Probucol aggravates long QT syndrome associated with a novel missense mutation M124T in the N-terminus of HERG

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ABSTRACT

Patients with LQTS (long QT syndrome) with a mutation in a cardiac ion channel gene, leading to mild-to-moderate channel dysfunction, may manifest marked QT prolongation or torsade de pointes only upon an additional stressor. A 59-year-old woman had marked QT prolongation and repeated torsade de pointes 3 months after initiation of probucol, a cholesterol-lowering drug. We identified a single base substitution in the\ HERG \ gene by genetic analysis. This novel missense mutation is predicted to cause an amino acid substitution of Met124 → Thr (M124T) in the N-terminus. Three other relatives with this mutation also had QT prolongation and one of them had a prolonged QT interval and torsade de pointes accompanied by syncope after taking probucol. We expressed wild-type HERG and HERG with M124T in Xenopus oocytes and characterized the electrophysiological properties of these HERG channels and the action of probucol on the channels. Injection of the M124T mutant cRNA into Xenopus oocytes resulted in expression of functional channels with markedly smaller amplitude. In both HERG channels, probucol decreased the amplitude of the HERG tail current, decelerated the rate of channel activation, accelerated the rate of channel deactivation and shifted the reversal potential to a more positive value. The electrophysiological study indicated that QT lengthening and cardiac arrhythmia in the two present patients were due to inhibition of I\textsubscript{Kr} (rapidly activating delayed rectifier K\textsuperscript{+} current) by probucol, in addition to the significant suppression of HERG current in HERG channels with the M124T mutation.

INTRODUCTION

Congenital LQTS (long QT syndrome) is characterized by prolonged ventricular repolarization and a propensity for life-threatening ventricular tachyarrhythmias, resulting in syncopal attacks and sudden death [1]. Mutations in various cardiac ion channel genes, including KCNQ1, KCNE1, HERG, KCNE2 and SCN5A [2–8], are known to cause this syndrome, and many LQTS mutations have been identified [9]. HERG encodes a voltage-gated K\textsuperscript{+} channel and the HERG current is similar to I\textsubscript{Kr} (rapidly activating delayed rectifier K\textsuperscript{+} current) [10] observed in the myocardium. Cellular expression studies revealed that mutations in different regions of the HERG gene elicit a variety of functional changes in the HERG channel [11], suggesting that each mutation should be examined...
to clarify the mechanism of suppression of the HERG current.

In patients with LQTS caused by a mutation, leading to mild-to-moderate dysfunction of a cardiac ion channel, marked QT prolongation or torsade de pointes may appear only upon the addition of a stressor, such as a drug, hypokalaemia or bradycardia. Probucol, a cholesterol-lowering drug, is known to induce QT prolongation in some patients, although ventricular arrhythmia or syncope due to the drug is rare [12].

We have recently identified in a Japanese family with LQTS a novel missense mutation in the N-terminus of the HERG gene that resulted in a putative amino acid substitution of Met$^{124}$ → Thr (M124T). While taking probucol, two family members with this mutation had QT prolongation and torsade de pointes with recurrent syncope. Since this particular mutation of the HERG gene has not been reported previously and since the relationship between QT prolongation and the action of probucol has not been clarified, it is of interest to identify whether this mutation causes HERG channel dysfunction and how probucol prolongs the QT interval.

Using the heterologous expression system of *Xenopus* oocytes, we examined the properties of this mutant channel and the effect of probucol on both WT (wild-type) and mutant M124T HERG channels. We demonstrate that the M124T mutation in HERG causes $I_{Kr}$ dysfunction, and that probucol decreases the current amplitudes of both WT and mutant HERG channels by altering the activation and deactivation kinetics and ion selectivity.

**METHODS**

**DNA isolation and mutation analysis**

Genetic analysis was performed after obtaining informed consent from all subjects. Genomic DNA was purified from the white blood cells of seven family members, after which PCR amplification was performed [13]. SSCP (single-strand conformational polymorphism) analysis of the amplified DNA was performed to screen for mutations in *KCNQ1*, *HERG*, *SCN5A*, *KCNE1* and *KCNE2* as described previously [14] with a slight modification [15]. Normal and aberrant SSCP products were isolated and sequenced by ABI PRISM 310 (PerkinElmer Applied Biosystems, Foster City, CA, U.S.A.). We sequenced all 15 exons of the *HERG* gene in the proband using 19 primer pairs as described previously [16]. To confirm the missense mutation that served as the basis of the present study, restriction enzyme analysis was performed. Using a mutagenic primer, on PCR gene amplification we introduced an artificial AccII site in the PCR product of the only T → C allele (M124T). Digestion of the PCR products derived from the mutant allele with AccII gave rise to a 119 bp fragment instead of a 138 bp fragment when resolved on a polyacrylamide gel.

**Site-directed mutagenesis and in vitro transcription of cRNA**

HERG cDNA in a pGH19 vector was kindly provided by Dr Gail A. Robertson (Department of Physiology, University of Wisconsin, Madison, WI, U.S.A.). The M124T HERG cDNA was constructed by an overlap extension strategy [17]. The sequence of the M124T HERG cDNA was confirmed by DNA sequence analyses using 11 primer pairs. WT HERG cDNA and M124T HERG cDNA were linearized by digestion with Not I, and cRNAs were prepared with the *mMESSAGE mMACHINE* kit (Ambion, Austin, TX, U.S.A.) using T7 RNA polymerase.

**Electrophysiology**

Defolliculated *Xenopus* laevis oocytes (stage V–VI) were isolated as described previously [18]. Each oocyte was injected with 50 nl of solution containing 1.0 ng of WT HERG cRNA, 1.0 ng of M124T HERG cRNA or a combination of 1.0 ng of WT HERG cRNA and 1.0 ng of M124T HERG cRNA. The oocytes were incubated at 16 °C in modified Barth’s solution (87.4 mM NaCl, 1 mM KCl, 2.4 mM NaHCO$_3$, 10 mM Hesper, 0.82 mM MgSO$_4$, 0.66 mM NaN$_3$ and 0.74 mM CaCl$_2$, pH 7.5) supplemented with penicillin (100 µg/ml) and streptomycin (100 µg/ml). The oocytes were used for experiments 2–4 days after injection.

Membrane currents were studied using the two-microelectrode voltage clamp technique with an amplifier (AXOCLAMP-2A; Axon Instruments, Foster City, CA, U.S.A.) at room temperature of 23–25 °C as described previously [19]. During recording, oocytes were perfused continuously with ND96 solution (96 mM NaCl, 2 mM KCl, 5 mM MgCl$_2$, 0.3 mM CaCl$_2$ and 5 mM Hesper, pH 7.6). Probucol (kindly supplied by Daiichi Pharmaceutical, Tokyo, Japan) was stored at 4 °C. On the day of the experiment, probucol was dissolved in ND96 solution and the pH was re-adjusted to 7.6. All measurements were made under steady-state conditions at least 3 min after total solution exchange.

Data acquisition and analysis were performed by a DigiDATA 1200 A/D converter (Axon Instruments) and pCLAMP (version 5.5.1; Axon Instruments).

**Voltage clamp protocols and data analysis**

All pulse protocols are described in the Figure legends. Data analysis was carried out using Clampfit (version 6.1; Axon Instruments). The voltage-dependence of HERG current activation was determined for each oocyte by fitting the peak value of $I_{tail}$ (tail current) against the test potential to a Boltzmann function in the following formula:

$$ I_{\text{tail}} = I_{\text{tail-max}} / \left[ 1 + \exp \left( \frac{(V_{1/2} - V_i)}{k} \right) \right] $$
where $I_{\text{tail-max}}$ is the peak $I_{\text{tail}}$, $V_t$ is the test potential, $V_{1/2}$ is voltage at which the current was half-activated (i.e. at which $I_{\text{tail}}$ is half of $I_{\text{tail-max}}$), and $k$ is the slope factor. All values are expressed as means $\pm$ S.E.M. Differences among these values were evaluated by ANOVA and the unpaired Student’s t-test where appropriate. $P < 0.05$ was considered to be significant.

### RESULTS

#### Clinical diagnosis

The proband was a 59-year-old woman who experienced recurrent syncope. She had started taking probucol (500 mg daily) for hypercholesterolaemia approx. 2 months prior to the episode of syncope. Figure 1(A, upper left panel) shows the ECG of the proband (Figure 2B, II-1) obtained approx. 8 years prior to initiation of probucol, which exhibited a prolonged QT interval. QTc (QT interval corrected for heart rate using Bazett’s formula $= \text{QT/}\sqrt{\text{RR interval in seconds}}$) was 495 ms. On the ECG obtained at admission, she had a significantly prolonged QTc interval of 550 ms (Figure 1A, upper-right panel) and polymorphic ventricular tachycardia (torsade de pointes; Figure 1A, lower panel), accompanied by fainting. The proband was diagnosed with LQTS. Probucol was discontinued soon after admission; however, the QTc interval remained prolonged (646 ms) 50 days after discontinuation of probucol. Figure 1(B, upper-left panel) shows the ECG of a niece of the proband (Figure 2B, III-4), which had been obtained when she was not taking any medication, indicating a prolonged QTc interval (478 ms). She was a 19-year-old woman who had been diagnosed with xanthomatosis and was prescribed 500 mg of probucol daily. Three months after she started taking probucol, she experienced an episode of syncope. An ECG revealed a markedly prolonged QTc interval (660 ms; Figure 1B, upper-right panel) and torsade de pointes (Figure 1B, lower panel). The concentration of probucol in her blood at the time of having cardiac arrhythmia was 26 µg/ml, which was within the therapeutic range. The two sisters (Figure 2B, II-2 and II-4) of the proband had prolonged QTc intervals of 524 ms and 478 ms respectively, on ECGs obtained when they were not taking any drugs. The average QTc interval of these four patients before taking probucol was 494 $\pm$ 11 ms.

#### Molecular genetic analyses

Screening for mutations in the HERG gene by SSCP analysis identified an abnormal SSCP conformer in the DNA samples from the proband. This SSCP anomaly

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were observed in DNA samples from more than 100 control subjects. Sequence analysis of the abnormal SSCP conformer revealed a single base substitution (T → C) at nucleotide position 371 (Figure 2A). On PCR RFLP (restriction fragment length polymorphism) analysis, all four individuals with prolonged QTc intervals showed both 138 bp and 119 bp fragments, indicating heterozygosity for the mutation (Figure 2C). The arrow in the pedigree indicates the proband. Numbers indicate the length in the proband and in three relatives.

Electrophysiological characterization of the M124T HERG mutant

Oocytes injected with 1.0 ng of WT HERG cRNA alone showed a slowly activating outward current that was associated with an inwardly rectifying property (Figures 3A and 3E), as described previously [10,20]. In contrast, oocytes injected with 1.0 ng of M124T HERG cRNA alone showed these currents, although the currents were smaller than those in oocytes injected with WT HERG cRNA alone (Figures 3B, 3E and 3F). The mean amplitude of the tail currents measured at −60 mV after a depolarizing test pulse to +20 mV was 521 ± 105 nA (n = 8) for the M124T HERG channels, which was significantly smaller than that of 1839 ± 216 nA (n = 10) for the WT HERG channels (P < 0.01). When M124T and WT HERG channels were co-expressed by injecting equal amounts of WT and M124T HERG cRNA into oocytes (i.e. 2.0 ng of cRNA in total; M124T/WT), the evoked currents were nearly the same as those in oocytes injected with WT HERG cRNA alone. The mean amplitude of the tail currents measured at −60 mV after a depolarizing test pulse to +20 mV was 2223 ± 161 nA (n = 16) for the WT channels and 2377 ± 159 nA (n = 14) for the M124T/WT channels (results not shown). Thus the M124T HERG channel did not suppress the WT HERG channel in a dominant-negative manner.

The amplitude of the tail currents was plotted as a function of the test potential and the curve was fitted to a Boltzmann function (Figure 4A). V1/2 was −17.6 ± 1.4 mV (n = 7) for the WT channels and −20.3 ± 1.2 mV (n = 7) for the M124T channels. The slope factor was 8.4 ± 0.6 mV (n = 7) for the WT channels and 7.8 ± 0.2 mV (n = 7) for the M124T channels. V1/2 and slope factor were comparable between the WT and M124T channels (P = 0.20 and P = 0.34 respectively). These data suggest that this mutation does not affect the voltage-dependence activation of the HERG channel.

Deactivation of currents following test pulses could be fitted to a double exponential function. The fast (at test potentials between −80 and −30 mV) and slow (between −60 and −20 mV) time constants of deactivation of the M124T HERG channels were similar to those of the WT HERG channels (Figures 4B and 4C). Our results are consistent with those of a previous report [21] in which some mutations close to the transmembrane in the N-terminus region of the HERG gene had little or no effect on the time course of deactivation of the expressed HERG channel.

Erev (reversal potential) was determined by clamping the tail currents at potentials ranging from −120 mV to +10 mV in 10 mV increments after a 3 s test pulse to
Figure 3  Voltage-clamp recordings of *Xenopus* oocytes expressing WT or M124T HERG (A–D) and *I–V* relationship of the peak currents (E,F) without and with probucol.

(A–D) Voltage-clamp recordings of *Xenopus* oocytes expressing WT or M124T HERG before and after bath superfusion with probucol. Representative whole-cell current traces of oocytes that had been injected with 1.0 ng of WT cRNA (A) or 1.0 ng of M124T cRNA (B) in the absence of probucol, or 1.0 ng of WT cRNA (C) or 1.0 ng of M124T cRNA (D) in the presence of 30 µM probucol, are shown. Depolarizing pulses were applied to a potential ranging between −80 and +50 mV for 3 s, followed by a hyperpolarizing pulse to −60 mV for 6 s. Holding potential of −60 mV was used. To show the current traces clearly, selected traces elicited by five depolarizing pulses (−60, −40, −20, +10 and +40 mV) are displayed. (E) *I–V* relationship of the peak currents during the test pulse. (F) *I–V* relationship of the amplitudes of the peak tail currents. (E and F) WT (■) and M124T (●) before bath superfusion with probucol; WT (□) and M124T (○) after bath superfusion with probucol. At each test potential, eight or ten different oocytes were used.

$E_{rev}$ of M124T HERG channels was $-87.4 \pm 2.8$ mV (n = 12), which was similar to that of WT channels ($-86.9 \pm 1.2$ mV (n = 13)).

**Effects of probucol on WT and M124T HERG channels**

We next investigated the effect of probucol on both the WT and M124T HERG channels. Current amplitudes and the activation and deactivation kinetics of HERG currents were analysed in both the absence and presence of 30 µM probucol. This concentration of probucol corresponded to the serum concentration of probucol at 8–12 weeks of continuous treatment with probucol (1 tablet of 500 mg twice daily) in patients with familial hypercholesterolaemia [22].

Figure 3(C and D) shows the original currents obtained after 3 s depolarizing pulses from a holding potential of −80 mV up to +50 mV in 10 mV steps in the presence of 30 µM probucol. In oocytes injected with either WT or M124T HERG cRNA, probucol did not affect the peak current amplitudes, but it shifted the voltage at the peak amplitude to a positive potential by approx. 10 mV in both the WT and M124T
channels (Figure 3E). This positive shift suggested that probucol affected the voltage-dependence of HERG channel activation. In contrast, probucol significantly decreased the peak tail current amplitude measured at −60 mV after a depolarizing test pulse to 0 mV by 38.3 ± 6.4% through the WT channels (from 1581 ± 158 nA for WT to 967 ± 146 nA for WT + probucol; n = 10 each; P < 0.01) and by 47.2 ± 9.3% through the M124T channels (from 509 ± 97 nA for M124T to 260 ± 81 nA for M124T + probucol; n = 8 each, P < 0.05; Figure 3F). Probucol blocked the WT and M124T channels by a similar degree (P = 0.44).

Normalized tail currents through the WT and M124T channels in the control and 30 µM probucol-treated conditions are shown in Figure 4(A). Perfusion of oocytes with probucol significantly shifted $V_{1/2}$ of activation of WT channels from −17.6 ± 1.4 mV to −8.2 ± 1.7 mV (n = 7; P < 0.01). Similarly, upon treatment with probucol, $V_{1/2}$ of activation of M124T HERG channels significantly shifted from −20.3 ± 1.2 mV to −9.5 ± 2.4 mV (n = 7; P < 0.01). Thus probucol decelerated the rate of HERG channel activation.

Probucol accelerated the deactivation rate of both the WT and M124T HERG channels (Figures 3C, 3D, 4B and 4C). Application of 30 µM probucol to both channels significantly decreased the fast time constants at test potentials between −70 and −40 mV and the slow time constants at test potentials between −60 and −30 mV. Thus probucol accelerated the deactivation of HERG channels.

Application of 30 µM probucol significantly shifted the $E_{rev}$ of WT HERG channels from −87.4 ± 2.8 mV (n = 12) to −77.6 ± 2.4 mV (n = 8; P < 0.05). Similarly, 30 µM probucol significantly shifted $E_{rev}$ of M124T HERG channels from −86.9 ± 1.2 mV (n = 13) to −74.2 ± 2.1 mV (n = 9; P < 0.01). These results suggest that probucol alters the selectivity for $K^+$. 

**DISCUSSION**

We identified in a Japanese family with LQTS a novel single base substitution (from T → C) at nucleotide position 371 in the HERG gene. This mutation is assumed to lead to the substitution of Met$^{249}$ → Thr, which is located in the N-terminus of HERG channels (Figure 2A). Because the M124T mutant was able to produce HERG outward currents when the corresponding cRNA was injected to express M124T HERG in *Xenopus* oocytes (Figure 3B), homomultimers of M124T subunits appeared to form functional channels. The amplitude of the outward tail current through M124T channels was less than one-third of that through the WT HERG channels. Moreover, upon co-expression of M124T with WT in oocytes, this mutant subunit did not have a dominant-negative effect on the WT subunit. Many properties of the WT and M124T HERG currents, including the peak of the current–voltage (I–V) relationship for activating currents recorded during depolarizing pulses (Figure 3E), the voltage dependence of activation (Figure 4A) and the time course of deactivation (Figures 4B and 4C), were similar. In previous reports [21,23], some mutations...
occurring around the PAS (Per-Arnt-Sim) domain in HERG accelerated HERG channel deactivation. For example, the rate of deactivation was significantly faster in HERG mutants with deletion of nucleotide residues 2–23 or 2–26 in HERG. However, mutations that were located near codon 124 barely altered HERG channel deactivation [21].

In the present study, all four patients with M124T mutation in HERG had prolonged QTc intervals (494 ± 11 ms), but were clinically asymptomatic prior to probucol administration; symptoms appeared in the proband and her niece after they received probucol. These clinical findings are consistent with the results in the electrophysiological study in that the M124T channels did not strongly suppress the WT channels in a dominant-negative manner. A previous report [24] found that LQTS patients with mutations in the non-pore regions of HERG had a lower frequency of arrhythmia-related cardiac events occurring at an earlier age than those with mutations in the pore region. In some individuals, congenital LQTS associated with a LQTS-related ion channel gene mutation, leading to mild-to-moderate channel dysfunction, is not detected until they are exposed to an additional stressor, such as particular drugs, hypokalaemia or bradycardia [8, 25–28].

The proband and her niece had a significantly prolonged QTc interval and torsade de pointes several months after initiation of probucol. Probucol is known to induce QT prolongation in some patients. In an analysis of 89 adult patients treated for hypercholesterolaemia with probucol, 23 patients (26 %) had QTc interval prolongation (over 0.450 s) on ECG [12]. However, the mechanism through which probucol prolongs the QTc interval had not been clarified. The present study showed that probucol blocks the HERG K⁺ channel. Probucol inhibited both the WT and mutant HERG tail currents (Figures 3C, 3D and 3F), induced a positive shift of the activation curve (Figure 4A), accelerated deactivation (Figures 4B and 4C) and shifted the reversal potential of HERG to a more positive value, which can decrease the net outward current during repolarization. In the proband and her niece, the M124T mutation in HERG caused I_Ks dysfunction and probucol further suppressed the I_Ks current; this would cause a delay in the repolarization phase of action potentials along cardiomyocytes and induce QT prolongation and cardiac arrhythmia. Moreover, the results of our electrophysiological study showed that probucol also inhibited WT HERG channels, suggesting that this drug might induce acquired LQTS by blocking the I_Ks. These drug actions are similar to those of propoxyphene, a relatively weak μ opioid receptor agonist, and its metabolite, norpropoxyphene. Ulens et al. [29] reported that propoxyphene and norpropoxyphene altered gating of HERG channels by decreasing the channel activation rate constant, increasing the channel deactivation rate constant and shifting the reversal potential of HERG currents to a more positive value. They also suggested that the interaction of propoxyphene and norpropoxyphene with amino acid Ser^631 of the HERG channel protein might contribute to the drug-induced alteration of ion selectivity. We therefore postulate that probucol may act on HERG channels in the same manner as propoxyphene and norpropoxyphene. Although HERG tail currents were decreased by probucol, the peak currents during the pulse were not. HERG channels possess the feature that rapid voltage-dependent inactivation is present during the pulse, but relieved during repolarization, giving rise to the resurgent tail current. The shift in inactivation kinetics may result in the difference between the two sets of measurements. It should be noted that the potency of drug inhibition of the HERG current obtained using an oocyte expression system may considerably underestimate the actual drug potency towards the HERG current in a mammalian expression system/native tissue [30]. Therefore the level of inhibition of HERG current by probucol obtained in the present study may be lower than its actual level.

The proband in the present study had a prolonged QTc interval (646 ms) 50 days after discontinuation of probucol. Probucol remains in the body for a long period of time. In a previous case report [31], probucol was still detected in the plasma of the patient 3 months after its discontinuation. The serum concentration of probucol in the proband in the present study was not measured; however, probucol may have still been present in her body and prolonged the QT interval at 50 days after its withdrawal.

In conclusion, we have identified a novel missense mutation in the N-terminus of HERG in a Japanese family with LQTS in which two family members developed torsade de pointes after taking probucol. The results of our electrophysiological studies indicate that QT prolongation and cardiac arrhythmia in the two patients were due to I_Ks dysfunction as a result of the M124T mutation in HERG and to the additional inhibitory effect of probucol on cardiac I_Ks current. Probucol also inhibited WT HERG channels and might induce acquired LQTS.

**Study limitation**

Probucol is a lipid-soluble drug and has low solubility in water. In the present study, we dissolved probucol in ND96 solution mechanically using an ultrasonic homogenizer, followed by heat treatment at over 120 °C. Although these solution treatments could almost completely dissolve the drug in water, the concentration of the drug in the solutions was not measured. However, it is hypothesized that this would not have a large effect on our results, because the conditions were similar for the WT and mutant HERG channels.
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