Structure-Guided Topographic Mapping and Mutagenesis to Elucidate Binding Sites for the Human Ether-a-Go-Go-Related Gene 1 Potassium Channel (KCNH2) Activator NS1643

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ABSTRACT

Loss-of-function mutations in human ether-a-go-go-related gene 1 (hERG1) is associated with life-threatening arrhythmias. hERG1 activators are being developed as treatments for acquired or genetic forms of long QT syndrome. The locations of the putative binding pockets for activators are still being elucidated. In silico docking of the activator 1,3-bis-(2-hydroxy-5-trifluoromethylphenyl)-urea (NS1643) to an S1-S6 transmembrane homology model of hERG1 predicted putative binding sites. The predictions of the in silico docking guided subsequent in vitro mutagenesis and electrophysiological measurements. The novel interacting site for NS1643 is predicted around Asn629 at the outer mouth of the channel. The applied N629H mutation is the sole amino acid replacement in the literature that abrogates the NS1643-induced left shift of the V_{1/2} of activation. In contrast, both N629T and N629D showed pharmacologic responses similar to wild type. Another important interacting pocket is predicted at the intracellular surface in the S4–S5 linker. Mutagenesis of the residues critical to interactions in this pocket had major effects on the pharmacologic response to NS1643. The inward conductance elicited by hyperpolarization of D540K hERG1 was abrogated by NS1643 treatment, suggesting that it alters the inward movement of the S4 segment. The neighboring E544L mutation markedly exaggerated tail-current responses to NS1643. However, an L564A substitution inhibited drug response. Structure-guided mutagenesis identified widespread clusters of amino acids modulating drug-induced shifts in inactivation; such modulation may reflect allosteric changes in tertiary structure. Model-guided mutagenesis led to the discovery of a range of novel interacting residues that modify NS1643-induced pharmacologic responses.

Introduction

Mutations in the human ether-a-go-go-related gene 1 (hERG1) are associated with long QT2 syndrome (Curran et al., 1995; Sanguinetti et al., 1995, 1996; Modell and Lehmann, 2006). The best-characterized drug-binding site within hERG1 is a promiscuous intracavitary pocket that produces a pharmacologic block (Lees-Miller et al., 2000; Mitcheson et al., 2000a; Mitcheson et al., 2005). An emerging strategy to reverse prolonged repolarization in long QT syndrome is the development of compounds that open (activate) the channel.

The locations of the putative binding pockets for hERG1 activators are still being elucidated. Different pharmacologic mechanisms of action have been reported for different hERG1 activators (Perry et al., 2010). Some activators shift the voltage-dependence activation to hyperpolarized potentials. These molecules have been termed type 1 activators (Perry et al., 2010). Type 2 activators affect deactivation or attenuate inactivation (Perry et al., 2010). 1,3-Bis-(2-hydroxy-5-trifluoromethylphenyl)-urea (NS1643) was considered to be a type 2 activator (Perry et al., 2010). The interacting sites for the type 1 activator contains supplemental material.

ABBREVIATIONS: hERG1, human ether-a-go-go-related gene 1; WT, wild type; EC, extracellular; IC, intracellular; VS, voltage sensor; TM, transmembrane; IFD, induced fit docking; SF, selectivity filter; NS1643, 1,3-bis-(2-hydroxy-5-trifluoromethylphenyl)-urea; RPR260243, (3R,4R)-4-[3-(6-methoxyquinolin-4-yl)-3-oxo-propyl]-1-[3-(2,3,5-trifluorophenyl)-prop-2-ynyl]-piperidine-3-carboxylic acid; PD-118057, 2-(4-[2-(3,4-dichlorophenyl)-ethyl]-phenylamino)-benzamide; E4031, 1-[2-(6-methyl-2-pyridyl)ethyl]-4-(4-methylsulfonylaminobenzoyl)piperidine.
ethyl methanethiosulfonate bromide and H2O2 treatments, included T613C and S631C during 2-(trimethylammonium) increase hERG1 currents (Xu et al., 2008). These mutations altering K+ opener PD118057 blocks hERG1 at a concentration of 30 μM (Casis et al., 2006).

It is established that mutations disrupting inactivation or altering K+ selectivity suppress the ability of the NS1643 to increase hERG1 currents (Xu et al., 2008). These mutations included T613C and S631C during [2-(trimethylammonium) ethyl] methanethiosulfonate bromide and H2O2 treatments, respectively. Casis et al. (2006) also evaluated the impact of S631A on NS1643-induced currents. They reported cell-to-cell heterogeneity in the context of disrupted inactivation. Cells with a loss of inward rectification had a diminished response to NS1643. Grunnet et al. (2011) examined the impact of a set of amino acid mutations in hERG1 with NS1643 treatment. Their study proposed a binding site in the vicinity of the extracellular (EC) end of the S5S6 segments of two adjacent hERG1 subunits. In addition, I567A, F619A, C643A and Y652A mutations enhanced the response to NS1643 treatment. To supplement electrophysiological recordings, they also performed molecular modeling studies. NS1643 was docked to a hERG1 homology model, and Leu650, Leu622, Leu646, Leu650, and Met651 residues were predicted to be in close proximity to the drug. However, experimentally identified residues did not form a single binding pocket in the proposed homology model. A potential bottleneck for many of the functional studies is an apparent absence of structural information of the S1–S6 transmembrane (TM) segments of hERG1. This lack of structural information renders mapping of the binding pocket very challenging.

One of the challenges of modeling the pore domain of hERG1 is the presence of an elongated S5 pore linker (turret) formed by approximately 40 amino acids. To circumvent this limitation, we reported a structural model of the S1–S6 domains of hERG1 based on the KvAP, Kv1.2, and Kv1.2-Kv2.1 chimera channels (Jiang et al., 2003; Long et al., 2005, 2007). Missing elements were modeled with the Rosetta de novo protein-modeling suite (http://www.rosettaconsomns.org/) and further refined with molecular dynamics simulations (Subbotina et al., 2010). We partially validated this model against available experimental information (Lees-Miller et al., 2009).

The present study aims to gain a deeper understanding of the structural and energetic factors governing favorable binding of an activator to hERG1. Using a developed model of hERG1, we are able to provide insights into the topology of putative interacting sites for the NS1643. We also discuss structural correlations with published experimental studies (Grunnet et al., 2011) and offer novel insights that may explain the pharmacological action of NS1643. Docking site predictions for NS1643 were prompted by site-directed mutagenesis and patch-clamp experiments to assess the effects of the drug on the mutated channel currents. The theoretical and experimental approaches used in this study may also help to unify the electrophysiological and biochemical data and connect them to the structural model for the hERG1. The existence of multiple binding sites for an activator, first predicted with modeling and then confirmed experimentally, suggested a complex mechanism of channel regulation by channel openers.

Materials and Methods

Structural Model of hERG1

Our model and validation of the S1–S6 domains of the hERG1 channel (RCN2H) have been reported previously (Subbotina et al., 2010). This model was used as the target for the investigation of potential binding site predictions for the hERG1 activator NS1643 by molecular docking simulations. In this model, K+ ions were present in the S2 and S4 sites of the selectivity filter. Because the target protein used in this study is a model (not the crystal structure), the partial and/or full flexibility of the target is used throughout the docking simulations.

Molecular Docking

The geometry of NS1643 was initially optimized by using Gaussian 03 (http://www.gaussian.com/) with density functional theory and the B3LYP/6-31G* basis set (Becke, 1993; Stephens et al., 1994). This combination of theory and basis set is known to reproduce the geometry of small organic molecules accurately. The derived coordinates of the drug were docked to the channel by using the following docking algorithms: Glide/induced fit docking (IFD), FlexX (Böhm, 1994), and generalized optimized ligand docking (GOLD) in a two-step procedure (Jones et al., 1997). In all of the studies, the entire receptor merged with the lipid bilayer was initially used as a target (i.e., blind docking where the whole receptor was used as an active site). Then, top docking poses were clustered with a 8- to 10-Å tolerance. The details of the docking algorithms are provided below.

Glide/IFD. The Glide-XP (extra precision) (version 5.0) (Friesner et al., 2006) and the IFD were used for the docking calculations. IFD uses the Glide docking program to account for the ligand flexibility in the refinement module. The Prime algorithm implemented in Glide was used to account for the flexibility of the receptor (Sherman et al., 2006). The Schrodinger’s IFD protocol model uses the following steps [the description that follows is from the IFD users’ manual (http://www.schrodinger.com/AcrobatFile.php?type=supportdocs&type2=&ident=6800): (1) constrained minimization of the receptor with a root mean square deviation cutoff of 0.18 Å; 2) initial Glide docking of each ligand using soft potentials (0.8 van der Waals radii scaling of nonpolar atoms of ligands and receptor using a partial charge cutoff of 0.15); 3) refinement of derived docking poses (i.e., minimization of docking poses within 20 Å of the ligand poses) by the Prime module of the Schrodinger suite (http://www.schrodinger.com/); and 4) Glide redocking of protein-ligand complexes. In the docking simulations, the inner and outer box sizes were selected as 21 and 36 Å, respectively. Glide uses two boxes to organize the calculations. The grids themselves are calculated within the space defined by the enclosing box or the outer box. This is the box within which all of the ligand atom positions at the binding site must be contained. The acceptable positions for the ligand center during the site point search lie within the bounding box or inner box.

GOLD. The GOLD program (version 5.0.1) (Jones et al., 1997) was used with two default docking scores (the GOLD Fitness and ChemScore). Partial flexibility of the receptor was gained with 10 selected amino acid residues at the active site. The side chains of these amino acid residues were selected as flexible rotamers. The default genetic algorithm parameters (100 for the population size, 5 for the number...
of islands, 100,000 for the number of genetic operations, and 2 for the niche size) were used. However, the maximum number of runs was set to 100 for each docking simulation.

**FlexX.** The FlexX program (version 4.0) from BioSolveIT (http://www.biosolveit.de/) was also used. The default algorithm parameters were used for the docking and construction of the active sites of the receptor. The solutions per fragment and solution per iterations both were set to 2000.

**In Silico Mutation Studies**

The previously developed hERG1 S1–S6 TM model was used in the mutation studies. After mutation of specific amino acid residues by the Maestro module (version 9; http://www.schrodinger.com/products/14/12) of the Schrodinger molecular modeling package, the mutated receptor was subsequently refined to remove any bad contacts by using a protein preparation algorithm (with 0.3 Å root mean square deviation cutoff) in the Schrodinger suite.

**Experimental Protocol**

**Heterologous Expression.** Site-directed mutagenesis was carried out by overlapping extension using the polymerase chain reaction as described previously (Ho et al., 1989). hERG1 was cloned into the pIREs-hr green fluorescent protein-1α vector (Agilent Technologies, Santa Clara, CA) for coexpression with humanized Renilla reniformis GPF. Human embryonic kidney (HEK) 293 cells were transfected by using calcium phosphate and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% horse serum (Invitrogen, Carlsbad, CA). Transfection was monitored by green fluorescence. HEK cells were chosen because their background potassium currents are small. More importantly, no dofetilide-sensitive tail current has been observed by using the voltage-clamp protocol in untransfected HEK cells.

**Electrophysiology.** Transfected HEK cells on glass coverslips were placed in a chamber mounted on a modified stage of an inverted microscope. The chamber was superfused at a rate of 2 ml/min with a normal external solution. Micropipettes were pulled from borosilicate glass capillary tubes on a programmable horizontal puller (Sutter Instrument Company, Novato, CA). Standard patch-clamp methods were used to measure the whole-cell currents of hERG1 mutants expressed in HEK 293 cells by using the Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) (Lees-Miller et al., 2009). The pipette solution contained the following: 10 mM KCl, 110 mM K-aspartate, 5 mM MgCl2, 5 mM Na2ATP, 10 mM ethylene glycol-bis[β-aminoethoxy]ether)-N,N',N″,N‴tetraacetic acid, 5 mM HEPES, and 1 mM CaCl2. The solution was adjusted to pH 7.2 with KOH. The EC solution contained the following: 140 mM NaCl, 5.4 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, and 5.5 mM glucose. The solution was adjusted to pH 7.4 with NaOH. For I∞ measurements, the whole-cell configuration of the patch-clamp method was used. The series resistance was less than 7 MΩ. The data were sampled at 1 kHz. The holding potential was −80 mV. The activation of hERG1 tail currents was induced by depolarization to a range of potentials from +50 to −100 mV in 10-mV steps for 1 s. This was followed by repolarization to −100 mV for 1 s to record the tail currents. If the tail current did not completely deactivate by −100 mV, a −120-mV, 1-s prepulse was applied to completely close the hERG1 channels before subsequent depolarization. All of the tail currents reported in this article represent dofetilide-sensitive currents.

The inactivation of hERG1 was measured by a previously reported triple-pulse protocol (Zou et al., 1998). The hERG1 channel was first depolarized to +50 mV for 1 s. Recovery from inactivation was achieved by using a short pulse (a 10-ms hyperpolarization) to −120 mV, followed by the application of test pulses from −100 to 0 mV for 1 s in 10-mV steps (Supplemental Fig. 1). A voltage of −100 mV was selected because it was on the flat part of the inactivation-voltage relationship. The inactivation ratio was measured as the ratio of the tail current level at 50 ms after onset of the test pulses to the theoretical peak tail amplitude. This was calculated by back-extrapolating the linear portion of the peak tail current. The inactivation ratio was plotted against the voltage of the test pulses. To resolve the time course of inactivation from deactivation, we chose to examine inactivation at voltages where the time constants of deactivation and inactivation were the most different. At the beginning of the third pulse, the hERG1 channels were mainly recovered from inactivation and in an open state. Thereafter, hERG1 currents simultaneously begin to deactivate and inactivate and depend on the membrane potential.

At potentials negative to E0, the current deactivates very rapidly. Even at voltages of −70 to −60 mV the kinetics of the current decay is determined by both deactivation and inactivation. To minimize the contribution of contamination by deactivation, we assessed the V∞ of a voltage where inactivation dominates. In our experiments, the relationship between the inactivation and the voltage varied substantially for the various mutations. Some mutations manifested positive shifts in the voltage dependence of inactivation, whereas others showed substantial negative shifts. Indeed, in some mutations, the maximum value of the ratio (I50/I∞) was below 0.5. To extract a parameter to represent the voltage dependence of inactivation (with little contamination of deactivation) in a large number of mutant channels we used the V0.3 measurement. Another parallel measurement of the voltage dependence of inactivation was used to validate the V0.3 metric of inactivation. The time constant of the inactivation process (tau of inactivation) was plotted against the voltage. Both measurements of the voltage dependence of inactivation accurately parallel each other.

There is a possibility that a positive shift in the voltage dependence of inactivation caused by a mutation would alter the NS1643-induced increase of the tail currents. To exclude this possibility, we plotted the relationship between the drug-induced change in amplitude of the tail current and the baseline drug-free inactivation V0.3 for all of the mutations examined (Supplemental Fig. 1). Using these conditions, no relationship was found.

Deactivation of the hERG1 tail current was measured at −100 mV. To simplify the evaluation of the effect of NS1643, the tail current at −100 mV was fitted to the single exponential function: $I = I_{\text{max}} \exp(-t/\tau) + C$.

In this equation, $\tau$ is the time constant. A single exponential function fitted the tail current well. The $\tau$ value for the fit ranged from 0.98 to 0.99. The tail currents recorded at −50 mV are best fit to a biexponential model. Accordingly, the tail currents in this study were recorded at −100 mV, where deactivation fits well to a single exponential. NS1643 (Tocris Bioscience, Ellisville, MO) was first dissolved in dimethyl sulfoxide (30 mM) and then diluted into the EC solution. All of the experiments were performed at room temperature. The data were sampled at 2 kHz and analyzed with Clampfit (Molecular Devices). A Student’s t test was used in the statistical analysis.

**Statistical Analysis**

Statview (Abacus Concepts, Berkeley, CA) was used to analyze the data. The data are presented as the mean ± S.E. An unpaired Student’s t test was used to compare the data. A two-tailed p value of 0.05 was designated as being significant.

**Results**

Here, we first summarize the predictions from theoretical studies. We then test modeling with biochemical experiments and electrophysiological recordings. The chosen strategy was to focus on the key amino acids that contributed the most to the ligand-receptor stabilization. Accordingly, we evaluated the per-residue decomposition of the binding energy and used the most promising candidate amino acids for experimental evaluation. As in all in silico
of the NS1643 ligand to the hERG1 channel. Previous theoretical studies (Mobley and Dill, 2009; Durdagi et al., 2010) on the membrane protein-drug interactions with free energy and molecular dynamics simulations highlighted an importance of correct accounting for the conformational dynamics of the substrate in the bulk phase and the receptor site. Despite the limitations of the molecular docking scores, it may provide insights into potential binding pockets. Moreover, one of the purposes of this study was to assess whether structure-guided topographic mapping could prospectively predict interacting sites that had not yet been identified by blind scanning mutagenesis. Figure 1 shows an overview of the top 100 predicted docking poses of NS1643 in the S1-S6 TM hERG1 homology model. Several distinct binding regions were predicted in simulations with three different docking software packages using different docking protocols. The first predicted binding pocket is localized at the outer mouth of the selectivity filter. The other two binding pockets are predicted to be in the S3S4 linker (EC site) and S4S5 linker [intracellular (IC) site]. In addition, we predicted binding of NS1643 to the IC cavity pocket. This binding site has been extensively reported to be involved in the blockade of hERG1 by a wide variety of small-molecule blockers. Some of the channel openers were also reported to have a modest blocking ability (Casias et al., 2006). When we performed targeted docking centered in a 20-Å radial cavity from the origin of the central cavity of the channel most of the predicted docking poses (99%) were distributed in the central cavity. The rest of the docking poses were predicted to be at the S4S5 IC site (Supplemental Figs. 2 and 3). The IC cavity binding pocket for NS1643 is predicted to form by Thr623, Ser624, Ser649, Thr650, Tyr652, and Phe656. The hydroxyl groups of Ser624 and Tyr652 are predicted to be involved in stable H-bonding with the drug. The aromatic moiety Phe656 is pivotal for drug stabilization in the cavity. Mutations in the position 656 (F/X) often inhibit the blocking ability of some of the activators such as RPR260243 and 3-nitro-N-(4-phenoxyphenyl)benzamide (ICA105574) (Casias et al., 2006). Because this article focuses on determinants of small-molecule activation (rather than blockade) of hERG1, and this binding pocket is very similar to that published previously for class III antiarrhythmic drugs, we do not describe this site in detail.

In Vitro Electrophysiologic Results: Dose-Response Studies. Figure 2 shows the pharmacologic responses to progressive increases in concentrations of NS1643 (0.1–100 μM). Figure 2A shows the increase in the magnitude of tail currents as a ratio of the control. Figure 2B and C shows the shift in the voltage dependence of activation and inactivation, respectively. Figure 2D shows the slowing of the deactivation tau. The concentration-response relationships for tail current amplitude and deactivation are clearly biphasic. The shift in voltage dependence in $V_{1/2}$ of activation has a trend to increase as a function of concentrations more than 1 μM. There is no significant shift in voltage dependence of activation at a concentration below 1 μM (Fig. 2B).

At the same time there is a statistically significant increase in the $V_{1/2}$ of inactivation at 0.3 μM (Fig. 2C). It seems that inactivation is altered by NS1643 at concentrations lower than that required to shift voltage dependence of activation. The biphasic nature of the concentration-response relationships

Fig. 1. An overview of the predicted docking poses of NS1643 in the S1–S6 TM hERG1 homology model. Shown are 100 predicted docking poses by blind docking (i.e., without defining the active site of the target) of the NS1643 ligand to the hERG1 channel.
NS1643 Interactions with hERG1: Structure-Guided Mutagenesis

In vitro studies revealed that a drug concentration of 10 μM was used. This concentration produces statistically significant results in all of the electrophysiological metrics. However, it also avoids high concentrations of dimethyl sulfoxide, which makes the membranes fragile during the voltage-clamp experiments (concentrations more than 30 μM).

In silico Protein-Ligand Contacts for SF-1 and SF-2 Sites: The Outer Mouth of the Selectivity Filter. The docking studies predict a potential binding site located at the outer mouth of the selectivity filter. The top-ranked docking poses are shown in Fig. 3. Clustering of the binding poses in this pocket led to the prediction of two possible binding modes for an activator. These are located around the top of the selectivity filter (SF-1) and between the outer mouth of the selectivity filter and the S3S4 (EC site) of the VS domain (SF-2) (see Fig. 3 top). Figure 3 bottom shows the binding poses as viewed from the top at higher magnifications. A list of predicted amino acids essential for drug binding in the SF-1 region includes Tyr597, Ser600, and Asn629 (Fig. 3). It is noteworthy that mutations of many of these sites have been reported previously to alter the baseline inactivation properties of hERG1. The predicted amino acids forming the SF-2 domain are Asn573, Lys595, Asn598, Lys610, Thr613, and Ser631 (Fig. 3). These amino acids were also suggested previously to be important for the pharmacological action of activators (Casis et al., 2006).

In Vitro Electrophysiologic Results for SF-1: The Outer Mouth of the Selectivity Filter. To test whether NS1643 targets amino acids within the putative binding site predicted by docking studies, patch-clamp studies compared the electrophysiology parameters of WT and mutant channels (Table 1). Site-directed mutagenesis was used to create these mutations for subsequent expression in HEK cells. Figure 4 shows the families of currents elicited by the pulse protocol (shown as an inset in C). Figure 4, A to C, right, shows the voltage dependence of activation before and after the application of NS1643. Figure 4D shows the NS1643-induced shift in the V_{1/2} of activation of the WT and mutant channels. In the WT hERG1, NS1643 produced a consistent left shift in the voltage dependence of tail current activation and slowing of deactivation (Fig. 4A). In contrast, the N629H mutant completely abrogated the NS1643-induced left shift in the voltage dependence of activation (Fig. 4C) and suppressed the NS1643-induced slowing of deactivation. Other substitutions at the Asn629 residue (N629T and N629D) were made at this site. The response of the N629T channel to NS1643 is shown in Fig. 4B. In contrast to N629H, in both N629T and N629D cells, NS1643 produced a left shift in the V_{1/2} of activation and slowed deactivation, similar to that observed in WT (Fig. 4). These data suggest that the bulkiness of the histidine mutation, or its partial charge at physiologic pH, alters the interaction of NS1643 with a domain involved in the drug-induced leftward shift in activation. This interaction may be allosteric in nature. At baseline, inactivation of N629H was disrupted over physiologic voltages (Fig. 4). Other residues in this neighborhood, which also alter/disrupt inactivation [such as S631A (Table 1) and N629C/S631C (data not shown)], did not suppress the ability of NS1643 to shift the activation to hyperpolarized potentials. To further assess whether histidine substitution at other sites in the SF-1 region would abrogate NS1643-induced shifts in the voltage dependence of activation, Y597H, S600H, and T613H mutations (data not shown) were created. None of these mutations abrogated the NS1643-induced shifts in the voltage dependence of activation.

Other mutations in SF-1 or SF-2, which were predicted to alter NS1643 interaction with hERG1, suppressed the ability of NS1643 to shift the voltage dependence of inactivation. For example, mutations of three amino acids (Y597V, T613C, and S631A) significantly decreased the drug-induced right shift in the V_{0.3} of inactivation (Table 1). The baseline drug-free
V_{0.3} of inactivation values for these mutants were $-58 \pm 3$, $-54 \pm 6$, and $+29 \pm 4$ mV, respectively (Table 1). The inability of NS1643 to induce a right shift in the $V_{0.3}$ of inactivation for these mutations was paralleled by the inability to shift the voltage dependence of the tau of inactivation (Supplemental Fig. 4). Other predicted mutations did not change the voltage dependence of activation or inactivation but instead altered the ability of NS1643 to increase the tail current amplitude (Table 1).

### In Silico Protein-Ligand Contacts for the EC-1 and EC-2 Sites: The S3S4 Linker

The blinded docking studies predicted a plausible binding pocket at the S3S4 linker. Two binding modes were predicted by clustering of the docking poses: 1) at the interface between the outer mouth of the selectivity filter and the EC site of the voltage sensor (VS) domain (labeled EC-1 in Fig. 5; this region partially overlaps with SF-2); 2) the S3S4 EC site of the VS domain (labeled EC-2 in Fig. 5).

Figure 5, top left, shows the predicted binding poses of NS1643 as viewed from the EC side. Fig. 5, top right, shows predicted 2D ligand interaction diagrams. Docking calculations predicted hydrogen bonds between amino acids Asn573 and Lys595 in the EC-1 region with NS1643. The top-docking pose predicts van der Waals contacts between the ligand and Thr436, Asn573, Met574, Asn598, and Thr613 amino acids.

### In Vitro Electrophysiological Results for Binding Site 2: The S3S4 Linker

Two mutations (M574A and N598T) of the six EC-1 potential interacting amino acids (T436A, N573Y, M574A, K595R, N598C, N598T, and T613C) showed an exaggerated NS1643-induced negative shift in the $V_{1/2}$ of activation ($p < 0.05$) compared with WT (Table 1; Fig. 4). Two of these six mutations (T436A and T613C) produced fewer NS1643-induced positive shifts of the voltage dependence of inactivation. Parallel results were observed when measuring the voltage dependence of inactivation as assessed by the $V_{0.3}$ or the inactivation tau (Supplemental Fig. 4). Three of these six mutations (M574A, N598T, and T613C) showed fewer drug-induced increases in the tail current amplitude (Table 1).

### In Silico Protein-Ligand Contacts for IC-1 and IC-2 Sites: The S4S5 Linker

The next cluster of binding poses was predicted around the S4S5 linker. The two potential binding regions are termed IC-1 and IC-2 (Fig. 6, top left). The vast majority of the interacting poses were observed in the IC-1 region. In this region, Leu532, Arg541, Glu544, Phe557, Met651, Leu654, and Tyr667 amino acid residues are predicted to form the potential binding site. The amino acid residues with NS1643 are Thr436, Asn573, Met574, Asn598, and Thr613 amino acids (Fig. 5).
acids Thr526, Leu529, and Ile567 are predicted as important residues to form the second predicted binding mode, IC-2 (Fig. 6). To systematically refine the search for amino acids interacting with NS1643 in these flexible domains, two regions (Leu523-Val535 and Leu553-Trp568) were scanned. The potential binding sites were created from the reference residue (e.g., residue x with a 10-Å radial cavity around the reference residue), and all of the regions were scanned by defining the binding site from residue x + 1, x + 2, x + 3, etc. (e.g., active sites within a 10-Å radial cavity from the reference amino acid Leu523; the reference amino acid was then shifted to Leu524 and iterated progressively) (Supplemental Fig. 5). In this manner, binding site predictions were scanned systematically, and the amino acids essential for NS1643 binding and the corresponding docking scores were calculated. A comparison of the GOLD fitness and ChemScore docking scores predicted that Val533-Val535 from the first binding site region and Leu564-Ala565 from the second binding site region interact with NS1643. These regions (Val533-Val535 and Leu564-Ala565 at intersubunits) are in close proximity to each other (Supplemental Fig. 5) in the developed model. Studies with the FlexX docking program predicted that Ile560 and Leu564 were potential residues for drug binding. Docking with Glide/IFD predicted two amino acids essential for drug stabilization: Glu544 and Leu564 (Supplemental Fig. 5).

**In Vitro Electrophysiologic Results: The S4S5 Linker.** The IC-1 amino acids including Arg541, Glu544, Leu564, Met645, Met651, and Tyr667 were chosen for evaluation. Of these six residues, three mutations (E544L, L564A and Y667D) significantly and substantially altered the pharmacologic response to NS1643 (see Table 1). The E544L mutation dramatically exaggerated most of the pharmacologic effects of NS1643. This mutation increased the right shift in inactivation and significantly increased the NS1643-induced increase in the tail current amplitude...
by 400% (Supplemental Fig. 6). In contrast, the L564A mutation inhibited the drug responses. This mutation suppressed the NS1643-induced voltage shift in inactivation and abrogated the ability of NS1643 to increase the tail current amplitude (Table 1; Supplemental Fig. 7). The IC-2 amino acids include Thr526 and Ile567. Mutations of these amino acids significantly modulated the response to NS1643 (Table 1). The T526M mutation facilitated the pharmacologic effects on both activation and inactivation. However, the I567A mutation produced statistically significant NS1643-induced increase in the tail current amplitude compared with WT.

**Other Residue Mutations Interacting with NS1643.** The previously reported hERG1 D540K mutation generates a time-dependent inward conductance during the application of hyperpolarizing pulses (Sanguinetti and Xu, 1999; Mitcheson et al., 2000b; Tristani-Firouzi et al., 2002; Perry et al., 2004). Therefore, this mutation was chosen for additional experimental studies. Figure 7 shows that NS1643 completely suppressed the inward conductance of D540K. Representative examples of the D540K currents elicited by the voltage-clamp protocol are shown in the insets in Fig. 7, A to D. The baseline data are shown in Fig. 7, A to D, left, and the NS1643 treatment is shown in Fig. 7, A to D, right. The cells in Fig. 7, A and B were held at a resting potential of −80 mV. These cells were pulsed to a range of depolarized potentials, and the tail current was recorded during a return to −60 mV. In contrast, Fig. 7, C and D shows representative examples of currents elicited by the voltage-clamp protocol shown in Fig. 7D, inset. The cells were then pulsed in a series of hyperpolarized potentials to −120 mV, followed by a return to −60 mV. During hyperpolarization, D540K manifested a large time-dependent inward conductance (Fig. 7C). Figure 7D shows that NS1643 suppressed the inward D540K current. In contrast, NS1643 increased the outward currents during the applied depolarized potentials (Supplemental Fig. 8).

To understand the molecular nature of the interaction of NS1643 with the D540K mutant, in silico-directed mutagenesis was performed (Fig. 8). NS1643 docking in the neighborhood of WT D540 predicts hydrogen bonds and van der Waals forces linking NS1643 with Asn470, Lys495, Arg537, Asp540, and Arg541 (Fig. 8). When in silico mutagenesis was performed to create D540K, NS1643 was predicted to interact with Asn470, Lys495, Lys540, and Arg541. Furthermore, the orientation of NS1643 in this binding site is altered by the D540K mutation.

It is noteworthy that when a different amino acid is substituted at the Asp540 position (i.e., D540C; data not shown), there was no inward conductance. In addition, the pharmacologic responses of this channel to NS1643 are similar to WT. These data suggest that NS1643 seems to have a specific effect on the inward conductance observed only with D540K. Although NS1643 has a significant effect on the inward conductance of the D540K channel, it did not alter the outward tail currents (Fig. 7). In addition, the ability of NS1643 to left-shift the $V_{0.3}$ of inactivation was not significantly changed by D540K (Supplemental Fig. 8). Previous studies in Sanguinetti’s laboratory (Sanguinetti and Xu, 1999; Mitcheson et al., 2000b; Tristani-Firouzi et al., 2002; Perry et al., 2004) have provided compelling evidence that the inward conductance elicited by hyperpolarizing pulses in the D540K mutant is related to an inward movement of the voltage-sensing S4 segment. D540K is located in the S4S5 linker near the cytosolic terminus of
the S4 region. The data suggest that NS1643 opposes the inward movement of the S4 segment.

**Discussion**

Potential interacting sites for NS1643 were predicted by in silico tools. These predictions prompted subsequent site-directed mutagenesis and patch-clamp assessments of the drug's effects on the mutated channel currents. Because the hERG1 modeling and molecular docking were not constrained by the existing experimental data from the literature, it should be considered as a blinded experiment. Using such an approach, we have mapped a number of entirely novel sites in hERG1 that interact with NS1643. The most important of these are D540K, E544L, and N629H. The N629H mutation is the sole mutation discovered that abrogates the NS1643-induced shift in the $V_{1/2}$ of activation. However, other substitutions at the same site (N629T and N629D) showed a pharmacologic response similar to the wild-type channel. In silico modeling predicts that the N629H mutation resulted in a different orientation of the ligand in this putative drug-binding pocket (Fig. 9) compared with the N629T mutant and WT hERG1. The D540K mutation generates a time-dependent inward conductance during the application of hyperpolarizing pulses. This is presumably caused by inward movement of the S4 region during hyperpolarization. The inward conductance of the D540K mutation was originally evaluated by Sanguinetti's laboratory (Sanguinetti and Xu, 1999; Mitcheson et al., 2000b; Tristani-Firouzi et al., 2002; Perry et al., 2004). The original contribution of the present study is that NS1643 completely abrogates this inward conductance, suggesting that it modulates movement of the S4 helix. The E544L mutation exaggerated most of the pharmacologic effects of NS1643. This mutation increased the magnitude of the NS1643-induced tail current amplitude by 400%. In contrast, the L564A mutation dramatically inhibited the NS1643-induced changes in $V_{0.3}$ of inactivation and prolongation of deactivation and abrogated the drug-induced increase in tail current density.

![Fig. 7.](image) NS1643 abrogates the inward currents induced by hyperpolarization in the D540K mutant. A to D, the raw D540K mutant currents before (control) and after NS1643 application. A and B, the depolarization-induced currents of the D540K mutant were increased by NS1643 treatment (B) compared with control (A). C and D, the hyperpolarization-induced currents (C) were eliminated by NS1643 application (D). E and F, the time-dependent currents (E) and the tail currents (F) of the D540K currents recorded when drug-free and then after treatment with 10 μM NS1643. The mean data are shown in Supplemental Fig. 8.

![Fig. 8.](image) Site-directed in silico mutagenesis around Asp540. NS1643 is held within a predicted binding site around D540K. The ligand is predicted to be in a different orientation and interacts with different amino acids compared with the WT (Asp540) channel.
Our in silico docking predictions and physiologic data indicate the presence of multiple pharmacologic effect sites. Inactivation occurs at a significantly lower concentration (0.3 μM) than required to increase tail currents or slow deactivation. Pharmacologic block develops at high concentration (> 10 μM). The shift in the voltage dependence of activation seems to progressively increase with increasing concentrations. These data strongly suggest the presence of multiple spatially separated binding sites producing diverse pharmacological effects.

An approach combining site-directed mutagenesis, functional voltage-clamp assays, and subsequent molecular modeling was used by Grunnet et al. (2011) to identify interacting sites for NS1643 on hERG1. They proposed a binding site for NS1643 in the vicinity of the EC end of the S5-S6 segments of two adjacent hERG1 subunits. The following mutations were reported to cause an enhanced response to the electrophysiologic activating effects of NS1643: I567A, F619A, C643A, Y652A, and Phe656 (to Met, Thr, or Val). Docking of NS1643 to the Kv1.2-based homology model of the hERG1 channel pore led to the prediction of Ile560, Leu622, Leu646, Leu650, and Met651 as important determinants of activator binding. Other residues experimentally identified as potentially important by scanning mutagenesis include Ala558 and Trp563. These residues were not exposed to the NS1643 molecule during previous molecular modeling studies. It is noteworthy that the binding site for NS1643 proposed by Grunnet et al. (2011) is made up of several amino acid residues predicted to be part of the IC-1 and IC-2 sites reported in the current study. The docking results reported in this study also predicted Ile567 as a site in hERG1 that interacts with NS1643. Thus, the reported data here and the experimental work published independently by Grunnet et al. (2011) are consistent.

The strategy used by Grunnet et al. (2011) is fundamentally different from the strategy used here. In their study a wide range of empiric scanning mutations were created and assessed electrophysiologically. These experiments were followed by the docking of the NS1643 activator to their model of hERG1, which was derived based on the crystal structure of the mammalian Shaker Kv1.2 channel. It must be noted that their model does not fully cover the S1–S4 voltage-sensing residues. In addition, their model does not include the S5 pore linker (turret), which is formed by approximately 40 amino acids. One of the key conclusions of a hERG1 modeling article published previously (Lees-Miller et al., 2009) was that de novo folding of the missing elements (traditionally avoided in a plethora of homology models including the one in Grunnet et al. (2011)) may have a critical impact on residue packing. This may affect the quality of the docking studies. Their approach was to assess whether important mutations discovered empirically had proximity to the docked NS1643 molecule evaluated in silico. In contrast, the strategy used in the present study differed because the in silico studies prospectively guided mutagenesis. Subsequently, electrophysiology studies were performed to test the predictions.

The hERG1 modulation by NS1643 binding seemed to be complex with multiple binding sites present in the receptor. These findings are consistent with recent studies by Wang et al. (2011), which mapped the sequence of conformational changes underlying C-type inactivation of hERG1 that involves cooperative movements of networks of residues. The sequence of conformational rearrangements occurs in the pore domain outer helix, then the EC turret region, the VS domain, the IC domains, and finally the pore domain inner helix. Thus, a complex spatial and temporal sequence of widespread domain motions through the entire channel underlies inactivation. Thus, it is not surprising that selected mutations in many regions of hERG1 might change inactivation.

Structure-Guided Mutagenesis Strengths and Shortcomings. A key limitation of our modeling is that it was based on a single state of the channel (the open state).
Because NS1643 modulates inactivation, it is likely that interrogation of an inactivated in silico state of the channel could predict other important residues. The presence of an elongated S5 pore linker in the partially validated S1–S6 TM hERG1 model used in the current study may increase the quality of the docking results.

The Potential Clinical Applicability of hERG1 Openers. Matsa et al. (2011) created cardiac myocytes derived from human iPS cells, isolated from a patient with the hERG1 LQT2 A561T mutation. Treatment of these myocytes with PD118057 caused a modest shortening of the action potential duration by 17%. Addition of PD118057 to myocytes with PD118057 caused a modest shortening of the hERG1 LQT2 A561T mutation. Treatment of these myocytes with PD118057 caused a modest shortening of the action potential duration by 17%. Addition of PD118057 to myocytes with PD118057 caused a modest shortening of the hERG1 LQT2 A561T mutation. Treatment of these myocytes with PD118057 caused a modest shortening of the action potential duration by 17%

In summary, this study suggests that PD118057 may be an antarrhythmic in one context and potentially prodysrhythm in another context.

The same study by Matsa et al. (2011) reported that PD118057 can partially reverse the action potential prolongation produced by 1-[2-(6-methyl-2-pyridyl)ethyl]-4-(4-methylsulfonylaminobenzoyl)piperidine. Likewise, Kang et al. (2005) pretreated cells expressing hERG1 with the activator RPR260243 and subsequently treated those cells with dofetilide. In this context, dofetilide treatment continued to block the channel but at a somewhat higher IC50 value (58 nM) compared with dofetilide treatment alone (11 nM). Hansen et al. (2008) also reported that in vivo treatment of guinea pigs with NS3623 produced a reversal of E4031-induced prolongation of the QT interval. In the absence of pharmacologic blockade of hERG1, Patel and Antzelevitch (2008) reported that the hERG1 activator PD118057 abbreviated the QT interval but increased transmural dispersion of repolarization. This leads to polymorphic ventricular tachycardia induction in a canine wedge preparation (Patel and Antzelevitch, 2008). Thus, it is still uncertain whether hERG1 activators will be antarrhythmic or prodsyrrhythmic.

In conclusion, structure-guided topographic mapping of potential interacting sites for the channel activator NS1643 correctly predicted some amino acids of hERG1 that have not been reported previously. They are important and previously unreported determinants of NS1643 effects. An understanding of the interacting sites for a specific hERG1 activator may open new avenues of rational drug design for novel hERG1 activators. The binding of an activator and its mode of action seem to be far from a “one drug/one binding site” paradigm and display considerable state dependence.

The three important and novel mutations in the SF-1 and IC-1 sites (D540K, E544L, and N629H) that alter response to NS1643 were discovered by this study. NS1643 completely abrogates this inward conductance of D540K, suggesting that it modulates inward movement of the S4 region. Many of the amino acids identified by structure-guided topographic mapping in this study are consistent with the results from previous electrophysiology experiments (Grunnet et al., 2011), providing further validation for our S1S6 TM hERG1 three-dimensional structure.


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SUPPLEMENTAL DATA

Structure-Guided Topographic Mapping and Mutagenesis to Elucidate Binding sites for the hERG1 Potassium Channel (KCNH2) Activator- NS1643

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Supplementary Figure 1 Methodology used to measure voltage dependence of inactivation. Currents are elicited using a triple pulse protocol (Zou, et al., 1998) as shown in Panel A. Currents before (A) and after (B) application of NS1643 are shown. Details as to the methods are presented in the text. Examples of the effects of NS1643 on WT hERG1 are shown in Panels C and D. (**, p<0.01) The relationship between the drug-induced change in amplitude of the tail current and the baseline drug-free inactivation $V_{0.3}$ for all the mutations examined is shown in Panel E. No relationship was found. Open circle at panel E represents the position of WT-hERG.
Supplementary Figure 2 (i) The top 100 predicted docking poses of NS1643 in the SF regions are superimposed. (ii) The top 100 predicted docking poses of NS1643 at the EC region are superimposed. (iii) The top 100 predicted docking poses of NS1643 at the IC region are superimposed.
Supplementary Figure 3 (i) Predicted docking poses of NS1643 by GOLD docking program when the binding sites are defined as a 20 Å radial cavity around the origin of the central cavity of the channel. (ii) Predicted binding interactions of NS1643 at the central cavity site. Yellow and green colored dashed bonds show hydrogen bonds and VDW close contacts, respectively. (In Figure, CPK coloring is used for the atoms. C, gray; F, green; H, white; N, blue; O, red). (iii) When the binding site is defined as a 20 Å radial cavity around the central cavity of the channel for the docking simulations, only one predicted docking pose (from top 100 predicted docking poses) is located outside of the central cavity (S4S5-linker site). Yellow and green colored dashed bonds show hydrogen bonds and VDW close contacts, respectively. (In Figure, CPK coloring is used for the atoms. C, gray; F, green; H, white; N, blue; O, red; S, yellow).
Supplementary Figure 4 Inactivation was measured using a second method. In WT, NS1643 shifted the voltage dependence of \( V_{0.3} \) of inactivation and this was paralleled by a NS1643-induced voltage shift in the tau of inactivation during application of the triple pulse protocol. The extent of shift in these two metrics of inactivation was similar. In addition, the T613C mutation abrogates this rightward shift both measured as \( V_{0.3} \) of inactivation and also measured by the tau of inactivation. Other mutations produced an exaggerated rightward shift in \( V_{0.3} \) of inactivation (Table 1) and this was also paralleled by an exaggerated rightward shift in the relationship between tau of inactivation and voltage. *, \( p<0.05 \).
Supplementary Figure 5 (i) Putative binding site predictions of NS1643 for the L523-V535 and the L553-W568 regions. (ii) GOLD Fitness and ChemScore calculations during serial binding site shifts. The GOLD Fitness score is dimensionless however, in each case the scale of the score gives a guide as to the goodness of fit for each pose. The higher the score, the better the binding interactions. The GOLD ChemScore function estimates the total free energy change that occurs upon ligand binding and was trained by regression against binding affinity data. (iii) The predicted top docking pose of NS1643 at the S4S5-IC region derived by Glide/IFD program. Predicted close contacts between ligand and the amino acids are shown with dashed lines.
Supplementary Figure 6  NS1643 dramatically increased current amplitude of E544L. The current traces of E544L before (A) and after (B) 10 µM NS1643. C, the time-dependent and tail currents of E544L in control and NS1643 in A and B. The tail currents were recorded at -100 mV to avoid any possible involvement of inactivation. D, The activation V1/2 in control and NS1643 of E544L, in comparing with that of WT. E, The amplitude of tail currents induced by +50 mV depolarization before and after NS1643, in comparing with that of WT. *, p<0.01.
**Supplementary Figure 7** Inactivation ratio was measured as current levels at 50 ms vs peaks of the tail current at each potential showed. The voltage-dependence of inactivation of L564A is shifted to the left by NS1643 (Panel A) compared to the rightward shift seen in WT. *, p<0.01 (Panel B). C, The inactivation time constants at control and in 10 µM NS1643. No significant difference was found. In WT, inactivation was significantly slowed by NS1643.
Supplementary Figure 8 A, activation $V_{1/2}$ of D540K in control and in NS1643, in comparing with that of WT. B, the increase of depolarization induced currents at 50 mV and the inhibition of hyperpolarization induced currents at -120 mV by 10 µM NS1643. C, NS1643-induced shifts of inactivation ratio of WT and D540K. *, $p<0.05$; **, $p<0.01$