hERG K+ Channel Binding Assays for Cardiac Safety Screening

Introduction

Torsades de pointes is a life-threatening ventricular arrhythmia that can be congenital or drug-induced; indeed, over 100 drugs from different classes have the potential to induce torsades de pointes[1]. Antihistaminic Hismanal (astemizole), Seldane (terfenadine), and the gastrointestinal prokinetic agent Propulcid (cisapride) are examples of such drugs that have been withdrawn from the U.S. market.

The primary cause of drug-induced arrhythmia is blockade of the K^+ channels involved in rapidly activating delayed rectifier K^+ currents (I_{Kr}). The human ether-a-go-go-related gene (hERG) encodes the pore-forming α-unit of the K^+ channel generating I_{Kr}[2] and heterologous expression of hERG in non-cardiac cells produces currents with all the characteristics of I_{Kr}[3,4].

Various methods exist for measuring hERG blockade. Whole cell patch clamp with hERG-transfected mammalian cells provides valuable information on the ability of a substance to block I_{Kr} but is labor intensive and requires specially trained personnel. New automated patch clamp instruments can screen a few thousand compounds a day but lack the flexibility of manual systems[5]. Atomic absorption spectroscopy measurements of Rb^+ efflux through hERG channels and detection of voltage sensor probes translocation in the plasma membrane allow higher screening throughput but suffer from limited sensitivity[6].

Radioligand binding assays rapidly and cost effectively identify compounds binding to hERG K^+ channels[7,8,9]. Furthermore, a strong correlation has been demonstrated between the binding affinity of hERG blockers in radioligand binding assays and their potency for blocking hERG currents[7,8,9,10,11]. The two most characterized assays, the [^{3}H]-astemizole and [^{3}H]-dofetilide competitive binding assays, appear equivalent for the identification of most hERG blockers. Indeed, when the binding affinities of 12 compounds from seven different drug classes were compared in the two assays (Finlayson et al., 2004), excellent correlation was obtained (r2 = 0.955). The radioligand [^{125}I]-BeKm-1, derived from a toxin produced by the scorpion *Buthus eupeus,* was also shown to be suitable for hERG binding assays[12].

Authors
Nathalie Bouchard[1]
Geneviève Hamann[1]
Soo-Hang Wong[1]
Terence E. Hebert[2]
Julie Guimond[2]
Lucille Beaudet[1]

[1]PerkinElmer
Life and Analytical Sciences
1744 William Street, Suite 600
Montreal, Québec Canada H3J 1R4

[2]Department of Pharmacology and Therapeutics
McGill University
McIntyre Medical Sciences Building
3655 Promenade Sir William Osler
Montréal, Québec Canada H3G 1Y6

We have developed a stable hERG-expressing cell line (PerkinElmer cat. RBHERGM400A) by transfecting HEK-293 cells with hERG cDNA. In this application note, we present the characterization of membrane preparations derived from this cell line in radioligand binding assays using [^{3}H]-astemizole and [^{125}I]-BeKm-1. hERG K^{+} channel membranes show high expression and a pharmacological profile in agreement with published data. As a useful complement to whole cell patch clamp, they make possible high-throughput binding assays that can assess potential cardiotoxicity of compounds early in the drug development cycle.

Materials and methods

Cold ligands were purchased from Sigma-Aldrich, with the exception of BeKm-1 purchased from Penta-Biotech (Union City, CA, USA) and dofetilide purchased from Sequoia Research Products (Pangbourne, UK). Consumables and media for cell culture were purchased from Invitrogen. Fetal bovine serum (FBS) was obtained from Wisent (St-Bruno, QC, Canada). All other chemicals were purchased from Sigma-Aldrich.

The hERG cDNA was cloned by RT-PCR from a brain cDNA library (Marathon-Ready™; Clontech Laboratories) and subcloned into pCR®-Blunt II-TOPO® (Invitrogen). The cDNA was transferred to the mammalian expression vector pcDNA™ 3.1(+) (Invitrogen) and transfected into HEK-293 cells using the transfection reagent Lipofectamine™ 2000. Clones were selected in the presence of 800 µg/mL Geneticin®. The sequence of the cloned cDNA was identical to the reference sequence for hERG (KCNH2 transcript variant 1, NM_000238). Clones were picked and cultured in minimal essential medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine and 400 µg/mL Geneticin. They were tested initially for expression in a [^{3}H]-astemizole binding assay and then characterized by whole cells patch clamp to identify a clone that showed both high expression and typical hERG currents.

hERG K^{+} membranes were prepared following the standard method used at PerkinElmer BioSignal for Membrane Target Systems reagents, with slight modifications from Angelo et al. (2004)[12]. Briefly, cells were lysed, and then spun at low speed to eliminate nuclei. Membranes were pelleted from the supernatant by ultracentrifugation, resuspended in storage buffer (50 mM Tris-HCl pH 7.2 at 4 ˚C, 10% sucrose), aliquoted and stored at -80 ˚C for further use.

Binding reactions were set up in 96-deep well polypropylene plates (VWR International cat. 267006) in a total volume of 200 µL. hERG blockers or assay buffer in 25 µL were added to 150 µL of hERG K^{+} channel membranes. The radioligand was added last in a volume of 25 µL and plates were incubated at 27 ˚C for 60 min. Binding reactions were terminated by filtration onto Filtermat A filters (PerkinElmer cat. 1450-421) pre-soaked in 0.3% PEI, followed by nine rapid washes of 500 µL of ice-cold buffer (20 mM Tris/HCl pH 7.3 and 150 mM NaCl). Meltilex solid scintillator (PerkinElmer cat. 1450-441) was applied onto the filters on a hot plate. Bound radioactivity was detected with a MicroBeta® scintillation counter (PerkinElmer).

Binding reactions for [^{125}I]-BeKm-1 (PerkinElmer cat. NEX-412, 2,200 Ci/mmol) were performed in 20 mM Hepes/Tris pH 7.2, 100 µM KCl, 0.1% BSA whereas binding reactions for [^{3}H]-astemizole (PerkinElmer cat. NET-1140, 86 Ci/mmol) were in 50 mM Hepes pH 7.4, 60 mM KCl, 0.1% BSA. For the determination of non-specific binding in saturation experiments, cold BeKm-1 was used at 100 nM and astemizole at 10 µM.

In competition binding experiments, radioligands were used at a concentration corresponding to their K_d value for hERG, which corresponds to 3 nM for [^{3}H]-astemizole and 0.1 nM for [^{125}I]-BeKm-1. Data are expressed as mean of triplicates ± standard deviation to the mean.

A homogeneous FlashBlue binding assay was developed for hERG K^{+} channels using the [^{125}I]-BeKm-1 radioligand. Binding reactions were set-up in clear well white matrix 96-well Isoplates (PerkinElmer cat. 1450-515) in a final volume of 80 µL. Each well contained cold BeKm-1 (100 nM) or buffer, 125 µg FlashBlue GPCR beads (PerkinElmer cat. FBB001), 5 µg hERG K^{+} channel membranes and [^{125}I]-BeKm-1. In competition experiments, the radioligand was used at a concentration of 0.23 nM. Assays were performed in buffer containing 20 mM Hepes/Tris pH 7.2, 100 µM KCl and 0.5% BSA. Plates were incubated for 1 h at room temperature and spun for 5 min at 1000 RPM prior to signal detection with a MicroBeta scintillation counter. Data were expressed as mean of triplicates ± standard deviation to the mean.

Results and discussion

Electrophysiological properties of hERG K^{+} channels expressed in HEK-293 cells

In order to demonstrate that the hERG K^{+} channels expressed in the stably transfected hERG-293 cell line were functional, patch clamp

characterization was performed. Cells were evaluated at different passages, and there was no decrease in signal amplitude after 40 passages. Representative results obtained at passage 40 are illustrated in Figure 1.

Currents recorded are characteristic of hERG channels and consistent with results presented in the literature where hERG is expressed using a similar heterologous expression system (Zhou et al., 1998).

[^{3}H]-astemizole binding to hERG K$^+$ channels

A total of nine saturation curves with [^{3}H]-astemizole were performed using three different lots of hERG K$^+$ channel membranes. Compilation of the data gave an average K_d value of 5.21 ± 2.41 nM with a B_{max} value of 7.2 ± 1.7 pmol/mg. Figure 2 shows representative results of a saturation experiment using 2.5 µg of membrane proteins per well.

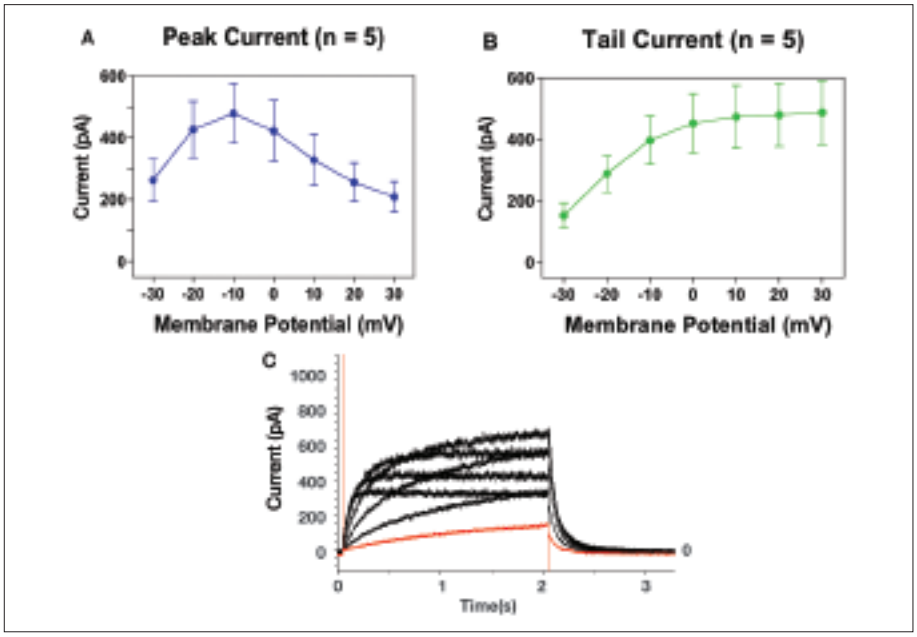


Figure 1. Electrophysiological properties of hERG K$^+$ channels expressed in HEK-293 cells. Panels A and B show step and tail currents from hERG expressed stably in HEK-293 cells at passage 40. Whole cell patch clamp was used to record currents at 37 °C. The holding potential was -80 mV and 2 sec depolarizing pulses were applied up to +30 mV. Tails were recorded at -50 mV. Data are presented as mean ± SEM. Panel C shows a family of hERG currents from an experiment described above.

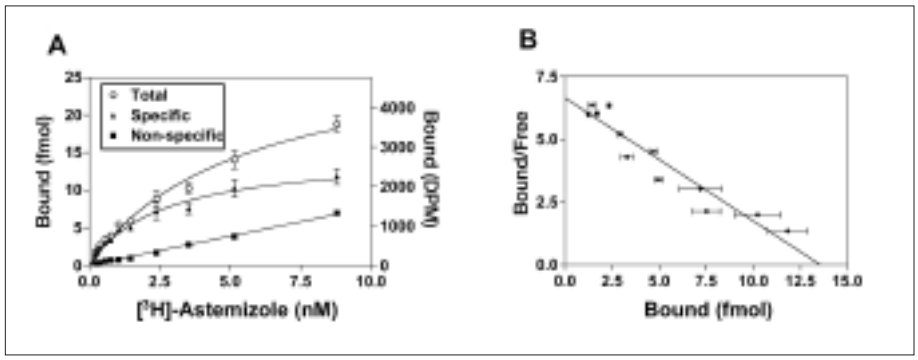


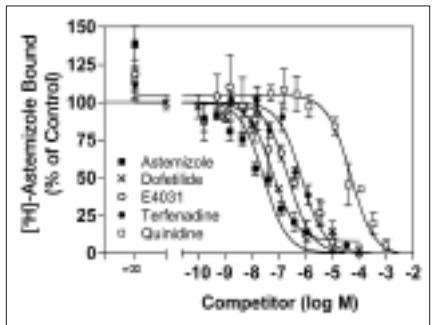
Figure 2. [^{3}H]-Astemizole saturation binding assay performed on hERG K$^+$ channel membranes. Panel A illustrates a representative saturation binding curve. The corresponding Scatchard analysis is shown in Panel B. The data presented is derived from single site fitting.

Competition experiments were performed with the antihistaminic drugs astemizole and terfenadine, and antiarrhythmic drugs dofetilide, E-4031 and quinidine (Figure 3). All K_i values were within 4-fold of published values, and the order of potency of the compounds was as expected[7,8,9,10].

[^{125}I]-BeKm-1 binding to hERG K$^+$ channels

A total of 18 saturation binding curves with [^{125}I]-BeKm-1 were performed using three different lots of hERG K$^+$ channel membranes, giving an average K_d value of 0.11 ± 0.03 nM and a B_{max} value of 0.48 ± 0.06 pmol/mg. Figure 4 shows typical results of a saturation binding experiment using 2.4 µg of membrane proteins per well.

Figure 5 shows competition curves with five known hERG blockers for [^{125}I]-BeKm-1: the unlabeled BeKm-1 toxin, antihistaminic drugs astemizole and terfenadine, and antiarrhythmic drugs dofetilide and E-4031. For all compounds but astemizole, K_i



Compound	K_i (nM)
Astemizole	18
Dofetilide	33
E-4031	134
Terfenadine	445.5
Quinidine	40,000

Figure 3. Competition of known hERG blockers for the binding of [^{3}H]-astemizole to hERG K$^+$ channels. A quantity of 2.5 µg of membrane proteins was used in each well. K_i values are the average of two independent experiments.

values for the displacement of [^{125}I]-BeKm-1 were within 2-fold of the values published for the [^{3}H]-astemizole binding assay[7], or from the Product Update for the BeKm-1 radioligand (NEX412 Product Update). The K_i value for astemizole in the [^{125}I]-BeKm-1 binding assay was shifted by about three logs when compared to the K_i value obtained in the [^{3}H]-astemizole binding assay. A likely mechanism for this shift is discussed below following Figure 7.

FlashBlue homogeneous hERG K$^+$ channel binding assay with [^{125}I]-BeKm-1

Figure 6 shows a typical [^{125}I]-saturation binding experiment using FlashBlue beads in which a signal to background ratio of ~ 4 was obtained with 1 nM radioligand. Considering a counting efficiency of 40%, a B_{max} of 0.5 pmol/mg protein was extrapolated. This value corresponds to the value obtained in filtration binding assays. The K_d value was slightly shifted to 0.17 nM, which is often the case in homogeneous assays.

Competition binding assays were performed using the hERG blockers BeKm-1, dofetilide, E-4031, terfenadine and quinidine.

The relative order of potency of BeKm-1, dofetilide, E-4031 and terfenadine (Figure 7) were identical to the one obtained in filtration binding assay (Figure 5). The K_i values were of the same order of magnitude, and comparable to data reported in the literature for the same compounds in the astemizole binding assay[7].

BeKm-1 interacts mostly with closed channels[13,14] and blocks K$^+$ current by interacting with the hERG channel's outer vestibule[15]. In contrast, dofetilide and astemizole are believed to interact with open and/or inactivated hERG channels[14,16], accessing the channel's vestibule from the inside of the cell. Displacement, or the lack of displacement, of the [^{125}I]-BeKm-1 radioligand by a hERG blocker can thus can give additional information on its binding properties. Figures 5 and 7 show that most blockers tested in [^{125}I]-BeKm-1 competition assays have K_i values of the same order compared to values obtained in the [^{3}H]-astemizole assay, with the exception of astemizole. This suggests that astemizole binds with lower affinity to hERG channels that are in a configuration suitable for BeKm-1 binding. A different situation is observed with dofetilide and E-4031: the two drugs compete for only a fraction of the [^{125}I]-BeKm-1 binding sites. This is evident in the homogeneous FlashBlue assay (Figure 7), but is also noticeable in the filtration assay (Figure 5). It is possible that the BeKm-1 radioligand binds to a fraction of hERG channels that are present in a configuration for which dofetilide and E-4031 have no affinity.

It is interesting to note that the B_{max} for the [^{3}H]-astemizole and [^{125}I]-BeKm-1 differ by a factor of ~ 10. This observation was also made with hERG membranes prepared from a cell line with a CHO background (data not shown) and is thus not an artifact of the hERG-293 cell line. The difference in B_{max} for the

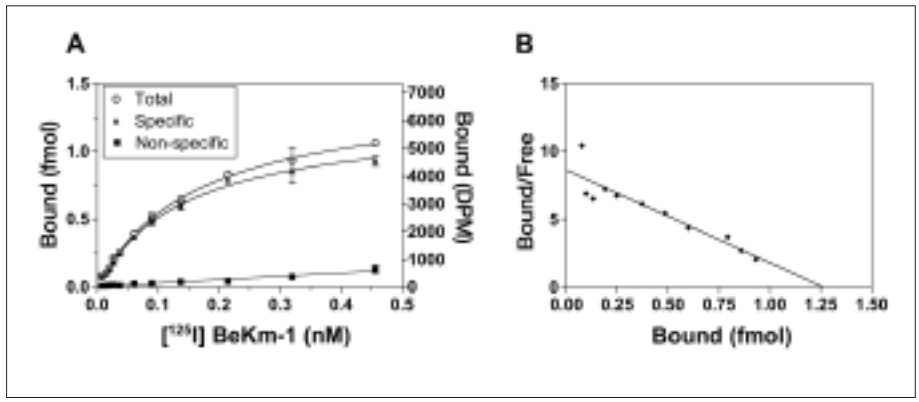
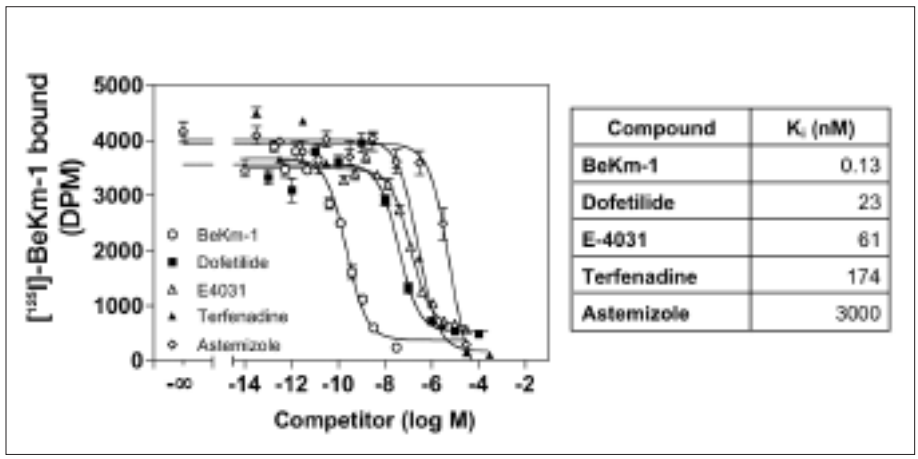


Figure 4. [^{125}I]-BeKm-1 saturation binding experiment performed on hERG K$^+$ channel membranes. Panel A illustrates the saturation binding curve. The corresponding Scatchard analysis is illustrated in panel B. The data presented is derived from single site fitting.



Compound	K_i (nM)
BeKm-1	0.13
Dofetilide	23
E-4031	61
Terfenadine	174
Astemizole	3000

Figure 5. Competition of known hERG blockers for the binding of [^{125}I]-BeKm-1 to hERG K$^+$ channels in filtration assay. A quantity of 2.4 µg of membrane proteins was used in each well. K_i values are the average of two independent experiments.

two radioligands substantiates the hypothesis that hERG channels exist in membrane preparations under a variety of conformational states.

Variability of the homogeneous FlashBlue [^{125}I]-BeKm-1 binding assay was estimated by determining the Z` value[17]. Signal from 24 assay wells for total binding and 24 assay wells for non-specific binding was compared. In 96-well format, a Z` value of 0.71 was obtained, demonstrating the robustness of the assay (Figure 8). Percent coefficients of variations (%CV) of 10% and 17% were observed for the total and the non-specific binding, respectively.

Conclusion

hERG K$^+$ channel membranes from PerkinElmer are an ideal tool for the sensitive, rapid and reliable detection of compounds interacting with hERG. hERG K$^+$ channel membranes have a B_{max} of ~7 pmol/mg of proteins for [^{3}H]-astemizole and ~0.5 pmol/mg of proteins for [^{125}I]-BeKm-1. K_i values and the order of potency of known hERG blockers have been shown to be in agreement with published data. Furthermore, the availability from PerkinElmer of two radioligands with distinct conformational specificity can differentiate with which of the distinct states of the hERG channel a compound is interacting. Together, this suite of products provides convenient and high throughput tools for screening for potential cardiotoxicity of compounds early in the drug development cycle.

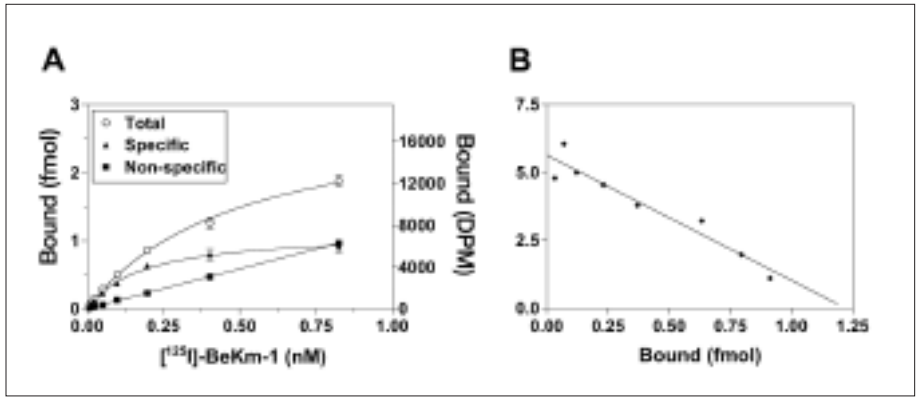


Figure 6. [^{125}I]-BeKm-1 saturation binding assay performed on hERG K$^+$ channel membranes using FlashBlue GPCR beads. A B_{max} value of 0.5 pmol/mg was obtained, with a K_d value of 0.17 nM. Panel A shows a saturation binding curve. The corresponding Scatchard analysis is illustrated in panel B. The data presented is derived from single site fitting.

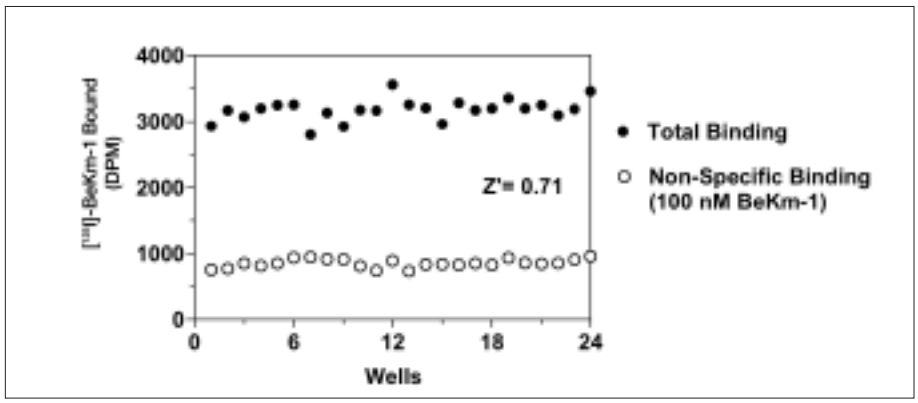
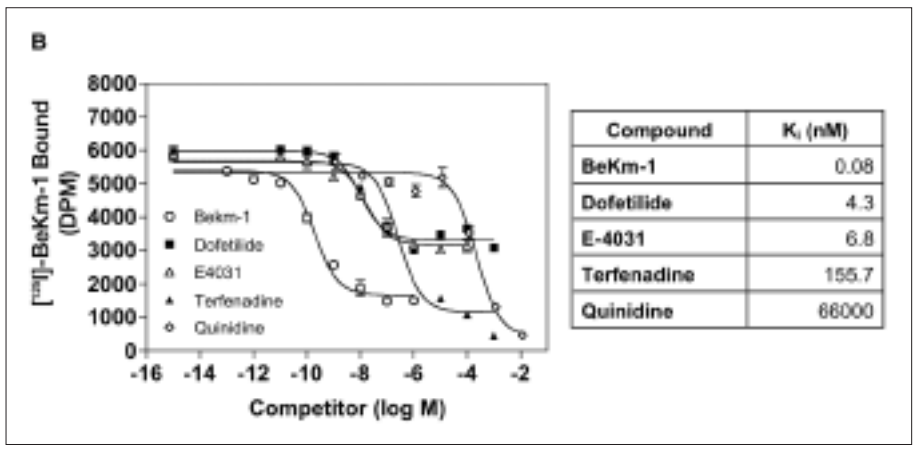


Figure 8. Z` value determination of the homogeneous [^{125}I]-BeKm-1 FlashBlue assay in 96-well format.



Compound	K_i (nM)
BeKm-1	0.08
Dofetilide	4.3
E-4031	6.8
Terfenadine	155.7
Quinidine	66000

Figure 7. Homogeneous [^{125}I]-BeKm-1 com-petition binding assays performed on hERG K$^+$ channel membranes using FlashBlue GPCR beads.

References

1. De Ponti F, Poluzzi E and Montanaro N. 2001. Organizing evidence on QT prolongation and occurrence of Torsades de Pointes with non-antiarrhythmic drugs: a call for consensus. *Eur J Clin Pharmacol* **57**(3):185-209.
2. Sanguinetti MC, Jiang C, Curran ME and Keating MT. 1995. A mechanistic link between an inherited and an acquired cardiac arrhythmia: HERG encodes the IKr potassium channel. *Cell* **81**(2):299-307.
3. Trudeau MC, Warmke JW, Ganetzky B and Robertson GA. 1995. HERG, a human inward rectifier in the voltage-gated potassium channel family. *Science* **269**(5220):92-95.
4. Zhou Z, Gong Q, Ye B, Fan Z, Makielski JC, Robertson GA and January CT. 1998. Properties of HERG channels stably expressed in HEK 293 cells studied at physiological temperature. *Biophys J* **74**(1):230-241.
5. Shah A. 2004. Planar patch clamping not an automatic choice. *Drug Discov & Dev* **7**(7):59-63.
6. Bennett PB and Guthrie HR. 2003. Trends in ion channel drug discovery: advances in screening technologies. *Trends Biotechnol* **21**(12):563-569.
7. Chiu PJ, Marcoe KF, Bounds SE, Lin CH, Feng JJ, Lin A, Cheng FC, Crumb WJ and Mitchell R. 2004. Validation of a [^{3}H] astemizole binding assay in HEK293 cells expressing HERG K^+ channels. *J Pharmacol Sci* **95**(3):311-319.
8. Finlayson K, Turnbull L, January CT, Sharkey J and Kelly JS. 2001. [^{3}H] dofetilide binding to HERG transfected membranes: a potential high throughput preclinical screen. *Eur J Pharmacol* **430**(1):147-148.
9. Finlayson K, Witchel HJ, McCulloch J and Sharkey J. 2004. Acquired QT interval prolongation and HERG: implications for drug discovery and development. *Eur J Pharmacol* **500**(1-3):129-142.
10. Diaz GJ, Daniell K, Leitza ST, Martin RL, Su Z, McDermott JS, Cox BF and Gintant GA. 2004. The [^{3}H]dofetilide binding assay is a predictive screening tool for hERG blockade and proarrhythmia: Comparison of intact cell and membrane preparations and effects of altering [K^+]o. *J Pharmacol Toxicol Methods* **50**(3):187-199.
11. Lautermilch N, Bishop A, Marcoe K, Bounds S, Lin C-H, Feng J-J, Lin A, Cheng F-C, Mitchel R, Chiu P and Baumgartner J. 2005. Comparison of a hERG Binding Assay with Automated Patch Clamp using the PatchXpress 7000A, in *SBS 11th Annual Conference & Exhibition.*
12. Angelo K, Korolkova YV, Grunnet M, Grishin EV, Pluzhnikov KA, Klaerke DA, Knaus HG, Moller M and Olesen SP. 2003. A radiolabeled peptide ligand of the hERG channel, [^{125}I]-BeKm-1. *Pflugers Arch* **447**(1):55-63.
13. Milnes JT, Dempsey CE, Ridley JM, Crociani O, Arcangeli A, Hancox JC and Witchel HJ. 2003. Preferential closed channel blockade of HERG potassium currents by chemically synthesised BeKm-1 scorpion toxin. *FEBS Lett* **547**(1-3):20-26.
14. Yao JA, Du X, Lu D, Baker RL, Daharsh E and Atterson P. 2005. Estimation of potency of HERG channel blockers: impact of voltage protocol and temperature. *J Pharmacol Toxicol Methods* **52**(1):146-153.
15. Korolkova YV, Bocharov EV, Angelo K, Maslennikov IV, Grinenko OV, Lipkin AV, Nosyreva ED, Pluzhnikov KA, Olesen SP, Arseniev AS and Grishin EV. 2002. New binding site on common molecular scaffold provides HERG channel specificity of scorpion toxin BeKm-1. *J Biol Chem* **277**(45):43104-43109.
16. Weerapura M, Hebert TE and Nattel S. 2002. Dofetilide block involves interactions with open and inactivated states of HERG channels. *Pflugers Arch* **443**(4):520-531.
17. Zhang JH, Chung TD and Oldenburg KR. 1999. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen* **4**(2):67-73.

PerkinElmer Life and Analytical Sciences
710 Bridgeport Avenue
Shelton, CT 06484-4794 USA
Phone: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

PerkinElmer®
precisely.

For a complete listing of our global offices, visit www.perkinelmer.com/lasoffices

007531_01 Printed in USA