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Adjudin disrupts spermatogenesis by targeting drug transporters

Lesson from the breast cancer resistance protein (BCRP)

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For non-hormonal male contraceptives that exert their effects in the testis locally instead of via the hypothalamic-pituitary-testicular axis, such as adjudin that disrupts germ cell adhesion, a major hurdle in their development is to improve their bioavailability so that they can be efficiently delivered to the seminiferous epithelium by transporting across the blood-testis barrier (BTB). If this can be done, it would widen the gap between their efficacy and general toxicity. However, Sertoli cells that constitute the BTB, peritubular myoid cells in the tunica propria, germ cells at different stages of their development, as well as endothelial cells that constitute the microvessels in the interstitium are all equipped with multiple drug transporters, most notably efflux drug transporters, such as P-glycoprotein, multidrug resistance-related protein 1 (MRP1) and breast cancer resistance protein (BCRP) that can actively prevent drugs (e.g., adjudin) from entering the seminiferous epithelium to exert their effects. Recent studies have shown that BCRP is highly expressed by endothelial cells of the microvessels in the interstitium in the testis and also peritubular myoid cells in tunica propria even though it is absent from Sertoli cells at the site of the BTB. Furthermore, BCRP is also expressed spatiotemporally by Sertoli cells and step 19 spermatids in the rat testis and stage-specifically, limiting to stage VII–VIII of the epithelial cycle, and restricted to the apical ectoplasmic specialization [apical ES, a testis-specific F-actin-rich adherens junction (AJ)]. Interestingly, adjudin was recently shown to be capable of downregulating BCRP expression at the apical ES. In this Opinion article, we critically discuss the latest findings on BCRP; in particular, we provide some findings utilizing molecular modeling to define the interacting domains of BCRP with adjudin. Based on this information, it is hoped that the next generation of adjudin analogs to be synthesized can improve their efficacy in downregulating BCRP and perhaps other drug efflux transporters in the testis to improve their efficacy to traverse the BTB by modifying their interacting domains.

Keywords: testis, adjudin, male contraception, ectoplasmic specialization, Bcrp, spermatogenesis

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Introduction

The impact of drug transporters in drug development is well established since multiple efflux and influx drug pumps are found in the epithelial and endothelial cells that create the blood-tissue barriers.[1-3] For the development of non-hormonal male contraceptives that exert their effects on developing germ cells in the seminiferous epithelium such as adjudin,[4,5] a better understanding of drug transporters in the testis and their interaction with the candidate contraceptive

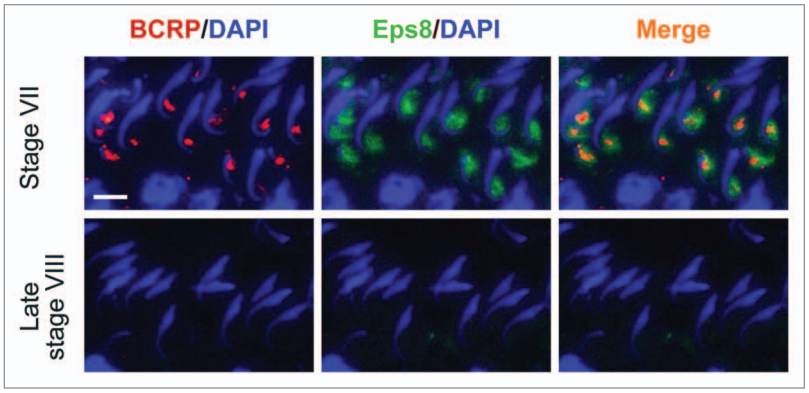

Figure 1. Stage-specific expression of BCRP at the apical ES during the epithelial cycle of spermatogenesis in the rat testis. BCRP (red fluorescence) was highly expressed at the apical ES, co-localized with a putative apical ES protein Eps8 (green fluorescence; epidermal growth factor receptor pathway substrate 8, an actin barbed end capping and bundling protein; it also displays stage-specific and spatiotemporal expression at the ES in the rat testis[23]) in stage VII tubules, but this expression rapidly subsided and diminished to a level almost undetectable at stage VIII when the release of sperm takes place at spermiation. Cell nuclei were visualized by DAPI (4′,6-diamidino-2-phenylindole) staining. Detailed of the information on antibodies and procedures used to obtain results on this study can be found in reference 18. Scale bar = 10 μm, which applies to all other micrographs.

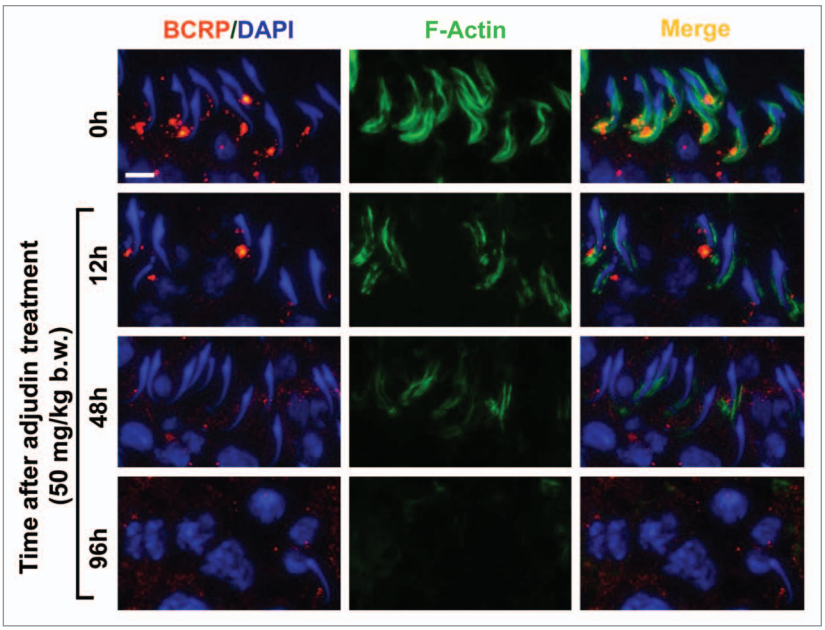

Figure 2. Adjudin effectively downregulates the expression of Bcrp at the apical ES. Rats (n = 3 for each time point) were treated with a single oral dose of adjudin at 50 mg/kg b.w. at time 0 h (hour) (control). Thereafter, rats were terminated at 12-, 48- and 96 h. BCRP (red fluorescence) was found to co-localize with F-actin (green fluorescence, visualized using FITC-phalloidin) in control testes and expressed prominently in this stage VII tubule. However, a rapid decline in BCRP expression at the apical ES was detected as soon as 12 h after adjudin treatment, and by 48 and 96 h, BCRP expression was completely abolished. Cell nuclei stained by DAPI. Scale bar = 10 μm, which applies to all other micrographs. Detailed of the information on antibodies and procedures used to obtain results on this study can be found in reference 18.

drugs are particularly important since the blood-testis barrier (BTB), created by specialized junctions between adjacent Sertoli cells near the basement membrane, is one of the tightest blood-tissue barriers in the mammalian body.[6-8] If the interacting domains between drug transporters and the candidate molecules are known, this information will be helpful to prepare better compounds that can have improved bioavailability, thereby widening the gap between their efficacy and toxicity.

Recent studies have shown that the testis is equipped with numerous drug transporters, many of which are highly expressed by Sertoli cells and different classes germ cells, including spermatogonia, spermatocytes, spermatids and spermatozoa,[9-16] and with different substrate specificity.[6,17] Several reports have shown that breast cancer resistance protein (BCRP), a member of the ABC (ATP-binding cassette) efflux drug transport (also known as ABCG2, ATP-binding cassette sub-family G member 2) that actively prevents drugs from entering into a mammalian cell or actively pumps drugs out of a cell that have somehow evaded the tissue barrier and got into the cell cytosol are also abundantly found in peritubular myoid cells and endothelial cells of the microvessels in the interstitium even though it is absent in the Sertoli cell at the BTB.[11,16,18] However, a recent study utilizing techniques of PCR and immunoblotting has shown that BCRP was expressed by Sertoli cells, even though at a level considerably lower than the peritubular myoid cells, germ cells and/or endothelial cells of the microvessel.[18] More important, BCRP is restricted to the Sertoli-spermatid interface in the adluminal compartment of the seminiferous epithelium known as the apical ectoplasmic specialization (apical ES), a testis-specific F (filamentous)-actin-rich adherens junction (AJ)[18] (**Fig. 1**). BCRP first appears and weakly expressed at the apical ES in stage VI tubules, however, it becomes prominently expressed at the apical ES at stage VII (**Fig. 1**), and it is rapidly downregulated in stage VIII and virtually non-detectable by late stage VIII of the epithelial cycle (**Fig. 1**).[18] These findings led us to speculate that BCRP might be crucial for the completion of spermiogenesis, having this drug efflux transporter to become highly expressed at this site at stage VII of the epithelial cycle to ensure the completion of spermiogenesis[18] (**Fig. 1**). Interestingly, the expression of BCRP is rapidly downregulated at the apical ES following exposure of adult rats

to adjudin, 1-(2,4-dichlorobenzyl)-1H-indazole-3-carbohydrazide, a potential male contraceptive under development in our laboratory[4-6] and it is also known to possess anti-inflammatory[30] and anticancer activity[31] (**Fig. 2**),[18] illustrating adjudin can specifically interact with BCRP. If this assumption is correct, better analogs of adjudin perhaps can be synthesized if the interacting domains between adjudin and BCRP can be identified, and that these new generations of drugs can be more potent in downregulating drug transporters such as BCRP in the testis and also the brain. Below is a summary of these findings based on the use of molecular modeling to explore the putative interacting domains between BCRP and adjudin.

Molecular docking of human and rat BCRP with adjudin. Active sites of proteins are often associated with structural pockets in the protein. The identification of such substrate binding sites in enzymes have helped us to understand their binding interactions with substrates and other small molecules. The drug-binding site of BCRP was obtained from literature. The docking simulation tool, Glide (Schrödinger, Inc.), was used to perform docking and to maintain uniformity with each of the two BCRP proteins and adjudin. The modeled proteins were prepared using the Protein Preparation Wizard, a workflow in the Schrödinger Suite of programs. Using this tool, all hydrogen atoms were added to the proteins, the protonation states for histidine residues were optimized, and the entire protein was minimized using OPLS (Optimized Potentials for Liquid Simulations)-2005 force field. The ligand, adjudin was prepared using Schrödinger Ligprep tool (Version 2.3, Schrödinger, LLC, New York, NY, 2009). LigPrep was used to find stereoisomers and to perform energy minimization using OPLS-2005 force field. The binding site was defined in terms of two concentric cubes: the bounding box, which contains the center of any acceptable ligand pose, and the enclosing box, which contains all ligand atoms of an acceptable pose. The ligand was docked flexibly to the two BCRPs using Simple Precision (SP) mode in Glide (Version 5.5, Schrödinger, LLC, New York, NY). To soften the potential for non-polar parts of the ligand, the van der Waals radii of ligand atoms were scaled with partial atomic charge (absolute value) less than the specified cutoff. Default scaling factor is 0.8 and the partial cutoff value is 0.15. The Glide docking algorithm generates 5,000 poses per ligand for the initial phase of docking and restricts to 400 poses for energy minimization. Upon completion of each docking calculation, the best docked structure was chosen using Glidescore (Gscore) function, a modified and extended version of the empirically based ChemScore scoring functions.

Homology modeling. An interesting feature of BCRP is that while most ABC transporters exist as heterodimers, BCRP is a single chain protein forming a homodimer which confers its functionality with a capability of undergoing oligomerization. Another unique feature of BCRP is that the domain topology is reversed here when compared with all other ABC transporters. The two domains viz. transmembrane helical domain (TM) and the nucleotide binding domain (NBD) are common to all ABC transporters. While other ABC transporters have TM at their N-terminal end followed by NBD at their C terminus (N-TM-NBD-C), in the case of BCRP, the protein starts with NBD at its N-terminal and then followed by TM domain at its C-terminal end (N-NBD-TM-C). This anomaly is a major obstacle for homology modeling of BCRP, which warrants for a different approach in the alignment of the template structure with BCRP protein. The structure and sequence of the template structure was rearranged to follow the N-NBD-TM-C pattern of BCRP protein. Hence, the template structure was chosen with all these factors involved. The amino acid sequence of BCRP of rat and human was used to find a suitable structural template using BLASTp against PDB database. The best homolog was selected based on functional similarity, sequence similarity score and crystal structures with better resolution. The template crystal structure best suited for BCRP of rat as well as human was multidrug resistance protein (P-glycoprotein) (PDB ID: 3G60).[19] The template structure was taken and the transmembrane and nucleotide binding domains were rearranged to mimic the membrane topology of BCRP. The resulting changes in residue numbers were fixed and the structure was optimized for use as template. The structure of BCRP of rat and human was modeled and the best reliable models were subjected to several steps of loop refinement by DS3.1 and validation by PROCHECK.

The Ramachandran Plot for human BCRP (**Fig. 3A**) and rat BCRP (**Fig. 3B**) showed all residues within the core and generously allowed region and no residues in the disallowed region. The validation of the two modeled BCRP structures shows that the stereochemical geometry as well as the overall structural geometry of the models is good. The structure of BCRP of human and rat represents an inward-facing conformation closely representing a 2-fold symmetry. The nucleotide binding domains (NBDs) are separated and the inward facing conformation is formed from two bundles of six helices. This results in a large internal cavity open to both the cytoplasm and the inner leaflet. The structure is consistent with the template crystal structure of mouse P-glycoprotein as well as the other ABC transporters. These modeled structures can now be further used to study interactions with small molecules, such as adjudin.

Molecular docking analysis. Molecular-docking was performed on the 3D model of BCRP, built by the homology modeling method. As BCRP is a multi-drug resistance protein, the drug binding pocket is a wide region forming a central cavity formed by membrane-spanning TM α-helices, which possess multiple drug binding sites. Site-directed mutagenesis has provided enough evidence supporting that Arg482 is a crucial residue for substrate specificity as well as transport activity.[20] Docking calculations done on BCRP and mitoxantrone (an antineoplastic agent for treating metastatic breast cancer, acute myeloid leukemia and non-Hodgkin's lymphoma) indicate that Arg482 might be directly involved in drug interaction.[21] Another study also indicated that His457 and Arg465 might be directly involved in substrate binding.[22] These residues were thus used for setting up docking grid. The modeled structure reveals that the inward facing conformation is competent to bind drugs. Since BCRPs

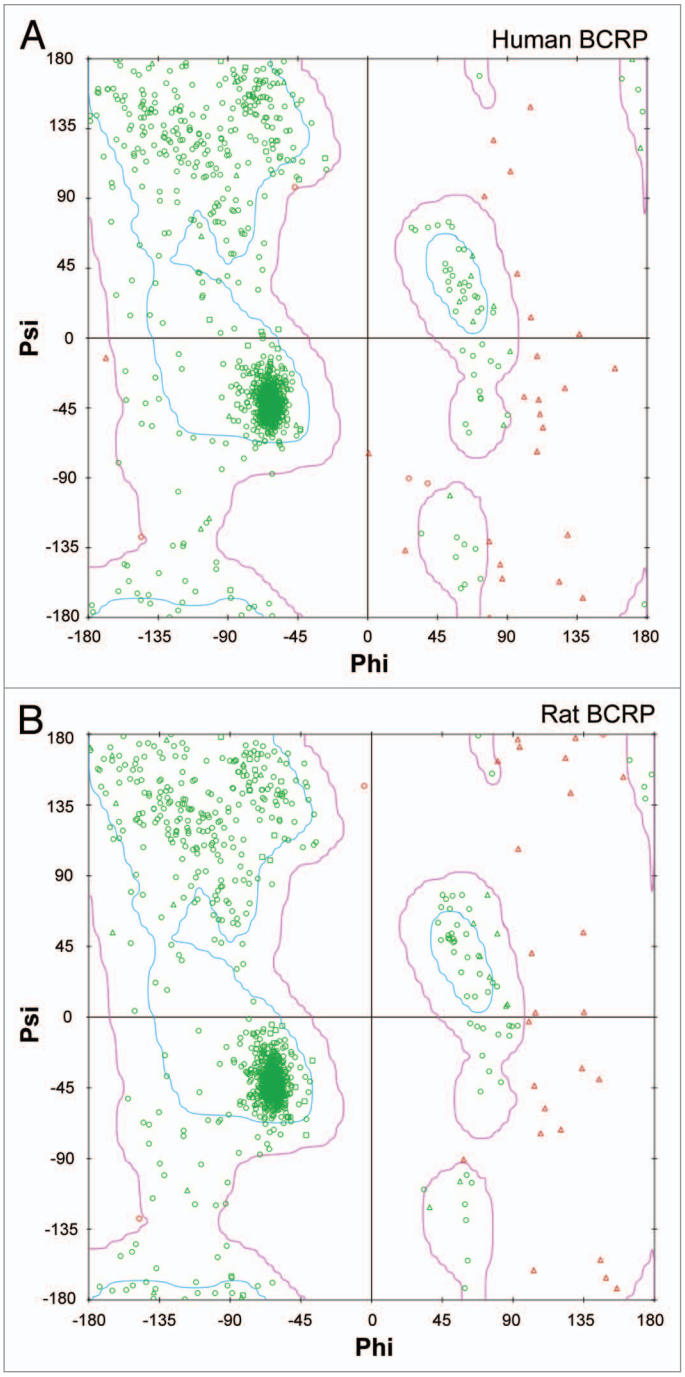

Figure 3. Ramachandran plot for the modeled human (**A**) and rat (**B**) BCRP. The amino acid sequences of breast cancer resistance protein (BCRP/ABCG2) of *Homo sapiens* (UniProtKB ID: Q9UNQ0) and of *Rattus rattus* (UniProtKB ID:Q80W57) were retrieved from UniProt (www.uniprot.org). A BLASTp[24] search was performed to find appropriate proteins with significant amino acid sequence and structural similarity to BCRP by searching the Protein Data Bank (PDB) database (PDB, www.rscb.org/pdb/).[25] The search was refined to find a suitable structural homolog for the modeling of BCRP in human and also in rat (see **Figs. 4 and 5**). The amino acid sequence of these two proteins and their template sequences were aligned using the web based interface MultAlin.[26] Based on the alignment generated, the tertiary structure of BCRP of human and rat were predicted using Modeler v9.11.[27] Discrete Optimized Protein Energy (DOPE) and Modeler Objective Function (MOF) scores of the resulting models were used to select the most reliable model. The predicted structures were energy minimized by Smart Minimizer algorithm in Discovery Studio 3.1. The minimization was performed in 500 steps by applying CHARMM (Chemistry at HARvard Macromolecular Mechanics) force field and then subjected to validation. Backbone conformation was evaluated by examining the Psi/Phi interactions in Ramachandran Plot, obtained from PROCHECK,[28] for human (**A**) and rat (**B**) BCRP and shown herein. Based on the plot, residues in the disallowed regions were refined using Loop Refinement (MODELER) module, from DS3.1. The final refined model was tested for their stability and reliability using ERRAT.[29]

of both rat and human share significant similarity in their sequence and structure, the same binding pocket residues with variation corresponding to their sequence position were specified for docking. Many of these residues face the drug binding pocket and are highly conserved, suggesting a common mechanism of polyspecific drug recognition.

Docking of BCRP with adjudin. The docking simulation of human and rat BCRP with adjudin shows that adjudin binds to human BCRP with a docking score of –3.859 kcal/mol and –4.856 kcal/mol, respectively. The docking energy and van der Waal's energy involved in docking are tabulated in **Table 1**. The docked complex of human as well as rat BCRP shows that Glu451 forms hydrogen bond with adjudin. In human BCRP, Phe470 plays a major role in forming Pi-Pi interactions with the ligand (**Fig. 4B and C**). The docked complex of rat BCRP shows that adjudin also forms two Pi-cation interactions with Arg465 (**Fig. 5B and C**). Two dimension plot created using DS3.1 for both the docked complexes illustrates a detailed view of the types of molecular interactions between adjudin and BCRP of human and rat (**Figs. 4C and 5C**).

Concluding Remarks and Future Perspective

It is clear that adjudin is interacting with BCRP via specific interacting domain at

Table 1. Molecular Interactions of BCRPs with adjudin

Receptor	Docking score (kcal/mol)	Docking energy (kcal/mol)	Van der Waal's energy (kcal/mol)	Hydrogen bond interacting residues	Van der Waal's interaction residues	Pi stacking interaction residues
Human BCRP	-3.859	-30.869	-27.111	Glu451 (2.03Å)	Glu319, Gln393, Val450, Ile460, Tyr464, Val533, Val534, Thr538	Phe470
Rat BCRP	-4.856	-30.620	26.748	Glu451 (2.01 Å)	Glu446, Leu447, Val450, Ser467, Tyr469, Phe470, Leu471, Val533	Arg465

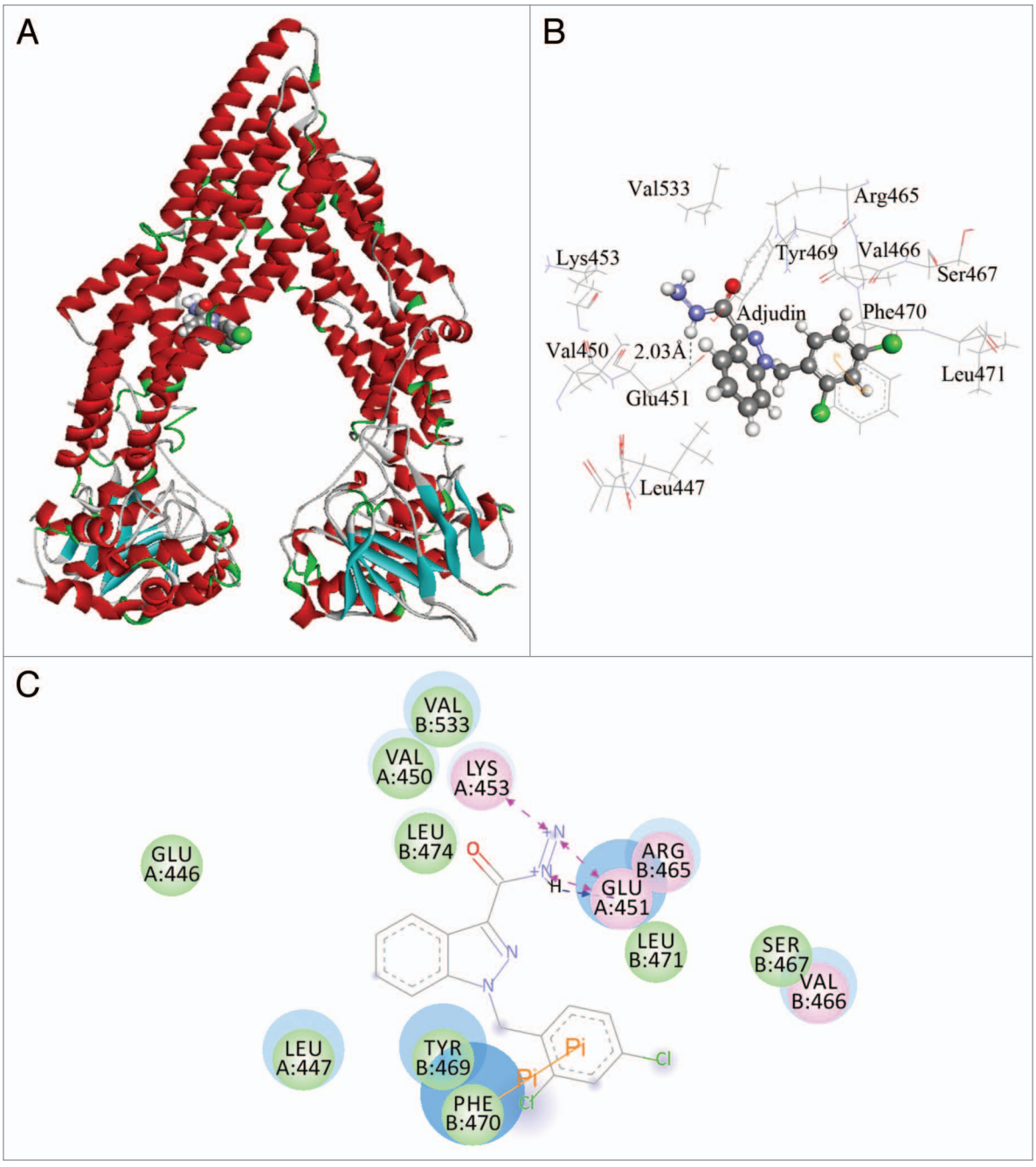

Figure 4. For figure legend, see page 6.

Figure 4. Docked complex of human BCRP with adjudin. (**A**) Entire modeled human BCRP in ribbon format, colored as per its secondary structure and the docked adjudin in CPK model. White for H; gray for C; blue for N; red for O; purple for P; green for Cl. (**B**) Enlarged view of the docking site in (**A**). Adjudin is depicted in scaled ball and stick model, interacting residues are in stick model and their interactions in its 3D conformation. (**C**) Two dimension representations of molecular interactions between adjudin and the human BCRP. Green circles represent residues involved in van der Waal's interactions; pink circles represent residues involved in hydrogen bond, polar or charge interactions; blue halo around residues represent solvent accessible surface of an interacting residue. Orange lines represent Pi-Pi and Pi-cation interactions between Phe470 and adjudin. Blue dotted line represents hydrogen bond formation with side chain of Glu451.

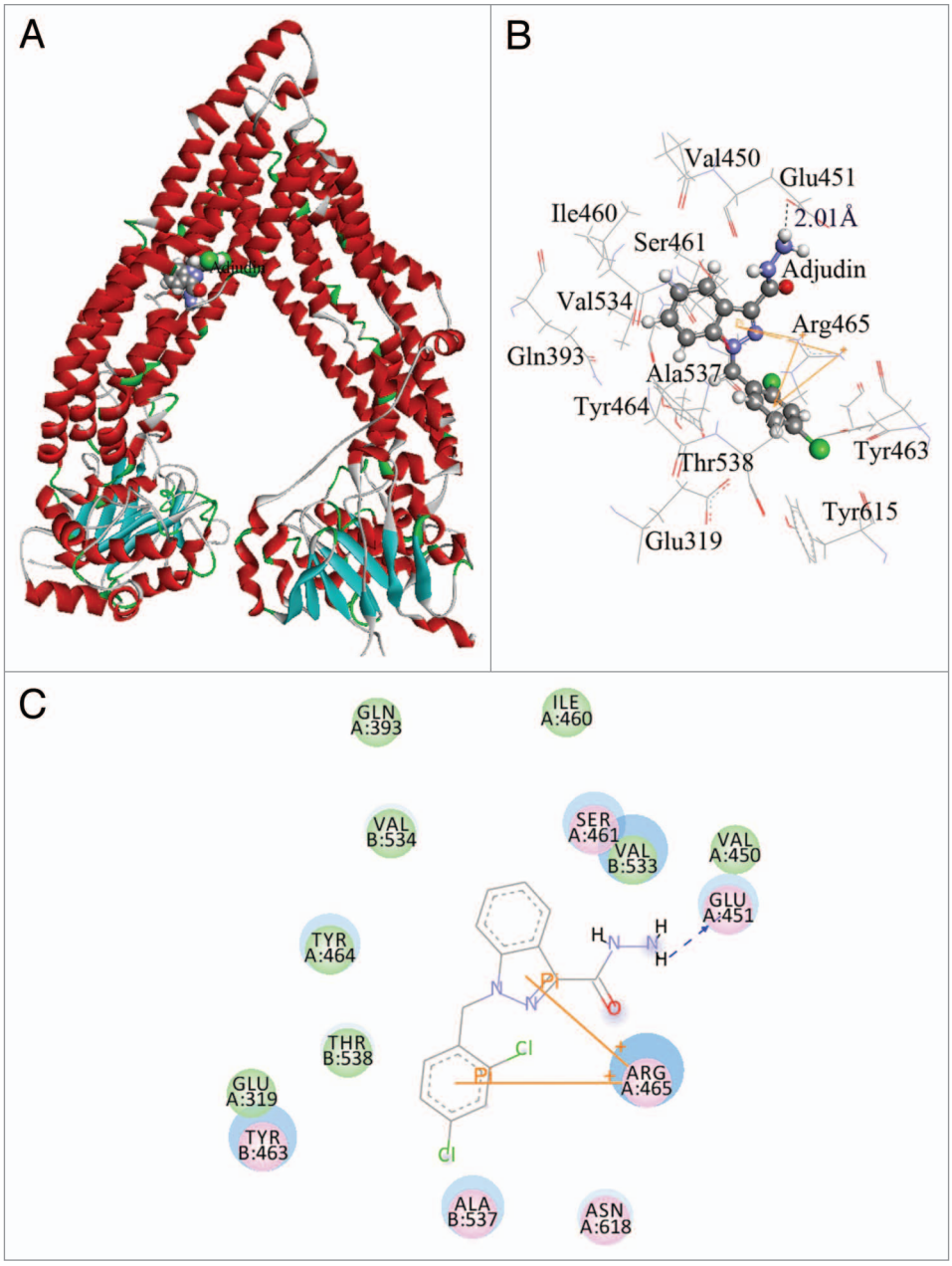

Figure 5. Docked complex of rat BCRP with adjudin. (**A**) This figure depicts the entire modeled protein docked with adjudin. (**B**) An enlarged view of the interacting residues in 3D conformation. (**C**) Orange line represents Pi-Pi interaction between Arg465 and adjudin. Blue dotted line represents hydrogen bond formation between side chain of Glu451 and donor oxygen atom of Adjudin. See **Figure 4C** for residue color code.

the likely docking pocket of BCRP from humans (**Fig. 4**) and rats (**Fig. 5**). This information will be helpful in our synthesis strategy of developing better analogs of adjudin to downregulate BCRP as well as other efflux drug transporters (e.g., P-glycoprotein) to further improve its efficacy in disrupting spermatogenesis.

Disclosure of Potential Conflicts of Interest

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