BeKm-1, a Peptide Inhibitor of Human ether-a-go-go-Related Gene Potassium Currents, Prolongs QTc Intervals in Isolated Rabbit Heart

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ABSTRACT

Drug-induced cardiac arrhythmia, specifically Torsades de pointes, is associated with QT/QTc interval prolongation, thus prolongation of the QT interval is considered as a biomarker for Torsades de pointes risk (N Engl J Med 350:1013–1022, 2004). Specific inhibition of human ether-a-go-go-related gene (hERG) potassium channels has been recognized as the main mechanism for QT prolongation (Cardiovasc Res 58:32–45, 2003). This mechanism has been demonstrated for a variety of small-molecule agents, which access the inner pore of the hERG channel preferentially from inside the cell. Peptide inhibitors of hERG, such as BeKm-1, interact with the extracellular amino acid residues close to the external pore region of the channel. In this study, the isolated rabbit heart was used to assess whether BeKm-1 could induce QTc prolongation like dofetilide and N-[4-[[1-[2-(6-methyl-2-pyridinyl)ethyl]-4-piperidinyl]carbonyl]phenyl]methanesulfonamide (E-4031). Five hearts were perfused with 10 and 100 nM BeKm-1 sequentially. ECG parameters and left ventricular contractility were measured with spontaneously beating hearts. Both concentrations of BeKm-1 prolonged QTc intervals significantly and concentration-dependently (4.7 and 16.3% at 10 and 100 nM, respectively). When evaluated for their inhibitory effect in a hERG functional assay, BeKm-1, dofetilide, and E-4031 caused QTc prolongation at concentrations that caused significant hERG channel inhibition. Lastly, two polyclonal anti-hERG antibodies were also assessed in the hERG channel assay and found to be devoid of any inhibitory effect. These results indicated that the isolated rabbit heart assay can be used to measure QTc changes caused by specific hERG inhibition by peptides that specifically block the external pore region of the channel.

Introduction

Drug-induced cardiac arrhythmias caused by induced QT prolongation are a well recognized and serious side effect that has led to withdrawal of several drugs from the market. Most drugs known to cause acquired long QT syndrome do so by preferentially blocking the rapidly activating delayed rectifier potassium current, I_Kr, in the heart (Sanguinetti et al., 1995). The human ether-a-go-go-related gene (hERG) encodes the pore forming α-subunit of I_Kr, a K^+ current involved in repolarization of the cardiac action potential. The hERG channels belong to an evolutionarily conserved multigenic family of voltage-activated K^+ channels, the Eng (ether-a-go-go) family. This channel has a tetrameric structure formed by coassembly of four identical α-subunits, each composed of six α-helical transmembrane domains (denoted S1-S6), with the S4 domain containing six positive charges, which is typical for voltage-gated K^+ channels (Warmke and Ganetzky, 1994). The S5 and S6 domains in each of the subunits together form the pore domain that contains the pore helix, the selectivity filter (extracellular end of the pore), and a lengthy S5-P linker. The charged S4 domain is the “voltage sensor” responding to changes in membrane potential.

Compounds with diverse chemical structures have been shown to block hERG current. Sites of binding between small-molecule “drug-like” compounds and the hERG channel have been inferred via site-directed mutagenesis. Much like voltage-gated sodium and calcium channels, two hydrophobic residues, Phe652 and Tyr656, in the S6 domain on the cytosolic side of the ion selectivity filter, are the sites of interaction for most small-molecule blockers with the hERG channel (Sanguinetti and Mitcheson, 2005). In contrast, peptide inhibitors of hERG, such as BeKm-1, have been shown to...
bind to the extracellular region on the N-terminal side of the re-entrant pore loop, spanning between transmembrane domains S5 and S6 (Korolkova et al., 2004). BeKm-1 consists of 36 amino acid residues and originally has been isolated from the venom of the Central Asian scorpion Buthus eupeus (Filippov et al., 1996). Recombinant BeKm-1 has been tested on a variety of potassium channels heterologously expressed in either Xenopus oocytes (Zhang et al., 2003) or HEK293 cells (Korolkova et al., 2001). It has been shown that BeKm-1 inhibited hERG currents with an IC50 in the single nanomolar range (Korolkova et al., 2001; Milnes et al., 2003); however, it did not have effects on the 14 other potassium channels tested, including hEAG, KCNQ1/KCNE (KvLQT1/mink), KCNQ2/KCNQ3, or KCNQ4. Like known small-molecule hERG channel blockers, BeKm-1 is another pharmacological tool with high specificity that could be used to assess the functional role that hERG channels play in the heart.

The isolated rabbit heart has been used for cardiac safety assessment to evaluate the propensity of small-molecule drugs to cause QT interval prolongation and arrhythmia (Lawrence et al., 2006), partly because the amino acid sequence of the rabbit Ikr channel shares 99% homology with the human Ikur channel sequence (Wymore et al., 1997). In the regions that are most important for interaction of BeKm-1 with hERG, i.e., the S5-P and P-S6 linker, homology is more than 98%. In addition, the late repolarization of rabbit cardiac action potential is similar to human in that it is strongly driven by Ikur (Carmeliet, 1992; Weirich and Antoni, 1998).

To date, there have been no reports on specific peptide inhibitors of hERG being tested in isolated rabbit heart for their ability to cause delayed cardiac repolarization. Therefore, we examined the inhibitory effect of BeKm-1 in the hERG channel assay and assessed this peptide’s ability to alter cardiac repolarization in the perfused isolated rabbit heart model. For comparative purposes, other agents known to bind or block various residues in the hERG channel [dofetilide, N-[4-[[1-2-(6-methyl-2-pyridinyl)ethyl]-4-piperidinyl]carbonyl]phenyl]methanesulfonamide (E-4031), and anti-hERG polyclonal antibodies] were evaluated.

**Materials and Methods**

Electrophysiological Recording of hERG Potassium Current. HEK293 cells stably transfected with hERG cDNA were licensed from the University of Wisconsin (Madison, WI). Whole-cell hERG currents were recorded using standard procedures in PatchXpress 7000A (Molecular Devices, Sunnyvale, CA). When a quality recording was established, cells were washed for 2 min, followed by applying control vehicle for 5 min. Then control and each concentration of test article were applied for 5 min (three additions for each concentration at 1-min intervals). The hERG current was recorded by automatic whole-cell voltage-clamp using the following solutions: extracellular, 137 mM NaCl, 4.0 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, and 10 mM glucose, pH 7.40 adjusted with NaOH; intracellular, 20 mM KF, 90 mM KCl, 10 mM NaCl, 10 mM EGTA, 5 mM K3ATP, 1 mM MgCl2, and 10 mM HEPES, pH 7.20 with KOH. A standardized protocol was used to elicit ionic current through the hERG potassium channel (see Fig. 1B, bottom). Cells were held at −80 mV. Membrane potential was first depolarized to −50 mV for 0.5 s. Because the hERG channel is not activated at −50 mV, this is used for baseline subtraction. A depolarization to +20 mV for 2 s was used to activate the hERG channel followed by a repolarization to −50 mV for 2 s to elicit a tail current. This stimulation paradigm was repeated once every 10 s (0.1 Hz). Currents were filtered at 3 kHz and acquired at 10 kHz in episodic mode. Experiments were conducted at room temperature (20–22°C).

**Isolated Rabbit Heart using Langendorff Perfusion.** Female rabbits (2.5–3.5 kg) were obtained from Charles River Laboratories (Montreal, Canada). All rabbit experiments were conducted in compliance with the Amgen Institutional Animal Care and Use Committee and U.S. Department of Agriculture regulations.

Rabbits were anesthetized with pentobarbital (50 mg/kg) by ear vein injection. Hearts were rapidly removed through a median sternotomy incision, cannulated via aorta, and retrograde-perfused according to the Langendorff technique (Langendorff, 1895) with a modified Krebs-Henseleit solution composed of 120 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 25 mM NaHCO3, 1.2 mM KH2PO4, 11.1 mM glucose, 2 mM Na-pyruvate, and 1.8 mM CaCl2 and bubbled with 95%/5% O2/CO2. Two lead ECGs were recorded with flexible unipolar electrodes (Harvard Apparatus Inc., Holliston, MA) placed on the heart, one over the epicardium of ventricles and the other over the epicardium of the left atria. For measuring left ventricular pressure (LVP), a metal cannula with a rubber balloon on the tip was inserted into the left ventricle (LV). For hemodynamic measurements, the latex balloon in the LV was expanded with water to achieve a LV diastolic pressure (LVPD) of approximately 5 to 10 mm Hg. Once the end diastolic pressure was stabilized at the baseline, it was not adjusted during the course of the experiment. The balloon was connected to a pressure transducer to measure LVPD and LV systolic pressure (LVSP). Coronary perfusion pressure (CPP) was measured with a pressure transducer connected to the aortic block. The heart was stabilized for at least 45 min in Krebs-Henseleit solution.
solution before baseline measurements. After baseline measurement, the hearts were perfused with 1% bovine serum albumin (BSA) in Krebs-Henseleit solution for 20 min (vehicle control). Thereafter, 10 and 100 nM BeKm-1 with 1% BSA were tested under constant flow condition, with each concentration applied for 12 min. For E-4031 and dofetilide, each concentration was applied for 20 min. The hearts were maintained at approximately 37°C.

**Data Acquisition, Analysis, and Statistics.** Electrophysiological data acquisition was performed using PatchXpress Commander v1.4 (Molecular Devices), and analyses were performed using DataXpress v1.4 (Molecular Devices). The average responses from the last five recorded hERG peak tail currents before the next application were measured and used to calculate the percentage of current inhibition at each concentration. For deriving IC_{50}, Sigmaplot 11.0 (Systat Software, Inc., San Jose, CA) was used.

For isolated rabbit heart assay, LVP and ECG measurements were continuously monitored with the NOTOCORD-hem v3.5 data capture system (Notocord, Croissy sur Seine, France). Digital markers were used to indicate drug application periods. For analysis, LVP and CPP were automatically done with NOTOCORD. Measurements from the final 30 s of the equilibration period and each dose exposure period were evaluated and used to determine effects. ECG was analyzed manually with the VME module of NOTOCORD, and 10 consecutive ECG waveforms (30 s before the next event marker) were analyzed for HR, PR, QRS, and QT intervals. Values from each individual heart were pooled to determine an average for each variable at individual concentrations. Average percentage changes of each variable between baseline and each concentration were also determined. In the isolated heart model, the Fridericia equation (QTcF = QT/RR^{0.37}) was used to correct QT intervals for heart rate change, because it is known that the QT interval varies with heart rate in many species, including rabbit (Kijtawornrat et al., 2006). Previous in-house studies (Supplemental Fig. 1) demonstrated that the slope of the QTcF versus heart rate regression had slight heart rate dependence (0.2–0.3), but was sensitive to detect QT interval prolongation caused by hERG blockade. In studies with BeKm-1, baseline QTc values were established before priming the perfusion system (20–40 min) with a modified Krebs-Henseleit solution containing 1% BSA. All numeric values derived in these studies are presented as mean ± S.D., and group comparisons were conducted using statistical software (Prism 4.01; GraphPad Software Inc., San Diego, CA). One-way repeated-measures ANOVA (with Dunnett’s multiple comparison test) was used for comparison, and p < 0.05 was required for significant change.

**Reagents.** Recombinant BeKm-1 and anti-hKv11.1 antibodies (Extra and Intra) were purchased from Alomone Labs (Jerusalem, Israel). The anti-Kv11.1 (Extra) was directed against an extracellular loop close to the C terminal corresponding to residues 1106 to 1159 of the hERG channel. BeKm-1 and antibodies were dissolved in distilled H_2O to produce stock solutions of 100 μM. Stock solutions were immediately added to either hERG assay buffer with 1% BSA or Krebs-Henseleit solution with 1% BSA to make up the concentrations tested. E-4031 was purchased from Enzo Life Sciences, Inc. (Plymouth Meeting, PA), and dissolved in distilled H_2O to make stock solutions. Dofetilide was synthesized at Amgen (San Francisco, CA) and dissolved in dimethyl sulfoxide to make stock solutions.

**Results**

**Inhibition of hERG Potassium Channels by BeKm-1 in HEK293 Cells.** BeKm-1 was tested on hERG potassium currents stably expressed in HEK293 cells using the PatchXpress 7000A system. Figure 1A shows the typical time course of BeKm-1’s inhibitory effect on hERG tail current for a given cell. The steady-state effects were both concentration-dependent and reversible upon washout.

The concentration response relationship for inhibition of hERG was obtained using the protocol in Fig. 1B. The curves were constructed as mean percentage of reduction in hERG tail current at −50 mV as shown in Fig. 3B (•). The BeKm-1 data could be reasonably described by a conventional Michaelis-Menten relationship (Fig. 3B, black solid curve) and demonstrated maximal inhibition of hERG K⁺ current of 85.9% with an IC_{50} value of 11.9 nM and a Hill coefficient of 0.9 (Fig. 3B).

**Fig. 2.** Effects of two anti-hERG antibodies on hERG current in HEK293 cells. Top, both antibodies and BeKm-1 were tested at 100 nM. Percentages of inhibitions were 16.1 ± 7.2% (five cells) for vehicle control (1% BSA), 12.8 ± 5.2% (five cells) by anti-Kv11.1 (Extra), 14.1 ± 4.4% by anti-Kv11.1 (Intra), and 16.1 ± 7.2% by vehicle. On the other hand, 100 nM BeKm-1 inhibits hERG by 73.8 ± 8.8%. Thus, neither pAbs produced significant inhibition of hERG currents even though they were developed for specific interaction with the channel.

**Prolongation of QTc Intervals by BeKm-1 in Isolated Rabbit Heart.** We examined the effect of BeKm-1 on the cardiac ECG intervals measured in isolated rabbit heart using the Langendorff method. To minimize peptide adherence to solution reservoirs and perfusion tubings, BeKm-1...
was made at its final concentrations in Hanks-Henseleit buffer with 1% BSA. Table 1 shows the absolute values (mean ± S.D.) for HR (bpm) and PR, QRS, QT, and QTc intervals (ms) from four hearts after vehicle treatment. In this study, application of 1% BSA for 40 min did not affect any of the parameters significantly.

When BeKm-1 was administered cumulatively (10 and 100 nM; n = 5), each concentration was administered for 12 min. QTc prolongation was observed at both concentrations (Table 2), and the effect was concentration-dependent. At 10 nM, QTcF was prolonged by 4.7% (absolute value increased by 15 ms), and at 100 nM, QTcF was prolonged by 16.3% (absolute value increased by 51 ms). In this study, BeKm-1 caused statistically significant cardiac repolarization delay.

From the absolute values, we calculated the absolute change compared with baseline (QTcF in the presence of BeKm-1 – QTcF in baseline) and overlaid the QTc change relative to hERG blockade (Fig. 3B). Clearly, BeKm-1 produced a concentration-dependent prolongation of QTc in the isolated rabbit heart. The QTc prolongation in the presence of BeKm-1 at both concentrations was significant compared with that in 1% BSA (p < 0.05 in repeated-measures ANOVA), these are indicated by * in Fig. 3B. The overlay plots in Fig. 3B show a good correlation between hERG inhibition in the whole-cell patch-clamp study and QTc prolongation in isolated rabbit heart.

In Table 2, PR and QRS intervals were not affected, indicating BeKm-1 did not affect atrio-ventricular conduction in this model. This further confirms that BeKm-1 is a very specific reagent for the hERG potassium channel. In addition, administration of 100 nM BeKm-1 decreased the heart rate significantly in the isolated rabbit heart (Table 2) with a mean decrease of 10.6%.

For a more complete understanding of its physiological effects in isolated rabbit hearts, we also measured left ventricular pressure. Application of 10 nM BeKm-1 had minimal effect on all of the hemodynamic parameters measured (Table 3); however, 100 nM BeKm-1 increased left ventricular contractility in terms of dP/dt+, LDevP, and LVSP (Table 3). dP/dt+ was increased by 6.5%, LDevP was increased by 7.3%, and LVSP was increased by 7.5%.

**Discussion**

This is the first demonstration that a specific peptide inhibitor of hERG, BeKm-1, can induce QTc prolongation in the isolated rabbit heart assay. We have shown BeKm-1 can cause QTc prolongation in a concentration-dependent manner that correlates with its hERG inhibition. It is known that BeKm-1 inhibits hERG specifically and does not interfere with the function of 14 other potassium channels (Korolkova et al., 2004). The ability of BeKm-1 to selectively antagonize hERG channel function suggests that hERG blockade is the principal mechanism underlying QTc prolongation in the rabbit heart. The best approach to confirm potassium channel block would have been to determine the potency of BeKm-1 against IKr in isolated rabbit ventricular myocyte, but because of the technical complications of this approach, the recording of hERG currents in HEK293 cells was used to infer IKr blockade. It is possible that BeKm-1 could alter IKr function by interacting with other components, e.g., auxiliary subunit of IKr, but that was not determined in this study.

Selective IKr peptide blockers have not been available for long (Filippov et al., 1996), and they are difficult to synthesize in large quantities, thus few descriptions of their pharmacological effects are found in the literature. In particular, there have been no reports on the effects of BeKm-1 in the isolated heart assay. In addition to BeKm-1, other hERG-specific peptides have been identified: Ergtoxin, CnErg1, or ErgTx1, were isolated from the venom of scorpion Centruridus noxius (Gurrola et al., 1999), ErgTx2 was isolated from the same source as BeKm-1 (Lecchi et al., 2002), CsEKerg1 was isolated from American scorpion Centruroides sculpturatus (Nastainczyk et al., 2002), and APETx1 was purified from the venom of the sea anemone Anthopleura elegantissima (Diochot et al., 2003). Among them, BeKm-1 and CnErg1 are the best-studied cases. Both of them block hERG by preferentially interacting with the closed (resting) state of the channel (Milnes et al., 2003). It is known that virtually all of the small-molecule drugs can cross the plasma membrane in an uncharged state, and when the channel is open, these drugs can access their binding site inside the hERG channel vestibule from the cytoplasmic side of the channel (Mitcheson et al., 2000). Compared with small-molecule
hERG blockers, the known peptides that potently and selectively block hERG are unusual in that they act from the extracellular side of the membrane (Pardo-Lopez et al., 2002). Mutagenesis studies suggest S5-P and P-S6 linkers of the hERG channel both are involved in interacting with the peptide inhibitors (Korolkova et al., 2004). Recordings of hERG currents expressed in mouse fibroblast L-929 cells (Margulis and Sorota, 2008) with combined application of cisapride and BeKm-1 support the mutagenesis results, i.e., cisapride and BeKm-1 are acting at independent sites. Despite the fact that small molecules and peptides inhibit hERG potassium channel by interacting with different sites, it is interesting that they share common pharmacological effects, i.e., cardiac repolarization delay, bradycardia, and increased left ventricular cardiac contractility.

**Comparison with Published Data.** Our data are consistent with literature descriptions. The IC_{50} values for hERG inhibition by BeKm-1 range from 3.3 nM (Korolkova et al., 2001) to 63.3 nM (Milnes et al., 2003) depending on the protocol, specifically, the amplitude and duration of conditioning pulse. It has been reported (Zhang et al., 2003) that BeKm-1 achieved 50% inhibition of hERG at 10 nM with the maximal effects of approximately 88% and the apparent concentration-response relationship could be described by a 1:1

![Fig. 3. BeKm-1 prolongs QTc intervals in the isolated rabbit heart in a concentration-dependent manner. A, ECG waveforms recorded in the absence and presence of 10 and 100 nM BeKm-1 with markings for beginning of P as green, beginning of Q as red, end of S as orange, and end of T as pink. Calibration: 2 mV, 150 ms. B, overlay of hERG inhibition and QTc prolongation plots for BeKm-1. For hERG inhibition, each concentration was tested in three to eight cells. For QTc measurement, each concentration was tested in five hearts. QTc prolongation is calculated as the increase of QTc intervals in absolute values (ms) compared with baseline. Blue symbols represent QTc changes in absolute values (compared with baseline) in 1% BSA for 20 and 40 min.](image)

**TABLE 3**

<table>
<thead>
<tr>
<th></th>
<th>LVSP (mm Hg)</th>
<th>LVDP (mm Hg)</th>
<th>dP/dt+ (mm Hg/s)</th>
<th>dP/dt− (mm Hg/s)</th>
<th>LVDevP (mm Hg)</th>
<th>CPP (mm Hg)</th>
</tr>
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<tbody>
<tr>
<td>Baseline</td>
<td>118 ± 14</td>
<td>9 ± 0</td>
<td>1416 ± 302</td>
<td>−1167 ± 120</td>
<td>108 ± 14</td>
<td>61 ± 12</td>
</tr>
<tr>
<td>10 nM BeKm-1</td>
<td>121 ± 13</td>
<td>10 ± 1</td>
<td>1451 ± 287</td>
<td>−1170 ± 108</td>
<td>111 ± 13</td>
<td>60 ± 10</td>
</tr>
<tr>
<td>100 nM BeKm-1</td>
<td>128 ± 15</td>
<td>10 ± 2</td>
<td>1308 ± 303</td>
<td>−1173 ± 112</td>
<td>116 ± 14</td>
<td>61 ± 12</td>
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the hERG channel, e.g., BeKm-1 binding region (Fig. 2). A specific binding interaction with the S1-S2 region was unable to access the cytoplasm and block the promiscuous inner pore of the hERG channel, in contrast with dofetilide and E-4031. The pAbs could potentially alter hERG function by blocking against an intracellular loop near the C terminal. Both of these reagents are “large protein molecules” with high molecular mass (>150,000 Da) that would not be able to penetrate the plasma membrane and therefore would be unable to access the cytoplasm and block the promiscuous inner pore of the hERG channel, in contrast with dofetilide and E-4031.

Polyclonal Antibodies against hERG Do Not Inhibit hERG Current Flow. In this study, two commercially available hERG pAbs were evaluated for their potential to inhibit potassium current flow through hERG channels expressed in HEK293 cells. Figure 2, bottom depicts the regions on the hERG channel that are the critical recognition domains (sites) for pAbs and BeKm-1 binding. One pAb was directed against extracellular epitopes between the S1 and S2 segments (voltage sensor region), and the other was directed against an intracellular loop near the C terminal. Both of these reagents are “large protein molecules” with high molecular mass (>150,000 Da) that would not be able to penetrate the plasma membrane and therefore would be unable to access the cytoplasm and block the promiscuous inner pore of the hERG channel, in contrast with dofetilide and E-4031. The pAbs could potentially alter hERG function by blocking nonspecifically the pore region between S5 and S6 in a manner like BeKm-1. Our results indicate, however, that neither pAb inhibited hERG currents measured in HEK293 cells at a concentration of 100 nM. For pAb (Extra), a “targeted” or specific binding interaction with the S1-S2 region was unable to cause functional block of the hERG channel. It can be concluded that hERG channel function can only be blocked by a specific inhibitory interaction with the outer vestibule of the hERG channel, e.g., BeKm-1 binding region (Fig. 2). Thus, the specific interaction of the S1-S2 extracellular re-
gion (anti-Kv11.1 pAb; Extra) or the use of an intracellular anti-Kv11.1 pAb to mimic nonspecific extracellular blockade were devoid of any effect on hERG channel function in vitro. These findings with the two pAb reagents demonstrate that large molecules, e.g., antibody type, are unlikely to have nonspecific effects at the hERG channel because such protein therapeutics are engineered to have high specificity for their molecular target/epitope and have little ability to block the hERG channel specifically in contrast with small-molecule-like drugs (Vargas et al., 2008).

In conclusion, isolated rabbit heart provides a convenient way to test direct cardiac effects of a small-molecule reagent as well as a peptide for its effects not only on electrocardiographic parameters but also on hemodynamic parameters. Our results provide the first evidence that a peptide inhibitor of hERG, BeKm-1, prolongs QT intervals in a concentration-dependent manner consistent with its inhibitory potency in the hERG assay.

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Figure 1. Plots of QT/QTc versus the HR (beats/min) for 2 individual rabbits in negative control experiments. The line of regression and its equation and \(R^2\) are shown. Beat-to-beat analysis was done for both hearts, for the heart in 20100513, more than 5000 beats were analyzed; and for the heart in 20091124, more than 7000 beats were analyzed.