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Allosteric modulators of the hERG K^+ channel
Radioligand binding assays reveal allosteric characteristics of dofetilide analogs

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ABSTRACT

Drugs that block the cardiac K^+ channel encoded by the human ether-à-go-go gene (hERG) have been associated with QT interval prolongation leading to proarrhythmia, and in some cases, sudden cardiac death. Because of special structural features of the hERG K^+ channel, it has become a promiscuous target that interacts with pharmaceuticals of widely varying chemical structures and a reason for concern in the pharmaceutical industry. The structural diversity suggests that multiple binding sites are available on the channel with possible allosteric interactions between them. In the present study, three reference compounds and nine compounds of a previously disclosed series were evaluated for their allosteric effects on the binding of [^{3}H]astemizole and [^{3}H]dofetilide to the hERG K^+ channel. LUF6200 was identified as an allosteric inhibitor in dissociation assays with both radioligands, yielding similar EC_{50} values in the low micromolar range. However, potassium ions increased the binding of the two radioligands in a concentration-dependent manner, and their EC_{50} values were not significantly different, indicating that potassium ions behaved as allosteric enhancers. Furthermore, addition of potassium ions resulted in a concentration-dependent leftward shift of the LUF6200 response curve, suggesting positive cooperativity and distinct allosteric sites for them. In conclusion, our investigations provide evidence for allosteric modulation of the hERG K^+ channel, which is discussed in the light of findings on other ion channels.

Introduction

The hERG K^+ channel encoded by the hERG gene (Keating and Sanguinetti, 2001) is responsible for the rapid delayed rectifier K^+ current (I_{Kr}) that plays a critical role in the repolarization of cardiomyocytes during the cardiac action potential (Hoppe et al., 2001). It is made up of large intracellular N- and C-terminal domains (Ng et al., 2011; Schönherr and Heinemann, 1996) and four identical α-subunits, each of which is formed by six α-helical transmembrane domains and a looping "pore region" (Finlayson et al., 2004; Sanguinetti and Tristani-Firouzi, 2006). In humans, blockade of the hERG K^+ channel by drugs can cause excessive lengthening of the action potential, which is reflected by a QT interval prolongation in the electrocardiogram (ECG) (Hancox et al., 2008; Vandenberg et al., 2001). The excessive action potential prolongation may combine to produce and sustain Torsade de Pointes (TdP), which can be self-limiting or degenerate into ventricular fibrillation rapidly leading to death (Hancox et al., 2008; Sanguinetti and Tristani-Firouzi, 2006). Therefore, it has become a routine practice in the pharmaceutical industry to test compounds for their hERG liability during early preclinical safety assessments according to the FDA guidelines (Sanguinetti and Mitcheson, 2005). In recent years, most attention has been paid to assess the affinity for the hERG K^+ channel of potential drug candidates in order to avoid and discard modest-to-high affinity compounds during the lead finding and optimization process (Gintant, 2011). However, allosteric modulation of the hERG K^+ channel as an alternative way of interaction has not been studied in any details until now.

With regard to G protein-coupled receptors (GPCRs), successful drugs that mediate their effects through the allosteric modulation of target activity have already reached the market, also in view of their potential greater selectivity, potency and/or safety profile when compared to orthosteric ligands (IJzerman et al., 2001; May et al., 2007). Radioligand binding assays, in particular kinetic radioligand dissociation assays, have been widely utilized to quantify the allosteric effects of GPCR ligands (Christopoulos, 2002). As for ion channels, allosteric modulators have been reported for ligand-gated ion channels in particular. For example, GW791343 might be applied to treat inflammatory disorders and pain due to its allosteric inhibition of the $P2X_7$ receptor (Michel et al., 2008a, 2008b). A negative allosteric modulator of the nicotinic acetylcholine receptor, UCI-30002, had been reported to have significant benefits as a strategy for treating nicotine addiction because of its high subtype-selectivity (Yoshimura et al., 2007). It has also become increasingly apparent that new potent and selective allosteric modulators of the GABA-A receptor and 5-HT_3 receptor may replace conventional

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antagonists and agonists for these targets (Sancar and Czajkowski, 2011; Trattnig et al., 2012). Allosteric modulation of voltage-gated ion channels such as several types of calcium, sodium and potassium channels and their possible clinical applications has been investigated as well, but to a lesser extent. As an example, a novel quinazolinone ligand (TTA-Q4) showed a positive allosteric interaction with the T-Type calcium channel since it enhanced radioligand binding, increased affinity in a saturable manner and slowed dissociation (Uebele et al., 2009).

Radioligand binding assays as a means of studying the hERG K^+ channel have been developed over the years. Two radioligands, [^{3}H] astemizole and [^{3}H]dofetilide, have mostly been used (Chadwick et al., 1993; Chiu et al., 2004; Finlayson et al., 2001). The purpose of the present study was to identify and investigate allosteric modulators of the hERG K^+ channel using these two radioligands. The antidepressant fluvoxamine (Mitcheson, 2003) and the channel opener PD118057 (Perry et al., 2009; Zhou et al., 2005) (Fig. 1), reported to have binding sites different from conventional hERG blockers, were selected as representative reference compounds. We identified a number of dofetilide analogs (Fig. 1) displaying allosteric modulation in a pilot experiment and these were further investigated as were the allosteric effects of potassium ions in the same binding assays. Our findings suggest potential applications for such allosteric modulators, which might provide novel solutions for drug cardiotoxicity due to blockade of the hERG K^+ channel.

Material and methods

Chemicals and reagents

Astemizole, terfenadine, fluvoxamine and PD118057 were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). Dofetilide and all the LUF compounds were synthesized in our own laboratory, as published previously (Shagufta et al., 2009). [^{3}H]Astemizole (specific activity 78.9 Ci mmol^{-1}) and [^{3}H]dofetilide (specific activity 70.0 Ci mmol^{-1}) were purchased from PerkinElmer (Groningen, The Netherlands). Bovine serum albumin (BSA, fraction V) was purchased from Sigma (St. Louis, MO, USA). G418 was obtained from Stratagene (Cedar Creek, USA). All the other chemicals were of analytical grade and obtained from standard commercial sources. HEK293 cells stably expressing the hERG K^+ channel (hERG/HEK293) were kindly provided by Dr. Eckhard Ficker (University of Cleveland, USA).

Cell culture

hERG/HEK293 cells were cultured in a humidified atmosphere at 37 °C and 7% CO_2 in DMEM, containing 10% fetal calf serum, 50 IU ml^{-1} penicillin, 50 μg ml^{-1} streptomycin and 1.25 μg ml^{-1} G418 for selection. Cells were subcultured twice a week (1:8). Then, the cells were subcultured 1:10 and transferred to large 15-cm diameter plates for membrane preparation.

Membrane preparation

hERG/HEK293 cells were grown to 80–90% confluence and detached from the plates by scraping them into 5 ml of PBS. Then, the detached cells were collected and centrifuged at 250 *g* for 10 min. The cell pellets were pooled and resuspended in 50 mM ice-cold Tris–HCl buffer containing 2 mM $MgCl_2$, pH 7.4. An UltraTurrax (Heidolph Instruments, Schwabach, Germany) was used to homogenize the cell suspension. Membranes and the cytosolic fraction were separated by centrifugation at 100,000 *g* in an Optima LE-80 K ultracentrifuge (Beckman Coulter, Fullerton, CA, USA) at 4 °C for 20 min. The pellets were resuspended in the Tris–HCl buffer, and the homogenization and centrifugation steps were repeated. The resulting pellets were resuspended in ice-cold incubation buffer (10 mM HEPES, 130 mM NaCl, 60 mM KCl, 0.8 mM $MgCl_2$, 1 mM EGTA, 10 mM glucose, 0.1% BSA, pH 7.4) using the UltraTurrax. Aliquots (125 or 250 μl) were stored at −80 °C. The protein concentration of the membranes was measured using the BCA method (Smith et al., 1985).

Kinetic radioligand association and dissociation assays

The incubation temperature and protein concentration were optimized for the kinetic studies. Three different temperatures (4, 15 and 25 °C) and five protein amounts (10, 15, 20, 30 and 50 μg) were investigated at the beginning of our research program. The optimal conditions for [^{3}H]astemizole kinetic studies proved to be using 30 μg of membrane protein at an incubation temperature of 15 °C. The association experiments of [^{3}H]astemizole were performed by incubating

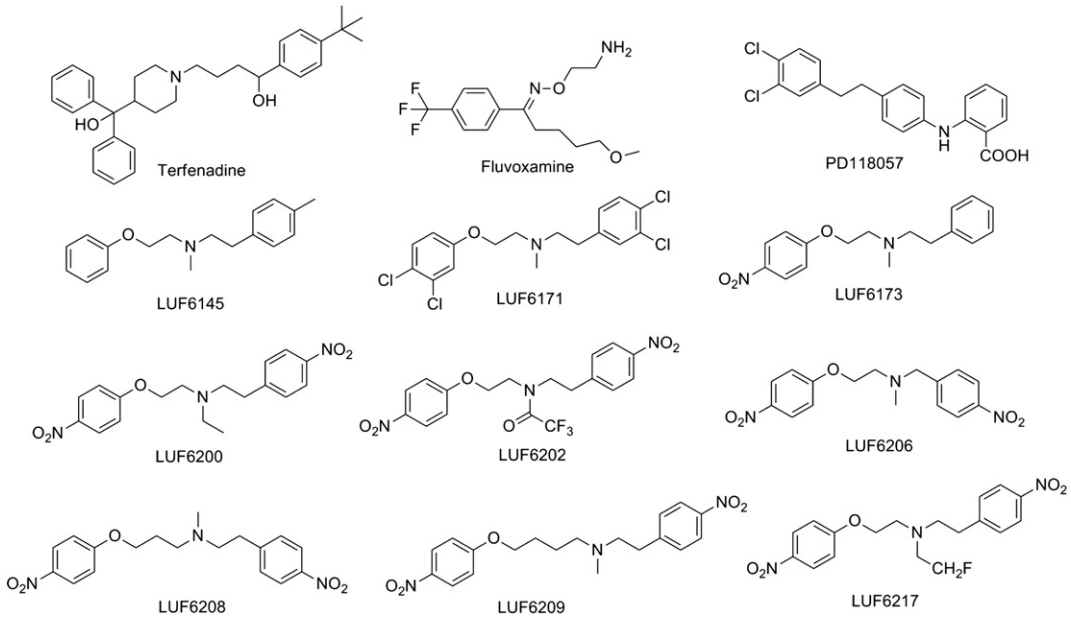

Fig. 1. Compounds evaluated in this study. Terfenadine is a high-affinity reference hERG blocker, fluvoxamine is a low-affinity reference hERG blocker, PD118057 is a reference hERG activator, and all the other LUF compounds are members of a previously disclosed compound series interacting with the hERG K^+ channel.

membrane aliquots containing 30 μg protein in a total volume of 100 μl incubation buffer at 15 °C for 90 min with 2 nM [^{3}H]astemizole. The amounts of radioligand bound to the receptor were measured at various time intervals during the incubation. The dissociation experiments were conducted by preincubating membrane aliquots containing 30 μg protein in a total volume of 100 μl incubation buffer at 15 °C for 90 min with 2 nM [^{3}H]astemizole. After preincubation, dissociation was initiated by addition of 10 μM astemizole for a total period of 60 min. The amounts of [^{3}H]astemizole still bound to the receptor were also measured at various time intervals. Separation of bound from free radioligand was performed by rapid filtration through Whatman GF/B filters using a Brandel harvester. Filters were subsequently washed six times with 2 ml ice-cold wash buffer (25 mM Tris–HCl, 130 mM NaCl, 60 mM KCl, 0.8 mM $MgCl_2$, 0.05 mM $CaCl_2$, 0.05% BSA, pH 7.4). Filter-bound radioactivity was determined by scintillation spectrometry using a liquid Scintillation Analyzer (Tri-Carb 2900TR) after addition of 3.5 ml Packard Emulsifier Safe and 2 h extraction.

For the kinetic studies of [^{3}H]dofetilide binding we used 20 μg of membrane protein at an incubation temperature of 25 °C with 5 nM [^{3}H]dofetilide. The association experiments were performed for 120 min, whereas the dissociation assays were induced by 10 μM dofetilide after a total period of 120 min preincubation and followed for another 60 min. Incubations were terminated, and samples were obtained and analyzed as described for [^{3}H]astemizole.

Kinetic radioligand dissociation assays — allosteric modulation

Single point dissociation assays. Dissociation experiments were mainly performed as described in the above paragraph. After preincubation, dissociation was initiated by addition of 10 μM astemizole or dofetilide in the absence (control) or presence of 10 μM of other ligands. After 10 min of dissociation, samples were separated by rapid filtration through a 96-well GF/B filter plate using a PerkinElmer Filtermate-harvester (PerkinElmer, Groningen, The Netherlands). Filters were subsequently washed twelve times with ice-cold wash buffer. The filter-bound radioactivity was determined by scintillation spectrometry using the 1450 Microbeta Wallac Trilux scintillation counter (PerkinElmer) after addition of 37.5 μl Microscint and 2 h extraction. The binding of [^{3}H]astemizole or [^{3}H]dofetilide in the control was set as 100%.

Dissociation assays. After preincubation, dissociation was initiated by addition of 10 μM astemizole or dofetilide in the absence (control) or presence of 10 μM of possible allosteric modulators for a total period of 60 min. The amounts of radioligand still bound to the receptor were measured at various time intervals. Incubations were terminated, and samples were obtained as described in 'Single point dissociation assays'.

Concentration dependency of allosteric modulators. In this setup the dissociation assay of [^{3}H]dofetilide was performed at 15 °C and the preincubation time to achieve radioligand association was 180 min; the dissociation assay for [^{3}H]astemizole was the same as described in 'Kinetic radioligand association and dissociation assays'. After preincubation, dissociation was initiated by addition of 10 μM astemizole or dofetilide in the absence (control) or presence of different concentrations of allosteric modulators. Incubations were terminated after 10 min of dissociation, and samples were obtained as described in 'Single point dissociation assays'.

Equilibrium radioligand binding assays. The [^{3}H]astemizole and [^{3}H]dofetilide equilibrium binding assays for the hERG K^+ channel were performed as described before (Chadwick et al., 1993; Chiu et al., 2004; Diaz et al., 2004; Finlayson et al., 2001) with minor modifications. In short, membrane aliquots containing 30 μg protein for [^{3}H]astemizole or 20 μg protein for [^{3}H]dofetilide were incubated in a total volume of 100 μl incubation buffer at 25 °C for 60 min. Binding experiments were performed using a series of concentrations of potassium ions in the presence of 2 nM [^{3}H]astemizole or 5 nM [^{3}H]dofetilide. Non-specific binding was determined in the presence of 10 μM astemizole and represented approximately 15% of the total binding. [^{3}H]Astemizole and [^{3}H]dofetilide did not show specific binding to membranes prepared from HEK293 cells lacking the expressed hERG K^+ channel. The binding of [^{3}H]astemizole and [^{3}H]dofetilide in the presence of 100 mM KCl was set at 100% in all experiments, whereas nonspecific binding was set at 0%. Incubations were terminated by dilution with ice-cold wash buffer, and samples were obtained as described in 'Kinetic radioligand association and dissociation assays'.

Cooperativity between different allosteric modulators. The dissociation assay of [^{3}H]dofetilide in the presence of different concentrations of KCl (2, 5, 10 and 60 mM) was performed as described in 'Concentration dependency of allosteric modulators'. After preincubation, dissociation was initiated by addition of 10 μM dofetilide in the absence (control) or presence of different concentrations of LUF6200. Incubations were terminated after 6 min of dissociation, and samples were obtained as described in 'Single point dissociation assays'.

Data analysis. All data of radioligand binding assays were analyzed with Prism v. 5.0 (GraphPad, San Diego, CA, USA). EC_{50} values for potassium ions were directly obtained from the non-linear regression concentration-effect curves. Dissociation rate constants, k_{off}, were obtained by computer analysis of the exponential decay of [^{3}H]astemizole or [^{3}H]dofetilide bound to the receptor. Association rate constants, k_{on}, were calculated according to the equation $k_{on} = (k_{obs} - k_{off}) / [L^*]$, where the k_{obs} was obtained by computer analysis of the exponential association of [^{3}H]astemizole or [^{3}H]dofetilide bound to the receptor and [L^*] was the amount of radioligand used for the association experiments. EC_{50} values from the dissociation experiments were calculated by non-linear regression analysis of concentration-effect curves of dissociation in the presence of different concentrations of unlabeled ligands. The differences of binding parameters between compounds and control were analyzed by an unpaired student t test. Significance was determined as $P < 0.05$. All values obtained are means of at least three independent experiments performed in duplicate.

Results

Optimization of assay conditions for [^{3}H]astemizole and [^{3}H]dofetilide kinetic study

Assay conditions were optimized according to a general radioligand binding protocol in our laboratory (Heitman et al., 2008a). Firstly, displacement assays for [^{3}H]astemizole and [^{3}H]dofetilide were performed at five different amounts of hERG/HEK293 membranes (10, 15, 20, 30 and 50 μg). A suitable window of specific [^{3}H]astemizole binding was obtained using 30 μg of membrane protein, whereas 20 μg of protein was required for [^{3}H]dofetilide binding to achieve a similar window. These membrane protein concentrations were also selected for the further kinetic study of two radioligands. Since it has been reported that some drugs can inhibit hERG currents via their interaction with AMP-dependent protein kinase (Almilaji et al., 2013), the binding of [^{3}H]astemizole and [^{3}H]dofetilide at membranes prepared from HEK293 cells lacking the expressed hERG K^+ channel was also determined. Neither of the two radioligands showed any specific binding, corroborating our assumption of a direct binding process of astemizole and dofetilide to the hERG K^+ channel. Subsequently, the association and dissociation of [^{3}H]astemizole and [^{3}H]dofetilide were determined at three different temperatures (4, 15 and 25 °C). The optimal incubation temperature was found to be 15 °C for [^{3}H]astemizole and 25 °C for [^{3}H]dofetilide

concerning the appropriate on- and off-rates and the practical convenience of the experiments. Nevertheless, these findings were not applied too stringently as concentration-dependent effects of allosteric modulators of [^{3}H]astemizole and [^{3}H]dofetilide binding to the hERG K^+ channel were later conducted at 15 °C and concentration-effect curves for K^+ ions were recorded at 25 °C in order to obtain comparable results between the two radioligand binding assays.

Association and dissociation of [^{3}H]astemizole from the hERG/HEK293 membranes

Because association and dissociation of [^{3}H]astemizole from the hERG/HEK293 membranes were both very fast at 25 °C, the kinetic study of [^{3}H]astemizole binding was performed at 15 °C using 30 μg hERG/HEK293 membrane protein. The data were best described by non-linear regression using a mono-exponential model. As shown in Fig. 2A and Table 1, the binding equilibrium was reached within 90 min with a k_{on} value of 0.068 ± 0.010 nM^{-1} min^{-1}. The dissociation rate and half-life time of [^{3}H]astemizole were 0.055 ± 0.010 min^{-1} and 11 ± 1.4 min, respectively (Fig. 2B and Table 1). The dissociation of [^{3}H]astemizole at 10 min was almost half of the total binding, so the 10-min dissociation time was selected for assessing the allosteric effects of compounds on the hERG K^+ channel in the follow-up experiments.

Association and dissociation of [^{3}H]dofetilide from the hERG/HEK293 membranes

As depicted in Fig. 3, the association of [^{3}H]dofetilide reached equilibrium at around 30 min, and it completely dissociated from the hERG K^+ channel within 60 min after addition of 10 μM dofetilide. By analyzing association and dissociation curves using a mono-exponential model, the k_{on} and k_{off} values were 0.032 ± 0.0026 nM^{-1} min^{-1} and 0.20 ± 0.026 min^{-1}, respectively (Table 1). Similar to [^{3}H]astemizole binding assay, a 10-min dissociation time point was chosen for the further study of allosteric modulators of the hERG K^+ channel.

Screening for allosteric modulators of [^{3}H]astemizole and [^{3}H]dofetilide binding

We screened three reference ligands and an in-house library of hERG blockers (LUF compounds) to identify their allosteric effects on [^{3}H]astemizole binding to the hERG K^+ channel using a single point dissociation assay. This protocol resulted in several compounds that potentially increased (allosteric inhibitors) and decreased (allosteric enhancers) the dissociation of the radioligand. All the data are listed in Table 2 and shown in Fig. 4. Fluvoxamine and PD118057 appeared to increase the specific binding of [^{3}H]astemizole by 29 and 33% above control after 10 min, suggesting that they might behave as allosteric enhancers

Table 1
The association rate (k_{on}), dissociation rate (k_{off}) and the kinetically derived K_d of [^{3}H]astemizole and [^{3}H]dofetilide binding to the hERG K^+ channel.

	k_{on} (nM^{-1} min^{-1})	k_{off} (min^{-1})	K_d (nM)
Astemizole [a]	0.068 ± 0.010	0.055 ± 0.010	0.85 ± 0.045
Dofetilide [b]	0.032 ± 0.0026	0.20 ± 0.026	6.4 ± 1.3

Values are means (± S.E.M.) of at least three independent assays performed in duplicate.
[a] The association and dissociation assays of [^{3}H]astemizole were performed at 15 °C.
[b] The association and dissociation assays of [^{3}H]dofetilide were conducted at 25 °C.

of [^{3}H]astemizole binding. On the contrary, some of the compounds synthesized in our laboratory including LUF6200, LUF6208, LUF6209 and LUF6217 substantially reduced the binding of [^{3}H]astemizole, supporting their role as allosteric inhibitors by accelerating the dissociation of the radioligand. Among them, LUF6200, 6208 and LUF6209 were the most potent allosteric inhibitors with only 39 ± 4, 36 ± 6 and 39 ± 2% specific binding left compared with 100% for the control. In addition, LUF6202 and LUF6206 also displayed significant allosteric inhibition effects, but to a lesser extent. Other compounds such as terfenadine, LUF6145, LUF6171 and LUF6173 had no significant allosteric modulatory effects in this experiment.

The effects of the same compounds in the single point dissociation experiments of [^{3}H]dofetilide binding to the hERG K^+ channel are shown in Fig. 5 and Table 2. The results obtained from this assay were almost the same as those derived from the [^{3}H]astemizole binding assay. The only two exceptions were that fluvoxamine exerted no allosteric enhancement here and that LUF6173 now showed significant allosteric inhibition of [^{3}H]dofetilide binding.

Subsequently, fluvoxamine, PD118057 and one potent representative allosteric inhibitor (LUF6200) were selected for the further experiments to validate their allosteric modulation effects.

Dissociation of [^{3}H]astemizole from the hERG K^+ channel: effects of fluvoxamine, PD118057 and LUF6200

The dissociation of [^{3}H]astemizole from the hERG K^+ channel was induced by 10 μM astemizole in the absence (control) or presence of 10 μM of fluvoxamine, PD118057 or LUF6200 during a 60-min time course at 15 °C. The results are depicted in Fig. 6. The dissociation rates of [^{3}H]astemizole in the presence of fluvoxamine and PD118057 were 0.040 ± 0.0091 min^{-1} and 0.055 ± 0.0053 min^{-1}, respectively, which were not significantly different from the control (k_{off} = 0.055 ± 0.010 min^{-1}). Moreover, the three curves in the absence or presence of fluvoxamine and PD118057 practically overlapped with each other. Thus, in this more detailed experiment, we verified that fluvoxamine and PD118057 did not allosterically enhance [^{3}H]astemizole binding at a concentration of 10 μM. Interestingly, LUF6200 increased the dissociation rate of radioligand binding notably

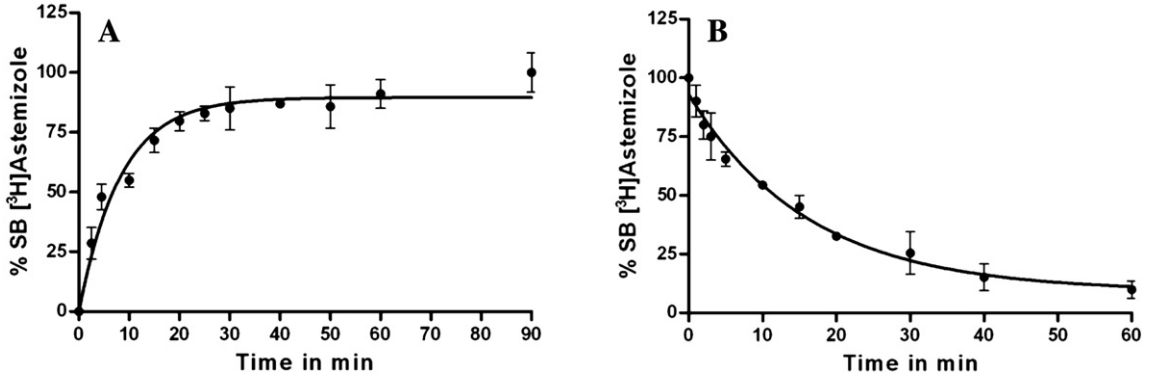

Fig. 2. Association and dissociation curves of [^{3}H]astemizole binding to the hERG K^+ channel. (A: association curve; B: dissociation curve) Dissociation was started by addition of 10 μM astemizole after 90 min preincubation. The experiment was performed at 15 °C using 30 μg of hERG/HEK293 membrane protein. Data shown are from three independent experiments performed in duplicate.

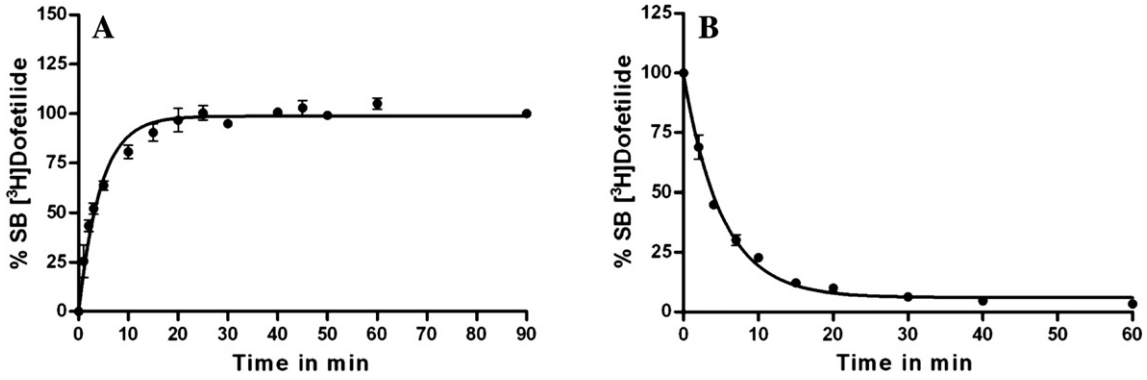

Fig. 3. Association and dissociation curves of [^{3}H]dofetilide binding to the hERG K^+ channel. (A: association curve; B: dissociation curve) Dissociation was started by addition of 10 μM dofetilide after 120 min preincubation. The experiment was performed at 25 °C using 20 μg of hERG/HEK293 membrane protein. Data shown are from three independent experiments performed in duplicate.

to a k_{off} value of 0.21 ± 0.011 min^{-1} (P = 0.0005), which corresponded to a 3.7-fold increase in the dissociation rate found for [^{3}H]astemizole alone.

Dissociation of [^{3}H]dofetilide from the hERG K^+ channel: effects of PD118057 and LUF6200

Since fluvoxamine was found to have no allosteric capacity in both the [^{3}H]astemizole and [^{3}H]dofetilide binding assays described above, only PD118057 and LUF6200 were tested for their allosteric effects on [^{3}H]dofetilide binding to the hERG K^+ channel (Fig. 7). The results demonstrated that LUF6200 accelerated the dissociation rate of [^{3}H]dofetilide from 0.20 ± 0.026 min^{-1} to 1.5 ± 0.17 min^{-1} (P = 0.0016), while the k_{off} of [^{3}H]dofetilide in the presence of 10 μM PD118057 was 0.20 ± 0.015 min^{-1}, displaying no enhancement effects on the binding of [^{3}H]dofetilide. This further proved that LUF6200 was an allosteric inhibitor but that PD118057 was not an allosteric enhancer of the binding of hERG radioligands.

Concentration dependence of allosteric effects of LUF6200 on [^{3}H] astemizole and [^{3}H]dofetilide binding

Fig. 8 shows the influence of increasing concentrations of LUF6200 on the dissociation of [^{3}H]astemizole and [^{3}H]dofetilide induced by 10 μM astemizole or dofetilide. After 10 min of dissociation, the process was terminated by washing and rapid filtration. LUF6200 accelerated the dissociation of [^{3}H]astemizole and [^{3}H]dofetilide in a concentration-dependent manner with similar, not statistically significantly different, EC_{50} values of 4.9 ± 0.52 and 3.4 ± 0.97 μM, respectively (P = 0.45).

Concentration dependent effects of potassium ions on [^{3}H]astemizole and [^{3}H]dofetilide binding

To assess the influence of increasing concentrations of potassium ions on the binding of [^{3}H]astemizole and [^{3}H]dofetilide, we determined the specific binding of [^{3}H]astemizole and [^{3}H]dofetilide at different concentrations of KCl after 60 min preincubation at 25 °C. The results are shown in Fig. 9. The binding of both [^{3}H]astemizole and [^{3}H]dofetilide was elevated with increasing concentrations of potassium ions and reached a plateau as of a concentration of 30 mM. The mean EC_{50} values in the two different radioligand binding assays were 7.2 ± 0.60 mM and 8.1 ± 0.47 mM for [^{3}H]astemizole and [^{3}H]dofetilide, respectively, which were not significantly different (P = 0.30). It is also worth noting that when no potassium ions were added to incubation mixture, the specific binding of [^{3}H]astemizole and [^{3}H]dofetilide was negligible (data not shown).

Table 2
The specific binding of [^{3}H]astemizole and [^{3}H]dofetilide to the hERG K^+ channel after 10 min of dissociation in the absence (control) or presence of 10 μM unlabelled ligands.

Compound	% SB of [^{3}H]astemizole	% SB of [^{3}H]dofetilide
Control	100 ± 0	100 ± 0
Terfenadine	98 ± 1	93 ± 7
Fluvoxamine	129 ± 13*	95 ± 12
PD118057	133 ± 14*	109 ± 2*
LUF6145	109 ± 5	93 ± 2
LUF6171	102 ± 6	104 ± 3
LUF6173	97 ± 8	71 ± 5*
LUF6200	39 ± 4***	50 ± 1***
LUF6202	70 ± 2***	62 ± 4*
LUF6206	86 ± 2***	70 ± 7*
LUF6208	36 ± 6***	47 ± 5**
LUF6209	39 ± 2***	43 ± 8*
LUF6217	53 ± 4***	54 ± 5*

Values are means (±S.E.M.) of three independent assays performed in duplicate.
* P < 0.05.
** P < 0.01.
*** P < 0.001 versus control.

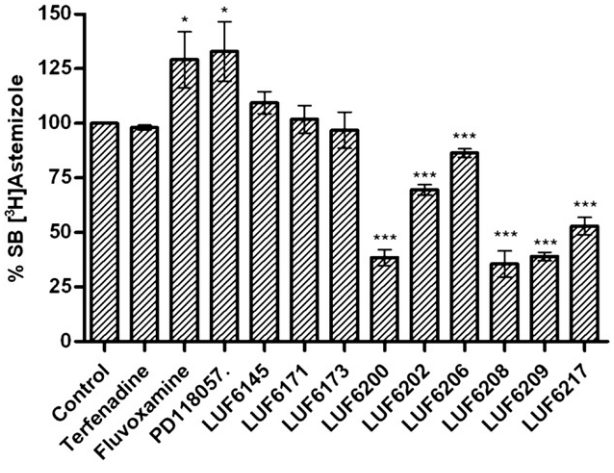

Fig. 4. Bar graphs of the percentage specific binding of [^{3}H]astemizole to the hERG K^+ channel after 10 min of dissociation induced by 10 μM astemizole in the absence (control) or presence of 10 μM of different compounds. The specific binding of [^{3}H]astemizole in the absence of compounds was set as 100%. The experiment was performed at 15 °C using 30 μg hERG/HEK293 membrane protein. Data shown are from three independent experiments performed in duplicate. Levels of significance vs control are *P < 0.05, **P < 0.01, ***P < 0.001.

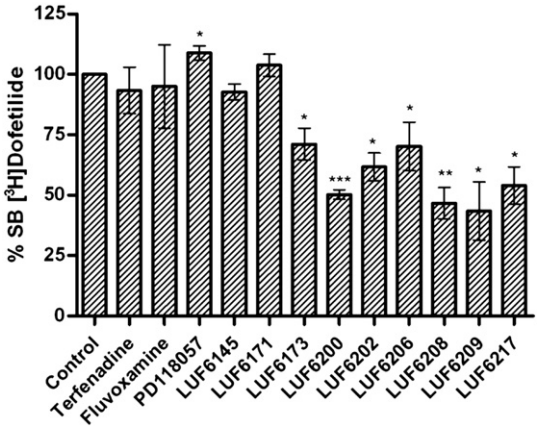

Fig. 5. Bar graphs of the percentage specific binding of [^{3}H]dofetilide to the hERG K^+ channel after 10 min of dissociation induced by 10 μM dofetilide in the absence (control) or presence of 10 μM of different compounds. The specific binding of [^{3}H]dofetilide in the absence of compounds was set as 100%. The experiment was performed at 25 °C using 20 μg hERG/HEK293 membrane protein. Data shown are from three independent experiments performed in duplicate. Levels of significance vs control are $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Effects of potassium ions on the acceleration of the [^{3}H]dofetilide dissociation rate induced by LUF6200

In order to investigate whether the two modulators described above acted at different sites to produce these effects, the concentration-dependent effect of LUF6200 on [^{3}H]dofetilide dissociation was studied in the presence of four different concentrations of KCl (Fig. 10). The data obtained are represented in two formats. Fig. 10A shows that addition of potassium ions decreased the dissociation, whereas LUF6200 dose-dependently enhanced that dissociation. Fig. 10B demonstrates that addition of potassium ions resulted in a concentration-dependent leftward shift of the LUF6200 concentration-effect curve. The EC_{50} values of LUF6200 in the presence of 2, 5, 10 and 60 mM potassium ions were 11 ± 1.7, 6.4 ± 0.74, 4.7 ± 0.53 and 3.5 ± 0.12 μM, respectively. This indicates that LUF6200 and potassium ions act at distinct allosteric sites on the hERG K^+ channel, and that potassium ions enhanced the allosteric effect of LUF6200.

Last, the cooperativity between LUF6200 and potassium ions was further analyzed according to a cooperativity model studied by us previously (Heitman et al., 2008b), and the curve connecting all the data is displayed in Fig. 10C. We discriminated three possible situations (Heitman et al., 2008b), in which two compounds showed positive ($\delta > 1$), neutral ($\delta = 1$) or negative cooperativity ($\delta < 1$). In the present study, our data points complied with $\delta > 1$, thus further supporting a positive cooperativity between the binding of LUF6200 and potassium ions.

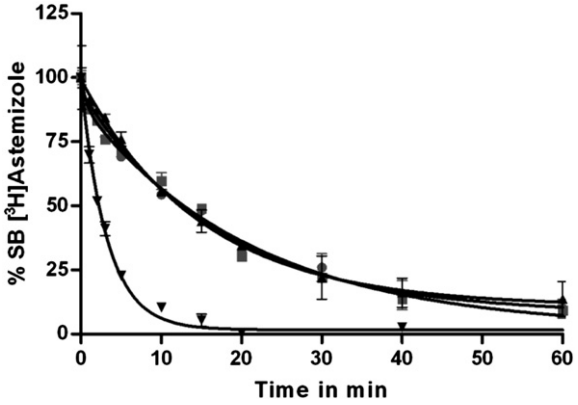

Fig. 6. Dissociation curves of [^{3}H]astemizole induced by 10 μM astemizole in the absence (control, ●) or presence of 10 μM fluvoxamine (■), PD118057 (▲) and LUF6200 (▼). The experiment was performed at 15 °C using 30 μg hERG/HEK293 membrane protein. Data shown are from three independent experiments performed in duplicate.

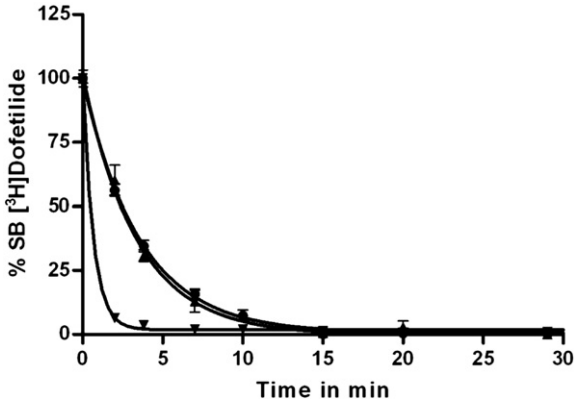

Fig. 7. Dissociation curves of [^{3}H]dofetilide by 10 μM dofetilide in the absence (control, ●) or presence of 10 μM PD118057 (▲) and LUF6200 (▼). The experiment was performed at 25 °C using 20 μg hERG/HEK293 membrane protein. Data shown are from three independent experiments performed in duplicate.

Discussion

In the present study, we evaluated the allosteric effects of a series of compounds with diverse chemical structures on the hERG K^+ channel using both [^{3}H]astemizole and [^{3}H]dofetilide binding assays, and also addressed the influence of potassium ions on the binding of two radioligands. To the best of our knowledge, this study is the first time to utilize radioligand binding assays for the characterization of allosteric modulators for the hERG K^+ channel. From these initial results, one potent allosteric inhibitor (LUF6200) was identified and several other compounds including LUF6208, LUF6209, LUF6217, LUF6202 and LUF6206 were also shown to possess allosteric inhibitory effects in two radioligand binding assays. Moreover, potassium ions increased the binding of [^{3}H]astemizole and [^{3}H]dofetilide to the hERG K^+ channel. Additionally, fluvoxamine and PD118057, known to have binding

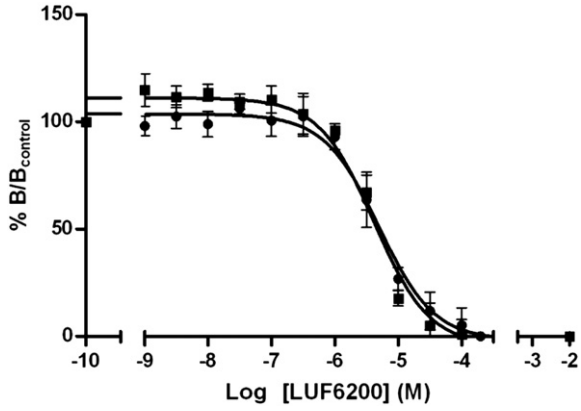

Fig. 8. Concentration dependence curves of LUF6200 for accelerating the [^{3}H]astemizole (●) or [^{3}H]dofetilide (■) dissociation from the hERG K^+ channel. Membranes were first pre-equilibrated with [^{3}H]astemizole or [^{3}H]dofetilide, then the dissociation was induced by 10 μM astemizole or dofetilide in the absence (control) or presence of different concentrations of LUF6200 and the reaction was terminated after 10 min. The results are expressed as the ratio of the specific binding of [^{3}H]astemizole or [^{3}H]dofetilide in the presence of 10 μM astemizole or dofetilide plus various concentrations of LUF6200 (B) over that in the presence of 10 μM astemizole or dofetilide alone ($B_{control}$). The experiments were performed at 15 °C using 30 μg hERG/HEK293 membrane protein for [^{3}H]astemizole and 20 μg membrane protein for [^{3}H]dofetilide binding. Data shown are from three independent experiments performed in duplicate.

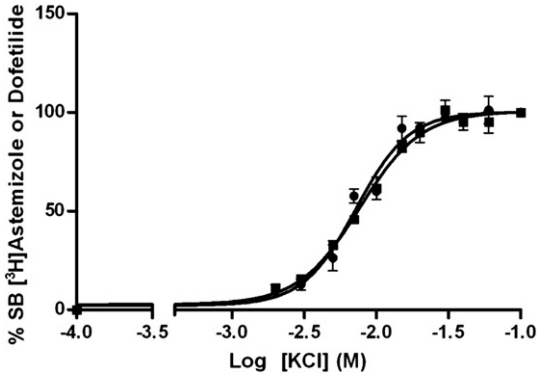

Fig. 9. Dose–response curves of the specific binding of [^{3}H]astemizole (●) and [^{3}H]dofetilide (■) at different concentrations of potassium ions. The experiments were performed at 25 °C using 30 μg hERG/HEK293 membrane protein for [^{3}H]astemizole binding and 20 μg membrane protein for [^{3}H]dofetilide binding. Data shown are from three independent experiments performed in duplicate.

sites distinct from most other hERG blockers, exhibited no allosteric effects in this investigation.

A common binding site for a wide spectrum of hERG blockers, including astemizole and dofetilide, has been implied through different methods (Fernandez et al., 2004; Kamiya et al., 2006). However, the amino acid residues involved in the interaction of these structurally diverse compounds may be different (Kamiya et al., 2006). Therefore, in this study two different radioligands ([^{3}H]astemizole and [^{3}H]dofetilide) were adopted in order to study, validate and compare the behavior of putative allosteric modulators. Since an altered dissociation rate is unequivocally indicative of an allosteric interaction (Chen et al., 2005), [^{3}H]astemizole and [^{3}H]dofetilide dissociation assays were firstly developed and then performed to reveal the allosteric effects of different ligands. The results in this paper show that both [^{3}H]astemizole and [^{3}H]dofetilide exhibit fast association rates and dissociate from the hERG K^+ channel after addition of an excess of unlabelled astemizole or dofetilide, indicative of the reversible binding of the two ligands. Additionally, both the association and dissociation curves of the two radioligands are better fitted with a single-site than a two-site model, which is different from the two binding sites model for [^{3}H]dofetilide reported by Duff et al. (1995). However, the latter experiments were performed on intact cells instead of membrane preparations used in our study. The diffusion into and possible trapping of [^{3}H]dofetilide inside the intact cells might affect the association and dissociation of the radioligand significantly.

Subsequently, single point dissociation assays were conducted to screen for allosteric modulators of the hERG K^+ channel. LUF6200 was selected as a representative allosteric inhibitor from the screening experiments due to its potent effects on both [^{3}H]astemizole and [^{3}H]dofetilide binding. Besides, terfenadine, which occupies the same binding site as astemizole and dofetilide (Zünkler and Friemel, 2009), did not display allosteric effects in either of the two radioligand screening assays, suggesting the validity and accuracy of our methods. To further characterize the allosteric effects of LUF6200, a full radioligand dissociation assay was performed in the presence of this compound. LUF6200 caused a 3.7- and 7.5-fold increase in the dissociation rates of [^{3}H]astemizole and [^{3}H]dofetilide, respectively. This further supports the notion that LUF6200 is an allosteric inhibitor of conventional hERG blockers binding to the hERG K^+ channel. The EC_{50} values from the two different radioligand dissociation assays were almost the same and in the micromolar range, which suggests that astemizole and dofetilide occupy the same binding site on the hERG K^+ channel. In a previous study from our laboratory, LUF6200 displaced [^{3}H]astemizole binding from the hERG K^+ channel under equilibrium conditions with a very low K_i value of 1.2 ± 0.6 nM (Shagufta et al., 2009). As evident from Fig. 8, there is little or negligible allosteric inhibition observed for

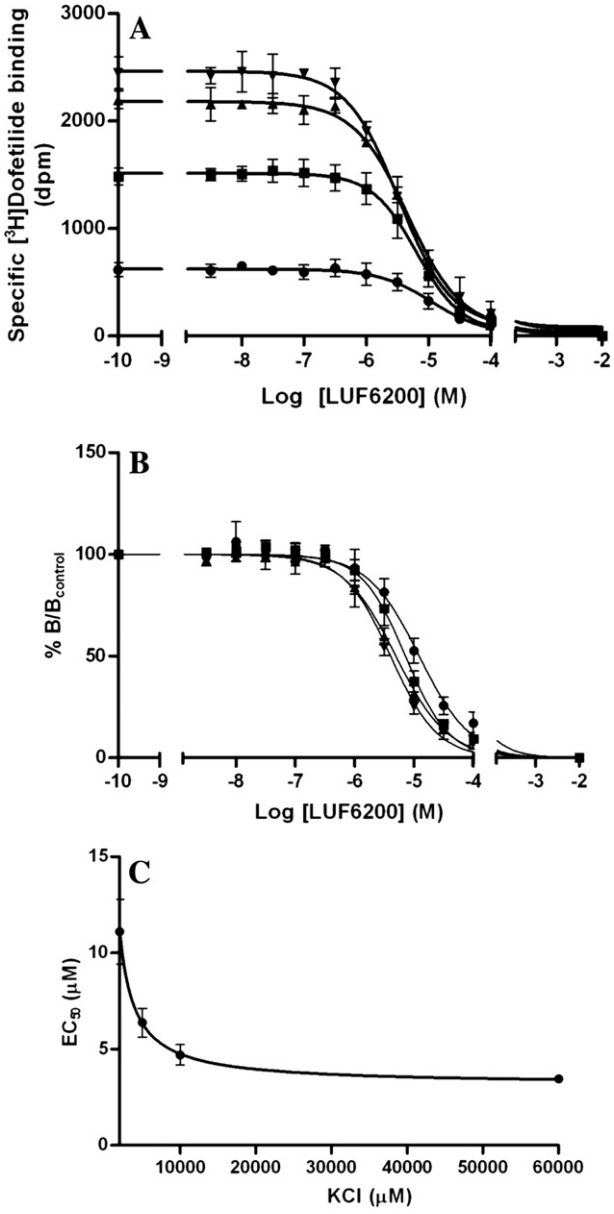

Fig. 10. Concentration-effect curves of LUF6200 for accelerating the [^{3}H]dofetilide dissociation from the hERG K^+ channel at 2 (●), 5 (■), 10 (▲) and 60 mM (▼) potassium ions before (A) and after normalization (B), and the positive cooperativity effect of potassium ions on LUF6200 in enhancing [^{3}H]dofetilide dissociation (C). Membranes were first pre-equilibrated with [^{3}H]dofetilide at different concentrations of potassium ions, then the dissociation was induced by 10 μM dofetilide in the absence (control) or presence of different concentrations of LUF6200 and the reaction was terminated after 6 min. The results in B are expressed as the ratio of the specific binding of [^{3}H]dofetilide in the presence of 10 μM dofetilide plus various concentrations of LUF6200 (B) over that in the presence of 10 μM dofetilide alone ($B_{control}$). The experiments were performed at 15 °C using 20 μg hERG/HEK293 membrane protein. Data shown are from three independent experiments performed in duplicate (A and B); the experimental data of different concentrations of potassium ions affecting the modulating potency of LUF6200 are exhibited with standard error of the mean from three independent experiments (C).

LUF6200 at a concentration lower than 0.1 μM. Taken together, we conclude that LUF6200 interacts with the hERG K^+ channel in a complicated fashion: it behaves as an orthosteric blocker at lower concentrations and as an allosteric inhibitor at higher concentrations.

Two major binding regions have been hypothesized for the hERG K^+ channel, one in the transmembrane region, the other extracellularly (Perry et al., 2010; Vargas et al., 2008). Most hERG blockers bind to the central transmembrane cavity of the channel, whereas agents described as facilitators, activators or enhancers of the hERG K^+ channel

usually interact with a region facing away from the inner cavity (Perry et al., 2010). For instance, A-935142 has recently been stated to possess a binding site responsible for hERG current enhancement which is different from the pore binding site for traditional hERG blockers (Liu et al., 2012). The binding sites of several peptide blockers like saxitoxin and BeKm-1 are located in the extracellular parts of the hERG K^+ channel (Milnes et al., 2003b; Wang et al., 2003) and some metal ions bind to different regions of the pore of the channel in comparison to most other hERG blockers (Elinder and Århem, 2003; Reinés et al., 2004). Additionally, it has been reported that a few nontoxin hERG blockers are unlikely to fit within the inner cavity and must bind elsewhere (Milnes et al., 2003a; Mitcheson, 2003). Thus, multiple binding sites of these diverse compounds or ions at the hERG K^+ channel imply the plausible allosteric modulation between them. They might allosterically increase (allosteric inhibitors/negative allosteric modulators) or decrease (allosteric enhancers/positive allosteric modulators) the dissociation rates of typical hERG blockers from the channel. The current study does not allow determining the location of the putative binding site of LUF6200. Its nanomolar potency as a classical blocker suggests that it interacts with the central cavity; for its allosteric property a different binding site or different conformation of the channel may play a role, and further studies such as chemical cross linker and/or photolabeling experiments are required to elucidate the exact binding sites of this compound.

The results in this paper also show that fluvoxamine does not behave as an allosteric modulator when tested in a rigorous kinetic assay. However, it has been reported that the binding of fluvoxamine to the hERG K^+ channel did not involve amino acids such as Y652 and F656 that are crucial to the binding of other general hERG blockers (Lees-Miller et al., 2000; Mitcheson et al., 2000); hence, it was suggested that fluvoxamine probably binds to an extracellular site at the outer mouth of the channel (Mitcheson, 2003). In addition, the activator PD118057 did not exert allosteric enhancement in both [^{3}H]astemizole and [^{3}H]dofetilide dissociation assays. PD118057 has been identified to bind to a hydrophobic binding pocket instead of the shared binding site for hERG blockers at the channel (Zhou et al., 2011). The results obtained for these two compounds suggest that even though a compound binds to a distinct region of the hERG K^+ channel, its allosteric effects on the binding of other hERG blockers are still uncertain and need to be validated in more detailed experiments.

Since it has been reported that the extracellular potassium concentration affected the binding of [^{3}H]dofetilide to neonatal mouse myocytes (Duff et al., 1997), we also studied the allosteric effects of potassium ions and found that these monovalent cations increase both [^{3}H]astemizole and [^{3}H]dofetilide binding in a concentration-dependent manner with very similar EC_{50} values. In earlier studies on a variety of ion channels, it was reported that metal ion binding to the pore of an ion channel may affect the occupancy of the binding site for other ligands and thus decrease their binding (Demo and Yellen, 1992; Reinés et al., 2004). This is in contrast to the observation in our study. Thus, we assume that K^+ ions possibly function as an allosteric enhancer of hERG blockers by changing the conformation of their binding site rather than competitively occupying their binding site. Since two distinct allosteric sites on the human GnRH receptor (Heitman et al., 2008b) and three different allosteric sites on the dopamine D_4 receptor (Schetz and Sibley, 2001) have been reported, we wondered whether LUF6200 and potassium ions exert their effect through two distinct allosteric sites on the hERG K^+ channel as well. Potassium ions both enhance the allosteric effect of LUF6200 and shift the LUF6200 dose–response curve to the left, supporting that the two modulators are indeed acting at different binding sites at the hERG K^+ channel. Furthermore, a curve described in Fig. 10C indicated a positive cooperativity between LUF6200 and potassium ions. Taken together, we anticipate that there are at least two different sites of allosteric modulation for hERG blockers at the channel and that they interact with each other as well.

It is hypothesized that the ceiling effect of allosteric modulators of ligand-gated ion channels has important advantages over classic orthosterically acting compounds or open channel blockers, because it offers a much larger safety margin when it comes to drug administration and patient compliance (Hogg et al., 2005). A similar scenario may also be feasible for the hERG K^+ channel. For allosteric inhibitors one might argue that when non-cardiovascular drugs (e.g. astemizole) are combined with allosteric inhibitors of the hERG K^+ channel, their cardiac side effects might be diminished. On the other hand, when allosteric enhancers are administered together with antiarrhythmic drugs such as dofetilide whose desired target is the hERG K^+ channel, the therapeutic dose might be lowered, thereby possibly preventing adverse drug reactions such as TdP. Taken together, the discovery of allosteric modulators for the hERG K^+ channel might open new avenues for treating clinical conditions related to the hERG K^+ channel as well as potentially reducing hERG cardiotoxicity of other non-cardiovascular drugs by combination therapy.

In summary, the present study provides evidence for the allosteric effects of dofetilide analogs on the binding of hERG blockers to the hERG K^+ channel using both [^{3}H]astemizole and [^{3}H]dofetilide binding assays. LUF6200 emerges as an allosteric inhibitor with micromolar potency. Potassium ions allosterically enhance the binding of typical hERG blockers in concentrations that are physiologically relevant. Moreover, LUF6200 and potassium ions act at distinct allosteric sites on the hERG K^+ channel, and these binding sites show positive cooperativity. In addition, neither fluvoxamine nor PD118057 exhibit significant allosteric effects on the binding of two radioligands. This new information may be relevant for a further analysis of hERG-related cardiotoxicity and provide novel solutions for proarrhythmic side effects induced by hERG blockers.

Conflict of interest statement

None.

Acknowledgments

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