Blockade of HERG and Kv1.5 by Ketoconazole

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ABSTRACT

Ketoconazole, a widely used fungicide in patients, has been associated with Q-T prolongation and torsade de pointes when co-administered with terfenadine (Seldane). Both compounds use the same cytochrome-P450 metabolic pathway, resulting in an increase in plasma concentration of terfenadine. We previously showed that terfenadine blocked HERG (Human Ether-a-Gogo Related Gene), an important component of the repolarizing cardiac delayed rectifier I\textsubscript{Kr} with concentration needed to obtain 50% of the block (IC\textsubscript{50} in the therapeutic range (300 nM). Another target is Kv1.5 (delayed outward rectifier potassium current), an important component of human atrial ultra-rapid delayed rectifier current. Whether Kv1.5 and HERG proteins are direct targets for ketoconazole has yet to be addressed. We heterologously expressed HERG and Kv1.5 in Xenopus oocytes and compared their sensitivities to ketoconazole. HERG and Kv1.5 currents were reduced comparably with apparent IC\textsubscript{50} values of 49 \textmu M and 107 \textmu M, respectively, when measured using the two-microelectrode recording technique. The differences in the IC\textsubscript{50} may help explain the preferential ventricular origin of the ketoconazole-associated arrhythmias during overdose. The mechanism of block was different between Kv1.5 and HERG. Cumulative application of terfenadine and ketoconazole at their respective IC\textsubscript{50} concentrations resulted in current reductions that suggest an additive rather than a competitive type of block by the two drugs. We conclude that ketoconazole may potentiate the effects of terfenadine first by an indirect pharmacokinetic action to elevate plasma levels and second by a direct pharmacodynamic action on HERG currents. These potential dual actions on HERG currents suggest that precautions should be taken in long-term ketoconazole treatment, particularly for patients who have decreased liver function or are on a drug regimen requiring simultaneous medications that use cytochrome-P450 for breakdown, such as terfenadine or erythromycin, or Class III antiarrhythmic drugs.

Ketoconazole is an antifungal agent used for treatment of disorders ranging from toe nail fungus to dandruff. Members of the conazole family inhibit the C-14 \textalpha-demethylase enzyme that converts lanosterol to ergosterol, an important component of fungal cell membranes (Kimura \textit{et al.}, 1992). Ketoconazole, however, is a potent inhibitor of hepatic P450 enzymes (Honig \textit{et al.}, 1993; Jurima-Romet \textit{et al.}, 1994), the most frequently reported side effects being related to endocrine physiology because P450 enzymes are involved in the synthesis of adrenal and gonadal steroid hormones (Santen \textit{et al.}, 1983; Loose \textit{et al.}, 1983; Pont \textit{et al.}, 1997). In spite of these side effects, ketoconazole is widely used in long-term treatment of blastomycosis, histoplasmosis and coccidioidomycosis (Simons and Simons, 1997) because of its low cost (Como and Dismukes, 1994).

Severe warnings are given against the concomitant administration of conazoles and compounds that use the same CYP3A4-P450 metabolic pathway. Among them, terfenadine (Seldane), historically one of the most widely prescribed antihistamines, has been linked to prolonged Q-T intervals in the EKG. Q-T prolongation may be associated with polymorphic ventricular arrhythmias such as torsade de pointes and death (Davies \textit{et al.}, 1989). The buildup of terfenadine to high plasma concentrations (~ 100 nM) (Davies \textit{et al.}, 1989) after reduction of its metabolism by conazoles seems to be the cause of its cardiotoxicity. A similar effect is also observed in patients who have taken macrolide antibiotics, such as erythromycin. Other causes of toxic plasma concentrations include excessive intake and impaired liver function.

Recently we reported that terfenadine blocks currents produced by two human cardiac potassium channel clones, HERG and Kv1.5, as expressed in Xenopus oocytes. Kv1.5 appears to be responsible for I\textsubscript{Kur} in human atrium, and block by terfenadine has an IC\textsubscript{50} of 3 \textmu M (Daneshmend and Warrack, 1983; Rampe \textit{et al.}, 1993; Roy \textit{et al.}, 1996). HERG, the human ether-a-go-go-related channel, is responsible for I\textsubscript{Kr} in ventricle (Sanguinetti \textit{et al.}, 1995; Curran \textit{et al.}, 1995) and is blocked by terfenadine with an IC\textsubscript{50} of 300 nM (Roy \textit{et al.}, 1996).
Ca defolliculated by collagenase treatment (2 mg/ml for 1.5 h) in a MgCl2 1, HEPES 5, gentamicin 100 mg/ml; pH 7.6 (NaOH-HCl). The oocytes were only injected with 46 nl of cRNA solution (in mM): NaCl 100, KCl 2, CaCl2 1.8, MgCl2 1, HEPES 5, pyruvic acid, pH 7.2 (TRIS), to give tip resistance of 0.2 to 0.5 M. Oocytes were placed in a chamber and perfused with Ringer’s solution containing (in mM): NaCl 120, KCl 2.5, CaCl2 1.1, EGTA 1.0, HEPES-agarose solution containing 3 M KCl, 10 mM HEPES and 10 mM EGTA, pH 7.4. Whole-cell currents were recorded from Xenopus oocytes using the conventional two-microelectrode voltage clamp technique. Beveled microelectrodes were filled with a 0.3% agarose solution containing 3 M KCl, 10 mM HEPES and 10 mM EGTA, pH 7.4 (TRIS), to give tip resistance of 0.2 to 0.5 MΩ. Oocytes were placed in a chamber and perfused with Ringer’s solution containing (in mM): NaCl 120, KCl 2.5, CaCl2 1.1, EGTA 1.0, HEPES-acid 10; pH 7.2 (NaOH). Stock solutions of ketoconazole (5 mM) and defollicination of cDNA was obtained by transformation into E. coli and overnight incubation at 37°C. HERG cDNA was linearized by digestion with EcoR1 for runoff transcription with the SP6 mMessage mMachine in vitro transcription kit (Ambion, Austin, TX). Kv1.5 was previously cloned in our laboratory (Fedida et al., 1993) and sub-cloned into A’-pCRII (Taglialatela et al., 1994). The final cRNA product was resuspended in 0.1 M KCl and stored at −80°C. The cRNA was diluted to the desired concentration (5–150 pg/ml) immediately before oocyte injection. Stage V to VI Xenopus oocytes were defolliculated by collagenase treatment (2 mg/ml for 1.5 h) in a Ca2+-free buffer solution containing (in mM): NaCl 82.5, KCl 2.5, MgCl2 1, HEPES 5, gentamicin 100 mg/ml; pH 7.6 (NaOH-HCl). The defolliculated oocytes were injected with 46 nl of cRNA solution (in 0.1 M KC1) and incubated at 19°C in culture medium containing (in mM): NaCl 100, KCl 2, CaCl2 1.8, MgCl2 1, HEPES 5, pyruvic acid, 2.5, gentamicin 100 mg/ml; pH 7.6. Electrophysiological measurements were made 3 to 7 days after cRNA injection. All experiments were conducted according to institutional animal care committee regulations.

Molecular biology. The HERG clone was a gift from Dr. M. T. Keating. The full-length CDNA was cloned into the pSP64 transcription vector as previously described (Sanguinetti et al., 1995). Amplification of cDNA was obtained by transformation into E. coli and overnight incubation at 37°C. HERG cDNA was linearized by digestion with EcoR1 for runoff transcription with the SP6 mMessage mMachine in vitro transcription kit (Ambion, Austin, TX). Kv1.5 was previously cloned in our laboratory (Fedida et al., 1993) and sub-cloned into A’-pCRII (Taglialatela et al., 1994). The final cRNA product was resuspended in 0.1 M KCl and stored at −80°C. The cRNA was diluted to the desired concentration (5–150 pg/ml) immediately before oocyte injection. Stage V to VI Xenopus oocytes were defolliculated by collagenase treatment (2 mg/ml for 1.5 h) in a Ca2+-free buffer solution containing (in mM): NaCl 82.5, KCl 2.5, MgCl2 1, HEPES 5, gentamicin 100 mg/ml; pH 7.6 (NaOH-HCl). The defolliculated oocytes were injected with 46 nl of cRNA solution (in 0.1 M KC1) and incubated at 19°C in culture medium containing (in mM): NaCl 100, KCl 2, CaCl2 1.8, MgCl2 1, HEPES 5, pyruvic acid, 2.5, gentamicin 100 mg/ml; pH 7.6. Electrophysiological measurements were made 3 to 7 days after cRNA injection. All experiments were conducted according to institutional animal care committee regulations.

Materials and Methods

Results

Although therapeutic concentrations of terfenadine (nM) have been shown to block HERG and I_{Kur} in human atrial myocytes, as well as Kv1.5 current expressed in transfectected human embryonic kidney cells or Xenopus oocytes (Daneshmand and Warnock, 1983; Rampe et al., 1993; Roy et al., 1996), no direct effects of ketoconazole on Kv1.5 or HERG currents have been reported. We therefore heterologously expressed human Kv1.5 and HERG in Xenopus oocytes to compare the block by ketoconazole with that of terfenadine in the same expression system.

HERG and Kv1.5 currents were reduced by ketoconazole in a dose-dependent manner and reached a steady-state level within 10 min when perfusing the oocytes at a rate of 3 ml/min. The external solution in the chamber was completely exchanged within 2 min as measured from the effects of changes of potassium concentration on holding and peak currents (not shown). We monitored the onset of the block by applying a standard 800-ms pulse to 0 mV from a holding potential of −80 mV every 30 s. After an initial perfusion of 1 min without stimulation, block was clearly observed, which showed that blockade does not require that channels be activated. Measurements were done once the block reached a steady state, usually within 10 min of perfusion with the drug. The block on HERG and Kv1.5 was fully reversible within 20 min for the lower doses and partially reversible (80%) in that time frame with 500 μM.

Ketoconazole reduced HERG current elicited by depolarization from a holding potential of −80 mV in a concentration-dependent manner, 100 μM ketoconazole blocking the HERG steady-state current by 60% (fig. 1A). Ketoconazole similarly reduced HERG maximal tail currents (fig. 1C), with respective 50% inhibition concentrations (IC_{50}) for steady-
state current and tail current of 49 ± 13 μM and 31 ± 2 μM, respectively, as fitted by a 1:1 binding isotherm (fig. 1C).

Kv1.5 currents elicited by the application of 350-ms depolarizing steps in 5-mV increments from a holding potential of −60 mV (fig. 1B) were as previously described (Daneshmend and Warnock, 1983; Rampe et al., 1993; Roy et al., 1996; Fedida et al., 1993; Wang et al., 1993), with activation developing between −20 and 20 mV (Fedida et al., 1993; Wang et al., 1993) and an ohmic increase in current at more depolarized potentials. As seen with HERG, ketoconazole reduced the Kv1.5 steady-state current in a dose-dependent fashion with 55% block at a concentration of 100 μM. We found an IC50 value of 107 ± 5 μM (fig. 1C).

Absence of voltage-dependent block. HERG currents recorded using the two-electrode voltage-clamp technique exhibited voltage-dependent properties as previously reported (Roy et al., 1996; Sanguinetti et al., 1995). Briefly, the current activated at potentials greater than −40 mV, reached a peak at 0 mV and then decreased at more positive potentials (fig. 1A; 2A) giving the steady-state I/V relationship its typical bell-shaped appearance (fig. 2A). The “tail” current, generated after the stimulating pulse has been completed, increased with voltage and then plateaued for test potentials positive to +10 mV as previously reported (Roy et al., 1996; Sanguinetti et al., 1995).

The possibility of voltage-dependent block in HERG was verified by comparing the I-V curves for different concentrations of ketoconazole. When individual peak test or tail currents were normalized to the maximal control amplitude, we did not find significant changes in the morphology of the I-V relationships (fig. 2A, and B). The fraction of control tail and peak test currents blocked by ketoconazole (fig. 2C, and D)

Fig. 1. Dose response of Kv1.5 and HERG currents expressed in Xenopus oocytes to ketoconazole. A) Representative family of currents from two-electrode voltage-clamp recordings generated using the pulse protocol shown. The oocyte was pulsed from a holding potential of −80 mV to test potentials from −60 to +40 mV, in 10-mV increments for 800 ms. In the same oocyte, 100 μM ketoconazole reduced steady-state and tail currents by 60% and 70%, respectively, within 7 min. B) Kv1.5 currents elicited by 350-ms depolarizing pulses from −40 to 50 mV (5-mV increments) were blocked by 60% after addition of 100 μM ketoconazole to the perfusion solution. C) HERG currents recorded at 0 mV in the presence of varying concentrations of ketoconazole were normalized to the control amplitude and plotted as a concentration of the drug to yield the dose response. Steady-state currents (filled circle) were measured at the end of the stimulating pulse (800 ms), and peak tail currents (filled down triangle) were measured upon return to the holding potential. Kv1.5 currents (open triangle) were measured at the end of the stimulating pulse and normalized in a similar fashion. The dose-response curves for both HERG steady-state and tail currents yielded IC50 values of 31 ± 2 μM and 49 ± 13 μM, respectively (n = 4) using a 1:1 binding isotherm to fit the data. We obtained 50% reduction of Kv1.5 current with 107 ± 5 μM (n = 5). Data are expressed as mean ± S.E.M.

Fig. 2. Ketoconazole block of HERG steady-state and tail current is not voltage-dependent. A) Relative current-voltage relationships for steady-state HERG current in control (n = 8), 2 μM (n = 4) and 100 μM (n = 6) ketoconazole. B) Tail currents are similarly plotted. C and D) Fraction of tail and steady-state control currents blocked at every given potential. The differences between the control and the treated current amplitudes were normalized to the control value. In each case, the current reduction at 40 mV was not significantly different from the block at −20 mV. E) Averaged activation curves, as calculated from the normalized peak tail current amplitudes under control, 2 μM, 20 μM and 100 μM (n = 3) ketoconazole. No significant shift of the mid-activation potential was apparent.
did not change significantly between -20 and 40 mV. Furthermore, we did not observe a significant shift of the mid-activation potential by ketoconazole (fig. 2E), obtained from a Boltzmann fit to the figure 2B data normalized to their respective peak values. From these results we concluded that the drug did not exert a significant voltage-dependent block on HERG.

The block of Kv1.5 current by ketoconazole produced a small bending (sigmoidicity) of the I-V relationship at potentials between -20 and 20 mV (fig. 3A). The fraction of the block closely followed the activation of the channels. It exponentially increased in a region of potentials (-20 to 20 mV) where the open probability ($P_o$) and the activation kinetics of Kv1.5 exponentially increase (Fedida et al., 1993; Wang et al., 1993). The block saturated at test potentials greater than 20 mV, where $P_o$ is maximal (fig. 3B). This result suggested that the binding of the drug might depend on the state of the channel. We next looked for changes in the waveform of Kv1.5 current for a possible block developing after the channels opened (fig. 3C). We did not observe changes in the current waveform after a depolarization step to 35 mV in presence of 100 μM ketoconazole (fig. 3C). Furthermore, there was no change in the current waveform when the normalized currents were superimposed (fig. 3D). We did not observe changes in the amplitude of the block when the holding potential was changed from -80 to -50 mV (not shown). These results demonstrated that there was no open-state block in Kv1.5 channels by ketoconazole and suggested that the voltage dependence of the block was due to changes in affinity of the channels in closed states before the opening of the channels.

In contrast, ketoconazole increased the rate of decay of HERG active current during the 800-ms test pulse shown in figure 4. This effect was concentration-dependent and was more evident at strongly depolarized potentials, where HERG inactivation became more pronounced. We first tested whether this small time-dependent block was due to potentiation of the inactivation. We specifically looked for changes in the kinetics of recovery and onset from inactivation and tested for changes in the steady-state inactivation.

The rectification of HERG current has been attributed to fast inactivation of the channels (Sanguinetti et al., 1995; Spector et al., 1996). According to this hypothesis, the outward current, elicited during a strong depolarization, never reach full activation because channels inactivate rapidly.

**Fig. 3.** Ketoconazole blocks Kv1.5 as heterologously expressed in *Xenopus* oocytes. A) Current-voltage relationship of Kv1.5 in the presence of 2, 20 and 100 μM. Currents were normalized to the maximum current recorded during a 350-ms pulse to 50 mV from a holding potential of -80 mV to yield the relative current amplitude. B) The fraction of current blocked was obtained from the difference between the amplitude of the control and the drug test current normalized to the amplitude of the control current for every given potential. The block followed the voltage dependence of the activation and plateaued when the channels were fully activated. C) Control currents during a 350-ms pulse to -35 mV were reduced by 56% after application of 100 μM ketoconazole (*). D) The traces from C were normalized to their respective maximal steady-state values and superimposed. There was no significant change in the activation kinetics of the current after application of ketoconazole.

**Fig. 4.** Time-dependent block of HERG. Ketoconazole potentiates the current decay in a dose-dependent fashion after extracellular application of 20 μM and 100 μM concentrations of the drug. Currents were recorded during a depolarizing step to 40 mV from a holding potential of -80 mV.
whether through transitions from the closed states preceding activation or from the open state (Sanguinetti et al., 1995; Sanguinetti and Jurkiewicz, 1990; Trudeau et al., 1995; Spector et al., 1996; Shibasaki, 1987). Furthermore, Sanguinetti et al., (1995) proposed that recovery from inactivation was faster than deactivation and occurred via a return to the open state, a mechanism previously proposed for I_{Ks} (Sanguinetti and Jurkiewicz, 1990; Shibasaki, 1987). These authors proposed that the fast recovery from inactivation was responsible for the increased amplitude of the tail current after strong depolarizations. According to this hypothesis, the onset of the tail current primarily represents the recovery of the channels from inactivation.

To test for effects of ketoconazole on recovery from inactivation, we measured the macroscopic time constant for the onset of the tail currents (fig. 5A). At more negative potentials (−120 to −60 mV), we used two exponential functions and took the time constant for the rising phase as representative of recovery from inactivation. As shown in figure 5C (filled symbols), we did not observe significant changes after application of 50 μM ketoconazole, which suggests that the drug did not have a different affinity for the inactivated channels. We did not observe changes for all the concentrations tested. In the five cells tested, the drug did not alter the deactivation rate after the peak of the tail current. We next tested for changes in the onset of inactivation.

The cells were first depolarized to 20 mV for 1 s to inactivate the channels (fig. 5B). After the first pulse, a short repolarization (30 ms) removed inactivation without introducing significant deactivation (Smith et al., 1996). The subsequent pulse back to more depolarized potentials forced the channels previously inactivated to “re-inactivate.” The time course of the current during the return pulses (fig. 5B) therefore represents the onset of inactivation, and the amplitude of the peak current represents the number of channels inactivated by the short repolarizing pulse. We tested for the effects of ketoconazole on the onset of inactivation of HERG by fitting these currents to a single exponential decay. For the five cells tested, ketoconazole did not significantly change the calculated macroscopic time constants for the onset of inactivation (open symbols fig. 5C). We next looked for changes in the steady-state inactivation (availability) of the channels.

We used a double-pulse protocol with varying interpulse potential amplitude first to inactivate the channels and then to remove inactivation (fig. 5D). The duration of the pulses is the same as that illustrated in fig. 5B. As previously mentioned, the maximal amplitude of the current during the second pulse to 20 mV represents the number of channels inactivated during the interpulse. To obtain a reliable measure of the maximal current, we extrapolated each single exponential fit on the currents to the end of the repolarizing interpulse (thin line fig. 5D). This procedure enabled us to minimize the contribution of the capacitive artifacts to our measurements. We then plotted the amplitude of the extrapolated peak current against the interpulse potential to obtain the steady-state inactivation (fig. 5E). We fitted the raw data to a Boltzmann distribution (fig. 5E) and used the calculated asymptotic value at the plateau to normalize the currents and plot the data in figure 5F. We obtained averaged mid-inactivation potentials of −57 ± 5 and −54 ± 5 mV for the normalized control and 50 μM ketoconazole data, respectively. These results demonstrated that ketoconazole did not change the availability of the channels, and we concluded that the drug did not exert specific blockade in the inactivated channels. We next measured the time course of onset of the time-dependent block on HERG.

The decay of current during steps to strongly depolarized potentials (fig. 4) contained a component linked to inactivation overlapping with the onset of the time-dependent block. To isolate the onset of the block, we measured the peak of the repolarizing tail current after depolarizing steps of increasing duration to 40 mV (fig. 6A). This protocol removed inactivation and allowed us to measure the effects of ketoconazole on the fully activated current. Figure 6B reveals the slow onset of HERG currents and the minimal contribution of inactivation to our measurements. Addition of 50 μM ketoconazole (fig. 6B) introduced a slow decay of the amplitude of the fully activated (tail) current. To measure the onset of the block, we normalized the currents to their maximal amplitude and fitted them with a sum of two exponential functions (fig. 6C). The control currents could be well fitted by a single exponential function with a time constant of 66 ± 9 ms (n = 3). With 50 μM ketoconazole in the bath, we obtained average values of 67 ± 3 and 298 ± 6 ms, using a sum of two exponential functions. There was no significant difference in the onset of activation between control and test conditions. Because the inactivation of HERG was not altered by the drug, these results strongly suggest that a different (lower) affinity of ketoconazole for the open channels is responsible for the slow decay of the current. We next tested for possible accumulation of the block during repetitive activation of the channels (use-dependent block).

Absence of use-dependent block. Use-dependent block was assessed by applying the monitoring pulse at frequencies of 0.1 to 1 Hz, after a steady-state block was attained. At 1 Hz, a small residual portion of activated channels was carried from one pulse to the other because of the slow deactivation of HERG channels, but the relative increase in active current was the same with or without 100 μM ketoconazole in the bath. Similar results were obtained for Kv1.5 in the same conditions. Kv1.5 current usually had a higher amplitude but did not deactivate as slowly as HERG, so we observed a decrease in the amplitude of the current at high frequencies (1 Hz) possibly as a result of the increased mean time at depolarized potentials or changes of the potassium reversal potential. Thus we did not observe use-dependent block of the two channels by ketoconazole in the range of frequencies studied. Because the cardioxicity of the terfenadine-ketoconazole interaction is presently linked to pharmacokinetics effects, namely competitive effects on the CYP3A4-P450 metabolic pathway, we next tested for competitive block by terfenadine and ketoconazole on HERG (I_{Kr}).

To test for a competitive block by the two compounds, we first challenged HERG with 50 μM ketoconazole (IC_{50} = 49 μM) and then added 300 nM terfenadine (IC_{50} = 350 nM) (Roy et al., 1996) to the perfusion solution (fig. 7). Ketoconazole alone blocked HERG control current by 48.3 ± 2.6% (n = 5). Cumulative addition of terfenadine blocked the residual current by 49.1 ± 1.4% (n = 5), a value expected from the IC_{50} value found in our previous measurements (Roy et al., 1996). The block matched the reduction of initial current expected from blockade by the first drug alone plus the block on the residual current expected from the second drug alone.
a result that suggests an additive rather than a competitive type of block by the two compounds.

Simulated prolongation of the cardiac action potential by ketoconazole. Chen and Woosley (1993) reported a 15% prolongation of the cat ventricular AP by ketoconazole, and Hey et al. (1996) reported a 5% prolongation by terfenadine alone in guinea pig. The contribution of I_{Ks} (HERG) to the ventricular AP is difficult to estimate primarily because of its overlap with I_{Kr}. Furthermore, the possibility of having other endogenous currents blocked by ketoconazole makes it
difficult to determine experimentally the contribution of HERG blockade to the prolongation of the AP. Therefore, we simulated the effects of the block of IKr (HERG) by ketoconazole alone and with terfenadine, using the ventricular AP routine of the Heart Oxsoft v. 4.4 program (Oxsoft, Oxford, England) and compared these values with experimental data previously reported. We first reduced the conductance of IKr (HERG) by 20% to mimic the block by 100 nM terfenadine (Roy et al., 1996). We set the contribution of IKr to IK to 50%, for the duration of the AP plateau on the basis of previous reports (Sanguinetti and Jurkiewicz, 1990; Zeng et al., 1995).

The block of the delayed rectifier current by terfenadine was therefore 10% (fig. 8B), a value in agreement with the inhibition of IK in cardiac human ventricle as reported by Berul and Morad (1995). On the basis of our results with ketoconazole, we also simulated its effects on the ventricular AP waveform after a 30% block of HERG (15% of IK, fig. 8B). This value corresponds to a circulating concentration between 10 and 12 μM, similar to the plasma concentration reported by von Moltke (von Moltke et al., 1994) during normal use (~10 μM).

Fig. 6. Full activation time course of HERG current as obtained from a tail current envelope protocol. A) Representative current traces obtained after a series of consecutive steps to 40 mV in 50-ms increments, followed by a 500-ms return to −50 mV to maximize the tail current (protocol). Holding potential was −80 mV. The tail current after repolarization at −50 mV gives the maximal amount of channels activated during the test pulse to 40 mV. The rapid recovery from inactivation ensures its minimal contribution to the tail current. The last pulse protocol of the train and its corresponding current trace are highlighted. Corresponding membrane potentials are indicated. B) The peak tail current after the return to −50 mV was plotted as a function of the test pulse duration. A monoexponential fit to the control data yielded the time constant τ = 62.9 ms. Application of 50 μM ketoconazole introduced a slow decay in the full activation of the channels and yielded time constants of 63.0 ms for the onset of activation and 480 ms for the time course of the block when data were fitted by a sum of two exponential functions (solid line). There were no significant changes in the onset of activation. C) The peak tail currents were normalized to their respective maximal values. The decaying phase of the normalized tail current plot shows the onset of the extra-block appearing after the opening of the channels, without contamination from the fast inactivation. The same phenomenon was observed in five cells (see text). Ctrl.: control, Keto.: ketoconazole, Δt: varying pulse duration.

Fig. 7. Additive block by ketoconazole and terfenadine. Current recordings after an 800-ms depolarizing pulse to 20 mV from a holding potential of −80 mV. Ketoconazole in concentrations close to its IC50 (49 μM) blocked 51% of the control current. Cumulative addition of 300 nM terfenadine reduced the residual current by 49% as expected from the IC50 for terfenadine (350 nM). This shows that adding ketoconazole does not shift the IC50 value for terfenadine, a result expected for an additive rather than a competitive blockade by the two drugs. The experiments were repeated in 12 cells, and all yielded similar results.

Fig. 8. Ketoconazole and terfenadine prolong the cardiac action potential (AP) in an additive fashion. A) Action potential simulated in control conditions and in the presence of therapeutic concentrations of ketoconazole (10 μM) and terfenadine (100 nM) with the Heart Oxsoft program (see text for details). Prolongation of the action potential plateau was measured at a repolarization potential of −20 mV (arrows). The base line (0 mV) is indicated by a dotted line, and the AP resulting from the additive effect of terfenadine and ketoconazole is indicated by Terf. + Keto. B) AP associated IK. The currents generated by the OxSoft Heart program had their conductances reduced by 10% and 15% to simulate the block of IKr by terfenadine and ketoconazole, respectively. The values for the block of HERG by both compounds were obtained from Roy et al. (1996) and this study. The maximal contribution of IKr to IK at the end of the pulse was set to 50% as previously reported (Sanguinetti and Jurkiewicz, 1990; Zeng et al., 1995).
As shown in figure 8A, terfenadine by itself prolonged the AP plateau from 232 ms (control) to 241 ms when the membrane was depolarized to -20 mV (arrows). Ketoconazole alone prolonged the plateau to 244 ms, and additive block of IKr by both compounds (fig. 8B) gave a plateau duration of 254 ms, a 9.5% increase of the AP plateau. Our results, within the limitations of the model, indicate that the blockade of other currents in the ventricle is needed to obtain Q-T increments as clinically observed.

### Discussion

We found that HERG and Kv1.5 currents were reduced by ketoconazole with IC50 values of 49 μM and 107 μM, respectively. These results were initially surprising, because there have been no formal reports in the literature of cardiac problems or side effects with ketoconazole treatment in human patients, except during concomitant administration with terfenadine or macrolide antibiotics. In one report analyzing the terfenadine-ketoconazole interactions, the investigators mention that such a study may be desirable, although they speculate that the effects of ketoconazole on EKG would be small (Honig et al., 1993). In a previous paper (Roy et al., 1996), we showed that 100 nM terfenadine, a physiologically relevant concentration (Clusin, 1983; Sorkin and Heel, 1985) is likely to block 20% of IKr (HERG). Interestingly, a comparison with the block of HERG by therapeutic concentrations (10 μM) of the ketoconazole (von Moltke et al., 1994) shows a similar reduction in the steady-state outward current. Chen and Woosley (1993) showed that ketoconazole blocked the tail current of IKr with an IC50 of 2.5 μM in cat ventricle, a value slightly lower than the one we report here for HERG. The prolongation of the action potential by terfenadine in our simulation was 3.5%, a value well within the indirect clinical observations (EKG) of 1% to 6% reported by Pratt et al. (1996) for normal use in human. The results of Hey et al. (1996), obtained in vivo from guinea pig heart, showed an increase of 5% (15 ms/275 ms) in the Q-T interval corrected for heart rate (Q-Tc), a value in agreement with our simulation. Ketoconazole alone prolonged the simulated AP plateau by 5.1%, slightly more than terfenadine, whereas the cumulative block of IKr by both compounds led to a prolongation of only 9.5%. Hey et al. (1996) however, showed an 18% increase of the Q-Tc interval in guinea pig.

Some of the discrepancies between our results with AP simulations and the experimental measurements might be explained by imperfections in the model. Alternatively, the oocyte expression system used in our study may give a higher IC50 value when compared with measurements in native cells or the mammalian expression system. Hydrophobic drugs such as ketoconazole often show a lower affinity for ionic channels heterologously expressed in frog oocytes. The difference in IC50 when compared with mammalian cells is strongly dependent on two factors: the hydrophobicity of the blocker and the lipid composition of the cytosolic membrane; the later influences the solubility of some blockers and their access to the ionic channel. This means that we may have underestimated the affinity of ketoconazole for HERG in native cells in our measurements. Because the IC50 we measured in oocytes is within the range of therapeutic plasma concentrations, a toxic effect of the drug might therefore be seen at a lower concentration in clinical settings. In this context, our results should be interpreted as an upper limit for the toxicity threshold and may in part explain the discrepancies observed with our AP simulation. Species differences may also result in different ratios of IKr and IKur in the cardiac ventricular wall (Liu and Antzelevitch, 1995). Moreover, isoforms of HERG with different affinities for the drug may also be found in other species. It is known that the RNA message for Kv1.5 (Amos et al., 1994; Tamkun et al., 1991; Brahmajothi et al., 1997) is present in the cardiac ventricles, but so far, no current in native ventricular cells has been specifically attributed to this gene. Given the propensity of voltage-dependent potassium channels to form heteromultimers, there is a possibility that Kv1.5 is part of a channel involved in the repolarization of the heart ventricle. Such a channel may therefore share some of the Kv1.5 sensitivity to ketoconazole and may also help to explain the discrepancies we found.

Kv1.5 has been linked to IKur (Fedida et al., 1993; Wang et al., 1993), a component of IKr involved in the repolarization of the atrium. Significant blockade of IKur will appear at higher concentrations of ketoconazole when compared with the block in HERG. Disturbance of sinus rhythm is unlikely to be the dominant effect of the drug, and the differences in the IC50 values may help explain the ventricular origin of the arrhythmogenic effects of the two drugs during light over-dose.

Our results showed that ketoconazole produces a tonic block on HERG, which accounts for most of the current reduction. Because this block does not require previous activation and is not voltage-dependent, this suggests that most of the tonic block is due to blockade in the closed (resting) states or to a fast open-state block. Ketoconazole also introduces a small time-dependent decrease in HERG current, which accounts for up to 10% of the peak current during an 800-ms pulse at strongly depolarized potentials. We found that this time-dependent block was not due to potentiation of the intrinsic inactivation of the channel but was probably due to a slowly developing block of the open channels. This last result suggests a lower affinity of the drug when the channels are in the open state. Blockade of HERG by ketoconazole is therefore composed of a high-affinity block, possibly in the resting (closed) states responsible for the tonic block, and a lower affinity block in the open states, primary responsible for the small time-dependent block.

In contrast, application of ketoconazole on Kv1.5 did not change the current time course, an indication that there was no change of affinity for the open state. But the block was voltage-dependent, increased with activation at weak depolarized potentials and saturated during full activation at strongly depolarized potentials. The increase of the block in a range of potentials where the channel rectifies (-30 and +20 mV) suggests that a transition rate during the activation process is acting as a limiting factor for the binding of the drug between -30 and +20 mV.

Our observations indicate that the interaction between the two drugs is not limited to the competitive pharmacokinetic effects at the level of the CYP3A4-P450 but may also encompass a pharmacodynamic effect of direct additive block on IKr.

Cardiac Kv1.5 and HERG ion channels were blocked by ketoconazole at concentrations reported in serum, and cardiovascular effects would ultimately depend on the amount of ketoconazole present in the heart tissues. Patients treated...
with ketoconazole may be using the drug over an extended period of time, and they should be monitored during treat-
ment, particularly in cases of compromised liver function or simultaneous use of a Class III antiarrhythmic medication or
CYP3A4-P450-requiring compounds.

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