Drugs That Induce Repolarization Abnormalities Cause Bradycardia in Zebrafish

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Background—Drug-induced QT prolongation and torsades de pointes remain significant and often unpredictable clinical problems. Current in vitro preclinical assays are limited by biological simplicity, and in vivo models suffer from expense and low throughput.

Methods and Results—During a screen for the effects of 100 small molecules on the heart rate of the zebrafish, Danio rerio, we found that drugs that cause QT prolongation in humans consistently caused bradycardia and AV block in the zebrafish. Of 23 such drugs tested, 18 were positive in this initial screen. Poor absorption explained 4 of 5 false-negative results, as demonstrated by microinjection. Overall, 22 of 23 compounds that cause repolarization abnormalities were positive in this assay. Antisense “knockdown” of the zebrafish KCNH2 ortholog yielded bradycardia in a dose dependent manner confirming the effects of reduction of repolarizing potassium current in this model. Classical drug-drug interactions between erythromycin and cisapride, as well as cimetidine and terfenadine, were also reproduced.

Conclusion—This simple high-throughput assay is a promising addition to the repertoire of preclinical tests for drug-induced repolarization abnormalities. The genetic tractability of the zebrafish will allow the exploration of heritable modifiers of such drug effects. (Circulation. 2003;107:1355-1358.)

Key Words: drugs ■ electrophysiology ■ arrhythmia ■ genes

Life-threatening arrhythmia in the setting of QT prolongation occurs as a result of inherited mutations in ion channel genes or, more commonly, as a consequence of drugs that affect cardiac repolarization.1,2 This latter mechanism is the focus of increasing regulatory attention as several pharmaceuticals have been withdrawn from the US market due to torsades de pointes (TdP). Despite their medical importance, drug-related repolarization abnormalities and related arrhythmias remain difficult to predict.3

Repolarization is complex, depending on individual channels, receptors, cytoskeletal elements, and the membrane. Additional complexity results from regional heterogeneity within the heart.4 Further, some drugs, although safe in isolation, cause repolarization abnormalities when given with other medications,1 through pharmacokinetic or pharmacodynamic interactions. Genetic variation may contribute to individual susceptibility to drug-induced arrhythmias.3 A tractable model system enabling the identification of genes responsible for such variation would represent a significant advance.

Virtually all QT-prolonging drugs identified to date inhibit the rapid component of the repolarizing potassium current (IKr). A channel composed of at least two subunits, KCNH2 and KCNE2, is responsible for this current. In vitro assays focusing on IKr are limited by biological simplicity, low throughput, and inability to detect drug-drug interactions.5,6 Animal models, although more physiological, have an even lower throughput, restricting their ability to screen systematically for drug-drug interactions.6

The zebrafish has a beating heart with both a complex repertoire of ion channels and functioning metabolism within 26 hours of fertilization.7 The transparency of the embryo facilitates rapid evaluation of heart rate (HR) and rhythm. In addition, dramatic effects on cardiac function are tolerated by the larval fish which survive for 4 to 5 days without a circulation.8 In the course of screening 100 small molecules for their effects on embryonic HR, we noted that compounds known to cause QT prolongation and TdP in humans consistently caused bradycardia in the zebrafish.

Methods

Aquaculture

TubingenAB zebrafish embryos (bred in-house) were reared in standard media. At 24 hours post fertilization (hpf), the embryos were distributed into 96 well plates, 3 to 5 embryos per well with 250 μL of media.

Determination of Small Molecule Effects on Heart Rate

At 48 hpf, compounds were added to the wells from dimethylsulfoxide stocks (final concentration <2%). Vehicle alone was used as a control. HR measurement: 15 second video recordings were obtained with a Nikon TE200 microscope and a Hamamatsu ORCA-ER camera. Analysis was performed with Metamorph Software (Universal Imaging). The average pixel density was measured...
for a region of interest over the heart and plotted against time. Fast-Fourier Transform was performed to determine HR. Because some compounds caused AV block, whereas others slowed both atrial and ventricular rates, the ventricular rate was chosen as the most sensitive index of HR effect. Each compound was tested at 1, 10, and 100 mcg/mL, and the highest, nonlethal concentration was reported. Two-tailed Student’s t-tests were performed.

Microinjections
Zebrafish larvae at 48 hpf were anesthetized with tricaine and 5 nL of 10 mg/mL stock solutions of each compound dissolved in Danieau’s solution (58 mmol/L NaCl, 7 mmol/L KCl, 0.4 mmol/L MgSO4, 6.0 mmol/L Ca(NO3)2, and 5.0 mmol/L HEPES pH 7.6) were injected into the yolk sac. The fish recovered for 4 hours before recording HR.

Approximately 5 nL of the morpholino antisense oligonucleotide (MO), diluted in Danieau’s solution, were injected into zebrafish embryos at the single cell stage. The KCN2 MO was directed against the initiator codon of the zebrafish KCN2 ortholog (5’-CCGTCGTAAGCGCCTGCGT-3’).

To explore interactions between known IKr blockers and the KCN2 MO, embryos were injected with the oligonucleotide at the single cell stage and exposed to drug at 24 hpf before HR was determined at 48 hpf.

## Results
Figure 1 shows the effects of 100 biologically active small molecules on zebrafish HR. Thirty-six compounds caused bradycardia, of which 18 were known in humans to result in QT prolongation and TdP. Five of the 23 compounds (erythromycin, N-acetyl-procainamide, pentamidine, procainamide, and sotalol) that cause QT prolongation or TdP did not cause bradycardia in the assay. We performed microinjection of four of these compounds to address the possibility that poor absorption was the limiting step. Figure 2A demonstrates the bradycardia seen on direct injection of each drug. Vehicle alone showed no significant effect on HR.

Antisense MO directed against zebrafish KCN2 demonstrated a dose-dependent effect on HR (Figure 2B). Control MO and vehicle alone had no effect. As higher doses of IKr blockade resulted in asystole, we explored drug-morpholino interactions with submaximal terfenadine doses. The effects of the MO and drug are additive (Figure 2C).

Increasing amounts of erythromycin potentiated the effects of cisapride, demonstrating evidence of drug-drug interaction (Figure 2D). A 10-fold decrease in the ED50 on HR for cisapride is seen with increasing doses of erythromycin. A second example demonstrates the interaction of cimetidine and terfenadine (Figure 2E).

## Discussion
The above results demonstrate that for a broad collection of small molecules, zebrafish bradycardia predicts clinical repo-
larization abnormalities and reproduces known drug-drug interactions.

**IKr and Bradycardia**

Bradycardia previously has been reported as a result of IKr blockade in several experimental and clinical situations. 6,10 This effect has been attributed to action potential prolongation.11 Of the 23 known IKr-blockers tested in our initial screen, 18 caused bradycardia. Five compounds (erythromycin, N-acetyl-procainamide, pentamadine, procainamide, and sotalol) failed to elicit the expected effect. We deduced that erythromycin is absorbed from its interaction with cisapride (Figure 2D). Poor absorption remained a possible explanation for the lack of effect of the four other compounds. Supporting this is the hydrophilicity of these molecules, each with a logarithm of the octanol:water partition coefficient $<1$. Microinjection revealed that these compounds cause bradycardia in the zebrafish once the absorption barrier is bypassed. When the microinjection experiments are included, 22 of 23 known IKr-blocking agents were positive in this assay.

This simple assay is useful as a screen, presenting an opportunity to test not only large numbers of molecules, but also their quantitative interactions with a throughput superior to current methods.

**Zebrafish KCNH2 “ Knock-Down” Experiments**

To demonstrate a mechanistic link between IKr blockade and bradycardia in the zebrafish, we performed “knock-down” experiments against the zebrafish ortholog of KCNH2. Antisense MO injected into the embryo elicited dose-dependent bradycardia. Combination of MO with submaximal terfenadine doses resulted in additive effects. Although not conclusive, these data are consistent with a model in which drug and MO act on the same target. Of note, submaximal doses of two IKr-blocking drugs have been shown to have additive properties.

The drugs we used are known to cause IKr blockade in a wide range of experimental models. In addition, IKr blockade causes bradycardia, and at high doses asystole, in whole animal and cellular systems. Taken together these data strongly suggest that IKr blockade is the cause of the bradycardia observed in the zebrafish. Some of the effects seen in the fish (as in other models) may reflect interactions with targets other than KCNH2. The zebrafish offers the potential to address such complexity.

**Drug-Drug Interactions**

The observation of two drug-drug interactions demonstrates another advantage of this zebrafish model. In humans, these interactions have been shown to be pharmacokinetic: the
inhibition of hepatic metabolism by one drug resulting in increased levels of the other. The CYP3A4 gene is present in the zebrafish, further work will be required to define the mechanism of these interactions in this model.

Limitations
Hydrophilicity affects drug absorption and can lead to false-negatives in this assay. However, this problem appears to be predictable from the physicochemical characteristics of these compounds, and can be overcome by injection. Like any model system, the zebrafish does not perfectly predict human clinical outcomes. For instance, erythromycin, which prolongs the QT interval and can cause TdP,

 did not affect zebrafish HR. An additional limitation is the lack of specificity of drug-induced bradycardia. Not all molecules that result in bradycardia do so through Ikur blockade; for example, propranolol and clonidine both reduce HR in this assay.

Future Directions
The observation that progesterone causes bradycardia raises the possibility that gender influences on drug-induced repolarization effects may be accessible. Possible differences in the mechanisms of action of Ikur-blockers could be addressed with this whole animal model. The development of sensitized or resistant zebrafish strains may improve the specificity of this system. Finally, human pharmacogenetic studies of cardiac repolarization have been limited to the evaluation of candidate genes, as more powerful segregation-based family studies of drug responses are not feasible. The ease of genetic manipulation in the zebrafish should allow the unbiased identification of inherited modifiers of drug responses.

Acknowledgments
Supported by the Clinical Investigator Training Program, Beth Israel Deaconess Medical Center, Harvard/MIT Health Sciences and Technology, in collaboration with Pfizer, Inc (D.J.M.), the Ned Sahin Research Fund for Restoring Developmental Plasticity (R.T.P.), and the National Institutes of Health HL68711 (C.A.M.). The authors thank Rachel Moore for expert technical assistance.

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_Circulation_. 2003;107:1355-1358; originally published online March 3, 2003;
doi: 10.1161/01.CIR.0000061912.88753.87
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circ.ahajournals.org/content/107/10/1355

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