Novel Chemical Suppressors of Long QT Syndrome Identified by an In Vivo Functional Screen

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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, spontaneous early afterdepolarizations, and 2:1 atrioventricular block in early stages of development. Because of 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 atrioventricular block at 72 hours in a 96-well format. Only compounds that suppressed the LQT phenotype in 3 of 3 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 with the voltage-sensitive dye di-4 ANEPPS was performed to measure compound effects on cardiac action potential durations. 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 action potential durations. 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 2 compounds, flurandrenolide and the novel compound 2-MMB, as small molecules that rescue the zebrafish LQT type 2 syndrome by shortening the ventricular action potential duration. We provide evidence that flurandrenolide functions via the glucocorticoid receptor–mediated pathway. These 2 molecules and future discoveries from this screen should yield novel tools for the study of cardiac electrophysiology and may lead to novel therapeutics for human LQT patients. (Circulation. 2011;123:00-00.)

Key Words: animal model ■ chemical screening ■ HERG arrhythmia ■ ion channels ■ long QT syndrome

Long-QT (LQT) syndrome (LQTS) affects 1 in 3000 live births and is responsible for ~4000 deaths yearly in the United States alone.1–3 The syndrome is due to a prolongation of the myocardial repolarization time4 and is diagnosed on an ECG by an increased duration of the QT interval. LQTS can be either 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 1 of 3 cardiac ion channel genes: KCNQ1, KCNH2, or SCN5a.4–6 Mutations in KCNQ1 or SCN5a lead to LQTS type 1 and 3, whereas defects in KCNH2 (also known as the human ether-a-go-go related gene [hERG]) lead to LQTS type 2.

Clinical Perspective on p ●●●

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,

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drugs that shorten the QT interval are rare and their effect is generally small. It has not been clear whether prolonged QT intervals could be corrected by pharmacological therapy.

Because the majority of LQTS type 2 mutations result in trafficking-defective KCNH2 ion channel proteins, considerable scientific effort has 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. Although these inhibitors can restore the proper subcellular localization of the ion channel, their therapeutic potential is nullified by their direct inhibition of channel function.

One major limitation in the study of LQTS has been the lack of a faithful animal model. The molecular details of murine and rat cardiac repolarization are markedly different from humans, and although faithful rabbit models of LQTS type 1 and 2 have been generated, rapid chemical screening in these animals is not currently feasible. Zebrafish recapitulate several key features of human cardiac repolarization, including drug-induced QT prolongation and models of LQTS types 2 and 3. The zebrafish breakdance (bkd) mutant carries an I59S mutation in KCNH2, the LQTS type 2 gene, and has an easily observed cardiac phenotype of 2:1 atrioventricular block. This 2:1 atrioventricular block is the direct result of ventricular action potential prolongation and mirrors the 2:1 atrioventricular block reported in pediatric cases of LQTS. Because cell-based chemical screens for correction of mutant KCNH2 trafficking defects may be susceptible to high false-positive rates because of the presence of KCN inhibitors in many chemical libraries, and because they focus on a single mechanism of rescue, we tested the hypothesis that whole-organism screening with the zebrafish LQTS type 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 with standard methods. For screening, tb218 −/− adults were crossed, and resultant embryos were reared in E3 buffer at 25°C. At 24 hours postfertilization (hpf), animals were dechorionated with pronase and plated 3 per well in 96-well plates in a final volume of 200 μL. At 48 hpf, library compounds (Prestwick, Illkirch, France and Chembridge Corp, San Diego, Calif) were added to a final concentration of 10 ng/μL. Zebrafish were visually scored at 72 hpf for the presence or absence of 2:1 atrioventricular block. For dose-response curves, tb218 fish at 48 hpf were treated for 24 hours with 2-methoxy-N-(4-methylphenyl) benzamide (2-MMB; Chembridge Corp, San Diego, Calif) or flurandrenolide (Sigma, St Louis, Mo) from 0.1 mol/L stock solutions in dimethyl sulfoxide to the indicated final concentration and scored for suppression of the breakdance phenotype at indicated times. The time-course studies were performed with 10 μmol/L flurandrenolide and 50 μmol/L 2-MMB. Dose-response curves for the steroids deoxycorticosterone acetate (DOCA), testosterone, and dexamethasone (Sigma) were performed at indicated concentrations.

**Figure 1.** A chemical screen for suppressors of LQTS resulted in the identification of 2 structurally unrelated compound classes. A, Flowchart indicating the small-molecule screening strategy and lead compounds. B, Dose-response curve of the 2 lead compounds, 2-MMB and flurandrenolide.

**Optical Mapping**

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

**Cloning of zERG-V5 and zERG-V5 I59S Constructs**

Polymerase chain reaction amplification of zebrafish KCNH2 (zERG) (AF532865; Open Biosystems, Huntsville, Ala) and subcloning into pcDNA3.2/V5/GW/D-TOPO (No. K2440-20; Invitrogen, Carlsbad, Calif) resulted in the sequence-verified clone zERG-V5 (forward, atgcccttgccgcgccggac; reverse, tgcttcgccgttgctggagctggc) and subcloning into pcDNA3.2/V5/GW/D-TOPO (No. K2440-20; Invitrogen, Carlsbad, Calif) to create the sequence-verified clone zERG-V5 (forward, atgcccttgccgcgccggac; reverse, tgcttcgccgttgctggagctggc). Site-directed mutagenesis (QuickChange II, No. 200523-12; Stratagene, La Jolla, Calif) resulted in the sequence-verified clone zERG-V5 (forward, atgcccttgccgcgccggac; reverse, tgcttcgccgttgctggagctggc) and subcloning into pcDNA3.2/V5/GW/D-TOPO (No. K2440-20; Invitrogen, Carlsbad, Calif) to create the sequence-verified clone zERG-V5 (forward, atgcccttgccgcgccggac; reverse, tgcttcgccgttgctggagctggc).

**Cell Culture Experiments**

HEK293 (No. CRL-1573; ATCC, Manassas, Va) and COS7 (No. CRL-1651; ATCC) cells were maintained in Dulbecco modified...
Figure 2. Flurandrenolide and 2-MMB treatment of the breakdance mutant partially corrects the repolarization defect. A, Ventricular APD curves (left) of wild-type (blue) and bkd−/− (red) fish treated with flurandrenolide (dashed lines). Bar graphs (right) indicate the mean±SD APD90 (n=7 to 8). B, Ventricular APD curves (left) of wild-type (blue) and bkd−/− (red) fish treated with 2-MMB (dashed lines). Bar graphs (right) indicate the mean±SD APD90 (n=7 to 16).

Morpholino Knockdown

Morpholino oligonucleotides (Gene Tools, LLC, Philomath, Ore) for the glucocorticoid receptor have been previously described.20 A splice-blocking antisense morpholino (GCACAGGCGCCCTCTTACCCTCCCAT) was designed against the zebrafish androgen receptor primary transcript targeting the first exon splice donor sequence. Morpholino oligonucleotides were suspended in distilled water, and ~1.5 ng was injected into single-cell Tb218 embryos.

Statistical Methods

Data are presented as mean±SD unless otherwise specified. For comparisons of continuous variables, the Mann-Whitney-Wilcoxon rank-sum (MATLAB, Natick, Mass) test was performed. For categorical variables, comparisons were made with the Fisher exact test. Electrophysiology data are presented as 25th/50th/75th percentiles and were compared by use of a Mann-Whitney-Wilcoxon rank-sum test with a significance-level α<0.017 (Sidak-corrected α<0.05 for 3 comparisons). Where no significant difference was found, the minimal detectable difference for power of 0.8 was determined with G’S Power.21

Results

Chemical Screen for Suppressors of Atrioventricular Block

Breakdance homozygotes (bd−/−) develop 2:1 atrioventricular block as a result of prolonged ventricular refractory periods.13 This phenotype is easily scored in vivo because of the transparency of the embryo, enabling a straightforward small-molecule suppressor screen. Breakdance homozygotes
are viable and fertile, with phenotypic penetrance of 92% to 96%. To minimize false-positive results, 3 bkd−/− embryos were placed in each well, and a compound was scored as a hit only if it suppressed the LQT phenotype in all 3 embryos. Embryos were treated after the onset of atrioventricular block to identify compounds that could treat LQTS rather than compounds that exert their effects primarily by altering cardiac development.

In a screen of 1200 chemicals, 2 hits were identified that suppressed 2:1 atrioventricular block, the LQT phenotype, in all 3 embryos: 2-MMB and the steroid flurandrenolide (Figure 1A). Nine compounds rescued 2 of 3 embryos in the well, and although they did not meet our strict definition of a chemical suppressor, the small number of compounds allowed further testing. Two of these, the steroids fluocinonide and fluorometholone (Figure 1A), were confirmed on repeat testing.

Flurandrenolide and 2-MMB display dose-related suppression of the breakdance phenotype with ED₅₀ values of ~2 and 10 µmol/L, respectively (Figure 1B). Time-series experiments revealed a higher percentage of drug effect at later time points for both flurandrenolide and 2-MMB but a slightly longer exposure requirement for flurandrenolide (Figure I in the online-only Data Supplement). To directly measure the effects of these compounds on myocardial repolarization, ventricular action potential durations (APDs) were measured with voltage-sensitive optical mapping in bkd−/− and wild-type fish treated with active compounds or diluent. Breakdance−/− embryos treated with 50 µmol/L flurandrenolide at 48 hpf for 48 hours displayed shorter ventricular APDs compared with untreated controls (338±44 versus 482±83 ms; P=0.0008; Figure 2A). Wild-type embryos treated with flurandrenolide did not have significantly shorter ventricular APDs than untreated wild-type controls (Figure 2A). Breakdance−/− embryos treated with 50 µmol/L 2-MMB at 48 hpf for 24 hours displayed shorter ventricular APDs compared with untreated bkd−/− embryos (376±66 versus 570±23 ms; P=0.00007; Figure 2B). Wild-type embryos treated with 2-MMB also had shorter ventricular APD90s (200±51 ms) compared with untreated wild type controls (290±85 ms; P=0.03; Figure 2B). Thus, both lead compounds achieve suppression of the LQT phenotype by shortening repolarization time.

Characterization of zERG I59S
Mutant Trafficking
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 and 155 kDa (Figure 3). These data demonstrate that, similar to the majority of known human mutations, the I59S zERG mutation results in a trafficking defect, which is not corrected by flurandrenolide or 2-MMB. Summary data from Western blots (n=3 to 4) indicating the amount of 155-kDa (top) cell surface isoform of zERG and zERG-I59S as a percentage of total zERG protein under various conditions are shown on a representative Western blot. Tubulin is shown as a loading control.

Western blot, with only a faint upper glycoform at 155 kDa (Figure 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 (Figure 4), whereas 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 with the wild-type zERG (13±7 versus 36±10 pA/pF; P=0.00009; Figure 5). The current-voltage relationship for the tail current was not otherwise appreciably different between wild-type and I59S mutant channels (the Table).

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 thapsigargin.\(^9\) 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 (Figure 3). These experiments demonstrate that, similar to many of the human trafficking-defective mutant KCNH2 proteins,\(^9\) the \( bkd \) trafficking defect can be partially corrected by KCNH2 inhibitors.

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 \( \mu \)mol/L 2-MMB or flurandrenolide for 24 hours did not appreciably change the percentage of the mature glycoform on Western blot (Figure 3) or affect the membrane localization measured by immunofluorescence (Figure 4). In addition, neither flurandrenolide or 2-MMB was able to rescue the defective trafficking of the human KCNH2 G601S mutant (Figure II in the online-only Data Supplement). These data suggest that 2-MMB and flurandrenolide do not rescue the \( breakdance \) phenotype by correcting defective I59S zERG trafficking, and they confirm the utility of this physiological 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. 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 DOCA did not result in any suppression of the \( breakdance \) phenotype, whereas treatment with testosterone and dexamethasone caused a dose-related rescue (Figure 6A).

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**Figure 4.** Subcellular localization of zERG and I59S zERG is shown in red and compared with a surface marker in green. The overlay demonstrates surface expression of wild type in yellow but not I59S zERG. Treatment with flurandrenolide or 2-MMB does not alter surface expression of I59S zERG. Scale bars are 10 \( \mu \)m.

**Figure 5.** zERG-I59S current density is reduced compared with wild type. Exemplar traces 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 with wild type (see the Table).
To distinguish between the androgen and glucocorticoid pathways, we used morpholino-mediated knockdown of the glucocorticoid and androgen receptors. Knockdown of the glucocorticoid receptor resulted in the anticipated reduction of dexamethasone rescue and reduced flurandrenolide-mediated rescue (Figure 6B). Conversely, knockdown of the androgen receptor reduced testosterone-mediated suppression without significantly altering flurandrenolide effects (Figure III in the online-only Data Supplement). Incomplete interruption of drug effects is likely due to incomplete receptor knockdown and/or waning effects of the morpholino at the later time points of drug exposure required for the experiment. The effect of 2-MMB was not interrupted by either androgen or glucocorticoid receptor knockdown (data not shown). Taken together, these data support a model in which 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 LQTS has been limited to β-blocker therapy, which does not shorten the QT interval but appears to reduce the triggers for a fatal arrhythmia. 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 the LQTS in which the readout was physiological suppression of the LQT phenotype independently of mutant ion channel trafficking. Two structurally unrelated compounds, flurandrenolide and 2-MMB, were discovered to rescue zebrafish LQTS. 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 LQT 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 with high-throughput screening. In cases when an established therapeutic target is known, in vitro or cell-based screening is frequently used. However, in many of the most complex and challenging diseases, including LQTS, effective drug targets are not well established. Additionally, the assay of an effect of a compound on a single ion channel in isolation may ultimately prove misleading because many compounds have activities against multiple ion channels. It is therefore not

| Voltage-Clamp Characterization of Wild-Type and I59S-zERG Expressed in COS7 Cells |
|---------------------------------|-----------------|-----------------|---|
| Wild Type | I59S | P |
|-------------------------------------------------------------------|
| Measurable zERG current, n | 12 | 10 |
| No measurable zERG current, n | 0 | 12 |
| Activation current-voltage relation (tails, fit to standard Boltzmann), 25th percentile/median/75th percentile | | |
| Maximal peak current density, pA/pF | 29.9/36.7/42.4 | 7.6/13.1/19.2 | 0.00009* |
| Half-maximal activation voltage, mV | -13.6/-7.9/-5.6 | -11.4/-7.4/-4.7 | 0.31 (7.3 mV)† |
| Slope factor, mV | 6.6/7.4/8.1 | 5.5/6.6/8.2 | 0.50 (2.7 mV)† |

*Significant at α<0.017 (Sidak corrected for 3 contrasts).
†Minimum detectable difference with power >0.8.

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 LQT by flurandrenolide and dexamethasone (n=26 to 31).
surprising that in vitro assays often lead to unforeseen results in vivo.

The alternative we used 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, ie, IKr-blocking drugs that rescue mutant KCNH2 channel trafficking but fail to shorten QT interval as a result of a 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 in which 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, diverse genetic mutations are responsible for LQTS type 2, and it will be important to determine whether these compounds will have activity in the setting of other KCNH2 mutations and 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. Although 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. The considerable side effects associated with long-term glucocorticoid administration will likely limit their therapeutic use, but there are circumstances in which 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. Although neither of the 2 compounds identified in this screen overcorrected the APD in this LQTS type 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 efforts will include studying whether these compounds shorten the ECG QT interval in higher organisms, other genotypes of LQTS type 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 LQT drug discovery could also be extended to a personalized approach, engineering patient-specific “humanized” zebrafish lines that could then be screened to discover compounds that rescue specific mutations.

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Disclosures

Dr January is a cofounder of Cellular Dynamics International, Inc, a Madison-based stem cell company. The other authors report no conflicts.

References


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**CLINICAL PERSPECTIVE**

Long QT syndrome (LQTS), caused by either genetic defects or pharmaceutical agents, carries a risk of sudden death. Despite significant medical advances in our understanding of the molecular and cellular mechanisms of these syndromes, there are still no therapies that directly address the underlying physiological problem of prolonged repolarization time. In this study, we used a zebrafish model of LQTS type 2 that harbors a mutation in the *KCNH2* gene. The diminutive physical size of zebrafish enabled a small-molecule screen for compounds that rescued the LQTS phenotype. We identified 2 compounds, 2-methoxy-N-(4-methylphenyl) benzamide and the steroid flurandrenolide, that reproducibly rescued the zebrafish LQT mutant in a dose-dependent fashion by shortening the prolonged ventricular action potentials. Neither of these compounds exerts their effects by rescuing the ion channel trafficking defect that results from the zebrafish LQTS mutation. We show that flurandrenolide acts through the glucocorticoid receptor to suppress LQTS in this model. This study highlights the power of using a nonbiased functional screen to identify compounds that may prove to be new tools for the study of cardiac electrophysiology and may lead to novel therapeutics for human LQTS patients.
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