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Biochemical and Biophysical Research Communications 351 (2006) 273–280
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# hERG $K^+$ channel blockade by the antipsychotic drug thioridazine: An obligatory role for the S6 helix residue F656

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Received 29 September 2006
Available online 23 October 2006

**Abstract**

The phenothiazine antipsychotic agent thioridazine has been linked with prolongation of the QT interval on the electrocardiogram, ventricular arrhythmias, and sudden death. Although thioridazine is known to inhibit cardiac hERG $K^+$ channels there is little mechanistic information on this action. We have investigated in detail hERG $K^+$ channel current ($I_{hERG}$) blockade by thioridazine and identified a key molecular determinant of blockade. Whole-cell $I_{hERG}$ measurements were made at 37 °C from human embryonic kidney (HEK-293) cells expressing wild-type and mutant hERG channels. Thioridazine inhibited $I_{hERG}$ tails at −40 mV following a 2 s depolarization to +20 mV with an $IC_{50}$ value of 80 nM. Comparable levels of $I_{hERG}$ inhibition were seen with physiological command waveforms (ventricular and Purkinje fibre action potentials). Thioridazine block of $I_{hERG}$ was only weakly voltage-dependent, though the time dependence of $I_{hERG}$ inhibition indicated contingency of blockade upon channel gating. The S6 helix point mutation F656A almost completely abolished, and the Y652A mutation partially attenuated, $I_{hERG}$ inhibition by thioridazine. In summary, thioridazine is one of the most potent hERG $K^+$ channel blockers amongst antipsychotics, exhibiting characteristics of a preferential open/activated channel blocker and binding at a high affinity site in the hERG channel pore.


*Keywords:* Antipsychotic; Arrhythmia; Thioridazine; hERG; QT interval; QT prolongation; Rapid delayed rectifier; *Torsade de pointes*

Diverse non-cardiac drugs are associated with a risk of the potentially fatal arrhythmia *Torsade de pointes* (TdP) and with prolongation of the QT interval on the electrocardiogram, e.g. [1,2]. Most QT interval prolonging drugs can inhibit $K^+$ channels mediating cardiac '$I_{Kr}$', which influences ventricular action potential repolarization [1,2]. *hERG* (*human ether-à-go-go-related gene*) encodes the pore-forming subunit of $I_{Kr}$ channels [1,3]. A variety of structure–function data (reviewed in [3]) provide evidence that the hERG channel possesses a larger pore-cavity than other Kv channels, and that aromatic amino acids present in the inner (S6) helices of hERG, but absent from Kv channels, form key components of a high-affinity drug binding site. These features appear to confer upon the hERG channel a unique susceptibility to pharmacological blockade [3].

Some antipsychotic drugs have been linked with QT interval prolongation and with a risk of pro-arrhythmia and sudden death, either in normal use or in overdose (e.g. [4–9]). The first such drug was the phenothiazine derivative thioridazine [9]. Subsequently other antipsychotic drugs have been linked to QT interval prolongation and pro-arrhythmia [4,7]. However, the link for thioridazine is particularly strong. In 1991, a Finnish study reported phenothiazines and especially thioridazine to be strongly

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doi:10.1016/j.bbrc.2006.10.039

represented in cases of psychotropic-linked sudden deaths [10]. In 2000, a study of QT prolongation with psychotropic drug use by Reilly and colleagues showed a robust association between thioridazine use and QT interval prolongation [4]. A subsequent case-control study of sudden unexplained deaths in patients receiving antipsychotic treatment demonstrated a significant association with thioridazine use [5]. In 2000 the FDA imposed labelling changes on Mellaril (thioridazine HCl) [11].

In common with other QT interval prolonging medications, thioridazine has been found to inhibit cardiac $I_{Kr}$ [12] and studies of hERG $K^+$ channels expressed in mammalian cell lines have reported inhibition of hERG current ($I_{hERG}$) with half-maximal inhibitory concentration ($IC_{50}$) values of between ~0.1 and 1 μM [13–19]. Surprisingly, however, no study has yet reported the mechanism of $I_{hERG}$ block by thioridazine. Accordingly, we set out to establish: (i) the effects of thioridazine on voltage- and time-dependent properties of $I_{hERG}$, (ii) the effects of command voltage waveform on level of observed $I_{hERG}$ inhibition, and (iii) the role in thioridazine's action of aromatic amino-acid residues in the inner (S6) helix that are considered to be key determinants of drug binding to the hERG channel [3,20].

## Methods

*hERG expressing cell lines*. Experiments were performed on human embryonic kidney (HEK-293) cells stably expressing wild-type (WT) hERG (provided by Professor Craig January [21]) or hERG mutants F656A and Y652A [22]. Maintenance of cell lines has been described previously [22]. Cells were incubated at 37 °C for a minimum of 2 days before use.

*Electrophysiological recordings*. Glass coverslips onto which cells had been plated were placed in a bath mounted on an inverted microscope and the cells were superfused with Normal Tyrode's solution containing (in mM): 140 NaCl, 4 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 5 Hepes, (pH 7.45 with NaOH). Heat-polished patch-pipettes had final resistances of 2.5–3.5 MΩ. For measuring hERG current ($I_{hERG}$), the pipette solution contained (in mM): 130 KCl, 1 $MgCl_2$, 5 EGTA, 5 MgATP, and 10 Hepes (pH 7.2 with KOH). Owing to low expression of the F656A mutant [20,22], experiments on the F656A-expressing cells and their corresponding WT controls (Fig. 4) were performed with 94 mM $[K^+]_{ex}$ and inward tails were measured at −120 mV [23].

Whole-cell patch-clamp recordings were made using an Axopatch-1D amplifier controlled by Clampex-8 (Axon). Between 80 and 90% of the electrode series resistance was compensated. Data were recorded on a computer *via* a Digidata 1200B. Data were digitized at 2–25 kHz with an appropriate bandwidth of 2–10 kHz set on the amplifier.

*Thioridazine*. Thioridazine (Sigma, UK) was dissolved in ethanol and stored at −20 °C. Stock was serially diluted in Tyrode's to a final vehicle concentration of 0.01%. During experiments, cell-superfusate was exchanged using a home-built solution application device capable of changing the solution bathing a cell in <1 s.

*Data presentation*. Data are presented as means ± standard error of the mean (SEM) or with 95% confidence intervals (CI). Graphical fits to the data were made using previously described, standard equations [22]. Statistical comparisons were made using a two-tailed Student's *t* test (paired or un-paired) or one-/two-way analysis of variance (ANOVA) with a Bonferroni post-test. *P* values of less than 0.05 were taken as statistically significant. NS = no significant difference.

## Results

### *Concentration-dependent inhibition of $I_{hERG}$ by thioridazine*

From a holding potential of −80 mV, $I_{hERG}$ was activated by repeated application of the protocol shown in inset of Fig. 1Ai (labelled "Std +20 mV" and used in previous studies, e.g. [22–24]). Fig. 1Ai shows the development of $I_{hERG}$ block during 9 min exposure to 100 nM thioridazine (THIO). Inhibition of $I_{hERG}$ reached steady state following ~8 min exposure to THIO and was poorly reversible on drug washout (not shown). Fractional block of $I_{hERG}$ tails by different THIO concentrations was quantified and a Hill plot (Fig. 1Aii) yielded an $IC_{50}$ of 80.3 nM (95% CI 54.1–119.3 nM) and a Hill coefficient ($n_H$) of 0.8 ± 0.1. Unless stated otherwise in the text, all other experiments used a concentration of 100 nM THIO (close to the $IC_{50}$).

Since it has been suggested that inhibition of $I_{hERG}$ by some drugs may vary with the stimulus protocol used [14,25], we compared the effect of 100 nM THIO on $I_{hERG}$ elicited by a range of voltage waveforms, including two-step and step-ramp protocols [14] and ventricular and Purkinje fibre cardiac action potentials (VAP and PAP, from guinea-pig and dog, respectively). Fig. 1Bi and Bii show $I_{hERG}$ records in the absence and presence of THIO elicited by step-ramp and PAP waveforms. Fig. 1C shows the mean level of $I_{hERG}$ inhibition produced by THIO by these and other stimulus protocols (for further details of the protocols, see legend of Fig. 1C). ANOVA showed no significant difference in $I_{hERG}$ block level between different stimulus protocols.

### *Thioridazine and voltage-dependent activation and inactivation of $I_{hERG}$*

The voltage dependence of $I_{hERG}$ blockade was determined using the protocol shown in the inset of Fig. 2A. Fig. 2A contains representative current traces in control (Fig. 2Ai) and in the presence of 100 nM THIO (Fig. 2Aii). At −40 mV THIO had less of an effect than at more positive potentials. Voltage-dependent activation of $I_{hERG}$ was assessed by measuring $I_{hERG}$ tail amplitude following the different voltage commands and standard Boltzmann fitting of the normalized $I_{hERG}$-tail–voltage relations (Fig. 2B). The half-maximal activation voltage ($V_{1/2}$) was −28.1 ± 0.6 mV in control and −30.3 ± 0.7 mV in THIO ($P < 0.05$ vs. control); corresponding slope factor ($k$) values were 6.0 ± 0.5 mV and 5.8 ± 0.7, respectively (NS). Thus, THIO produced only a small, though statistically significant, negative shift in the voltage dependence of $I_{hERG}$ activation. Fig. 2C shows a plot of mean fractional block of $I_{hERG}$ tails against test potential ($n = 4$). Fractional block of $I_{hERG}$ at −40 mV was significantly different from those following commands to −30 mV and more positive voltages; there was no significant difference over the range −30 to +60 mV. Thus,

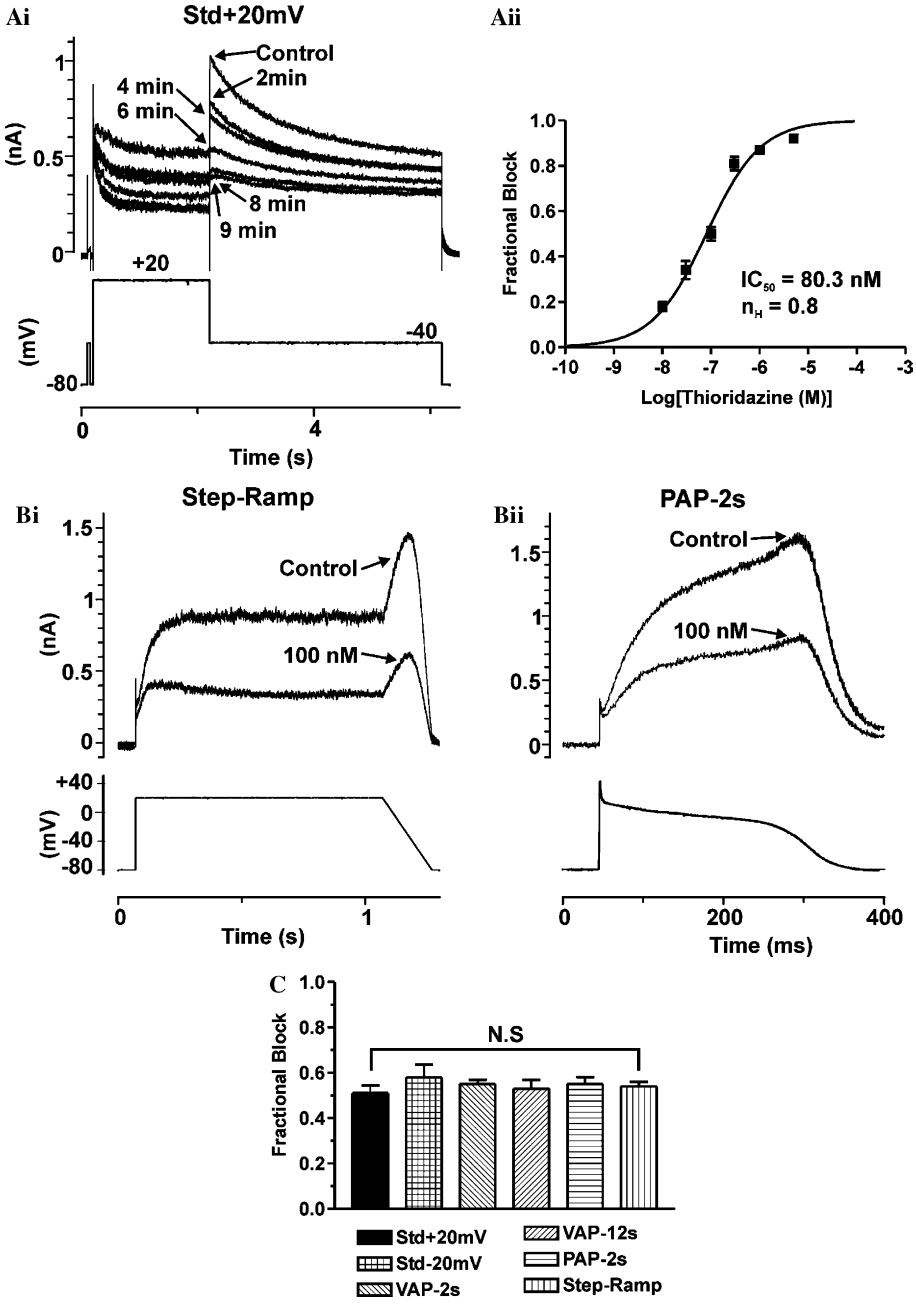


Fig. 1. Concentration dependence of $I_{hERG}$ inhibition. (Ai) $I_{hERG}$ traces (upper) recorded in response to the voltage protocol shown (lower, Start-to-Start interval = 12 s). Control current and stable current in the presence of 100 nM THIO following 9 min exposure to drug. (Aii) Mean fractional block data for THIO (±SEM) for experiments similar to that in (Ai) following 8 min exposure to drug ($n = 5$–12 cells at each of six concentrations). (B) Typical $I_{hERG}$ traces recorded in responses to either a step-ramp or Purkinje AP waveform in control and following 8 min exposure to 100 nM THIO. (C) Mean fractional block (±SEM, $n = 6$–7 cells for each protocol) of $I_{hERG}$ following 8 min exposure to 100 nM THIO. $I_{hERG}$ was evoked by: a standard two-step protocol to +20 or −20 mV (Std + 20 mV, Std − 20 mV); a ventricular or Purkinje AP applied at either 2 or 12 s intervals (VAP-2s, PAP-2s, and VAP-12s); or by a step-ramp waveform (S-to-S interval = 10 s).

$I_{hERG}$ blockade by THIO was only weakly dependent on voltage.

The effect of THIO on the voltage dependence of inactivation of $I_{hERG}$ was assessed using the three-step protocol shown in the inset of Fig. 2D [23,24]. Mean data from seven cells are plotted in Fig. 2D and were fitted with a modified Boltzmann equation [22], yielding inactivation $V_{1/2}$ values of $-62.6 \pm 0.7$ mV in control and $-64.1 \pm 1.0$ mV in THIO (NS). Corresponding $k$ values were $-19.2 \pm 0.7$ in control and $-20.7 \pm 0.9$ in THIO (NS). The time-course of development of inactivation was also assessed, using the protocol shown in the inset of Fig. 2E. Mono-exponential curve-fitting of the inactivation time-course gave time-constant values (plotted against voltage in Fig. 2E; $n = 10$). At all test potentials, the time constants of inactivation were smaller in the presence than in the absence of THIO.

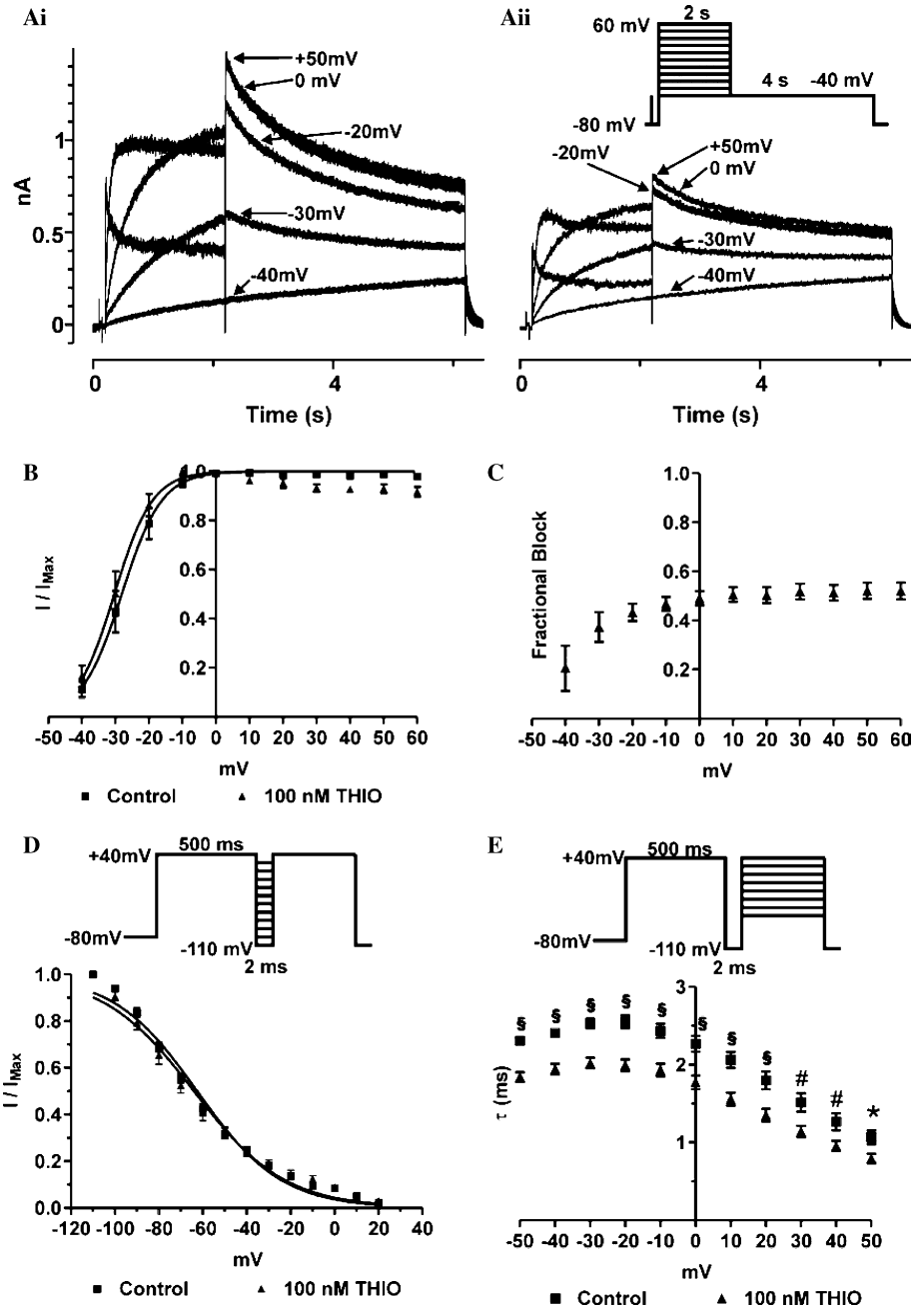


Fig. 2. Voltage dependence of $I_{hERG}$ inhibition. (A) Typical $I_{hERG}$ traces (upper) recorded in absence (Ai) and presence (Aii) of 100 nM THIO. Membrane potential was held at −80 mV and stepped to range of potentials (−40 to +60 mV for 2 s) prior to recording tails at −40 mV (4 s). Some current traces are omitted to aid clarity of display. (B) Means (±SEM) normalized tail current amplitude plotted as a function of test potential. Data were fitted with a Boltzmann equation and $V_{1/2}$ and $k$ values are given in Results. (C) Mean (±SEM, $n = 4$) tail current block plotted against step potential. (D) Voltage dependence of $I_{hERG}$ inactivation (protocol shown in inset). The mean ($n = 7$) normalized data ($I/I_{max}$) were plotted against the test potential and fitted with a modified Boltzmann function; $V_{1/2}$ and $k$ values are given in Results. (E) Voltage dependence of inactivation time-course (protocol shown in inset). Mean (±SEM) time-constant values yielded from mono-exponential fits to rapidly inactivating $I_{hERG}$ on depolarization from −110 mV were plotted against voltage ($n = 10$). Data were compared using a two-way ANOVA with Bonferroni post-test. *, #, and $ denote $P < 0.05$, 0.01, and 0.001.

*Time-course of $I_{hERG}$ blockade by thioridazine*

This was investigated using a sustained (10 s) depolarizing voltage command from −80 to 0 mV. Fig. 3Ai shows a current trace in control solution and the first current trace recorded following equilibration (>8 min) in 100 nM THIO in the absence of pulsing. Similar data are shown in Fig. 3Aii for 1 μM THIO. Fractional block was calculated at 100 ms intervals and is shown plotted throughout the 10 s voltage command in Fig. 3Bi and on an expanded time-base for the first 2 s in Fig. 3Bii. Block of $I_{hERG}$ developed progressively over the first ~500 ms for both concentrations tested. Mono-exponential curve-fitting to the data gave rate constants ($K$) of 6.5 ± 1.8 and 10.3 ± 1.3 $s^{-1}$ for

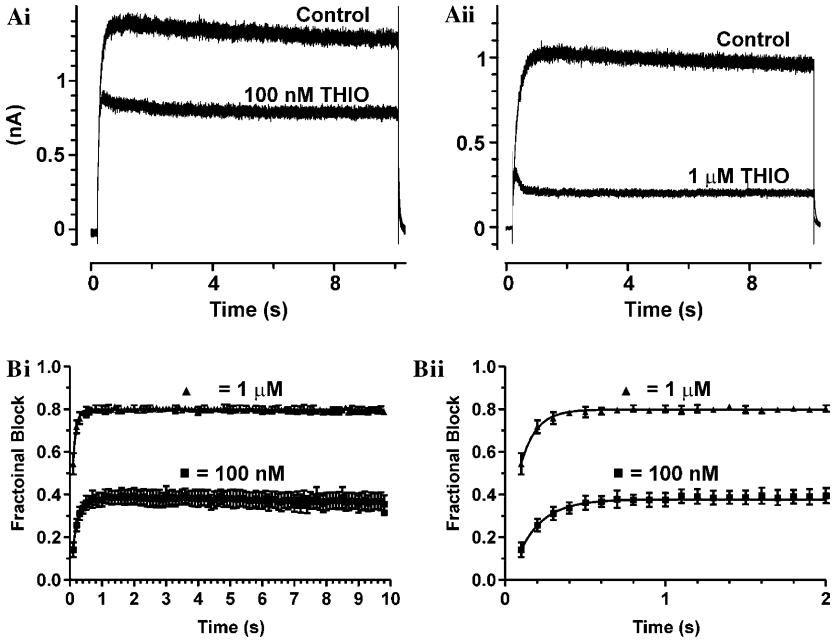


Fig. 3. Time dependence of $I_{hERG}$ inhibition. (Ai,Aii) Typical $I_{hERG}$ traces recorded during a 10 s depolarization from −80 to 0 mV. Control current and the first record following equilibration in either 100 nM or 1 μM THIO ((Ai) and (Aii), respectively). (Bi) Mean fractional block data (±SEM) for the full 10 s voltage step calculated at 100 ms intervals. $I_{hERG}$ inhibition data during a sustained depolarization were fitted with a mono-exponential function. From this, rate constant ($K$) and time-constant ($\tau = 1/K$) values (see Results) were calculated. For 100 nM THIO, $n = 7$ for 1 μM THIO, $n = 5$. (Bii) Mean fractional block data over the first 2 s of depolarization.

100 nM ($n = 7$) and 1 μM ($n = 5$), respectively (equivalent to time-constant ($\tau$) values of 154 and 97 ms) Thus, at both THIO concentrations studied, $I_{hERG}$ blockade developed relatively rapidly on membrane depolarization.

### *Molecular determinants of thioridazine block of $I_{hERG}$*

Mutation of two aromatic amino-acid residues (Y652 and F656) on the S6 helices of the hERG channel has been shown to attenuate dramatically $I_{hERG}$ block by a number of drugs, shifting the $IC_{50}$ by over 100-fold (e.g. [20]). The role of these two amino-acid residues in THIO block of $I_{hERG}$ was investigated using the alanine-mutant hERG channels Y652A and F656A and protocols identical to those in other recent studies of hERG-blocking drugs from this laboratory [22,23]. Fig. 4Ai and Aii show representative currents recorded from cells expressing wild-type hERG and Y652A-hERG, respectively. A THIO concentration producing a profound level of inhibition of WT-hERG was employed (1μM: >10-fold the $IC_{50}$ value in Fig. 1). $I_{hERG}$ tails on repolarization to −40 mV from +20 mV were measured (protocol shown in inset). Fig. 4Ai shows that 1 μM THIO almost completely abolished WT-$I_{hERG}$ current, whilst the Y652A mutation partially attenuated blockade (Fig. 4Aii). The effect of the F656A mutation was examined using the protocol shown in the inset of Fig. 4Bi (see also [22]). Whilst THIO produced a substantial inhibition of WT-hERG (Fig. 4Bi), it produced virtually no inhibition of the F656A-hERG mutant (Fig. 4Bii). Mean data for Y652A-hERG and F656A-hERG are shown in Fig. 4C (five cells each group). Both mutations produced a significant decrease in observed level of $I_{hERG}$ blockade by THIO compared to their respective WT controls, with the effect of the F656A mutation being particularly prominent.

## Discussion

### *The mechanism of $I_{hERG}$ block by thioridazine*

Despite the long-standing association between thioridazine and QT interval prolongation and pro-arrhythmia [4,8,9], until now there has been little detailed information available regarding its hERG-blocking action in comparison to other antipsychotic agents such as chlorpromazine [26,27], mesoridazine [28] or ziprasidone [29]. One previous study that has provided limited data on the blocking action suggested $I_{hERG}$ blockade by THIO to be voltage-dependent [16]. Under our conditions $I_{hERG}$ block by THIO was only weakly voltage-dependent and we saw no significant alteration by THIO of the voltage dependence of $I_{hERG}$ inactivation (Fig. 2D). Although the time-course of inactivation appeared to be accelerated (Fig. 2E), this result could also be explained by rapidly developing open-channel block as the drug bound on depolarization during this protocol. The rapid time dependence of onset of blockade during a sustained membrane depolarization (Fig. 3) contrasts with the time dependence of $I_{hERG}$ block by high-affinity methanesulphonanilides, which typically shows little block initially, followed by a progressive development of block with time (e.g. [30,31]). The comparatively rapid association between THIO and the hERG channel on

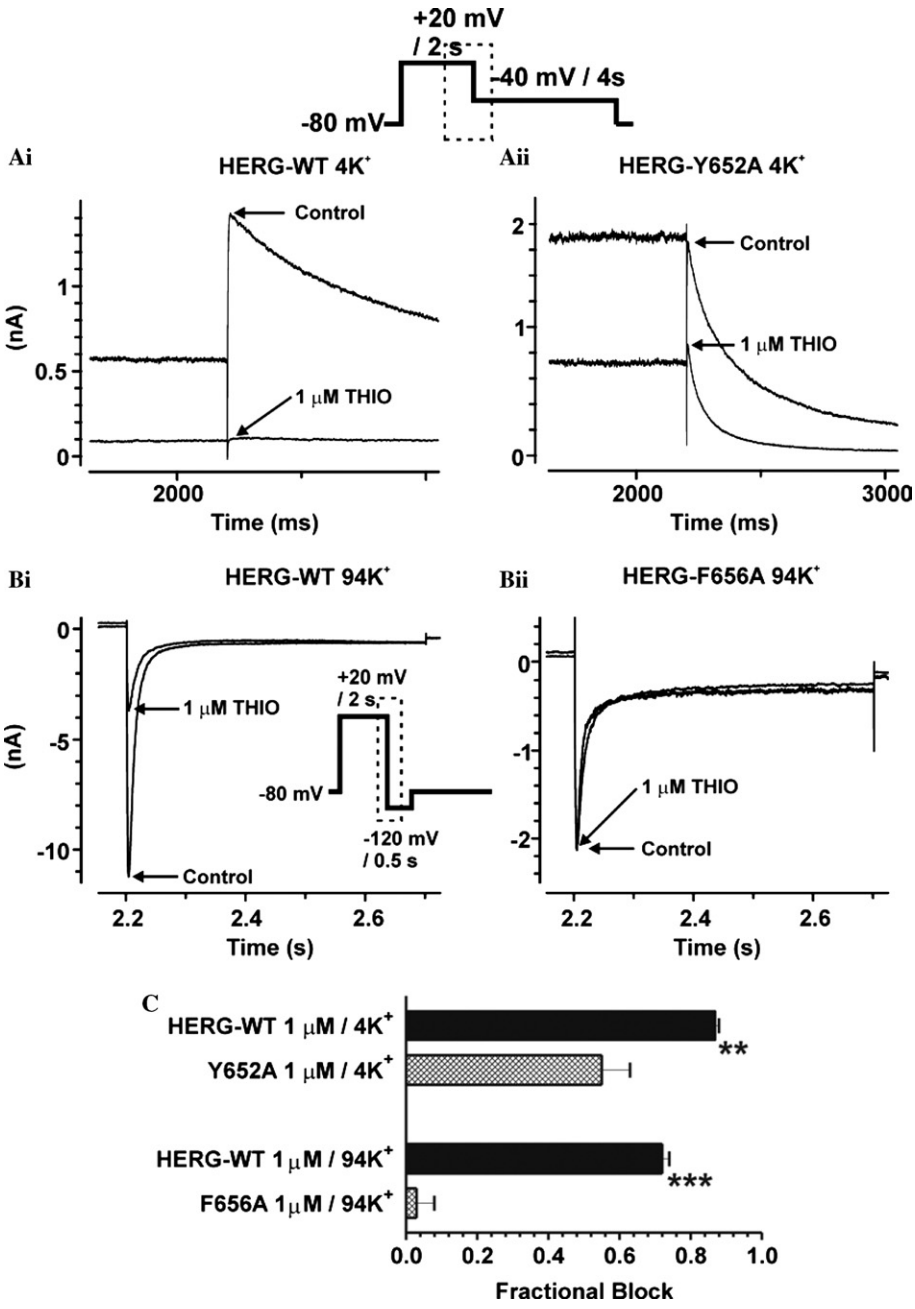


Fig. 4. Effect of S6 aromatic amino-acid mutations. (A) Representative WT-hERG (Ai) and Y652A-hERG (Aii) currents in the absence and presence of 1 μM THIO, elicited by the voltage protocol shown in inset (S-to-S interval 12 s). (B) Representative WT-hERG (Bi) and F656A-hERG (Bii) tail currents in the absence and presence of 1 μM THIO, elicited by the voltage protocol (S-to-S interval 12 s). (C) Mean fractional block produced by THIO for WT and mutant hERG ($n = 5$ cells for each group). Comparisons made using a $t$-test. ** and *** denote $P < 0.01$ and $P < 0.0001$, respectively.

depolarization also contrasts with that reported with sertindole [32], but is similar to that reported for other phenothiazines including haloperidol [33], mesoridazine [28], and chlorpromazine [26]. Significantly, whilst it was possible to exchange the extracellular superfusate rapidly in our experiments, $I_{hERG}$ block accumulated more slowly, reaching steady state at ~8 min. This suggests: (i) slow membrane permeation; (ii) that THIO gained access to its binding site on the hERG channel from the cell interior on channel gating.

To our best knowledge, no information has been published previously on molecular determinants of $I_{hERG}$ inhibition by thioridazine or other phenothiazine antipsychotic drugs. Our findings indicate that THIO binding occurs within the hERG channel vestibule, at a site incorporating two amino acids demonstrated to be important in high-affinity block for a number of compounds (for reviews, see [3,34]). It is notable that the almost complete abolition of THIO blockade of $I_{hERG}$ by the F656A mutation is shared with a phenothiazine *antihistamine* agent clemastine [23].

Considering the effects of the Y652A and F656A mutations alongside the characteristics of WT $I_{hERG}$ blockade by THIO, it is reasonable to propose that THIO blockade of $I_{hERG}$ involves: (i) drug passage from the exterior to the interior of the cell, (ii) drug entry into the channel vestibule on channel gating, and (iii) binding at a site to which the aromatic amino-acid residues Y652 and F656 contribute, and of which F656 is an obligatory component.

### *Comparison of $I_{hERG}$-blocking potency of THIO with previous studies*

THIO is a drug for which the published hERG $IC_{50}$ values appear to vary greatly: from 33 to 1070 nM [12–16,18,19]. One possible cause of such variability might be a strong protocol dependence of block. However, we observed little variation in the effect of THIO using widely differing protocols (Fig. 1B and C). This may be explained by the rapid time dependence and relatively weak voltage dependence of $I_{hERG}$ inhibition by this drug. Therefore, wide variations between studies in observed $IC_{50}$ for $I_{hERG}$ inhibition by THIO are difficult to explain purely on the basis of different protocols used. Other variables that might influence observed blocking potency include: choice of experimental temperature [14]; use of different expression systems; and high $\log P$ (5.3–5.19 [35]), which could lead to variable drug–plastic binding between laboratories.

### *Significance of potency of blockade by THIO*

In considering the relationship between $I_{hERG}$ blockade, QT prolongation, and TdP arrhythmia Redfern and colleagues have provisionally recommended a 30-fold safety margin between maximal effective plasma concentration and observed hERG $IC_{50}$ [36]. The therapeutic free plasma concentration of THIO is 20.6 nM (assuming 99% plasma protein binding) [37], which with our observed hERG $IC_{50}$ would yield a safety margin of ~3.9. This would place THIO at the lower end of the 0.03 to 35-fold range quoted by Redfern et al. for drugs associated with a measurable incidence of TdP in humans and is consistent with the known risk of arrhythmia with this drug. That said, were the value of ~1.25 μM for guinea-pig $I_{Kr}$ inhibition reported by Drolet et al. [12] to be used instead, this would yield a safety margin for THIO of ~61, well outside the range expected to be associated with TdP arrhythmia. This disparity highlights a continuing need to improve understanding of which cellular assays are likely to be most predictive of QT prolongation/TdP liability in humans.

## Acknowledgments

The authors thank Mrs. Lesley Arberry for technical assistance and also acknowledge financial support from Pfizer and the British Heart Foundation.

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