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Effects of probucol, a typical hERG expression inhibitor, on *in vivo* QT interval prolongation in conscious dogs

Hisashi Nogawa *, Tomoyuki Kawai, Miho Yajima, Masahiro Miura, Tetsurou Ogawa, Kouji Murakami

Discovery Research Laboratories, Kyorin Pharmaceutical Co., Ltd., 2399-1 Nogi, Nogi-machi, Shimotsuga-gun, Tochigi 329-0114, Japan

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ABSTRACT

The cholesterol-lowering drug, probucol, is known to induce QT interval prolongation and *torsades de pointes* in patients. Recent *in vitro* studies have indicated that probucol reduces hERG expression in the plasma membrane and does not directly block human ether-a-go-go-related gene (hERG) channels. The present study was performed to investigate the effects of probucol on *in vivo* QT interval prolongation. Epicardial electrocardiograms were recorded in conscious dogs given oral single or repeated (7 days) doses of probucol (100 mg/kg), and in combination with moxifloxacin (20 mg/kg). QTc intervals were analyzed by a probabilistic method with individual rate collection formulae. Values of change in QTc (< DELTA > QTc) interval and its integration from 1 to 21 h ($AUC_{1-21\ h}$) were calculated to evaluate drug-induced QT prolongation. A single dose of probucol slightly but significantly increased the $AUC_{1-21\ h}$ QTc interval on days 2 and 3. The QT prolongation was markedly augmented by repeated doses of probucol in a time-dependent manner, despite the lack of increase in plasma concentration. The combination of probucol and moxifloxacin produced additive effects on QT interval prolongation. These results suggest that long-term exposure to the hERG expression inhibitor, probucol, is required to evaluate its maximal effects on *in vivo* QT interval prolongation. A combination of direct and indirect hERG inhibitors may produce simple additive effects on QT interval prolongation.

1. Introduction

It was first reported in the 1990s that several non-cardiovascular drugs, including antihistamines, antibiotics and anti-arrythymics, induced QT interval prolongation, a cardiac repolarisation disorder that can lead to life-threatening polymorphic ventricular arrhythmia, *torsades de pointes* (TdP) and sudden cardiac death (Redfern et al., 2003; Straus et al., 2005; Yap and Camm, 2003). This drug-induced QT interval prolongation was reported to be mainly due to direct blockade of the rapidly activating delayed rectifier K^+ current (I_{Kr}) encoded by the human ether-a-go-go-related gene (hERG) by the drug (Sanguinetti and Tristani-Firouzi, 2006), and the direct blockade of hERG potassium channels by drugs has become a central target of drug safety programs in cardiotoxicity.

On the other hand, some drugs were recently shown to indirectly block hERG potassium channels, such as by disrupting hERG expression in the plasma membrane, representing another mechanism for drug-induced long QT syndrome (Ficker et al., 2004; Guo et al., 2007; Kuryshev et al., 2005; Rajamani et al., 2006). The mechanisms by which these drugs disrupt hERG expression have not yet been clarified, but some of these drugs were reported to inhibit trafficking of hERG protein to the plasma membrane or accelerate degradation of hERG channel protein in the plasma membrane (Ficker et al., 2003; Guo et al., 2011). In addition, a recent screen for drug-disrupted hERG expression identified a number of direct hERG blockers (~40%) that carry the additional risk of hERG trafficking inhibition (Wible et al., 2005). These drugs represent dual hERG risk, *i.e.*, inhibition and direct channel blockade, and would make the heart more sensitive to QT interval prolongation and vulnerable to the development of arrhythmia. However, little information is available about the *in vivo* QT interval prolongation effects of hERG expression inhibitors, and there have been no previous reports regarding the combination of such drugs with direct inhibitors.

The cholesterol-lowering drug, probucol, is known to cause QT interval prolongation and TdP in patients (Hayashi et al., 2004; Matsuhashi et al., 1989; Tamura et al., 1994). Recently, Guo et al. (2007) reported that probucol reduced hERG protein level in the plasma membrane and did not directly block hERG channels expressed in human embryonic kidney 293 cells. In addition, the mechanism underlying the probucol-induced reduction of hERG expression was shown to involve accelerated degradation of

* Corresponding author. Tel.: +81 280 56 2201; fax: +81 280 57 1293.
E-mail address: hisashi.nogawa@mb.kyorin-pharm.co.jp (H. Nogawa).

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mature hERG channels that associate with caveolon-1 (Guo et al., 2011). However, drug-disrupted hERG expression was studied only by *in vitro* experiments. In the present study, we investigated whether a single dose or repeated doses over 7 days of probucol resulted in QT interval prolongation by probabilistic QT analysis in conscious dogs. Moreover, to investigate the QT interval prolongation effects of a combination of hERG expression inhibitor and direct inhibitor, we examined the effects of moxifloxacin, a typical direct hERG channel inhibitor, on QT interval prolongation in combination with probucol.

2. Materials and methods

2.1. Animal use and care

Animals were cared for throughout the study in accordance with the institutional protocol and standard operating procedures approved by the Institutional Animal Care and Use Committee of Kyorin Pharmaceutical Co., Ltd. (Tochigi, Japan). Four male beagle dogs (weight 10–13 kg) were purchased from Kitayama Labs (Yamaguchi, Japan). All dogs were individually housed in stainless steel cages and kept under controlled environmental conditions with a temperature of 20–26 °C, relative humidity of 40–75% and 12-h lighting schedule (light on: 07:00–19:00). The animals were given approximately 200–300 g/day of solid food and allowed free access to tap water.

2.2. Surgical procedure

The animals were pre-treated with acepromazine (0.05 mg/kg intramuscular (i.m.)), followed by anesthesia with thiopental (20 mg/kg intravenous (i.v.)), and maintained with isoflurane (1–3% in O_2 and N_2O, 1:1) under artificial ventilation after tracheal intubation. A radiotelemetry transmitter (TL11M2-D70-PCT; Data Sciences International, St. Paul, MN, USA) was implanted subcutaneously into the left dorsal region. The positive electrocardiogram (ECG) electrode was sutured directly to the left ventricular epicardium near the ventricular apex, and the negative electrode was secured to the left auricle. A telemetric transducer for measuring blood pressure was inserted into the left femoral artery, and body temperature was monitored by a sensor located within telemetry implant. The animals received intercostal nerve block with a local anesthetic (lidocaine; 2%, 2 mL/dog) and then treated with antibiotic (penicillin; 200,000 units/dog) for several days after surgery. Experiments were performed after at least one month of post-surgical recovery.

2.3. Drug preparation

Probucol (Sinlestal®; Daiichi Sankyo Co., Ltd., Tokyo, Japan) was purchased as fine granules. The dose of probucol was set at 100 mg/kg. Moxifloxacin hydrochloride (Avelox®; Bayer Health Care, Osaka, Japan) tablets were ground with a pulverizing mill, and the dose of moxifloxacin was set at 20 mg/kg. Each drug was added to gelatine capsules, and administered orally to the animals.

2.4. Experimental protocol

Fig. 1 illustrates the experimental procedure. We performed six experiments to clarify the effects of probucol on *in vivo* QT interval prolongation and investigate the QT interval prolongation effect of a combination of probucol and moxifloxacin. In the four beagle dogs: (1) non-drug treatment for 5 days; (2) single dose of vehicle; (3) single dose of probucol; (4) single dose of moxifloxacin; (5) single dose of moxifloxacin after a single dose of probucol; (6) single dose of moxifloxacin after repeated dose of probucol for 7 days. Probucol was administered orally once a day in repeated dose experiments. Recovery from the effects of probucol was evaluated after experiment (6). Each experiment was performed with at least a one-week interval after the last administration, and the stability of ECG parameters had continued for 3 months, including all of the present experimental period. ECG, blood pressure and body temperature signals were recorded with a Dataquest ART data acquisition system (Data Sciences International).

2.5. Analysis of cardiovascular parameters

We assessed *in vivo* QT intervals using the probabilistic QT analysis method of Holzgrefe et al. (2007a, 2007b). ECG parameters were analyzed on a beat-to-beat basis throughout the data with Ponemah template analysis modules for P3 plus (Data Sciences International). The individual rate-corrected QT interval (QTc) was derived by a modification of the probabilistic method described by Holzgrefe et al. (2007a, 2007b). Each individual QTc was obtained according to one of the following three expressions:

$$QTc = QT_{raw} - \beta(RR_{raw} - RR_{ref}) \quad (1)$$

$$QTc = QT_{raw} - \beta\exp(-\alpha) + \beta\exp(-\alpha RR_{raw}/RR_{ref}) \quad (2)$$

$$QTc = QT_{raw}/(RR_{raw}/RR_{ref})^{\beta} \quad (3)$$

where QT_{raw} is the uncorrected raw QT interval (in ms), RR_{raw} is RR interval associated with the raw QT interval (in ms) and RR_{ref} is the reference cycle length (in ms). RR_{ref} was set to a cycle length of 1000 ms because it reflects the prevalent resting heart rate in beagle dogs. The β and α values of three expressions for each animal were determined from the relationship between QT and RR data, which were collected under conditions without drug treatment (vehicle control). All beat-to-beat RR and QT data were collected as average values at a logging rate of 10 s over 20 h, and were grouped into bins at successive RR increments of 10 ms. The mean values of the QT interval distribution for each RR bin were calculated using all 3 rate-correction expressions. The most suitable expression for individual animals was selected by comparing the correlation coefficient (R^2) calculated for the three expressions. Data were expressed as mean value for 10 min at each time point.

2.6. Pharmacokinetic study

Blood samples were collected from the cephalic vein. In repeated probucol dosing experiments, blood sampling was performed once a day just before drug administration. The blood was treated with sodium heparin, and plasma was obtained from by centrifugation at 4 °C at 1200 × *g* for 5 min. The plasma samples stored frozen at −30 °C until determination of plasma concentration.

The plasma concentration of probucol was measured by electrospray liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis with a Waters Quattro Ultima quadrupole mass spectrometer equipped with an Agilent 1100 high-performance liquid chromatography (HPLC) pump and CTC Analytics HTC PAL autosampler. For chromatographic separation, a Capcell Pak C1 UG120 column (35 mm × 2.0 mm ID, particle size 5 μm; Shiseido, Tokyo, Japan) was used with a mobile phase consisting of water containing 0.05% formic acid (A) and acetonitrile containing 0.05% formic acid (B). The flow rate was 0.4 mL/min and the gradient program was as follows: (min, B%)=(0, 20), (0.5, 20), (3, 100), (5, 100), (5.1, 20), (7.0, 20). The column temperature was set at 40 °C. Mass spectra were acquired in the negative ionization mode by multiple reaction monitoring (MRM). The capillary voltage was