British Journal of Pharmacology (2009), 156, 226–236

0007-1188/09
www.brjpharmacol.org

# RESEARCH PAPER

# Selective noradrenaline reuptake inhibitor atomoxetine directly blocks hERG currents

Daniel Scherer[1]*, David Hassel[1]*, Ramona Bloehs[1], Edgar Zitron[1], Katharina von Löwenstern[1], Claudia Seyler[1], Dierk Thomas[1], Franziska Konrad[1], Heiner F. Bürgers[2], Gunnar Seemann[3], Wolfgang Rottbauer[1], Hugo A. Katus[1], Christoph A. Karle[1] and Eberhard P. Scholz[1]

[1]*Department of Internal Medicine III, University Hospital Heidelberg, Heidelberg, Germany,* [2]*Department of Physiology and Pathophysiology, University of Heidelberg, Heidelberg, Germany, and* [3]*Institute of Biomedical Engineering, University of Karlsruhe, Karlsruhe, Germany*

**Background and purpose:** Atomoxetine is a selective noradrenaline reuptake inhibitor, recently approved for the treatment of attention-deficit/hyperactivity disorder. So far, atomoxetine has been shown to be well tolerated, and cardiovascular effects were found to be negligible. However, two independent cases of QT interval prolongation, associated with atomoxetine overdose, have been reported recently. We therefore analysed acute and subacute effects of atomoxetine on cloned human *Ether-à-Go-Go*-Related Gene (hERG) channels.
**Experimental approach:** hERG channels were heterologously expressed in *Xenopus* oocytes and in a human embryonic kidney cell line and hERG currents were measured using voltage clamp and patch clamp techniques. Action potential recordings were made in isolated guinea-pig cardiomyocytes. Gene expression and channel surface expression were analysed using quantitative reverse transcriptase polymerase chain reaction, Western blot and the patch clamp techniques.
**Key results:** In human embryonic kidney cells, atomoxetine inhibited hERG current with an $IC_{50}$ of 6.3 μmol·$L^{-1}$. Development of block and washout were fast. Channel activation and inactivation were not affected. Inhibition was state-dependent, suggesting an open channel block. No use-dependence was observed. Inhibitory effects of atomoxetine were attenuated in the pore mutants Y652A and F656A. In guinea-pig cardiomyocytes, atomoxetine lengthened action potential duration without inducing action potential triangulation. Overnight incubation with high atomoxetine concentrations resulted in a decrease of channel surface expression.
**Conclusions and implications:** Whereas subacute effects of atomoxetine seem negligible under therapeutically relevant concentrations, hERG channel block should be considered in cases of atomoxetine overdose and when administering atomoxetine to patients at increased risk for the development of acquired long-QT syndrome.
*British Journal of Pharmacology* (2009) **156**, 226–236; doi:10.1111/j.1476-5381.2008.00018.x; published online 19 January 2009

**Keywords:** hERG channels; atomoxetine; acquired long-QT syndrome; selective noradrenaline reuptake inhibitor; SNRI; repolarization; arrhythmia; attention-deficit/hyperactivity disorder; ADHD

**Abbreviations:** ADHD, attention-deficit/hyperactivity syndrome; APD, action potential duration; HEK, human embryonic kidney cells; hERG, human *Ether-à-Go-Go*-Related Gene; qRT-PCR, quantitative reverse transcriptase PCR; SNRI, selective noradrenaline reuptake inhibitor

## Introduction

Traditionally, stimulants have been the first-line treatment for children and adults with attention-deficit/hyperactivity disorder syndrome (ADHD) (Spencer *et al.*, 2002). However, as up to 50% of the patients do not respond to therapy or suffer from side-effects of stimulants, there is a growing interest in alternative medication (Wernicke *et al.*, 2003). For ADHD, a model has been proposed, in which a low level of noradrenaline in the prefrontal cortex constrains cognitive function and leads to immature behaviour (Spencer *et al.*, 2002). Atomoxetine (Figure 1) (formerly known as tomoxetine) is a phenoxypropylamine derivative and is structurally related to the antidepressant fluoxetine. Atomoxetine exhibits a high affinity to noradrenaline transporters, leading to an increase of noradrenaline levels via inhibition of presynaptic noradrenaline uptake. Originally developed as antidepressant, atomoxetine has been shown to be effective in ADHD therapy (Spencer *et al.*, 2002). Compared with stimulants, atomoxetine has been found to be a safe and well-tolerated drug and

Correspondence: Eberhard P. Scholz, Department of Internal Medicine III, University Hospital Heidelberg, Im Neuenheimer Feld 410, Heidelberg 69120, Germany. E-mail: eberhard.scholz@med.uni-heidelberg.de
*These authors contributed equally.
Received 24 July 2008; revised 21 August 2008; accepted 2 September 2008

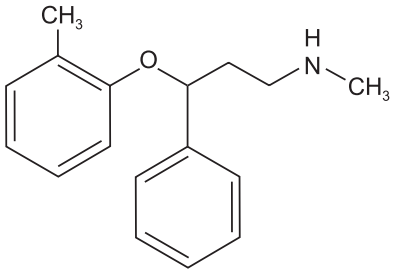


**Figure 1** Chemical structure of selective noradrenaline reuptake inhibitor atomoxetine.

decreased appetite and dizziness have been found to be the main adverse effects (Wernicke and Kratochvil, 2002). Cardiovascular effects have been studied in great detail (Wernicke *et al.*, 2003; Simpson and Plosker, 2004). Atomoxetine was found to be associated with a mild but significant increase in heart rate and blood pressure (Wernicke *et al.*, 2003). Taking these findings into account, QT intervals were analysed using different corrections for heart rate. Using the Bazett correction, atomoxetine was associated with a mild, but highly significant QT prolongation (Wernicke *et al.*, 2003). However, applying the Fridericia formula as well as correcting the QT intervals, using derived data, did not support this finding (Wernicke *et al.*, 2003).

Drug-induced QT prolongation is an adverse effect of many drugs not targeted to the heart. Virtually all of those substances exert their cardiac side-effects via inhibition of the delayed rectifier potassium current ($I_{Kr}$), which is one of the most important components of cardiac repolarization and is thought to be responsible for the termination of the plateau phase in humans (Kiehn *et al.*, 1999). The human *Ether-à-Go-Go*-Related Gene (hERG) encodes the α-subunit underlying $I_{Kr}$ (Curran *et al.*, 1995; Sanguinetti *et al.*, 1995; Trudeau *et al.*, 1995; channel nomenclature follows Alexander *et al.*, 2008). Decreased $I_{Kr}$ due to pharmacological inhibition can result in QT prolongation and facilitate the development of cardiac arrhythmia, especially Torsade de Pointes (Roden *et al.*, 2002; Redfern *et al.*, 2003). Here we report that atomoxetine exerts acute effects on cloned cardiac hERG potassium channels and causes action potential prolongation at relevant concentrations and therefore should be avoided in patients at increased risk for the development of an acquired long-QT syndrome.

## Methods

*Solutions and drug administration*

Two microelectrode voltage clamp measurements of *Xenopus* oocytes were performed in a $K^+$ solution containing (in mmol·$L^{-1}$) KCl 5, NaCl 100, CaCl2 1.5, MgCl2 2 and HEPES 10 (pH adjusted to 7.4 with NaOH). Current and voltage electrodes were filled with 3 M KCl solution. The volume of the bath chamber was 150 μL; after solution switch, it took about 4 s for the new solution to reach the bath; at a flow rate of 1 mL·$min^{-1}$, solution in the bath was totally exchanged within 9 s. In general, recordings were started 30 s after solution switch. All measurements were performed under steady-state conditions at least 2 min after total solution exchange.

For whole-cell patch clamp studies in the human embryonic kidney (HEK) cell line, the bath solution contained (in mmol·$L^{-1}$) NaCl 140, KCl 5, $MgCl_2$ 1, HEPES 10, $CaCl_2$ 1.8 and glucose 10 (pH adjusted to 7.4 with NaOH). The pipette solution contained (in mmol·$L^{-1}$) K-aspartate 100, KCl 10, $MgCl_2$ 2, $CaCl_2$ 1, EGTA 10, HEPES 10 and glucose 40 (pH adjusted to 7.2 with KOH). For action potential measurements, the bath solution contained (in mmol·$L^{-1}$) KCl 4, NaCl 140, $CaCl_2$ 2, $MgCl_2$ 1, $NaH_2PO_4$ 0.33, glucose 10 and HEPES 10 (pH adjusted to 7.3 with NaOH). The internal solution contained (in mmol·$L^{-1}$) K-glutamate 120, KCl 10, $MgCl_2$ 2, EGTA 10 and HEPES 10 (pH adjusted to 7.20 with KOH). All measurements were carried out at room temperature (22°C).

*Electrophysiology and data analysis*

All animal procedures and investigations conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996).

The double-electrode voltage clamp configuration was used to record currents from *Xenopus laevis* oocytes. Microelectrodes had tip resistances ranging from 1 to 5 MΩ. Data were low pass-filtered at 1 to 2 kHz (–3 dB, four-pole Bessel filter) before digitalization at 5 to 10 kHz. Recordings were performed using a commercially available amplifier (Warner OC-725A, Warner Instruments, Hamden, USA) and pCLAMP software (Axon Instruments, Foster City, CA, USA) for data acquisition and analysis. No leak subtraction was performed during the experiments. For activating currents, absolute values were used for analysis. For analysis of tail currents, currents were calculated relative to baseline measurements at the holding potential (–80 mV).

The HEK293 cell line (#CRL-1573, ATCC, Manassas, VA, USA) expressing hERG channels was generously provided by Barbara A. Wible (Cleveland, OH, USA). Cells were transferred from the incubator into a recording chamber that was continuously superfused with bath solution. For measurements, single-HEK cells were chosen. Pipettes had resistances of 3–4 MΩ and whole-cell patch clamp currents were measured with an RK-400 amplifier (Bio-Logic SAS, Claix, France), recorded on hard disk and analysed with pCLAMP6 software (Axon Instruments, Foster City, CA, USA). For the quantification of hERG channel expression, current density measurements were performed in a HEK cell line stably expressing hERG channels. After overnight drug pre-incubation, cells were washed thoroughly with control medium and placed in non-drug-containing external solution 1 h prior to the experiments. Whole-cell current was quantified using the patch clamp technique and current density was estimated by dividing current amplitude by cell capacity.

Left ventricular cardiomyocytes were isolated from four adult guinea pigs (200–300 g) as reported by Scholz *et al.* (2003). After isolation, cells were kept at 4°C for about 2 h. All experiments were performed within 6 h after isolation of the cells. Action potential recordings were performed with the RK-400 amplifier in the current clamp mode. Patch clamp pipettes had a tip resistance of 3–4 MΩ. After disruption of the

patch, cell parameters were adjusted. Mean values of cell capacitance and equivalent resistance revealed 77 ± 16 pF and 12 ± 6.6 MΩ ($n$ = 10). Only cells with a membrane potential below –60 mV were selected for further analysis. A short supra-threshold stimulus (3 ms, 0.5 Hz) was applied to elicit action potentials. Before each measurement, the cells were stimulated for 5 min (0.5 Hz) to reach a steady state. Stimulation and data acquisition were performed using pCLAMP6 software and digitized with a Digidata 1200 (Axon Instruments). All action potential measurements were performed at room temperature to allow a direct comparison with the current measurements.

Concentration response curves were fitted to the function: $Y = A_1 + \{[A_2 - A_1]/[1 + 10\text{^}\log(X_0 - X) \times n_4]\}$ with $A_1$ being the bottom asymptote, $A_2$ the top asymptote, log$X_0$ being the centre and $n_H$ being the Hill slope.

Activation and inactivation curves were fitted to a Boltzmann function: $Y = \{(A_1 - A_2)/[1 + (e\text{^}(X - X_0)/k)]\} + A_2$ with $A_1$ being the initial value, $A_2$ being the final value, $X_0$ representing the half-maximal activation potential, $Y$ the degree of activation and $k$ the slope factor. Statistical data are expressed as mean ± standard error with $n$ representing the number of experiments performed. Statistical significance was evaluated using either the independent Student's $t$-test or ANOVA followed by $t$-test with Bonferroni correction. Differences were considered to be significant when the $P$-value was <0.05.

*Western blot analysis*

For the analysis of channel trafficking, a HEK cell line stably expressing hERG channels was used. Increasing atomoxetine concentrations (1, 3, 10, 30 and 100 μmol·L$^{-1}$) as well as 100 μmol·L$^{-1}$ $As_2O_3$ were diluted in DMEM (Invitrogen) medium and added to the cells for 24 h at 37°C prior to protein extraction. For control measurements, an untransfected HEK cell line was incubated without atomoxetine-containing medium. The cells were washed with phosphate-buffered saline and subsequently harvested with TNN buffer (50 mmol·L$^{-1}$ Tris, 0.25 mol·L$^{-1}$ NaCl, 5 mmol·L$^{-1}$ EDTA, 0.5% NP-40) supplemented with 1 mmol·L$^{-1}$ dithiothreitol, 1 mmol·L$^{-1}$ NaF, 1 mmol·L$^{-1}$ sodium orthovanadate, 1 mmol·L$^{-1}$ phenylmethylsulphonyl fluoride and complete protease inhibitor (Roche, Mannheim, Germany). Protein concentration was determined using the BCA technique (Pierce Chemical, Rockfort, IL, USA). Proteins (50 μg per sample) were separated by 8% SDS-PAGE and transferred to a polyvinylidene fluoride membrane. For detection of hERG channels, blots were probed with a rabbit polyclonal Anti-h$K_v$11.1 antibody (Alomone Labs, Jerusalem, Israel, 1:400) and signals were detected by chemiluminescence. The blots were quantified by using ImageJ (NIH, USA). Equal protein loading was confirmed by Ponceau S staining (data not presented).

*RNA isolation, reverse transcription and quantitative reverse transcriptase polymerase chain reaction*

RNA isolation and reverse transcription reaction was performed as described previously (Hassel *et al.*, 2008). hERG mRNA expression levels were quantified by quantitative reverse transcriptase polymerase chain reaction (PCR) using an ABIPrism 7000 Sequence Detection System (Applied Biosystem, Foster City, CA, USA). cDNA derived from HEK cells stably expressing hERG was amplified using ABsolute SYBR Green ROX Mix (Thermo Scientific, Surrey, UK) in the presence of primer oligonucleotides specific for hERG and β-actin. The quantification was performed by the comparative cycle threshold method, using the β-actin expression level as internal control and by subsequent ratio determination, relative to samples from untreated hERG-expressing HEK cells.

*Mutagenesis and expression of hERG channels in* Xenopus *oocytes*

The hERG clone (Accession No. u04270) was a gift from M.T. Keating (Boston, MA, USA). hERG complementary RNA was prepared from the hERG cDNA in the pSP64 plasmid with the mMESSAGE mMACHINE *in vitro* transcription kit (Ambion, Spitfire Close Huntingdon, Cambridgeshire, UK) by use of SP6 Polymerase after linearization with EcoRI (Roche, Mannheim, Germany). The amino acid mutations were generated by PCR with synthetic mutant oligonucleotide primers using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, USA). Production of mutant hERG channels Y652A and F656A has been reported previously (Scholz *et al.*, 2003). The mutations Y652A and F656A were verified by sequencing (SeqLab, Goettingen, Germany). Injection of RNA (50 to 500 ng·μL$^{-1}$) into stage-V and -VI defolliculated oocytes was performed by using a Nanoject automatic injector (Drummond, Broomall, USA). The volume of injected cRNA solution was 48 nL per oocyte, and measurements were made 2–10 days after injection.

## Results

*Atomoxetine inhibited cloned hERG channels in* Xenopus *oocytes*

In order to analyse inhibitory effects of atomoxetine on cloned hERG potassium channels, double-electrode voltage clamp experiments were performed in *Xenopus* oocytes heterologously expressing hERG potassium channels. From a holding potential of –80 mV, variable test pulses from –80 to +80 mV in 10 mV increments (400 ms) were applied at a frequency of 0.2 Hz to measure activating currents. Each pulse was followed by a constant return pulse to –60 mV (400 ms) evoking typical outward tail currents. After having obtained a control measurement (Figure 2A), atomoxetine (10 μmol·L$^{-1}$) was washed into the bath for 10 min. hERG current under control conditions had a typical activation threshold of approximately –40 mV, and reached a current maximum at +10 mV before a considerable current reduction at higher test pulse potentials could be observed due to inward rectification. Figure 2C displays the current–voltage relationship at the end of the first test pulse (first dashed line in Figure 2A,B). Application of 10 μmol·L$^{-1}$ atomoxetine reduced peak current amplitude to 48% of initial values ($n$ = 7).

Tail currents reached a plateau after a test pulse potential of approximately +30 mV (Figure 2D). Atomoxetine at a concentration of 10 μmol·L$^{-1}$ reduced peak tail current amplitude

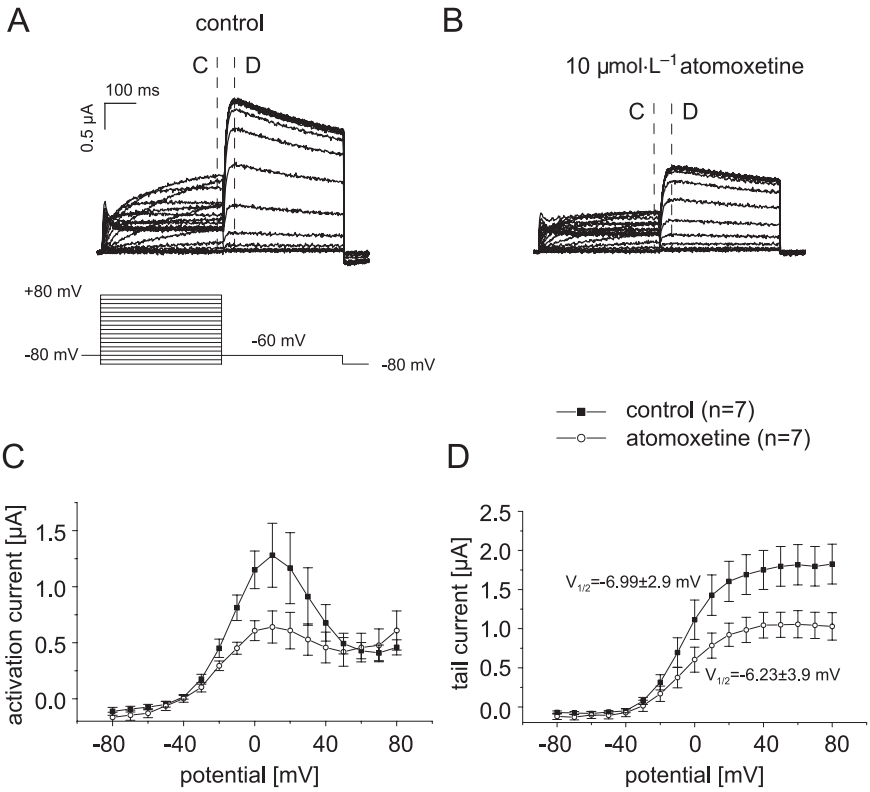


**Figure 2** Atomoxetine inhibits cloned human *Ether-à-Go-Go*-Related Gene (hERG) potassium channels. Typical outward hERG currents elicited by a two-stage voltage protocol (A). Addition of atomoxetine led to a significant inhibition of cloned hERG currents (B). (C) Corresponding activating current amplitude at the end of the first test pulse as a function of the test pulse potential. The mean current amplitude at +10 mV was reduced to 48% of initial current. (D) Tail current amplitudes as a function of the preceding test pulse potential. Mean peak tail current after the test pulse to +40 mV was reduced to 54% of initial tail current. Protocol: holding potential –80 mV, test pulse –80 to +80 mV (400 ms) in 10 mV increments, return pulse constant –60 mV (400 ms).

(after a test pulse to +40 mV) to 54% of initial values ($n = 7$). hERG activation curves were fitted to a Boltzmann function to obtain half-maximal activation potentials. No significant shift of the half-maximal activation potential could be observed ($P > 0.05$, $n = 7$).

*Concentration-dependence of block of hERG channels established in a HEK cell line*

Concentration-dependence of atomoxetine-induced hERG block was analysed in a HEK cell line stably expressing hERG channels using the whole-cell patch clamp technique. From a holding potential of –80 mV, a constant test pulse to +20 mV (400 ms) was applied, followed by a return pulse to –120 mV (400 ms) to elicit large inward tail currents (Figure 3, inset). Atomoxetine, over a range of concentrations (0.1–100 µmol·L$^{-1}$), reduced peak tail currents ($n = 5$–7, Figure 3). The dose–response curve was fitted using the Hill equation and yielded an $IC_{50}$ of 6.26 ± 0.92 µmol·L$^{-1}$ ($n_H = 0.6 \pm 0.06$).

*S6 domain mutations F656A and Y652A in hERG channels attenuate inhibitory effects of atomoxetine*

Time courses of development of block and washout were evaluated over a period of 15 min. From a holding potential

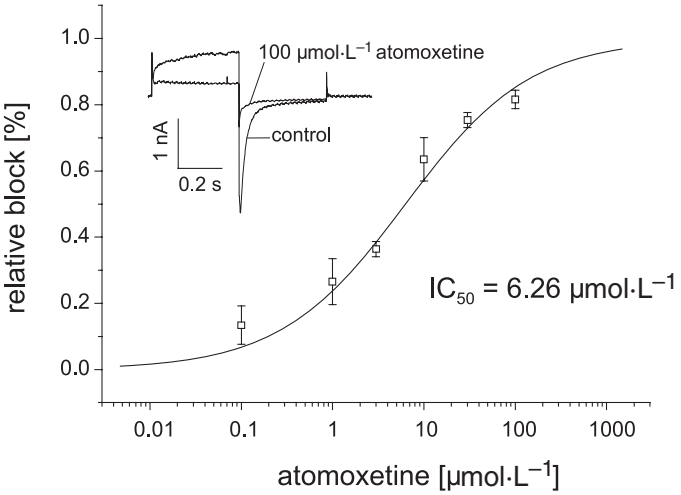


**Figure 3** Concentration-dependence of block established in a human embryonic kidney cell line. To investigate the concentration-dependence of atomoxetine-induced block, a standard voltage protocol was used, eliciting large inward tail currents (see inset). Atomoxetine inhibited human *Ether-à-Go-Go*-Related Gene channels in a concentration-dependent manner. The $IC_{50}$ was 6.26 µmol·L$^{-1}$ and the Hills slope 0.6. Protocol: repetitive pulsing at a frequency of 0.1 Hz, holding potential –80 mV, test pulses to +20 mV (400 ms) and return pulses to –120 mV (400 ms) ($n = 5$–7).

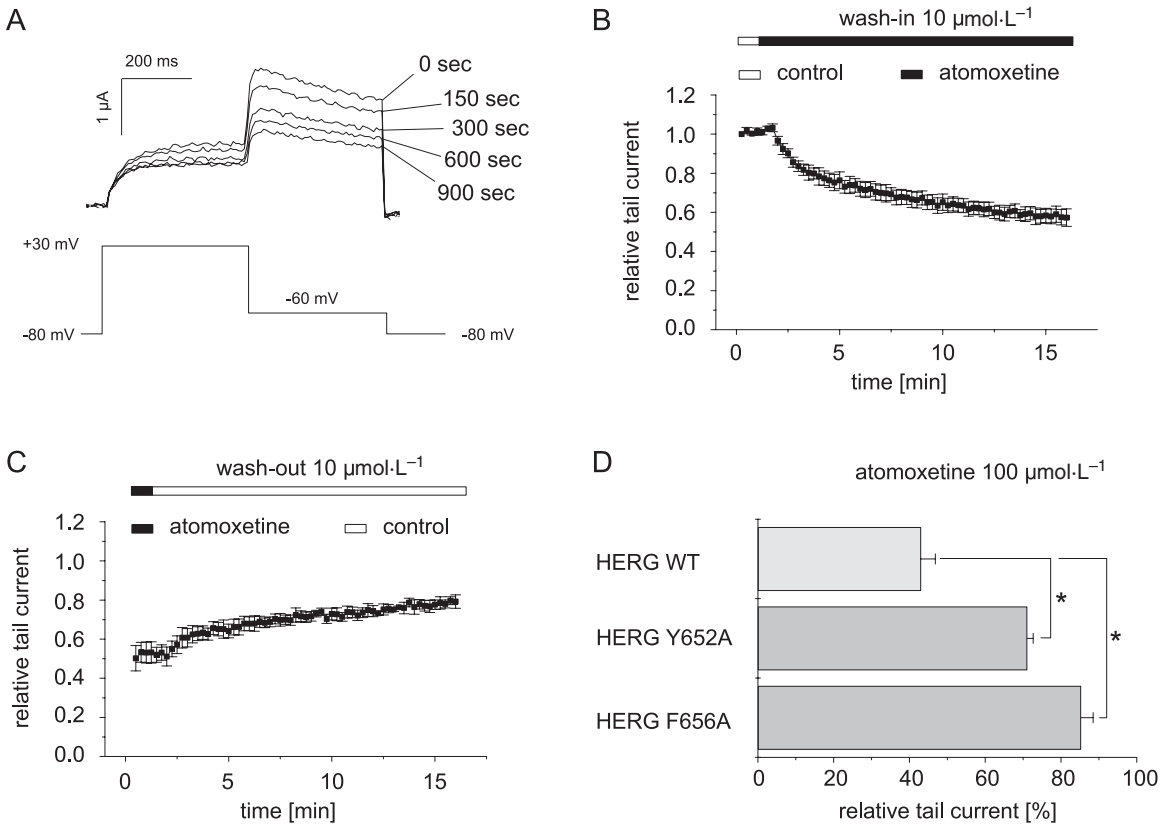


**Figure 4** S6 domain mutations Y652A and F656A attenuate human *Ether-à-Go-Go*-Related Gene (hERG) block by atomoxetine. A and B. Time course of development of block was recorded over a period of 15 min, using a two-stage voltage protocol. Loss of inhibitory effects upon washout was recorded (C). Time constant of development of block was 3.60 ± 0.39 min ($n = 6$). Loss of inhibitory effects was found to be slower, yielding a time constant of 7.67 ± 2.14 min ($n = 6$). D. Compared with hERG wild-type, inhibitory effects of atomoxetine were significantly attenuated in mutant (F656A and Y652A) hERG channels, suggesting an involvement of the aromatic residues in binding properties ($P < 0.01$, $n = 5$). Protocol: A–C. Repetitive pulsing at a frequency of 0.07 Hz, holding potential –80 mV, first pulse to +30 mV (400 ms), followed by a second pulse to –60 mV (400 ms), representative experiment. D. Holding potential –80 mV. Constant first test pulse to +30 mV (400 ms), followed by a constant step to –120 mV (400 ms).

of –80 mV and at a frequency of 0.07 Hz, cells were subject to a two-stage voltage protocol with a first depolarizing voltage step to +30 mV (400 ms), followed by a second step to –60 mV (400 ms) eliciting outward tail currents. Time courses of peak tail currents were tracked to determine the degree of block (Figure 4A). After a period of 1 min, demonstrating the stability of experimental conditions, cells were exposed to atomoxetine (10 µmol·L$^{-1}$) (Figure 4B). Washout of inhibitory effects was recorded analogously (Figure 4C). Time constants were estimated by fitting the latter part of the data set to single exponential functions. Time constants of development of block and washout yielded 3.60 ± 0.39 and 7.67 ± 2.14 min respectively ($n = 6$).

The two aromatic amino acids Y652 and F656 located in the S6 domain of hERG play a major role in binding properties of most hERG inhibitors (Mitcheson *et al.*, 2000; Scholz *et al.*, 2003). It has been assumed that they form a binding site for lipophilic drugs within the hERG channel pore cavity (Mitcheson *et al.*, 2000). In order to investigate binding properties of atomoxetine, we compared inhibitory effects of 100 µmol·L$^{-1}$ atomoxetine on wild-type and mutant (F656A and Y652A) hERG channels. From a holding potential of –80 mV and at a frequency of 0.07 Hz, test pulses to +30 mV (400 ms) were applied, followed by a second pulse to –120 mV (400 ms) eliciting large inward tail currents. Peak tail current amplitudes were measured in order to quantify the inhibitory effects. In hERG wild type, atomoxetine (100 µmol·L$^{-1}$) caused a current reduction to 43 ± 3.8% of initial tail current ($n = 5$). In F656A as well as in Y652A mutant channels, atomoxetine effects were attenuated significantly, resulting in a decreased inhibition to 85 ± 3.2% and 71 ± 1.6% of initial tail current respectively ($P < 0.05$, ANOVA, $n = 5$).

### *Atomoxetine inhibits hERG channels in their open and inactivated states*

In order to analyse the state-dependence of hERG block by atomoxetine, two different voltage protocols were utilized. In a first single-stage protocol, cells were subject to a depolarizing test pulse of 0 mV (6 s), eliciting activation currents. Having obtained a control measurement, a holding potential of –80 mV was applied to keep the channels in the closed state. Meanwhile, the cells were exposed to atomoxetine (10 µmol·L$^{-1}$) for 10 min to allow an equilibration of drug concentrations within the bath and the cell. Then, the step protocol was repeated (Figure 5A). By division of currents and presentation in a normalized form, the time course of relative block was obtained (Figure 5B). Fractional block showed a

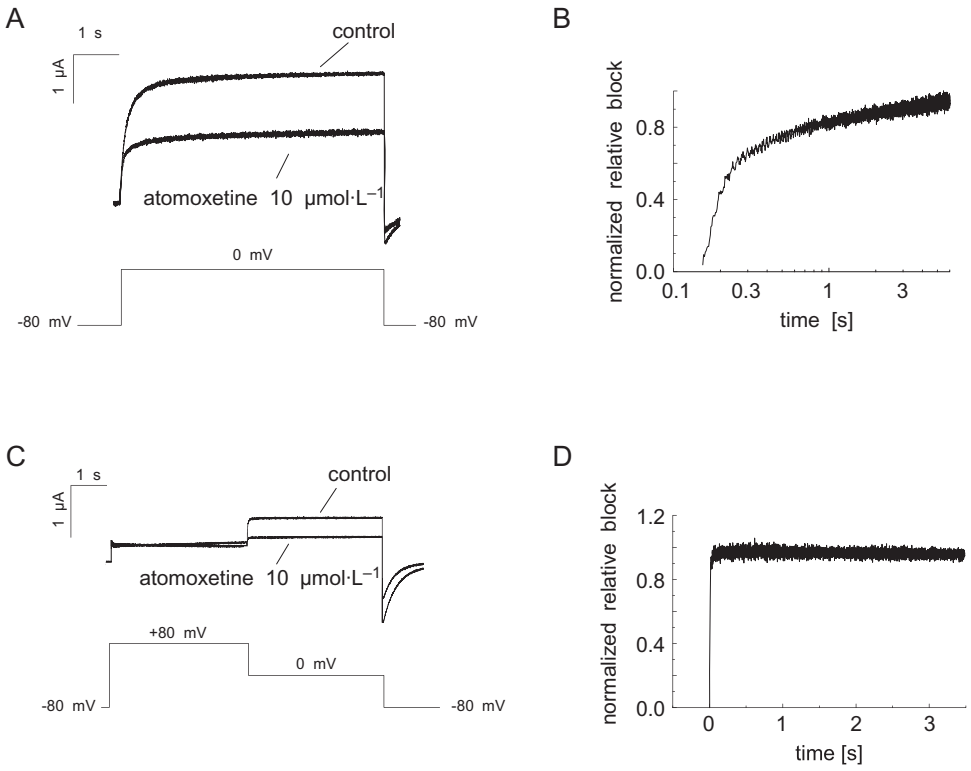


**Figure 5** Atomoxetine inhibits open and inactivated channels. Channels were held at –80 mV, that is, in their closed state, before an activating test pulse to 0 mV (for 6000 ms) was applied in the presence of atomoxetine. (A) Typical activating currents are displayed for control and after incubation with atomoxetine (10 µmol·L$^{-1}$) (for 15 min). (B) Fractional block was calculated by division and is plotted versus time. Fractional block developed in a time-dependent manner within the first seconds, suggesting an open channel block by atomoxetine (C). In a second approach, human *Ether-à-Go-Go*-Related Gene channels were inactivated by a first voltage step to +80 mV (3500 ms), before opening by a second step to 0 mV (3500 ms) followed. Corresponding normalized relative block is shown in (D). In contrast to (B), inhibition by atomoxetine showed no marked time-dependence.

time-dependence within the first second, suggesting channel inhibition as soon as the channel opened, without a relevant proportion of pre-existing closed channel block ($n = 6$).

In order to address the question whether hERG channels were also blocked in their inactivated state, cells were subject to a long inactivating test pulse (+80 mV, 3500 ms). The inactivating step was followed by a return pulse to 0 mV, eliciting large reactivating currents (3500 ms) ($n = 6$). Typical current traces under control conditions and after 10 min of incubation with 10 µmol·L$^{-1}$ atomoxetine while holding the cell at –80 mV are displayed in Figure 5C. Fractional block is displayed in Figure 5D. In contrast to Figure 5B, there was no time-dependent development of block, suggesting no further inhibition while channels were re-opening. Thus, we conclude that the development of inhibition by atomoxetine is possible during the inactivated state of hERG channels. Frequency-dependence of block was further analysed using a short two-stage voltage protocol that was repeated at a frequency of 1 Hz and 0.07 Hz (for 80 s). From a holding potential of –80 mV, a depolarizing test pulse to 20 mV (300 ms) was used to activate hERG channels, and a second repolarizing step to –40 mV (300 ms) elicited outward tail currents. After a control measurement, the oocyte was exposed to 10 µmol·L$^{-1}$ atomoxetine for 10 min without pulsing. Only one oocyte was used at each rate. Relative block was determined by comparing peak tail current amplitudes with and without atomoxetine ($n = 6$). Neither the development of block nor the amount of steady-state block was frequency-dependent ($P > 0.05$) (data not shown).

### *Atomoxetine did not significantly alter hERG channel inactivation kinetics*

The effect of atomoxetine on the time course of channel inactivation was investigated using a three-stage voltage protocol. From a holding potential of –80 mV, a long constant test pulse to +40 mV (900 ms) was applied to inactivate the channels. Channels were then reopened by a short repolarizing step to –100 mV (16 ms). Subsequently, variable voltage steps to potentials ranging from –60 to +40 mV (150 ms, in 20 mV increments) were applied, eliciting large re-inactivating currents. Control re-inactivating currents (Figure 6A) and currents with 10 µmol·L$^{-1}$ atomoxetine (Figure 6B) were fitted to single exponential functions to obtain time constants of inactivation (Figure 6C, $n = 6$). Time constants of inactivation showed a voltage-dependence in both groups and there was no significant difference between time constants of channel inactivation ($P > 0.05$), due to atomoxetine.

Second, the degree of steady-state inactivation was measured with the following protocol: channels were inactivated at a holding potential of +20 mV before short test pulses

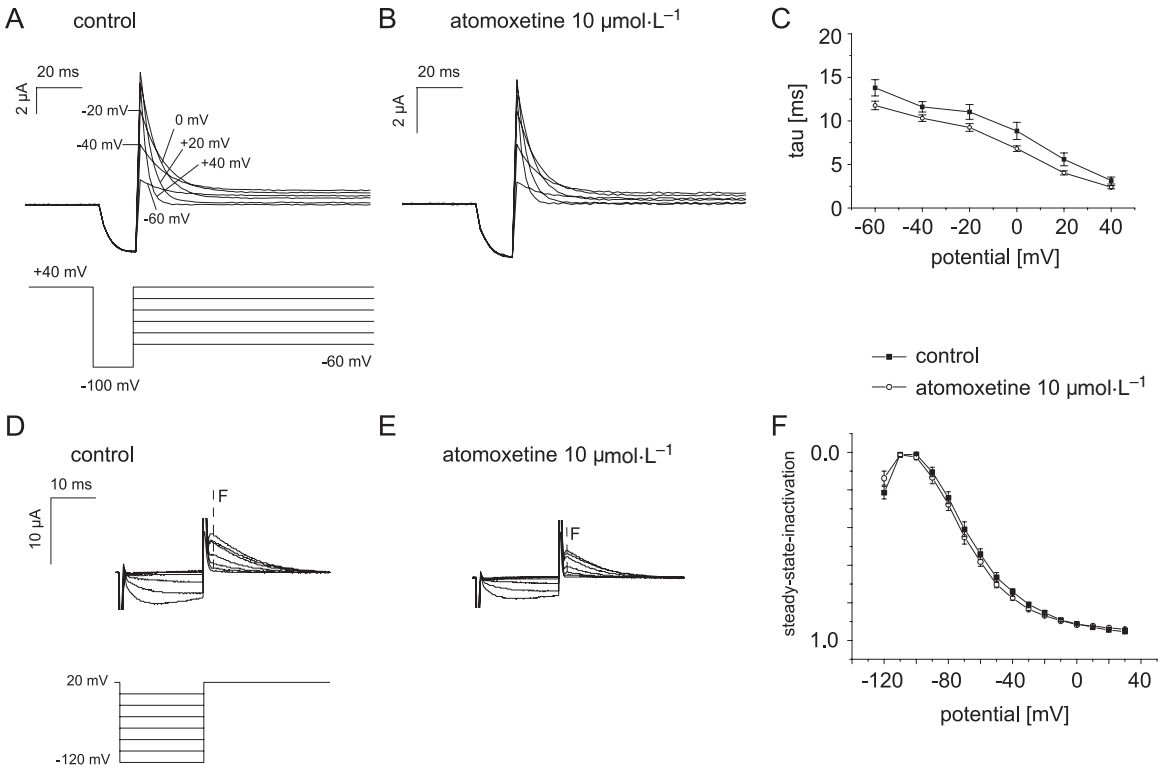


**Figure 6** hERG inactivation kinetics were not altered by atomoxetine. Direct inactivating currents were measured under control conditions and with atomoxetine (A,B). Current traces were fitted to single exponential functions and time constants of channel inactivation were plotted as a function of the test pulse potential (C). Atomoxetine caused no significant changes in time constants of direct channel inactivation ($P > 0.05$). Protocol: first test pulse +40 mV (900 ms), followed by a second test pulse to –100 mV (16 ms). Re-inactivating currents were elicited by a third test pulse to potentials ranging from –60 to +40 mV (150 ms) in 20 mV increments. The holding potential was –80 mV. Second, the degree of steady-state inactivation was determined. From a holding potential of +20 mV, cells were subject to a single-stage voltage protocol. Typical current traces are displayed for control conditions and after application of atomoxetine (D,E). Only selected current traces are presented in (D) and (E) in order to achieve a clearer presentation. Outward current amplitudes were measured 2 ms after return to +20 mV (see dashed line), normalized and plotted as a function of the preceding test pulse potential in (F). No significant shift of the inactivation curve was observed. Protocol: holding potential +20 mV, test pulse potentials ranging from –120 to +30 mV in 10 mV increments (15 ms). Return pulses to +20 mV, evoking outward inactivating currents.

to potentials ranging from –120 to +30 mV (15 ms, 10 mV increments) were applied to recover the channels from inactivation. Returning to the holding potential of +20 mV after those test pulses evoked large outward inactivating currents (Figure 6D,E). The oocyte was clamped at a holding potential of –80 mV during the wash-in period (10 min, 10 µmol·L$^{-1}$ atomoxetine). This was necessary to avoid destruction of the cell during long periods of depolarization. Outward current amplitudes measured 2 ms after return to the holding potential (dashed lines in Figure 6D,E) were normalized and fitted to a Boltzmann function. Atomoxetine did not significantly affect the half-maximal inactivation voltage of hERG channels ($n = 5$, Figure 6F).

*Atomoxetine prolonged action potential duration in guinea pig isolated cardiomyocytes*

To analyse the physiological relevance of atomoxetine-induced hERG block, action potentials were recorded in freshly isolated guinea pig left ventricular cardiomyocytes (Figure 7A). Under control conditions, mean action potential duration ($APD_{90}$) was 662 ± 107 ms ($n = 10$). Application of 1 and 3 µmol·L$^{-1}$ atomoxetine prolonged APD at 20%, 50% and 90% repolarization in a dose-dependent manner (Figure 7B). Interestingly, action potential prolongation was more pronounced at early repolarization ($APD_{20}$), suggesting the absence of atomoxetine-induced action potential triangulation.

*Atomoxetine affects hERG channel surface expression in a HEK cell line*

Reduced expression of hERG channels on the cell surface is increasingly recognized as a mechanism of drug-induced QT prolongation and has been reported for a variety of QT prolonging drugs (Ficker *et al.*, 2004; Kuryshev *et al.*, 2005; Rajamani *et al.*, 2006; Takemasa *et al.*, 2008). Effects of atomoxetine on channel surface expression were analysed using the Western blot technique. HEK cells were incubated with either control medium or atomoxetine-containing medium (1, 3, 10, 30, 100 µmol·L$^{-1}$) for 24 h. Figure 8A (upper panel) shows a representative result of the Western blot analysis. Under control conditions, a protein band at ~135 kD (core glycosylated hERG protein) and a protein band at ~155 kD (complex glycosylated hERG protein) could be detected. $As_2O_3$ (100 µmol·L$^{-1}$; a positive control for drug-induced traf-

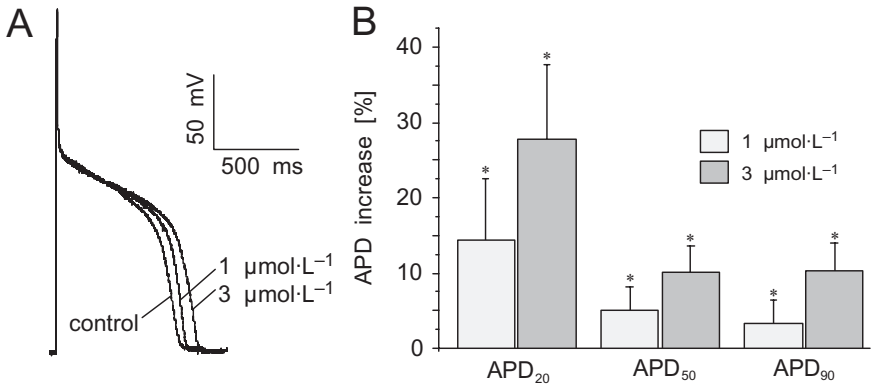


**Figure 7** Action potential prolongation by atomoxetine. A. Application of increasing atomoxetine concentrations induced action potential prolongation in freshly isolated left ventricular cardiomyocytes from guinea pigs. Cells were stimulated with a short supra-threshold stimulus at a frequency of 0.5 Hz. B. Application of increasing atomoxetine concentrations (1 and 3 µmol·L$^{-1}$) resulted in a dose-dependent action potential prolongation. Action potential prolongation was measured for 20%, 50% and 90% repolarization. Application of 1 µmol·L$^{-1}$ atomoxetine resulted in an action potential prolongation at 20%, 50% and 90% repolarization. Effects of 3 µmol·L$^{-1}$ atomoxetine resulted in increases at 20%, 50% and 90% repolarization. *$P < 0.05$, *t*-test; $n = 6$–8. Action potential prolongation by atomoxetine was more pronounced for the plateau phase than phase 3 repolarization, thereby inducing no triangulation of the action potential.

ficking defects) resulted in a significant reduction of the fully glycosylated form of the hERG channel protein (155 kD) (Figure 8A, lane 1, $n = 5$ blots). Compared with control conditions, increasing atomoxetine concentrations resulted in a dose-dependent reduction of the 155 kD hERG form (Figure 8A, lanes 2 to 7, $n = 6$ blots). However, this effect did not reach the level of significance within the range of therapeutically relevant atomoxetine concentrations. Of note, there was no significant difference between the core glycosylated forms (135 kD) of all groups (Figure 8A, lanes 1 to 7, $P > 0.05$, $n = 5$–6 blots).

To confirm that incubation with high atomoxetine concentrations results in a reduction of hERG channel surface expression, current density was measured in HEK cells stably expressing hERG channels. Cells were incubated with either control medium or atomoxetine-containing medium (3 and 100 µmol·L$^{-1}$) over 12 h. On the day of the experiment, cells were washed with control medium and placed in standard external solution for electrophysiological recordings. Current density yielded 16 ± 3.9 pA/pF in cells pre-incubated with control medium (Figure 8B, $n = 4$). Overnight pre-incubation with 3 µmol·L$^{-1}$ atomoxetine did not significantly affect current density ($n = 6$, $P > 0.05$). However, in cells pre-incubated with 100 µmol·L$^{-1}$ atomoxetine, current density was significantly reduced, confirming the observed drug-induced reduction of cell-surface expression of hERG channels, at high atomoxetine concentrations (Figure 8B, $n = 6$, $P < 0.05$).

Results from Western blot experiments and current density recordings both point to a reduction of channel surface expression under high atomoxetine concentrations. To further analyse the effect of atomoxetine on hERG channel processing, the influence of atomoxetine on hERG mRNA levels was quantified. HEK cells were treated with either control medium or atomoxetine-containing medium (3 or 100 µmol·L$^{-1}$) for 24 h. hERG mRNA levels were determined with the quantitative reverse transcriptase PCR technique, using β-actin as an internal control. Whereas 3 µmol·L$^{-1}$ atomoxetine did not affect gene expression, 100 µmol·L$^{-1}$ atomoxetine resulted in a significant reduction of hERG mRNA (Figure 8C; $n = 3$).

## Discussion

To our knowledge, this is the first study to demonstrate pharmacological effects of the selective noradrenaline reuptake inhibitor atomoxetine on the electrophysiology and cell-surface expression of cloned hERG potassium channels. The results add to the understanding of the cardiovascular risk profile of atomoxetine.

*Atomoxetine concentrations* in vivo *and* in vitro

When administered orally, atomoxetine is rapidly absorbed from the gastrointestinal tract (Simpson and Plosker, 2004). However, bioavailability strongly depends on the ability of the cytochrome P450 (CYP) 2D6 enzyme to metabolize atomoxetine to its major active metabolite 4-hydroxy-atomoxetine (Simpson and Plosker, 2004). Investigating plasma kinetics of atomoxetine, marked differences in CYP2D6 activity were found between individuals, due to polymorphic enzyme expression (poor and extensive metabolizers). About 7% of the Caucasians were found to be poor metabolizers, yielding 8- to 10-fold higher plasma levels of atomoxetine (Simpson and Plosker, 2004). A study in healthy subjects yielded peak plasma levels of atomoxetine between 1 and 3 µmol·L$^{-1}$, depending on CYP2D6 activity, whereas plasma levels of up to 27 µmol·L$^{-1}$ have been reported in cases of atomoxetine overdose. At therapeutic dosages, approximately 98% of atomoxetine is bound to plasma proteins (Simpson and Plosker, 2004). Thus, levels of up to 0.5 µmol·L$^{-1}$ of unbound atomoxetine are possible in cases of atomoxetine overdose. Taking these values into account, the $IC_{50}$ measured in HEK cells seems high. However, based on large samples of *in vitro* data and clinical reports about a broad spectrum of

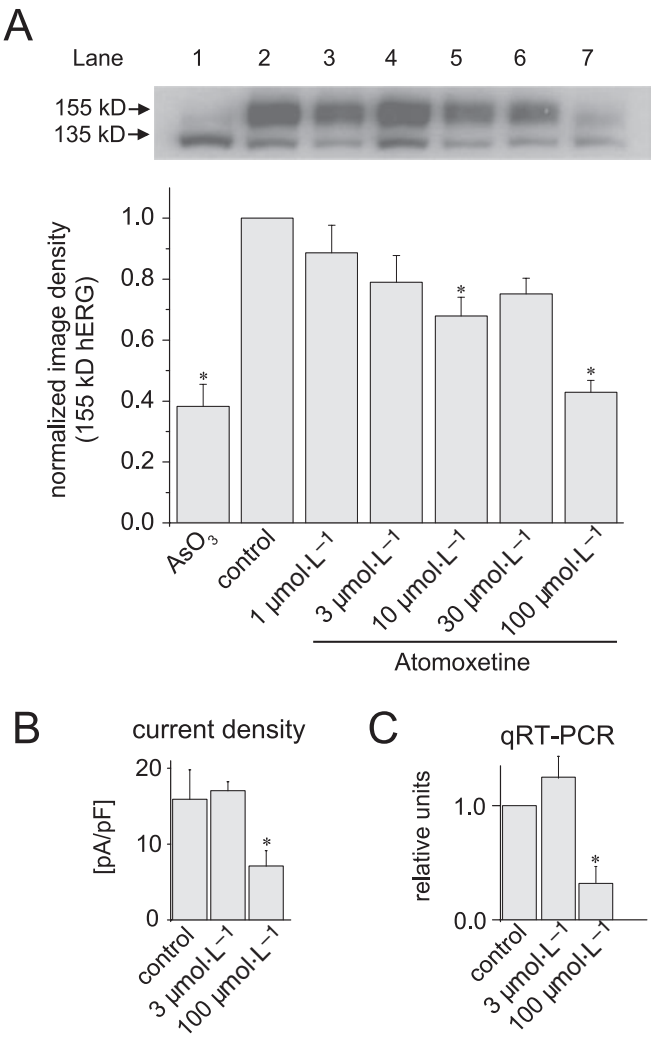


**Figure 8** Atomoxetine, at high concentrations, reduced cell-surface expression of human *Ether-à-Go-Go*-Related Gene (hERG) channels. (A) Effects of atomoxetine on channel surface expression analysed by the Western blot technique. Image density of the 155 kD hERG form was determined to quantify cell-surface expression of channels. Incubation with 100 µmol·L$^{-1}$ $As_2O_3$ in a significant reduction of the fully glycosylated hERG form (lane 1). Increasing atomoxetine concentrations resulted in a dose-dependent decrease of channel expression that was significant for 10 and 100 µmol·L$^{-1}$. *$P < 0.05$, ANOVA, $n = 5–6$. (B) Effects of high atomoxetine concentrations on channel surface expression were further analysed by current density measurements in a human embryonic kidney cell line after overnight pre-incubation with atomoxetine. While therapeutically relevant atomoxetine levels (3 µmol·L$^{-1}$) showed no effect on current density, high atomoxetine concentrations significantly reduced hERG current density. (C) Whereas hERG mRNA was not significantly reduced after overnight pre-incubation with 3 µmol·L$^{-1}$ atomoxetine, quantitative reverse transcriptase polymerase chain reaction revealed a reduction of hERG mRNA at high atomoxetine concentrations (100 µmol·L$^{-1}$). *$P < 0.05$, ANOVA, $n = 3$.

QT-prolonging drugs, there is a proposal that a safety factor of 30-fold is needed between plasma concentrations and the $IC_{50}$ of block in mammalian cells to make the induction of arrhythmias unlikely (Redfern *et al.*, 2003). Analysing the effects of atomoxetine on action potentials from freshly isolated guinea-pig cardiomyocytes, a moderate but significant action potential prolongation was observed. It has been suggested that triangulation of the action potential is one of the main determinants of drug-induced pro-arrhythmia (Hondeghem *et al.*, 2001). We show that atomoxetine preferentially prolongs plateau phase with only minor effects on phase 3 repolarization, thereby inducing no triangulation of the action potential. Considering these effects as well as the lack of reverse use-dependence of atomoxetine-induced block, the pro-arrhythmic potential of atomoxetine seems rather low. As all current recordings in this pharmacological study were performed at room temperature, we decided to conduct the action potential recordings at the same temperature to allow a direct comparison between the pharmacological effects. However, considering that baseline values of action potential recordings are shorter at physiological temperatures, it cannot be excluded that pharmacological effects of atomoxetine might differ at body temperature. However, our results are in good concordance with the observed moderate, but highly significant, QT prolongation observed in a pre-clinical safety trial (Wernicke *et al.*, 2003). Considering these results, it remains to be seen if atomoxetine is able to induce malignant arrhythmia and severe repolarization changes in patients without any additional risk factors for acquired long-QT syndrome. However, atomoxetine use should be avoided or carefully monitored in patients with pre-existing QT prolongation, a history of drug-induced QT prolongation or additional risk factors for the development of repolarization changes.

*Acute effects of atomoxetine*

We found that hERG channels were blocked by atomoxetine in a dose-dependent manner ($IC_{50}$ of 6.3 µmol·L$^{-1}$). Onset of inhibition was found to be rapid and partly reversible upon washout. In order to elucidate the biophysical properties of hERG block, we assayed the state-dependence of block. For hERG channels, a five-state model has been suggested, including an open, an inactivated and several closed states (Kiehn *et al.*, 1999). The transition from the closed to the open states is thought to be mediated by an intracellular activation gate, whereas channels inactivation seems to depend on an extracellular gate (Vandenberg *et al.*, 2004). For many drugs tested so far, inhibition was shown to occur in the open and inactivated, but not in the closed states (Zitron *et al.*, 2002; Scholz *et al.*, 2003; Scholz *et al.*, 2005). Thus, it is tempting to speculate that these drugs, like atomoxetine, enter the channel cavity from the intracellular site via the activation gate. In order to further analyse binding properties of atomoxetine within the channel cavity, we utilized two hERG mutants lacking in the aromatic amino acids Y656 or F652 respectively. It is widely accepted that these aromatic residues play a major role in drug binding properties of numerous molecules (Mitcheson *et al.*, 2000). So far, for most of the drugs tested, there was a significant loss of inhibitory effects found in mutant hERG channels lacking either aromatic residue. For atomoxetine also, there was a significant loss of inhibitory effects in both the Y652A and F656A mutants of the hERG channels.

*Subacute effects of atomoxetine*

hERG channel block by cardiac and non-cardiac drugs is a well-established mechanism for the development of acquired

long-QT syndrome (Redfern *et al.*, 2003). However, over the last few years it has become clear that a reduction of channel surface expression can result in a prolongation of the QT interval as well (Ficker *et al.*, 2004; Kuryshev *et al.*, 2005; Rajamani *et al.*, 2006; Takemasa *et al.*, 2008). Recently described examples of this dual effect on hERG channels include $As_2O_3$, pentamidine, ketoconazole and fluoxetine (Ficker *et al.*, 2004; Kuryshev *et al.*, 2005; Rajamani *et al.*, 2006; Takemasa *et al.*, 2008). Considering the close structural relationship between atomoxetine and fluoxetine, we analysed effects of atomoxetine on gene expression and surface expression of hERG channels. In contrast to fluoxetine, atomoxetine did not affect channel surface expression within the range of physiological relevance. However, using high atomoxetine concentrations a significant reduction of channel surface expression could be detected by Western blot analysis and by current density measurements. This effect might be due to defective protein synthesis, defective channel trafficking or an increased channel degradation. Analysing hERG channel mRNA levels, we found a reduction of hERG gene expression after incubation with high atomoxetine concentrations. Taken together, our findings point to a reduction of both, mRNA and protein synthesis, by high atomoxetine concentrations. However, our results show that atomoxetine does not affect cell-surface expression of hERG channels, within the range of therapeutically relevant atomoxetine concentrations.

*Clinical implications*

Recently, there have been two independent case reports linking acute atomoxetine overdose to a marked QTc prolongation (Barker *et al.*, 2004; Sawant and Daviss, 2004). However, in both cases, atomoxetine therapy was combined with a complex co-medication with either inhibitory effect on hERG channels or on CYP2D6 activity. Furthermore, the contribution of adrenergic stimulation through inhibition of noradrenaline reuptake in the CNS on the QT interval has not been systematically analysed yet. Clinically, administration of noradrenaline reuptake inhibitors results in a decrease of RR, QRS and QT intervals, whereas the effects on corrected QT intervals seem small (Wernicke *et al.*, 2007). Although the relevance of hERG channel block seems to be negligible under therapeutic concentrations, it might become more important under specific conditions. High atomoxetine intake, slower metabolism due to enzyme polymorphisms or pharmacological CYP2D6 inhibition, as well as decreased atomoxetine clearance (impaired hepatic function, renal disease) may result in elevated atomoxetine plasma levels (Simpson and Plosker, 2004). Thus, hERG block by atomoxetine may be clinically relevant under aggravating circumstances – as demonstrated by the case reports mentioned above – and should be taken into account in individuals receiving complex co-medication.

In a large sample of more than a thousand patients, effects of atomoxetine on QT interval have been analysed (Wernicke *et al.*, 2003). By application of the Bazett correction, a mild but significant QTc prolongation was observed in children, adolescents and adults (Wernicke *et al.*, 2003). However, as no QTc prolongation was detectable when applying the Fridericia formula. Moreover, after QT interval correction using derived data, the authors stated that the QT prolongation was an artefact (Wernicke *et al.*, 2003). To our knowledge, there has been no case report linking therapeutic atomoxetine levels to marked QT prolongation or to the induction of lethal arrhythmia so far. Thus, it remains unclear whether the reported QT prolongation was an artefact due to an overcorrection for heart rate or mediated via hERG channel blockade of atomoxetine.

In conclusion, this study provides a plausible molecular basis for the QT prolongation observed in cases of atomoxetine intoxication. So far, inhibitory effects of atomoxetine on hERG-conducted, $I_{Kr}$ current have not been reported. These results might add to the understanding of cardiac adverse effects in cases of atomoxetine overdose. hERG channel block by atomoxetine should be considered especially when treating ADHD patients with additional risk factors for acquired long-QT syndrome.

## Acknowledgements

The skilful assistance of Ramona Bloehs is gratefully acknowledged. This work was supported by a grant of the Deutsche Forschungsgemeinschaft KA 1714/1-2 to Dr. Karle and TH1120/1-1 to Dr. Thomas. ES, CS and EZ were supported by the Young Investigator Award of the Medical Faculty Heidelberg.

## Conflicts of interest

None.

## References

Alexander SPH, Mathie A, Peters JA (2008). Guide to receptors and channels (GRAC), 3rd edn. *Br J Pharmacol* **153** (Suppl. 2): S1–S209.

Barker MJ, Benitez JG, Ternullo S, Juhl GA (2004). Acute oxcarbazepine and atomoxetine overdose with quetiapine. *Vet Hum Toxicol* **46**: 130–132.

Curran ME, Splawski I, Timothy KW, Vincent GM, Green ED, Keating MT (1995). A molecular basis for cardiac arrhythmia: hERG mutations cause long QT syndrome. *Cell* **80**: 795–803.

Ficker E, Kuryshev YA, Dennis AT, Obejero-Paz C, Wang L, Hawryluk P *et al.* (2004). Mechanisms of arsenic-induced prolongation of cardiac repolarization. *Mol Pharmacol* **66**: 33–44.

Hassel D, Scholz EP, Trano N, Friedrich O, Just S, Meder B *et al.* (2008). Deficient zebrafish ether-à-go-go-related gene potassium channel gating causes short-qt syndrome in zebrafish reggae mutants. *Circulation* **117**: 866–875.

Hondeghem LM, Carlsson L, Duker G (2001). Instability and triangulation of the action potential predict serious proarrhythmia, but action potential duration prolongation is antiarrhythmic. *Circulation* **103**: 2004–2013.

Kiehn J, Lacerda AE, Brown AM (1999). Pathways of hERG inactivation. *Am J Physiol* **277**: H199–210.

Kuryshev YA, Ficker E, Wang L, Hawryluk P, Dennis AT, Wible BA *et al.* (2005). Pentamidine-induced long QT syndrome and block of hERG trafficking. *J Pharmacol Exp Ther* **312**: 316–323.

Mitcheson JS, Chen J, Lin M, Culberson C, Sanguinetti MC (2000). A

structural basis for drug-induced long QT syndrome. *Proc Natl Acad Sci USA* **97**: 12329–12333.

Rajamani S, Eckhardt LL, Valdivia CR, Klemens CA, Gillman BM, Anderson CL *et al*. (2006). Drug-induced long QT syndrome: hERG K+ channel block and disruption of protein trafficking by fluoxetine and norfluoxetine. *Br J Pharmacol* **149**: 481–489.

Redfern WS, Carlsson L, Davis AS, Lynch WG, MacKenzie I, Palethorpe S *et al*. (2003). Relationships between preclinical cardiac electrophysiology, clinical QT interval prolongation and torsade de pointes for a broad range of drugs: evidence for a provisional safety margin in drug development. *Cardiovasc Res* **58**: 32–45.

Roden DM, Balser JR, George ALJ, Anderson ME (2002). Cardiac ion channels. *Annu Rev Physiol* **64**: 431–475.

Sanguinetti MC, Jiang C, Curran ME, Keating MT (1995). A mechanistic link between an inherited and an acquired cardiac arrhythmia: hERG encodes the IKr potassium channel. *Cell* **81**: 299–307.

Sawant S, Daviss SR (2004). Seizures and prolonged QTc with atomoxetine overdose. *Am J Psychiatry* **161**: 757.

Scholz EP, Zitron E, Kiesecker C, Lueck S, Kathöfer S, Thomas D *et al*. (2003). Drug binding to aromatic residues in the hERG channel pore cavity as possible explanation for acquired long QT syndrome by antiparkinsonian drug budipine. *Naunyn Schmiedebergs Arch Pharmacol* **368**: 404–414.

Scholz EP, Zitron E, Kiesecker C, Lück S, Thomas D, Kathöfer S *et al*. (2005). Inhibition of cardiac hERG channels by grapefruit flavonoid naringenin: implications for the influence of dietary compounds on cardiac repolarisation. *Naunyn Schmiedebergs Arch Pharmacol* **371**: 516–525.

Simpson D, Plosker GL (2004). Atomoxetine: a review of its use in adults with attention deficit hyperactivity disorder. *Drugs* **64**: 205–222.

Spencer TJ, Biederman J, Wilens TE, Faraone SV (2002). Novel treatments for attention-deficit/hyperactivity disorder in children. *J Clin Psychiatry* **63** (Suppl. 12): 16–22.

Takemasa H, Nagatomo T, Abe H, Kawakami K, Igarashi T, Tsurugi T *et al*. (2008). Coexistence of hERG current block and disruption of protein trafficking in ketoconazole-induced long QT syndrome. *Br J Pharmacol* **153**: 406–409.

Trudeau MC, Warmke JW, Ganetzky B, Robertson GA (1995). hERG, a human inward rectifier in the voltage-gated potassium channel family. *Science* **269**: 92–95.

Vandenberg JI, Torres AM, Campbell TJ, Kuchel PW (2004). The hERG K+ channel: progress in understanding the molecular basis of its unusual gating kinetics. *Eur Biophys J* **33**: 89–97.

Wernicke J, Lledó A, Raskin J, Kajdasz DK, Wang F (2007). An evaluation of the cardiovascular safety profile of duloxetine: findings from 42 placebo-controlled studies. *Drug Saf* **30**: 437–455.

Wernicke JF, Kratochvil CJ (2002). Safety profile of atomoxetine in the treatment of children and adolescents with ADHD. *J Clin Psychiatry* **63** (Suppl. 12): 50–55.

Wernicke JF, Faries D, Girod D, Brown J, Gao H, Kelsey D *et al*. (2003). Cardiovascular effects of atomoxetine in children, adolescents, and adults. *Drug Saf* **26**: 729–740.

Zitron E, Karle CA, Wendt-Nordahl G, Kathöfer S, Zhang W, Thomas D *et al*. (2002). Bertosamil blocks hERG potassium channels in their open and inactivated states. *Br J Pharmacol* **137**: 221–228.