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Interaction of Oligomeric Breast Cancer Resistant Protein (BCRP) with Adjudin: A Male Contraceptive with Anti-Cancer Activity

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Abstract

Breast cancer resistant protein (BCRP, ABCG2) is an ATP-binding cassette (ABC) transporter, which together with two other ABC efflux drug pumps, namely P-glycoprotein (P-gp, ABCB1) and multidrug resistance-related protein 1 (MRP1, ABCC1) is the most important multidrug resistance protein found in eukaryotic cells including cells in the testis. However, unlike P-gp and MRP1, which are components of the Sertoli cell blood-testis barrier (BTB), BCRP is not expressed at the BTB in rodents and human testes. Instead, BCRP is expressed by peritubular myoid cells and endothelial cells of the lymphatic vessel in the tunica propria, residing outside the BTB. As such, the testis is equipped with two levels of defense against xenobiotics or drugs, preventing these harmful substances from entering the adluminal compartment to perturb meiosis and post-meiotic spermatid development: one at the level of the BTB conferred by P-gp and MRP1 and one at the tunica propria conferred by BCRP. The presence of drug transporters at the tunica propria as well as at the Sertoli cell BTB thus poses significant obstacles in developing non-

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

hormonal contraceptives if these drugs (*e.g.*, adjuvin) exert their effects in germ cells behind the BTB, such as in the adluminal (apical) compartment of the seminiferous epithelium. Herein, we summarize recent findings pertinent to adjuvin, a non-hormonal male contraceptive, and molecular interactions of adjuvin with BCRP so that this information can be helpful to devise delivery strategies to evade BCRP in the tunica propria to improve its bioavailability in the testis.

Keywords

Adjuvin; male contraceptive; anti-cancer drug; breast cancer resistance protein; ectoplasmic specialization; spermatogenesis; testis

1. INTRODUCTION

Adjuvin, 1-(2,4-dichlorobenzyl)-*1H*-indazole-3-carbohydrazide, is an analog of lonidamine synthesized in the 2000s [1, 2]. Lonidamine, 1-(2,4-dichlorobenzyl)-1*H*-indazole-3-carboxylic acid, is an indazole-containing anti-cancer drug synthesized in the 1970s which, along with its structurally related compounds, was shown to have potent anti-spermatogenic activity [3, 4]. However, lonidamine, unlike most other anti-cancer drugs, is not anti-mitotic, instead, it is a mitochondrial hexokinase inhibitor, most notably an inhibitor of aerobic glycolysis in tumor cells [5–7]. It is noted that due to the shortage of oxygen during tumorigenesis, tumor cells utilize glucose to undergo aerobic glycolysis, generating ATPs *via* the production of pyruvate, which is then metabolized to lactate instead of going through the Krebs cycle to generate the maximal number of ATPs per glucose molecule – the hallmark of tumor cell metabolism known as the Warburg effect. This metabolic behavior of selecting aerobic glycolysis limited to the production of pyruvate and lactate is maintained in cancer cells even when they are exposed to sufficient oxygen. Interestingly, the Sertoli cell in the testis, somewhat similar to cancer cells, also prefers to metabolize glucose to pyruvate *via* aerobic glycolysis, which is then converted to lactate instead of having the pyruvate to enter the Krebs cycle, undergoing oxidative respiration in the mitochondria for an entirely different reason [8, 9]. During spermatogenesis, each Sertoli cell supports ~30–50 developing germ cells at different stages of their development by providing nourishment, as well as functional, structural, and hormonal supports [10]. However, germ cells are incapable of utilizing glucose (or other hexoses) to generate energy for their survival, instead they rely on Sertoli cells solely for the supply of lactate [8, 9]. Thus, germ cells obtain lactate from the Sertoli cells, which is then converted to pyruvate by a germ cell-specific LDH called LDH-X (or LDH-C4) [11]. Pyruvate can then be metabolized to generate ATPs *via* the Krebs cycle in the mitochondria *via* oxidative phosphorylation [8, 9]. Thus, while lonidamine is a mitochondrial hexokinase inhibitor, its anti-spermatogenic effect is not targeted specifically to germ cell metabolism since germ cells in the testis do not utilize hexokinase to generate ATPs. Instead, lonidamine exerts its effects primarily on mitochondrial hexokinase II in tumor cells with condensed mitochondria, most notably following their exposure to irradiation which is known to induce the formation of condensed mitochondria, or when lonidamine is being used in combination with other chemotherapeutic drugs, such as paclitaxel [6, 12–14]. During exposure of rats to adjuvin or lonidamine, the most noticeable effect is the rapid depletion of spermatids from the

seminiferous epithelium, which usually occurs within ~6–8 hr and more than 50% of the tubules examined display signs of spermatid loss [15–18]. However, spermatozoa in the epididymis are not affected since rats treated with adjuvin at doses capable of inducing infertility remain fertile for up to ~4-wk before the sperms stored in the epididymis are exhausted [19]. It is also known that exposure of Sertoli cells to lonidamine induces vacuolization, signs of Sertoli cell focal damage, as well as reducing Sertoli cell aromatase activity, thereby reducing its ability to produce estradiol-17 β , but lonidamine has no apparent effects on Sertoli cell protein and nucleic acid synthesis activity [20]. Also, lonidamine has no apparent effect on Sertoli cell aerobic glycolysis and energy metabolism [21]. However, acute doses of lonidamine are found to disrupt mitochondrial membranes of germ cells, causing mitochondrial degeneration, mostly notably in the maturation phase of spermatids during spermiogenesis in the rat [22].

2. ADJUDIN AND ITS BIOAVAILABILITY IN THE TESTIS

Adjuvin, being an analog of lonidamine, its administration to rats, mice, and rabbits induces phenotypic changes in the testis similar to lonidamine, causing exfoliation of germ cells from the seminiferous epithelium, most notably elongating/elongated spermatids, to be followed by round spermatids and eventually spermatocytes but apparently not undifferentiated spermatogonia or spermatogonial stem cells since fertility rebounds in adjuvin treated animals [19, 23–25]. Subsequent studies have shown that adjuvin exerts its effects primarily at the apical ES (ectoplasmic specialization), a testis-specific and actin-rich adherens junction typified by the presence of actin microfilaments that lie perpendicular to the Sertoli cell plasma membrane, which are grouped into bundles and sandwiched in-between cisternae of endoplasmic reticulum and the Sertoli cell plasma membrane [26–28]. For instance, adjuvin causes defragmentation of actin microfilaments, which in turn leads to depletion of spermatids from the seminiferous epithelium, this is followed by desmosome that elicits adhesion at the Sertoli-early spermatid and Sertoli-spermatocyte interface [29–32], and then the basal ES at the blood-testis barrier (BTB) [33]. However, these disruptive effects are transient, as such, the antifertility effect is reversible unless an acute high dose is used, which is estimated to be ~3–5 times of the effective dose, at ~125–250 mg/kg b.w. *vs.* 50 mg/kg b.w., and such high doses can induce permanent damage to the BTB, making the treated rats incapable of re-initiating spermatogenesis even though the population of spermatogonial stem cells and/or undifferentiated spermatogonia in the tubules is largely unaffected [33]. Thus, this drug has been actively investigated as a potential male contraceptive. However, one of the obstacles of developing adjuvin, or any contraceptives that exert their effects in the testis in the adluminal compartment, is the presence of the blood-testis barrier (BTB) that severely limits its bioavailability [34]. For instance, using [³H]-adjuvin, it was shown that fewer than 1% of orally administered adjuvin reached the testis [19], illustrating that if this drug can be specifically targeted at the testis, its effective dose can be significantly lowered. Even though studies based on a proof-of-concept approach have shown that conjugation of adjuvin *via* its hydrazide group to an FSH mutant which has its intrinsic FSH activity inactivated while retaining its FSH receptor binding affinity is a viable approach such that the conjugate can be specifically targeted to the testis [2]. However, the cost of preparing the conjugate plus the need for using a needle for

administering the conjugate into the male subject renders this approach not an attractive choice for further development. Thus, the biology of BTB must be better understood before a non-hormonal contraceptive that exerts its effects locally in the testis can possibly be developed [35]. Recent studies have shown that the testis is equipped with numerous drug transporters, such as P-glycoprotein, breast cancer resistance protein (BCRP) and several others that actively pump adjuvin out of the Sertoli cell, or deny its entry to the adluminal compartment [35–37]. Many of these drug transporters are expressed by Sertoli cells at the BTB that guard the entry of foreign drugs into the adluminal compartment including male contraceptive adjuvin. Interestingly, while BCRP is not expressed by the Sertoli cell at the BTB in the rat testis, it is highly expressed by peritubular myoid cells and endothelial cells of the lymphatic vessel in the tunica propria, as well as endothelial cells of the microvessels in the interstitial space [38–40]. Furthermore, BCRP is also expressed by step 19 spermatids at stage VII and also at early stage VIII tubules, displaying a strict spatiotemporal expression at the concave side of elongated spermatid heads at the Sertoli-spermatid interface during the epithelial cycle [38]. Collectively, these findings suggest that BCRP is the first line of defense to limit the access of any drug to the adluminal compartment behind the BTB. In short, BCRP would pump drugs out of the testis before they could even reach the Sertoli cell BTB, illustrating that an extensive network of drug transporters is present in the testis to protect the well-being of spermatogenesis, in particular post-meiotic development, from harmful substances including male contraceptives. However, if some residual chemicals/drugs (*e.g.*, adjuvin) manage to pass the Sertoli cell BTB, entering the adluminal compartment, elongated spermatids that express BCRP at stage VII-early VIII prior to their release at spermiation can pump the contraceptive adjuvin out, attempting to maintain spermatogenesis.

Recent studies have shown that treatment of adult rats with adjuvin by oral gavage at 50 mg/kg b.w. down-regulates the expression of BCRP significantly based on immunoblot analysis using testis lysate [38]. Also, there are considerable changes in the spatiotemporal expression of BCRP in the seminiferous epithelium in these rats. For instance, the predominant expression of BCRP at the concave (ventral) side of spermatid heads at the Sertoli-spermatid interface, namely the apical ectoplasmic specialization, in stage VII-early VIII tubules is considerably diminished [38]. However, its expression at the peritubular myoid cell layer in the tunica propria remains high and relatively unaltered following adjuvin treatment [38]. Taking collectively, these findings suggest that there are putative interactions between adjuvin and BCRP, which in turn induce signaling function changes downstream to elicit changes on BCRP expression, at least at the apical ES. Thus, we sought to perform molecular modeling analysis to identify the interacting domain of BCRP with adjuvin, and this information is likely to be helpful in ongoing studies to identify better analogs of adjuvin for male contraception, so that BCRP fails to recognize and engage adjuvin by pumping it (or its derivatives) out of the testis.

3. BCRP (BREAST CANCER RESISTANT PROTEIN)

BCRP, also known as ABCG2 (ATP-binding cassette subfamily G member 2) and CDw338 (cluster of differentiation w338) is a 70 kDa integral membrane protein in the superfamily of ATP-binding cassette (ABC) transporters and a member of the White subfamily [41–43].

BCRP is an efflux drug transporter found in multiple organs including the gut barrier along the small and large intestine, tight junction (TJ) of the proximal tubule and Bowman's capsule brush border in the kidney, and the blood-brain barrier [40, 42, 43]. BCRP, together with P-glycoprotein (P-gp/ABCB1) and multidrug resistance-associated protein 1 (MRP1/ABCC1, also known as multidrug resistance-related protein 1) is the most important and best studied eukaryotic ABC transporter involved in multidrug resistance. Interestingly, BCRP is not a component of the Sertoli cell BTB in the rodent testis and no BCRP expression was detected at the BTB [39, 40]. Instead, BCRP is highly expressed by peritubular myoid cells and endothelial cells of the lymphatic vessel at the tunica propria [38–40], lying outside the Sertoli cell BTB. While Sertoli and germ cells also expressed some BCRP in the rat testis, it is localized and expressed spatiotemporally by these cells at the Sertoli-spermatid interface known as the apical ectoplasmic specialization (apical ES), which is located at the opposite end of the BTB in the seminiferous epithelium, and restrictively expressed at stage VII of the epithelial cycle [38]. These findings thus suggest that unlike other efflux drug transports, such as P-glycoprotein, which are localized to the Sertoli cell BTB near the basement membrane in the seminiferous epithelium [39, 44, 45], BCRP provides the testis with an added layer of protection by being strategically localized to the tunica propria [38]. Since tunica propria is located outside the BTB, and the BTB is already guarded by P-glycoprotein [39, 44, 45] and other drug transporters (*e.g.*, Oatp3) [46], the strategic localization of BCRP at the tunica propria thus provides the adluminal compartment of the seminiferous epithelium with another line of defense, in the light of possible assault from xenobiotics and toxicants. However, such extensive defense system also poses a major obstacle for the development of a non-hormonal male contraceptive, in particular, if it exerts its effects in the adluminal compartment of the seminiferous epithelium behind the BTB, such as adjuvin. Adjuvin must first be able to penetrate through the endothelial cells of the lymphatic vessel and the peritubular myoid cells without being stopped by drug transporters, such as BCRP. If it succeeds to pass the barrier imposed by BCRP at the tunica propria, adjuvin faces another barrier imposed by P-glycoprotein at the Sertoli cell BTB besides the tight junction-barrier at the site. Thus, in an effort to better deliver adjuvin to the adluminal compartment, we perform molecular modeling analysis to understand the interaction between adjuvin and BCRP. The 70 kDa BCRP polypeptide is a half ABC transporter with one NBD (nucleotide binding domain) near its terminus, followed by one TMD (transmembrane binding domain) [47], and the TMD consists of six TM (transmembrane) α helices [48]. Thus, a functional BCRP ABC transporter is a homodimer. In a recent report, we used molecular modeling approach to examine the interaction between adjuvin and the functional homodimer of BCRP [49]. However, BCRP is also known to assume a tetrameric configuration in which four functional homodimers of BCRP create an oligomeric drug efflux transporter of BCRP [50, 51] in mammalian cells. We herein present some findings based on molecular modeling on the likely interaction between adjuvin and the human BCRP oligomer. This information will be helpful in future synthesis experiments to prepare better derivatives of adjuvin to maximize its bioavailability in the testis by reducing their interactions with drug transporters in the testis such as BCRP to improve bioavailability.

4. INTERACTION OF BCRP WITH ADJUDIN

(i) Three dimensional structure of human BCRP homodimer with an inward and outward conformation and molecular interaction with adjudin

The structural organization of BCRP protein shows that nucleotide binding domain (NBD) is at its N-terminal, followed by transmembrane (TM) domain at their C-terminal (NH₂-NBD-TM-COOH). The three dimensional structure of the BCRP homodimer in their inward conformation was predicted using the template (PDB ID: 3G60) [52], as described in our previous study [49], and the outward 'closed' conformation of BCRP was predicted using the template ABC transporter (Sav1866) from *Staphylococcus aureus* (PDB ID: 2HYD) [53]. In both cases, the domain regions of templates were swapped to match the domain organization of BCRP. Predicted models were refined and validated as defined in our earlier study [49].

The predicted model depicts that in the inward conformation, BCRP adopts a characteristic 'inverted V' shape (Fig. 1A–C; Fig. 2A) whereas in the outward facing conformation it adopts an 'X' shape as seen in other ABC transporters (Fig. 2A). The ability of adjudin to bind with both conformations of BCRP was then evaluated using molecular docking studies (Fig. 2A–C).

The receptor grid for outward conformation of BCRP was defined around residue Arg482, which is crucial for substrate specificity and transport [54]. Molecular docking analysis of human BCRP in outward conformation shows that adjudin interacts with both monomers and forms an hydrogen bond with Asp418 of monomer B (orange), and it also forms pication interactions with Arg482 and Lys616 of monomer A (green) and with Arg378 of monomer B (orange) (Fig. 2C). Adjudin forms hydrophobic interactions with Lys417, Thr421, Gly422, L479, Val533, Lys616, Ile619 and Asp620 of monomer A (green). It also establishes charged interactions with Asn418, Ala529, Gly530 of monomer A (green) and Arg378, Asn418, Thr421 and Gln424 of monomer B (orange). In comparison with inward and outward conformation, adjudin is observed to switch between two different binding sites (Fig. 2B,C). Initially, it binds with BCRP through strong electrostatic interaction with R465, which is experimentally proven to be crucial for substrate binding. Later it switches to the second binding site, where it forms electrostatic interaction with R482, which is proven vital for drug transport activity [51]. The switch between the binding sites is shown to be observed by various other drug molecules in ABC drug transporters. In both the cases, the 3-carbohydrazide moiety of adjudin remains important for active positioning of adjudin within the BCRP, which indicates that replacement at these positions by a suitable hydrophobic group can effectively prevent adjudin from interacting with BCRP.

(ii) Three dimensional structure of human BCRP in tetrameric arrangement of homodimers

The homodimer model from our previous study [49] was used to generate the tetramer, which was formed by the association of 4 functional homodimers that spanned the membrane constituting 8 individual monomers of BCRP (Fig. 3A). We also generated the tetrameric arrangement of BCRP complexed with adjudin (Fig. 3B). Since the arrangement of NBD and TM in BCRP are different from the usual organization of ABC transporters, the

inter- domain interactions between adjacent homodimers are unclear and require extensive MD (molecular dynamics) simulations. Herein, we reported our findings for the BCRP tetramer arrangement using *Coot*, an application for model building and validation of biological macromolecules, as described [55].

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In this short review, we briefly summarize latest findings on adjuvin that is being actively investigated as a male contraceptive in our laboratory. It is noted that adjuvin is an analog of lonidamine, and adjuvin was synthesized among more than two dozens analogs, aiming to identify a less toxic version of lonidamine, yet maintaining the potent antispermatogenic effects. Since lonidamine is an anticancer drug, thus, it is not surprisingly that adjuvin also possesses anti-cancer activity [56]. Interestingly, adjuvin also has anti-neuroinflammatory and anti-neurodegenerative activity [57, 58]. Collectively, these other data illustrate the added health benefits of adjuvin. Thus, it is important to better understand the biology of drug transports in the testis so that second generation adjuvin-based drugs can be synthesized to evade the drug transporters at the Sertoli cell BTB and tunica propria. Herein, we review some recent data on BCRP and present our findings on molecular modeling of adjuvin interactions with functional BCRP homodimer and the tetrameric configuration. This information will be used by synthetic chemists to modify the side chains of adjuvin to obtain better analogs for their delivery to the testis by avoiding or reducing interactions with drug transports such as BCRP. Perhaps, it is necessary to perform additional studies to examine the role of BCRP on the bioavailability of adjuvin and its related derivatives/ analogs including the use of *Bcpr*^{-/-} mice [59], such as from Taconic Biosciences. Furthermore, interaction of BCRP with adjuvin can also be biochemically analyzed by co-immunoprecipitation since an anti-adjuvin antibody is available [60].

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Biography

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Fig. (1). Predicted structure of human BCRP oligomer

(A) Displays the functional unit of BCRP (Homodimer: Green – Monomer A, Orange – Monomer B) in their open conformation. (B, C) depict the orientation of the individual BCRP monomer as present in the homodimer. Three dimensional structure of the homodimer was predicted using the template (PDB ID: 3G60) as described [49]. The structural organization of BCRP protein starts with nucleotide binding domain (NBD) at its N-terminal, followed by transmembrane (TM) domain at their C-terminal (NH₂-NBD-TM-COOH), which is in contrast to the organization of other ABC transporters.

Fig. (2). Predicted structure of human BCRP dimer and molecular interaction with adjudin
(A) Displays the functional unit of BCRP in (Homodimer: Green – Monomer A, Orange – Monomer B) in their open ‘inward facing’ conformation and closed ‘outward facing’ conformation bound to adjudin. The NBDs of the monomeric protein are in the cytosolic side, with the TM regions spanning the plasma membrane and the loops connecting the TMs in the extracellular space. The binding site of adjudin is represented as mesh with adjudin in stick model. **(B, C)** Three dimensional view of the molecular interactions between adjudin and human BCRP in inward **(B)** and outward **(C)** conformation, respectively. Adjudin is depicted in scaled ball and stick model and interacting residues are in stick model. Black and green dotted lines represent hydrogen bond formation, and orange lines represent Pi-Pi and Pi-cation interactions between BCRP and adjudin. These models were generated using PyMol (The PyMOL Molecular Graphics System, Version 1.5.0.3 Schrödinger, LLC.).

Fig. (3). Tetrameric arrangement of BCRP traversing the plasma membrane

(A) The tetramer is formed by the definite association of 4 functional homodimers spanning the membrane thereby constituting 8 individual monomers of BCRP. The NBDs of the monomeric protein are into the cytosol, with the TM regions spanning the plasma membrane and the loops connecting the TMs in the extracellular space. The homodimers are arranged such that they traverse across the plasma membrane on four sides thereby developing a considerably large pore-like cavity at its center. The monomers are colored according to their functional units. (B) Molecular docking analysis of human BCRP functional oligomer with adjuvin. Tetrameric arrangement of human BCRP in complex with adjuvin and the binding site of adjuvin are depicted as mesh regions. The interactions between adjuvin and BCRP are stabilized by H-bonds and Pi interactions. The work was done using *Coot*, an application for model building and validation of biological macromolecules [55]. Images were generated using PyMol (The PyMOL Molecular Graphics System, Version 1.5.0.3 Schrödinger, LLC.).