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Mechanism of hERG K^+ channel blockade by the fluoroquinolone antibiotic moxifloxacin

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1 The fluoroquinolone antibiotic moxifloxacin has been associated with the acquired long QT syndrome and is used as a positive control in the evaluation of the QT-interval prolonging potential of new drugs. In common with other QT-prolonging agents, moxifloxacin is known to inhibit the hERG potassium K^+ channel, but at present there is little mechanistic information available on this action. This study was conducted in order to characterise the inhibition of hERG current (I_{hERG}) by moxifloxacin, and to determine the role in drug binding of the S6 aromatic amino-acid residues Tyr652 and Phe656.

2 hERG currents were studied using whole-cell patch clamp (at room temperature and at 35–37°C) in an HEK293 cell line stably expressing hERG channels.

3 Moxifloxacin reversibly inhibited currents in a dose-dependent manner. We investigated the effects of different voltage commands to elicit hERG currents on moxifloxacin potency. Using a 'step-ramp' protocol, the IC_{50} was 65 μM at room temperature and 29 μM at 35°C. When a ventricular action potential waveform was used to elicit currents, the IC_{50} was 114 μM.

4 Block of hERG by moxifloxacin was found to be voltage-dependent, occurred rapidly and was independent of stimulation frequency.

5 Mutagenesis of the S6 helix residue Phe656 to Ala failed to eliminate or reduce the moxifloxacin-mediated block whereas mutation of Tyr652 to Ala reduced moxifloxacin block by ~66%.

6 Our data demonstrate that moxifloxacin blocks the hERG channel with a preference for the activated channel state. The Tyr652 but not Phe656 S6 residue is involved in moxifloxacin block of hERG, concordant with an interaction in the channel inner cavity.
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Abbreviations: ECS, extracellular solution; hERG, human *ether-a-go-go* related gene

Introduction

Therapeutic doses (400 mg daily) of the fluoroquinolone broad spectrum antibiotic, moxifloxacin (Avelox®), have been associated with modest changes (~6 ms) in the QT interval and, rarely, with the ventricular arrhythmia Torsades de Pointes (Shah, 2005). Other members of the fluoroquinolone family, such as levofloxacin and grepafloxacin, have been associated with cases of Torsades de Pointes (Frothingham, 2001; Iannini *et al*., 2001; Iannini, 2002; Owens Jr & Ambrose, 2002). In particular, grepafloxacin has been withdrawn from the market due to its higher incidences of this type of arrhythmia. Delays in cardiac repolarisation and prolongation of the QT interval induced by a wide variety of compounds has meant that during the drug development process all new drugs are assessed for their propensity to prolong QT as part of the routine screening cascade employed in the pharmaceutical industry (de Ponti *et al*., 2002; Redfern *et al*., 2003; Finlayson *et al*., 2004; Recanatini *et al*., 2005). Due to its recognised QT-prolonging effect, moxifloxacin is recommended by regulatory authorities as a non-cardiac positive control drug in clinical studies evaluating the QT-prolonging potential of new drugs (Barriere *et al*., 2004; Morganroth *et al*., 2004; Serra *et al*., 2005). A recent publication illustrates that there is good concordance between pre-clinical models such as electrocardiograms in conscious dogs and clinical data for moxifloxacin (Chen *et al*., 2005).

It is well established that the majority of cases of drug-induced QT prolongation are due to blockade of the rapidly inactivating delayed rectifier potassium current, I_{Kr}, one of the principal repolarising currents activated during the plateau phase of an action potential (Sanguinetti & Jurkiewicz, 1990; Sanguinetti *et al*., 1995). The human cloned counterpart of I_{Kr} is known as the hERG channel (human *ether-a-go-go*-related gene) and is the target of the class III antiarrhythmic drugs such as dofetilide (Snyders & Chaudhary, 1996; Trudeau *et al*., 2005). Similar to other QT-prolonging agents, moxifloxacin is known to inhibit the hERG K^+ channel (Bischoff *et al*., 2000;

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Lacroix *et al*., 2003; Chen *et al*., 2005), but to-date no mechanistic information is available on this effect. Therefore, the aim of this study was to characterise the mechanism of hERG channel block by moxifloxacin and to determine the role in drug binding of the S6 aromatic amino-acid residues Tyr652 and Phe656, which are proposed to be key determinants of block by a range of compounds (Mitcheson *et al*., 2000; Sanguinetti & Mitcheson, 2005).

Methods

Cell culture

HEK293 cells stably expressing the HERG potassium channel were obtained from Dr C.T. January (University of Wisconsin, U.S.A.) and the methodology for construction of this stable line has been described previously (Zhou *et al*., 1998). Construction of mutant hERG-expressing stable cell lines were as previously described (Milnes *et al*., 2003). Mutated hERG sequences were subcloned into a vector based on pGW1H (British Biotechnologies, Oxford, U.K.). Wild-type (WT) cells were maintained in MEM supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 × nonessential amino acids (in mg l^{-1}: 8.9 L-alanine, 13.2 L-asparagine, 13.3 L-aspartic acid, 14.7 L-glutamic acid, 7.5 glycine, 11.5 L-proline, 10.5 L-serine) and 400 μg ml^{-1} G418 and kept at 37°C in a humidified atmosphere of 5% CO_2. Mutant hERG cell lines were maintained using the same media except that G418 was replaced with 100 μg ml^{-1} hygromycin. For patch clamp experiments, cells were plated onto 3-mm glass coverslips placed in Petri dishes and used within 72 h of plating.

Electrophysiology

Cells were continuously superfused by gravity feed with extracellular solution (ECS) containing (in mM): 130 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 5 HEPES (pH 7.4 with NaOH). Whole-cell currents were recorded with a MultiClamp 700 patch clamp amplifier (Axon Instruments Molecular Devices Corporation, Foster City, CA, U.S.A.), filtered at 1 kHz, digitised at 5 kHz (or 50 kHz for inactivation protocols) and data acquired using a Digidata 1322A and the pClamp9.2 suite of software (Axon Instruments Molecular Devices Corporation). Patch pipettes had resistances of 1.5–4 MΩ when filled with intracellular solution containing (in mM): 130 KCl, 5 MgATP, 1 $MgCl_2$, 10 HEPES, 5 EGTA (pH 7.2 with KOH). A liquid junction potential of 4 mV was calculated (JPCalc; Barry, 1994), but since this value was small, no corrections to the data were made. Series resistance compensation was routinely applied to at least 75% and tips of pipettes were coated in a Parafilm and mineral oil emulsion to reduce stray capacitance. Experiments were conducted at room temperature (22°C) unless otherwise stated. For experiments at elevated temperatures, an automatic temperature controller (TC-344B, Warner Instruments Corporation) was used to maintain temperature at 35–37°C or at 41–43°C. Once the whole-cell mode had been achieved, a step-ramp protocol (see Figure 1a) was always applied to ensure a stable baseline. Unless otherwise stated, all protocols were applied repeatedly until steady state had been achieved. Since the magnitude of rundown of hERG currents was minimal (<0.75%/min at room temperature), rundown of currents was not corrected for.

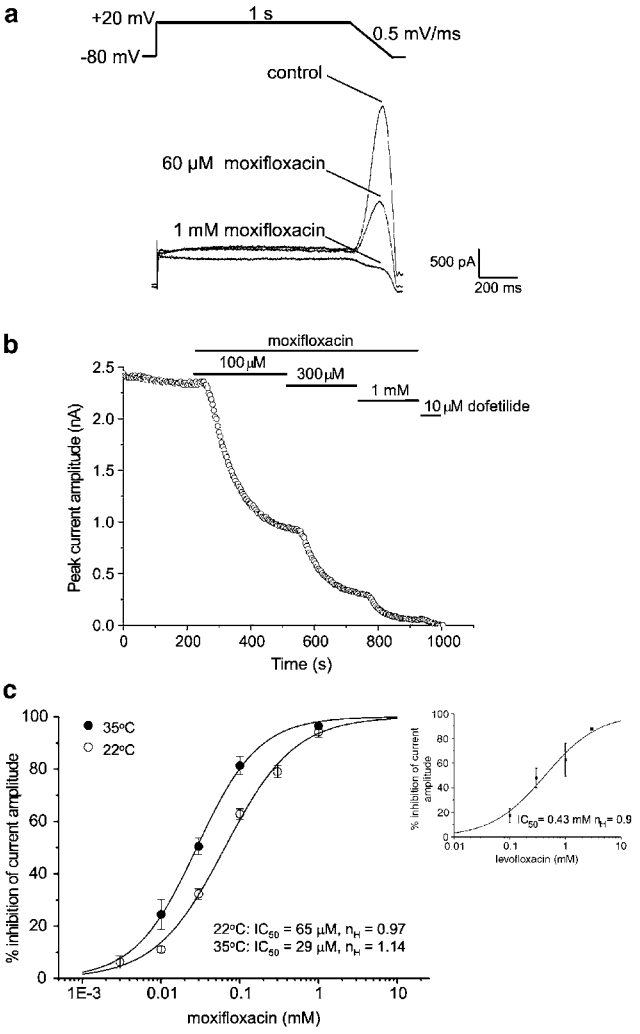

Figure 1 Moxifloxacin inhibition of hERG currents is weakly temperature-sensitive. (a) 'Step-ramp' voltage command used to evoke hERG currents. Cells were voltage-clamped at −80 mV and depolarised to +20 mV for 1 s followed by a repolarising ramp back to −80 mV at a rate of 0.5 mV ms^{-1}. The command was applied every 4 s. Peak hERG current amplitude, and the effects of 60 μM and 1 mM moxifloxacin thereon, was measured during the repolarising phase as indicated in the example traces shown. (b) This graph illustrates the effects of cumulative concentrations of moxifloxacin (0.1, 0.3 and 1 mM) on peak hERG amplitude with time. Dofetilide (10 μM) was added at the end of the experiment as a positive control. (c) A moxifloxacin concentration–response curve was generated for experiments conducted at 22°C and at 35°C. Data are shown fitted to a Hill equation of the form $y = [(A_1 - A_2)/(1 + (x/C)n_H)] + A_2$ where A_1 and A_2 are 0 and 100% inhibition, respectively, C is the IC_{50} concentration and n_H is the Hill slope. The IC_{50} values yielded under these two experimental conditions were statistically different from one another ($P < 0.001$). The inset shows the concentration–response curve at 22°C for levofloxacin generated using the step-ramp voltage protocol. Data are from six cells and a Hill equation was fitted as for moxifloxacin.

Experiments using hERG Y652A or F656A stable lines were conducted at 35–37°C. Due to the low expression of hERG F656A, experiments were performed in elevated (94 mM) external potassium and the voltage command to evoke hERG

currents consisted of a 2 s step to +20 mV from a holding potential of −80 mV, followed by a step to −120 mV to observe inward tail currents (Milnes *et al*., 2003; Ridley *et al*., 2004a).

Data are presented as mean ± s.e.m., where *n* indicates the number of cells recorded from. Current traces were analysed using pClamp9 Clampfit and further analysed with Excel 2000 (Microsoft) and Origin v.6.0 (Microcal) software. Data were analysed for statistical significance using either Student's *t*-test or one way repeated measures ANOVA tests with post-Bonferroni or Dunnett's test as appropriate (* indicates $P \leqslant 0.05$, ** indicates $P \leqslant 0.01$ and *** indicates $P \leqslant 0.001$).

Drugs and reagents

Moxifloxacin and levofloxacin were easily dissolved in ECS up to a concentration of 10 mM. Stock concentrations were diluted down in ECS where necessary to the final working concentrations. Dofetilide was made up as a 10 mM stock in 100% DMSO and diluted down to a final working concentration of 10 μM. Solutions never contained more than 0.1% DMSO and this was found in control experiments to have no effects on hERG currents. Both dofetilide and moxifloxacin were either made up fresh each experimental day or small aliquots were stored at −80°C and defrosted only once. Tissue culture reagents were obtained from Invitrogen (Paisley, U.K.) or Sigma (Gillingham, Dorset, U.K.).

Results

Concentration–response relationship for moxifloxacin and levofloxacin inhibition of hERG channels

We first defined the concentration-dependence with which moxifloxacin blocks the hERG channel. We employed a 'step-ramp' protocol to evoke hERG currents where peak hERG current was measured during the repolarising ramp phase of the voltage protocol, as illustrated in Figure 1a. After entering the whole-cell configuration cells were superfused with ECS for at least 5 min to achieve a stable baseline. The effects of moxifloxacin were rapid and block could be reversed by washing the cells with ECS – currents recovered to 81.3 ± 2.1% ($n = 3$) of control amplitude after wash-out of 1 mM moxifloxacin with ECS. To establish a dose–response relationship, increasing concentrations of moxifloxacin were cumulatively bath-applied until steady-state effect was achieved and a supra-maximal concentration of dofetilide (10 μM) was applied at the end of each experiment as a positive control. For each concentration of moxifloxacin (applied to 3–8 cells), steady-state percentage inhibition was evaluated when three consecutive sweeps were superimposed on one another. A representative example of the effects of cumulative application of 100, 300 and 1000 μM moxifloxacin is shown in Figure 1b. A dose–response curve was constructed and data fit to a Hill equation, yielding an IC_{50} of 65 μM and Hill slope, n_H, of 0.97 (Figure 1c). Moxifloxacin also blocked the current evoked during the depolarising step to a similar extent to the peak hERG current. A dose–response curve and fit to a Hill equation yielded an IC_{50} of 74 μM and n_H of 1.09, similar to those values obtained for the peak hERG current. A large concentration of dofetilide (10 μM) was added at the end of each experiment as a positive control. In order to determine whether or not the residual current remaining after pharmacological blockade of I_{hERG} by the supra-maximal concentration of dofetilide might influence the observed IC_{50}, we also analysed moxifloxacin inhibition of peak hERG currents by expressing the data as percentage inhibition of dofetilide-sensitive currents. This analysis yielded an IC_{50} of 62.0 ± 3.2 μM (and Hill slope, n_H, of 0.98), which was not significantly different to that obtained using % inhibition of total current for analysis (65.0 ± 4.2 μM; $P = 0.15$).

We then examined whether the potency of moxifloxacin was altered when recording at a near-physiological temperature of 35–37°C using the same voltage command as described above. We observed a moderate, but significant, shift in potency of moxifloxacin, with an IC_{50} of 29 μM (Figure 1c). It has recently been demonstrated by Guo *et al*. (2005) that raising the temperature to 42°C, a temperature observed in febrile states, led to an increase in the level of I_{hERG} block produced by the macrolide antibiotic, erythromycin. We conducted similar experiments but found no difference in moxifloxacin potency between 35 and 42°C (data not shown).

The potency of another fluoroquinolone drug, levofloxacin, was assessed to provide a comparator for moxifloxacin. Levofloxacin has been reported to cause QT prolongation and Torsades de Pointes (Frothingham, 2001; Iannini *et al*., 2001; Iannini, 2002; Owens Jr & Ambrose, 2002). The inhibitory effects of levofloxacin on peak hERG currents were measured using the step-ramp voltage protocol at room temperature. Under these conditions, levofloxacin inhibited hERG currents with an IC_{50} of 0.43 mM and a Hill slope, n_H, of 0.90. These data are shown as an inset in Figure 1c.

As there has been recent debate regarding the nature of the voltage protocol used to elicit hERG currents as part of new drug screening studies (Kirsch *et al*., 2004; Yao *et al*., 2005), we next employed different voltage commands to stimulate hERG channels and examined whether these resulted in any differences in potency. We used a 'step-step' protocol and a physiological ventricular action potential (VAP) waveform recorded from guinea-pig papillary muscle to elicit hERG currents. The effects of moxifloxacin on the peak tail currents were measured and dose–response relationships generated (Figure 2). We observed a significant reduction in the potency of moxifloxacin using the VAP protocol (IC_{50} 114 μM, n_H 0.94; $P < 0.001$) but not with the 'step-step' protocol (IC_{50} 61 μM, n_H 0.96; $P = 0.96$). The dose–response curves generated are compared in Figure 2c with that for the standard step-ramp protocol from Figure 1c. We also used the VAP voltage protocol to examine levofloxacin potency and found that similarly to moxifloxacin, the potency of levofloxacin was also reduced using this protocol (IC_{50} 1.45 mM, n_H 1.09; data not shown).

Voltage-dependence of moxifloxacin block

Some hERG channel-blocking compounds have been shown to exhibit a clear voltage-dependence in their action, illustrated by a shift in activation curves and consequent $V_{1/2(act)}$ values. However, it has been reported by some experimenters that a time-dependent hyperpolarising shift in channel activation may occur in the absence of drug (Ferreira *et al*., 2001; Milnes *et al*., 2003; Stanat *et al*., 2003). To establish whether this was an issue in our hands, we recorded hERG currents evoked

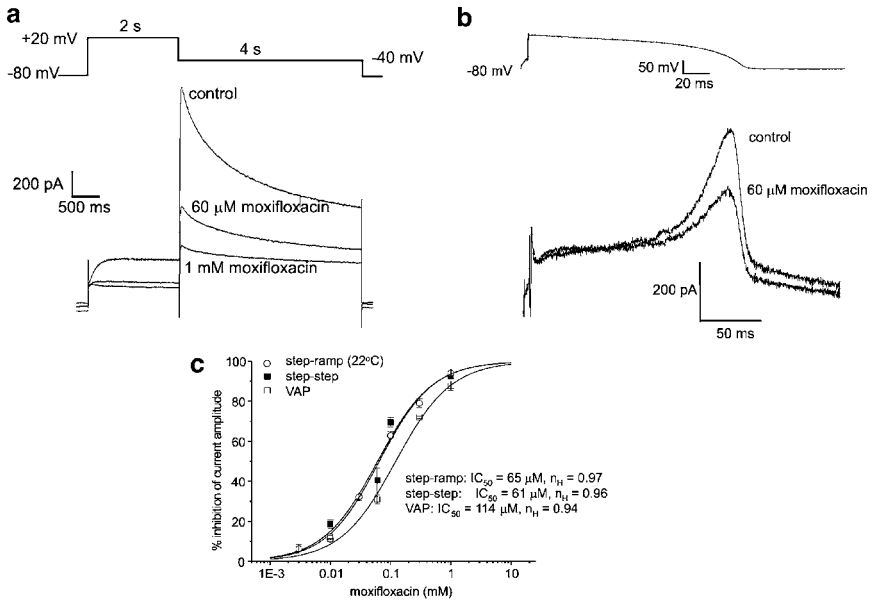

Figure 2 Potency of moxifloxacin is reduced by using a physiological type waveform. (a) This panel illustrates an example of currents evoked using the 'step-step' voltage protocol, as illustrated. Cells were depolarised to +20 mV for 2 s followed by a step to −40 mV for 4 s to elicit tail currents; this voltage command was applied every 12 s. The effect of moxifloxacin on peak tail current amplitude was measured and multiple concentrations were cumulatively applied to each cell. (b) A ventricular action potential (VAP) waveform was also used to evoke hERG currents. This protocol, illustrated, was applied every 2 s. Example current traces elicited using this waveform are shown, and the effect of moxifloxacin on peak repolarising currents was measured. (c) Dose–response curves using the 'step-step' and VAP protocols are shown with the 'step-ramp' dose–response (at 22°C) curve for comparison. As in Figure 1c, data were fitted to a Hill equation where % inhibition was constrained between 0 and 100%. Values for IC_{50}s and Hill slopes, n_H, are indicated.

using a voltage protocol, which consisted of steps from a holding potential of −80 mV to test potentials between −60 and +50 mV in 10 mV increments. For each cell, mean $V_{1/2(act)}$ was calculated at 2 min intervals over a 20-min period. A negligible change in the $V_{1/2(act)}$ was observed (1.6±1.3 mV, $n=9$) and $V_{1/2(act)}$ at $t=2$ min was not significantly different to that at $t=20$ min ($P=0.75$). The reasons for the time-dependent shift observed by some experimenters are not clear but did not appear to be an issue during the present study.

The voltage-dependence of moxifloxacin was then investigated. Both depolarising step current amplitudes and tail current amplitudes upon repolarisation to −40 mV were measured in the absence and presence of 60 μM moxifloxacin (employed as an approximate IC_{50} concentration). Representative examples of evoked current traces are shown in Figure 3a and mean current–voltage relationships for both depolarising step and tail currents from six cells were plotted in Figure 3b. A Boltzmann relation was fitted to the tail current data obtained in individual cells and $V_{1/2(act)}$ and slope values derived. The mean $V_{1/2(act)}$ was −14.1±2.2 mV under control conditions and in the presence of moxifloxacin was significantly shifted to −18.2±2.0 mV ($n=6$, $P=0.015$), while the slope values were not significantly different from one another 6.9±0.4 and 7.2±0.4 mV, respectively. Figure 3c shows the magnitude of tail current and depolarising step current inhibition by 60 μM moxifloxacin at different test potentials. At −20 mV, significant block of tail currents was observed (21.1±7.3%; $n=6$). The extent of block was significantly enhanced as the membrane became more depolarised and reached a plateau level at +10 mV (43.9±4.9%) suggesting that block is voltage-dependent.

Time-dependence of moxifloxacin inhibition of the hERG channel

To investigate the time-dependence of the actions of moxifloxacin, we activated channels for 5 s at 0 mV under control conditions and then repeated this after application of moxifloxacin (60 μM). Cells were voltage clamped at −80 mV during moxifloxacin application and the moxifloxacin trace shown is the first evoked hERG current after drug application (Figure 4a). The degree of block at various time points during the depolarising step was calculated and is shown in the graph in Figure 4a. Block developed rapidly within the first 1 s and the extent of block remained constant throughout the remaining 4 s. To investigate further the time-dependence of moxifloxacin block we used an 'envelope of tails' protocol where cells were depolarised to +20 mV in steps of incrementing duration resulting in increasing tail current amplitudes. Moxifloxacin (60 μM) was applied while the cells were voltage-clamped at −80 mV, similarly to above, and the protocol repeated. Figure 4b shows a typical example of evoked currents in the absence and presence of moxifloxacin when the cell was depolarised to +20 mV for time periods of 100–1400 ms. A plot of peak tail current amplitudes *versus* pulse duration for that cell is shown in Figure 4c, with corresponding single exponential fits. The rate of current activation was measured by fitting a single exponential function to the tail current amplitudes in each cell. Moxifloxacin had the effect of accelerating the mean time course of activation, as observed by a significant reduction in the time constant from 191.4±21.7 to 107.5±10.7 ms ($n=7$, $P=0.002$). The extent of block obtained for each pulse

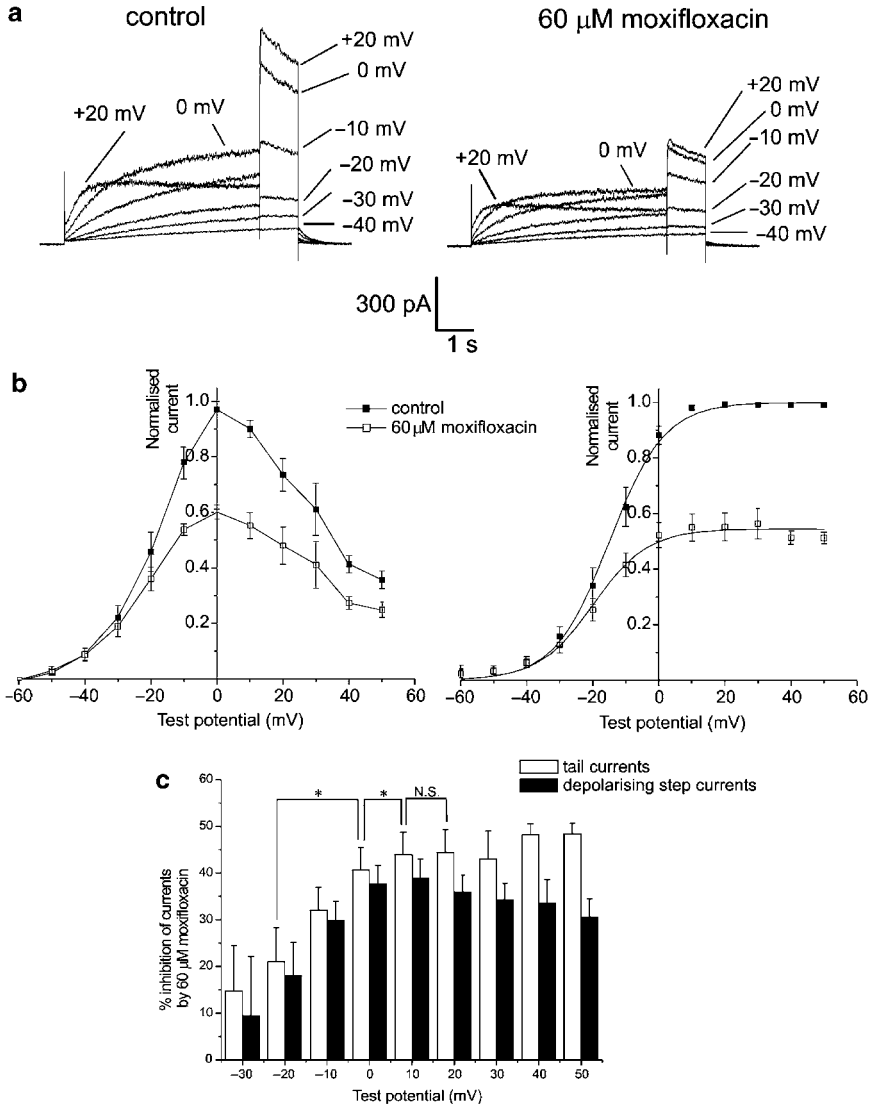

Figure 3 Voltage-dependence of moxifloxacin block. (a) Representative examples of current traces recorded in the absence (left-hand panel) and presence (right-hand panel) of 60 μM moxifloxacin. Currents were evoked by stepping from −80 mV to test potentials between −60 and +50 mV for 5 s followed by a repolarising step to −40 mV. For clarity, some of the voltage traces have been omitted in this figure. (b) This shows the mean current–voltage relationship for currents measured at the end of the depolarising step (left-hand panel) and for tail currents (right-hand panel) in control and in the presence of 60 μM moxifloxacin. Tail currents for each individual cell were normalised to the maximum obtained in the absence of drug, and fitted with a Boltzmann function of the form $y = [(A_1 - A_2)/(1 + e^{(x-V_{1/2})/k}] + A_2$ where A_1 and A_2 are 0 and 1 respectively, $V_{1/2}$ is the half-activation voltage and k is the slope factor. (c) Mean % inhibition of both tail currents (open bars) and depolarising step currents (solid bars) were calculated at each test potential and are summarised here.

duration was calculated and the mean data are shown in Figure 4d. In the presence of moxifloxacin, significant block was achieved even with a 40 ms depolarising step (26.8 ± 4.8%, $n = 5$, $P = 0.005$). Increasing the pulse duration further did not result in a statistically significant increase in the magnitude of block; however, there was a trend for block to increase with pulse duration and maximal block was achieved at 700 ms.

Effects of moxifloxacin on inactivated channels and state-dependence of moxifloxacin block

The kinetic actions of moxifloxacin demonstrated in the previous section are consistent with a degree of rapid open channel block and possibly an additional component of closed channel block. Therefore, to investigate further the state-dependence of moxifloxacin's action, additional voltage protocols were applied. We examined the voltage-dependence of hERG current inactivation in the absence and presence of moxifloxacin. Channels were inactivated by holding at +40 mV before being recovered from inactivation at test potentials between −140 and +40 mV (10 ms steps in 10 mV increments). Subsequent peak outward tail current amplitudes at +40 mV were then measured and plotted as a function of the preceding test potential to generate a steady-state inactivation curve, as shown in Figure 5A, in the absence and presence of 60 μM moxifloxacin. Moxifloxacin produced a small negative shift of the inactivation curve shifting $V_{1/2(\text{inact})}$ from −68.7 ± 1.7 to −75.5 ± 2.9 mV ($n = 7$, $P = 0.03$), but

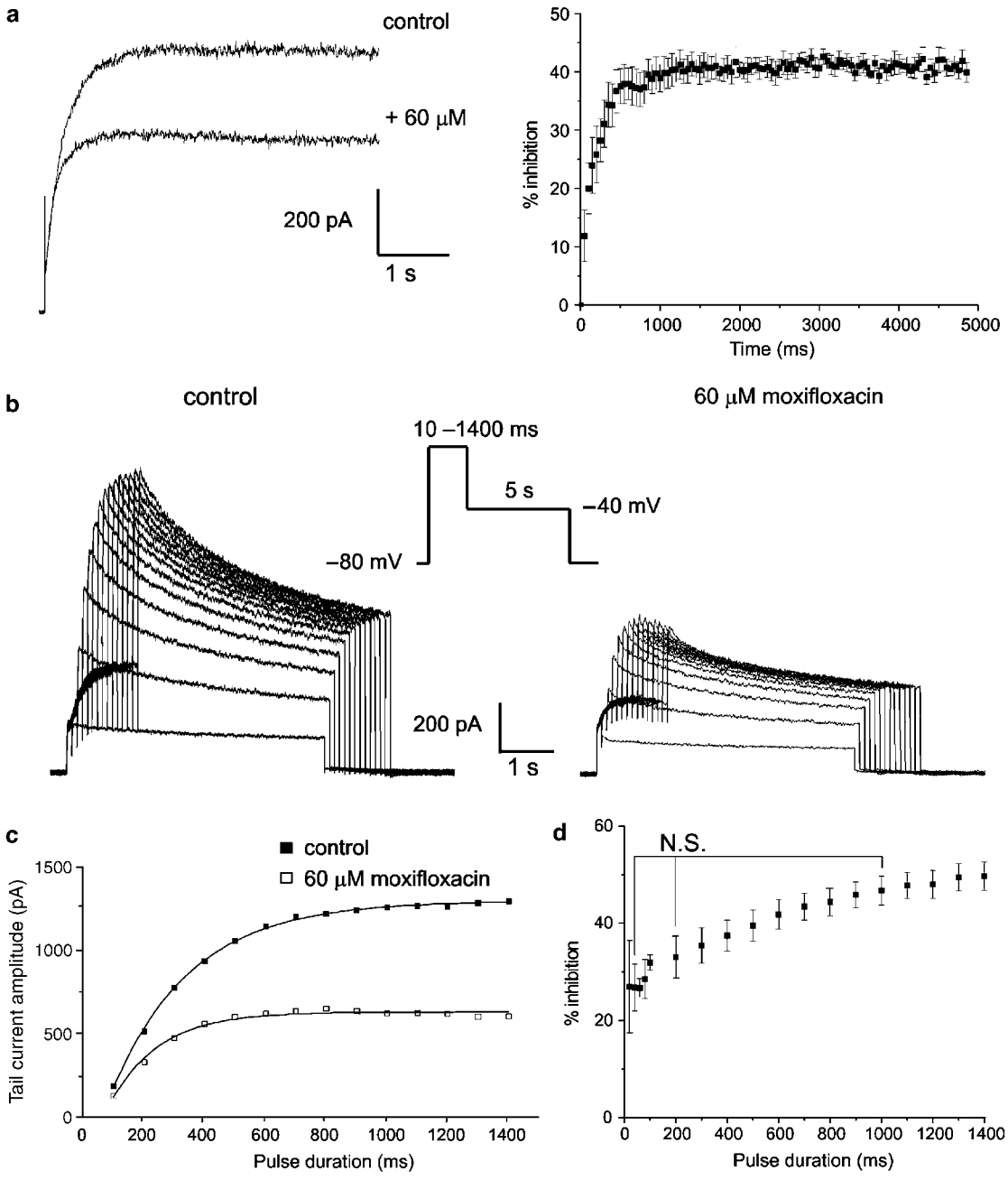

Figure 4 Time-dependence of hERG block by moxifloxacin. (a) Cells were depolarised from the holding potential of −80 mV to 0 mV for 5 s until a steady-state response was observed. Moxifloxacin was then bath-applied for 3 min while the channels were held in a closed state by voltage-clamping the cell at −80 mV. The voltage command was then again repeated in the presence of moxifloxacin. The representative traces in the left-hand panel indicate the last pulse prior to, and the first pulse after, application of 60 μM moxifloxacin. The right-hand panel shows the mean development of block over time by 60 μM moxifloxacin from five experiments. Full extent of block was observed within 1 s and was maintained for the 5 s duration. The development of block could be fit by a single exponential yielding a mean τ of 243.5±9.5 ms. (b) An envelope of tails protocol, as illustrated in the inset, was used to investigate time-dependence of channel block. Cells were depolarised to +20 mV for periods of time from 10 to 1400 ms before repolarising to −40 mV to elicit tail currents. Peak tail currents were measured in control conditions and after equilibration of cells in 60 μM moxifloxacin. For clarity, not all current traces are shown. (c) This panel shows the increase in tail current with length of the depolarising step for the cell illustrated in (b), in the absence and presence of moxifloxacin. (d) Summary of mean data from envelope of tails experiments where mean % inhibition of tail currents is plotted as a function of pulse duration. Significant inhibition (26.8±4.8%; $n=5$) was observed with a 40 ms step. Inhibition appeared to further increase as pulse duration was incrementally lengthened; however, the changes were not statistically significant.

without affecting the slope factor (18.1±1.0 and 20.7±1.3 mV, respectively; $n=7$, $P=0.11$).

To examine whether moxifloxacin affected the rate of onset of inactivation, similarly to the previous protocol, channels were forced into the inactivated state at +40 mV (as per the previous voltage protocol) before being briefly stepped to −100 mV to allow for recovery from inactivation (Wu *et al.*, 2003; Su *et al.*, 2004). A subsequent step to test potentials

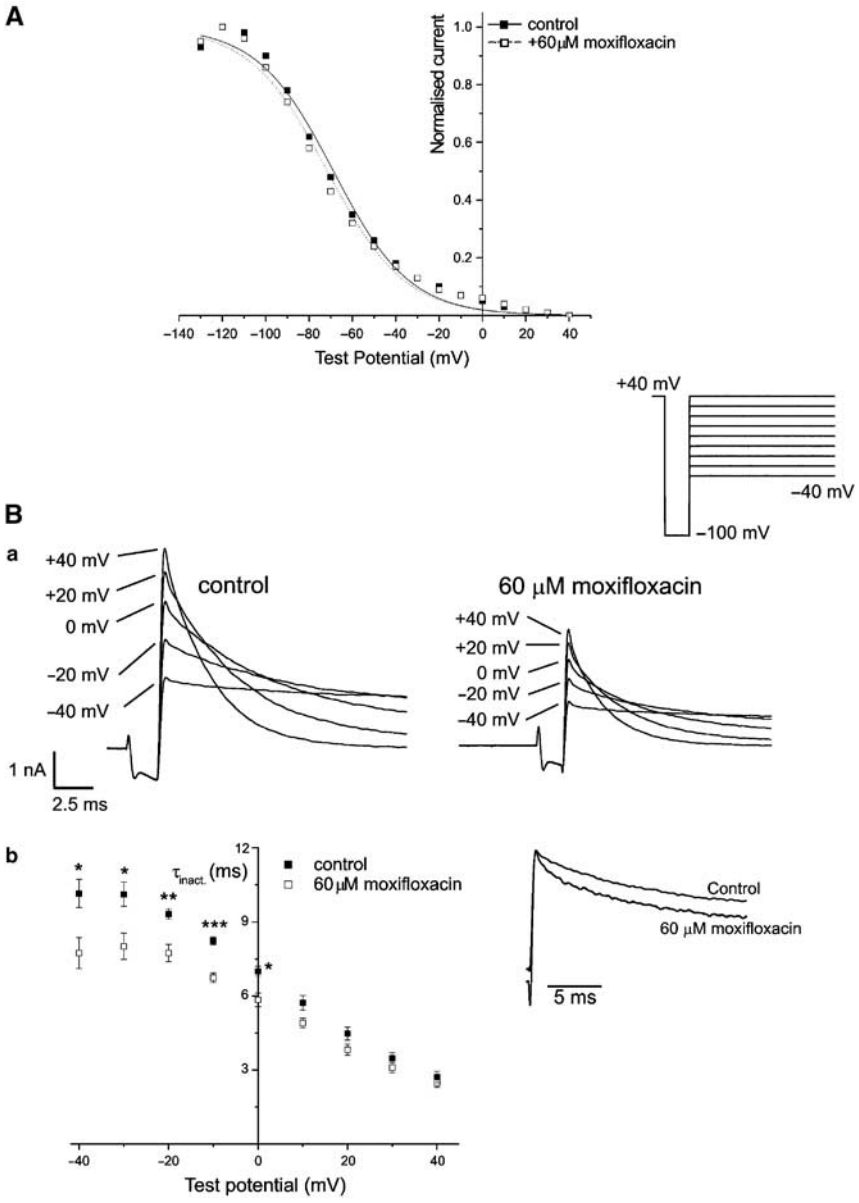

Figure 5 Effects of moxifloxacin on hERG current inactivation. (A) This panel shows examples of steady-state inactivation curves under control conditions (closed symbols) and in the presence of 60 μM moxifloxacin (open symbols). To elicit steady-state inactivation, following a 500 ms step to +40 mV, 10 ms pulses were applied to potentials between −130 and +40 mV in 10 mV increments, followed by a second 500 ms step to +40 mV. Current amplitudes elicited during the final step to +40 mV were measured, normalised and plotted as a function of the preceding test potential to yield a steady-state inactivation curve. In this cell, the mean $V_{1/2(\text{inact})}$ under control conditions was −69.3 mV (slope 15.4 mV) and in the presence of moxifloxacin, −73.4 mV (slope 16.2 mV). (B) Example traces in the upper panel (a) illustrate the effects of moxifloxacin on inactivation kinetics. Part of the voltage command is shown in the inset: cells were depolarised to +40 mV for 500 ms and then briefly stepped to −100 mV followed by a subsequent step to test potentials ranging from −40 to +40 mV. An example recording under control conditions is shown in the left-hand panel and in the presence of 60 μM moxifloxacin in the right-hand panel. For clarity, not all the current traces are shown. The outward currents elicited during this final step were then fitted to a single exponential function to yield a time constant at each test potential. (b) Time constants were plotted *versus* test potential as shown in the graph. The inset shows an example of currents in the absence and presence of moxifloxacin at −20 mV, which have been normalised and overlayed.

ranging from −40 to +40 mV was then applied to elicit outward inactivating tail currents. This protocol, illustrated as an inset to Figure 5B(a), was applied under control conditions and after equilibration in 60 μM moxifloxacin. Outward tail currents, examples of which are shown in Figure 5B(a), could be fitted well by a single exponential function to yield time constants at each test potential under both control and drug conditions (Figure 5B(b)). We found that moxifloxacin accelerated the time course of inactivation at potentials negative to, and including, 0 mV. The inset to Figure 5B(b) shows currents in the absence and presence of 60 μM moxifloxacin obtained at −20 mV, which have been normalised and overlayed. This illustrates the shift in inactivation time course.

Frequency-independence of moxifloxacin block of hERG channels

To investigate whether moxifloxacin exhibited frequency-dependence, we used the step-ramp protocol (shown in Figure 1a) applied with different start-to-start intervals (2, 4, 8 and 12 s) and examined both the extent and the development of block by moxifloxacin. We first observed that the magnitude of inhibition by 60 μM and 1 mM moxifloxacin was unaffected by the frequency of voltage protocol application (Figure 6a). We then plotted the development of channel block in the presence of moxifloxacin *versus* time and observed that the time course of current inhibition was unaffected by the start-to-start interval employed (Figure 6b).

Use of hERG channel pore mutants to understand moxifloxacin block

Finally, we examined whether or not the degree of hERG channel block by moxifloxacin is affected by mutation of the pore-S6 residues, Tyr652 and Phe656, to Alanine residues. These experiments utilised cell lines stably expressing the mutant channels as previously described (Milnes *et al*., 2003). Experiments examining the effect of the Y652A mutation on moxifloxacin potency were conducted at 35–37°C using the standard step-step protocol as illustrated in Figure 2a. At a high concentration of 600 μM (used in order to produce a large blockade of WT hERG), moxifloxacin blocked hERG Y652A channels by 24.2±6.0% ($n=5$), which was significantly less than inhibition of WT channels (71.8±5.6%, $n=5$, $P<0.001$; Figures 7A(a) and (b)). From these data (and assuming an n_H of 1), the projected IC_{50} concentration for moxifloxacin inhibition of Y652A channels would be ~1.9 mM, a 30- to 70-fold change in affinity. The Y652 residue has been implicated in voltage-dependence of I_{HERG} blockade by some drugs (Sanchez-Chapula *et al*., 2002; 2003). Therefore, additional experiments were performed in which the protocol used in Figure 7A(a) (lower panel) was modified, in order to apply successive voltage commands at 20 mV intervals between −20 and +40 mV. The mean values for fractional block of the Y652A I_{HERG} tails following the steps to each test potential produced by 600 μM moxifloxacin was then determined and was plotted against test potential as shown in Figure 7A(c) ($n=5$ at each voltage). There was no significant difference in the level of fractional block for the differing voltage commands ($P>0.8$; ANOVA), thereby implicating the Y652 residue in voltage-dependence of I_{HERG} block by moxifloxacin. We also examined the effect of the mutant F656A on moxifloxacin-mediated inhibition by comparing the effects of 600 μM moxifloxacin on WT and F656A channels. We found that this mutant had no significant effect on the magnitude of inhibition of currents by moxifloxacin (72.0±2.6%, $n=5$ *versus* WT: 70.0±12.4%, $n=5$, $P=0.89$). Representative example traces and mean summary data are shown in Figure 7B.

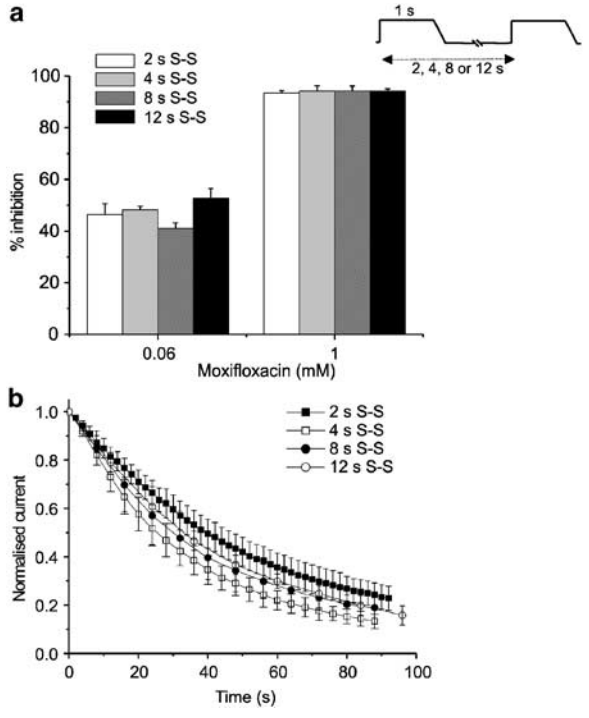

Figure 6 Frequency-dependence of moxifloxacin block. (a) Summary of the effects of 60 μM and 1 mM moxifloxacin on % inhibition of tail current amplitudes obtained from experiments using the step-ramp protocol applied with different start-to-start intervals of 2, 4, 8 or 12 s, as shown in the voltage protocol (inset). The effects of 60 μM moxifloxacin with a 4 s start-to-start interval are predicted from the dose–response curve shown in Figure 1c. (b) To investigate frequency-dependence, the rate of decline of current in response to 1 mM moxifloxacin (normalised to the current immediately prior to the onset of drug effects) was plotted *versus* time for each experimental condition (i.e. 2, 4, 8 or 12 s start-to-start interval).

Discussion

This study represents the first characterisation of the mechanism of block of hERG channels by the clinically used antibiotic moxifloxacin, which is also used as a positive control in human QT clinical studies. We found that moxifloxacin exhibits reversible channel block with fast kinetics, that its actions are voltage-dependent, that block is independent of stimulation frequency and that it is sensitive to mutation of the S6 helix residue Tyr652 but not Phe656. Under our conditions, moxifloxacin inhibited hERG currents with an IC_{50} of 65 μM using a step-ramp protocol, similar to values previously reported in CHO and HEK293 cells (Bischoff *et al*., 2000; Lacroix *et al*., 2003; Chen *et al*., 2005). Potency of inhibition was decreased (IC_{50} of 114 μM) when an action potential voltage-clamp waveform was used, which may be attributable to the shorter duration of membrane depolarisation during the AP compared to step-ramp or step-step protocols. From a drug-safety perspective, the use of step-step or step-ramp protocols are therefore unlikely to underestimate moxifloxacin potency, rather they are likely to yield a conservative IC_{50} value.

Moxifloxacin is widely used in the treatment of bacterial infections such as *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Haemophilus influenzae* (Caeiro & Iannini, 2003). Structurally it is very different to the macrolide antibiotics, such as erythromycin, which is a large, complex alicyclic molecule. Erythromycin block of hERG channels is strongly temperature-dependent: it has been shown to exhibit a seven-fold increase in potency between 22 and 35°C (Kirsch *et al*.,

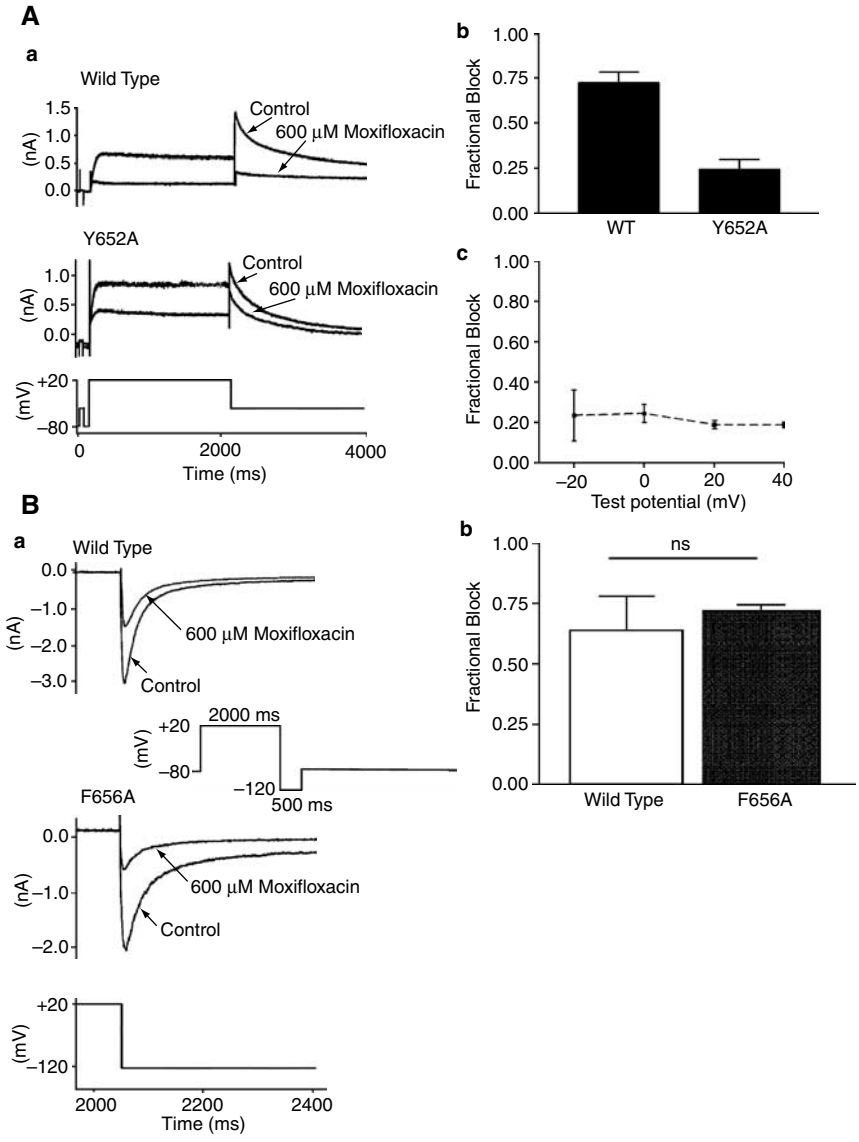

Figure 7 Effects of the S6 mutations Y652A and F656A on moxifloxacin block of hERG channel. (A) Effects of the Y652A mutation on the action of moxifloxacin. (a) Representative current traces. Left-hand panel shows example current traces of the effects of a high (~20-fold the IC_{50}) concentration of moxifloxacin (600 μM) on wild-type hERG current (upper traces) and on Y652A hERG current (middle traces). Lower trace shows the voltage-protocol used. In each case, hERG tail amplitude was measured as the difference between the peak outward tail current at −40 mV and the instantaneous current activated by the brief depolarisation from −80 to −40 mV. (b) Mean ± s.e.m. fractional-block data for Y652A hERG currents and its corresponding control ($n = 5$ cells for each). (c) Mean ± s.e.m. fractional block data for Y652A hERG tails following voltage-commands to −20, 0, +20 and +40 mV. Data are from five cells. (B) Effects of the F656A mutation on the action of moxifloxacin. (a) The voltage protocol used to elicit currents is shown as an inset to the figure, while the lower trace in (a) shows an expanded portion of the protocol, comprised of the repolarising step from +20 to −120 mV. Upper set of current traces contains example records of WT I_{HERG} in the absence (Control) and presence of 600 μM moxifloxacin; lower current traces show similar data for the F656A HERG mutant. (b) Mean ± s.e.m. fractional block data ($n = 5$ cells for each) for F656A I_{HERG} and its corresponding control.

2004) and a doubling in potency from 36°C to 42°C (Guo *et al*., 2005). Although we observed that moxifloxacin did exhibit some temperature-dependence between 22 and 35°C (a ~2.2-fold change in IC_{50}), this was less marked than that observed for erythromycin by Kirsch *et al*. (2004), nor did we observe any further enhancement of block by moxifloxacin at 42°C. Nevertheless, this observation indicates that when moxifloxacin is used as a positive control for agents undergoing preclinical safety screening: (a) consistency in experimental temperature for different compounds should be employed within a single study and (b) data for moxifloxacin at physiological/near-physiological temperatures will provide a conservative IC_{50} value for comparison with data from other compounds.

We compared the potency of moxifloxacin inhibition of I_{hERG} with that of levofloxacin, another fluoroquinolone antibiotic but one which has been shown to cause QT prolongation and Torsades de Pointes (Frothingham, 2001; Iannini *et al*., 2001; Iannini, 2002; Owens Jr & Ambrose, 2002). We found that levofloxacin was less potent than moxifloxacin

using both our standard step-ramp (IC_{50} 0.43 mM) and VAP (IC_{50} 1.45 mM) voltage protocols. A previous study by Kang *et al*. (2001) has reported that levofloxacin was ~7 times less potent than moxifloxacin, and our data were consistent with this finding: we also observed a ~7-fold shift between the two compounds using our step-ramp protocol.

Clinically, moxifloxacin is typically given as a 400 mg daily dose; furthermore, it is this dose which is used in QT clinical studies as a positive control. Since moxifloxacin exhibits ~50% binding to plasma proteins (Stass *et al*., 1998), then after a 400 mg dose, the free concentration is estimated at approximately 3 μM. Levofloxacin exhibits less plasma protein binding (24–38%; Levaquin® product information) such that the free concentration following a typical 500 mg daily dose is around 10 μM. Thus, in terms of safety margins, that is, the ratio between IC_{50} and plasma concentrations (Redfern *et al*., 2003), levofloxacin would appear to have a greater safety margin than moxifloxacin (38 *versus* 22 for levofloxacin and moxifloxacin, respectively). However, there have been sixteen reported cases per 10 million prescriptions of Torsades de Pointes following administration of levofloxacin, yet no cases with moxifloxacin (Frothingham, 2001). That said, it should be noted that for both compounds the risks of Torsades are very low and in some reported cases, confounding additional risk factors such as drug–drug interactions or hypokalaemia were present (Gandhi *et al*., 2003; Amankwa *et al*., 2004).

Our data suggest that, like many compounds, moxifloxacin exhibits mixed state-dependence of channel block. A small shift in the half-maximal activation voltage (~4 mV) was observed and the degree of inhibition was accentuated with depolarisation: these data are consistent with a requirement for the channel to be open for moxifloxacin to block. We also observed that moxifloxacin was able to exert its effects within 40 ms of channel opening and that full block developed over the subsequent 700 ms. The first pulse elicited after application of moxifloxacin at −80 mV (while the channels were closed) showed significant inhibition of current amplitude, suggesting that moxifloxacin may also possess a component of closed channel block. However, from our data we cannot differentiate between closed channel and very rapid open channel block. On the basis of the small shift in the hERG inactivation curve with moxifloxacin (Figure 5), it appears that moxifloxacin did not act strongly to stabilise the inactivated state of the channel, although we do not rule out the possibility that the drug can interact with both activated and inactivated channels.

The magnitude of effects of 60 μM and 1 mM moxifloxacin was independent of stimulation frequency when voltage commands were applied with start-to-start intervals of 2, 4, 8 or 12 s. Furthermore, the time courses for onset of block under each of these conditions were also independent of stimulation frequency. While on initial consideration, this may appear to be in contrast to observations of prolongation of action potentials in isolated canine Purkinje fibres in a reverse rate-dependent manner (Gintant *et al*., 2001), there are a number of compounds which exhibit a similar pattern of reverse-rate dependence of action potential prolongation but no rate-dependence of hERG block. One such example is the methanesulphonanilide dofetilide, the effects of which were demonstrated in isolated ventricular myocytes by Jurkiewicz & Sanguinetti (1993). It is thought that reverse-rate dependence of action potential prolongation reflects differences in contribution of I_{Kr} in relation to I_{Ks}, to repolarisation at different stimulation rates (Rocchetti *et al*., 2001). The increased magnitude of I_{Ks} at faster stimulation rates may act to decrease the relative importance of I_{Kr}, thus reducing the potential impact of I_{Kr} block and therefore contributing to reverse rate-dependence of action potential prolongation (Bosch *et al*., 1998).

Tyr652 and Phe656 are reported to be canonical sites for binding of many compounds in the pore region of the hERG channel (Mitcheson *et al*., 2000) and mutation of these residues (to Ala or Val) has been shown to negate the blocking actions of a number of compounds (Lees-Miller *et al*., 2000; Sanchez-Chapula *et al*., 2002). We observed that moxifloxacin was sensitive to mutation of Tyr652 since its inhibitory effects were reduced by ~66% in the Y652A line and for Y652A-HERG there was no voltage-dependence of inhibition. The elimination of the voltage-dependence of moxifloxacin-induced block by mutation of Y652 fits with previous observations from a range of drugs, including chloroquine (Sanchez-Chapula *et al*., 2002), quinidine, quinine and vesnarinone (Sanchez-Chapula *et al*., 2003), that mutation of this amino-acid residue can eliminate or even reverse the voltage-dependence of hERG blockade. We estimated that the IC_{50} of moxifloxacin in the Y652A cell line would be approximately 1.9 mM, which would equate to a 30- to 65-fold decrease in affinity compared to WT hERG. In contrast, in channels containing the mutation F656A, the effects of moxifloxacin were unaffected: 600 μM inhibited both WT and F656A channels by approximately 70%.

The potency of almost every compound tested so far has been reduced dramatically by mutation of either Tyr652 or Phe656. For example, the blocking affinity of MK-499 was reduced by 94- and 650-fold, respectively, with these mutations (Mitcheson *et al*., 2000) and the effects of chloroquine were reduced by approximately 500-fold by each mutation (Sanchez-Chapula *et al*., 2002). Often, the effects of Phe656 mutation on drug potency are more pronounced than the effects of mutation of Tyr652, for example, quinidine (Sanchez-Chapula *et al*., 2003) and MK-499 (Mitcheson *et al*., 2000), but this is not the case for moxifloxacin. Recently, some compounds have been reported to be relatively insensitive to mutation of these residues for example, dronedarone (Ridley *et al*., 2004b) and fluvoxamine (Milnes *et al*., 2003). Our results provide evidence that whilst Phe656 is not a molecular determinant for channel block by moxifloxacin, Tyr652 participates in moxifloxacin binding, although the estimated 30- to 65-fold reduction in blocking potency is somewhat less than for agents such as MK-499 (Mitcheson *et al*., 2000).

In summary, we have investigated the mechanism of hERG channel block by moxifloxacin. Inhibition of hERG was dependent upon voltage but not on stimulation frequency and its kinetics of action were rapid. The data presented in this paper suggest that moxifloxacin binds to the open state of the channel and, to a lesser extent, the inactivated state and that drug binding occurs at the aromatic residue Tyr652 but not Phe656 in the inner cavity of the channel.

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