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„Computational Docking study offers supportive evidence
of the presence of pockets in hERG K⁺ Channels“

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Milica Gaćeša

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1. ABSTRACT

Purpose: The study was conducted to test the hypothesis that hydrophobic pouches observed in the newly available cryo-EM structure of the hERG K⁺ channel facilitate drug binding. This study will provide new information about the molecular basis of drug block in hERG K⁺ channels, which is a major risk factor for acquired long QT syndrome.

Methods: 15 snapshots were extracted from molecular dynamics simulations of the hERG channel, and used to perform computational docking studies. Residues from 546 to 661 of each subunit were analyzed. Twelve well known hERG blockers: MK-499, (–)-Cisapride, (+)-Cisapride, R/S-Terfenadine, R/S-Ibutilide, Clofilium, Sertindole, Astemizole, Dofetilide and Grepafloxacin were analyzed.

Results: This study offers supportive evidence of the presence of hydrophobic pockets in hERG, which seem to facilitate drug binding of a wide variety of structurally different hERG blockers.

Conclusion and implications: The results showed that indeed hydrophobic pouches contribute to drug binding and might explain the unique promiscuity of the hERG K⁺ channel, with respect to binding of structurally different drugs. Further this study highlights the importance of binding site dynamics, which was obtained by extracting MD snapshots.

1. Zusammenfassung

Zielsetzung:

in der vorliegenden Studie wurde die Bedeutung der sogenannten “hydrophoben Taschen” für die Inhibierung von hERG K⁺ Kanälen erforscht, welche in der kürzlich veröffentlichten cryo-EM Struktur des Kanals beobachtet wurden.

Methoden:

15 Konformationen des Kanals wurden aus einer Moleküldynamik Simulation extrahiert und für Docking Studien mit ausgewählten Inhibitoren herangezogen. Aminosäuren 546 bis 661 in jeder Untereinheit wurden analysiert. Die folgenden bekannten hERG K⁺ Blocker wurden für die Studie ausgewählt: MK-499, (–)-Cisapride, (+)-Cisapride, R/S-Terfenadine, R/S-Ibutilide, Clofilium, Sertindole, Astemizole, Dofetilide und Grepafloxacin.

Ergebnisse:

Die Studie konnte zeigen, dass die hydrophoben Taschen in der hERG Struktur tatsächlich eine dynamische Interaktion mit zahlreichen, strukturell unterschiedlichen hERG Kanal Inhibitoren ermöglicht.

Zusammenfassung:

Die Docking Ergebnisse legen den Schluss nahe, dass die neu entdeckten, für den hERG Kanal einzigartigen hydrophoben Taschen eine wichtige Rolle bei der Promiskuität des hERG Kanals spielen und die Bindung mit einer Vielzahl strukturell unterschiedlicher Arzneistoffen ermöglicht. Diese Studie zeigt außerdem die Wichtigkeit der Flexibilität der Bindungstasche, welche durch Einbeziehung von MD generierten Strukturen ermöglicht wurde.

2.INTRODUCTION

2.1 The hERG K⁺ channel and drug block

hERG (Kv11.1) channels conduct the rapid delayed rectifier K⁺ current (I_{Kr}), a critical current in the phase 3 repolarization of the cardiac action potential[1], [2]. Control of the repolarization phase of the cardiac action potential is critical for normal excitability [3] . Abnormalities in channel function such as those due to congenital defects, or that are due to acquired mechanisms (i.e., drug-induced), can compromise the action potential waveform and potentially lead to life-threatening arrhythmias[3]. When hERG is defective, the cardiac action potential is prolonged, resulting in a hallmark lengthening of the QT interval on the electrocardiogram (ECG)[4], [5]. Inherited mutations of hERG were found to cause type 2 long QT syndrome, a disorder of cardiac action potential repolarization that predisposes affected patients to arrhythmias and sudden death[6]. Acquired LQTS is more common than inherited LQTS and is usually caused by preferential block of I_{Kr} by common medications [7]. This undesirable side effect has prompted removal of several drugs from the market and is now recognized as a significant hurdle in the development of new and safer drugs[7].LQT is considered a major risk factor for torsades de pointes, a life-threatening arrhythmia[8].

The Comprehensive in vitro Proarrhythmia assay (CiPA) has been proposed as a new safety paradigm in understanding TdP and assessing proarrhythmia risk[9]. CiPA has two primary objectives:

(1) Detailed in vitro electrophysiological characterization of drug interaction with Kv11.1 (and other cardiac ion channels) and integration of this data into in silico models to predict proarrhythmia in simulations of the cardiac action potential and (2) Validation of in silico models using human induced pluripotent stem cell derived cardiac cardiomyocytes [10].

It has been estimated that 50–70% of all lead compounds are eliminated at early stages due to hERG-related safety issues[11], thereby limiting the number of drugs that enter the development pipeline[12].

In April 2017, the laboratory of Mackinnon published the first single-particle cryo –EM structure of the hERG K⁺ channel [13], revealing unique structural features. In particular, hydrophobic pouches were observed in the structure, leading to the hypothesis that they might play a role in drug block. However, the structure was solved in the apo state only, limiting insights into inhibition. Figure 1 shows hydrophobic pouches used in this study.

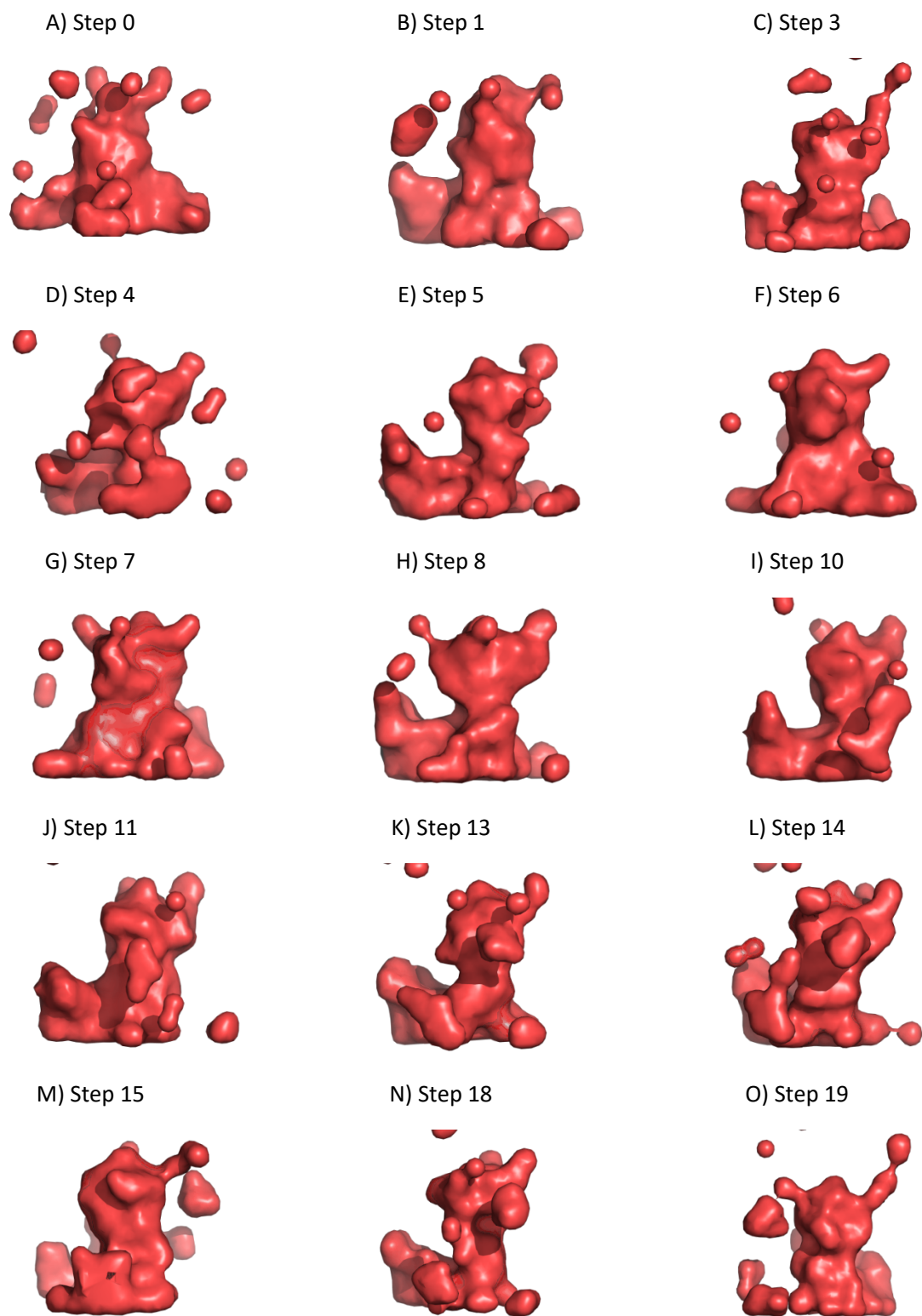


Figure 1: Samples of hydrophobic pockets from each step, representing different MD snapshots. Hydrophobic pockets are created by a Hollow program[14].

Thus, in this master's thesis, the hypothesis about the presence and importance of the hydrophobic pouches for drug block in hERG was tested by using computational docking and inspection of the molecular mechanisms between hERG blockers and the human ether-a-go-go (hERG) voltage dependent potassium channel.

3. MATERIALS AND METHODS

3.1 Hollow program

To illustrate the size of the hydrophobic drug binding pocket in the hERG channel of different conformational states, we used the program Hollow[14]. HOLLOW takes a "casting" of the cavities by filling the voids, channels and pockets of a protein with dummy atoms defined on a grid [14]. At small grid-spacing, the surface of the casting is shown to match the molecular surface of the protein [14]. In the automated mode, overlapping dummy atoms are defined on a rectangular grid surrounding the molecular structure [14]. By default, the probe radius of the dummy atoms is set to 1.4 Å and the grid-spacing to 0.5 Å [14]. The dummy atoms are eliminated from the grid if they overlap with the molecular structure, or they are found outside the exterior envelope of the protein [14]. To reduce the number of dummy atoms while not affecting the overall volume, dummy atoms from the interior of the block are selectively removed if this does not affect the overall volume of the block [14]. These dummy atoms are then written to a PDB file as oxygen atoms by default, or any other atom type as specified in the configuration file[14]. In this study, the probe radius of the dummy atoms was set to 1.4 Å and grid-spacing to 0.5 Å. The output was illustrated as a transparent surface by the visualization program PyMol[15]

3.2 Docking studies

Docking was performed using the program Gold 5.6 [16]. In a previous unpublished study carried out by our group, based on the open cryo-EM structure of hERG twelve well-known hERG blockers were used as ligands. Since this was a continuation of work ie a computational docking study on 15 snapshots extracted from molecular dynamics simulations of the hERG channel, the same drugs were used. As reported there, the ligand selection was based on the previous structure-activity data[17], [18], the proposed pharmacophore for drugs that bind to hERG[13], [17], as well as the recognised inhibition of IKr current. Twelve drugs : MK-499, the two known diastereomers of cisapride, both enantiomers of the racemic blockers terfenadine and ibutilide, clofilium, sertindole, astemizole, dofetilide and grepafloxacin were docked into 15 different snapshots extracted from an MD simulation of the hERG channel. The Cryo-EM structure of the human ether-a-go-go related K⁺ Channel was obtained from the Protein Data Bank (pdb code: 5VA1)[13]. Residues from 546 to 661 of each subunit were analyzed.

In all docking runs using the 15 distinct MD steps, Tyr 652 was chosen as the center of the binding site. Only for step 19 namely 19 MD snapshot in terms of binding site Tyr 652 and Gly 648 were used. Three sets of docking poses were conducted under the same conditions for each step and two additional sets for 19 MD snapshots were performed in terms of binding site and radius as it was mentioned before.

- The first set was performed with protein side chains regarded as rigid and with an approximate radius of 10 Å around the center of the binding site.
- The second set was carried out under the same conditions with a different radius-6 Å was used.
- The third set was conducted by utilising residues of interest, which line proposed hydrophobic pouches and set their side chains to freely rotate, while the approximate adjusted radius was 8 Å.

In addition to three sets of docking poses with Tyr 652 as the binding site for each step, two sets of docking poses were made using step 19 with Gly 648 as the binding site. One was conducted with protein side chains regarded as rigid and using a radius of 6 Å. A second set of docking poses with 19 MD snapshot was performed using a radius of 10 Å and with Phe 557 A,B, Leu 622 B, Thr 623 A,B, Ser 624 B, Met 645 B, Tyr A,B side chains set to freely rotate.

As for the ligands, in every set, each ligand to be docked was selected separately.

The number of independent generic algorithm runs was adjusted to 20 docking runs. Considering the number of used conformational protein snapshots, the number of ligands and the number of sets, a lot of samples were obtained in this study. Ligand flexibility was set to enable flipping pyramidal N, flipping amide bonds, detecting internal H bonds, flipping ring corners, flipping all planar R-NR1R2. Rotations were also set for Ring-NHR, Ring-NR1R2, flip protonated carboxylic acids.

The purpose of this study was to detect whether the ligands occupy the pocket that was suggested to be a possible binding site[13]. Another goal was to identify possible binding poses of the ligands in one or more pouches. The poses were not compared and analyzed based on their fitness score, so the fitness score was not observed.

The computational time was around a few minutes for each blocker on an Intel Core i7-6900K CPU computer with a GeForce GTX 1080 graphics card.

For sets with protein side chains regarded as rigid all docking poses were checked with the Pymol Viewer.

For sets with certain residues with side chains set to rotate freely only poses, which positioned their functional group toward or into the pouches were selected and then exported via Gold option and analyzed via Pymol Viewer. Others were rejected. The programs VMD (Visual Molecular Dynamic software) [19] and Hollow [14] were used to see a possible occupation of pockets with functional groups of drugs.

To get data about the percentage of occupation of the pockets, all poses that showed occupation of functional groups of ligands in the pouch were counted, without taking the type of functional group into account. A percentage was calculated based on the 20 dockings that were performed for every snapshot. Even though the number of independent generic algorithm runs was set to 20 docking runs, sometimes the number of obtained poses was smaller. In this case the percentage was counted based

on 20 docking runs, because the ranking of individual percentages would stay the same, no matter if the number of poses obtained was smaller. The aim was to have the same metric. In that case, for the sake of clarity percentages in the table were marked by larger numbers (see tables in Supplementary Information). The poses were selected manually based on visual inspection. It is important to highlight that sometimes ligand occupied pouches, but still had parts of the molecule slightly outside the pocket. In that case they were counted as inside for the calculation of the percentages. If the ligands were reaching too far outside the pocket, they were excluded. Certain observation will be mentioned, but it won't be explained in detail for the sake of clarity. For each ligand the step with the highest percentage of occupancy in the pocket was chosen. The poses of these steps were visually inspected and further analyzed. The interaction patterns of ligands with the protein were calculated by the MOE[20]. It is important to underline that the comparison of the steps for each ligand based on highest percentage was performed in the first and second sets of docking poses with protein side chains regarded as rigid, regardless of whether the radius of 6 Å or 10 Å was used. If there were two or more steps with the equal percentage selection was made based on visual inspection.

In the third docking set, the poses with the highest percentage were inspected with PyMol for every ligand. The “best” pose was chosen based on this visual inspection. This pose was used for the investigation of the molecular basis of hERG binding. It is also important to emphasize that if ligands occupied the pocket of an adjacent chain, these results were also included into the calculation of the percentage of ligands in the binding pocket, as shown in tables in Supplementary Information. This is indicated with bold letters.

Additionally, tables in Supplementary Information shows the options “The ligand occupies half of the pocket” and “The ligand occupies initial part of the pocket”, since choosing the answer Yes or No would completely discard the poses that partly, but not entirely, insert their functional groups into pouches.

Snapshots were extracted from MD simulations available in the group. These snapshots were also used in a recent publication (for details about MD, see [21])(<https://www.ncbi.nlm.nih.gov/pubmed/29550817>).

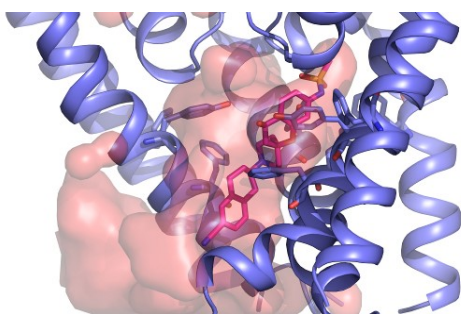
4. RESULTS

The key results for each drug are described separately in the following section.

4.1 MK-499

In the first set of docking with protein side chains regarded as rigid and with an approximate radius of 10 Å around the center of the binding site, the number of steps where the poses showed the occupancy of the pockets was considerably smaller in comparison with the second set. The occupancy rate was also lower.

A) Step 14 (5%)



B) Step 18 (5%)

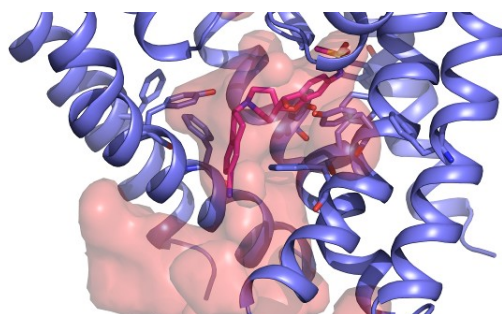


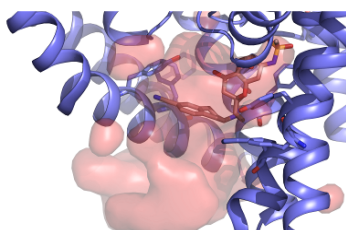
Figure 2: Binding poses of MK-499 in stick representation, based on snapshots of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which functional groups are positioned inside the hydrophobic pockets:

A) methanesulfonanilide of MK-499 in step 14

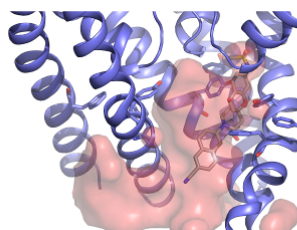
B) methanesulfonanilide of MK-499 in step 18

In the second set of docking poses where a different radius of 6 Å was used, the following percentages of pouch occupation with the methanesulfonanilide moiety of MK-499 were obtained for the next steps:

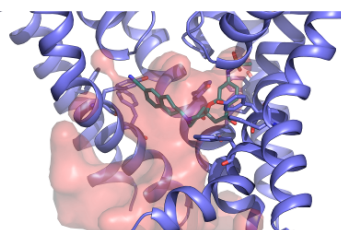
A) Step 4 (20%)



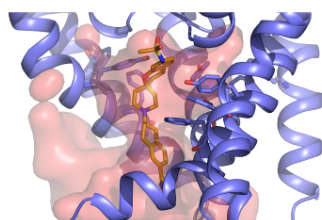
B) Step 5 (35%)



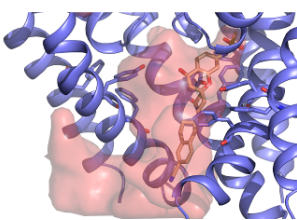
C) Step 8 (55%)



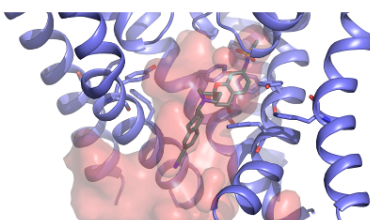
D) Step 8 (55%)



E) Step 10 (20%)



F) Step 11 (5%)



G) Step 14 (15%)

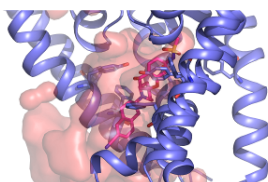


Figure 3: Binding poses of MK-499 in stick representation, based on snapshots of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which functional groups are positioned inside the hydrophobic pockets:

A) methanesulfonanilide of MK-499 in step 4 B) methanesulfonanilide of MK-499 in step 5 C) methanesulfonanilide of MK-499 in step 8

D) methanesulfonanilide of MK-499 in step 8 (it is important to highlight that two more poses occupy the pouch of the neighboring chain to the chain where docking was performed, but only with methyl group, the rest of the molecule was outside the proposed cavity) E) methanesulfonanilide of MK-499 in step 10 F) methanesulfonanilide of MK-499 in step 11

G) methanesulfonanilide of MK-499 in step 14. In the table the percentage is marked with a bigger number, because the number of solutions obtained was less than 20.

The first set of docking poses with 19 MD snapshot was performed using the radius of 6 Å and Gly 648 as the binding site and with protein side chains regarded as rigid. Under these conditions MK-499 occupied the pocket with cyano-tetrahydronaphthyl group (80%). Most of these poses showed reduced volume of the pocket, when investigated in Hollow, but not too reduced and because of this, these poses were included into the percentage. Under this condition, the highest percentage of occupation of the pocket was obtained for MK-499. It is displayed in Figure 4. This pose was used for the investigation of the molecular mechanisms of interactions between MK-499 and hERG. Figure 5 shows 2D ligand-protein interaction diagram.

Step 19 (Binding site Gly 648)(80%)

6 Å RIGID

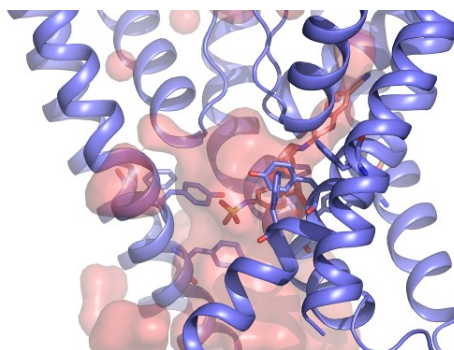


Figure 4. Binding pose of MK-499 in stick representation, based on 19 snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which cyano-tetrahydronaphthyl group is positioned inside the hydrophobic pocket.

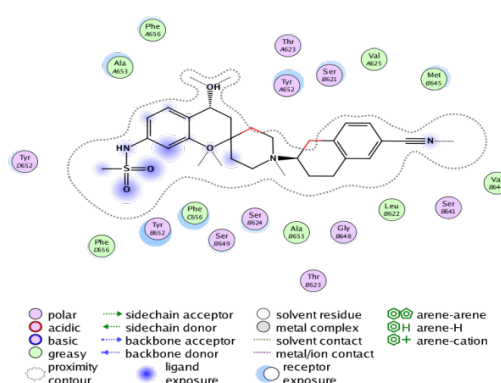


Figure 5: 2D ligand interactions of MK-499 from the step with the highest percentage of occupation (19 Gly) at the hydrophobic pocket of the hERG structure, based on snapshot. 2D ligand-protein interaction diagrams were generated using MOE (Chemical Computing Group Inc., Molecular Operating Environment (moe)).

The ligand–protein interaction diagram did not illustrate any π - π stacking interactions or hydrogen-bonding interactions between MK-499 and hERG structure. Ligand stays in the vicinity of other hydrophobic (i.e., Phe 656 A,C,D, Ala 653 A,B, Leu 622 B, Val 625 A, Val 644 B, Met 645B) residues. This can suggest potential hydrophobic interactions. The molecule is in a close contact with polar residues (i.e. Thr 623 A,B, Ser 621B, Ser 624 B, Ser 641 B, Ser 649 B, Gly 648B). Residues exposure with the blue shadows indicates amino acids whose side chains are partially solvent exposed (i.e., Tyr 652 A,B,D etc.). The proximity contour is the grey dotted line surrounding the ligand and it indicates the shape of the binding site and available space for ligand. The red colored part of the ligand clashes with protein,

meaning it is very close. Ligand exposure indicates that the blue-colored part of the ligand molecule is solvent accessible (methanesulfonanilide group, cyano group, etc.).

Previous alanine-scanning mutagenesis study has identified the key binding site for drug block of hERG by MK-499, a methanesulfonanilide antiarrhythmic drug. The rank order of importance of HERG channel residues that affect binding of MK-499 was F656 >> Y652 >G648 =V625 >T623 >S624 = V659[22]. The importance of a π - π stacking interactions between MK-499 and the aromatic side groups of Y 652 and F 656 could not be confirmed based on this diagram, meaning this observation is in contrast with previous studies [17], [22]. The number of steps where the poses showed the occupancy of the pockets with methanesulfonanilide compared to cyano-tetrahydronaphthyl group was significantly larger. Probably we would get more hydrogen bonds between the residues of interest (i.e. Thr 623, Ser 624) as already mentioned in the quoted article above, if we used the poses where methanesulfonanilide group occupies pockets for further calculation of interactions. Docking study based on homology model of hERG suggests that the mutation of V 625 and G 648 to Ala disrupts the MK-499 binding site by altering the size or shape of the binding pocket formed between the pore helix and the S6 domain of a single subunit[22].

In the third set of docking poses methanesulfonanilide of MK-499 positioned within the hydrophobic pocket only in step 8. The percentage of pouch occupation was 20 %. It is interesting to emphasize that here protein side chain flexibility did not show up as an important factor for pocket occupation of MK-499, because it is evident that in the first and second sets of docking poses with protein side chains regarded as rigid the number of steps with a ligand bound within hydrophobic pouches was significantly higher. Also MK-499 occupied half of the pocket with methanesulfonanilide moiety in step 1 (15%) and the initial part of the pocket in step 7 with methanesulfonanilide and cyano group (15 %). In tables you can find additional information about residues and their side chains, which are set to rotate freely.

Step 8 (20%)

Flexible sidechains 8 Å: Phe 557 C, Leu 622 C, Thr 623 C, Ser 624 C, Tyr 652 B,C, Phe 656 C

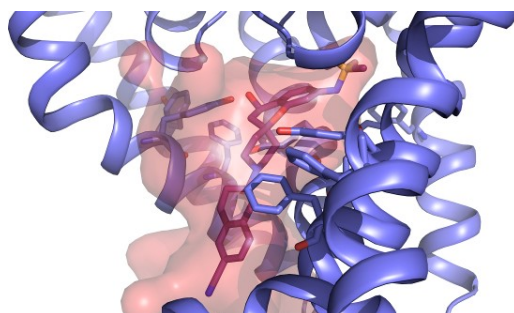


Figure 6. Binding pose of MK-499 in stick representation, based on snapshot 8 of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in

which methanesulfonanilide group is positioned inside the hydrophobic pocket.

In the first set of docking poses MK-499 partially (5%) occupied the pocket with methyl group in step 13. In the second set of docking poses MK-499 partially occupied the pocket in

- Step 3 (10%) with methanesulfonanilide moiety
- Step 6 (5%) with methanesulfonanilide moiety
- Step 7 (30%) with cyano moiety
- Step 13 (5%) with methanesulfonanilide moiety
- Step 15 (5%) with methanesulfonanilide moiety.

Summarizing, the docking studies suggest that different functional groups of the MK-499 compound can occupy the hydrophobic pouches. The results are however quite sensitive to the settings used, including the selected binding radius, side-chain treatment (rigid/flexible), and MD snapshot used.

4.2 (+)-Cisapride

In the first set of docking poses (+)-cisapride with 4-amino-5-chloro-2-methoxybenz-1yl moiety positioned within the hydrophobic pocket only in step 18. Occupation rate was 10 %.

Step 18 (10%)

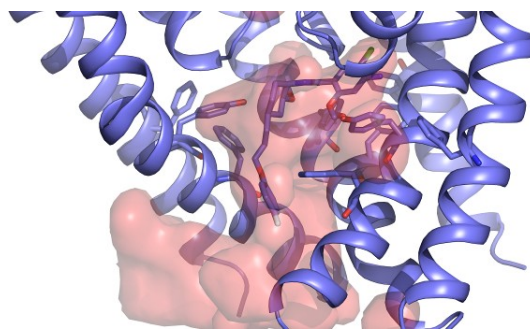


Figure 7. Binding pose of (+)-Cisapride in stick representation, based on snapshot 18 of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which 4-Fluorophenoxy moiety is positioned inside the hydrophobic pocket.

As well as for MK-499, by using smaller radius (6 Å) better results were obtained compared with the first set of docking poses. Inspection in Hollow, for most of the poses, displayed reduced volume of the

pocket. Functional groups such as 4-amino-5-chloro-2-methoxybenz-1yl and 4-Fluorophenoxy were positioned within the hydrophobic pouch in the next steps in relation to the second set of docking poses:

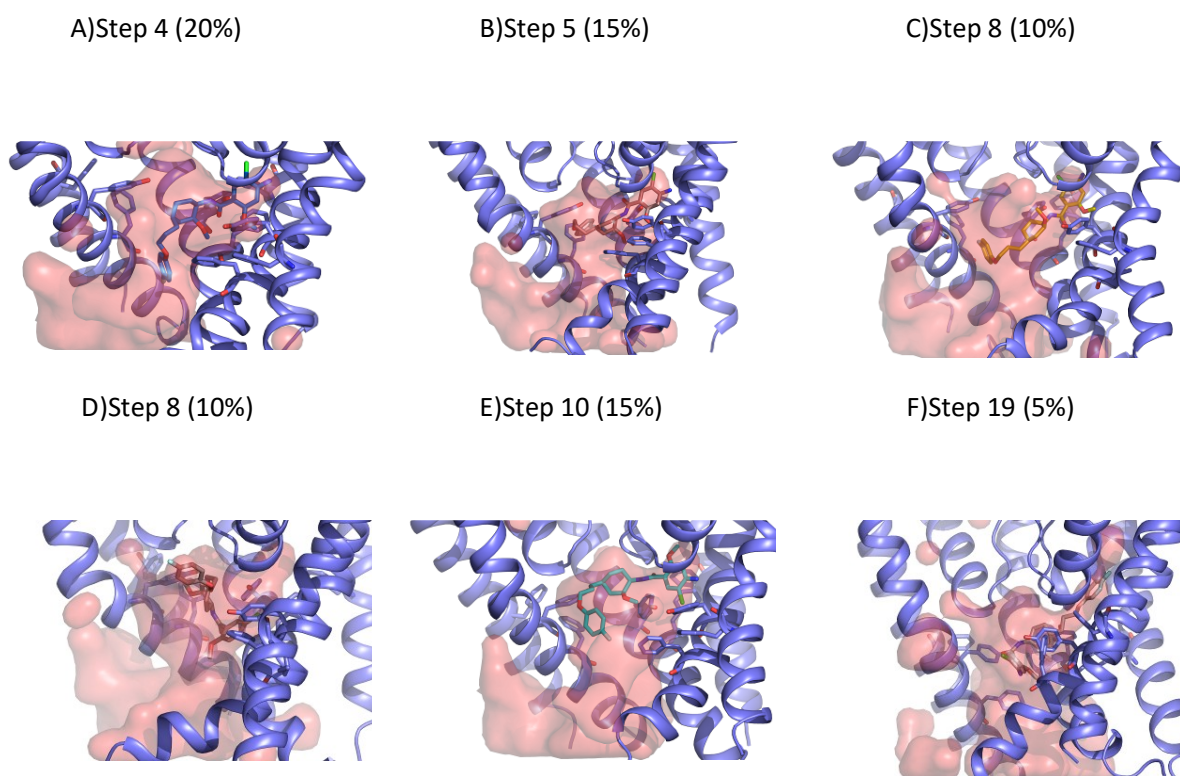


Figure 8: Binding poses of (+)-Cisapride in stick representation, based on snapshots of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which functional groups are positioned inside the hydrophobic pockets:

A) 4-amino-5-chloro-2-methoxybenz-1yl of (+)-Cisapride in step 4

B) 4-amino-5-chloro-2-methoxybenz-1yl of (+)-Cisapride in step 5

C) 4-amino-5-chloro-2-methoxybenz-1yl of (+)-Cisapride in step 8

D) One more pose occupied the pouch of the neighboring chain to the chain where docking was performed with 4-Fluorophenoxy, but not completely.

E) 4-amino-5-chloro-2-methoxybenz-1yl of (+)-Cisapride in step 10

F) 4-Fluorophenoxy of (+)-Cisapride in step 19

(4-Fluorophenoxy)propyl of (+)-cisapride occupied the pocket in the first set of docking poses with 19 MD snapshot with 80 % of pouch occupation. This was the highest percentage of occupation of pockets for (+)-cisapride, therefore, the poses were further visually investigated. It is important to highlight that the reduced volume of the pocket for most poses was observed. The “best” pose was chosen based on this

visual inspection and represented in the figure 9. This pose was used for the investigation of the molecular basis of drug block in hERG K⁺ channels. Figure 10 shows 2D ligand-protein interaction diagram.

Step 19 (Binding site Gly 648)(80%)

6 Å RIGID

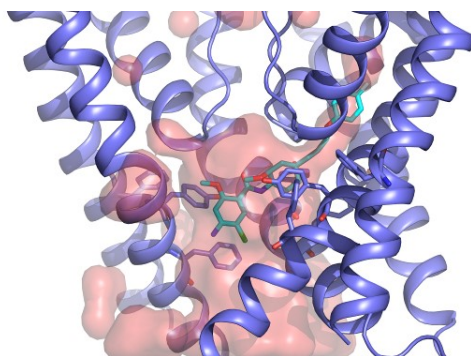


Figure 9. Binding pose of (+)-Cisapride in stick representation, based on 19 snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which (4-Fluorophenoxy)propyl group is positioned inside the hydrophobic pocket.

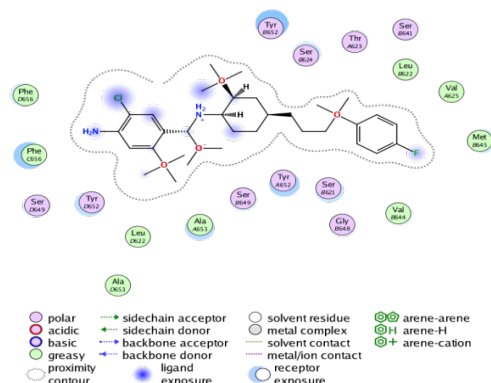


Figure 10: 2D ligand interactions of (+)-cisapride from the step with the highest percentage of occupation (19 Gly) at the hydrophobic pocket of the hERG structure, based on snapshots. 2D ligand-protein interaction diagrams were generated using MOE (Chemical Computing Group Inc., Molecular Operating Environment (moe)).

The diagram did not reveal any π - π stacking interactions or hydrogen-bonding interactions between (+)-cisapride and hERG structure. The ligand is close to the following hydrophobic residues (Phe 656 D, Ala 653 D, Leu 622 B,D, Val 625 A, Val 644BA). The molecule is in close contact with polar residues (Ser 621B, Ser 624 B, Ser 641 B, Ser 649 B,D, Thr 623 A, Gly 648B). Residues exposure with the blue shadows indicates amino acids whose side chains are partially solvent exposed (i.e. Tyr 652 B,D, Phe 656 C,D, Ala 653 A, Met 645 B, Ser 621 B, Ser 624 B). The proximity contour is the grey dotted line surrounding the ligand. Ligand exposure indicates that the blue-colored part of the ligand molecule is solvent

accessible(F,Cl,etc.).

It is important to emphasize that π - π stacking interactions or hydrogen-bonding interactions are not observed between (+) - cisapride and residues of interest in the diagram (see figure 10) , which is contrary to the previous docking studies[17], [23], [24]. Also previous Ala-scanning mutagenesis approach has identified 4 residues as critical for block of hERG channels by terfenadine and cisapride: T623 and S624 located at the base of the pore helix and Y652 and F656 located in the S6 domain [25]. Unlike methanesulfonanilides, the block of hERG by terfenadine or cisapride was not affected by mutation of the K⁺ signature sequence residue V625 or the S6 residues G648 and V659 [25].

In the third set of docking poses, 4-Fluorophenoxy moiety of (+)-cisapride positioned within the hydrophobic pouch only in step 8. The percentage of pouch occupation was 10 %. Again protein side chain flexibility was not show up as an important factor for binding the ligand inside the pocket.

Step 8 (10%)

Flexible sidechains 8 Å:Phe 557 C, Leu 622 C, Thr 623 C, Ser 624 C, Tyr 652 B,C, Phe 656 C

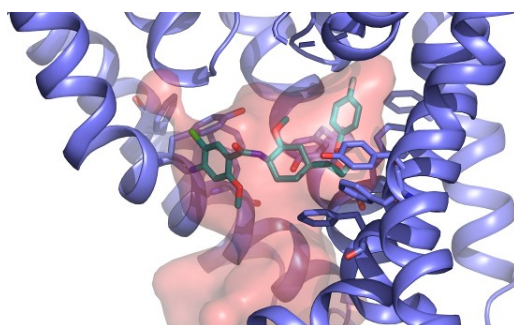


Figure 11. Binding pose of (+)-Cisapride in stick representation, based on 8 Å snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 656 residues are in slate stick representation), in which 4-Fluorophenoxy moiety is positioned inside the hydrophobic pocket.

(+)-Cisapride partially occupied the hydrophobic pouch in the next steps:

- 10 (10 %) with 4-amino-5-chloromoiety for the first set of docking poses
- 13 (5 %) with 4-amino-5-chloro-2-methoxybenz-1yl for the second set of docking poses
- 4 (10%) with 4-amino-5-chloro-2-methoxybenz-1yl for the third set of docking poses

4.3 (-)-Cisapride

(-)-Cisapride showed a similar behavior pattern of binding as (+)-cisapride, with the difference that in the first set of docking poses, no poses showed occupancy of the pocket. In most cases reduced volume of the pocket featured all poses. In the second set of docking poses (-)-cisapride occupied the pocket for the next steps with the following percentages of pouch occupation :

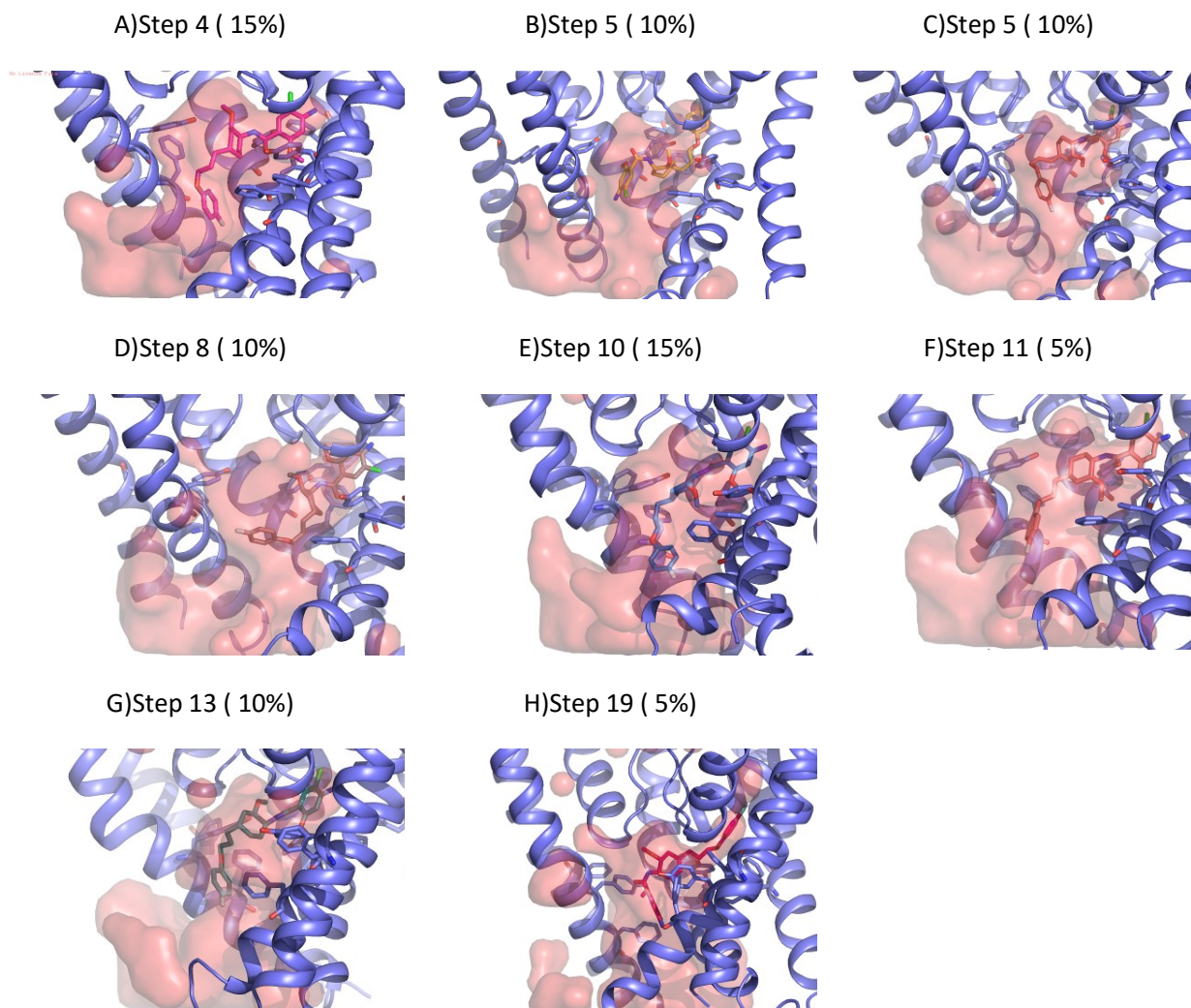


Figure 12: Binding poses of (-)-Cisapride in stick representation, based on snapshots of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which functional groups are positioned inside the hydrophobic pockets:

A) 4-amino-5-chloro-2-methoxybenz-1yl of (-)-Cisapride in step 4

B) 4-Fluorophenoxy of (-)-Cisapride in step 5

C) 4-amino-5-chloro-2-methoxybenz-1yl of (-)-Cisapride in step 5

D)4-amino-5-chloro-2-methoxybenz-
1yl of (-)-Cisapride in step 8

E)4-amino-5-chloro-2-methoxybenz-
1yl of (-)-Cisapride in step 10

F)4-amino-5-chloro-2-
methoxybenz-1yl of (-)-Cisapride in
step 11

G)4-amino-5-chloro-2-methoxybenz-
1yl of (-)-Cisapride in step 13

H) 4-Fluorophenoxy of (-)-Cisapride in
step 19

In the first set of docking with 19 MD snapshot (4-Fluorophenoxy)propyl moiety of (-)-cisapride was positioned within the hydrophobic pouch. The rate of occupation was 15%. This can be seen in the figure 13. We had the same percentage of occupancy of pockets in steps 4 and 10 in the second set of docking and in the step 8 for the third set of docking. Step 19 was chosen for further investigation of molecular interaction mechanisms between (-)-cisapride and hERG. Figure 14 shows 2D ligand-protein interaction diagram.

Step 19 (Binding site Gly 648)(15%)

6 Å RIGID

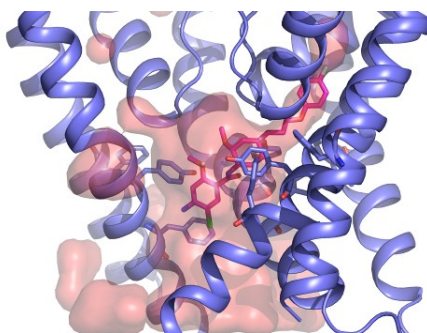


Figure 13. Binding pose of (-)-Cisapride in stick representation, based on 19 snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which (4-Fluorophenoxy)propyl group is positioned inside the hydrophobic pocket.

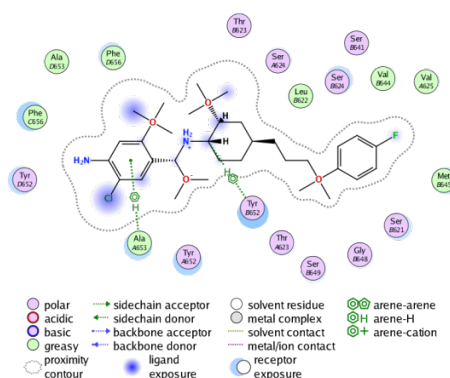


Figure 14: 2D ligand interactions of (-)-cisapride from the step with the highest percentage of occupation (19 Gly) at the hydrophobic pocket of the hERG structure, based on snapshot. 2D ligand-protein interaction diagrams were generated using MOE (Chemical Computing Group Inc., Molecular Operating Environment (moe)).

The ligand-protein interaction diagram shows two H-bonding to π surfaces, the first one between π system 4-amino-5-chloro-2-methoxybenz-1yl of (-)-cisapride and H of Ala 653 A. The second arene-H interaction was observed between π surfaces of Tyr 652 B and H of methoxypiperidin of (-)-cisapride. The ligand stays in the vicinity of other hydrophobic residues (Ala 653 D, Leu 622 B, Val 644 B, Val 625 A). The ligand stays in the vicinity of polar residues (Thr 623 A,B, Ser 624 A, Ser 641 B, Ser 649 B, Gly 648 B). The proximity contour is the grey dotted line surrounding the ligand. Ligand exposure indicates that the blue-colored part of the ligand molecule is solvent accessible (F, Cl, etc.). Residues exposure with the blue shadows indicates amino acids whose side chains are partially solvent exposed (i.e. Phe 656 C,D, Tyr 652 A,B,D, Ser 621 B, Ser 624 B, Met 645 B).

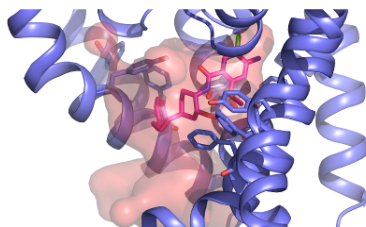
It is interesting to note that although the selected poses for interaction testing with respect to the two known diastereomers of cisapride have been similarly positioned within the pocket, in the case of (-)-cisapride two H-bonding to π surfaces were observed. In the previous docking study CH- π interactions were observed between Tyr 652 in the adjacent subunit with the CH group adjacent to the cationic nitrogen in the central piperidine ring [23]. π - π stacking interactions or hydrogen-bonding interactions were not observed between (-)-cisapride and key residues for hERG drug block as already mentioned above in the case of (+)-cisapride.

In the third set of docking poses, poses which inserted functional groups inside the pocket were obtained only in step 8. In this case they were 4-Fluorophenoxy moiety and 4-amino-5-chloro-2-methoxybenz-1yl moiety with 15% of occupation.

Step 8 (15%)

Flexible sidechains 8 Å:Phe 557 C, Leu 622 C, Thr 623 C, Ser 624 C, Tyr 652 B,C, Phe 656 C

A)



B)

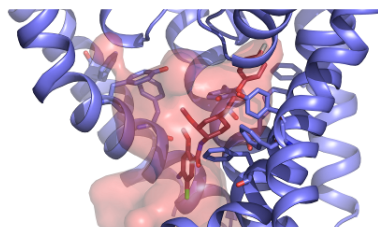


Figure 15: Binding poses of (+)-Cisapride in stick representation, based on 8 snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which functional groups were positioned inside the hydrophobic pockets:

A) 4-amino-5-chloro-2-methoxybenz-1yl of (-)-Cisapride in step 8

B) 4-Fluorophenoxy of (-)-Cisapride in step 8

(-)-Cisapride partially occupied the hydrophobic pouch in the next steps:

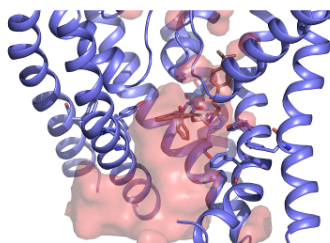
- 3 (10 %) with 4-amino-5-chloro-2-methoxybenzamide for the second set of docking poses
- 6 (5%) with methoxy group for the second set of docking poses
- 14 (35%) with methoxy group for the second set of docking poses
- 10 (10 %) with 4-amino-5-chloro group for the first set of docking poses
- 13 (5 %) with 4-amino-5-chloro group for the first set of docking poses
- 14 (35 %) with methoxy group for the second set of docking poses
- 18 (20%) with 4-amino-5-chloro-2-methoxybenz-1yl moiety for the first set of docking poses
- 1 (10%) with 4-amino-5-chloro-2-methoxybenz-1yl moiety for the third set of docking poses

Summarizing, similar to the results obtained for MK-499, different functional groups of ($\pm$)-Cisapride are predicted to be able to protrude into the hydrophobic pockets. However the results are very diverse, suggesting that detailed analyses of the binding modes, possibly with long molecular dynamics simulations will be required to further analyze the importance of the pouches for inhibition.

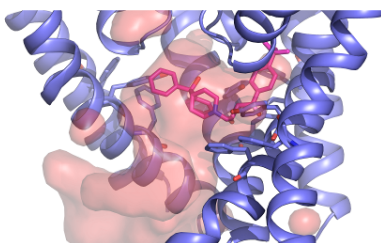
4.4 R-Terfenadine

In the first set of docking poses, R-terfenadine did not show any poses that occupy the presumable pouch. Most of the poses showed reduced volume of the pocket. Some poses demonstrated occupancy of the pockets, but at the same time they got out of the pocket significantly, therefore, these poses were rejected. In the second set of docking poses R-terfenadine occupied the pocket for the next steps and with the following percentages of pouch occupation :

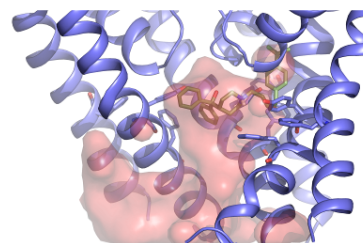
A)Step 3 (30 %)



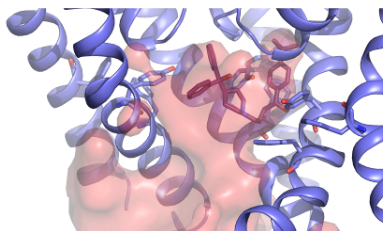
B)Step 4 (5 %)



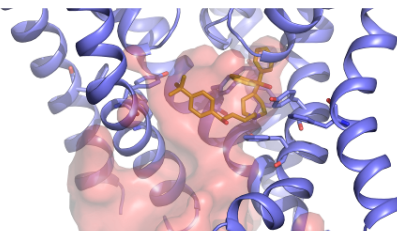
C)Step 5 (15 %)



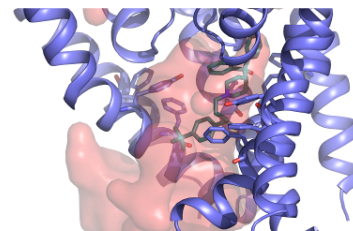
D)Step 8 (30 %)



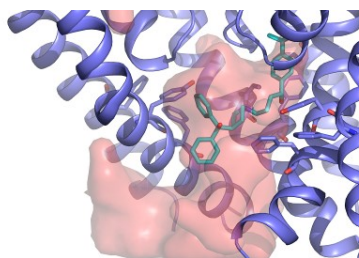
E)Step 8 (30 %)



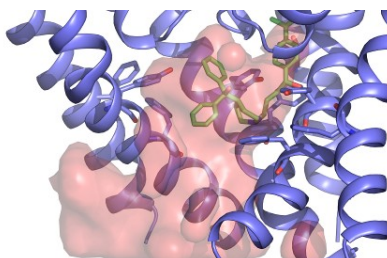
F)Step 10 (25 %)



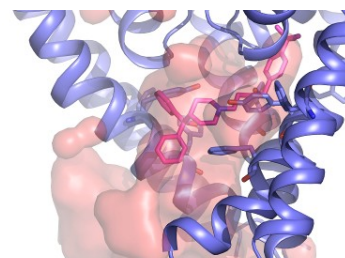
G)Step 10 (25 %)



H)Step 11 (25 %)



I)Step 14 (25 %)



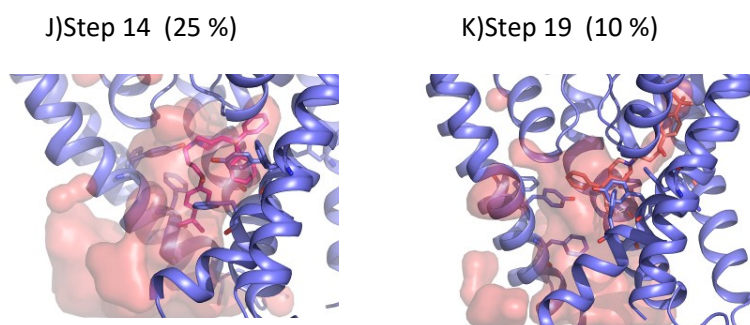


Figure 16: Binding poses of R-Terfenadine in stick representation, based on snapshots of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which functional groups are positioned inside the hydrophobic pockets:

- | | | |
|--|--|--|
| A) 4-tert-butylphenyl moiety of R-Terfenadine in step 3 | B) 4-tert-butylphenyl moiety of R-Terfenadine in step 4 | C) 4-tert-butylphenyl moiety of R-Terfenadine in step 5 |
| D) 4-tert-butylphenyl moiety of R-Terfenadine in step 8 | E) Phenyl moiety of R-Terfenadine in step 8 | F) Phenyl moiety of R-Terfenadine in step 10 |
| G) 4-tert-butylphenyl moiety of R-Terfenadine in step 10 | H) 4-tert-butylphenyl moiety of R-Terfenadine in step 11 | I) 4-tert-butylphenyl moiety of R-Terfenadine in step 14 |
| J) Phenyl moiety of R-Terfenadine in step 14 | K) 1-(4-tert-butylphenyl)butan-1-ol moiety of R-Terfenadine in step 19 | |

We had the same percentage of occupancy of pockets in steps 3 (30%) and 8(30%) in the second set of docking and in step 19 Gly (30%) with protein side chains regarded as rigid. The pose from step 8 shown in Figure 16 under the letter D was selected for further calculating interactions in the MOE program. Figure 17 shows 2D ligand-protein interaction diagram.

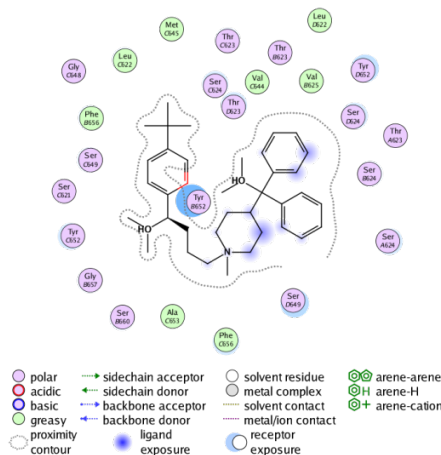


Figure 17: 2D ligand interactions of R-Terfenadine from the step with the highest percentage of occupation (step 8) at the hydrophobic pocket of the hERG structure, based on snapshots. 2D ligand-protein interaction diagrams were generated using MOE (Chemical Computing Group Inc., Molecular Operating Environment (moe)).

The diagram did not reveal any π - π stacking interactions or hydrogen-bonding interactions between R-Terfenadine and hERG structure. Ligand stays in the vicinity of other hydrophobic residues (Ala 653 C, Leu 622 C,D, Val 625 B, Val 644 C, Met 645 B, Phe 656 B). The molecule is in close contact with polar residues (Thr 623 A,B,C, Ser 621 C, Ser 624 B,C, Ser 649 C, Ser 660 B, Gly 648 C, Gly 657B). There are several residues in the close proximity of the ligand with receptor exposure shadows (i.e. Tyr 652 B,D,C, Thr 623D, Ser 624 A,D, Ser 649D). The proximity contour is the grey dotted line surrounding the ligand. The red colored part of the ligand clashes with protein, meaning it is very close. Ligand exposure indicates that the blue-colored part of the ligand molecule is solvent accessible (i.e. part of the benzene ring, etc.)

The trend of absence of π - π –stacking interactions with Y 652 and F 656 continues, which is in contrast to previous docking studies [17], [23], [24], [26]. The absence of hydrogen bonds is also contradictory to previous studies [17], [24].

The first set of docking poses with 19 MD snapshot was performed using radius of 6 Å around the center of the binding site. Gly 648 was chosen as the center of the binding site and with protein side chains regarded as rigid. Under these conditions R-terfenadine occupied the pocket with 1-(4-tert-butyphenyl)butan-1-ol group (30%). Most of these poses showed reduced volume of the pocket.

Step 19 (Binding site Gly 648)(30%)

6 Å RIGID

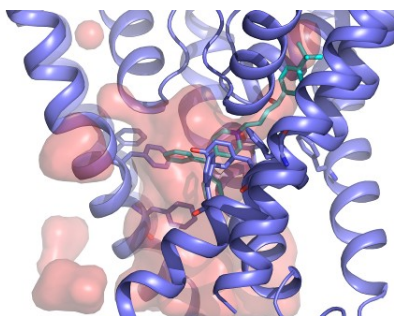


Figure 18. Binding pose of R-terfenadine in stick representation, based on 19 snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which 1-(4-tert-butyphenyl)butan-1-ol group is positioned inside the hydrophobic pocket.

In the third set of docking poses R-terfenadine occupied the pocket for the next steps and with the following percentages of pouch occupation :

- Step 1 (5 %) with phenyl group

- Step 3 (5 %) with phenyl group
- Step 8 (15 %) with phenyl group
- Step 18 (5 %) with phenyl group

In Figure 14 only step 8 will be shown as an example.

Step 8 (15%)

Flexible sidechains 8 Å:Phe 557 C, Leu 622 C, Thr 623 C, Ser 624 C, Tyr 652 B,C, Phe 656 C

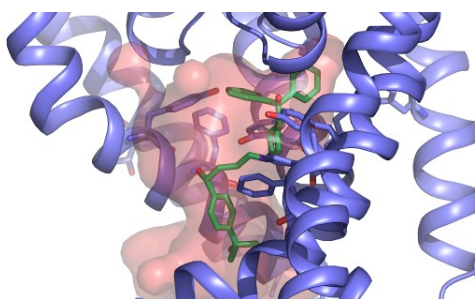
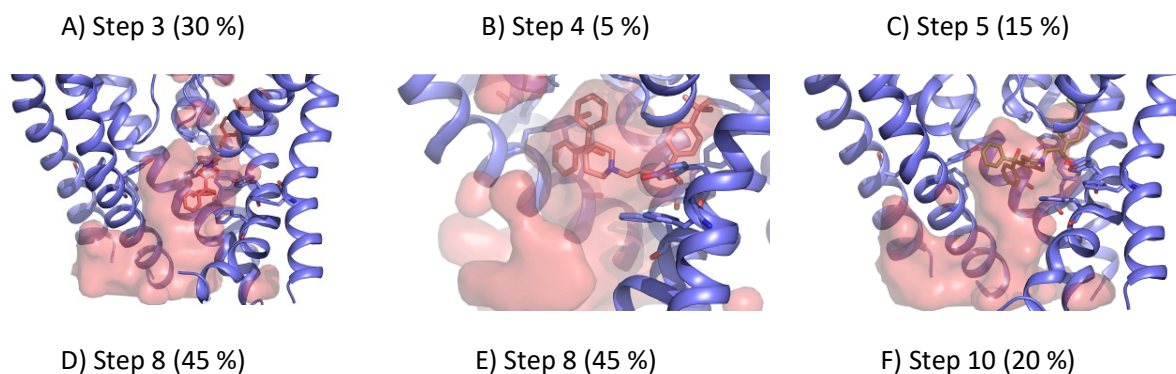


Figure 19: Binding poses of R-terfenadine in stick representation, based on 8 snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which phenyl group is positioned inside the hydrophobic pockets:

4.5 S-Terfenadine

As in the case of R-Terfenadine in the first set of docking poses, S-terfenadine did not have any poses that occupied the presumable hydrophobic pouches. In the second set of docking poses S-Terfenadine positioned functional groups within the hydrophobic pocket in the next steps:



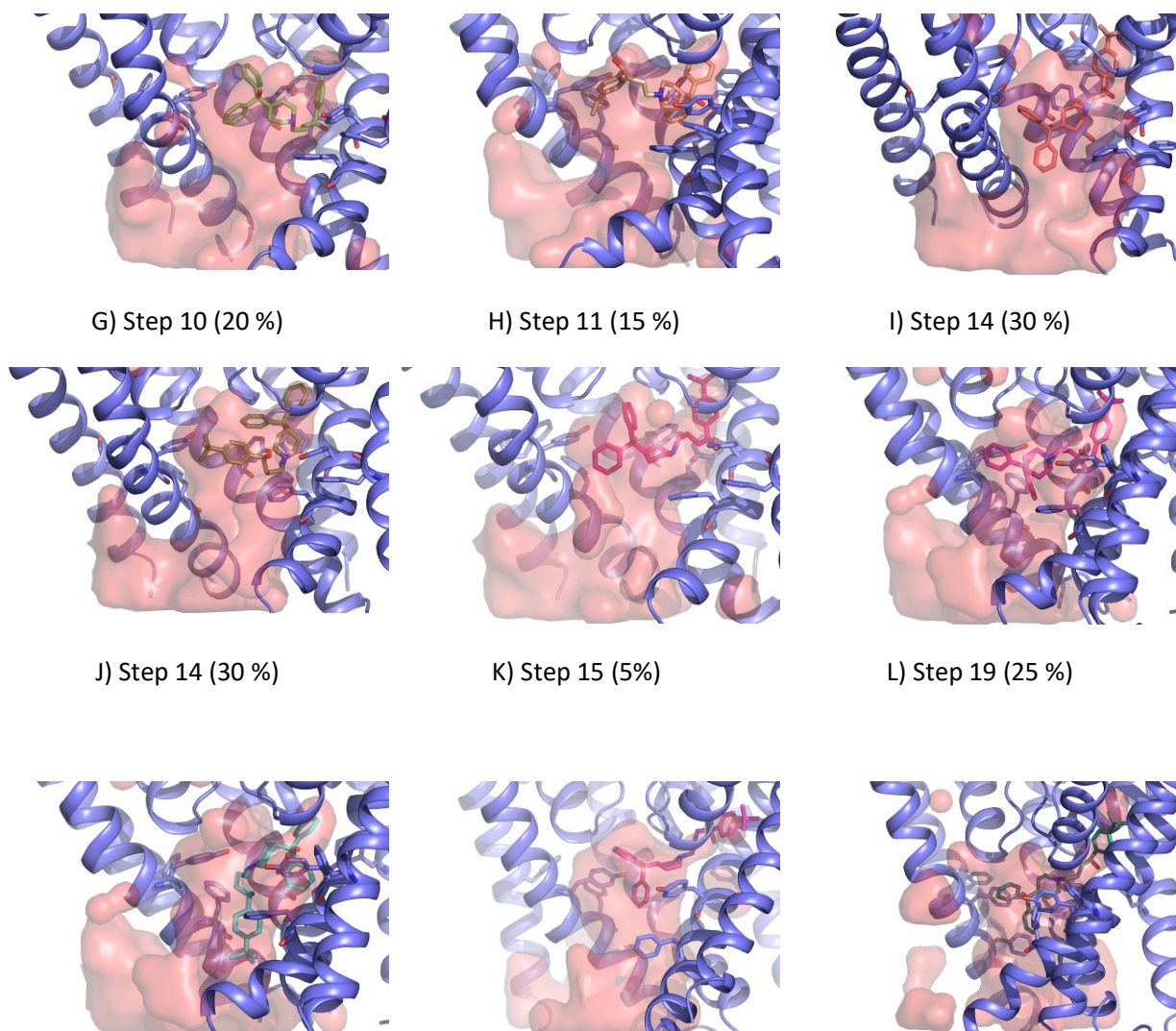


Figure 20: Binding poses of *S*-Terfenadine in stick representation, based on snapshots of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which functional groups are positioned inside the hydrophobic pockets:

- | | | |
|---|---|---|
| A) 4-tert-butylphenyl moiety of <i>S</i> -Terfenadine in step 3 | B) 4-tert-butylphenyl moiety of <i>S</i> -Terfenadine in step 4 | C) 4-tert-butylphenyl moiety of <i>S</i> -Terfenadine in step 5 |
| D) 4-tert-butylphenyl moiety of <i>S</i> -Terfenadine in step 8 | E) Phenyl moiety of <i>S</i> -Terfenadine in step 8 | F) 4-tert-butylphenyl moiety of <i>S</i> -Terfenadine in step 10 |
| G) Phenyl moiety of <i>S</i> -Terfenadine in step 10 | H) 4-tert-butylphenyl moiety of <i>S</i> -Terfenadine in step 11 | I) 4-tert-butylphenyl moiety of <i>S</i> -Terfenadine in step 14 |
| J) Phenyl moiety of <i>S</i> -Terfenadine in step 14 | K) 1-(4-tert-butylphenyl)butan-1-ol of <i>S</i> -Terfenadine in step 15 | L) 1-(4-tert-butylphenyl)butan-1-ol of <i>S</i> -Terfenadine in step 19 |

The diagram did not reveal any π - π stacking interactions or hydrogen-bonding interactions between S-Terfenadine and hERG structure. Ligand is near hydrophobic residues (i.e. Ala 653 C, Phe 656 B, Leu 622 C, Val 625 B, Val 644 C, Met 645 C). Ligand stays in the vicinity of polar residues (i.e. Thr 623 A,B, C, Ser 621 C, Ser 624 B, Ser 649 C, Ser 660 B, Gly 648 C, Gly 657 B). There are several residues in the close proximity of the ligand marked by receptor exposure shadows (i.e. Tyr 652 B,D,C, Ser 624 A,C,D, Thr 623 D, Ala 653 A, Phe 656 C...). The proximity contour is the grey dotted line surrounding the ligand. Ligand exposure indicates that the blue-colored part of the ligand molecule is solvent accessible (i.e. a part of the benzene ring, etc.). The red colored part of the ligand clashes with protein, meaning it is very close.

Step 19 (Binding site Gly 648)(15%)

6 Å RIGID

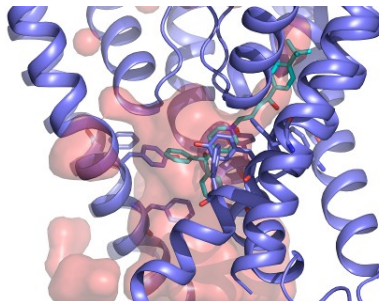


Figure 22. Binding pose of *S*-Terfenadine in stick representation, based on 19 snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which 1-(4-tert-butylphenyl)butan-1-ol group is positioned inside the hydrophobic pocket.

In the third set of docking, results are:

- Step 1 (5%), phenyl moiety
- Step 5 (5%), phenyl moiety
- Step 8 (10%), phenyl moiety

In Figure 17 only step 5 will be shown as an example.

Step 5 (5%)

Flexible sidechains 8 Å: Phe 557 C, Leu 622 C, Thr 623 C, Ser 624 C, Tyr 652 B,C, Phe 656 C

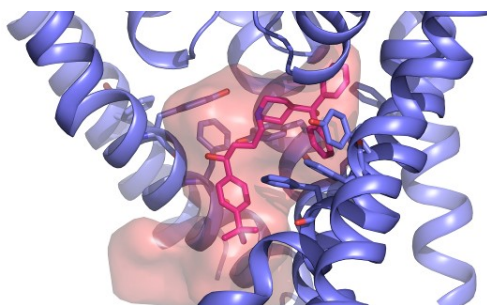


Figure 23: Binding poses of (*S*)-Terfenadine in stick representation, based on 8 snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which phenyl group is positioned inside the hydrophobic pocket.

S-Terfenadine partially occupied the hydrophobic pouch in the next steps:

- Step 18 (15%) with phenyl moiety for the first set of docking poses
- Step 3 (5%) with phenyl moiety for the third set of docking poses

S-Terfenadine and R-Terfenadine showed a similar pattern of binding with hERG structure and approximately identical percentages of occupation and representation of the same steps and groups. Some of the poses showed reduced volume of the pocket.

4.6 Clofilium

The protein side chain flexibility was extremely important for Clofilium binding inside the pocket, because in the first and second sets of docking poses, no poses showed occupancy of the presumable pouches. In most cases Gold could not find a solution or solutions themselves were ineffective in validating the role of the pockets.

Unlike the first two sets of docking poses, in the third set clofilium occupied the hydrophobic pocket in the next steps:

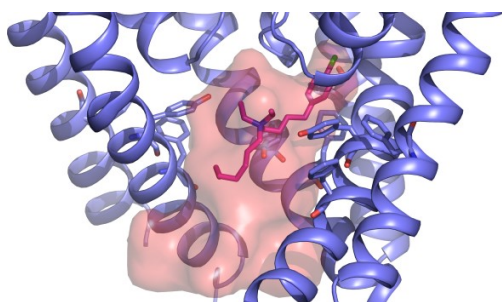
- Step 1 (15%) with 4-chlorophenyl and heptyl moiety
- Step 3 (5%) with heptyl moiety
- Step 8 (5%) with heptyl moiety
- Step 10 (5%) with 4-chlorophenyl

In Figure 24 only steps 8 and 1 will be shown as examples.

Step 1 (15%)

Flexible sidechains 8 Å: Phe 557 C, Leu 622 C, Thr 623 B,C, Tyr 652 B,C, Ser 624 C,D, Phe 656 B,C

A)



Step 8 (5%)

Flexible sidechains 8 Å: Phe 557 C, Leu 622 C, Thr 623 C, Ser 624 C, Tyr 652 B,C, Phe 656 C

B)

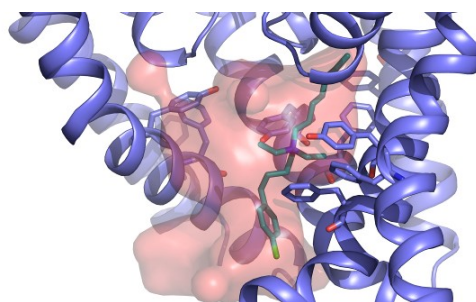


Figure 24: Binding poses of Clofilium in stick representation, based on 8 snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which functional groups are positioned inside the hydrophobic pockets:

A) 4-Chlorophenyl of Clofilium in step 1

B) Heptyl moiety of Clofilium in step 8

The pose from step 1 shown in Figure 24 under the letter A was selected for further calculating interactions in the MOE program. Figure 25 shows 2D ligand-protein interaction diagram.

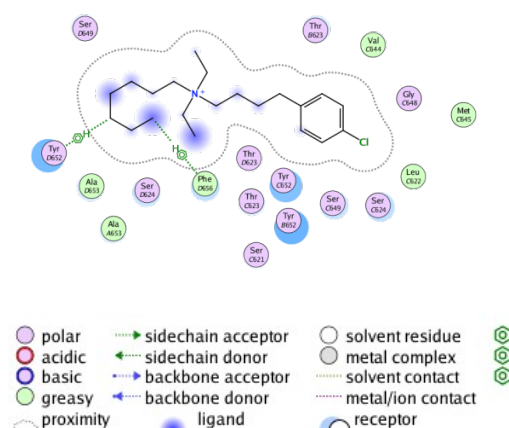


Figure 25: 2D ligand interactions of Clofilium from the step with the highest percentage of occupation (1) at the hydrophobic pocket of the hERG structure, based on snapshot. 2D ligand-protein interaction diagrams were generated using MOE (Chemical Computing Group Inc., Molecular Operating Environment (moe)).

The ligand–protein interaction diagram shows two H-bonding to π surfaces, the first one between π surfaces of Tyr 652 D and H of heptyl moiety of Clofilium. The second arene-H interaction was observed between π surfaces Phe 656 D and H of heptyl moiety of Clofilium. The diagram did not reveal any π - π stacking interactions between Clofilium and hERG structure. Ligand is near hydrophobic residues (i.e., Ala 653 A, D, Leu 622 C, Val 644 C, Met 645 C). Ligand stays in the vicinity of polar residues (i.e. Thr 623 C, D, Ser 621 C, Gly 648 C). There are several residues in the close proximity of the ligand marked by receptor exposure shadows (i.e. Ser 624 C, D, Ser 649 C, D, Thr 623 B, Tyr 652 B, C, D, Phe 656 D). The proximity contour is the grey dotted line surrounding the ligand. Ligand exposure indicates that the blue-colored part of the ligand molecule is solvent accessible (i.e. butyl-diethyl-heptylazanium of Clofilium).

The decreased drug sensitivity of S624A channels and the rapid recovery from block suggest that clofilium interactions with Ser624 are important for drug binding at depolarized potentials and for the exceptionally slow rates of recovery from block in WT HERG channels [27].

Polar interactions between the -OH of Ser and the Cl of clofilium may help stabilize clofilium binding[27]. They were noticed interactions between the chlorine atom and the -OH group of Ser624

and π -stacking interactions between the phenyl ring of clofilium and Tyr652[27], but diagram did not detect any H-bonding or π - π interactions, only two H-bonding to π surfaces were recorded. Previous study suggested that the aromatic and polar groups of clofilium and ibutilide are oriented toward the selectivity filter end of the central cavity, and the aliphatic tail of the drugs are pointed toward the intracellular opening of the inner helices [27]. Based on the obtained results, which is in contrast to the previous study [27], the chlorophenyl group or heptyl moiety of clofilium were positioned within hydrophobic pockets.

The results from this docking study suggest that the mode of binding was difficult to determine, and that further research in this field is necessary.

Clofilium partially occupied the hydrophobic pouch in the third set with 4-chlorophenyl in step 0.

4.7 Sertindole

In the second set of docking poses, sertindole positioned 1-imidazolidin-2-one moiety within the presumed side pockets in step 8. The rate of occupation was 55%. All poses displayed reduced volume of the pocket. The poses, which occupied the pocket of the chain adjacent to the chain where the docking was conducted are included into the percentage.

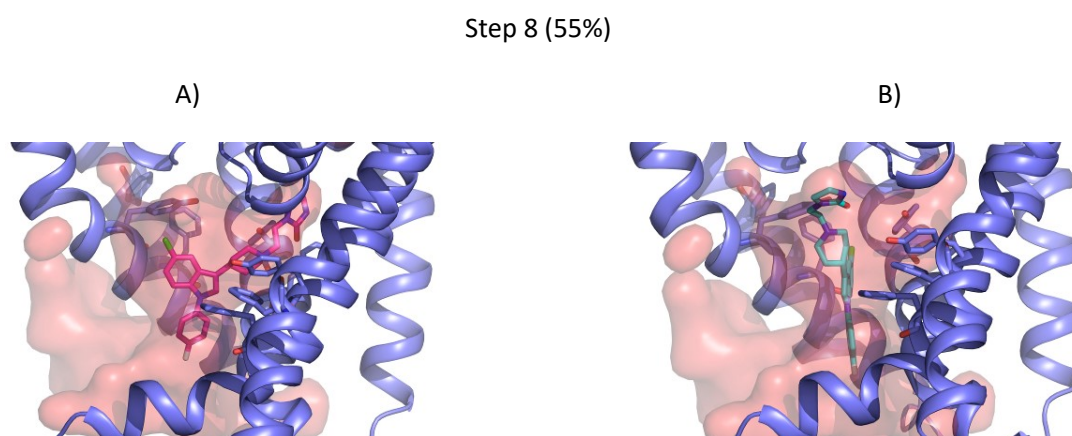


Figure 26: Binding poses of Sertindole in stick representation, based on 8 snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which functional groups are positioned inside the hydrophobic pockets:

A) 1-imidazolidin-2-one moiety of Sertindole in step 8.

B) 1-imidazolidin-2-one moiety of Sertindole in step 8, the pose was positioned in the pocket of the chain adjacent to the chain where docking was performed.

The pose from step 8 shown in Figure 26 under the letter A was selected for further investigation of molecular interaction mechanisms between sertindole and hERG. In contrast to previous studies[17], π - π interactions were not observed. Figure 27 shows 2D ligand-protein interaction diagram.

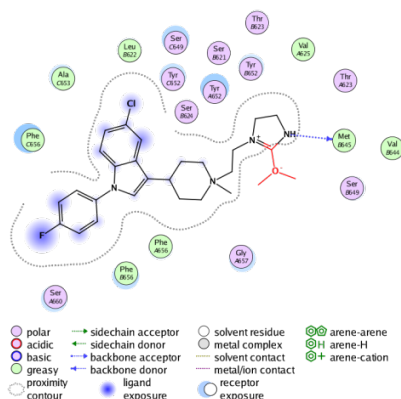


Figure 27: 2D ligand interactions of Sertindole from the step with the highest percentage of occupation (8) at the hydrophobic pocket of the hERG structure, based on snapshot. 2D ligand-protein interaction diagrams were generated using MOE (Chemical Computing Group Inc., Molecular Operating Environment (moe)).

The diagram did not reveal any π - π stacking interactions between Sertindole and hERG structure. Hydrogen-bonding interactions occur between ligand (imidazolidin-2-one) and Met 645 B (backbone acceptor). The molecule is in close contact with polar residues (Thr 623 A,B, Ser 621 B, Ser 624 B, Ser 649 B). Ligand is near hydrophobic residues (i.e. Phe 656 A, Leu 622 B, Val 625 A, Val 644 B). There are several residues in the close proximity of the ligand marked by receptor exposure shadows (i.e. Tyr 652 A,B,C, Ser 649 C, Ser 660 A, Gly 657 A, Phe 656 C,B, Ala 653 C). The proximity contour is the grey dotted line surrounding the ligand. Ligand exposure indicates that the blue-colored part of the ligand molecule is solvent accessible (i.e. part of the benzene ring F, Cl, etc.). The red colored part of the ligand clashes with protein.

In the first set of docking with 19 MD snapshot, 1-(2-piperidin-1-yl)ethyl-imidazolidin-2-one of sertindole occupied the pocket by 20%. Also the reduced volume of the pocket was detected.

Step 19 (Binding site Gly 648)(20%)

6 Å RIGID

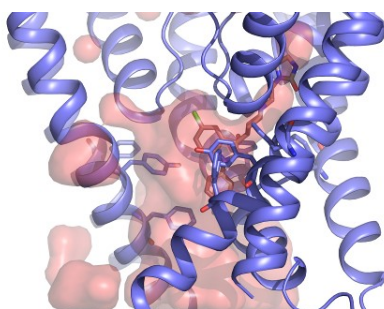


Figure 28. Binding pose of Sertindole in stick representation, based on 19 snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which 1-(2-piperidin-1-yl)ethyl-imidazolidin-2-one of sertindole positioned inside the hydrophobic pocket.

In the third set of docking poses sertindole occupied the pocket in step 13 with 1-imidazolidin-2-one moiety by 20%. The protein side chain flexibility was highlighted as an important feature, because under this condition, the best poses were obtained, which occupied side pouch without unwanted characteristics as reduced volume of the pocket.

Step 13 (20%)

Flexible sidechains 8 Å:Phe 557 C, Leu 622 C, Thr 623 C, Ser 624 C, Tyr 652 B,C, Phe 656 C

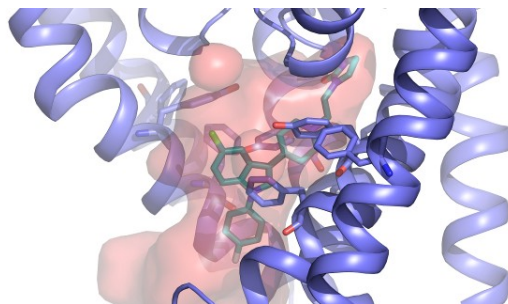


Figure 29: Binding poses of Sertindole in stick representation, based on 13 snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which 1-imidazolidin-2-one of sertindole is positioned inside the hydrophobic pocket.

In the second set with 19 MD snapshot docking was performed using 10 Å radius and Gly 648 as the binding site and using certain residues, whose side chains were set to rotate freely. Under these terms, 1-imidazolidin-2-one moiety of sertindole was positioned within hydrophobic pocket by 15 %.

Sertindole partially occupied the hydrophobic pouch in the following steps:

- Step 4 (5%) with 1-imidazolidin-2-one moiety in the second set of docking
- Step 5 (5%) with 1-imidazolidin-2-one moiety in the second set of docking
- Step 10 (60%) with 1-imidazolidin-2-one moiety in the second set of docking
- Step 14 (15%) with 1-imidazolidin-2-one moiety in the second set of docking
- Step 14 (15%) with 1-imidazolidin-2-one moiety in the first set of docking, but one pose was positioned in the pocket of the chain adjacent to the chain where docking was performed
- Step 1 (5%) with 4-fluorophenyl in the third set of docking
- Step 8 (25%) with 1-imidazolidin-2-one moiety in the third set of docking

4.8 Astemizole

Astemizole inserts functional groups inside the hydrophobic pocket in the following steps and in the first set of docking poses:

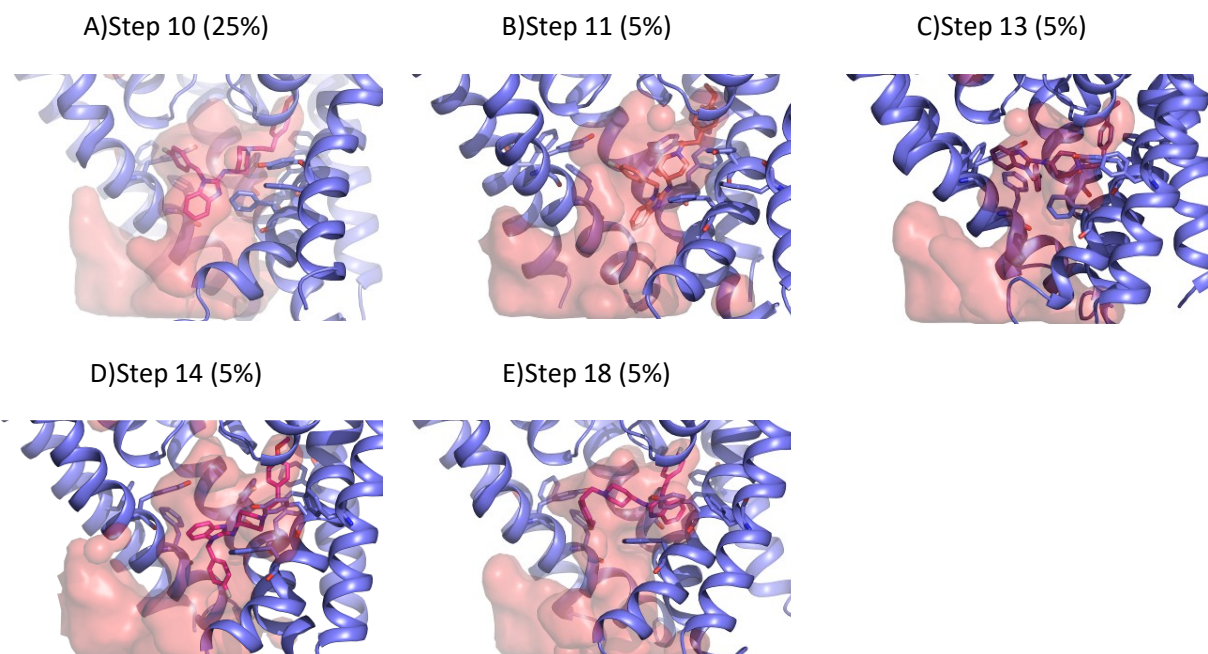


Figure 30: Binding poses of Astemizole in stick representation, based on snapshots of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which functional groups are positioned inside the hydrophobic pockets:

A) 4-methoxyphenyl of Astemizole in step 10 (reduced volume)

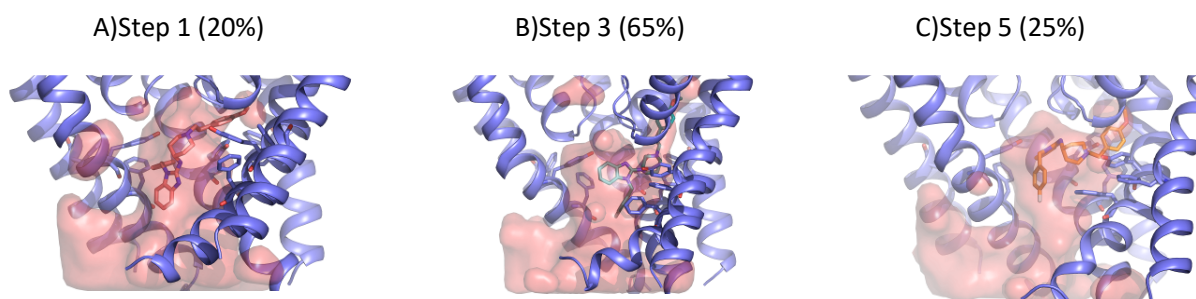
B) 4-methoxyphenyl of Astemizole in step 11 (reduced volume)

C) 4-methoxyphenyl of Astemizole in step 13

D) 4-methoxyphenyl of Astemizole in step 14

E) 4-fluorophenyl of Astemizole in step 18

Again using smaller radius, better results were obtained. In the second set of docking poses the results were:



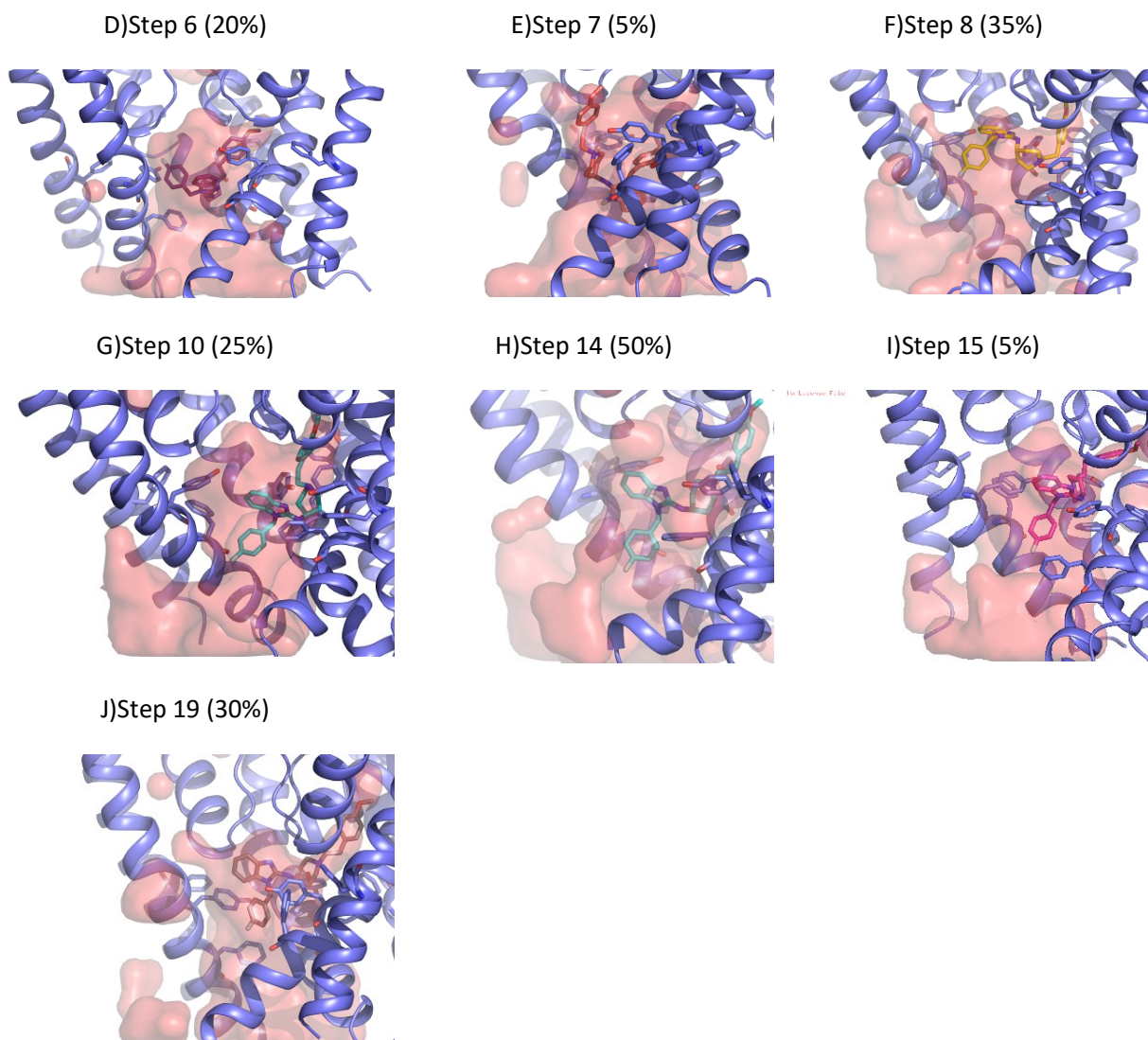


Figure 31: Binding poses of Astemizole in stick representation, based on snapshots of hERG structure in slate cartoon representation (One chain is hidden and Phe 557,Tyr 652,Phe 556 residues are in slate stick representation), in which functional groups are positioned inside the hydrophobic pockets:

A) 4-methoxyphenyl of Astemizole in step 1 (reduced volume)

B) N-(1-(2-(4-methoxyphenyl)ethyl)piperidin-4-yl) of Astemizole in step 3 (reduced volume)

C) 4-methoxyphenyl of Astemizole in step 5 (reduced volume)

D)) methoxy group of Astemizole in step 6

E) 4-methoxyphenyl of Astemizole in step 7(the pose positioned in the pocket of the chain adjacent to the chain where docking was

F) 4-methoxyphenyl of Astemizole in step 8 (reduced volume)

performed)(reduced
volume)

G) 4-methoxyphenyl of Astemizole in
step 10

H) 4-methoxyphenyl of Astemizole
in step 14 (reduced volume)

I) 4-methoxyphenyl of Astemizole
in step 15 (reduced volume)

J) 2-(4-methoxyphenyl)ethyl) of
Astemizole in step 19(reduced volume)

The highest percentage of occupancy of pockets was in step 3 in the second set of docking poses. The pose shown in the figure 31 under the letter B is used for further investigation of molecular interaction mechanisms between astemizole and hERG .Based on this diagram π - π interactions with 557 were not observed, which is contrary to the previous study [24]. Figure 32 shows 2D ligand-protein interaction diagram.

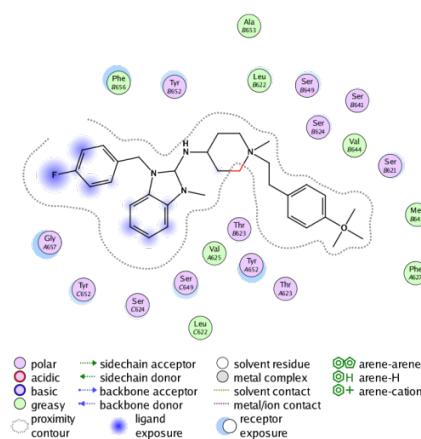


Figure 32:2D ligand interactions of Astemizole from the step with the highest percentage of occupation (3) at the hydrophobic pocket of the hERG structure,based on snapshots.2D ligand-protein interaction diagrams were generated using MOE (Chemical Computing Group Inc., Molecular Operating Environment (moe)).

The diagram did not reveal any π - π stacking interactions or hydrogen-bonding interactions between Astemizole and hERG structure. Ligand stays in the vicinity of other hydrophobic residues (i.e. Ala 653 B, Leu 622 B,C, Val 625 A, Val 644 B, Met 645 B,Phe 627 A). The molecule is in close contact with polar residues (i.e. Thr 623 A, B, Ser 624 B,C, Ser 641 B). There are several residues in the close proximity of the ligand marked by receptor exposure shadows (i.e. Tyr 652 A,B,C, Gly 657 A, Ser 621 B, Ser 649B,C, Phe 656 B). The proximity contour is the grey dotted line surrounding the ligand. Ligand exposure indicates that the blue-colored part of the ligand molecule is solvent accessible (i.e. part of the benzene ring,F, etc.). The red colored part of the ligand clashes with protein.

In the first set of docking with 19 MD snapshot (1-(2-(4-methoxyphenyl)ethyl)piperidin-4-yl) moiety of astemizole was positioned within the hydrophobic pouch. The rate of occupation was 20 %.(reducedvolume).

Step 19 (Binding site Gly 648)(20%)

6 Å RIGID

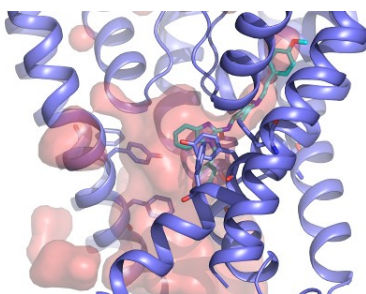


Figure 33. Binding pose of Astemizole in stick representation, based on 19 snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which (1-(2-(4-methoxyphenyl)ethyl)piperidin-4-yl) of astemizole was positioned inside the hydrophobic pocket.

In the third set of docking poses astemizole occupied the pocket in the next steps and by the next of percentages of pouch occupation :

- Step 1 (10%) with benzimidazol moiety and with 4-methoxyphenyl
- Step 4 (5%) with methoxy group
- Step 8 (10%) with methoxy group

In Figure 25 only step 8 will be shown as an example.

Step 8 (10%)

Flexible sidechains 8 Å: Phe 557 C, Leu 622 C, Thr 623 C, Ser 624 C, Tyr 652 B,C, Phe 656 C

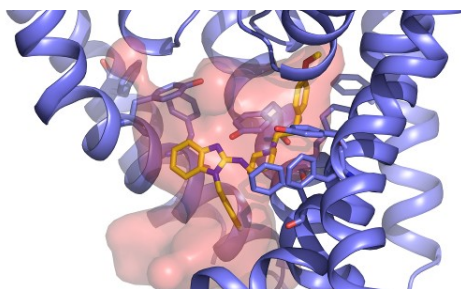


Figure 34: Binding poses of astemizole in stick representation, based on 8 snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557,Tyr 652,Phe 556 residues are in slate stick representation), in which methoxy group is positioned inside the hydrophobic pocket.

Astemizole partially occupied the hydrophobic pouch in step 4 with 4-methoxyphenyl in the first set of docking. The rate of occupation was 5%.

4.9 Dofetilide

Dofetilide positioned methanesulfonanilide moiety inside pockets in the first set of docking poses in 13 (5%) and 14 (10%).

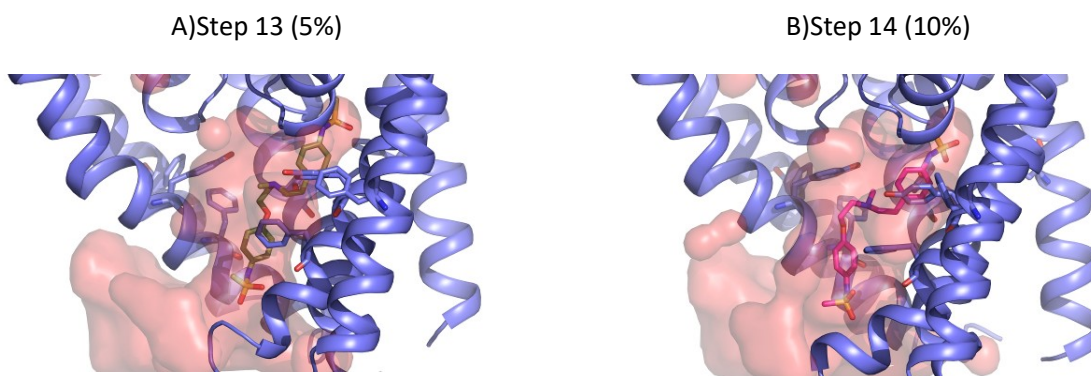
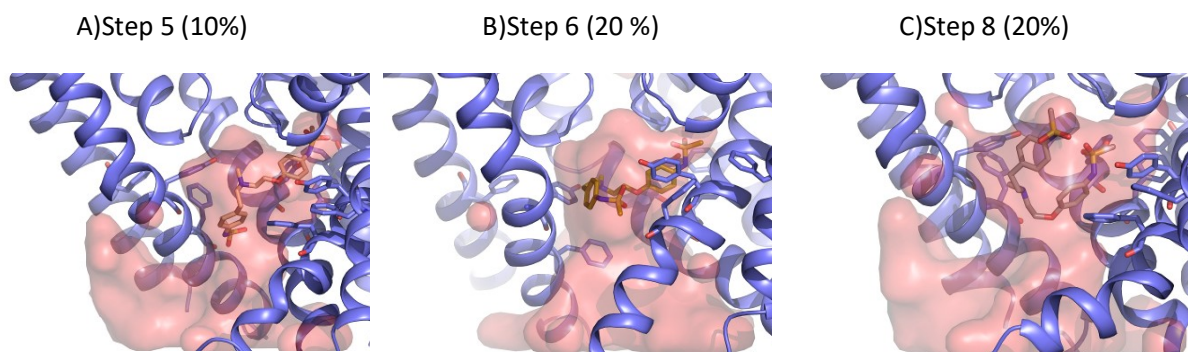


Figure 35: Binding poses of Dofetilide in stick representation, based on snapshots of hERG structure in slate cartoon representation (One chain is hidden and Phe 557,Tyr 652,Phe 556 residues are in slate stick representation), in which methanesulfonanilide moiety is positioned inside the hydrophobic pockets:

A) Methanesulfonanilide moiethy of Dofetilide in step 13 B) Methanesulfonanilide moiethy of Dofetilide in step 14

In the second set of docking poses dofetilide occupied hydrophobic pouch in the next steps:



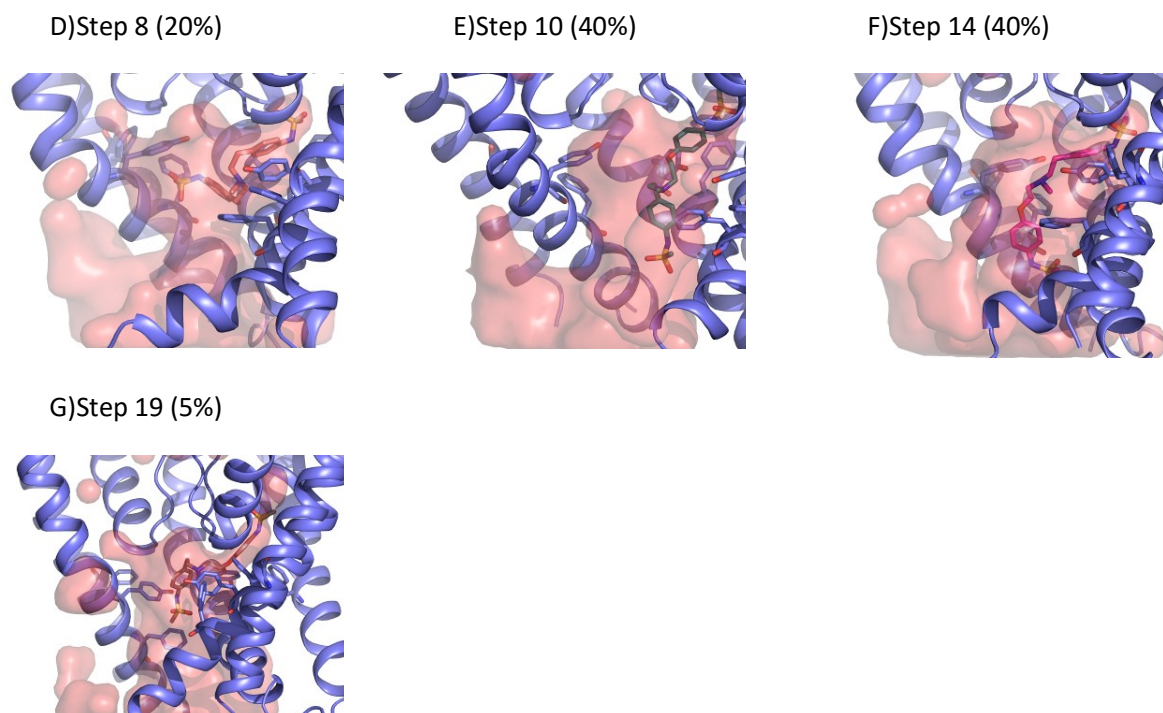


Figure 36: Binding poses of Dofetilide in stick representation, based on snapshots of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which functional group is positioned inside the hydrophobic pockets:

A) Methanesulfonanilide moiety of Dofetilide in step 5

B) Methanesulfonanilide moiety of Dofetilide in step 6

C) Methanesulfonanilide moiety of Dofetilide in step 8 (the pose positioned in the pocket of the chain adjacent to the chain where docking was performed) (reduced volume)

D) Methanesulfonanilide moiety of Dofetilide in step 8

E) Methanesulfonanilide moiety of Dofetilide in step 10

F) Methanesulfonanilide moiety of Dofetilide in step 14

G) 4-(methanesulfonamido)phenoxy of Dofetilide in step 19 (reduced volume)

In the first set of docking with 19 MD snapshot (4-(methanesulfonamido)phenoxy)ethyl-methylamino moiety and with N-(4-(2-(methylamino)ethyl)phenyl)methanesulfonamide of dofetilide was positioned within the hydrophobic pouch. The rate of occupation was 60%. For further investigation of molecular interaction mechanisms between dofetilide and hERG step 19 was chosen. Figure 38 shows 2D ligand-protein interaction diagram.

Step 19 (Binding site Gly 648)(60%)

6 Å RIGID

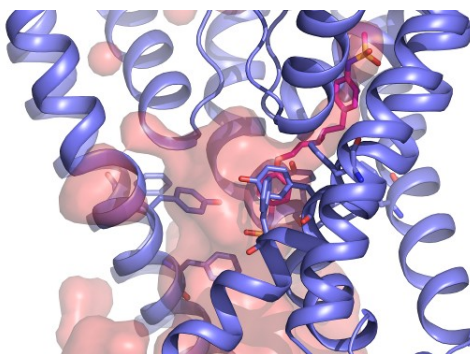


Figure 37. Binding pose of Dofetilide in stick representation, based on 19 snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which N-(4-(2-(methylamino)ethyl)phenyl)methanesulfonamide of dofetilide is positioned inside the hydrophobic pocket (reduced volume).

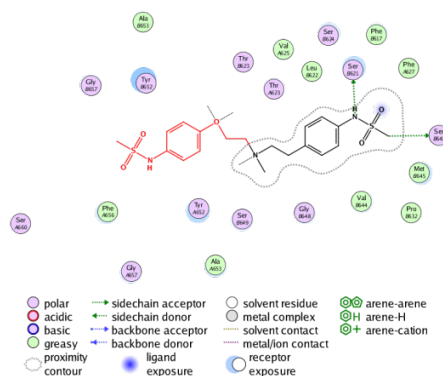


Figure 38: 2D ligand interactions of Dofetilide from the step with the highest percentage of occupation (19 Gly) at the hydrophobic pocket of the hERG structure, based on snapshots. 2D ligand-protein interaction diagrams were generated using MOE (Chemical Computing Group Inc., Molecular Operating Environment (moe)).

The diagram did not reveal any π - π stacking interactions between Dofetilide and hERG structure. The amino acids important for the Dofetilide were the following: H bonding, Ser 621 B and Ser 641 B (side chain acceptor), hydrophobic, Val 625 A, Val 644 B, Leu 622 B, Phe 617 B, Phe 627 A, Pro 632 B, Ala 653 B, polar, Thr 623 A, B, Ser 649 B, Ser 660 A, Gly 648 B, Gly 657 A, B. There are several residues in the close

proximity of the ligand marked by receptor exposure shadows: Tyr 652 A,B, Ser 624 B, Met 645 B, Phe 656 A). The proximity contour is the grey dotted line surrounding the ligand. Ligand exposure indicates that the blue-colored part of the ligand molecule is solvent accessible.

The mutation (to Ala) of T623, S624 and V625 located at the base of the pore helix or G648, Y652, F656 and V659 located in the S6 inner helix attenuated block by dofetilide [28]. However, hydrogen bonds with Thr 623 and Ser 624 were not detected in this diagram, but H bonds were noticed with Ser 621 and Ser 641, this is also in contrast to the previous study [24]. Extensive ring stacking or hydrophobic interactions with several aromatic side chains including Y652, F656 and F557 were observed in the aforementioned study [24], but we must mention once again that based on this diagram, π - π stacking interactions have not been recorded.

In the third set of docking poses dofetilide methanesulfonanilide group moved into pockets:

- Step 3 (5%)
- Step 5 (5%)
- Step 8 (35%)

In Figure 39 only step 8 will be shown as an example.

Step 8 (10%)

Flexible sidechains 8 Å: Phe 557 C, Leu 622 C, Thr 623 C, Ser 624 C, Tyr 652 B,C, Phe 656 C

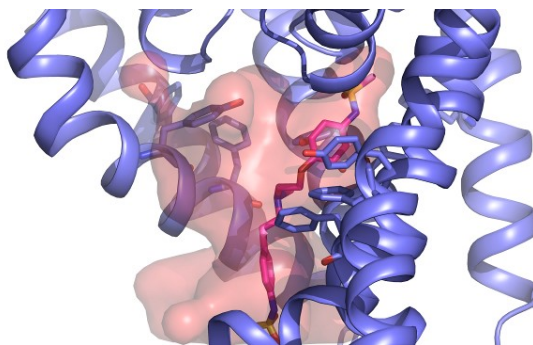


Figure 39: Binding pose of dofetilide in stick representation, based on 8 snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which methanesulfonanilide group is positioned inside the hydrophobic pocket.

Dofetilide partially occupied the hydrophobic pouch in the next steps:

- Step 10 (5%) with methanosulfanilide group in the first set of docking poses
- Step 18 (5%) with methanosulfanilide group in the first set of docking poses
- Step 1 (5%) with methanosulfanilide group in the second set of docking poses
- Step 11 (10%) with methyl group in the second set of docking poses

4.10 Grepafloxacin

The poorest results were obtained for grepafloxacin. The plausible explanation is the presence of different modes of binding between hERG and grepafloxacin.

Only in step 8 grepafloxacin with carboxylic acid occupied the pocket of the chain adjacent to the chain where docking was performed in the second set of docking. The percentage of occupation was 15 %.

Step 8 (15%)

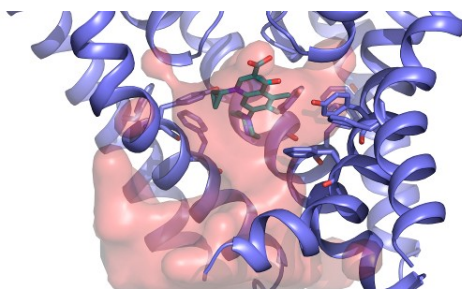


Figure 40: Binding poses of Grepafloxacin in stick representation, based on 8 snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which carboxylic acid is positioned inside the hydrophobic pocket. Poses were positioned in the pocket of the chain adjacent to the chain where docking was performed.

This pose was further used for calculating interactions in the MOE program. Figure 41 shows 2D ligand-protein interaction diagram.

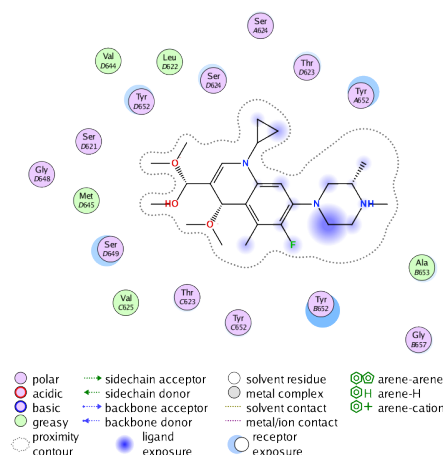


Figure 41: 2D ligand interactions of grepafloxacin from the step with the highest percentage of occupation(8) at the hydrophobic pocket of the hERG structure, based on snapshots. 2D ligand-protein interaction diagrams were generated using MOE (Chemical Computing Group Inc., Molecular Operating Environment (moe)).

The diagram did not reveal any π - π stacking interactions or hydrogen-bonding interactions between grepafloxacin and hERG structure. Ligand stays in the vicinity of other hydrophobic residues (i.e. Ala 653 B, Val 625 C, Val 644 D, Leu 622 D, Met 645 D). The molecule is in the close contact with polar residues (i.e. Thr 623 C, Tyr 652 C, Ser 621 D, Ser 624 A, Gly 648 D, Gly 657 B). There are several residues in the close proximity of the ligand marked by receptor exposure shadows (i.e. Tyr 652 A, B, D, Ser 624 D, Ser 649 D, Thr 623 D). The proximity contour is the grey dotted line surrounding the ligand. Ligand exposure indicates that the blue-colored part of the ligand molecule is solvent accessible (F, cyclopropyl, 3-methylpiperazin, etc.).

Previous docking studies, for another fluoroquinolone, levofloxacin and sparfloxacin, suggested that the protonated carboxyl group in the compounds strongly interacts with Phe656 as a π acceptor [29], but the diagram did not detect any interactions between the carboxyl group and Phe656. Also contradicting the previous study, for another fluoroquinolone, levofloxacin and sparfloxacin [29], cation- π interaction or CH- π interaction with Tyr 652, as well as hydrogen-bonding with Ser 624 and grepafloxacin were not observed in the diagram.

Grepafloxacin partially occupied the hydrophobic pouch in the next steps:

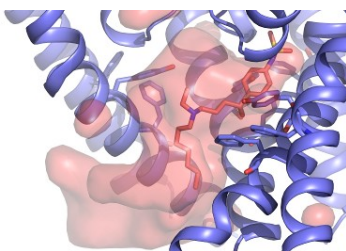
- Step 6 with carboxyl moiety in the first set of docking poses
- Step 7 (55%) with carboxyl moiety in the second set of docking poses
- Step 10 (15%) with 3-methylpiperazin-1-yl moiety in the second set of docking poses
- Step 1 (5%) with methyl moiety in the third set of docking poses
- Step 8 (5%) with 3-methylpiperazin-1-yl moiety in the third set of docking poses

- Step 19 (10%) with carboxy moiety in the second set of docking poses with 19 MD snapshot (Gly 648 as the binding site and with certain residues, whose side chains were set to rotate freely)

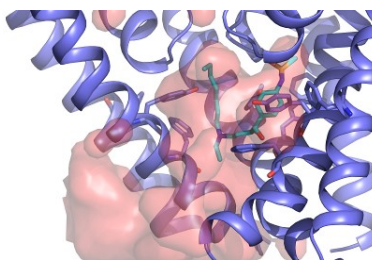
4.11 R-Ibutilide

The steps where the poses showed the occupancy of the pockets in terms of first set of docking poses:

A) Step 4 (5%)



B) Step 14 (10%)



C) Step 18 (5%)

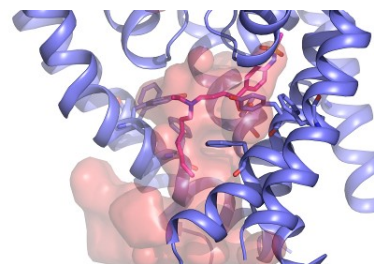


Figure 42: Binding poses of R-Ibutilide in stick representation, based on snapshots of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which functional groups are positioned inside the hydrophobic pockets:

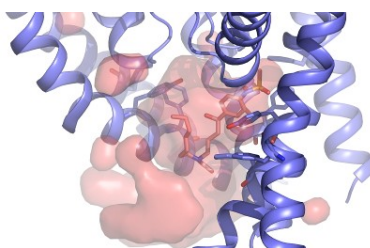
A) Methanesulfonanilide moiety of R-Ibutilide in step 4 (reduced volume)

B) Methanesulfonanilide moiety of R-Ibutilide in step 14

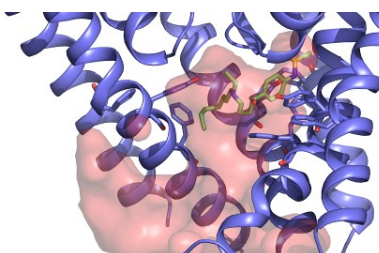
C) Methanesulfonanilide moiety of R-Ibutilide in step 18 (reduced volume)

R-Ibutilide inserted functional group into hydrophobic pocket in the second set of docking poses in the following steps:

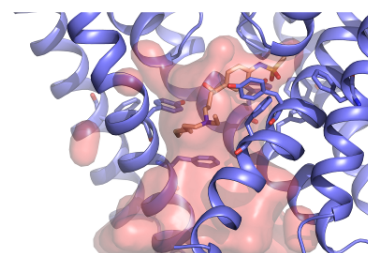
A) Step 4 (5%)



B) Step 5 (30%)



C) Step 7 (5%)



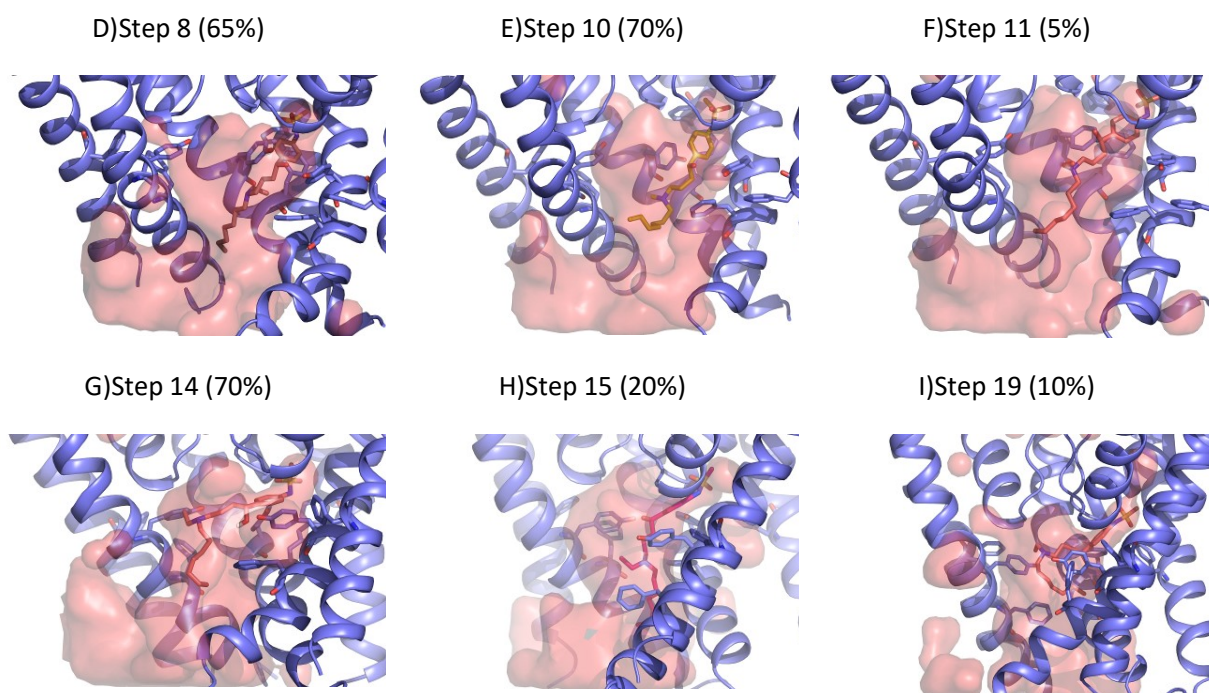


Figure 43: Binding poses of R-Ibutilide in stick representation, based on snapshots of hERG structure in slate cartoon representation (One chain is hidden and Phe 557,Tyr 652,Phe 556 residues are in slate stick representation), in which functional groups are positioned inside the hydrophobic pockets:

- A) Methanesulfonanilide group of R-Ibutilide in step 4 B) Methanesulfonanilide group of R-Ibutilide in step 5 C) Methanesulfonanilide group of R-Ibutilide in step 7
- D) Methanesulfonanilide group of R-Ibutilide in step 8 E)) Methanesulfonanilide group of R-Ibutilide in step 10 F) Methanesulfonanilide group of R-Ibutilide in step 11(reduced volume)
- G) Methanesulfonanilide group of R-Ibutilide in step 14 H)) Methanesulfonanilide group of R-Ibutilide in step 15(reduced volume) I) N-(phenyl)methanesulfonamide of R-Ibutilide in step 19

We had the same percentage of occupancy of pockets in steps 10 (70%) and 14 (70%) in the second set of docking. For further investigation of molecular interaction mechanisms between (R)-Ibutilide and hERG step 10 was chosen. Figure 44 shows the 2D ligand-protein interaction diagram.

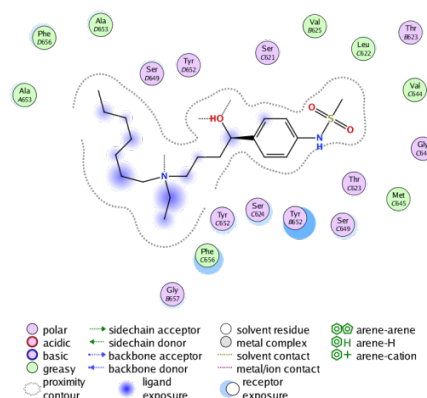


Figure 44: 2D ligand interactions of R-Ibutilide from the step with the highest percentage of occupation(10) at the hydrophobic pocket of the hERG structure, based on snapshots. 2D ligand-protein interaction diagrams were generated using MOE (Chemical Computing Group Inc., Molecular Operating Environment (moe)).

The diagram did not reveal any π - π stacking interactions or hydrogen-bonding interactions between R-Ibutilide and hERG structure. The amino acids important for the R-Ibutilide were the following: hydrophobic, Leu 622 B,D, Val 625 A, Val 644 D, polar, Thr 623 B,C, Ser 621 C, Ser 649 D, Tyr 652 D, Gly 648 C. There are several residues in the close proximity of the ligand marked by receptor exposure shadows (Tyr 652 B,C, Ser 624 C, Ser 649 C, Phe 656 C,D, Ala 653 A,D). The proximity contour is the grey dotted line surrounding the ligand. Ligand exposure indicates that the blue-colored part of the ligand molecule is solvent accessible (i.e. ethyl, heptyl, etc.).

As it can be seen from the figures shown in most cases, the methanesulfonanilide group of R-Ibutilide was positioned within the hydrophobic pocket, but it can also be seen in Figure 45 that the aliphatic tail of the drug occupied the pocket. This observation is in contrast to the previous study [27], which has already been mentioned in the case of clofilium. The suggested orientation allows π -stacking and cation- π interactions between Tyr652 and Phe656 residues and the phenyl ring and charged amine groups on the drugs [27], but π - π stacking interactions are not observed in the diagram. They also suggest that interactions between Thr623 and Ser624 with polar moieties on the drug molecules are an important component of the drug-channel interaction [27], but H-bonding was not detected.

In the first set of docking with 19 MD snapshot, N-(4-(1-hydroxybutyl)phenyl)methanesulfonamide of R-Ibutilide and 4-(ethyl(heptyl)amino-1-hydroxybutyl) occupied the pocket by 65%. Also the reduced volume of the pocket was detected.

Step 19 (Binding site Gly 648)(65%)

6 Å RIGID

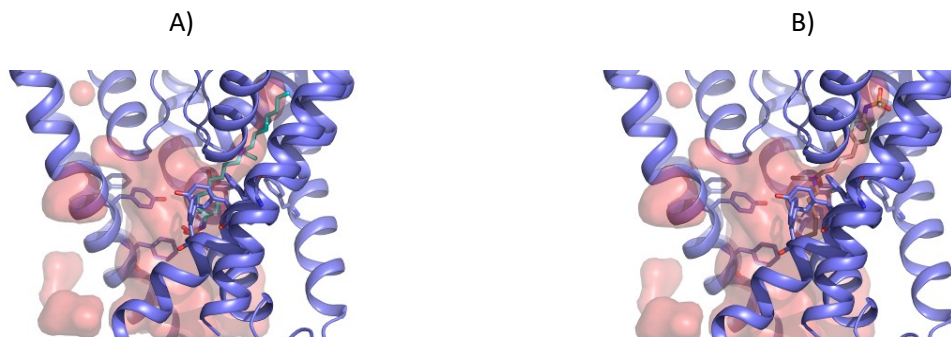


Figure 45. Binding pose of R-Ibutilide in stick representation, based on 19 snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which functional groups of dofetilide are positioned inside the hydrophobic pocket. (reduced volume)

A) 4-(ethyl(heptyl)amino-1-hydroxybutyl) of R-Ibutilide

B) N-(4-(1-hydroxybutyl)phenyl)methanesulfonamide of R-Ibutilide

With methanesulfonanilide moiety in the third set of docking poses R-Ibutilide occupied pockets in the step 1 (5%) and in the step 8 (70%). In Figure 29 only step 8 will be shown as an example.

Step 8 (10%)

Flexible sidechains 8 Å: Phe 557 C, Leu 622 C, Thr 623 C, Ser 624 C, Tyr 652 B,C, Phe 656 C

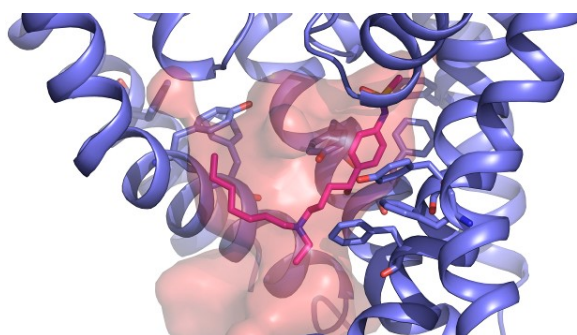


Figure 46: Binding pose of R-Ibutilide in stick representation, based on 8 snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which methanesulfonanilide group is positioned inside the hydrophobic pocket.

R-Ibutilide partially occupied the hydrophobic pouch in the next steps:

- Step 10 (5%) with methanosulfonanilide group in the first set of docking poses
- Step 1 (15%) with methanosulfonanilide group in the second set of docking poses
- Step 3 (20%) with methanosulfonanilide group in the second set of docking poses
- Step 6 (20%) with methanosulfonanilide group in the second set of docking poses
- Step 13 (5%) with methanosulfonanilide group in the second set of docking poses
- Step 18 (30%) with methanosulfonanilide group in the third set of docking poses

4.12 S-Ibutilide

S-ibutilide moved its methanosulfonanilide group into pocket in step 14 under the terms of the first set of docking. The rate of occupation was 5%.

Step 14 (5%)

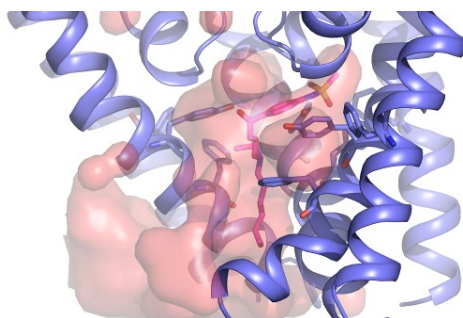
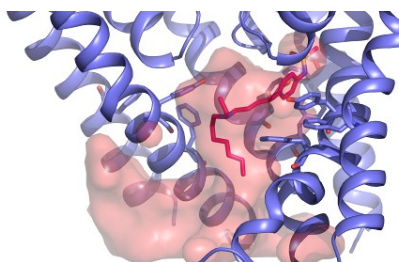


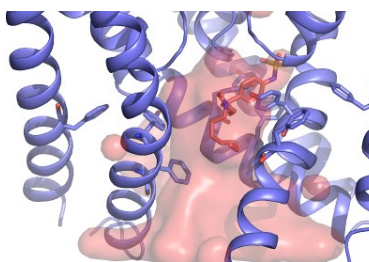
Figure 47: Binding pose of S-Ibutilide in stick representation, based on 14 snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557,Tyr 652,Phe 556 residues are in slate stick representation), in which methanosulfonanilide group is positioned inside the hydrophobic pocket.

In the second set of docking poses S-Ibutilide occupied hydrophobic pocket in the following steps:

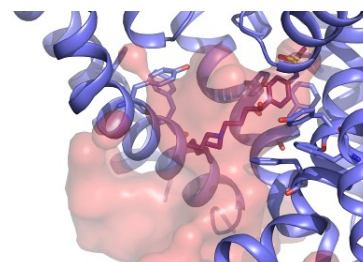
A)Step 5 (25%)



B)Step 6 (5%)



C)Step 8 (75%)



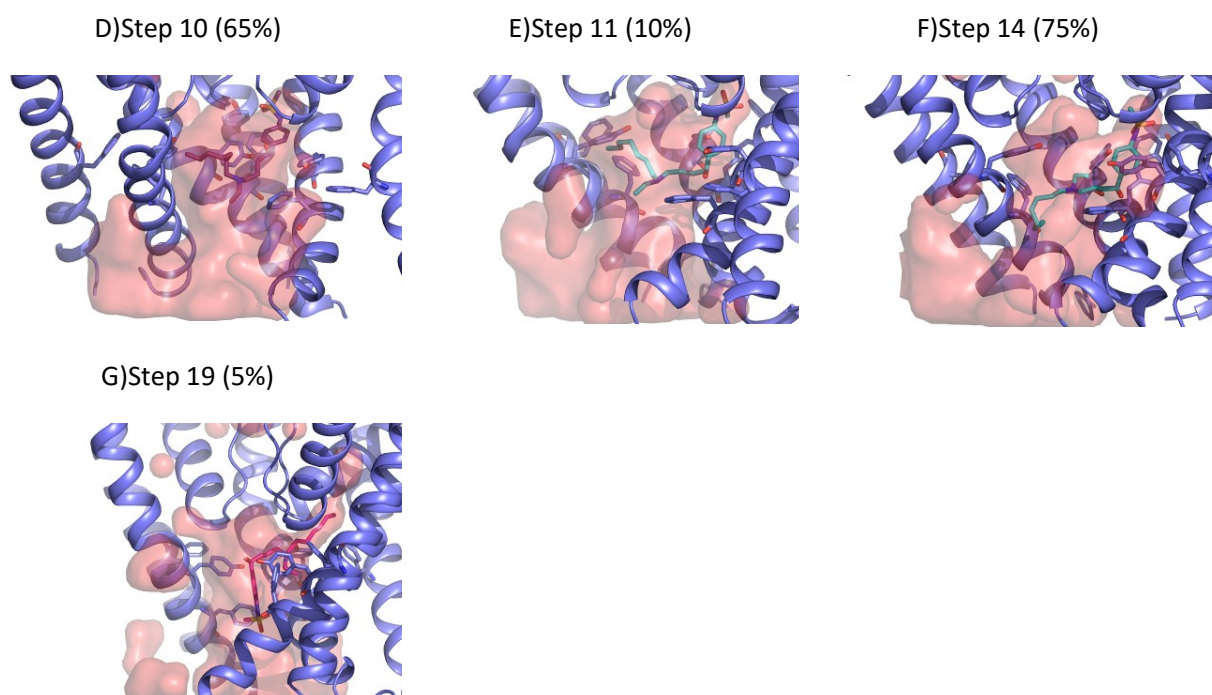


Figure 48. Binding pose of *S*-Ibutilide in stick representation, based on snapshots of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which functional groups of *S*-Ibutilide are positioned inside the hydrophobic pocket.

A) Methanesulfonanilide moiety of *S*-Ibutilide in step 5

B) Methanesulfonanilide moiety of *S*-Ibutilide in step 6 (reduced volume)

C) Methanesulfonanilide moiety of *S*-Ibutilide in step 8

D) Methanesulfonanilide moiety of *S*-Ibutilide in step 10 (reduced volume)

E) Methanesulfonanilide moiety of *S*-Ibutilide in step 11

F) Methanesulfonanilide moiety of *S*-Ibutilide in step 14

G) Heptyl moiety of *S*-Ibutilide in step 19

We had the same percentage of occupancy of pockets in steps 8 (75%) and 14 (75%) in the second set of docking. For further investigation of molecular interaction mechanisms between (*S*)-Ibutilide and hERG step 8 was chosen. Figure 49 shows the 2D ligand-protein interaction diagram.

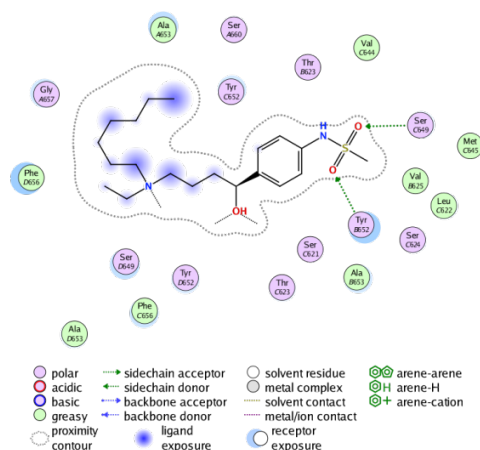


Figure 49: 2D ligand interactions of S-Ibutilide from the step with the highest percentage of occupation(8) at the hydrophobic pocket of the hERG structure, based on snapshots. 2D ligand-protein interaction diagrams were generated using MOE (Chemical Computing Group Inc., Molecular Operating Environment (moe)).

The diagram did not reveal any π - π stacking interactions between S-Ibutilide and hERG structure. The amino acids important for the S-Ibutilide were the following: H bonding, Ser 649 C and Tyr 652 B (side chain donor), hydrophobic, Ala 653 D, Val 625 B, Val 644 C, Leu 622 C, Met 645 C, polar, Thr 623 B, C, Ser 621 C, Ser 624 C, Ser 660 A. There are several residues in the close proximity of the ligand marked by receptor exposure shadows (i.e. Tyr 652 B, C, D, Ser 649 D, Gly 657 A, Ala 653 A, B, Phe 656 C, D). The proximity contour is the grey dotted line surrounding the ligand. Ligand exposure indicates that the blue-colored part of the ligand molecule is solvent accessible (i.e. ethyl, heptyl, etc.).

Hydrogen-bonding interactions between S-Ibutilide and hERG structure were observed based on this diagram, but the amino acids involved in the interaction were Ser 649 and Tyr 652, contrary to previous studies [17], [27]. The two enantiomers of ibutilide demonstrated similar positioning functional groups within hERG hydrophobic pockets and the similar observations mentioned above may also be applied in the case of S-Ibutilide.

In the first set of docking with 19 MD snapshot, N-(4-(1-hydroxybutyl)phenyl)methanesulfonamide of R-Ibutilide and 4-(ethyl(heptyl)amino-1-hydroxybutyl) occupied the pocket by 40%. Also the reduced volume of the pocket was detected.

Step 19 (Binding site Gly 648)(40%)

6 Å RIGID

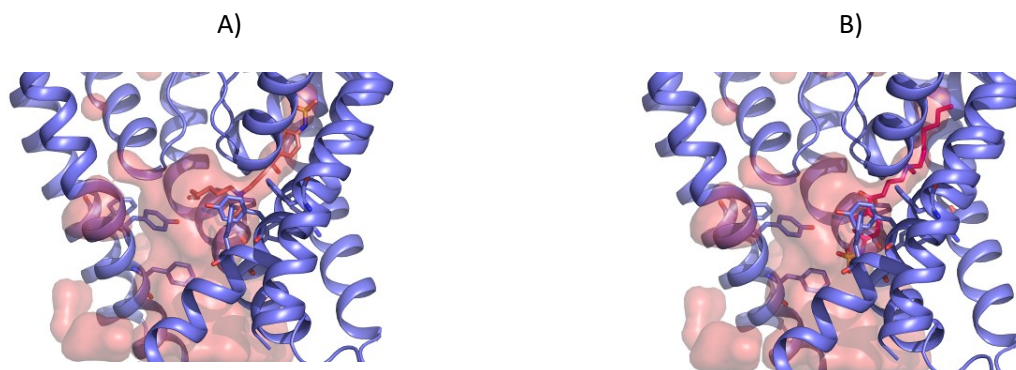


Figure 50. Binding pose of S-Ibutilide in stick representation, based on 19 snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557, Tyr 652, Phe 556 residues are in slate stick representation), in which functional groups of S-Ibutilide are positioned inside the hydrophobic pocket (reduced volume).

A) N-(4-(1-hydroxybutyl)phenyl)methanesulfonamide of S-Ibutilide B) 4-(ethyl(heptyl)amino-1-hydroxybutyl of S-Ibutilide

In the third set of docking the results were:

- Step 4 (10%) with methanesulfonamide group
- Step 5 (5%) with methanesulfonamide group
- Step 8 (45%) with methanesulfonamide group

In Figure 39 only step 8 will be shown as an example.

Step 8 (10%)

Flexible sidechains 8 Å:Phe 557 C, Leu 622 C, Thr 623 C, Ser 624 C, Tyr 652 B,C, Phe 656 C

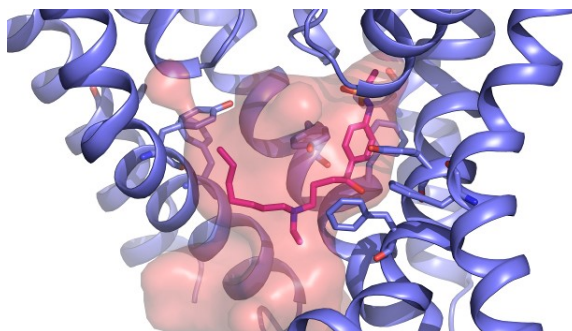


Figure 51: Binding pose of S-Ibutilide in stick representation, based on 8 snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557,Tyr 652,Phe 556 residues are in slate stick representation), in which methanosulfonanilide group is positioned inside the hydrophobic pocket.

S-Ibutilide partially occupied the hydrophobic pouch in the next steps:

- Step 4 (5%) with methanesulfonanilide group in the first set of docking poses
- Step 11 (5%) with methyl group in the first set of docking poses
- Step 1 (5%) with methanesulfonanilide group in the second set of docking poses
- Step 3 (30%) with methanesulfonanilide group in the second set of docking poses
- Step 4 (15%) with methanesulfonanilide group in the second set of docking poses
- Step 7 (45%) with methanesulfonanilide group in the second set of docking poses
- Step 1 (15%) with methanesulfonanilide group and with heptyl moiety in the third set of docking poses
- Step 3 (5%) with methanesulfonanilide group in the third set of docking poses

5. DISCUSSION

This docking study with different MD snapshots offers supportive evidence of the presence of pockets in hERG and of their importance for drug binding, because, we obtained poses which displayed occupancy of the pouches. Interestingly, a previous unpublished study of our group based on the open cryo-EM structure of hERG, was ineffective in proving the presence and significance of the hydrophobic pouches for drug binding. Importantly, pouch analysis of different MD snapshots with the program HOLLOW

revealed that the shape of the pockets is changing significantly over simulation time. **Consequently, in the docking study presented here, we observe a wide range of drug-pouch interactions in different MD snapshots. It can be said that except for grepafloxacin, all tested blockers readily interact with the hydrophobic pouches. The frequency of interaction and mode of interaction however varies, depending on the snapshot used, and the treatment of binding site radius and side chain flexibility during docking.**

5.1 The role of the state of the structure

The hERG channel cryo-EM structure is considered to be in an open conformation, with the voltage sensors in a depolarized conformation[13]. However, the structure displays only one structural snapshot and since proteins are not static, but flexible macromolecules, which can change their shape or conformation in different conditions, it is important to consider protein dynamics, when addressing drug block. It might be speculated that the geometry of the hydrophobic side pockets depends on the geometry of the helices and the rotameric state(s) of the aromatic side chains, suggested by MD simulations. By changing the shape and volume of the pockets more possibilities are provided for the adaptation of the drug structure or functional groups of drugs within the pockets themselves, as shown in this docking study. In this study some poses displayed positioning of functional groups within these pockets especially in the following steps 8,19,10,5,14,4,11.

The question that arises is the state of the structure. And why the poorer results were obtained in the third trial, although some residues had the freedom of rotation? At depolarizing potentials, hERG channels can exist in either an open or an inactivated state [1], [30], yet it has not been established whether the open or the inactivated state is preferred for drug binding[31]. From the previous studies we know that the inactivated state is important for the binding of certain drugs[32]–[36], unfortunately, it is currently unclear, how the structure of the inactivated state would look like. However, another study provides strong evidence that drug binding to hERG K⁺ channels is influenced by whether the channels are in the inactivated or the open state [31]. They have demonstrated that this phenomenon is drug-dependent, with the ratio of affinities for the open to inactivated state varying from 1:1 (i.e., not state-dependent: quinidine, erythromycin, and perhexiline) to 1:70 (dofetilide)[31]. They showed that preferential binding to the inactivated state is necessary but not sufficient for high-affinity binding to hERG channels[31]. Also it was demonstrated that the position of aromatic residues along the S6 α helix and not inactivation per se is the main determinant of hERG and eag channel sensitivity to block by cisapride [7].

Taking into consideration everything above given, we will be able to fully illuminate the importance of hydrophobic pockets as the site of drug binding only when we determine the structure of the hERG channels in the inactivated form. Only then will we be able to make a comparison, i.e. to determine whether the hydrophobic pockets are present in the inactivated conformational state. In any case, further research is necessary to determine the frequency of interaction and the mode of interaction between hERG channels and drugs and the conditions contributing to it.

5.2 The role of aromatic residues and hydrophobic pockets and protein flexibility

“Substantial progress has been made by identifying amino acids essential for drug block. The majority of hERG inhibitors are interacting with the pore module. This homo tetrameric module consists of an outer S5 helix, a pore helix P1 and an inner helix S6. They include T623, S624 and V625 from the P1, and residues G648, Y652 and F656 located on the helix S6 [22], [24], [25], [35], [37]”. In the structure they are located near the central cavity and in the selectivity filter adjacent to the central cavity [13]. The structure offers an explanation as to why these particular amino acids are important: they line the surfaces of elongated, relatively hydrophobic pockets that extend from the central cavity[13]. The hydrophobic pockets are roughly cylindrical in shape with diameter of about 8 Å and depth about 11 Å (measurements between atomic centers)[13].The authors propose that substituted aromatic rings of astemizole and dofetilide fit snugly into the pockets [13].If this occurs, these drugs occupy the center of the cavity and insert a functional group into one of the hydrophobic pockets [13]. Consistent with this hypothesis in this docking study we obtained poses which displayed occupancy of the pouches. From the ligand-protein interaction diagrams we can see that we did not detect any π - π interactions between ligands and Tyr 652 or Phe 656 side chains. Although the importance of these residues in direct drug binding has been emphasized in many previous studies, as noted during the results and discussion, on the basis of what is presented in this study, it is more likely that their role in sculpting these hydrophobic pouches may be as significant as suggested in previous works[13], [38] . The same could apply to the importance of Ser624 and Thr623 in direct binding, since we did not detect any hydrogen bonds with these residues. H bonding interactions were detected in the case of S-ibutilide, dofetilide and sertindole but did not include Ser624 and Thr623. Arene-H interactions were detected in the case of (-)-cisapride and clobetolium. Side chains of Tyr 652 and Phe 656 participated twice and ones respectively in the formation of arene-H bonds. In any case, the role of these residues in direct drug binding cannot be excluded on the basis of this study, as more detailed analyzes are needed. We also need to take into account that this disagreement with previous studies may be due to different drug positioning within hERG K⁺ channels. For example, it is possible that we would have obtained similar results in terms of interactions as reported in the study by Saxena et al., 2016 that we used to further explore the patterns of ligand-protein interactions poses, which are positioned between the two subunits or near Phe 557 (see Figure 51).

In the previous research it was pointed out that the potency for block of hERG channels by three structurally diverse drugs (MK-499, cisapride, terfenadine) was well correlated with the two-dimensional approximation of the van der Waals hydrophobic surface area for the side chain of residue 656 and was not related to aromaticity per se [37]. In contrast, an aromatic side group at residue 652 was essential for high affinity block, suggesting the importance of a cation- π interaction between Tyr-652 and the basic nitrogen of the drugs [37]. However , a study with unnatural amino acids showed that cation- π interactions do not play a role at either Tyr652 or Phe656 for hERG block by terfenadine, quinidine, or dofetilide in the steady state [39]. Also a recent study with lubeluzole reported that no cation- π interactions were observed in any of the docking poses[21]. Based on the diagrams we have shown in this study, we have not noticed any cation- π interaction with the centrally located basic nitrogen of the ligands and aromatic residues. As we can see from the given facts, there is controversy concerning the

significance of cation- π interactions for the hERG block by drugs. The step with the highest percentage was chosen for each ligand, and the pose from this step looking the best based on visual inspection was used for further calculating interactions. It is likely that by carrying out a more detailed analysis of interaction calculations for all poses that were positioned inside the pocket we would have gotten different results. On the other hand, there is the possibility that the results will fit more closely with the previous two articles cited, as well as the possibility of several different ways of binding for hERG blockers.

5.3 The role of protein flexibility

The first and second docking poses did not allow protein flexibility in comparison to the third trial, but the second docking poses displayed the best docking outputs, that confirm the importance of hydrophobic pockets. The only difference between the first and second trials was the radius of the binding site considered. Using a smaller radius has proven to be the most important factor in obtaining poses that confirms the significance of hydrophobic pouches. The question whether this happens in reality or only in virtual conditions when we force the situation, ie the conditions such as radius or the binding site remains open. Step 19 in which the same radius, but a different binding site is used serves as an example of the above mentioned in this paper. Using Gly 648 produced significantly better results. Certainly the reasons for the liability of the hERG, as already mentioned, may be different mechanisms and models of binding different groups of drugs. For Clofilium, protein flexibility has been proven to be the most important factor in obtaining poses that confirm the significance of the hydrofobic pockets. The flexibility of Y652 and F656 rotamers in hERG is probably required to accommodate the diversity of hERG blockers, and rotamer sampling in docking calculations is likely to be important for correctly defining drug poses[18], but in this docking study we could not confirm side chain flexibility as an important feature in getting poses for most drugs which occupy the hydrophobic pockets. We selected residues of significance, within the desired radius and set the side chains to rotate freely. Although the role of side chain flexibility of Tyr 652 [40] and Phe 656[26] is confirmed in the binding of drugs, the significance of other residues, i.e. their flexibility has not yet been clarified . A possible explanation for not obtaining a higher percentage of desired docking outputs in these conditions is that we have caused adverse conformational changes by choosing more residues and allowing them to rotate freely.

In some steps, during the third trial and especially in the 15th step during the performance of the docking under the conditions of the second set it has been noted many poses for some drugs are positioned between the two subunits or near Phe 557 (see Figure 51) as it was suggested in the previous study which claims that all studied blockers bind in the cavity, below the selectivity filter, partially protruding into lateral, hydrophobic pore openings[24]. This was important, because ,a novel aromatic high affinity binding determinant for blockers located in helix S5, F557, which is equally potent as Y652 was identified[24]. These openings, also known as fenestrations, have first been identified in bacterial sodium channels[41], [42]. This can be interesting for further research.

Step 15

6 Å RIGID

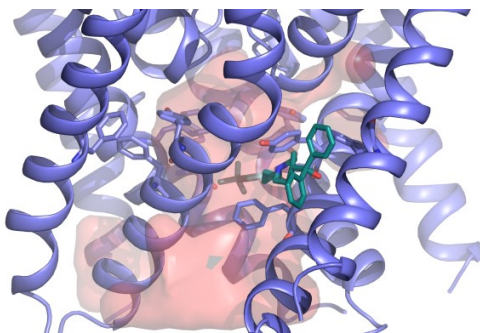


Figure 51: Binding pose of R-Terfenadine in stick representation, based on 15 snapshot of hERG structure in slate cartoon representation (Phe 557, Tyr 652, Phe 556 residues are in slate stick representation). R-Terfenadine is positioned between the two subunits or near Phe 557.

Contrary to previous studies where drug binding was established within the central cavity of the pore region of hERG[22], [40], [43], during this study we have documented some docking solutions for most blockers that were located outside the cavity . This was especially noticeable during the first set of docking poses, then on a smaller scale during the second and third sets.

Sometimes it is noticed, but it is rare that the ligand, in this case S-Ibutilide, in one position during the second docking set in step seven has spread from one pocket to another, but of course not completely.

Step 7

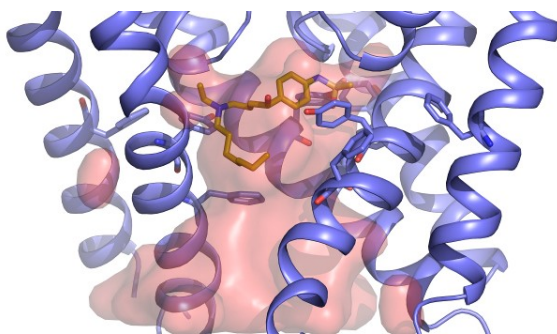


Figure 52: Binding pose of S-Ibutilide in stick representation, based on 7 snapshot of hERG structure in slate cartoon representation (One chain is hidden and Phe 557,Tyr 652,Phe 556 residues are in slate stick representation), in which the ethyl and methanesulphonanilide group is placed in the starting parts of the pockets.

5.4 Conclusions and Recommendations

MD simulations, docking studies and analysis the molecular mechanisms of hERG drug binding displayed that the hydrophobic pouches could be explanation for hERG liability. This study demonstrated that drugs insert functional groups into these pockets. In any case, this study was valuable because it has

confirmed the presence of hydrophobic pockets in the hERG channel, and also the importance of backbone dynamics in the changing of the shape of the hydrophobic pockets, and consequently the places for the binding of drugs. Further analyses of the binding modes will be necessary, possibly with long molecular dynamics simulations, docking studies with different settings, detailed analyses of protein-ligand interaction patterns. Again, we need more structures and MD simulations of mutant channels, ideally in the presence of drugs[38].

5.5 The role of hERG in the future

Although Kv11.1 channels have been most extensively studied with respect to cardiac function, they are also quite widely expressed in brain regions, smooth muscle cells of the gastrointestinal and genitourinary tracts, as well as a range of endocrine cells [40]. Taking into account the distribution of KV11.1 channels in the organism, the potential role and significance of the hERG remains an unexplained field. One of the articles that supports the potential perspectives of hERG has reported, that exposing human mammary gland adenocarcinoma to the hERG1 channel activator NS1643 leads to arrest of the cell cycle in G0/G1 phase by activating a senescence program[44]. This work provides the first evidence of a role of hERG1 potassium channel in promoting cellular senescence in breast cancer [44]. Furthermore, it offers an opportunity to design an antiproliferative pharmacological strategy for cancer cells expressing hERG1 channel[44]. At present, insufficient information is available regarding the efficacy and safety of hERG1 agonists [45]. However, this new class of drugs could potentially cause a paradigm shift in the clinical management of LQTS or perhaps other disorders where hERG1 channel function is implicated[45]. Previous research identified the first KCNH2 mutation to produce a remarkable gain of function and provide for the first time a genetic basis for the SQTS, a disease characterized by marked abbreviation of the QT interval and a high incidence of atrial and ventricular arrhythmias and sudden death [46]. They also tested the hypothesis that blocking IKr with class III antiarrhythmic drugs could be a potential therapeutic approach for the treatment of SQTS [46]. However, N588K reduced the ability of D-sotalol to block the channel, a result consistent with the clinical findings[46]. A N588K missense mutation is shown to abolish rectification of the current and reduce the affinity of the channel for drugs with class III antiarrhythmic action[46]. Another study identified astemizole as a high-affinity blocker of the mutant N588K-hERG channel and propose it as a possible therapeutic candidate for treatment of the life-threatening SQTS 1 [31]. On the basis of everything exposed, we see that potential therapeutic benefits as well as limitations related to the hERG channel as a site of action of a large number of drugs are significant and uneven. Therefore, the research related to this problem continues.

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7. APPENDIX

7.1 Supplementary Information

	STEP0 CHAIN A		STEP 1 CHAIN C				STEP 3 CHAIN C			
	6 Å RIGID	10 Å RIGID	6 Å RIGID			10 Å RIGID	6 Å RIGID			10 Å RIGID
LIGAND	Does ligand occupies pocket?	Does ligand occupies pocket?	Does ligand occupies pocket?	Moiety	%	Does ligand occupies pocket?	Does ligand occupies pocket?	Moiety	%	Does ligand occupies pocket?
MK-499	No	No	No			No	The ligand occupies half of the pocket	Methanesulfonamide	10%	No
(+)-Cisapride	No	No	No			No	No			No
(-)-Cisapride	No	No	No			No	The ligand occupies half of the pocket?	4-amino-5-chloro-2-methoxybenzamide	10%	No
R-Terfenadine	No	No	No			No	Yes	4-tert-butylphenyl	30%	No
S-Terfenadine	No	No	No			No	Yes	4-tert-butylphenyl	30%	No
Clofilium	No	No	Gold could not find solution			Gold could not find solution	Gold could not find solution			Gold could not find solution
Sertindole	No	No	No			No	No			No
Astemizole	No	No	Yes	4-methoxyphenyl	20%	No	Yes	N-{1-[2-(4-methoxyphenyl)ethyl]piperidin-4-yl}	65%	No
Dofetilide	No	No	The ligand occupies initial part of the pocket	Methanesulfonamide	5%	No	No			No
Grepafloxacin	No	No	No			No	No			No
R-Ibutilide	No	No	The ligand occupies initial part of the pocket	Methanesulfonamide	15%	No	The ligand occupies half of the pocket	Methanesulfonamide	20%	No
S-Ibutilide	No	No	The ligand occupies initial part of the pocket	Methanesulfonamide	5%	No	The ligand occupies half of the pocket	Methanesulfonamide	30%	No

Table S1. Step 0, 1, 3 in terms of the first and second set of docking poses.

	STEP 4 CHAIN C						STEP 5 CHAIN C			
	6 Å RIGID			10Å RIGID			6 Å RIGID			10 Å RIGID
LIGAND	Does ligand occupies pocket?	Moiety	%	Does ligand occupies pocket?	Moiety	%	Does ligand occupies pocket?	Moiety	%	Does ligand occupies pocket?
MK-499	Yes	Methanesulfonanilide	20%	No			Yes	Methanesulfonanilide	35%	No
(+)-Cisapride	Yes	4-amino-5-chloro-2-methoxybenz-2yl	20%	No			Yes	4-amino-5-chloro-2-methoxybenz-1yl	15%	No
(-)-Cisapride	Yes	4-amino-5-chloro-2-methoxybenz-2yl	15%	No			Yes	1)4-amino-5-chloro-2-methoxybenz-1yl 2)4-fluorophenoxy	10%	No
R-Terfenadine	Yes	4-tert-butylphenyl	5%	No			Yes	4-tert-butylphenyl	15%	No
S-Terfenadine	Yes	4-tert-butylphenyl	5%	No			Yes	4-tert-butylphenyl	15%	No
Clofilium	Gold could not find solution			Gold could not find solution			Gold could not find solution			Gold could not find solution
Sertindole	The ligand occupies half of the pocket	1-imidazolidin-2-one	5%	No			The ligand occupies initial part of the pocket	1-imidazolidin-2-one	5%	No
Astemizole	No			The ligand occupies half of the pocket	4-methoxyphenyl	5%	Yes	4-methoxyphenyl	25%	No
Dofetilide	No			No			Yes	Methanesulfonanilide	10%	No
Grepafloxacin	No			No			No			No
R-Ibutilide	Yes	Methanesulfonanilide	5%	Yes	Methanesulfonanilide	5%	Yes	Methanesulfonanilide	30%	No
S-Ibutilide	The ligand occupies initial part of the pocket	Methanesulfonanilide	15%	The ligand occupies initial part of the pocket	Methanesulfonanilide	5%	Yes	Methanesulfonanilide	25%	No

Table S2. Step 4, 5 in terms of the first and second set of docking poses.

STEP 6 CHAIN B					STEP 7 CHAIN B				STEP 8 CHAIN C			
	6 Å RIGID			10 Å RIGID	6 Å RIGID			10 Å RIGID	6 Å RIGID			10 Å RIGID
LIGAND	Does ligand occupies pocket?	Moiety	%	Does ligand occupies pocket?	Does ligand occupies pocket?	Moiety	%	Does ligand occupies pocket?	Does ligand occupies pocket?	Moiety	%	Does ligand occupies pocket?
MK-499	The ligand occupies half of the pocket	Methanesulfonanilide	5%	No	The ligand occupies initial part of the pocket	cyano	30%	No	Yes	Methanesulfonanilide	55%	No
(+)-Cisapride	No			No	No			No	Yes	1)4-amino-5-chloro-2-methoxybenz-1yl 2)4-fluorophenoxy	10%	No
(-)-Cisapride	The ligand occupies initial part of the pocket	Methoxy	5%	No	No			No	Yes	4-amino-5-chloro-2-methoxybenz-1yl	10%	No
R-Terfenadine	No			No	No			No	Yes	1)4-tert-butylphenyl 2)phenyl	30%	No
S-Terfenadine	No			No	No			No	Yes	1)4-tert-butylphenyl 2)phenyl	45%	No
Clofilium	Gold could not find solution			Gold could not find solution	Gold could not find solution			No	Gold could not find solution			No
Sertindole	No			No	No			No	Yes	1-imidazolidin-2-one	55%	No
Astemizole	Yes	Methoxy group	20%	No	Yes	4-methoxyphenyl	5%	No	Yes	4-methoxyphenyl	35%	No
Dofetilide	Yes	Methanesulfonanilide	20%	No	No			No	Yes	Methanesulfonanilide	20%	No

Grepafloxacin	No			The ligand occupies initial part of the pocket	The ligand occupies initial part of the pocket	Carboxyl	55 %	No	Yes	carboxylic acid	15 %	No
R-Ibutilide	The ligand occupies initial part of the pocket	Methanesulfonanilide	20 %	No	Yes	Methanesulfonanilide	5 %	No	Yes	Methanesulfonanilide	65 %	No
S-Ibutilide	Yes	Methanesulfonanilide	5 %	No	The ligand occupies initial part of the pocket	Methanesulfonanilide	45 %	No	Yes	Methanesulfonanilide	75 %	No

Table S3. Step 6, 7, 8 in terms of the first and second set of docking poses.

	STEP10 CHAIN C						STEP11 CHAIN C					
	6 Å RIGID			10Å RIGID			6 Å RIGID			10Å RIGID		
LIGAND	Does ligand occupies pocket?	Moiety	%	Does ligand occupies pocket?	Moiety	%	Does ligand occupies pocket?	Moiety	%	Does ligand occupies pocket?	Moiety	%
MK-499	Yes	Methanesulfonanilide	20%	No			Yes	Methanesulfonamide	5%			
(+)-Cisapride	Yes	4-amino-5-chloro-2-methoxybenzyl	15%	The ligand occupies initial part of the pocket	4-amino-5-chloro	10%	No			No		
(-)-Cisapride	Yes	1)4-amino-5-chloro-2-methoxybenzyl	15%	The ligand occupies initial part of the pocket	4-amino-5-chloro	10%	Yes	4-amino-5-chloro-2-methoxybenzyl	5%	No		
R-Terfenadine	Yes	1)4-tert-butylphenyl 2)phenyl	25%	No			Yes	4-tert-butylphenyl	25%	No		
S-Terfenadine	Yes	1)4-tert-butylphenyl 2)phenyl	20%	No			Yes	4-tert-butylphenyl	15%	No		

Clofilium	Gold could not find solution			Gold could not find solution			Gold could not find solution			Gold could not find solution		
Sertindole	The ligand occupies half of the pocket	1-imidazolidin-2-one	60%	No			No			No		
Astemizole	Yes	4-methoxyphenyl	25%	Yes	4-methoxyphenyl	25%	No			Yes	4-methoxyphenyl	5%
Dofetilide	Yes	Methanesulfonanilide	40%	The ligand occupies half of the pocket	Methanesulfonanilide	5%	The ligand occupies initial part of the pocket	Methyl	10%	No		
Grepafloxacin	The ligand occupies initial part of the pocket	3-methylpiperazin-1-yl	15%	No			No			No		
R-Ibutilide	Yes	Methanesulfonanilide	70%	The ligand occupies half of the pocket	Methanesulfonanilide	5%	Yes	Methanesulfonanilide	5%	No		
S-Ibutilide	Yes	Methanesulfonanilide	65%	No			Yes	Methanesulfonanilide	10%	The ligand occupies initial part of the pocket	Methyl group	5%

Table S4. Step 10, 11 in terms of the first and second set of docking poses.

	STEP13 CHAIN C						STEP14 CHAIN C					
	6 Å RIGID			10Å RIGID			6 Å RIGID			10Å RIGID		
LIGAND	Does ligand occupies pocket?	Moiety	%	Does ligand occupies pocket?	Moiety	%	Does ligand occupies pocket?	Moiety	%	Does ligand occupies pocket?	Moiety	%
MK-499	The ligand occupies half of the pocket	Methanesulfonamide	5%	The ligand occupies initial part of the pocket	Methyl	5%	Yes	Methanesulfonanilide	15%	Yes	Methanesulfonanilide	5%

(+)-Cisapride	The ligand occupies half of the pocket	4-amino-5-chloro-2-methoxybenz-1yl	5%	No			No			No		
(-)-Cisapride	Yes	4-amino-5-chloro-2-methoxybenz-1yl	10%	The ligand occupies initial part of the pocket	4-amino-5-chloro	5%	The ligand occupies initial part of the pocket	Methoxy	35%	No		
R-Terfenadine	No			No			Yes	1)4-tert-butylphenyl 2)phenyl	25%	No		
S-Terfenadine	No			No			Yes	1)4-tert-butylphenyl 2)phenyl	30%	No		
Clofilium	Gold could not find solution			Gold could not find solution			Gold could not find solution			Gold could not find solution		
Sertindole	No			No			The ligand occupies half of the pocket	1-imidazolidin-2-one	15%	The ligand occupies half of the pocket	1-imidazolidin-2-one	15%
Astemizole	No			Yes	4-methoxyphenyl	5%	Yes	4-methoxyphenyl	50%	Yes	4-methoxyphenyl	5%
Dofetilide	No			Yes	Methanesulfonanilide	5%	Yes	Methanesulfonanilide	40%	Yes	Methanesulfonanilide	10%
Grepafloxacin	No			No			No			No		
R-Ibutilide	The ligand occupies half of the pocket	Methanesulfonanilide	5%	No			Yes	Methanesulfonanilide	70%	Yes	Methanesulfonanilide	10%
S-Ibutilide	No			No			Yes	Methanesulfonanilide	75%	Yes	Methanesulfonanilide	5%

Table S5. Step 13, 14 in terms of the first and second set of docking poses.

	STEP15 CHAIN D				STEP18 CHAIN C			
	6 Å RIGID			10Å RIGID	6 Å RIGID	10Å RIGID		
LIGAND	Does ligand occupies pocket?	Moiety	%	Does ligand occupies pocket?	Does ligand occupies pocket?	Does ligand occupies pocket?	Moiety	%
MK-499	The ligand occupies half of the pocket	Methanesulfonanilide	5%	No	No	Yes	Methanesulfonanilide	5%
(+)-Cisapride	No			No	No	Yes	4-amino-5-chloro-2-methoxybenz-1yl	10%
(-)-Cisapride	No			No	No	The ligand occupies half of the pocket	4-amino-5-chloro-2-methoxybenz-1yl	20%
R-Terfenadine	No			No	No	No		
S-Terfenadine	Yes	1(4-tert-butylphenyl)butan-1-ol	5%	No	No	The ligand occupies initial part of the pocket	phenyl	15%
Clofilium	Gold could not find solution			No	No	Gold could't find solution		
Sertindole	No			No	No	No		
Astemizole	Yes	4-methoxyphenyl	5%	No	No	Yes	4-fluorophenyl	5%
Dofetilide	No			No	No	The ligand occupies half of the pocket	Methanesulfonanilide	5%
Grepafloxacin	No			No	No	No		
R-Ibutilide	Yes	Methanesulfonanilide	20%	No	No	Yes	Methanesulfonanilide	5%
S-Ibutilide	No			No	No	No		

Table S6. Step 15, 18 in terms of the first and second set of docking poses.

	STEP19 CHAIN B (Binding site Tyr 652)		STEP19 CHAIN B (Binding site Gly 648)
	6 Å RIGID	10Å RIGID	6 Å RIGID

LIGAND	Does ligand occupies pocket?	Moiety	%	Does ligand occupies pocket?	Does ligand occupies pocket?	Moiety	%
MK-499	No			No	Yes	cyano-tetrahydronaphthyl	80%
(+)-Cisapride	Yes	4-fluorophenoxy	5%	No	Yes	(4-Fluorophenoxy)propyl	80%
(-)-Cisapride	Yes	4-fluorophenoxy	5%	No	Yes	(4-Fluorophenoxy)propyl	15%
R-Terfenadine	Yes	1-(4-tert-butylphenyl)butan-1-ol	10%	No	Yes	1-(4-tert-butylphenyl)butan-1-ol	30%
S-Terfenadine	Yes	1-(4-tert-butylphenyl)butan-1-ol	25%	No	Yes	1-(4-tert-butylphenyl)butan-1-ol	15%
Clofilium	Gold could not find solution			No	No		
Sertindole	No			No	Yes	1-(2-piperidin-1-yl)ethyl-imidazolidin-2-one	20%
Astemizole	Yes	2-(4-methoxyphenyl)ethyl	30%	No	Yes	(1-(2-(4-methoxyphenyl)ethyl)piperidin-4-yl)	20%
Dofetilide	Yes	(4-(methanesulfonamido)phenoxy)	5%	No	Yes	1)4-(methanesulfonamido)phenoxy ethyl-methylamino) 2)N-(4-(2-(methylamino)ethyl)phenyl)methanesulfonamide	60%
Grepafloxacin	No			No	No		
R-Ibutilide	Yes	N-(phenyl)methanesulfonamide	10%	No	Yes	1)N-(4-(1-hydroxybutyl)phenyl)methanesulfonamide 2)4-(ethyl(heptyl)amino-1-hydroxybutyl)	65%
S-Ibutilide	Yes	Heptyl	5%	No	Yes	1)N-(4-(1-hydroxybutyl)phenyl)methanesulfonamide 2)4-(ethyl(heptyl)amino-1-hydroxybutyl)	40%

Table S7. Step 19 in terms of the first and second set of docking poses. Additional set of docking poses was made using 19 step with Gly 648 as the binding site. It was conducted with protein side chains regarded as rigid and using a radius of 6 Å.

	STEP 0 CHAIN A		STEP 1 CHAIN C			STEP 3 CHAIN C			STEP 4 CHAIN C		
	FLEXIBLE SIDECHAINS 8 Å		FLEXIBLE SIDECHAINS 8 Å			FLEXIBLE SIDECHAINS 8 Å			FLEXIBLE SIDECHAINS 8 Å		
	Phe 557 A;Leu 622 A;Thr 623 A,D;Tyr 652 A,B,D;Phe56A		Phe 557 C;Leu 622 C;Thr 623 B,C;Ser 624 C,D;Tyr 652 B,C;Phe 656 B,C			Phe 557 C;Leu 622 C;Thr 623 C;Ser 624 C;Tyr 652 C;Phe 656 C			Phe 557 C;Leu 622 C;Thr 623 C;Ser 624 C;Tyr 652 B,C;Phe 656 C		
LIGAND	Does ligand occupies pocket ?	Moiety	Does ligand occupies pocket?	Moiety	%	Does ligand occupies pocket ?	Moiety	%	Does ligand occupies pocket?	Moiety	%
MK-499	No		The ligand occupies half of the pocket	Methanesulfon anilide	15 %	No			No		
(+)-Cisapride	No		No			No			The ligand occupies initial part of the pocket	4-amino-5-chloro-2-methoxybenz-1yl	10%
(-)-Cisapride	No		The ligand occupies initial part of the pocket	4-amino-5-chloro-2-methoxybenz-1yl	10 %	No			No		
R-Terfenadine	No		Yes	Phenyl moiety	5 %	Yes	Phenyl moiety	5 %	No		
S-Terfenadine	No		Yes	Phenyl moiety	5 %	The ligand occupies half of the pocket	Phenyl moiety	5 %	No		
Clofilium	The ligand occupies initial part of the pocket	4-chlorophenyl	Yes	1)4-chlorophenyl 2)-heptyl moiety	15 %	Yes	Heptyl moiety	5 %	No		
Sertindole	No		The ligand occupies initial part of the pocket	4-fluorophenyl	5 %	No			No		
Astemizole	No		Yes	1)benzimidazol 2)4-methoxyphenyl	10 %	No			Yes	methoxy moiety	5%

Dofetilide	No		No			Yes	Methanesulfonamide	5 %	No		
Grepafloxacin	No		The ligand occupies initial part of the pocket	methyl moiety	5 %	No			No		
R-Ibutilide	No		Yes	Methanesulfonamide	5 %	No			No		
S-Ibutilide	No		The ligand occupies half of the pocket	1)heptyl moiety 2)methanesulfonamide	15 %	The ligand occupies half of the pocket	Methanesulfonamide	5 %	Yes	Methanesulfonamide	10%

Table S8. Step 0, 1, 3, 4 in terms of the third set of docking poses.

	STEP 5 CHAIN C			STEP 6 CHAIN B	STEP 7 CHAIN B		STEP 8 CHAIN C		
	FLEXIBLE SIDECHAINS 8 Å			FLEXIBLE SIDECHAINS 8 Å	FLEXIBLE SIDECHAINS 8 Å		FLEXIBLE SIDECHAINS 8 Å		
	Phe 557 C;Leu 622 C;Thr 623 C;Ser 624 C;Tyr 652 B,C;Phe 656 C			Phe 557 B;Leu 622 B;Thr 623 B;Ser 624 B,C;Tyr 652 A,B;Phe 656 B	Phe 557 B;Leu 622 B;Thr 623 B;Ser 624 B,C;Tyr 652 A,B;Phe 656 B		Phe 557 C;Leu 622 C;Thr 623 C;Ser 624 C;Tyr 652 B,C;Phe 656 C		
LIGAND	Does ligand occupies pocket?	Moiety	%	Does ligand occupies pocket?	Does ligand occupies pocket?	Moiety	Does ligand occupies pocket?	Moiety	%
MK-499	No			No	The ligand occupies initial part of the pocket	1)Methanesulfonanilide 2)Cyano moiety	Yes	Methanesulfonanilide	20%
(+)-Cisapride	No			No	No		Yes	4-fluorophenox y	10%
(-)-Cisapride	No			No	No		Yes	1)4-fluorophenox y 2)4-amino-5-chloro-2-methoxybenz-1yl?	15%
R-Terfenadine	No			No	No		Yes	Phenyl moiety	15%

S-Terfenadine	Yes	Phenyl moiety	5%	No	No		Yes	Phenyl moiety	10%
Clofilium	No			No	No		Yes	Heptyl moiety	5%
Sertindole	No			No	No		The ligand occupies initial part of the pocket	1-imidazolidin-2-one	25%
Astemizole	No			No	No		Yes	methoxy moiety	10%
Dofetilide	Yes	Methanesulfonanilide	5%	No	No		Yes	Methanesulfonanilide	35%
Grepafloxacin	No			No	No		The ligand occupies initial part of the pocket	3-methylpiperazin-1-yl	5%
R-ibutilide	No			No	No		Yes	Methanesulfonanilide	70%
S-ibutilide	Yes	Methanesulfonanilide	5%	No	No		Yes	Methanesulfonanilide	45%

Table S8. Step 5,6,7,8 in terms of the third set of docking poses.

	STEP 10 CHAIN C			STEP 11 CHAIN C		STEP 13 CHAIN C			STEP 14 CHAIN C	STEP 15 CHAIN D
	FLEXIBLE SIDECHAINS 8 Å			FLEXIBLE SIDECHAINS 8 Å		FLEXIBLE SIDECHAINS 8 Å			FLEXIBLE SIDECHAINS 8 Å	FLEXIBLE SIDECHAINS 8 Å
	Phe 557 C;Leu 622 C;Thr 623 C;Ser 624 C;Tyr 652 C;Phe 656 C			Phe 557 C;Leu 622 C;Thr 623 C;Ser 624 C;Tyr 652 C;Phe 656 C		Phe 557 C;Leu 622 C;Thr 623 C;Ser 624 C;Tyr 652 B,C;Phe 656 C			Phe 557 C;Leu 622 C;Thr 623 C;Ser 624 C;Tyr 652 B,C;Phe 656 C	Phe 557 D;Leu 622 D;Thr 623 D;Ser 624 A,D;Tyr 652 D;Phe 656 D
LIGAND	Does ligand occupies pocket?	Moiety	%	Does ligand occupies pocket?		Does ligand occupies pocket?	Moiety	%	Does ligand occupies pocket?	Does ligand occupies pocket?
MK-499	No			No		No			No	No

(+)-Cisapride	No			No	No			No	No
(-)-Cisapride	No			No	No			No	No
R-Terfenadine	No			No	No			No	No
S-Terfenadine	No			No	No			No	No
Clofilium	Yes	4-chlorophenyl	5%	No	No			No	No
Sertindole	No			No	Yes	1-imidazolidin-2-one	20%	No	No
Astemizole	No			No	No			No	No
Dofetilide	No			No	No			No	No
Grepafloxacin	No			No	No			No	No
R-Ibutilide	No			No	No			No	No
S-Ibutilide	No			No	No			No	No

Table S9. Step 10, 11, 13, 14, 15 in terms of the third set of docking poses.

	STEP 18 CHAIN C			STEP 19 CHAIN B(Binding site Tyr 652)	STEP 19 CHAIN B(Binding site Gly 648)		
	FLEXIBLE SIDECHAINS 8 Å			FLEXIBLE SIDECHAINS 8 Å	FLEXIBLE SIDECHAINS 10 Å		
	Phe 557 C;Leu 622 C;Thr 623 C;Ser 624 C,D;Tyr 652 B,C;Phe 656 C			Phe 557 B;Leu 622 B;Thr 623 B;Ser 624 B,C;Tyr 652 A,B,C;Phe 656 A,B	Phe 557 A,B;Leu 622 B;Thr 623 A,B;Ser 624 B;Met 645 B;Tyr 652 A, B;		
LIGAND	Does ligand occupies pocket?	Moiety	%	Does ligand occupies pocket?	Does ligand occupies pocket?	Moiety	%
MK-499	No			No	No		
(+)-Cisapride	No			No	No		

(-)-Cisapride	No			No	No		
R-Terfenadine	Yes	Phenyl moiety	5%	No	No		
S-Terfenadine	No			No	No		
Clofilium	No			No	Gold couldn't find solution		
Sertindole	No			No	Yes	1-imidazolidin-2-one	15%
Astemizole	No			No	No		
Dofetilide	No			No	No		
Grepafloxacin	No			No	The ligand occupies initial part of the pocket	carboxy moiety	10%
R-Ibutilide	The ligand occupies initial part of the pocket	Methanesulfonamide	30%	No	No		
S-Ibutilide	No			No	No		

Table S9. Step 18, 19 in terms of the third set of docking poses. Additional set of docking poses was made using 19 step with Gly 648 as the binding site. It was conducted by utilising residues of interest, which line proposed hydrophobic pouches and set their side chains to freely rotate, while the approximate adjusted radius was 10 Å.

