voltage-dependent activation of the hERG channels. The data obtained under the control conditions were well-fitted by the Boltzmann Equation, with a half-maximal activation ($V_{1/2}$) at $-25.7$ mV. The peak $I_{\text{tail}}$ amplitude decreased with increasing promethazine concentration, which indicates that the maximum conductance of the hERG channels is decreased by promethazine. In addition, in the presence of promethazine, $I_{\text{tail}}$ does not reach the steady-state level but decreases at more positive potentials, indicating that the block is more pronounced at the positive potentials.

The values shown in Fig. 2C were normalized to the respective maximum values at each concentration to determine if promethazine shifts the activation curve. The activation curves in the control oocytes, as well as those treated with 1–100 μM promethazine basically overlapped, whereas the curves representing the highest concentration of the drug (300 μM) were shifted leftward (Fig. 2D). The $V_{1/2}$ calculations are consistent with this finding, yielding values of $-25.7 \pm 0.30$ mV, $-24.0 \pm 0.39$ mV, $-24.4 \pm 0.38$ mV, $-25.9 \pm 0.94$ mV, $-26.8 \pm 1.20$ mV, $-27.7 \pm 1.71$ mV, $-28.1 \pm 1.54$ mV, and $-29.5 \pm 0.61$ mV in the control and 1 μM, 3 μM, 10 μM, 20 μM, 50 μM, 100 μM and 300 μM promethazine-treated groups, respectively ($n=5, P<0.05$). Therefore, the $V_{1/2}$ values in the presence of 1–100 μM promethazine were similar, indicating that promethazine does not alter the activation gating at this concentration range.

### 3.3. Voltage-dependent block of WT hERG channel by promethazine

The promethazine-induced decrease in $I_{\text{tail}}$ at different potentials was compared in order to determine if the effect of promethazine was voltage-dependent (Fig. 3). The results showed that a higher degree of block was present at the higher positive voltages (Fig. 3A). The percentage inhibition in the hERG current by 20 μM promethazine at −20 mV, 0 mV, +20 mV, and +40 mV was 26.8 ± 2.7%, 31.5 ± 3.2%, 34.4 ± 1.2%, and 38.9 ± 1.1%, respectively ($n=3$, Fig. 3B). Dose–response relationships were obtained at +40 mV, +20 mV and −20 mV. The data were fitted by Hill equations, giving IC$_{50}$ values of 52.1 ± 6.14 μM, 41.9 ± 2.13 μM and 35.0 ± 2.23 μM, respectively, and Hill coefficients of 0.88 ± 0.10, 0.91 ± 0.04 and 0.87 ± 0.05 at −20 mV, +20 mV and +40 mV, respectively ($n=3$, data not shown). This suggests that the promethazine-induced block of the hERG currents progressively increases with increasing depolarization. Overall, these findings indicate that the promethazine-induced block of the hERG current is voltage-dependent.

### 3.4. Time-dependence of WT hERG channel block by promethazine

The currents were activated using a protocol containing a single depolarizing step to 0 mV for 8 s to determine if the channel was blocked in the closed or activated (i.e. open and/or inactivated) state...
Fig. 3. Voltage dependence of promethazine-induced hERG current block. (A) Current traces from a cell depolarized to −20 mV (left panel), +20 mV (middle panel) and +40 mV (right panel), before and after exposure to 20 μM promethazine, showing increased block of hERG current at the more positive potential. The protocol consisted of 4 s depolarizing steps to −20 mV, +20 mV or +40 mV from a holding potential of −70 mV, followed by repolarization to −60 mV. (B) Promethazine-induced hERG current inhibition at different voltages. At each depolarizing voltage step (−20 mV, +10 mV, +20 mV or +40 mV), the tail currents in the presence of 20 μM promethazine were normalized to the tail current obtained in the absence of drug. Bars with error bars represent mean ± S.E.M. (n = 3).

After obtaining the control measurement, 50 μM promethazine was applied and the recordings were made. Fig. 4B shows the degree of inhibition (i.e. (1 − promethazine current/control current) × 100 (in %)). Analysis of the test pulse after the application of promethazine revealed a time-dependent increase in block in this representative cell to 58% at 2 s (Fig. 4B). In addition, at the beginning of the pulse, the fractional sustained current, which was obtained by normalizing the currents with promethazine relative to control currents, was 0.91 ± 0.02 of the control (n = 5). This indicates that the hERG channels were only slightly blocked by promethazine while remaining at the holding potential. In this series of experiments, 50 μM promethazine reduced the hERG outward currents at the end of the 0 mV pulse by 54.8 ± 9.4% (n = 5). Overall, promethazine inhibits the hERG channels mainly in the open and inactivated state rather than in the closed state.

3.5. Promethazine block of WT hERG currents expressed in HEK cells

The IC50 values of many hERG channel blockers have been shown to differ depending on whether the hERG channels are expressed in Xenopus oocytes or mammalian cells, an effect probably due to the sequestration of blockers in the large ooplasm of oocytes [19]. We therefore tested the effects of promethazine in HEK293 cells expressing hERG channels at 36°C (Fig. 5A). hERG currents were activated by a 4.8 s depolarizing pulse to +20 mV from a holding potential of −80 mV, and the tail current was recorded at −60 mV. The cells were treated with steadily increasing concentrations of promethazine from 0.001 μM to 10 μM (Fig. 5B). We observed that during each depolarizing pulse, the current amplitude gradually declined as the drug blocked the hERG channels, until a steady state was reached. Once the current amplitude reached steady state, we increased the drug concentration. Promethazine inhibited the hERG channel tail currents dose-dependently.

The dose–response curve for the promethazine-induced block of the normalized tail current showed an IC50 of 1.46 μM and a Hill coefficient of 1.07 (n = 4) (Fig. 5B). These results indicate that the promethazine-induced inhibition of hERG channels stably expressed in HEK293 cells occurred at a concentration ~35 times lower concentration than that required by hERG channels expressed in Xenopus oocytes.

3.6. Promethazine block of WT and mutant hERG channels expressed in oocytes

Previous studies reported that two aromatic residues, Tyr-652 and Phe-656, which are located in S6 domain and face the pore
Fig. 4. Blocking of activated hERG channels by promethazine. (A) An original recording of currents under control conditions (control) and after exposure to 50 μM promethazine (for 13 min, without any intermittent test pulse). (B) The degree of hERG-current inhibition in percentages (%). Current inhibition increased time-dependently to 58% at 2 s in this representative cell, indicating that mostly open and/or inactivated channels were blocked. (C) Inhibition of inactivated channels by 50 μM promethazine. hERG channels were inactivated by a first voltage-step to +80 mV, followed by channel opening at 0 mV. (D) The corresponding relative block during the 0 mV step is displayed. Maximum inhibition was achieved in the inactivated state during the first step, and no further time-dependent block occurred upon channel opening during the second voltage step.

cavity of the channel, are important components of the binding site for a number of compounds [15]. The potency of a channel block for the wild type and two mutant hERG channels (Y652A and F656A) were compared in order to determine if these key residues are also important in the promethazine-induced blocking of the hERG channel. The effect of promethazine on WT was quantified during pulses to −140 mV after 4 s activating pulse to 0 mV (Fig. 6A–C). As shown in Fig. 6, the inhibitory effect of promethazine (100 μM) was partially attenuated by a Y652A mutation (Panels A and B) or abolished by a F656A mutation (Panels A and C). The IC50 values were consistent with this finding as shown in Fig. 6D. The wild type hERG channel current was blocked by promethazine with an IC50 of 91.8 ± 2.4 μM (n = 3–4), while the IC50 values were 519.3 ± 0.01 μM (n = 6), and 3254.6 ± 0.01 μM (n = 8) for the Y652A and F656A hERG mutants, respectively. This indicates that a mutation of Phe-656 located in the S6 domain of the hERG channel reduced the potency of the channel block by promethazine more than a mutation of Tyr-652 in the same region.

4. Discussion

These results suggest that promethazine inhibits the hERG channels and increases the APD90. The IC50 value of promethazine was 35.0 μM (at +40 mV) for the hERG channels expressed heterologously in Xenopus oocytes. However, the IC50 value of promethazine was 1.46 μM and 0.73 μM, respectively, for the hERG channels stably expressed in the HEK cells and for the prolongation of APD90 in guinea pig ventricular myocytes. A higher extracellular concentration of the drug was required to block the hERG channels due to the properties of the Xenopus oocyte-expression system. For example, the block of hERG by trifluoperazine [19] resulted in IC50 values 10 times higher when the drug was applied to Xenopus oocytes rather than to mammalian cells. This might be due to a decrease in drug concentration at the cell membrane caused by the vitelline membrane and egg yolk.

Promethazine is a histamine H1-receptor antagonist with moderate muscarinic dopamine-receptor-blocking effects. The clinical effects became apparent within 20 min after oral, rectal, or intra-muscular administration, and the effects can last from 4 h to 6 h [25]. The therapeutic plasma concentrations of promethazine typically range from 9.3 nM to 150 nM [26,27], and the elimination half-life after oral administration was estimated to be 12–15 h [28]. The free plasma concentration might range from 1.9 nM to 30 nM when the rate of protein binding reaches 80% for promethazine [26]. Also, it was reported that in heart tissues, basic amphiphilic substances, e.g. phenothiazines including promethazine, can reach an up to 4 times higher plasma concentration [29], indicating the accumulated concentration may be assumed to be in the range from 7.6 nM to 120 nM in the tissue. The class III antiarrhythmic effect of promethazine manifests as changes to the ECG in rat and feline models at the supra-therapeutic level, such as a prolonged PR interval and increased QT interval [10,11]. There is one case report of fatal polymorphous ventricular tachycardia and torsades de pointes related to the therapeutic level of promethazine [30]. It was suggested that drugs with an IC50 at least 30 times greater than the highest achievable free plasma concentration will not cause torsades de pointes, a potentially fatal ventricular tacharrhythmia [31]. The hERG channels in the heart might be blocked by promethazine at clinically relevant plasma concentrations because the IC50 value (~1.5 μM) obtained from HEK cells in this study was approximately 13 times the expected highest achievable concentration in heart (120 nM). In addition, the myocardial concentrations of
Symbols with error bars represent mean ± S.E.M. (n = 4 each).

Promethazine-induced block of hERG channels stably expressed in HEK293 cells. (A) Representative current traces recorded from HEK293 cells after application of promethazine. (B) Concentration-dependent block of hERG current. hERG tail-current amplitude was normalized to the control and plotted as a function of promethazine concentration. Data were fitted with the Hill equation, giving a half-maximal inhibitory concentration (IC_{50}) of 1.46 μM and a Hill coefficient of 1.07. Symbols with error bars represent mean ± S.E.M. (n = 4 each).

Promethazine in patients with hepatic disease, renal insufficiency or overdoses may increase the possibility of block the I_{Ks} current in the heart, which could induce its proarrhythmic effects such as the prolongations of the APD_{90} (Fig. 1) and QT interval [11], and torsades de pointes [30].

A large number of drugs from a range of classes, including antihistamines, antipsychotics, antiarrhythmics, antibiotics, and gastrointestinal prokinetic agents, have been associated with torsades de pointes and a long QT syndrome. Many of the drugs block the hERG channels in a voltage-dependent manner, which suggests that these drugs bind to the open or inactivated state of the hERG channels. For example, two histamine-receptor antagonists, terfenadine and astemizole [32], bind preferentially to the inactivated state of hERG, whereas the gastrointestinal prokinetic agent, cisapride, blocks the channel in its open state [33]. This shows that promethazine decreases the amplitudes of the maximum outward current and maximum peak tail current (Fig. 2). Moreover, the magnitude of the block increased with increasing positive voltage, which increased the open probability and enhanced inactivation (Fig. 3). In addition, promethazine block may be state-dependent: the hERG channels are blocked mainly in the open and inactivated states but not in the closed state (Fig. 4). Finally, promethazine did not significantly alter the V_{1/2} values of the activation curve, which suggests that the drug blocks the hERG channels without altering the activation properties (Fig. 2). These open channel block property and voltage dependence of promethazine might result in increased block at higher heart rate although our results has the limitation in that the drug increased APD_{90} at unphysiologically low stimulation rate of 0.33 Hz (Fig. 1). In other view of such a voltage dependence of a promethazine block of the hERG channels, the drug may well increase the APD and make heart more prone to arrhythmia, particularly under the pathological conditions associated with rapid heart actions and partially depolarized membranes.

Other antihistamines have been reported to block hERG channel. In Xenopus oocytes, the 1st generation antihistamine, chlorpheniramine and diphenhydramine blocked the channel with lower potency compared to the 2nd generation antihistamine, terfenadine and astemizole [32]. Also, Katchman et al. [34] have shown that loratadine, the nonselecting 2nd generation antihistamine has blocked hERG current with IC_{50} value of 4 μM, which is lower than that of chlorpheniramine (IC_{50} value of 13 μM) in HEK cell. In this context, our results indicate that promethazine have relatively high potency for the block of hERG channel compared with other 1st generation antihistamines.

Promethazine, a phenothiazine H1 antihistamine, was reported to modify the cardiac electrical activity. It has a negative chronotropic effect on the guinea pig atria [6], and blocks the histamine-induced positive chronotropic response in rabbit atria [7]. The class III antiarrhythmic effect of the drug manifests as changes to the ECG in isolated perfused hearts from rats [10] and felines [11], such as a prolonged PR interval and increased QT interval. The prolongation of cardiac repolarization by phenothiazines is unlikely to be the result of their ability to block H1-histamine receptors because there is little correlation between their receptor-binding affinities and their ability to prolong the QT interval [35]. The present results suggest that promethazine blocks the hERG channel directly and slows the repolarization of the action potential, possibly resulting in cardiac arrhythmia. In addition, it is possible that the modification of the cardiac electrical activity by phenothiazine is due to non-specific membrane effects, such as perturbation of the membrane lipid–protein interaction. This is because phenothiazines bind easily to the phosphatidylincholine/cholesterol bilayer membrane through both hydrophobic and electrostatic interactions [36,37], which leads to perturbed cell functions [38]. Nevertheless, the direct inhibition of hERG by promethazine would be a major cause of APD prolongation because the pore mutations of the channel protein, Y652A and F656A, significantly attenuate the drug-induced hERG block (Fig. 6). However, the relatively low incidence of arrhythmia during the promethazine treatment [30] can be explained by the inhibition of the fast Na+ channels [39]. This might counteract the potential arrhythmogenic effects of the hERG block even though the IC_{50} value for the block of the fast Na+ channels obtained from their study was approximately 6 times higher than the value for the APD_{90} prolongation observed in the present study (Fig. 1).

Alanine-scanning mutagenesis study has revealed several amino acids are important for the drug inhibition of the hERG channel [15]. More mutagenesis study has suggested that Y652 makes a π–π interaction with an aromatic moiety of the hERG channel blocker and F656 makes a hydrophobic interaction with a hydrophobic group of the hERG channel blocker [40]. Indeed our mutagenesis study confirmed that the two amino acids are important for the block of the hERG channel by promethazine (Fig. 6). We have performed virtual docking simulation to understand the blocking mode of the hERG channel by the drug. Because the crystal structure of the hERG channel is not known yet, we made a homology model of the hERG channel using the KvAP channel structure as the template. The KvAP channel has high homology with the hERG channel and the structure is presumably closed state. Then a virtual docking was performed designating Y652 and F656 as active sites. The highest score result showed the binding mode that the protonated nitrogen makes a hydrogen bond with the oxygen of T623 (Fig. 7B). In addition to the hydrogen bond, the aromatic group of promethazine makes π–π interactions with the side chain aromatic rings of hERG residues Y652(I), Y652(II), and Y652(IV). These results are consistent with our previous model of hERG channel block by hERG current inhibitors [41]. However we could not see any interactions...
between the promethazine and the side chain of hERG residue F656. This result is not in agreement with the mutants study that F656A showed reduced sensitivity to promethazine.

In conclusion, the H₁ antihistamine, promethazine, increases the APD₉₀ in guinea pig ventricular myocytes and blocks the hERG channels, which is the pore-forming subunit of the channel underlying Iₖᵢᵣ in Xenopus oocytes and HEK cells. In addition, Tyr-652 and Phe-656 in the S6 domain are important molecular determinants for promethazine-induced blocking of the hERG channel. These results suggest a potential mechanism by which promethazine...
may increase the possibility of cardiac arrhythmia in drug-treated patients.

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References