Journal of Pharmacological and Toxicological Methods 59 (2009) 29–34

Contents lists available at ScienceDirect

Journal of Pharmacological and Toxicological Methods

journal homepage: www.elsevier.com/locate/jpharmtox

Original article

Test article concentrations in the hERG assay: Losses through the perfusion, solubility and stability

Jessica C. Brimecombe *, Glenn E. Kirsch, Arthur M. Brown

ChanTest Corporation, 14656 Neo Parkway, Cleveland, OH 44128, USA

ARTICLE INFO

Article history:
Received 10 July 2008
Accepted 27 October 2008

Keywords:
hERG
Dose solution analysis
Preclinical methods
Purkinje
Solubility
Stability

ABSTRACT

Introduction: Drug-induced prolongation of the electrocardiographic QT interval (long QT syndrome) has been associated with increased risk of a serious ventricular arrhythmia, torsade de pointes. Inhibition of hERG, a cardiac potassium channel that controls action potential repolarization, is the most common cause of QT prolongation by non-cardiac drugs. The ICH S7B describes preclinical safety testing required for new drugs, including the determination of the hERG IC_{50}. Actual and target concentrations may differ due to solubility, stability, or loss of compound. Significant differences will invalidate quantitative concentration–response curves which may be critical to interpretation of drug safety. To examine the frequency and significance of these differences, we conducted an analysis of studies where both the electrophysiology and the dose solution analysis were conducted in-house. We have investigated the actual concentrations of test article in vehicle solution as compared to the target concentrations in an attempt to determine the reasons behind differences between the two values. **Methods:** Studies that involved both electrophysiology and dose solution analysis performed at ChanTest Corporation were evaluated. The effects of stability, solubility and loss through the perfusion apparatus on actual dosing concentrations were investigated. **Results:** There was a large range in the loss of the test article attributed to the perfusion apparatus (range from 0 to 74% loss). For 12 of the 22 studies evaluated, the IC_{50} was>2-fold more potent when using actual values as determined by HPLC versus the target concentrations. Twenty-two percent of the test articles were not stable 24 h after room temperature storage; 16% after 24 h frozen conditions. **Discussion:** The best practices when considering dose solution concentration verification of test article solutions are to: determine the solubility of the compound in a physiological buffer, analyze samples collected from the perfusion chamber, and analyze samples the same day as sample collection (e.g., same day as hERG assay).

1. Introduction

Drug-induced prolongation of the electrocardiographic QT interval (long QT syndrome) has been associated with increased risk of a serious ventricular arrhythmia, torsade de pointes. Inhibition of I_{Kr}, a potassium current that controls cardiac action potential repolarization, is the most common cause of QT prolongation by non-cardiac drugs (Brown & Rampe, 2000; Weirich & Antoni, 1998; Yap & Camm, 1999). The cardiac potassium channel, hERG, is responsible for a rapid delayed rectifier current (I_{Kr}) in human ventricles (Curran et al., 1995; Sanguinetti, Jiang, Curran, & Keating, 1995). Drugs that inhibit hERG have the potential to prolong the cardiac action potential and the QT interval, and cause torsade de pointes. A number of drugs, including antibiotics, antipsychotics, and gastrointestinal prokinetic agents, have been withdrawn from the market due to delaying ventricular repolarization and increasing the risk of torsade de pointes (Roy, Dumaine, & Brown, 1996; Suessbrich, Waldegger, Lang, & Busch, 1996; Mohammad, Zhou, Gong, & January, 1997; Rampe, Roy, Dennis, & Brown, 1997; Iannini, 2002; Ray et al., 2004). New chemical entities investigated for use in humans are now required to be evaluated for this potentially lethal side effect. The International Conference of Harmonisation issued the S7B guideline "The Non-Clinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals" in 2005 which describes non-clinical testing strategies to evaluate new chemicals for the potential to prolong the QT interval (www.ich.org). This document supports the evaluation of a new chemical entity in an *in vitro* electrophysiological hERG assay and in an *in vivo* QT assay. In the hERG assay, tests are conducted over a range of concentrations sufficient to determine the IC_{50} or the maximum inhibitory effect at the highest achievable concentration within the physico-chemical limits of the assay (e.g., solubility or cytotoxicity).

Section 58.113 of 21 CFR Part 58 (Good Laboratory Practice for Non Clinical Laboratory Studies) of the Food & Drug Administration Code of Federal Regulations requires that the uniformity, stability,

* Corresponding author. Tel.: +1 216 332 1665; fax: +1 216 332 1706.
E-mail address: jbrimecombe@chantest.com (J.C. Brimecombe).

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doi:10.1016/j.vascn.2008.10.004

and concentration of a test and/or control article in a solvent/vehicle be determined. This is to ensure that the actual concentrations of test article applied to the test system are comparable to the target concentrations. A validated analytical method for each test compound in the vehicle is developed to accurately measure the test article concentrations in the vehicle solution. Poor solubility, stability and/or binding to the study equipment can lower the actual drug concentration applied to the test system (Herron, Tower, & Templeton, 2004). Thus, if analytical methodology is not used to determine the actual concentrations in the vehicle solution applied to the test system, the possibility exists of overestimating or underestimating the concentrations applied. This would result in an inaccurate IC_{50}, with the reported IC_{50} being higher or lower than the actual value. In the case of the hERG safety test, errors would occur in the assessment of hERG risk and the calculated safety margin (Redfern et al., 2003).

We conducted an analysis of studies where both the electrophysiology and the dose solution analysis were conducted in-house. We have investigated the actual concentrations of test article in vehicle solution as compared to the target concentrations in an attempt to determine the reasons behind differences between the two values.

2. Methods

2.1. Cell culture

Human embryonic kidney (HEK293) cells were stably transfected with hERG cDNA. Stable transfectants were selected by coexpression with the G418-resistance gene incorporated into the expression plasmid. Selection pressure was maintained by including G418 in the culture medium. Cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (D-MEM/F-12) supplemented with 10% fetal bovine serum, 100 U/mL penicillin G sodium, 100 μg/mL streptomycin sulfate and 500 μg/mL G418. Cell culture stock plates were prepared each week. Cells for electrophysiological recording were cultured in 35-mm plastic dishes.

2.2. Vehicle solution

HEPES-buffered physiological saline solution (HB-PS) consisted of (composition in mM): NaCl, 137; KCl, 4.0; $CaCl_2$, 1.8; $MgCl_2$, 1; HEPES, 10; Glucose, 10; pH adjusted to 7.4 with NaOH (prepared weekly and refrigerated until use), supplemented with 0.3% DMSO. Canine Purkinje fiber buffer was composed of (composition in mM): NaCl, 131; KCl, 4.0; $CaCl_2$, 2.0; $MgCl_2$, 0.5; $NaHCO_3$, 18.0; NaH_2PO_4, 1.8; Glucose, 5.5, supplemented with 0.3% DMSO. Rabbit Purkinje fiber buffer was composed of (composition in mM): NaCl, 131; KCl, 5.4; $CaCl_2$, 2.0; $MgCl_2$, 0.5; $NaHCO_3$, 18.0; NaH_2PO_4, 1.8; Glucose, 5.5, supplemented with 0.3% DMSO.

2.3. Electrophysiology

Cells in plastic culture dishes were transferred to the stage of an inverted phase-contrast microscope. Cells were superfused with vehicle control solution (HB-PS). Micropipette solution for whole cell patch clamp recordings was composed of (mM): potassium aspartate, 130; $MgCl_2$, 5; EGTA, 5; ATP, 4; HEPES, 10; pH adjusted to 7.2 with KOH. Micropipette solution was prepared in batches, aliquoted, stored frozen, and a fresh aliquot thawed each day. The recording was performed at a temperature of 35±2 °C using a combination of in-line solution pre-heater, chamber heater, and feedback temperature controller. Temperature was measured using a thermistor probe in the recording chamber. Micropipettes for patch clamp recording were made from glass capillary tubing using a P-97 micropipette puller (Sutter Instruments, Novato, CA). A commercial patch clamp amplifier was used for whole cell recordings. Before digitization, current records were low-pass filtered at one-fifth of the sampling frequency.

Cells stably expressing hERG were held at −80 mV. Onset and steady state inhibition of hERG potassium current due to the test article were measured using a pulse pattern with fixed amplitudes (conditioning prepulse: +20 mV for 1 s; repolarizing test ramp to −80 mV (−0.5 V/s) repeated at 5 s intervals. Each recording ended with a final application of a supramaximal concentration of the reference substance (E-4031, 500 nM), to assess the contribution of endogenous currents. The remaining uninhibited current was subtracted off-line digitally from the data to determine the potency of the test substance for hERG inhibition. One or more test article concentrations were applied sequentially (without washout between test substance concentrations) in ascending order, to each cell. Peak current was measured during the test ramp. A steady state was maintained for at least 20 s before applying test article. Peak current was measured until a new steady state was achieved or 12 min of exposure time had elapsed.

2.4. Electrophysiology patch clamp data analysis

Data were stored on the ChanTest computer network (and backed-up nightly) for off-line analysis. Data acquisition and analyses were performed using the suite of pCLAMP (Version 8.2) programs (MDS-AT, Sunnyvale, CA). Steady state was defined by the limiting constant rate of change with time (linear time dependence). The steady state before and after test article application was used to calculate the percentage of current inhibited at each concentration. Concentration–response data were fit to an equation of the form:

$$\%\,\text{Inhibition} = \{1-1/[1+([Test]/IC_{50})N]\}*100$$

Where [Test] is the test article concentration, IC_{50} is the test article concentration at half-maximal inhibition, N is the Hill coefficient, and % Inhibition is the percentage of current inhibited at each test article concentration. Nonlinear least squares fits were solved with the Solver add-in for Excel 2000, or later (Microsoft, WA). The IC_{50} was calculated if the test article produced greater than 50% inhibition at the highest concentration.

2.5. Analytical chemistry methods

Analytical methods were developed for each test article. High Performance Liquid Chromatography (HPLC) instruments using UV detection from Waters Corporation were used. The test method procedure was validated to establish stability, linearity, accuracy and intra-assay precision (repeatability), and lack of interference in the chromatographic region of interest. Data collection was performed and peak areas of the test articles were measured using Empower software from Waters Corporation. Descriptive statistics including arithmetic mean (average) and standard deviation, % CV (coefficient of variance) and % RE (relative error) were conducted using Microsoft Excel, version 2000, or later (Microsoft, WA). The accuracy of the dose solution samples was measured as the % RE using the equation % RE = 100 × (nominal concentration − actual concentration)/nominal concentration. The % change was used for the calculation of the stability of the samples using the equation % change = 100 × (measured concentration at T_n − mean concentration at T_0)/mean concentration at T_0, where T_n = time of the non-zero time point. The acceptance criterion for accuracy of the dose solution concentrations was defined as ±15% or in some studies, ±10% of the target concentration. Likewise, the acceptance criterion for the stability of the formulations was defined as ≤15%, or in some studies, ≤10% change in concentration between time points.