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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

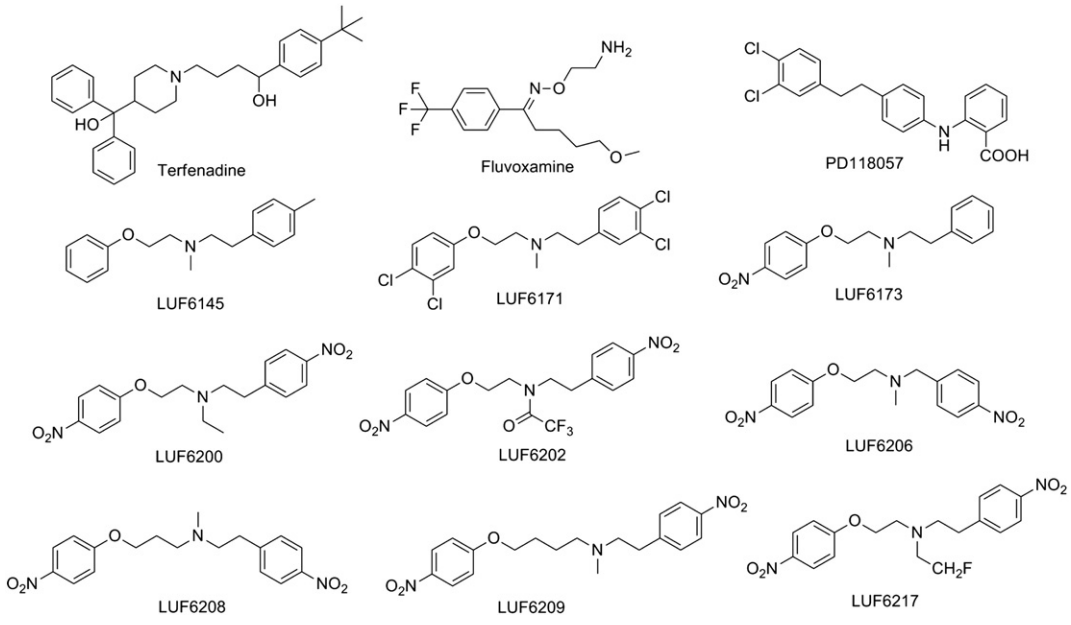

Fig. 1. Compounds evaluated in this study. Terfenadine is a high-affinity reference hERG blocker, fluvoxamine is a low-affinity reference hERG blocker, PD118057 is a reference hERG activator, and all the other LUF compounds are members of a previously disclosed compound series interacting with the hERG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