Several noncardiovascular drugs have the potential to induce Torsades de Pointes cardiac arrhythmias via blockade of the rapid component of the cardiac delayed rectifier K⁺ current (I_{Kr}), which is encoded by human ether-à-go-go–related gene (hERG). The aim of the present study was to characterize possible interactions between terfenadine, binding to a site located inside the central cavity, and the following substances with various binding sites: dofetilide, fluvoxamine, chlorobutanol, and a hERG-specific toxin isolated from scorpion venom (CnErg1). The whole-cell configuration of the patch-clamp technique was employed on hERG channels stably expressed in human embryonic kidney 293 cells. Terfenadine does not interact with dofetilide or fluvoxamine at hERG channels. Slight subadditive inhibitory effects on hERG peak tail currents were observed when terfenadine and CnErg1 were administered in combination. Terfenadine and chlorobutanol synergistically inhibit hERG peak tail currents and enhance each other’s inhibitory effect in a concentration-dependent way. In conclusion, terfenadine interacts with CnErg1 and chlorobutanol, but not with dofetilide or fluvoxamine, at hERG channels. It is shown that interactions between chlorobutanol and a hERG channel blocker binding inside the central cavity (terfenadine) produce synergistic effects on hERG currents.

Key Words: hERG channel; interactions; terfenadine; chlorobutanol; CnErg1.
Binding sites at the outer mouth of the channel have been identified for hERG-specific toxins found in scorpion venom (ergotoxin-like peptides, e.g., CnErg1, isolated from scorpions of the genus *Centruroides*; Gurrola *et al.*, 1999). hERG channels possess an unusually long S5-P linker (43 amino acids in comparison with 12–23 residues in other K_\text{v} channels), which presumably forms an amphipathic \( \alpha \)-helix, which, together with the P-S6 linker, is responsible for a hydrophobic CnErg1–binding site (Gurrola *et al.*, 1999; Pardo-Lopez *et al.*, 2002; reviews in Korolkova *et al.*, 2004, and in Wanke and Restano-Cassulini, 2007).

Chlorobutanol, which is used as a preservative in parenteral formulations, inhibits hERG currents at millimolar concentrations (Kornick *et al.*, 2003). Its binding site on hERG channels is unknown. Since it has a much lower affinity to hERG channels than the blockers binding inside the central cavity, it might be suggested that chlorobutanol inhibits hERG currents via interaction with another binding site.

The existence of different binding sites raises the question whether these binding sites interact. If so, how does this influence the effects of simultaneously administered drugs acting at different binding sites. This has been investigated for methadone and chlorobutanol, each inhibiting hERG currents in a concentration-dependent manner; moreover, chlorobutanol enhances the ability of methadone to block hERG currents (Kornick *et al.*, 2003). Some opioid agonists (L-\( \alpha \)-acetyl-methadol (levacetylmethadol, LAAM) and methadone) are inhibitors of hERG currents (Katchman *et al.*, 2002), but their binding sites are not yet known.

The aim of the present study was to characterize possible interactions at hERG channels between a blocker binding inside the central cavity (terfenadine) and substances with various binding sites (dofetilide, fluvoxamine, CnErg1, and chlorobutanol).

**MATERIALS AND METHODS**

**Culture of human embryonic kidney 293 cells stably transfected with hERG channels.** Human embryonic kidney (HEK) 293 cells stably expressing hERG channels were kindly provided by Prof C. January (University of Wisconsin). HEK 293 cells were plated at a density of \( 1 \times 10^5 \) cells per dish (35-mm diameter) and cultured (at 10% \( \text{CO}_2 \)) in Dulbecco’s modified Eagle’s medium with 1 g/l glucose supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 \( \mu \)g/ml streptomycin, and 2 mM glutamine.

**Electrophysiological recording.** hERG currents were recorded using the whole-cell patch-clamp configuration (Hamill *et al.*, 1981) and an EPC 10 patch clamp amplifier (Heka Elektronik, Lambrecht, Germany). Patch pipettes were pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) and had resistances between 3 and 5 M\( \Omega \) when filled with pipette solution B (see section Drugs and solutions). Stimulation protocols and data acquisition were carried out using Pulsitell software (Heka Elektronik). The current sample frequency was 1 kHz. At least 50% series resistance compensation was achieved in all experiments. Outward currents flowing from the pipette to the bath solution are indicated by upward deflections. Experiments were performed at room temperature (20–22°C). The bath had a volume of 1 ml and was perfused at a rate of 2 ml/min; about 0.5 min was needed for the exchange of the bath solution.

Determination of the inhibitory effects of test substances on hERG currents was based on the following voltage-clamp protocol: a holding potential of –80 mV and a voltage step to +20 mV applied for 2 s to evoke hERG currents, followed by a repolarization step to –40 mV for 2 s to induce hERG tail currents (stimulation frequency of 0.1 Hz). hERG tail currents were leak corrected. Reactivating tail currents were fitted with two exponential functions and extrapolated to the beginning of the repolarization step in order to calculate the peak tail current amplitude.

The voltage dependence of hERG channel activation was determined from peak tail currents measured at –40 mV following 2-s depolarizations to membrane voltages ranging from –40 to +40 mV in 10 mV steps. Peak tail current amplitudes were normalized to the maximum peak tail current amplitudes. The voltage dependence of channel availability was determined by fitting the values of the normalized peak tail currents with a Boltzmann equation of the form:

\[
\frac{I}{I_{\text{max}}} = \frac{1}{1 + \exp \left( \frac{V - V_1}{k} \right)},
\]

where \( I \) was the peak tail current amplitude following the test potential \( V \), \( I_{\text{max}} \) was the maximal peak tail current amplitude, \( V_1 \) was the potential at which channel availability was determined, and \( k \) was the slope factor describing channel activation.

The time dependence of hERG current blockade by terfenadine, chlorobutanol, and the terfenadine and chlorobutanol combination was evaluated by using three different voltage protocols. First, an envelope of four steps: cells were held at –80 mV and stepped to +40 mV for 50–600 ms in 50–ms increments; peak tail currents after repolarization to –40 mV at each time point were measured as described above in the absence (control) and presence of test substances. Second, a long step pulse protocol: HEK 293 cells were stepped from a holding potential of –80 mV to +20 mV for 10 s; stimulation was terminated and test substances were applied for >5 min; and then the voltage protocol was applied again. Third, development and recovery of block were determined by applying 2-s voltage steps to +20 mV from a holding potential of –80 mV, followed by 2-s repolarization steps to –40 mV at a stimulation frequency of 0.1 Hz; peak tail currents were determined during the application of test substances until steady-state block was achieved and during the 10-min washout of test substances.

**Data analysis and statistical procedures.** The concentration-response relationships for the inhibition of peak tail current amplitudes by test substances were calculated according to the logistic form of the Hill equation:

\[
\frac{I}{I_{\text{c}}} = \frac{1}{1 + \left( \frac{\text{IC}_{50}}{\alpha} \right)^n},
\]

where \( I_c \) is the peak tail current amplitude during the control periods before application of the test substances and \( I \) is the peak tail current amplitude in the presence of test substances, \( \alpha \) is the fraction of the hERG peak tail current sensitive to inhibition by the test substance (a measure of the intrinsic activity; this value was set to 1 in the case of terfenadine, dofetilide, fluvoxamine, and chlorobutanol since these substances inhibit hERG currents completely), \( n \) is the slope parameter (Hill coefficient), \( x \) is the concentration of test substances, and \( \text{IC}_{50} \) is the midpoint of the curve with \( x = 0 \) and \( pK_x = -\log_{10}(\text{IC}_{50}) \).

For calculation of the concentration-response relationships for the inhibition of hERG peak tail current amplitudes, the test substances were applied cumulatively at increasing concentrations and studied at the following concentrations: 3, 10, 30, 100, and 300\( \mu \)M terfenadine; 1, 3, 10, 30, and 100\( \mu \)M dofetilide; 0.3, 1, 3, 10, and 30\( \mu \)M fluvoxamine; 0.3, 1, 3, 10, and 30\( \mu \)M chlorobutanol; 0.3, 1, 3, 10, 30, and 100\( \mu \)M CnErg1. The number of observations ranged from 3 to 12 per test concentration.

The combined effects of two substances A and B in the case of additivity were calculated according to van den Brink (1977):
where $I_c$ is the hERG peak tail current amplitude during the control periods before application of the test substances and $I_{AB}$ is the hERG peak tail current amplitude in the presence of the test substances, $A$ and $B$, applied in combination, $\alpha$ and $\beta$ are the intrinsic activities, and $IC_{50A}$ and $IC_{50B}$ are the half-maximally inhibitory concentrations of $A$ and $B$.

According to the diagonal constant ratio combination design proposed by Chou (2006), the combination ratio of two test substances applied simultaneously was kept at an equipotency ratio ($IC_{50}$ ratio) since the contribution of each test substance to the combination is approximately equal under these conditions. The combination index (CI) for the quantification of synergism or antagonism for two test substances administered simultaneously was calculated according to Chou (2006):

$$CI = \frac{A_1}{A_{(x1)}} + \frac{B_1}{B_{(x1)}},$$

where the denominators $A_{(x1)}$ and $B_{(x1)}$ stand for the concentrations of test substances $A$ and $B$, with each inhibiting hERG peak tail currents by $x\%$, and the numerators $A_1$ and $B_1$ in combination also inhibit hERG peak tail currents by $x\%$. The $A_{(x1)}$ and $B_{(x1)}$ values were calculated from Equation 2. A CI equalling 1 indicates additivity, a CI smaller than 1 indicates synergism, and a CI greater than 1 indicates antagonism.

The results are expressed as means with the 95% confidence intervals given in parentheses. Equations were fitted using the Sigma Plot Windows program (Jandel Scientific). Significances were calculated by the two-tailed nonpaired $t$-test for single comparisons and by ANOVA with Bonferroni correction for multiple comparisons. $p < 0.05$ was considered to be significant.

**Drugs and solutions.** The bath (solution A) contained (in mM): 140 NaCl, 5.6 KCl, 1.2 MgCl$_2$, 2.6 CaCl$_2$, and 10 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) titrated to pH $= 7.40$ with NaOH. The pipette (solution B) contained (in mM): 140 KCl, 5 MgCl$_2$, 10 Ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 2 CaCl$_2$, 5 ATP, and 5 HEPES titrated to pH $= 7.15$ with KOH (free [Ca$^{2+}$] $= 50$nM; free [Mg$^{2+}$] $= 0.7$mM). Terfenadine and fluvoxamine were purchased from Sigma (St Louis, MO), dofetilide from Sequoia (Pangbourne, UK), and CnErg1 from Alomone Labs (Jerusalem, Israel). Stock solutions of 30mM terfenadine and 3mM dofetilide were prepared in dimethylsulfoxide and stock solutions of 30mM fluvoxamine, 30mM chlorobutanol, and 1$\mu$M CnErg1 in solution A.

**RESULTS**

Table 1 shows the $IC_{50}$ values, Hill coefficients ($n$), and intrinsic activities ($\alpha$) calculated for the concentration-dependent inhibition of hERG peak tail currents by terfenadine, dofetilide, fluvoxamine, chlorobutanol, and CnErg1. Figure 1 shows representative experiments demonstrating the effects of the combined administration of terfenadine with high-affinity

![FIG. 1. Effects on hERG currents of terfenadine combined with dofetilide, fluvoxamine, chlorobutanol, or CnErg1, each tested at half of their respective $IC_{50}$ values. The voltage protocol is shown above. Arrows indicate zero current levels. The block of hERG peak tail currents is 38.6% at 13.8nM terfenadine + 6.5nM dofetilide, 41.2% at 13.8nM terfenadine + 1.8$\mu$M fluvoxamine, 62.7% at 13.8nM terfenadine + 3.7mM chlorobutanol, and 30.7% at 13.8nM terfenadine + 3.2nM CnErg1.](https://academic.oup.com/toxsci/article-abstract/114/2/346/1673141)
hERG channel blockers with $IC_{50}$ values in the nanomolar concentration range (dofetilide and CnErg1), a low-affinity hERG channel blocker with an $IC_{50}$ value in the micromolar concentration range (fluvoxamine), or a very low-affinity hERG channel blocker with an $IC_{50}$ value in the millimolar concentration range (chlorobutanol) on hERG peak tail currents. The results of several experiments are summarized in Table 2. The inhibitory effects of combinations of terfenadine with dofetilide or fluvoxamine on hERG peak tail currents did not deviate markedly from the expected values in cases of additivity (Fig. 1, Table 2). The combination of terfenadine and CnErg1 had subadditive inhibitory effects on hERG peak tail currents at low concentrations (Table 2). The interaction between terfenadine and CnErg1 was studied in detail, and CI values were calculated according to Chou (2006): CI values of 0.9–1.1 indicate nearly additive effects of a combination of test substances; 1.10–1.20, 1.20–1.45, and > 1.45 indicate slight antagonism, moderate antagonism, and antagonism, respectively; 0.85–0.9, 0.7–0.85, 0.3–0.7, 0.1–0.3, and < 0.1 indicate slight synergism, moderate synergism, synergism, strong synergism, and very strong synergism, respectively. When hERG peak tail currents were half-maximally preblocked by terfenadine and CnErg1 at the increasing concentrations of 1, 3, and 10nM was administered simultaneously, the calculated CI values were 1.13 (n = 6), 1.11 (n = 7), and 0.83 (n = 5), respectively, indicating additive effects on hERG channels; by contrast, when first CnErg1 was administered and then terfenadine at increasing concentrations of 1, 3, and 10nM was administered simultaneously, the calculated CI values were 1.23 (n = 5), 1.46 (n = 5), and 1.20 (n = 7), respectively, indicating subadditive effects. The effects of the combination of terfenadine and chlorobutanol on hERG peak tail currents showed superadditivity at high concentrations (Fig. 1, Table 2). These interactions between terfenadine and chlorobutanol on hERG channels were examined in detail (Fig. 2). hERG currents were preblocked by chlorobutanol at

**FIG. 2.** CI-Fa plot for the effects of terfenadine-chlorobutanol combinations on hERG peak tail current amplitudes. The CI value on the y-axis is plotted as a function of the effect levels (Fa: hERG peak tail current amplitude affected, $1 - I/I_c$) on the x-axis. CI < 1, = 1, and > 1 indicate synergism, additive effect, and antagonism, respectively. (A) The CI-Fa plot for the inhibition of hERG peak tail currents by simultaneous administration of 2.5 and 7.4mM chlorobutanol and increasing concentrations (1, 3, 10, 30, and 100nM) of terfenadine. The slopes of the regression lines are −0.62 and −1.35 in the presence of 2.5 and 7.4mM chlorobutanol, respectively, and increasing concentrations of terfenadine. (B) The CI-Fa plot for the inhibition of hERG peak tail currents by simultaneous administration of 9.2 and 27.7nM terfenadine and increasing concentrations of chlorobutanol at increasing concentrations (0.1, 0.3, 1, 3, and 10nM) of chlorobutanol. The slopes of the regression lines are −0.86 and −1.54 in the presence of 9.2 and 27.7nM terfenadine, respectively, and increasing concentrations of chlorobutanol. The number of observations was 6–14 per concentration of the combination tested.
concentrations inhibiting the peak tail currents by 25 or 50% (2.5 and 7.4mM); additionally, terfenadine was administered at increasing concentrations. The slopes of the regression lines of the CI-Fa plot increased with increasing chlorobutanol concentrations (from −0.62 at 2.5mM to −1.35 at 7.4mM chlorobutanol), indicating that chlorobutanol enhanced the inhibitory effects of terfenadine concentration dependently. Similarly, hERG currents were preblocked by terfenadine at concentrations inhibiting the peak tail currents by 25 or 50% (9.2 and 27.7nM); additionally, chlorobutanol was administered at increasing concentrations. The slopes of the regression lines of the CI-Fa plot increased with increasing terfenadine concentrations (from −0.86 at 9.2nM to −1.54 at 27.7nM terfenadine), indicating that terfenadine enhanced the inhibitory effects of chlorobutanol concentration dependently.

In order to further characterize the mechanism of the synergistic effects of terfenadine and chlorobutanol, the voltage- and time-dependent inhibitory effects were studied for separate administration of terfenadine and chlorobutanol at their half-maximally inhibitory concentrations (IC50 values) of 27.7nM and 7.4mM, respectively, and of the combination at half their IC50 values (13.8nM and 3.7mM). Block of hERG currents was strongly voltage dependent for chlorobutanol (stronger at more positive membrane potentials), whereas terfenadine-induced block demonstrated only slight voltage dependence. For the combination of terfenadine and chlorobutanol, voltage dependence was intermediate (Fig. 3). Voltage dependence of steady-state hERG current activation had the following V½ and k values: 9.1 ± 0.3 mV and 7.7 ± 0.3 mV in the absence of test substances (control; n = 28); 5.7 ± 1.1 mV and 6.8 ± 0.9 mV for terfenadine (n = 8); −15.2 ± 0.6 mV and 8.4 ± 0.5 mV for chlorobutanol (n = 11); and −4.9 ± 0.7 mV and 7.4 ± 0.6 mV for the terfenadine and chlorobutanol combination (n = 9). Chlorobutanol and the terfenadine and chlorobutanol combination induced statistically significant hyperpolarizing shifts in the voltage dependence of channel activation (p < 0.05); the k values did not significantly vary between the different experimental conditions (p > 0.05). The voltage dependence of the effects of chlorobutanol and the terfenadine and chlorobutanol combination coincided with the voltage range over the rising phases of the steady-state activation curves corresponding to the membrane potential ranges over which hERG channel opening occurs (Fig. 3B).

The time-dependent block by terfenadine, chlorobutanol, and the terfenadine and chlorobutanol combination was first determined by an envelope of tails protocol for the study of the effects of progressive hERG channel activation on current blockade (Fig. 4). The time dependences of the fractional block of hERG current amplitudes (after depolarization to a membrane voltage of +40mV) by both the chlorobutanol and the terfenadine and chlorobutanol combination were fitted with mono-exponential functions, yielding the following half-life (t½) values for the onset of hERG current blockade: 251 ms for chlorobutanol and 277 ms for the terfenadine and chlorobutanol combination. The terfenadine-induced hERG current block was not significantly different (p > 0.05) at depolarizing pulses between 200 and 600 ms duration.

Second, a long step pulse protocol was used (Fig. 5). The time dependences of the fractional block of hERG current amplitudes (during the long pulse protocol) by both the chlorobutanol and the terfenadine and chlorobutanol combination were fitted with mono-exponential functions, yielding the following values for t½: 1.1 s for chlorobutanol and 1.7 s for the terfenadine and chlorobutanol combination. Terfenadine-induced block increased slightly but not significantly (p > 0.05) between seconds 1 and 10.

Third, when cells were repetitively stimulated at a frequency of 0.1 Hz (Fig. 6), development of hERG current blockade occurred at t½ values of 5.4 ± 0.5 min for terfenadine (n = 6), 1.8 ± 0.2 min for chlorobutanol (n = 5), and 4.0 ± 0.8 min for the terfenadine and chlorobutanol combination (n = 7). On washout, the effects of chlorobutanol recovered rapidly with a t½ value of 1.3 ± 0.3 min. By contrast, the effects of both the terfenadine and the terfenadine and chlorobutanol combination showed poor reversibility after a 10-min washout and the block was still 43.5 ± 4.7% and 29.3 ± 5.2%, respectively, which was not significantly different (p > 0.05).

In order to study the effects of chlorobutanol applied intracellularly, chlorobutanol was added to the pipette solution at a concentration of 7.4mM to diffuse into the cell. Intracellularly applied chlorobutanol had no effects on hERG peak tail current amplitudes, which were 100.8 ± 2.9% (n = 7) of the control 10 min after the whole-cell configuration was established.

**DISCUSSION**

It is shown in the present study that terfenadine does not interact with dofetilide or fluvoxamine at hERG channels. Slight subadditive inhibitory effects on hERG peak tail currents were observed when terfenadine and CnErg1 were administered in combination. Terfenadine and chlorobutanol in combination inhibited hERG peak tail currents synergistically, and each substance enhanced the inhibitory effect of the other substance in a concentration-dependent manner.

The IC50 value for block of hERG peak tail currents by terfenadine (27.7nM, Table 1) obtained in the present study was slightly lower than the values reported previously (56–204nM; review in Stansfeld et al., 2007). The lower IC50 value might be due to the long period of administration of terfenadine (about 5–7 min). The administration was prolonged because of the recent observation (Stork et al., 2007) that at a stimulation frequency of 0.1 Hz, steady-state inhibition of hERG currents by terfenadine is reached after about 5–10 min of drug application. The IC50 value for block of hERG peak tail currents by dofetilide (12.9nM, Table 1) is within the range of the previously reported values (9.5–15nM; Snyders and
Chaudhary, 1996; review in Stansfeld et al., 2007). hERG peak tail currents were blocked by fluvoxamine with an IC50 value of 3.6 μM (Table 1), thus similar to the previously reported value (3.8 μM; Milnes et al., 2003). Also, the IC50 value for the block of hERG peak tail currents by chlorobutanol (7.4 mM, Table 1) is similar to the previously reported value (4.4 mM; Kornick et al., 2003). Fitting the concentration-response relationship for the inhibition of hERG peak tail current amplitudes by chlorobutanol according to Equation 2 yielded a value for α of 1.1 (0.6–1.6), which was not significantly different from unity; therefore, it was assumed that high concentrations of chlorobutanol inhibit hERG currents completely. The value for α was fixed to unity to calculate the IC50 value and the Hill coefficient given in Table 1. The scorpion toxin CnErg1 binds to hERG channels with a 1:1 stoichiometry (Gurrola et al., 1999; Hill et al., 2007; Pardo-Lopez et al., 2002), so that the value for the Hill coefficient was fixed to 1 in order to calculate the concentration-response relationship for the inhibition of hERG peak tail current amplitudes by CnErg1 according to Equation 2. The IC50 value (6.4 nM, Table 1) is within the range of the previously reported values (6–16 nM; Gurrola et al., 1999; Hill et al., 2007; Pardo-Lopez et al., 2002). Peptide toxins usually occlude the pore of the channel; however, CnErg1 did not completely inhibit hERG currents at

FIG. 3. Voltage-dependent modulation of hERG currents by terfenadine, chlorobutanol, and the combination of terfenadine and chlorobutanol. The voltage protocol is shown above. (A) Representative hERG current traces in the absence (control, left traces) and presence of 27.7 nM terfenadine (upper traces), 7.4 mM chlorobutanol (middle traces), or the combination of 13.8 nM terfenadine and 3.7 mM chlorobutanol (lower traces). Arrows indicate zero current levels. (B) The normalized Boltzmann functions describing the voltage-dependent activation of hERG currents in the presence of 27.7 nM terfenadine, 7.4 mM chlorobutanol, and the combination of 13.8 nM terfenadine and 3.7 mM chlorobutanol and the mean data for the voltage dependence of fractional block of hERG peak tail currents indicated by the bars. Symbols represent means and the vertical lines the SE. The number of experiments was 8 for terfenadine, 11 for chlorobutanol, and 9 for the combination.
high concentrations (Table 1), in accordance with previous observations that the maximal effect of CnErg1 is about 90% suppression of the hERG current (Hill et al., 2007; Pardo-Lopez et al., 2002).

The combination of terfenadine and dofetilide had additive inhibitory effects on hERG peak tail currents (Fig. 1, Table 2), which seems to be due to overlapping binding sites in the central cavity of the hERG channel for both terfenadine and dofetilide (Mitcheson et al., 2000; review in Stansfeld et al., 2007).

The lack of interactions between terfenadine and fluvoxamine (Fig. 1, Table 2) was surprising. Binding of fluvoxamine at the outer mouth of the channel (as suggested by Milnes et al., 2003, and Mitcheson, 2003) was expected to interfere with the transition from open to inactive (i.e., inactivation), which involves closure of an extracellular inactivation gate. This in turn was expected to antagonize terfenadine’s inhibitory effects on hERG currents. However, using a molecular modeling approach, fluvoxamine was recently docked into the hERG channel, so that its protonated nitrogen binds below the Phe656 residues and outside the central cavity, which permits the trifluoromethyl group of the compound to interact with the nonaromatic binding site formed by Thr623 and Ser624.

FIG. 4. Envelope of tails protocol used to study the time dependence of hERG current block induced by terfenadine, chlorobutanol, and the combination. The voltage protocol is shown above. (A) Representative current traces evoked by an envelope of tails protocol under control conditions (control, left traces) and in the presence of 27.7 nM terfenadine (upper traces), 7.4 mM chlorobutanol (middle traces), and the combination of 13.8 nM terfenadine and 3.7 mM chlorobutanol (lower traces). Arrows indicate zero current levels. (B) Mean data (± SE) for fractional block of hERG currents by 27.7 nM terfenadine, 7.4 mM chlorobutanol, and the combination of 13.8 nM terfenadine and 3.7 mM chlorobutanol plotted as a function of the depolarizing pulse durations. Block developed mono-exponentially for both the chlorobutanol and the terfenadine and chlorobutanol combination. The number of experiments was 11 for terfenadine, 7 for chlorobutanol, and 10 for the combination.
This model suggests overlapping binding sites for fluvoxamine and terfenadine. The observation that the simultaneous administration of terfenadine and fluvoxamine has additive inhibitory effects on hERG peak tail currents may be compatible with both models. Further studies are necessary for the elucidation of the fluvoxamine-binding site on hERG channels.

Recently, Margulis and Sorota (2008) found that cisapride with a binding site located within the central cavity has additive inhibitory effects on hERG currents when combined with (1) quinidine with an overlapping binding site, (2) fluvoxamine, and (3) the ergtoxin BeKm-1. Whereas the first two interactions correspond to those obtained in the present study, the third does not. At low concentrations, terfenadine and CnErg1 administered in combination had subadditive inhibitory effects on hERG peak tail currents (Table 2). In order to examine the interaction between terfenadine and CnErg1 in detail and to calculate a CI-Fa plot, hERG currents were preblocked by one test substance and the other test substance was administered at increasing concentrations in the presence of the first test substance. When CnErg1 was administered in the presence of terfenadine, the calculated CI values were between 0.83 and 1.13, indicating a nearly additive effect of a combination of test substances according to the Chou (2006) criteria. However, when terfenadine was administered in the presence of CnErg1, the calculated CI values were between 1.20 and 1.46, indicating a moderate antagonism of two test substances applied in combination according to the Chou (2006) criteria. In summary, the interactions of terfenadine and CnErg1 at hERG channels seem to be complex and cannot be described simply by additive effects of two test substances. The observations may indicate that CnErg1 slightly antagonizes the inhibitory effects of terfenadine on hERG peak tail currents.

The simultaneous administration of terfenadine and chlorobutanol induced superadditive inhibitory effects on hERG peak tail currents (Fig. 1, Table 2). The observation that synergism increased at increasing test concentrations of the combination can be explained by the higher probability that both molecules bind simultaneously to the same channel at higher test concentrations. Chlorobutanol and terfenadine enhanced the inhibitory effects of the other substance concentration dependently (Fig. 2).

Experiments studying the voltage- and time-dependent inhibitory effects of terfenadine and chlorobutanol on hERG currents demonstrated that they have different mechanisms of action and, consequently, different binding sites on hERG
channels. hERG channel blockade by terfenadine showed weak voltage dependence (Fig. 3), did not increase with depolarizing pulses > 200-ms duration during the envelope of tails protocol (Fig. 4), and increased only slightly during the long step pulse protocol (Fig. 5). The observation that the inhibitory effect of terfenadine on hERG currents is very slowly reversed on drug washout (Fig. 6), an observation also made recently by Stork et al. (2007), might be explained by a tight binding of application of 27.7nM terfenadine, 7.4mM chlorobutanol, and the combination of 13.8nM terfenadine and 3.7mM chlorobutanol.

The binding site of chlorobutanol on hERG channels is not known. It might be suggested that hERG current block by chlorobutanol is caused by a modification of the composition of membrane lipids. Hydroxypropyl β-cyclodextrins, which are cyclic oligosaccharides used to enhance drug solubility, inhibit hERG currents at a concentration of 6%; cyclodextrins modify the lipid environment and cholesterol composition of the plasma membrane, and it has been suggested that channels that are located in the lipid raft domains tend to be sensitive to the interaction with cyclodextrins (Mikhail et al., 2007). However, the concentration-dependent enhancement of chlorobutanol’s inhibitory effects by terfenadine (Fig. 2) seems to argue against nonspecific effects of chlorobutanol on hERG channels mediated via a modification of the composition of membrane lipids and might point toward a specific binding site of chlorobutanol on hERG channels. Two observations seem to indicate a binding site for chlorobutanol on the extracellular side of the hERG channel: first, intracellular application of chlorobutanol had no effects on hERG currents, which argues against a binding site accessible via the lipid phase of the plasma membrane; second, the effects of chlorobutanol on hERG currents were rapidly reversible on washout (Fig. 6). Further studies using mutagenesis approaches are required to characterize the chlorobutanol-binding site on hERG channels.

Chlorobutanol is used in parenteral dosages forms as an antimicrobial preservative at concentrations of up to 0.5% (about 30mM). Plasma concentrations of about 0.5mM chlorobutanol were reported in a patient receiving iv morphine preserved with 0.5% chlorobutanol (DeChristoforo et al., 1983). The ratio of the IC50 value for the block of hERG currents (7.4mM) to the plasma concentration of chlorobutanol is about 15 and, therefore, below the margin of 30, which seems to be the line of demarcation between substances associated with TdP and those which are not (Redfern et al., 2003). However, the torsadogenic potential induced by block of hERG currents can be counterbalanced by effects on other types of cardiac ion channels (e.g., Na+ and L-type Ca2+ channels; Redfern et al., 2003). Further in vitro and in vivo electrophysiological studies are required to test the torsadogenic potential of chlorobutanol. Depending on both the site and the speed of injection, it is not unlikely that the concentration of chlorobutanol in the heart may approach the level (2.5mM) at which synergistic inhibitory effects on hERG currents were observed after simultaneous administration of increasing terfenadine concentrations (Fig. 2B).

In conclusion, it is shown in the present study that terfenadine, which binds to the central cavity of the hERG channel, does not interact with a substance with an overlapping binding site ( dofetilide) or with a substance with an unknown binding site (fluvoxamine). Its inhibitory effects are slightly antagonized by the presence of a substance binding at the outer mouth (CnErg1). Terfenadine and the preservative chlorobutanol synergistically inhibit hERG peak tail currents. It is not unlikely that similar synergistic effects on hERG currents are generated by interaction between chlorobutanol, which may be an open state blocker.
binding to the extracellular side of the hERG channel, and other hERG channel blockers binding inside the central cavity.

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