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Original article

Early evaluation of compound QT prolongation effects: A predictive 384-well fluorescence polarization binding assay for measuring hERG blockade

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Abstract

Introduction: A large number of drugs from a variety of pharmacological classes have been demonstrated to cause adverse effects on cardiac rhythm, including the life-threatening arrhythmia *Torsades de Pointes*. These side effects are often associated with prolongation of the QT interval and are mediated via blockade of the human ether-a-go-go related gene (hERG) encoded potassium channel. In order to manage this risk in the pharmaceutical industry it is desirable to evaluate QT prolongation as early as possible in the drug discovery process. **Methods:** Here we describe the development of a 384-well fluorescence polarization (FP) binding assay compatible with high-throughput assessment of compound blockade of the hERG channel during the lead optimisation process. To characterise the fluorescent ligand that was developed, competition binding studies, kinetic studies and electrophysiology studies were performed. Furthermore, to validate the assay as a key screening method a series of competition binding studies were performed and correlated with functional data obtained via patch-clamp. **Results:** Evaluation of the assay indicates that high quality data is obtained ($Z' > 0.6$), that the K_i values determined are equivalent to more traditional radiometric methods and that it is predictive for functional hERG blockade as assessed by patch clamp. **Discussion:** Whilst FP assays, utilizing a variety of fluors, have become well established for the evaluation of G-protein-coupled receptor (GPCRs) and kinase ligand interactions, this technique has not been applied widely to the study of ion channels. Therefore, this represents a novel assay format that is amenable to the evaluation of thousands of compounds per day. Whilst other assay formats have proven predictive or high throughput, this assay represents one of few that combines both attributes, moreover it represents the most cost effective assay, making it truly amenable to early assessment of hERG blockade.
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1. Introduction

Over the last few years drug-induced cardiac arrhythmia has become a key safety concern for the pharmaceutical industry and its regulatory bodies, with a wide variety of pharmacological agents having been found to cause potentially lethal prolongation of the QT phase of the electrocardiogram (Calderone, Testai, Martinotti, Del Tacca, & Breschi, 2005; De Ponti, Poluzzi, Cavalli, Racanatini, & Montanaro, 2002; Fermini & Fossa, 2003; Haverkamp et al., 2000). Furthermore, it is well established that such acquired long QT syndrome (ALQTS) is predominantly mediated through blockade of potassium channels encoded by the human ether-a-go-go related gene (hERG), which comprise the delayed rectifier potassium current involved in cardiac repolarization (I_{Kr}) (Curran et al., 1995; Pearlstein, Vaz, & Rampe, 2003; Sanguinetti, Jiang, Curran, & Keating, 1995; Vandenberg, Walker, & Campbell, 2001). With recent emphasis on the pharmaceutical industry to decrease costs and improve efficiency by minimising attrition, it is clearly

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desirable to evaluate compounds for their potential to prolong QT as early as possible and ideally during early lead optimisation (Fermini & Fossa, 2003; Kola & Landis, 2004). Whilst molecular models that predict hERG blockade could be employed as an early filter, these models are only as effective as the biological data used to define them and as more information is known about non-blockers than blockers, at present false predictions could be obtained (Aronov, 2005). Therefore, to facilitate early evaluation of the potential for compound QT prolongation a high-throughput, low cost assay is required. Furthermore, it is essential that this assay is predictive of other *in vitro* downstream studies such as electrophysiology so that, in combination with appropriate pharmacokinetic/dynamic considerations and *in vivo* data, accurate integrated assessments can be made regarding the safety margin of compounds entering the clinic (Redfern et al., 2003; Webster, Leishman, & Walker, 2002).

There are a number of "high-throughput" assay technologies that have been evaluated for profiling compound activity at hERG, including radioligand binding (Chiu et al., 2004; Diaz et al., 2004; Finlayson, Turnbull, January, Sharkey, & Kelly, 2001), membrane potential (Dorn et al., 2005), rubidium efflux (Cheng et al., 2002; Tang et al., 2001) and automated patch clamp (Bridgland-Taylor et al., 2006; Dubin et al., 2005). However, as highlighted in recent reviews (Netzer, Bischoff, & Ebneth, 2003; Wood, Williams, & Waldron, 2004; Zheng, Spencer, & Kiss, 2004) the currently available techniques have limitations either in terms of cost, throughput or discrepancies in the pharmacological profile that could result in false negatives. Here we describe the development and characterisation of a new binding assay for the hERG channel, which is compatible with high-throughput 384-well screening, using the inexpensive, non-radiometric fluorescence polarization (FP) technology. The assay demonstrates an excellent correlation with historical radiometric binding and electrophysiology data, indicating that it will be a valuable drug discovery screen which is compatible with the early elimination of compounds that have the potential to induce QT effects in patients.

2. Methods

2.1. Manual electrophysiological characterisation studies

Testing was carried out in HEK 293 cells transfected with the hERG gene (Zhou et al., 1998) maintained at 37 °C in Minimum Essential Medium with Earle's Salts and L-glutamine, 10% fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids and 0.4 mg/mL geneticin. Membrane currents of cells continuously perfused with 35 °C extracellular recording saline (in mM: 137 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 D-glucose, pH 7.4, 335 mOs) were measured using whole-cell patch clamp (Hamill, Marty, Neher, Sakmann, & Sigworth, 1981) with a MultiClamp 700A amplifier (Molecular Devices) and 3–5 MΩ glass pipettes filled with intracellular recording saline (in mM: 130 KCl, 1 $MgCl_2$, 10 HEPES, 5 Mg-ATP, 5 EGTA, pH 7.2, 320 mOs). The liquid junction potential was 4 mV. Access resistance was compensated by at least 80%, gain was set to 1 mV/pA, and Bessel filters were set to 3 and 10 kHz.

For initial characterization of C6–Cy3B ligand potency, the hERG current was activated with a voltage step to +20 mV for 1 s followed by a ramp to −80 mV at 0.5 V/s delivered at 0.25 Hz (2.8-s interpulse interval). Following 5 min of stable control recording in which rundown of the tail current peak was <2%/min, Cy3B ligand solution was continuously perfused into the recording chamber for evaluation of current blockade, and reversal was evaluated by perfusion with saline upon reaching an apparent steady state inhibition. In order to investigate the use-dependent nature of block by the C6–Cy3B ligand (100 nM), the hERG current was activated with a voltage step to 0 mV for 4 s followed by a ramp to −80 mV at 0.4 V/s delivered at 0.14 Hz (2.8-s interpulse interval). The magnitude of block was determined and compared to data obtained with the +20 mV voltage step for 1 s with 100 nM C6–Cy3B ligand. To further explore use-dependent block of the hERG current, the rate of onset of block by the C6–Cy3B ligand (1 μM) was evaluated at different frequencies of channel activation. The hERG current was activated using a voltage step to +20 mV for 1 s followed by a ramp to −80 mV at 0.5 V/s, and interpulse intervals of 1.0, 2.2 and 6.8 s were investigated. Peak currents in the presence of compound were normalized to the rundown adjusted control current amplitude, plotted as a function of time, and fit with a single exponential equation to derive a time constant (τ) for onset of block.

2.2. Fluorescence polarization

Membrane homogenates of HEK 293 (Cell line #15-08) cells expressing the hERG product supplied by (PGRD) Sandwich Laboratories were prepared as follows. Cell pellets were thawed at room temperature and kept on ice. Buffer (50 mM Tris–HCl, 1 mM $MgCl_2$, 10 mM KCl, pH 7.4, 4 °C) was added to each cell pellet (10 mL of buffer per 10 g of packed cell pellet) and the mixture homogenised using an Omni LabTek homogeniser (20,000 rpm for 30 s). The homogenate was centrifuged at 48,000×*g* for 20 min between 3 and 5 °C in a Sorvall Evolution RC centrifuge and the supernatant discarded. The pellet was resuspended, homogenised (20,000 rpm for 10 s), and centrifuged as before. The resultant supernatant was discarded and the final pellet resuspended (100 mL of the above buffer per 10 g of packed cell pellet), homogenised (20,000 rpm for 10 s), dispensed in to tubes in 1, 2 and 5 mL aliquots and stored between −75 °C and −85 °C until use. Protein concentration was determined using a Coomassie Blue kit as per manufacturer's instructions (Sigma 610A and 610-11).

The Cy3B ligand was stored in 100% DMSO and diluted to 6 nM in assay buffer (50 mM Tris–HCl, 1 mM $MgCl_2$, 10 mM KCl, 0.05% Pluronic F127, pH 7.4, 4 °C) on the day of the experiment. Test samples and controls were diluted in 6% DMSO, 0.05% Pluronic F127. Cell membranes were removed from the −80 °C freezer and placed on ice after defrosting. When required the defrosted membranes were homogenised using a polytronic device for no more than 10 s, they were then diluted in the above assay buffer to produce a working solution of 0.3 mg/mL. The assay was compiled by adding 10 μL of test compound or control solution, 10 μL of the Cy3B ligand and 10 μL of cell

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