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Novel Chemical Suppressors of Long QT Syndrome Identified by an *in vivo* Functional Screen

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Abstract

Background—Genetic long QT (LQT) syndrome is a life-threatening disorder caused by mutations that result in prolongation of cardiac repolarization. Recent work has demonstrated that a zebrafish model of LQT syndrome faithfully recapitulates several features of human disease including prolongation of ventricular action potential duration (APD), spontaneous early after-depolarizations, and 2:1 atrioventricular (AV) block in early stages of development. Due to their transparency, small size, and absorption of small molecules from their environment, zebrafish are amenable to high throughput chemical screens. We describe a small molecule screen using the zebrafish *KCNH2* mutant *breakdance* to identify compounds that can rescue the LQT type 2 phenotype.

Methods and Results—Zebrafish *breakdance* embryos were exposed to test compounds at 48 hours of development and scored for rescue of 2:1 AV block at 72 hours in a 96-well format. Only compounds that suppressed the LQT phenotype in three of three fish were considered hits. Screen compounds were obtained from commercially available small molecule libraries (Prestwick and Chembridge). Initial hits were confirmed with dose response testing and time course studies. Optical mapping using the voltage sensitive dye di-4 ANEPPS was performed to measure compound effects on cardiac APDs. Screening of 1200 small molecules resulted in the identification of flurandrenolide and 2-methoxy-N-(4-methylphenyl) benzamide (2-MMB) as compounds that reproducibly suppressed the LQT phenotype. Optical mapping confirmed that treatment with each compound caused shortening of ventricular APDs. Structure activity studies and steroid receptor knockdown suggest that flurandrenolide functions via the glucocorticoid signaling pathway.

Conclusions—Using a zebrafish model of LQT type 2 syndrome in a high throughput chemical screen, we have identified two compounds, flurandrenolide and the novel compound, 2-MMB, as small molecules that rescue the zebrafish LQTS 2 by shortening the ventricular action potential duration. We provide evidence that flurandrenolide functions via the glucocorticoid receptor mediated pathway. These two molecules, and future discoveries from this screen, should yield

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Conflict of Interest Disclosures

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novel tools for the study of cardiac electrophysiology and may lead to novel therapeutics for human LQT patients.

Keywords

long QT syndrome; animal models of human disease; ion channels; chemical screening

Introduction

LQTS affects one in 3,000 live births and is responsible for approximately 4,000 deaths yearly in the United States alone[1, 2]. The syndrome is due to a prolongation of the myocardial repolarization time[3] and is diagnosed on an electrocardiogram by an increased duration of the QT interval. LQTS can either be congenital or acquired as a result of medication or metabolic disturbance. Many genetic causes of LQTS have been identified, but the majority of cases are the result of mutations in one of three cardiac ion channel genes: *KCNQ1*, *KCNH2*, or *SCN5a*[4–6]. Mutations in *KCNQ1* or *SCN5a* lead to LQTS 1 and 3, while defects in *KCNH2* (also known as the *human ether-a-go-go related gene, hERG*) lead to LQTS 2.

Currently there are no therapies for the treatment of LQTS that address the fundamental problem of prolonged myocardial repolarization time. Chemical modulators of myocardial repolarization are well known: QT prolongation as an unintended side effect of pharmaceutical agents is a common and troubling regulatory problem[7]. However, despite myriad examples of compounds that prolong myocardial repolarization, drugs that shorten the QT interval are rare and their effect generally small[8]. It has not been clear whether prolonged QT intervals could be corrected by pharmacologic therapy.

Because the majority of LQTS 2 mutations result in trafficking defective KCNH2 ion channel proteins[9], there has been considerable scientific effort focused on correcting the trafficking defect. In many cases mutant KCNH2 protein trafficking can be remedied by treatment with KCNH2 inhibitors which presumably bind to and stabilize the mutant protein in its properly folded state facilitating its transport to the cell surface[10]. While these inhibitors can restore the proper sub-cellular localization of the ion channel, their therapeutic potential is nullified by their direct inhibition of channel function[11].

One of the major limitations in the study of long QT syndromes has been the lack of a faithful animal model. The molecular details of murine and rat cardiac repolarization are markedly different from humans, and while faithful rabbit models of LQTS type 1 and 2 have been generated[12], rapid chemical screening in these animals is not currently feasible. Zebrafish recapitulate several key features of human cardiac repolarization, including drug-induced QT prolongation as well as models of LQTS types 2 and 3[13–16]. The zebrafish *breakdance* (*bkd*) mutant carries an I59S mutation in KCNH2, the LQTS 2 gene, and has an easily observed cardiac phenotype of 2:1 AV block[15]. This 2:1 AV block is the direct result of ventricular action potential prolongation and mirrors the 2:1 AV block reported in pediatric cases of LQTS[17–19]. Since cell-based chemical screens for correction of mutant KCNH2 trafficking defects may be susceptible to high false positive rates due to the presence of I_{Kr} inhibitors in many chemical libraries, and because they focus on a single mechanism of rescue, we tested the hypothesis that whole organism screening using the zebrafish LQTS 2 model *breakdance* could provide a functional phenotypic screen for compounds that shorten myocardial repolarization. We hypothesized that as an *in vivo* assay, zebrafish would be less susceptible to KCNH2 blockers as false positives, and would enable the discovery of compounds that act by mechanisms other than trafficking rescue.

Methods

Fish Husbandry and Chemical Treatment

Tubingen AB and tb218 (*bkd*$^{-/-}$) fish were maintained using standard methods. For screening, tb218$^{-/-}$ adults were crossed, and resultant embryos were reared in E3 buffer at 25°C. 24 hours post-fertilizaton (hpf) animals were dechorionated with pronase and plated 3 per well in 96-well plates in a final volume of 200 uL. At 48 hpf, library compounds (Prestwick and Chembridge) were added to a final concentration of 10 ng/uL. Zebrafish were visually scored at 72 hpf for presence or absence of 2:1 AV block. For dose response curves, tb218 fish at 48hpf were treated for 24 hours with 2-methoxy-N-(4-methylphenyl) benzamide (Chembridge Corporation, San Diego CA) or flurandrenolide (Sigma) from 0.1M stock solutions in DMSO to the indicated final concentration and scored for suppression of the *breakdance* phenotype at indicated times. The time-course studies were performed using 10 uM flurandrenolide and 50 uM 2-MMB. Dose response curves for the steroids deoxycorticosterone acetate, testosterone, and dexamethasone (Sigma) were performed at indicated concentrations.

Optical Mapping

Optical voltage mapping of embryonic hearts was performed as described previously12. Mapping was performed on tb218 or TuAB hearts from embryos that had been treated with 100 uM of each compound starting at 48 hpf. Explanted hearts were mapped at 72–75 hpf for 2-MMB treatment, and at 96–99 hpf for flurandrenolide treatment. Control fish for each time point were treated with 0.1% DMSO.

Cloning of zERG-V5 and zERG-V5 I59S Constructs

PCR amplification of zebrafish *KCNH2* (zERG) (Open Biosystems, AF532865) and subcloning into pcDNA3.2/V5/GW/D-TOPO (Invitrogen, #K2440-20) resulted in the sequence verified clone zERG-V5 (Forward:atgcccgtgcgccgcggac, Reverse:tgcttccgggtaagactggatcgg). Site-directed mutagenesis (QuikChange II, Stratagene, #200523-12) using F1-cgcgggcggagaGcatgcagcagtcctgc, R1-gcaggactgctgcatgCtctccgcccgcg resulted in zERG-V5 I59S.

Cell Culture Experiments

HEK293 (ATCC, #CRL-1573) and COS7 (ATCC, #CRL-1651) cells were maintained in DMEM with 10% FBS, 1% Glutamine and 1% Pen-Strep. Cells were transfected using Lipofectamine LTX Reagent (Invitrogen, #15338) according to the manufacturer's instructions. For immunofluorescence, transfected cells were rinsed with ice-cold PBS and treated with 2.5 ug/mL of FITC-labeled wheat germ agglutinin (WGA, Sigma, #L4895) for 3 minutes on ice. Cells were rinsed with PBS, fixed in 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. Standard immunofluorescence using a murine anti-V5 antibody (Invitrogen, #46-1157, 1:5000), and an Alexafluor 555 conjugated secondary (Invitrogen, #A21424, 1:5000) was performed and cells were imaged using a Zeiss LSM 5 Pascal confocal microscope. Transfected HEK293 cells were treated with 2-MMB or flurandrenolide from DMSO stock solutions to the final concentrations indicated for 24 hours for Western Blots and immunofluorescence. Western blots were performed using standard techniques with the mouse anti-V5 primary described above (1:5000) and an HRP conjugated secondary antibody (Thermo Scientific #31444 at 1:5000) for V5 epitope detection. Tubulin controls were measured with mouse anti-tubulin antibody (Millipore, #05-829, 1:5000) and the same secondary. Blots were visualized using a chemiluminescent substrate (Pierce, #32106) and subsequent film exposure. Results from three to four separate

experiments were used to quantify changes in glycosylation patterns in response to the treatments shown in figure 3.

Patch Clamp Electrophysiology

Whole-cell currents were recorded using the disrupted patch technique in COS7 cells transiently expressing wildtype or I59S zERG. Recordings were performed at room temperature 48 hours after transfection. The bath solution contained (in mM) 150 NaCl, 5.4 KCl, 1.8 CaCl2, 1 MgCl, and 5mM HEPES (pH adjusted to 7.4 with NaOH). Fire-polished patch electrodes had 2–5MΩ tip resistance when filled with (in mM) 150 KCl, 5 Mg2ATP3, 2 MgCl, 5 BAPTA, and 10 HEPES (pH adjusted to 7.2 with KOH). Recordings were low-pass filtered at 5kHz with an Axopatch 200B amplifier and digitized at 10kHz with a Digidata 1322A A/D converter, both controlled using Clampex software, and later analyzed using Clampfit software (Axon Instruments, CA). Capacitive transients were electronically subtracted, and cell capacitance noted. Cells were clamped at a holding potential of −80mV, sequentially stepped to test potentials ranging from −90 and +50mV for 4 seconds, followed by a step to −50mV for 500 milliseconds to elicit typical zERG tail currents. Current density-voltage relations were derived from peak tail current values divided by cell capacitance, and were fit to a standard Boltzmann function.

Morpholino Knockdown

Morpholino oligonucleotides (MOs, Gene Tools, LLC) for the glucocorticoid receptor has been previously described20. A splice blocking antisense morpolino (AGCAGAGCCGCCTCTTACCTGCCAT) was designed against the zebrafish androgen receptor primary transcript targeting the first exon splice donor sequence. MOs were suspended in distilled water and approximately 1.5 ng were injected into single cell Tb218 embryos at the single cell stage.

Statistical Methods

Data are presented as means plus or minus standard deviations, unless otherwise specified. For comparisons of continuous variables, the Mann-Whitney-Wilcoxon rank-sum (MATLAB, Natick MA) test was performed. For categorical variables comparisons were made using Fisher's exact test. Electrophysiology data are presented as 25th/50th/75th percentiles and were compared using a Mann–Whitney–Wilcoxon rank-sum test with a signifcance level alpha<0.017 (Sidak-corrected alpha<0.05 for 3 comparisons). Where no significant difference was found, the minimal detectable difference for power = 0.8 was determined using G*Power21.

Results

Chemical Screen for Suppressors of AV Block

Breakdance homozygotes (*bkd* $^{-/-}$) develop 2:1 AV block as a result of prolonged ventricular refractory periods13. This phenotype is easily scored *in vivo*, owing to the transparency of the embryo, enabling a straightforward small molecule suppressor screen. *Breakdance* homozygotes are viable and fertile, with phenotypic penetrance of 92%–96%. In order to minimize false positive results, three *bkd*$^{-/-}$ embryos were placed in each well and a compound was only scored as a hit if it suppressed the LQT phenotype in all three embryos. Embryos were treated after the onset of atrioventricular block in order to identify compounds that could treat LQTS rather than compounds that exert their effects primarily by altering cardiac development.

In a screen of 1,200 chemicals, two hits were identified that suppressed 2:1 AV block, the long QT phenotype, in all three embryos: 2-methoxy-N-(4-methylphenyl) benzamide (2-

MMB) and the steroid flurandrenolide (Fig. 1a). Nine compounds rescued two of three embryos in the well and although these did not meet our strict definition of a chemical suppressor, the small number of compounds allowed further testing. Two of these confirmed on repeat testing and were found to be the steroids, fluocinonide and fluorometholone (Fig. 1a).

Flurandrenolide and 2-MMB display dose related suppression of the *breakdance* phenotype with ED50s of approximately 2 uM and 10 uM respectively (Fig. 1b). Time series experiments revealed higher percentage of drug effect at later timepoints for both flurandrenolide and 2-MMB, but a slightly longer exposure requirement for flurandrenolide (Supplementary Fig. 1). In order to directly measure the effects of these compounds on myocardial repolarization, ventricular action potential durations were measured using voltage sensitive optical mapping in *bkd*$^{-/-}$ and wildtype fish treated with active compounds or diluent. *Breakdance*$^{-/-}$ embryos treated with 50 uM flurandrenolide at 48 hours post fertilization (hpf) for 48 hours displayed shorter ventricular action potential durations, 338 +/− 44 msec (mean +/− standard deviation) compared to untreated controls, 482 +/− 83 msec ($p = 0.0008$, Fig. 2a). Wild type embryos treated with flurandrenolide did not have significantly shorter ventricular APDs than untreated wild type controls (Fig. 2a). *Breakdance*$^{-/-}$ embryos treated with 50 uM 2-MMB at 48 hpf for 24 hours displayed shorter ventricular action potential durations, 376 +/− 66 msec, compared to untreated *breakdance*$^{-/-}$ embryos (570 +/− 23 msec, $p = 0.00007$, Fig. 2b). Wild type embryos treated with 2-MMB also had shorter ventricular APD90s of 200 +/− 51ms compared to untreated wild type controls (290 +/− 85 msec, $p = 0.03$, Fig. 2b). Thus, both lead compounds achieve suppression of the long QT phenotype by shortening repolarization time.

Characterization of zERG I59S Mutant Trafficking

In order to better understand the mechanisms of these compounds in *breakdance* embryos, we characterized the nature of the *bkd* I59S mutation. Transfection of a C-terminal V5 epitope tagged zebrafish ERG (zERG) in HEK cells produced a protein that runs as a doublet at 133 kDa and 155 kDa (Fig. 3). This pattern is similar to the human KCNH2 protein which also runs as a doublet with a lower band that is the core glycosylated protein while the upper band is a mature glycoform that traffics to the membrane. In the majority of human KCNH2 mutations studied to date the mature glycoform fails to develop and the protein does not traffic appropriately to the cell membrane. Expression of I59S zERG in HEK cells results in a protein that runs at 133kDa on Western blot, with only a faint upper glycoform at 155 kDa (Fig. 3), an 83% reduction in the cell surface isoform. Immunofluorescence of transfected HEK cells demonstrated that the wild type zERG colocalizes with a cell surface marker (Fig. 4), while the mutant I59S zERG does not. These data demonstrate that, similar to the majority of known human mutations, the I59S zERG mutation results in a trafficking defective protein.

Characterization of zERG I59S Mutant Electrophysiology

To further characterize the I59S zERG mutation, we performed patch clamp electrophysiology on HEK cells transfected with either wild type zERG or I59S zERG. Although both the wild type and mutant give rise to an outward potassium current, there was a marked reduction in maximal current density in the I59S zERG mutant compared to the wild type zERG (13 +/−7 pA/pF versus 36 +/−10 pA/pF, $p=0.00009$, Fig. 5). The current voltage relationship for the tail current was not otherwise appreciably different between wild type and I59S mutant channels (Table 1).

Effects of Drugs on zERG I59S Mutant Trafficking

Several distinct manipulations have been shown to correct the trafficking defect of various subsets of mutant hERG proteins, including incubation at lower temperature, treatment with I_{Kr} blocking drugs, and treatment with thapsigargin9. The upper protein band of the zERG mutant I59S was partially restored by incubation with dofetilide, a potent KCNH2 inhibitor, but not by growth at low temperature, or treatment with thapsigargin (Fig. 3). These experiments demonstrate that, similar to many of the human trafficking defective mutant KCNH2 proteins9, the *bkd* trafficking defect can be partially corrected by KCNH2 inhibitors.

In order to determine how 2-MMB and flurandrenolide act to suppress the *bkd* phenotype, we tested whether treatment with 2-MMB or flurandrenolide would correct I59S zERG trafficking. Treatment of HEK cells transfected with I59S zERG with 100 uM 2-MMB or flurandrenolide for 24 hours did not appreciably change the percentage of the mature glycoform on Western blot (Fig. 3) or affect the membrane localization measured by immunofluorescence (Fig. 4). In addition, neither flurandrenolide or 2-MMB was able to rescue the defective trafficking of the human KCNH2 G601S mutant (Supplementary Fig. 2). These data suggest that 2-MMB and flurandrenolide do not rescue the *breakdance* phenotype by correcting defective I59S zERG trafficking, and confirm the utility of this physiologic screen in identifying chemical suppressors that function via alternative mechanisms to trafficking rescue.

Flurandrenolide acts via the glucocorticoid receptor

Little is published about the specificity of flurandrenolide with respect to glucocorticoid, mineralocorticoid, or androgen effects. In order to further define the signaling pathway by which flurandrenolide suppresses the *bkd* phenotype we examined the effects of a pure mineralocorticoid, a glucocorticoid, and androgen on the *bkd* mutant. Treatment with the pure mineralocorticoid, deoxycorticosterone acetate (DOCA) did not result in any suppression of the *breakdance* phenotype, while treatment with testosterone and dexamethasone caused a dose related rescue (Figure 6a). To distinguish between the androgen and glucocorticoid pathways, we employed morpholino mediated knockdown of the glucocorticoid and androgen receptors. Knockdown of the glucocorticoid receptor resulted in the anticipated reduction of dexamethasone rescue and also reduced flurandrenolide mediated rescue (Figure 6b). Conversely, knockdown of the androgen receptor reduced testosterone mediated suppression without significantly altering flurandrenolide effects (Supplementary Figure 3). Incomplete interruption of drug effects are likely due to incomplete receptor knockdown and/or waning effects of the morpholino at the later timepoints of drug exposure required for the experiment. The effect of 2MMB was not interrupted by either androgen or glucocorticoid receptor knockdown (data not shown). Taken together, these data support a model where flurandrenolide, acting through the glucocorticoid receptor, shortens ventricular action potentials by a mechanism that is distinct from trafficking rescue of the defective zERG channel.

Discussion

To date, pharmacotherapy of the long QT syndrome has been limited to beta-blocker therapy, which does not shorten the QT interval but appears to reduce the triggers for a fatal arrhythmia22. In many cases implantable cardioverter defibrillators are used which do not prevent arrhythmias, but can effectively terminate them. A drug treatment that restores normal repolarization would represent a novel therapeutic tool in LQTS.

We have described a screen for chemical suppressors of long QT syndrome in which the readout was physiologic suppression of the long QT phenotype independent of mutant ion channel trafficking. Two structurally unrelated compounds, flurandrenolide and 2-MMB, were discovered to rescue zebrafish long QT syndrome. These compounds work by a mechanism that is distinct from correction of defective channel trafficking. We further demonstrate that flurandrenolide functions via the glucocorticoid signaling pathway, and that the canonical pure glucocorticoid dexamethasone can rescue the long QT phenotype as well.

Recent developments in the availability of large compound libraries combined with automated and rapid assays have enabled the study of thousands of chemical compounds using high throughput screening. In cases where an established therapeutic target is known, *in vitro*, or cell based screening is frequently employed. However, in many of the most complex and challenging diseases, including LQTS, effective drug-targets are not well established. Additionally, the assay of a compound's effect on a single ion channel in isolation may ultimately prove misleading as many compounds have activities against multiple ion channels. It is therefore not surprising that *in vitro* assays often lead to unforeseen results *in vivo*.

The alternative we employed is a phenotype driven screen in the context of the whole organism. Target based approaches can discover compounds that modify a given target, but may ultimately fail to modify the disease phenotype i.e., I_{Kr} blocking drugs that rescue mutant KCNH2 channel trafficking, but fail to shorten QT interval due to concurrent KCNH2 channel block. In contrast, phenotype-based approaches can discover compounds that modify the disease trait in a mechanistically agnostic fashion. Our results indicate that for diseases such as LQTS where complexity is high and no single validated therapeutic target exists, phenotype-based screens may be a promising avenue of investigation.

The greatest limitation to these results remains the question of whether the benefits of these chemicals will translate to higher organisms and ultimately human LQT patients. The discovery of these compounds in an embryonic assay raises the possibility that we may be observing developmental effects in the zebrafish. Additionally, there are diverse genetic mutations responsible for LQTS 2 and it will be important to determine whether these compounds will have activity in the setting of other KCNH2 mutations as well as in other types of LQTS including drug induced LQTS. Finally, it will be critical to understand the mechanism(s) of action and toxicology of these compounds. While validation in higher species remains to be established we believe that these results may lead to novel therapeutic approaches to this serious unmet clinical need. While the considerable side effects associated with long term glucocorticoid administration will likely limit their therapeutic use, there are circumstances where short term therapy could be expected to aid in the acute management of LQTS patients, including episodes of arrhythmic storm, or pediatric cases of 2:1 atrioventricular block, although the timing of drug effects will be a critical factor in determining their clinical benefit. Additional consideration must be given to the possibility of creating a drug-induced short QT syndrome which might introduce proarrhythmia. While neither of the two compounds identified in this screen overcorrected the action potential duration in this LQTS 2 model, treatment of wild type embryos with 2-MMB did result in shortening of the APD. The effects of these compounds in LQTS cases with only mildly prolonged QT intervals would need to be carefully studied to address this concern.

Future studies will include studying whether these compounds shorten the electrocardiographic QT interval in higher organisms, other genotypes of LQTS 2, and drug-induced and other genetic LQT types. We are studying the mechanisms of action of these compounds by several methods including direct recordings from zebrafish cardiomyocytes.

Ultimately, this method for long QT drug discovery could also be extended to a personalized approach, engineering patient-specific “humanized” zebrafish lines that could then be screened in order to discover compounds that rescue specific mutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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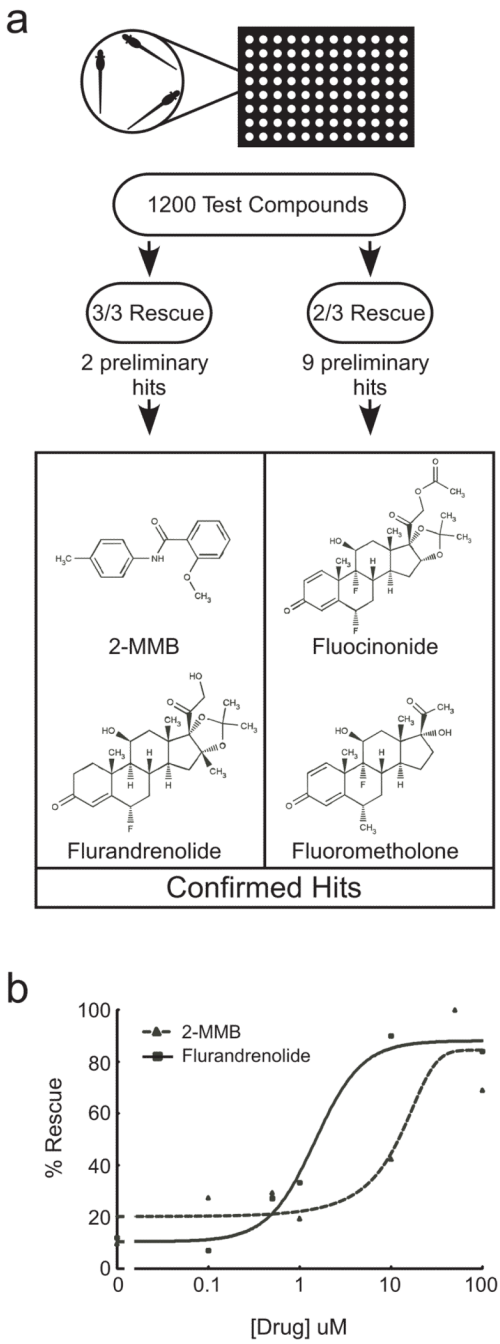

Figure 1.

A chemical screen for suppressors of long QT syndrome resulted in the identification of two structurally unrelated compound classes. (a) Flow chart indicating the small molecule screening strategy and lead compounds. (b) Dose-response curve of the two lead compounds, 2-MMB and flurandrenolide.

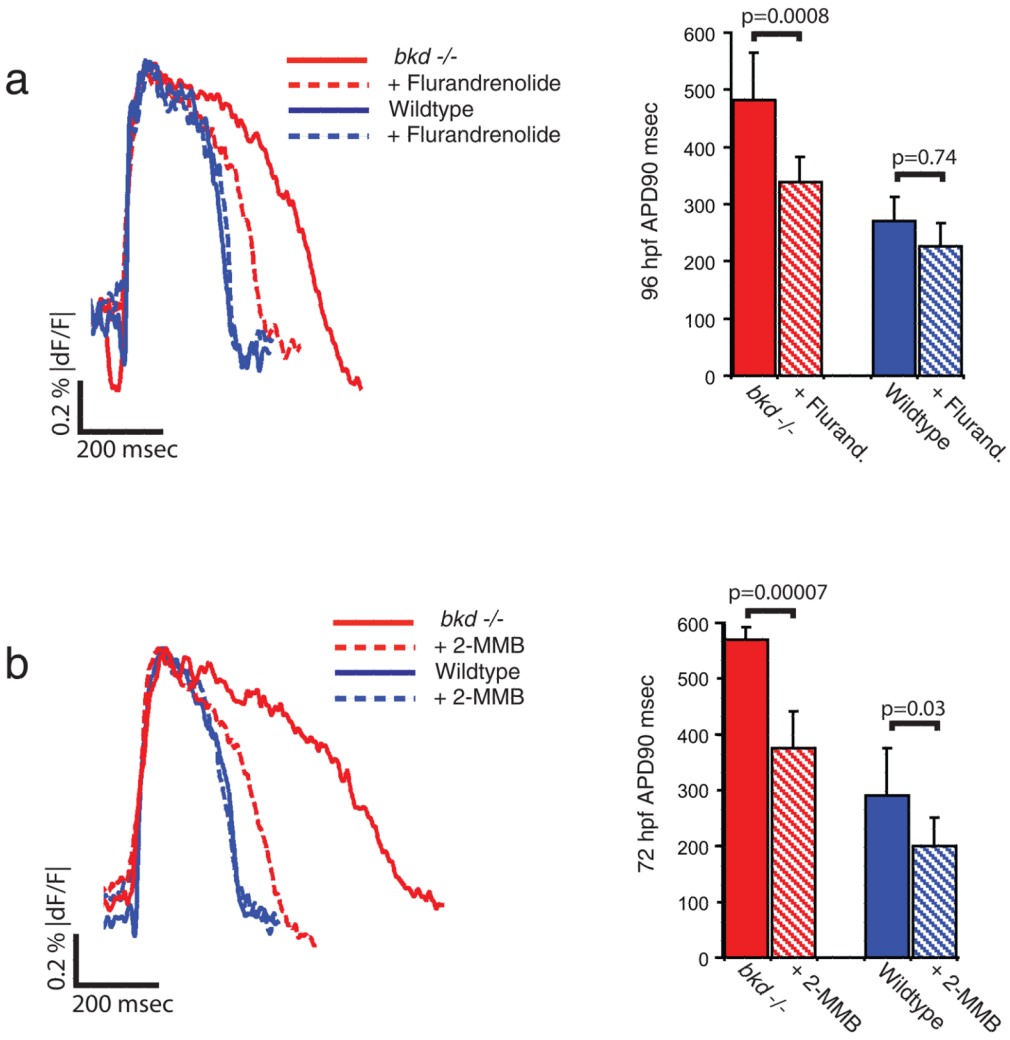

Figure 2.

Flurandrenolide and 2-MMB treatment of the *breakdance* mutant partially correct the repolarization defect. (a) Ventricular action potential duration (APD) curves (left) of wildtype (blue) and $bkd^{-/-}$ (red) fish treated with flurandrenolide (dashed lines). Bar graphs (right) indicate the mean APD90 +/− the standard deviation (n=7–8). (b) Ventricular APD curves (left) of wildtype (blue) and $bkd^{-/-}$ (red) fish treated with 2-MMB (dashed lines). Bar graphs (right) indicate the mean APD90 +/− the standard deviation (n=7–16).

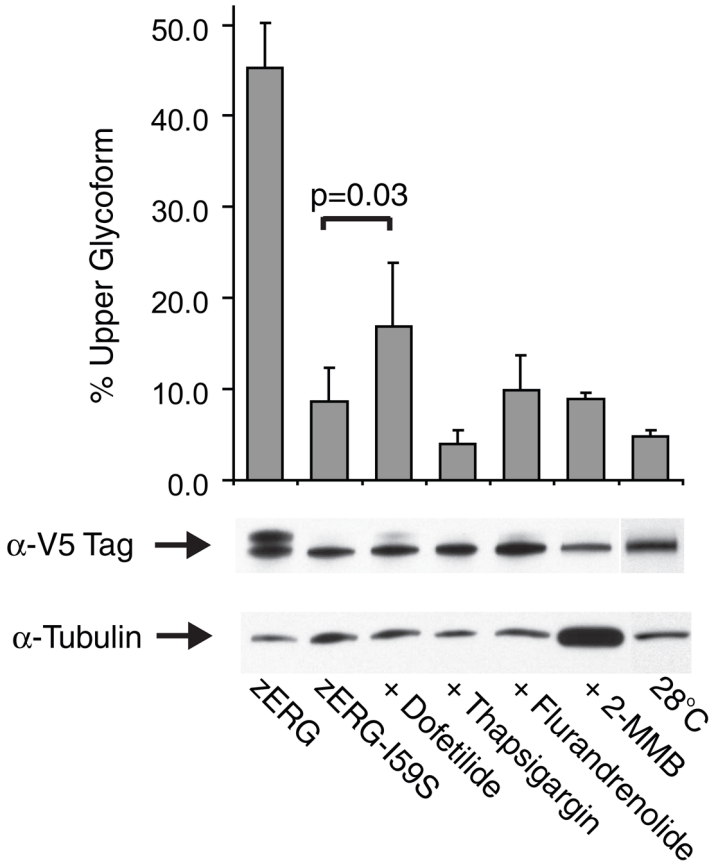

Figure 3.

zERG I59S mutation leads to a trafficking defect, which is not corrected by flurandrenolide or 2-MMB. Summary data from Western blots (n = 3–4) indicating the amount of 155 kd (upper) cell surface isoform of zERG and zERG-I59S as a percentage of total zERG protein under various conditions is shown above on a representative Western blot. Tubulin is shown as a loading control.

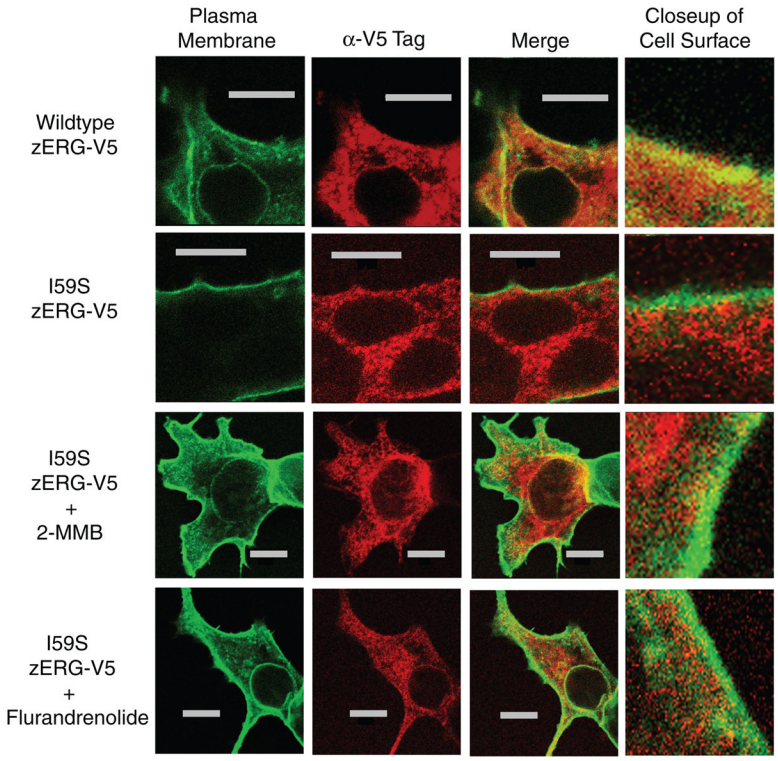

Figure 4.

Subcellular localization of zERG and I59S zERG is shown in red and compared to a surface marker in green. The overlay demonstrates surface expression of wildtype in yellow but not I59S zERG. Treatment with flurandrenolide or 2-MMB does not alter surface expression of I59S zERG. Scale bars are 10 μm.

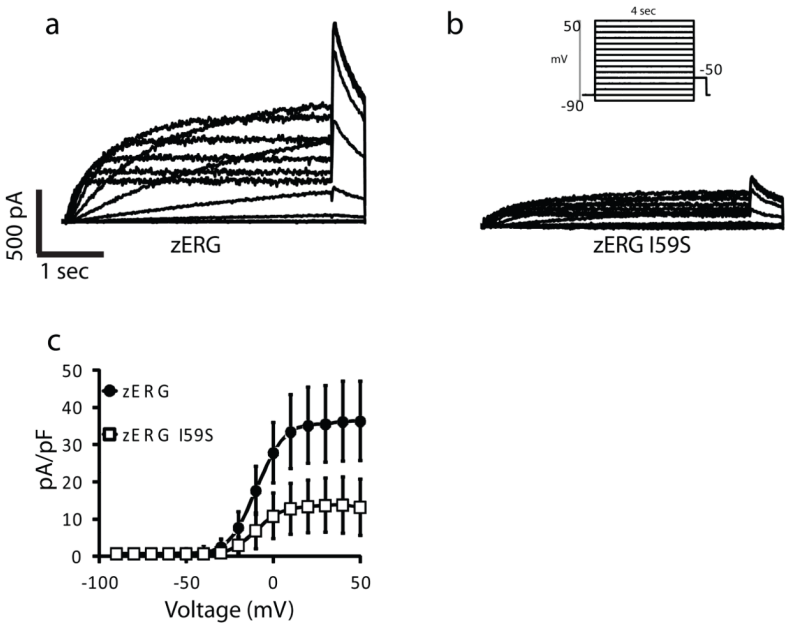

Figure 5.

zERG-I59S current density is reduced compared to wildtype. Exemplar traces from from zERG (a) and zERG-I59S (b) transfected cells. (c) The current-voltage relationship for zERG (black circles) and zERG-I59S (white squares). The voltage clamp protocol is shown as an inset. There is a significant reduction in peak current density in I59S zERG compared to wild type (see Table 1).

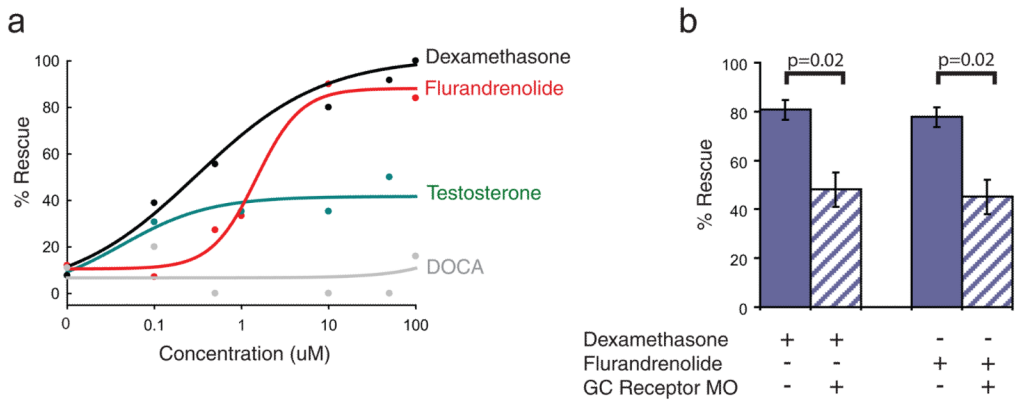

Figure 6.

Flurandrenolide acts via the glucocorticoid signaling pathway. (a) Dose-response curve using steroids known to act through specific signaling pathways: dexamethasone (glucocorticoid pathway), testosterone (androgen pathway), and DOCA (mineralocorticoid pathway). (b) Knockdown of the glucocorticoid receptor blocks rescue of long QT by flurandrenolide and dexamethasone (n= 26–31).