Novel characteristics of a misprocessed mutant HERG channel linked to hereditary long QT syndrome

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Ficker, Eckhard, Dierk Thomas, Prakash C. Viswanathan, Adrienne T. Dennis, Silvia G. Priori, Carlo Napolitano, Mirella Memmi, Barbara A. Wible, Elizabeth S. Kaufman, Sudha Iyengar, Peter J. Schwartz, Yoram Rudy, and Arthur M. Brown. Novel characteristics of a misprocessed mutant HERG channel linked to hereditary long QT syndrome. Am J Physiol Heart Circ Physiol 279: H1748–H1756, 2000.—Hereditary long QT syndrome (hLQTS) is a heterogeneous genetic disease characterized by prolonged QT interval in the electrocardiogram, recurrent syncope, and sudden cardiac death. Mutations in the cardiac potassium channel HERG (KCNH2) are the second most common form of hLQTS and reduce the delayed rectifier K+ currents, thereby prolonging repolarization. We studied a novel COOH-terminal missense mutation, HERG R752W, which segregated with the disease in a family of 101 genotyped individuals. When the mutant cRNA was expressed in Xenopus oocytes it produced enhanced rather than reduced currents. Simulations using the Luo-Rudy model predicted minimal shortening rather than prolongation of the cardiac action potential. Consequently, a normal or shortened QT interval would be expected in contrast to the long QT observed clinically. This anomaly was resolved by our observation that the mutant protein was not delivered to the plasma membrane of mammalian cells but was retained intracellularly. We found that this trafficking defect was corrected at lower incubation temperatures and that functional channels were now delivered to the plasma membrane. However, trafficking could not be restored by chemical chaperones or E-4031, a specific blocker of HERG channels. Therefore, HERG R752W represents a new class of trafficking mutants in hLQTS. The occurrence of different classes of misprocessed channels suggests that a unified therapeutic approach for altering HERG trafficking will not be possible and that different treatment modalities will have to be matched to the different classes of trafficking mutants.

HEREDITARY LONG QT SYNDROME (LQTS) is a heterogeneous genetic disease that predisposes affected individuals to life-threatening arrhythmias (25, 26). The disease is caused by mutations in five known genes, of which four encode potassium channels (1, 19, 19a, 19b). The second most common form, LQT2, is linked to mutations in the cardiac potassium channel HERG on human chromosome 7 (22). Mutations in HERG have been described in both cytoplasmic termini (2, 5) as well as transmembrane domains (e.g., Refs. 10, 21, 30) and result in three classes of defects: 1) channels with abnormal gating and reduced currents (21, 30), 2) channels that reach the plasma membrane but do not conduct current (30), and 3) channels that are retained in the endoplasmic reticulum (ER) (10, 30, 31). Mutations in classes 1 and 2 reduce expression levels even more if there is dominant-negative suppression of functional channels produced by the wild-type (WT) allele (21). Some mutations in class 3 are hypomorphic, i.e., they show severely impaired trafficking where the majority of channel protein is retained in the ER and few channels leak out to the plasma membrane producing small currents at 37°C (10, 31).

Mutations defective in trafficking have been reported for other membrane proteins including minK (3), P-glycoprotein (14), aquaporins (27) and the cystic fibrosis transmembrane conductance regulator (CFTR) (11, 12). Loss of function associated with trafficking mutations in CFTR (8), P-glycoprotein (14), and aquaporins (27) may be rescued by synthesis at reduced temperature (8, 14, 31), by overexpression (6), by chemical chaperones such as glycerol and dimethylsulfoxide (4, 24, 27), or by specific drugs binding to misprocessed membrane proteins (15, 31). For the hypomorphic HERG mutation N470D, it was shown more recently...
that trafficking can be improved not only by incubation at low temperature but also in the presence of osmolytes or the specific channel ligand E-4031 (31).

In a large LQT2 family we found a novel COOH-terminal missense mutation, HERG R752W, which was retained in the ER. The mutant protein was “rescued” and delivered to the plasma membrane after incubation at reduced temperatures in mammalian cells or after expression in *Xenopus* oocytes. However, trafficking could not be restored by incubation with chemical chaperones or E-4031, a specific blocker of HERG channels. When trafficking was corrected, HERG R752W channels conducted enhanced currents that slightly shortened the cardiac action potential simulated by the Luo-Rudy model of a mammalian ventricular myocyte.

**MATERIALS AND METHODS**

**Molecular biology and electrophysiology.** HERG R752W was prepared by overlap extension PCR, verified by sequencing, and subcloned as a Xho I-BamHI fragment into either full-length HERG-pSP64 or HERG-pCDNA3. In vitro RNA synthesis and oocyte injections were performed as described (9). Production and size of full-length cRNA was verified by denaturing agarose gel electrophoresis, and cRNA concentrations were determined with Ribogreen RNA quantitation kit (Molecular Probes). In *Xenopus* oocytes, currents were recorded 2–7 days after injection using a Dagan 8500 two-electrode voltage-clamp amplifier. Current and voltage electrodes were filled with 3 M KCl and had resistances of ~1 MΩ. The composition of the bath solution used to perfuse oocytes was (in mM) 96 NaCl, 5 KCl, 1.8 CaCl₂, 1.0 MgCl₂, and 5 HEPES (K⁺ Ringer solution, pH 7.4). Cell-attached macropatch recordings in oocytes were performed as described previously (9). Patch pipettes (resistance, 0.2–0.6 MΩ) were filled with K⁺ Ringer solution, and the depolarizing bath solution had the following composition (in mM): 100 KCl, 10 EGTA, 1 EDTA, and 10 HEPES (pH 7.4). For electrophysiological experiments in HEK293 cells, transient transfections were carried out with the Ca²⁺-transferase (GST) fusion protein, corresponding to HERG residues 1102–1121 (TLTLD-SLSQVSQFMACEELP) or the entire fusion protein.

**Simulations.** The simulations in this study were conducted using the Luo-Rudy model, a theoretical dynamic model of a mammalian ventricular action potential. The model includes membrane ionic channel currents that are formulated mathematically using the Hodgkin-Huxley approach as well as ionic pumps and exchangers. It also includes processes that regulate intracellular concentration changes of Na⁺, K⁺, and Ca²⁺ (7, 16, 29). For the purpose of this study, the formulation of the fast delayed rectifier potassium current, I_Kr, was modified to fit our experimental voltage clamp data obtained from HERG-WT and HERG-R752W channels. All kinetic parameters were normalized to 37°C with a Q_10 of 3. The basic protocol for action potential simulations involved stimulation of the model cell 10 times at a constant cycle length (CL) of 1,000 ms.

**Antibodies.** The anti-HERG polyclonal antibody used in Western blots and for immunostaining was generated in rabbits against a glutathione-S-transferase (GST) fusion protein containing the last 112 amino acids of HERG (residues 1048–1159). HERG antiserum was either purified on an affinity column consisting of a short COOH-terminal peptide corresponding to HERG residues 1102–1121 (TLTLD-SLSQVSQFMACEELP) or the entire fusion protein.

**Western blot analysis and immunostaining.** For Western blotting, HEK 293 cells were transfected with Lipofectamine/Plus as recommended by manufacturer (Life Technologies). Forty-eight hours after transfection, cells were solubilized for 1 h at 4°C in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100], containing protease inhibitor mix (Complete, Roche Biochemicals). To isolate a crude membrane fraction, oocytes were homogenized in 0.3 M sucrose and 10 mM NaPO₄ (pH 7.4) supplemented with protease inhibitor mix (Complete). After removal of debris and nuclei (3,000 g, 10 min), the supernatant was spun at 50,000 g for 1 h at 4°C to pellet membranes. Protein concentrations were determined by the bicinchoninic acid method (Pierce). For Western blotting, proteins were separated on 8% SDS polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked overnight with 5% nonfat dry milk in PBS plus 0.1% Tween and immunoblotted with polyclonal rabbit anti-HERG antibody (1:200 dilution; 1 h at RT) followed by horseradish peroxidase-conjugated secondary antibody (1:3,000; 1 h at RT; Amersham Pharmacia Biotech). Western blots were developed with the ECL-Plus detection system (Amersham Pharmacia Biotech). For immunostaining, transiently transfected COS cells were grown on coverslips and fixed with 4% paraformaldehyde in PBS 48 h after transfection. After fixing, cells were permeabilized with 0.1% Triton X-100 in PBS, blocked with 5% nonfat dry milk in PBS, and stained at 4°C overnight with anti-HERG antibody (1:100). The tetramethylrhodamine isothiocyanate-conjugated secondary antibody (1:100; Jackson Labs) was added for 2 h at room temperature. For observation, coverslips were mounted on slides with Vectashield (Vector Labs).

**RESULTS**

HERG R752W was identified in a 31-year-old woman with a history of syncope who died suddenly. Twenty-six of 101 genotyped family members were found to be carriers. Of these, 17 had prolonged QT intervals (QTc > 0.44 ms) and 6 had a history of syncope.

**Characterization of HERG R752W currents in *Xenopus* oocytes.** The cellular phenotype of LQT mutations is often studied by measuring currents expressed in *Xenopus* oocytes. After injection of equivalent amounts of cRNA, we were surprised to find that mutant HERG R752W channels produced larger outward currents than WT channels (Fig. 1, A and B). Both WT and R752W produced typical bell-shaped isochronal current-voltage (I-V) relationships (Fig. 1C). For R752W, the amplitudes were larger and the I-V curve was shifted to more hyperpolarized potentials. To evaluate the shift, maximum tail currents elicited at −90 mV were used to calculate a correction factor to account for
Fig. 1. HERG R752W is located in the COOH-terminal linker between transmembrane domain S6 and the cyclic-nucleotide binding fold common to all members of the ether-a-go-go gene family with the arginine at this position being highly conserved. Macroscopic current recordings from *Xenopus* oocytes injected with 0.5 ng each of HERG wild-type (WT; A) or HERG R752W cRNA (B; RW). *Inset*: voltage protocol; holding potential, −85 mV, test pulses, from −100 to +60 with 10-mV increments; return potential −90 mV. C: isochronal current-voltage (*I*-*V*) relationship for HERG WT (0.5 ng cRNA, mean values from 5 oocytes, ●), HERG RW (0.5 ng, n = 6, ■), and 1:1 coinjection HERG WT/HERG RW (0.5 + 0.5 ng, n = 5, ▲). Currents were elicited with voltage protocol shown in *inset* to A. Amplitudes were analyzed at the end of the depolarizing 650-ms voltage commands. A single batch of oocytes was used for C. All recordings were made 2 days after injection. D: maximal tail current amplitudes elicited on return to −90 mV after a test pulse to 60 mV were back-extrapolated and used to assess the average number of functional channels expressed in oocytes injected with WT or R752W cRNA. The fractional difference in tail current expression between HERG WT and RW was used to obtain the normalized WTcorr *I*-*V* relationship in (C, ▲). E and F: steady-state activation and inactivation properties of HERG WT (●, ▲) and HERG RW channels (■, △). Steady-state activation was analyzed in cell-attached macropatches by plotting tail current amplitudes at −110 mV against 30-s depolarizing test pulses between −80 and +40 mV. Steady-state inactivation was assessed with brief hyperpolarizing steps from +20 mV (E); plotted are normalized peak currents on return to +20 mV. Dashed line indicates zero current level. Data were fitted to Boltzmann equations of the form: $y = D/(1+\exp(V_h-V/k))$. Half-maximal inactivation $V_{1/2}$ was $-63.3 \pm 1.1$ mV for WT and $-55.5 \pm 1.6$ mV for HERG R752W (n = 4). Half-maximal activation $V_{h}$ was $-49.6 \pm 0.7$ mV (n = 4) and $-37.7 \pm 0.2$ mV (n = 7) for HERG WT and HERG RW, respectively.
small differences in expression levels (Fig. 1D). Compared with the corrected WT, I-V curve threshold and peak for R752W remained shifted (Fig. 1C), suggesting that altered gating properties contributed importantly to the larger currents of R752W. Coinjection of equal amounts of WT and R752W cRNA produced a summation of current amplitudes at potentials more positive than −20 mV. At more hyperpolarized potentials, current amplitudes from the combined injection were not significantly changed from R752W alone despite the increased amount of cRNA injected. One explanation might be that below −20 mV, activation rates for R752W/WT heteromultimers are very different from homomultimeric channels (see Fig. 2C), thereby producing a complex interaction with current amplitudes as measured in Fig. 1C. At more positive potentials, however, activation rates converge, and consequently current amplitudes increase as expected for coinjection experiments. Nonetheless, our results show clearly that mutant channels do not suppress WT currents in a dominant-negative manner.

To analyze the gating changes introduced by R752W, we studied steady-state activation and inactivation (Fig. 1, E and F). Compared with WT, the activation and inactivation curves of R752W were shifted by +11.9 and +7.8 mV, respectively (Fig. 1F). The shifts in voltage dependence of activation and inactivation were accompanied by changes in activation and deactivation kinetics (Fig. 2) while inactivation kinetics and removal of inactivation were not altered (data not shown). R752W channels activated much faster than WT channels, and this acceleration contributed to increased outward currents with shorter depolarizations. To test for interactions of WT and mutant proteins, we coinjected cRNA in equal amounts. Activation time constants were intermediate between WT and R752W at −20 and 0 mV but approximated values measured for R752W alone at +20 and +40 mV (Fig. 2C). Current deactivation was fit by the sum of two exponentials. Both time constants were accelerated in mutant channels (Fig. 2, E and F). Coinjections of equal amounts of WT and R752W produced intermediate

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**Fig. 2. HERG R752W accelerates current activation (A).** Activation time constants were measured using an envelope of tails protocol to separate current activation from inactivation. A test pulse of variable duration (in this case to 0 mV) was given to open and/or inactivate channels before stepping back to −100 mV. B: peak tail current amplitudes at −100 mV were back extrapolated for time 0 and plotted against test pulse duration. The resulting activation time course could be fitted to a single exponential function. C: activation time constants plotted for different voltages (n = 8–10). Note that activation time constants are significantly different between HERG WT (●) and HERG RW (●) even at +20 mV [WT, 105.3 ± 8.1 ms (n = 8); RW, 67.9 ± 3.7 ms (n = 10)] and at +40 mV [WT, 71 ± 4.8 ms (n = 8); RW, 45.8 ± 2.9 ms (n = 10)]. HERG R752W speeds channel deactivation (D). Currents were activated during a 750-ms depolarizing test pulse to 20 mV before stepping back to potentials ranging from −40 to −120 mV to measure current deactivation. Deactivation kinetics were fitted to double exponential functions. Fast (E, n = 16–19) and slow deactivation time constants (F, n = 5 or 6) are shown as a function of membrane potential. ●, Time constants measured on coinjection of equal amounts of WT and R752W cRNA.
values for slow time constants. For the faster process, coinjections approached values measured for R752W alone at potentials between −90 and −120 mV. The faster deactivation rates measured in mutant channels might reduce outward currents during the terminal repolarization phase of the cardiac action potential and cause prolongation of the QT interval. On the other hand, the faster activation rates of R752W predicted more current during the plateau and a tendency to shorten the cardiac action potential.

**Simulations of the effects of WT HERG R752W current on the cardiac action potential.** Because the kinetic differences were ambiguous with respect to changes of the QT interval, the effects were simulated with the Luo-Rudy model of the cardiac ventricular action potential (7, 16, 29). The mathematical formulation of the rapid delayed rectifier, $I_{Kr}$, reproduced the kinetic data of Figs. 1 and 2 for WT and mutant channels. Figure 3 shows action potentials and $I_{Kr}$ currents computed from WT and mutant cells. Due to faster activation kinetics, mutant $I_{Kr}$ was larger than WT $I_{Kr}$ and produced a minor shortening of action potential duration.

Reconciling mutant HERG currents with QT prolongation. The discordance between the cellular phenotype in Xenopus oocytes and the prolonged QT interval observed clinically recalled the temperature sensitivity of the CFTR ΔF508 mutant (8). Therefore, we expressed HERG R752W in mammalian HEK293 cells at a physiological temperature of 37°C and used whole cell patch clamp recordings to characterize mutant currents. We were not able to detect any HERG-like outward current in HEK293 cells transfected with R752W, although WT current was obtained routinely under identical experimental conditions (Fig. 4, A and B). Because mutant currents were elicited in Xenopus oocytes and because oocytes are maintained at lower temperatures than mammalian cells, we tested the effect of reduced incubation temperature on expression of R752W. After incubation for 2 days at 26°C, large whole cell HERG currents were recorded in cells transfected with R752W cDNA and had an electrophysiological profile similar to that described in Xenopus oocytes (Fig. 4, C and D).

As in oocytes, the $I-V$ relationship was shifted to more hyperpolarized potentials and current activation and deactivation were accelerated (Fig. 4, E and F). These results suggested that R752W was not processed correctly at 37°C and processing resumed when the incubation temperature of mammalian cells was lowered to 26°C.

To test for interactions of WT and mutant proteins at 37°C, we coexpressed both cDNAs in equal concentrations (Fig. 4G). Compared with transfections of WT cDNA alone, current densities and kinetics were not found to be different, suggesting that WT and mutant proteins are not likely to interact at physiological temperatures.

We confirmed our electrophysiological observations with biochemical and immunocytochemical methods. On Western blots from lysates of transiently transfected COS-7 cells, WT HERG was present in two forms. A 135-kDa band represents a core-glycosylated immature form of the channel protein localized to the ER. A more diffusely stained band of ∼160 kDa represents the mature glycosylated channel protein ultimately delivered to the plasma membrane (30). In contrast, HERG R752W channel protein synthesized at 37°C was present only in the core-glycosylated 135-kDa form and a somewhat smaller form representing either an unglycosylated form (18) or more likely a degradation product (Fig. 5C). Accordingly, immunostaining of R752W-transfected cells with anti-HERG antibodies produced strong perinuclear staining consistent with the accumulation of protein in the ER. For WT cells, additional immunostaining of the trans-Golgi network was observed, indicating progression of channel protein to the plasma membrane (Fig. 5, A and B).

When cells transfected with R752W were incubated at 26°C, we detected the 160-kDa band corresponding to the fully mature protein as well as the immature bands. The same bands were observed in Western blots of crude membrane fractions from Xenopus oocytes injected with either WT or R752W cRNA. Taken to-

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Fig. 3. Action potentials (A) and corresponding delayed rectifier current $I_{Kr}$ (B) simulated using the Luo-Rudy model. Thick lines correspond to WT cells; thin lines correspond to mutant cells. A: the last AP from a train of 10 action potentials. Because of the faster activation kinetics of HERG RW, $I_{Kr}$ reaches a higher magnitude, thereby causing minor shortening of the mutant cell action potential duration. However, quantitatively the action potential duration change is negligible and should not affect electrophysiological function.
gether with our current recordings, we conclude that at reduced temperatures, mutant protein is able to exit the ER, traverse the Golgi complex, and insert into the plasma membrane.

Because defects in processing of the temperature-sensitive ΔF508 CFTR protein were partially corrected by chemical chaperones such as glycerol (4, 24) or transcriptional regulators such as 4-phenylbutyrate (4-PB) (20), we tested whether these agents might correct the malfunction of R752W processing. We evaluated the effects of 7.5, 10, and 12.5% glycerol added to the incubation medium using Western blots. In none of these experiments did we observe any change in the glycosylation pattern of R752W incubated at 37°C, whereas protein synthesis was progressively decreased with higher glycerol concentrations (Fig. 5C). Patch clamp recordings also failed to detect R752W currents in cells incubated for 2 days at 37°C at the different glycerol concentrations. However, cell viability was not dramatically impaired, because HERG WT currents could be recorded from cells incubated with 10% glycerol (data not shown). Similarly, incubation with DMSO (140 and 280 mM) and trimethylamine N-oxide (100 and 200 mM) did not improve channel trafficking (data not shown). Furthermore, treatment of R752W transfected cells with 2.5 mM 4-PBA at 37°C had no effect on processing and no currents were detectable although protein synthesis was substantially increased.
More recently it was reported that the anti-arrhythmic drug E-4031 improves trafficking of the hypomorphic mutation HERG N470D (31). In contrast, HERG R752W channel protein synthesized at 37°C was present only in the core-glycosylated form when transfected cells were treated for 24 h in the presence of 5 μM E-4031 (Fig. 5C).

**DISCUSSION**

The electrophysiological phenotype of the processed R752W mutant is characterized by mild changes in gating properties that do not translate into prolongation of the cardiac action potential and hence the QT interval as judged by our simulations. The accelerated activation and deactivation kinetics of R752W resembles mutations described previously in the NH2-terminal PAS (Per, Arnt, and Sim) domain of the channel protein, especially HERG R56Q (5). In prokaryotes, PAS domains serve as sensors for light and redox potential (32) and in HERG channels it is thought that the PAS domain is attached to the intracellular mouth of the channel protein, thereby regulating channel gating (17, 28). Accordingly, structure-function studies suggested that mutations in the PAS domain disrupted interactions with amino acid residues in the cytoplasmic S4-S5 linker (23). We speculate that R752 may interact in a similar manner with the activation machinery of HERG or, alternatively, may stabilize the interaction of the PAS domain with the S4-S5 linker.

Our electrophysiological studies show that mutant channels function with slightly altered kinetics. The Luo-Rudy simulations suggest that these channels generate a normal action potential once they reach the plasma membrane, indicating that the clinically observed prolonged QT interval does not reflect altered channel kinetics. At physiological temperatures, however, mutant channels cannot pass quality control processes of the ER and do not reach the plasma membrane. At reduced temperatures where protein folding might be altered, HERG R752W attained a conformation that ultimately permitted exit from the ER. The molecular mechanism responsible for this conformational stabilization is not understood and remains to be analyzed in the future.

The coexpression experiments suggest that at 37°C, mutant channels are arrested in conformations that do not allow for the assembly of HERG tetramers, because neither dominant-negative current suppression nor a significant increase in current amplitudes has been observed. This interpretation is in line with previous reports that the COOH terminus is critical for HERG assembly and expression (13).

We explored whether chemical chaperones known to stabilize temperature-sensitive mutants were effective in correcting the trafficking defect associated with HERG R752W. Glycerol has been described as the most effective chemical chaperone to correct misfolding of CFTR, P-glycoprotein, or aquaporin mutant channels (14, 24, 27), and at molar concentrations we found no increase in the delivery of mutant protein to the cell membrane. In contrast, glycerol seemed to decrease the overall synthesis of mutant protein. An explanation might be that the efficacy of chemical chaperones not only depends on the particular cell line used for heterologous expression but also on the protein domain affected by a mutation and on the chemical nature of the amino acid side chain introduced (24). In CFTR it was shown that glycerol promoted the maturation of mutants K464R and K464A but not of K464W, a mutation with an amino acid substitution similar to the one analyzed in HERG R752W.
The failure of glycerol treatment to facilitate trafficking of HERG R752W was surprising because trafficking of HERG N470D, a hypomorphic mutation in the transmembrane domain of HERG, was improved by incubation in 10% glycerol (31). Similarly, the anti-arrhythmic drug E-4031 facilitated trafficking of HERG N470D, but was ineffective on the R752W mutant. These differences suggest that HERG R752W represents a new and different class of misprocessed mutants with distinct mechanisms responsible for retention of mutations localized to different functional domains of the HERG channel protein. This observation is important because it makes a unifying approach for altering HERG trafficking unlikely. On the contrary, functionally different classes of mutations will have to be targeted separately in any attempt to discover novel and useful chaperones for misprocessed channel proteins.

Because chemical chaperones were not effective in HERG R752W, we complemented our experiments by treating HEK cells expressing HERG R752W with 4-PB, an analog of the transcriptional regulator bupredesulfoxindole. In analogy to experiments in CFTR, we tried to boost expression of HERG R752W to force the mutant by mass action past the ER to the plasma membrane (20). Although synthesis of HERG R752W was substantially enhanced, we were not able to detect fully glycosylated mature protein. This might indicate that at 37°C, only a small portion, if any, of the mutant protein was exportable and that the quality control system was not saturated.

In summary, HERG R752W is the first member of a novel class of temperature-sensitive trafficking mutants that do not express any current at 37°C and are resistant to rescue by osmolytes or specific channel ligands. The clinically observed prolonged QT interval is explained best by a reduction of the delayed rectifier current \( I_{Kr} \) caused by the inability of the mutant protein to reach the plasma membrane at body temperature. Thus, although HERG R752W behaves as a loss-of-function mutation, there is one major difference from hLQT mutations reported heretofore: HERG R752W encodes a fully functional channel protein if it can be made to reach the plasma membrane. For this reason, further efforts should aim at discovering novel and useful chemical chaperones.

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