Comparison of in-cell immunoassay and automated electrophysiology for assessment of hERG trafficking liability for new drugs



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Abstract

hERG-Lite® is an antibody-based chemiluminescent assay that identifies compounds that disrupt trafficking or suppress expression of hERG channel protein. Electrophysiological recording from single cell using the manual patch-clamp technique is more direct way to quantify hERG expression in cell membrane but this method has critical limitations: low throughput and selection bias during choice of cells for patching. Application of the automated population patch clamp technique for the hERG expression analysis provides a dramatic increase in throughput and eliminates the cell selection bias. We analyzed a panel of drugs (Amiodarone, Amitriptyline, Bepridil, Diphenhydramine, Dofetilide, Fluoxetine, Imipramine, Ouabaine, Pentamidine, Pimozide, Sunitinib and Verapamil) for chronic effects on hERG channel trafficking and expression by the hERG-Lite® method and with IonWorks Barracuda (IWB), a population-based automated patch clamp platform. We find that the hERG-Lite® assay is the more accurate method to detect direct drug effects on hERG trafficking with lower probability of false positive and false negative hits. In the population patch clamp-based assay, all of the hERG-Litepositive drugs also reduced hERG currents after chronic (overnight) treatment followed by washout. However, certain hERG-Lite-negative drugs, followed by washout, reduced hERG activity detected by IWB. Such drugs also displayed classic hERG block after acute application to cells on IWB, and reduced hERG currents after incubation times (1 h) too short to affect trafficking. We conclude that population electrophysiological assays, and likely manual patch clamp assays, are prone to falsepositives with lipophilic hERG-blocking compounds, which appear to be retained in cell membranes at sufficient concentrations after washout to directly block hERG channel activity.



Methods

IonWorks™ Barracuda based assay

Cells and Treatment. HEK293 cells were transfected with KCNH2 encoding cDNA. Stable transfectants were selected using the G418-resistance gene incorporated into the expression plasmid. 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 g/mL streptomycin sulfate and 500 g/mL G418. Test article concentrations were diluted in the cell culture media and applied to the cells in 60-mm dishes for 1 or 24 hours; final concentration DMSO was 0.3%. Vehicle control cells were incubated with 0.3% DMSO for 1 or 24 hours. To verify the sensitivity of the assay, one concentration of the positive control (pentamidine: 30 μM; 0.3% DMSO) was applied to the cells for 1 or 24 hours. Before testing, cells in culture dishes were washed three times with Hank's Balanced Salt Solution (HB-PS) to remove the culture medium and test articles then treated with Accutase for up to 60 minutes. Immediately before use in IonWorks system, the cells were washed twice in HB-PS to remove the Accutase and re-suspended in the final volume of HB-PS.

Electrophysiological Procedures. In preparation for a recording session, the intracellular solution (in mM: 70 KCl, 70 KF, 2 MgCl₂, 1 EGTA, 10 HEPES) was loaded into the intracellular compartment of the population patch clamp (PPCTM) planar electrode. Extracellular buffer (HEPES-buffered Physiological Saline; HB-PS) was loaded into the PPC plate wells (11 μl per well). Cell suspensions (vehicle control, positive control and test compound treated cells) were pipetted into the wells of the PPC planar electrode (9 μl per well). After establishment of a whole-cell configuration (the perforated patch: Escin), membrane currents were recorded using patch clamp amplifier in the lonWorksTM Barracuda system (IWB). Each IWB experiment had included: two (2) treatments – 1 and 24 hours with two (2) test articles per treatment (4 concentrations, N = 16 per concentration), the positive control (one concentrations, N = 32) and the vehicle control (N = 32).

hERG current was elicited using a pulse pattern with fixed amplitudes (conditioning pre-pulse: 20 mV for 2000 ms; test pulse: -40 mV for 500 ms) from a holding potential of -80 mV. hERG current amplitude was measured as a peak current at the test step to -40 mV.

hERG-Lite®

hERG-Lite® experiments were performed as described previously: Wible BA, Hawryluk P, Ficker E, Kuryshev YA, Kirsch G, Brown AM. HERG-Lite: a novel comprehensive high-throughput screen for drug-induced hERG risk. J Pharmacol Toxicol Methods. 2005 Jul-Aug;52(1):136-45.



Results

Voltage clamp population patch: functional assay for measuring relative changes in membrane surface hERG

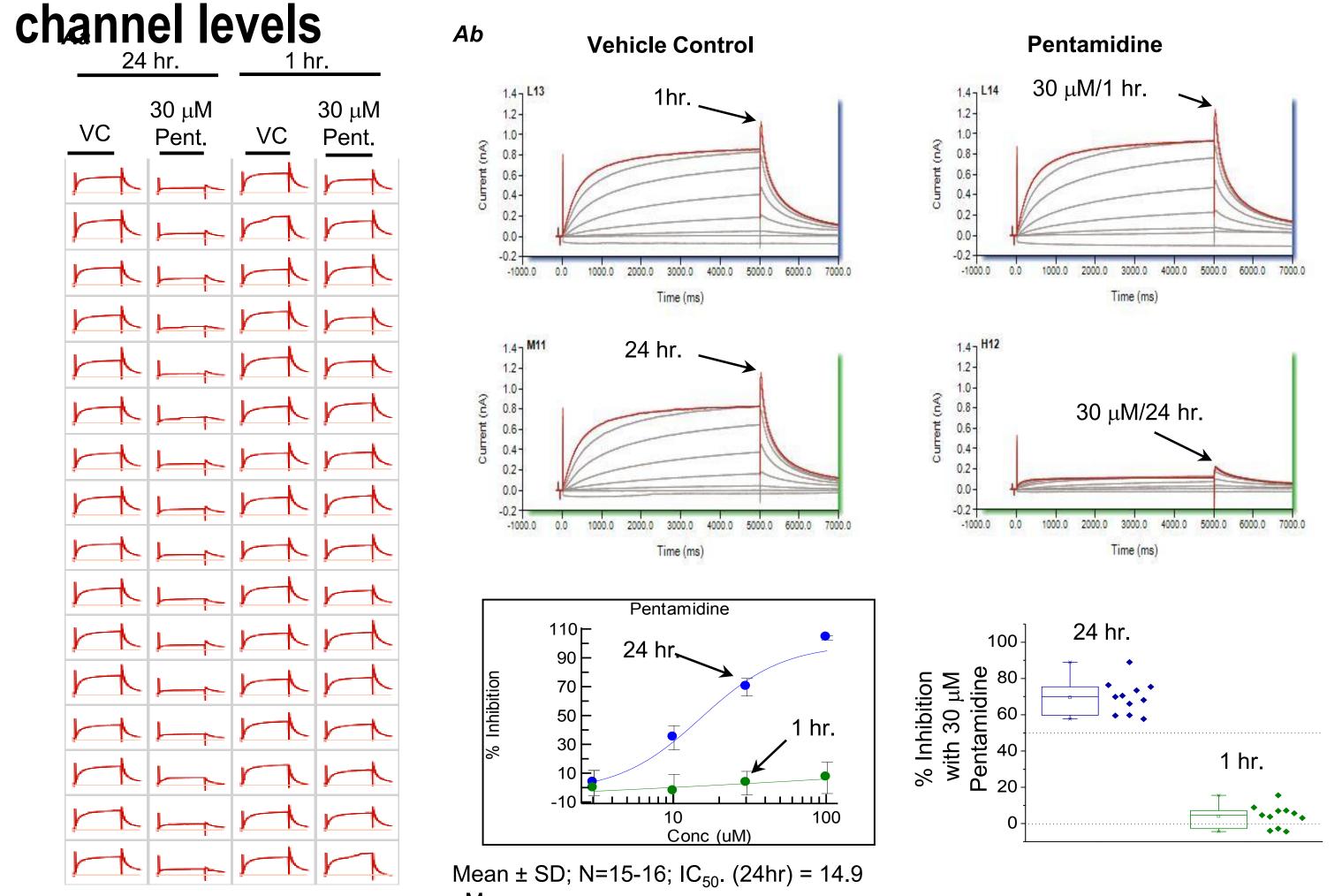


Figure 1. hERG currents on IWB platform; PPC mode. Effect of Pentamidine – 1 hour vs. and 24 hours treatment **A**, representative hERG current traces. **B**, Dose-response of Pentamidine produced inhibition of hERG expression. **C**, reproducibility of Pentamidine effect on hERG expression; each point represent mean of N = 14-16 wells from independent experiments.

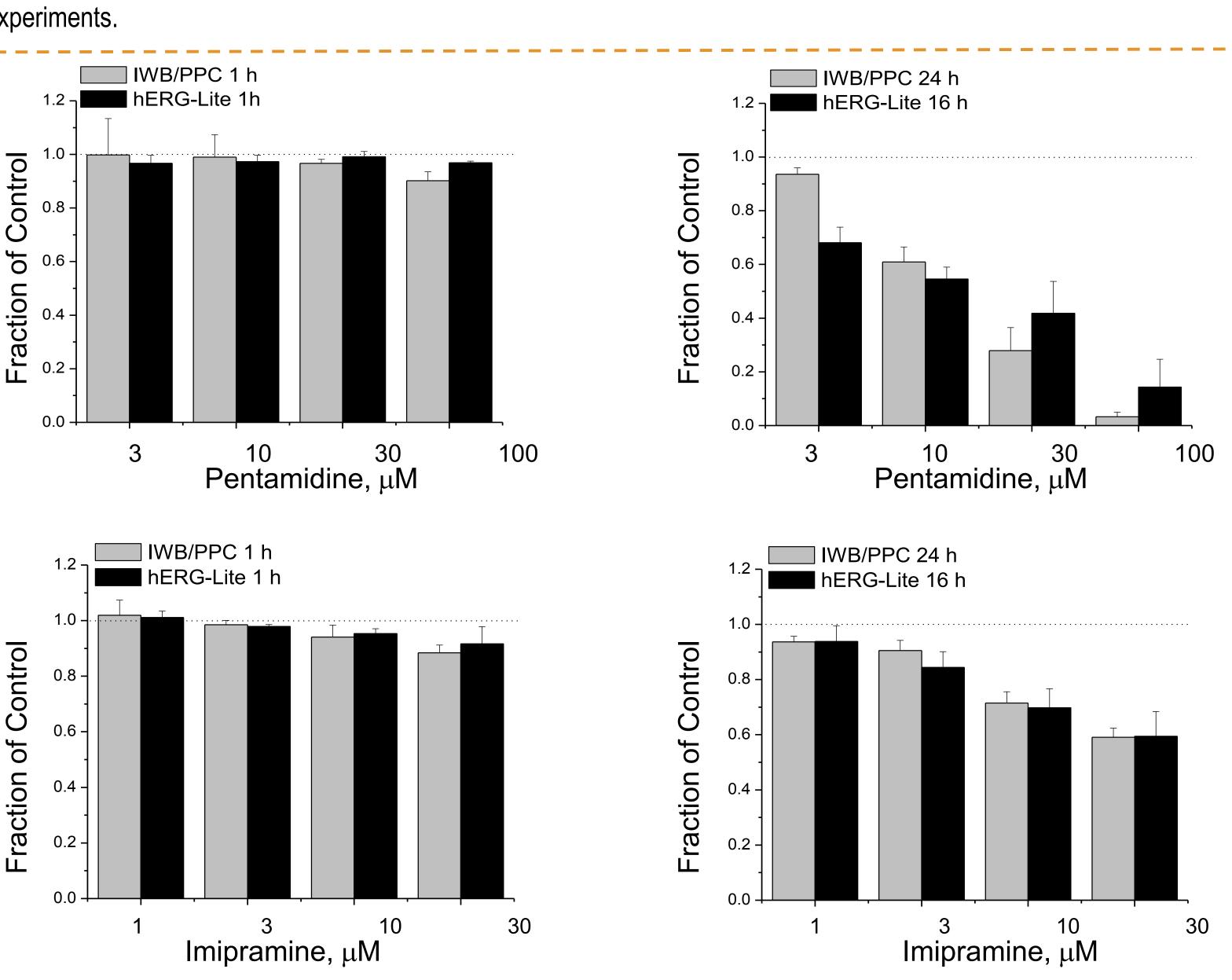


Figure 2. Measured effects of hERG inhibition with Pentamidine and Imipramine IWB/PPC (gray) vs. hERG-Lite (black); 24 hours (left) vs. 1 hour (right). Averaged data from 3 IWB and 4 hERG-Lite experiments; Mean + SD.

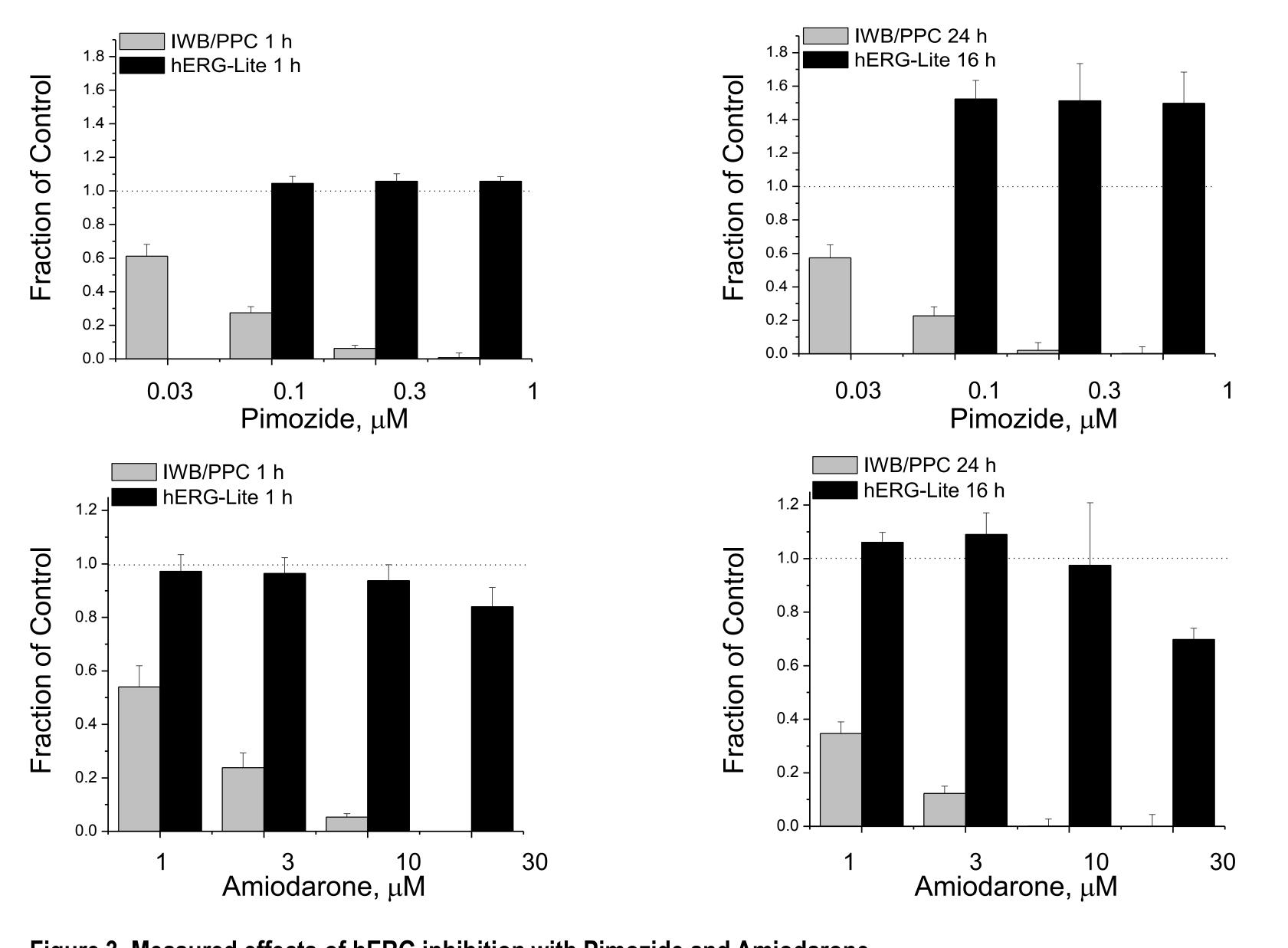


Figure 3. Measured effects of hERG inhibition with Pimozide and Amiodarone IWB/PPC (gray) vs. hERG-Lite (black); 24 hours (left) vs. 1 hour (right). Averaged data from 3 IWB and 4 hERG-Lite experiments; Mean + SD.

Western Blot: Biochemical assay for measuring relative changes of compartmentalized intracellular and surface hERG channel forms

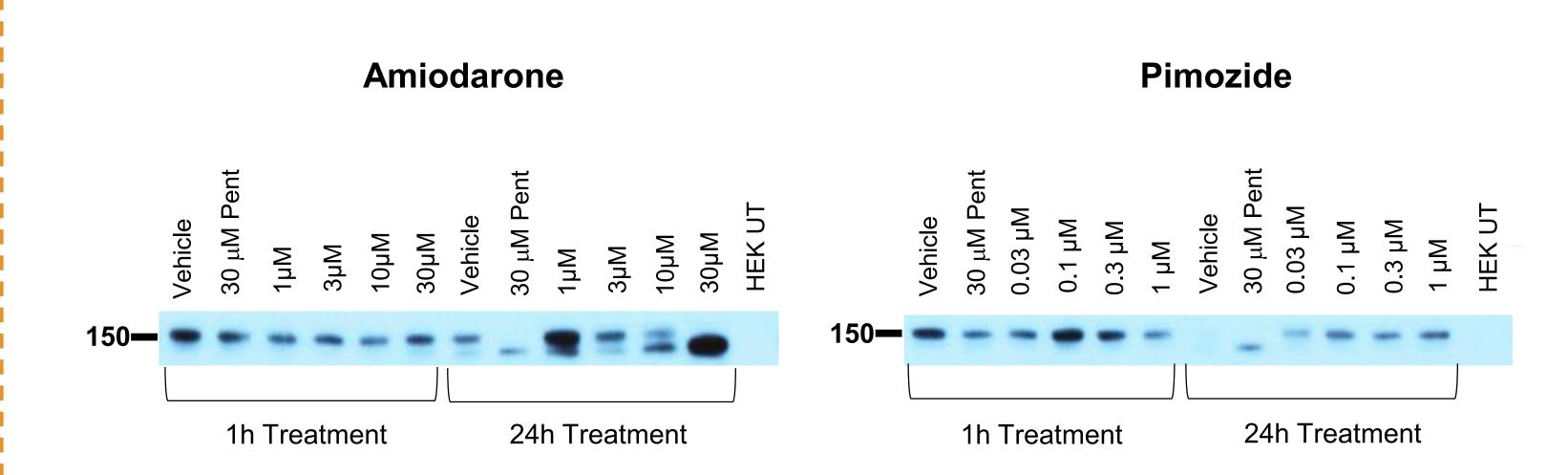


Figure 4. Amiodarone inhibits hERG trafficking – Western Blot Analysis

Western blot showing effect of 1- and 24-hour treatment of HEK 293 [H71] cells stably expressing hERG with increasing concentrations of Pimozide and Amiodarone. Amiodarone-treated cells exhibited an increase in coreglycosylated hERG (135 KDa) with increasing treatment time and drug concentration. Pimozide – treated cells exhibited fully glycosylated hERG (150 KDa) under all conditions, indicating that Pimozide has no effect on hERG trafficking to the cell surface.

Western Blot Protocol: Cells were rinsed in ice cold DPBS, followed by direct lysis in TmPER (FIVEPhoton) for 5 minutes on ice. Protein concentrations were assayed by Coomassie Plus Bradford Assay Kit. SDS-PAGE was run with 10 µg whole cell lysate per lane on a 4-12% Bis-Tris gel. Proteins were transferred to PVDF overnight at a 12 V constant. Antibodies: Polyclonal Rabbit anti-hERG (Alomone, APC-109), followed by incubation with Goat anti-Rabbit HRP-conjugated secondary antibody (Santa Cruz). Blots were developed in SuperSignal West Pico ECL reagent and signal was detected with X-ray film.



Summary

hERG-Lite®: in-cell immunoassay for measuring relative changes in hERG channel surface levels

	hERG-Lite	WB*	IWB/PPC
Amiodarone	\downarrow	\downarrow	N.D.**
Amitriptyline	\downarrow	\downarrow	\downarrow
Diphenhydramine	N.E.	-	N.E.
Dofetilide	N.E.	N.E.	N.D.**
Fluoxetine	N.E.***	\downarrow	\downarrow
Imipramine	↓	-	\downarrow
Pentamidine	↓	\downarrow	↓
Pimozide	N.E.	N.E.	N.D.**
Sunitinib	N.E.	N.E.	N.D.**
Verapamil	N.E.	N.E.	N.E.

For all compound results are obtained from 3-4 independent experiments (each assay). *Positive* or *Negative* results were consistent or all 3-4 experiments

N.D., Trafficking effects were not detected; N.E., No effect

*Wester Blot data from the present study or published Western Blot and long-treatment data

**Significant dose-dependent inhibition was detected after 1 hour and 24 hour treatments

***Non-significant trafficking inhibition effects were obtained in hFRG-Lite experiments where solutions.

***Non-significant trafficking inhibition effects were obtained in hERG-Lite experiments where solutions contained FBS but inhibition was seen in absence of FBS in the media

	Assay Limitation	IWB/PPC	hERG-Lite
-	Trafficking effects not detected due to compound retention	Yes	No



Conclusions

- hERG-Lite® is the only medium throughput, cost effective assay to identify drugs that decrease hERG trafficking.
- The IWB/PPP method identifies hERG trafficking inhibition and drug accumulation.

