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Discovering a Reversible Male Contraceptive Agent Derived from Lonidamine

Shengnan Gong,‡ Shiyao Zhu,‡ Peng Zhou, C. Yan Cheng, Wenqing Li,* Weiwei Yao,* and Fei Sun*

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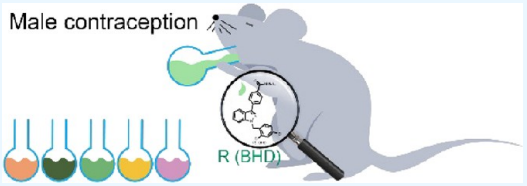
ABSTRACT: There is a huge demand for safe and effective non-hormonal male contraceptives to prevent unintended pregnancy, but research on male contraceptive drugs lacks far behind the pills for women. Lonidamine and its analog adjudin are two of the best studied potential male contraceptives. However, the acute toxicity of lonidamine and the subchronic toxicity of adjudin had impeded their development for male contraception. Here, we designed and synthesized a whole new series of molecules derived from lonidamine according to a structure ligand-based design strategy and obtained a new effective and reversible contraceptive agent (BHD), and their efficacy was demonstrated in male mice and rats. Results showed that BHD had a 100% contraceptive effect on male mice after 2 weeks following a single oral dose of BHD at 100 mg/kg body weight (b.w.) or 500 mg/kg b.w. treatments. The fertility of mice was reduced to 90 and 50% after 6 weeks with a single oral dose of BHD-100 and BHD-500 mg/kg b.w. treatments, respectively. We also revealed that BHD induced the apoptosis of spermatogenic cells rapidly and disrupted the blood−testis barrier effectively. It appears to be a new potential male contraceptive candidate for future development.



1. INTRODUCTION

Population control is critically needed across the globe including industrial nations,[1] in particular male contraceptives, since some women are not suitable candidates to be the recipients of the pills.[2,3] Furthermore, studies have shown that long-term use of hormonal-based contraceptives in women has emerging health risks,[4] at least in specific groups of women. Also, there is an increasing need to have men share the burden of family planning in both developed and developing countries.[5] As such, there is an urgent need for safe and reversible non-hormonal male contraceptives.[6,7] Based on studies of the past two decades, an ideal agent for the control of male fertility is one that has a selective inhibitory action on the germinal epithelium of the testes by blocking spermatogenesis,[8,9] long before mature spermatozoa are formed. Adjudin[10] was an anti-spermatogenic compound that exerted its effects on the seminiferous tubules, which disrupted the adhesion of spermatids to Sertoli cells in the testis.[11] Briefly, adjudin causes premature release of spermatids, analogous to spermiation, thereby leading to infertility in adult rats without changes in serum testosterone, FSH, or LH concentrations.[12] Unfortunately, liver inflammation was detected in 10% of the mice under subchronic toxicity study, thereby preventing adjudin from being moved to the clinical trial.[13] H2-gamendazole[14] was another similar compound to adjudin, which also exerted its antifertility effects by impairing the apical ectoplasmic specialization function in male rats. In a toxicology study, three out of five rats died after administration with a dose of 200 mg/kg H2-gamendazole.[15] Therefore, it remains a huge challenge to identify a contraceptive pill for men since long-term use of such a drug should have minimal toxic side effects.

Given the drawbacks of currently available contraception,[16] it is necessary to develop new methods of male contraception.[17,18] A potential non-hormonal male contraceptive, the small molecule lonidamine,[19] has attracted much attention from investigators in the field. Lonidamine was found to have potent anti-spermatogenesis effects and was initially explored to serve as an anti-spermatogenic agent.[20] Lonidamine also targets energy metabolisms of mammalian cells,[21] such as the inhibition of the monocarboxylate transporter, mitochondrial pyruvate carrier, respiratory chain complex I/II, mitochondrial permeability transition pore, and hexokinase II.[22] It is based on such bioactivity of lonidamine, and it was developed for cancer therapy.[23] Lonidamine has definitely the potential to become a male contraceptive based on studies in the past few decades. Nonetheless, the irreversible anti-spermatogenic and some undesirable side effects when lonidamine was used at high and acute doses had limited its use for male contraception.[9] Since the early years of studying lonidamine, Cheng and his colleagues had developed an effective and reversible male contraceptive agent and discovered adjudin,[10] which could serve as a better male contraceptive with a lower effective dose

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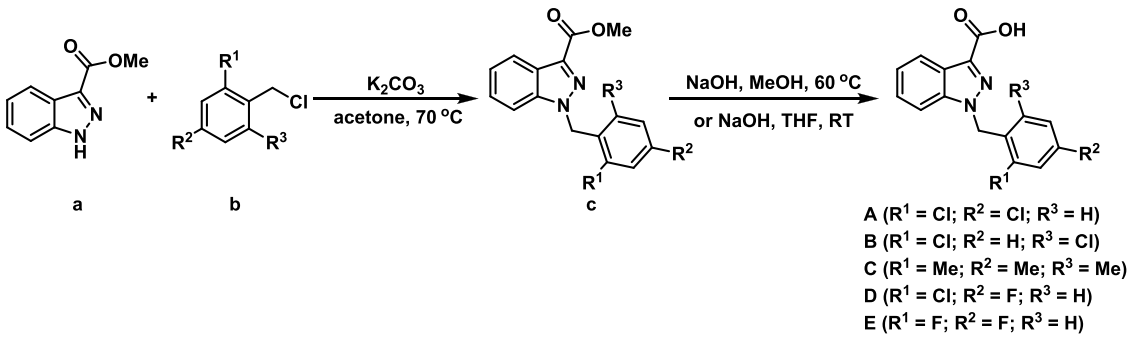

Figure 1. General synthesis procedure of **A**−**E**.

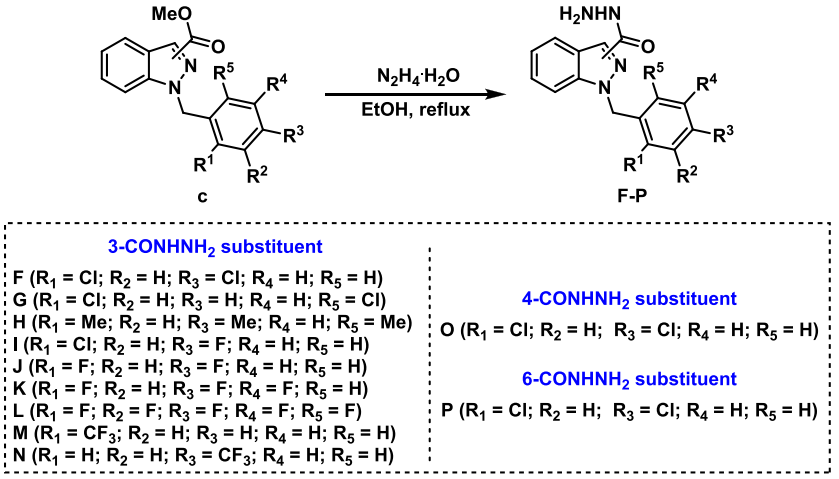

Figure 2. General synthesis procedure of **F**−**P**.

in male rats, mice, and rabbits. However, the liver inflammation and muscle atrophy toxicity of adjudin had prevented its further clinical applications.[24]

In this report, we presented findings based on the use of a novel strategy of structure and function drug design strategy by synthesizing a series of analogs of compounds based on the core structure of lonidamine due to its excellent characteristics such as simple preparation, low cost, and easy modification. We reported our findings of a new reversible male contraceptive agent (BHD) in mice and rats, which was optimized based on the structure of lonidamine according to a ligand-based design strategy. We also investigated the male contraceptive mechanism of BHD. Our findings suggest that BHD may develop into a new potential male contraceptive agent for human use.

2. MATERIALS AND METHODS

2.1. General Procedures. All solvents and chemicals to be used for synthesis were used as purchased without further purification. All compounds during the synthesis steps were purified through column chromatography and recrystallization. The purity of compounds **A**−**S** was identified by NMR spectroscopy, and the NMR spectra are listed in the Supporting Information.

2.2. Synthesis of Compounds A−S. *2.2.1. Compounds **A**−**E**.* A mixture of **a** (1.0 equiv), **b** (1.1 equiv), and K_2CO_3 (4.5 equiv) in acetone was refluxed overnight at 70 °C. The reaction mixture was cooled to room temperature and filtered, and the residue was washed with acetone. The combined filtrate was concentrated under vacuum. The solid was dissolved in CH_2Cl_2 and filtered to remove any undissolved solid. The residue was crystallized (CH_2Cl_2/hexane) to afford the pure product **c** as a white solid. To a solution of **c** (1.0 equiv) dissolved in methanol or THF was added 1 M NaOH. The mixture was stirred for 24 h at 60 °C or ambient temperature. The reaction was terminated by washing two times with ethyl acetate, saving the aqueous layer. The aqueous layer was acidified slowly by adding conc. HCl in an ice bath, which led to the separation of a white product (**A**−**E**, Figure 1).

*2.2.2. Compounds **F**−**P**.* To a solution of **c** (1.0 equiv) in ethanol at room temperature was added hydrazine hydrate (50.0 equiv). The reaction mixture was refluxed for 4 h. The volatiles were removed under reduced pressure, the crude mass was diluted with dichloromethane, washed with water and brine, and dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure to obtain the crude product. The residue was crystallized (CH_2Cl_2/hexane) to afford the pure product (**F**−**P**) as a white solid (Figure 2).

*2.2.3. Compound **Q**.* Indazole (**d**, 5 mmol) was added to a stirred mixture of KOH (10 mmol, 2.0 equiv) and I_2 (10 mmol, 2.0 equiv) in DMF (15 mL). After 2 h at room temperature, the organic layer was washed successively with aq $Na_2S_2O_3$ and brine, dried over anhydrous sodium sulfate, filtered, and evaporated in vacuo. Then, the crude material was dissolved in acetone, and to the mixture, **b** (5.5 mmol, 1.1 equiv, R^1 = Cl; R^2 = Cl; R^3 = H) and K_2CO_3 (22 mmol, 4.4 equiv) were added. The reaction mixture was refluxed overnight at 70 °C. Then, it was cooled to room temperature and filtered, and the residue was washed with acetone. The

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combined filtrate was concentrated under vacuum. The solid was dissolved in CH_2Cl_2 and filtered to remove any undissolved solid. The residue was crystallized (CH_2Cl_2/hexane) to afford the pure product **f** as a white solid in 81% yield.

A glass pressure tube was charged with **f** (0.5 mmol, 1.0 equiv), Pd $(OAc)_2$ (0.05 mmol, 10 mol %), PPh_3 (0.1 mmol, 20 mol %), and anhydrous DMF (2 mL). The reaction mixture was stirred and degassed by purging the nitrogen. $^iPr_2NEt$ (1.5 mmol, 3.0 equiv) and methyl acrylate (5 mmol, 10.0 equiv) were added, and the tube was sealed. It was stirred at 120 °C for 24 h. The reaction mixture was cooled and diluted with ethyl acetate. It was washed with water and dried over Na_2SO_4. The residue was evaporated and purified by column chromatography (eluents: petroleum ether/ethyl acetate = 20:1) to obtain the product **g** in 83% yield.

To a solution of **g** (1 mmol, 1.0 equiv) in ethanol at room temperature was added hydrazine hydrate (50 mmol, 50.0 equiv). The reaction mixture was stirred at room temperature overnight. The volatiles were removed under reduced pressure, the crude mass was diluted with dichloromethane, washed with water and brine, and dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure to obtain the crude product. The residue was purified by column chromatography (eluents: CH_2Cl_2/MeOH = 30:1) to afford the pure product **Q** as a white solid in 94% yield (Figure 3).

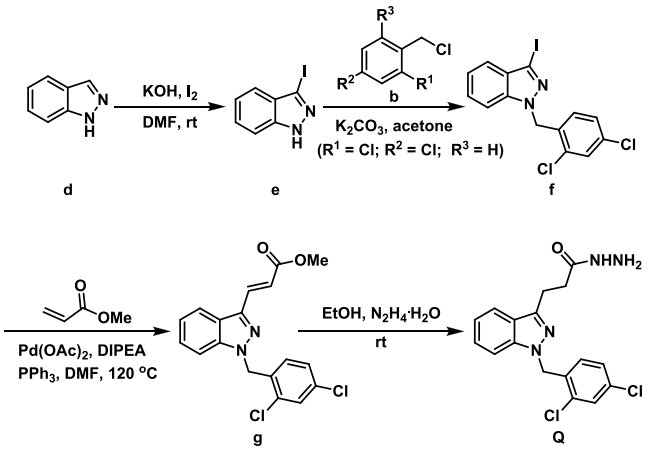

Figure 3. Synthesis procedure of **Q**.

*2.2.4. Compounds **R** and **S**.* A mixture of **f** (1 mmol, 1.0 equiv), methoxycarbonylphenylboronic acid (1.5 mmol, 1.5 equiv), Pd(dppf)Cl_2 (0.1 mmol, 10 mol %), and saturated aqueous Na_2CO_3 solution (4 mL) in ethanol (1 mL) and toluene (10 mL) was stirred at 80 °C for 12 h. Upon completion of the reaction (TLC), the reaction mixture was extracted with CH_2Cl_2. The combined organic layers were washed with water and brine, dried, and concentrated in vacuo. The residue was purified by silica column chromatography (eluents: petroleum ether/ethyl acetate = 10:1) to give compound **h** as a white solid.

To a solution of **h** (1.0 equiv) in ethanol at room temperature was added hydrazine hydrate (50.0 equiv). The reaction mixture was refluxed for 4 h. The volatiles were removed under reduced pressure, the crude mass was diluted with dichloromethane, washed with water and brine, and dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure to obtain the crude product. The residue was purified by column chromatography to afford the pure product (**R** and **S**) as a white solid (Figure 4).

2.3. Animals. Six to eight week-old ICR male mice (~30 g) and adult male Sprague−Dawley rats (~250 g) were obtained from Laboratory Animal Center of Nantong University. Animals were housed in a temperature-controlled environment (22−24 °C) with a 12 h light−dark cycle and sterile water and food ad libitum. All the procedures were approved by the Institutional Ethics Committee of Laboratory Animal Center of Nantong University.

2.4. Testing the Anti-Spermatogenic Effect of the Compounds A−S on Male Mice. Six week-old ICR male mice were randomly assigned to 20 groups: control and compound A to S groups (n = 3 per group). The male mice were intragastrically administered with a single dose of a compound (10% DMSO/corn oil) at 100 mg/kg, respectively. After 2 weeks, the mice were sacrificed and the testes and epididymis were collected for assessment.

2.5. Anti-Spermatogenic Effects with Different Doses of BHD. For optimizing different doses of BHD, 6 week-old ICR male mice were randomly divided into five groups: control and BHD-50, BHD-100, BHD-200, and BHD-500 mg/kg (n = 10 per group). The male mice were intragastrically administered with a single dose of BHD at 0, 50, 100, 200, and 500 mg/kg. During the experimental period, the body weights of mice were recorded. After 2 weeks, the mice were sacrificed. The testes, epididymis, liver, and kidney were collected for assessment.

For the rescue study, 45 mice were randomly divided into three groups: control, BHD-100 mg/kg, and BHD-500 mg/kg with intragastric administration with a single dose of BHD, respectively. The mice were sacrificed at 2 and 6 weeks, and the testes were collected.

For the mechanism of BHD, BHD was given at 100 mg/kg and 500 mg/kg by gavage. The mice were sacrificed at 12 and

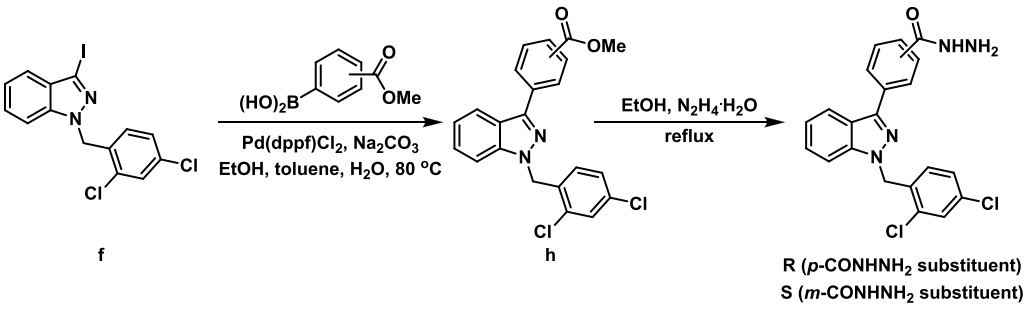

Figure 4. Synthesis procedure of **R** and **Q**.

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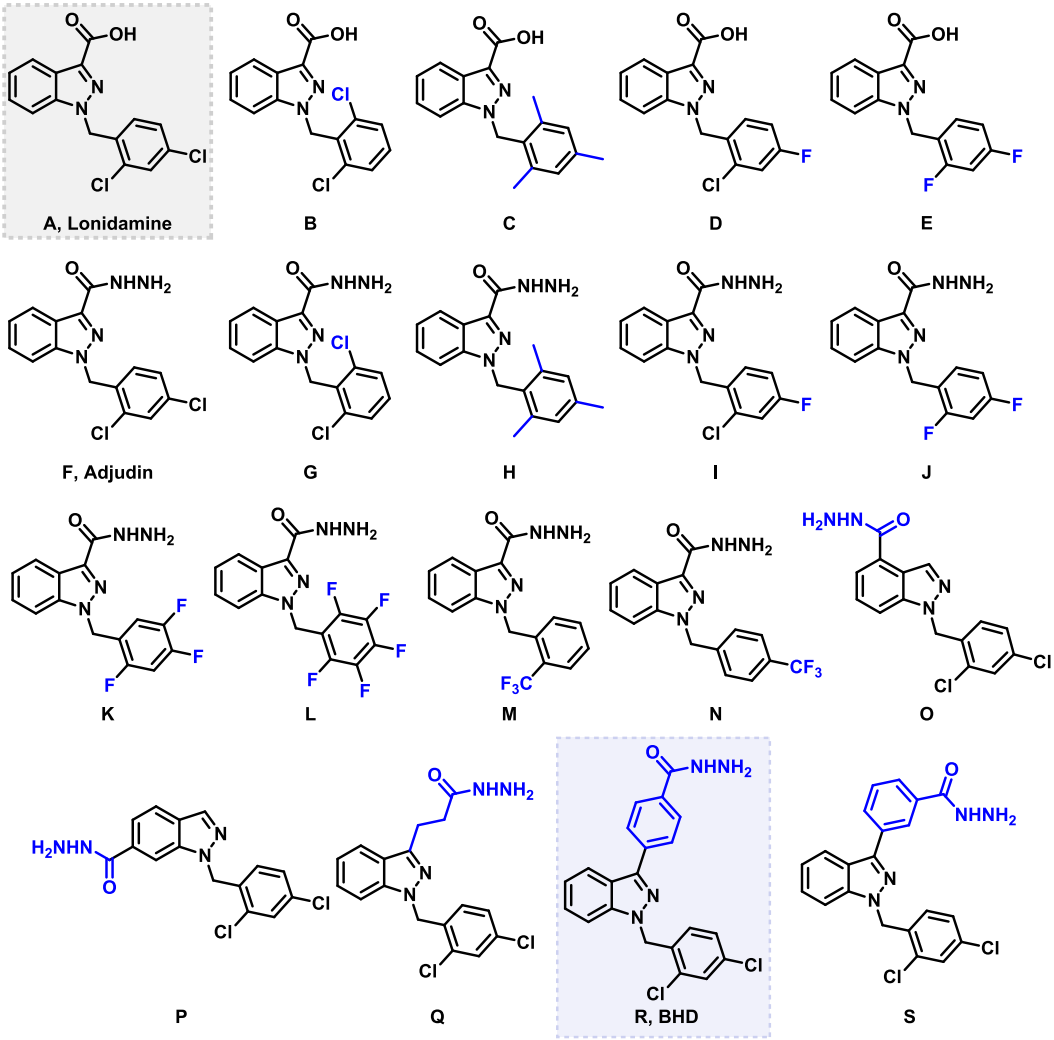

Figure 5. Synthesis of new molecules derived from lonidamine. The structures of compounds **A**–**S** are shown. **A** is lonidamine, **F** is adjudin, and **R** is BHD (4-(1-(2,4-dichlorobenzyl)-1*H*-indazol-3-yl)benzohydrazide).

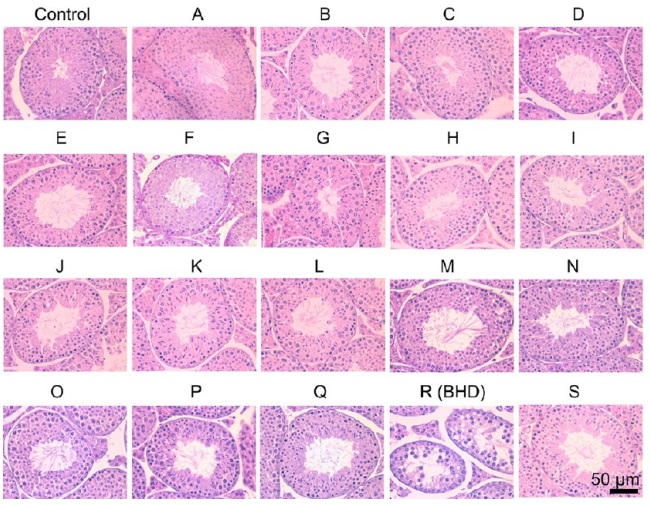

Figure 6. Anti-spermatogenic effects of compounds **A**–**S** on male mice with a single oral dose of 100 mg/kg. Testis cross sections in each group were stained with hematoxylin–eosin (HE). The control group was orally administered with vehicle only (n = 3 for each group).

48 h, and the testis and epididymis were collected for HE staining, western blot analyses, and TUNEL assay.

2.6. Histological Examination. The histology of major organs including liver, kidney, testes, and epididymis was analyzed. Briefly, testes were fixed in MDF fixatives and other organs were fixed in 4% PFA. Then, they were embedded into paraffin and cut into 5 μm sections. The sections were then deparaffinized and rehydrated. HE staining was carried out using the hematoxylin–eosin (HE) staining kit (Sangon Biotech, E607318).

2.7. Fertility Test of mice. Control, BHD-100 mg/kg, and BHD-500 mg/kg were treated as before. After 2 and 6 weeks, the male and female mice were mated with a ratio of 1:2. After 1 week, the female mice were separated to observe their fertility, and the pregnancies and pups were observed and recorded.

2.8. TUNEL Assay. Testes were fixed in MDF fixatives, embedded, and sectioned. The sections were deparaffinized, rehydrated, and then incubated with protease K (20 μg/mL) at room temperature for 30 min. The TUNEL assay was performed using the One-Step TUNEL Apoptosis Assay Kit (Beyotime, C1090) by following the instructions of the manufacturer. The nuclei were stained with DAPI. Images

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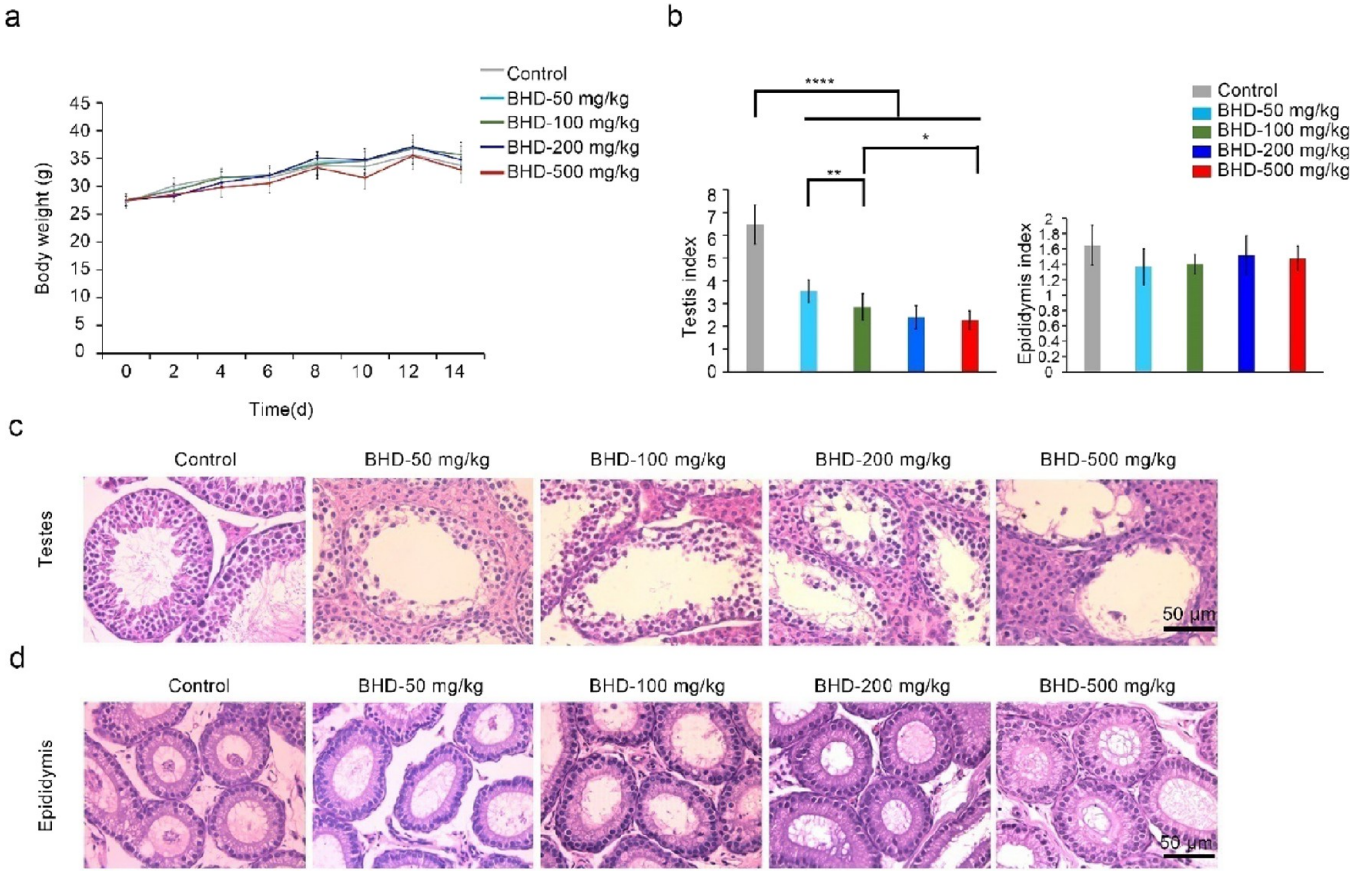

Figure 7. BHD induced anti-spermatogenic effects in a dose-dependent manner. (a) Body weight changes of male mice with a single oral dose of control, BHD-50 mg/kg, BHD-100 mg/kg, BHD-200 mg/kg, and BHD-500 mg/kg (n = 10 for each group). (b) Testis index and epididymis index of male mice with a single oral dose of control, BHD-50 mg/kg, BHD-100 mg/kg, BHD-200 mg/kg, and BHD-500 mg/kg (n = 10 for each group). Testis index = (testis weight/ body weight) × 100. Epididymis index = (epididymis weight/ body weight) × 100. Statistical analysis was performed using Student's two-tailed t test. *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$. (c) Cross sections of testes and (d) cross sections of the epididymis of male mice with a single oral dose of control, BHD-50 mg/kg, BHD-100 mg/kg, BHD-200 mg/kg, and BHD-500 mg/kg (n = 10 for each group) in each group were stained with HE.

were obtained using a ZEISS Axiocam 503 mono fluorescence microscope.

2.9. Western Blot Analyses. Testes were lysed in RIPA lysis buffer (Beyotime, P0013C) containing cOmplete, EDTA-free protease inhibitor tablets (Roche, 04693 132001) and PMSF (Beyotime, ST506) and homogenized. The lysates were lysed for 30 min, and the supernatants were collected. The protein concentrations were determined using the BCA Protein Assay Kit (Beyotime, P0010S). Western blotting was performed as a standard protocol. Primary antibodies used were caspase-3 (#19677-1-AP, Proteintech, America) and GAPDH (#600041-Ig, Proteintech, America). Secondary antibodies (ab216777, Abcam; ab216776, Abcam, America) were used separately after washing with TBST. Signals were captured by an Amersham Typhoon (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

2.10. BTB Integrity Assay. ICR male mice were intragastrically administered with a single dose of BHD at 500 mg/kg for 12 and 48 h. Then, the mice were testicularly injected with 50 μL (10 mg/mL) of EZ-Link Sulfo-NHS-SS-Biotin (Thermo Scientific, 21331). After 1 h, the mice were euthanized. The testes were cut into 6 μm-thick frozen sections in a cryostat at −22 °C. Testicular tissue sections were fixed with acetone at room temperature and then stained with Alexa Fluor 488-Streptavidin at 1:250 dilution for 1 h, and nuclei were stained with DAPI. Fluorescence signals were examined using a ZEISS Axiocam 503 monofluorescence microscope.

2.11. Immunofluorescence Staining. The testicular tissue sections were deparaffinized with xylene, rehydrated with gradient alcohol, and then incubated with protease E (0.1 μg/mL) (Thermo Fisher, P8811) at 37 °C for 10 min. Sections were microwave-heated in 10 mM sodium citrate buffer (Sangon Biotech, E673000) for antigen retrieval. Then, sections were permeabilized with 0.1% Triton X-100 and blocked in 3% bovine serum albumin (Sangon Biotech, A500023-0100). The sections were incubated with the Zo-1 (Proteintech, 21773-1-AP) and Occludin (Proteintech, 27260-1-AP) antibodies at 4 °C overnight, followed by the addition of Cy3 fluorescently labeled secondary antibodies at room temperature for 1 h, and nuclei were stained with DAPI. Fluorescence signals were examined using a ZEISS Axiocam 503 monofluorescence microscope.

2.12. Fertility Test of Rats. Sixteen Sprague−Dawley rats were randomly divided into two groups: control and BHD-500 mg/kg with intragastric administration with a single dose of BHD, respectively. The rats were euthanized with CO_2 in a euthanasia chamber. The rats were sacrificed at 2 and 6 weeks, and the testes were collected. To test a rat's fertility, eight Sprague−Dawley rats were randomly divided into two groups: control and BHD-500 mg/kg with intragastric administration

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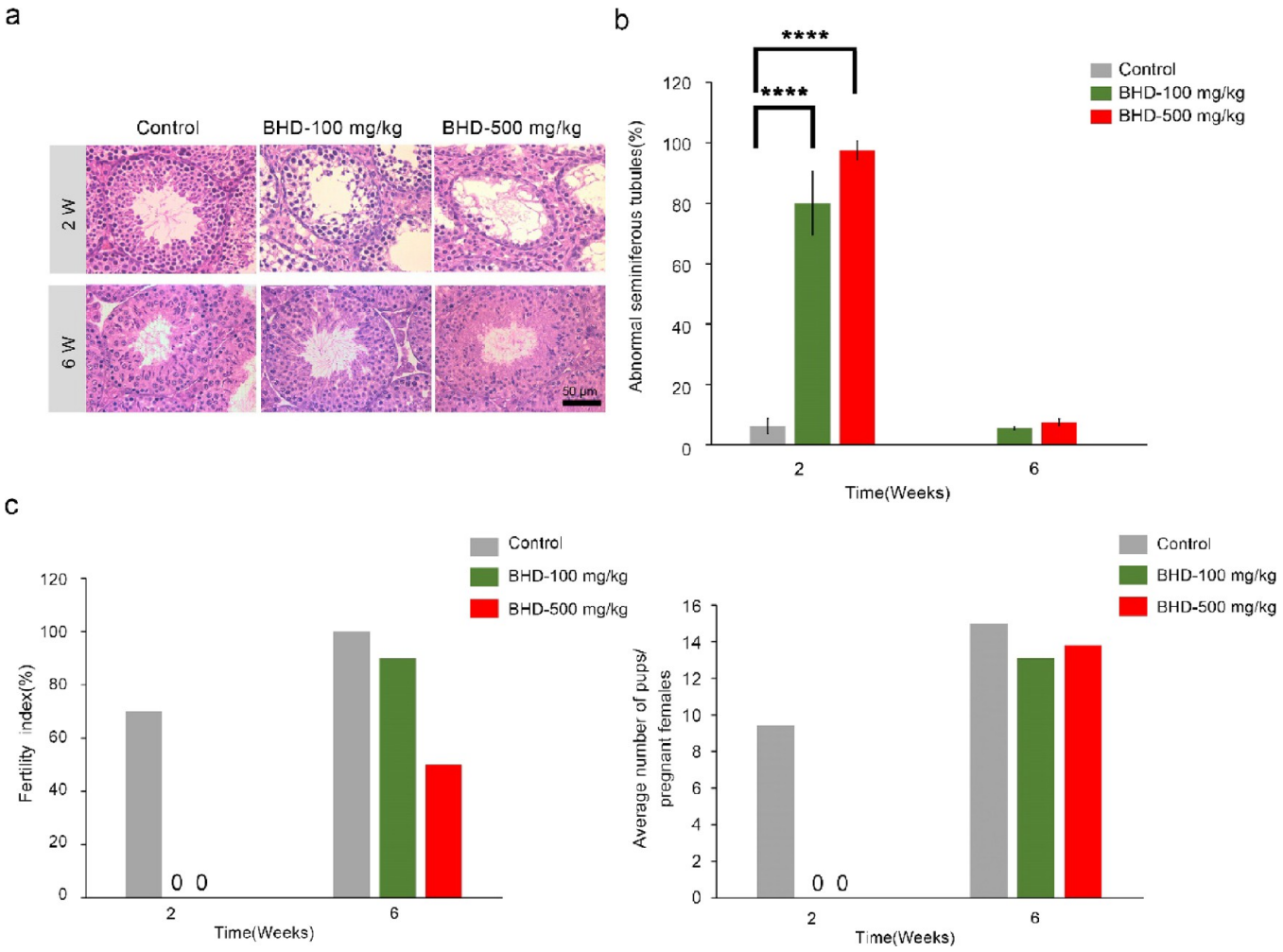

Figure 8. BHD induced reversible male contraception in mice. (a) Cross sections of testes of male mice with a single oral dose of control, BHD-100 mg/kg, and BHD-500 mg/kg after 2 and 6 weeks (n = 5 for each group). (b) Percentage of abnormal seminiferous tubules of male mice with a single oral dose of control, BHD-100 mg/kg, and BHD-500 mg/kg after 2, 6, and 11 weeks, which were counted from Figure 8a and showed that at least 200 seminiferous tubules were counted in a mouse (n = 5 for each group). (c) Fertility index and average number of pups/pregnant female mice with a single oral dose of control, BHD-100 mg/kg, and BHD-500 mg/kg after 2 and 6 weeks (n = 5 for each group).

with a single dose of BHD. After 2 weeks, the male and female rats were mated with a ratio of 1:2. Then, after 1 week, the female mice were separated to observe their fertility, and the pregnancies and pups were observed and recorded.

2.13. Analysis. Student's t test was used to examine the statistical analysis. Data were shown as the mean ± S.D., unless otherwise indicated. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

3. RESULTS AND DISCUSSION

3.1. Designed and Synthesized New Molecules Derived from Lonidamine. Lonidamine was first used as an anti-spermatogenic agent and developed as a chemotherapeutic drug for tumor therapy. Then, it is found that lonidamine has a contraceptive effect but with unwanted high toxicity, unless it was given to patients with late-stage cancers. Here, we tried to design and synthesize some new molecules to promote the male contraceptive effect but reduce their toxicity through functional group modification based on the core structure of lonidamine. Previous studies reported that the benzyl group of lonidamine was essential to confer its male contraceptive effect, and one or more halogen or methyl groups in the benzyl ring were important to augment its efficacy. First, we changed the position of the *para*-chlorine atom in lonidamine (**A**) to the *ortho* position, replaced the 2,4-dichlorobenzyl group with the mesitylene benzyl group, and obtained compounds **B** and **C** (Figure 5). Given that fluorine and the fluorinated functional group probably improved the biological activity of drug molecules, we developed compounds **D** and **E** (Figure 5) with a fluorine substituent in the aromatic ring of lonidamine. Adjudin (**F**, a lonidamine derivative) performed a good contraceptive effect on rats, which showed that the hydrazide functional group owned an increased male contraceptive effect. We designed and synthesized adjudin and its derivatives (**G**−**P**). Compounds **G**−**N** were synthesized by modifying benzyl groups with methyl or fluorinated functional groups (Figure 5) based on adjudin. Changing the hydrazide group in adjudin to the 4- or 6-positions of indazole obtained compounds **O** and **P** (Figure 5), possibly reducing its toxicity. Furthermore, previous reports showed that H2-gamendazole[10] and AF-2785[6] presented an excellent contraceptive effect than lonidamine, which showed that the chain length extension of carboxyl in the 3-position of indazole might promote the contraceptive effect. Thus, we synthesized compound **Q**

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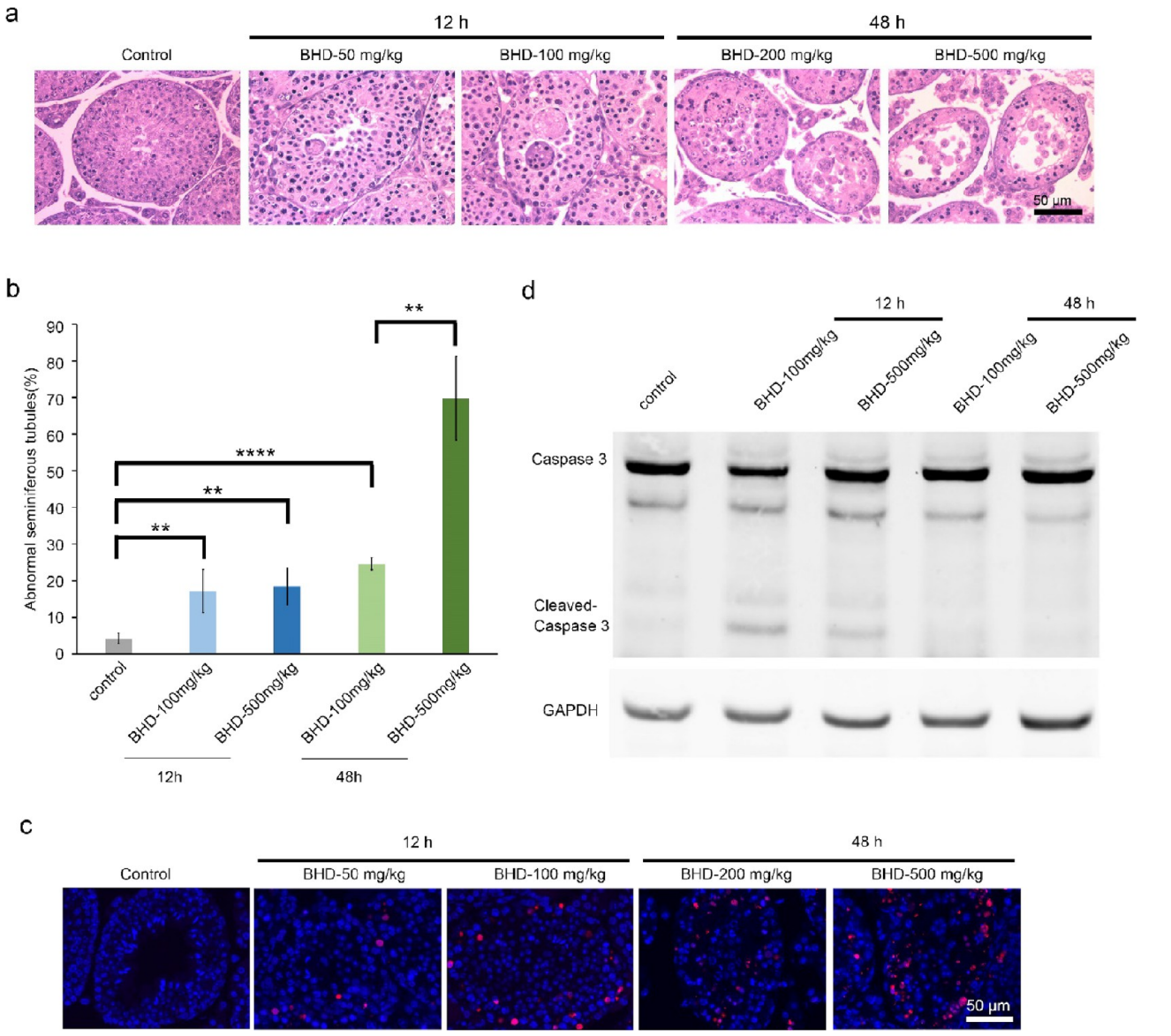

Figure 9. BHD induced spermatogenic cell apoptosis. (a) Cross sections of testes of male mice with a single oral dose of control, BHD-100 mg/kg, and BHD-500 mg/kg after 12 and 48 h (n = 5 for each group). (b) Percentage of abnormal seminiferous tubules of male mice with a single oral dose of control, BHD-100 mg/kg, and BHD-500 mg/kg after 12 and 48 h, which were counted from Figure 9a and showed that at least 200 seminiferous tubules were counted in a mouse (n = 5 for each group). Statistical analysis was performed using Student's two-tailed t test. $^{**}p < 0.01$ and $^{****}p < 0.0001$. (c) TUNEL assay showed the apoptosis effect of spermatogenesis of male mice with a single oral dose of control, BHD-100 mg/kg, and BHD-500 mg/kg after 12 and 48 h (n = 5 for each group). (d) Western blot for caspase-3 and GAPDH of male mice with a single oral dose of control, BHD-100 mg/kg, and BHD-500 mg/kg after 12 and 48 h (n = 5 for each group).

(Figure 5). Considering that the chain between the hydrazide functional group and indazole in **Q** was a flexible alkyl chain group, the hydrazide functional group in **Q** would swing back and forth. A rigid chain group would play an active role in fixing the hydrazide functional group. We got two similar compounds **R** (BHD, 4-(1-(2,4-dichlorobenzyl)-1*H*-indazol-3-yl)benzohydrazide) and **S** (Figure 5).

3.2. Investigating the Anti-Spermatogenic Effects of the Compounds on Male Mice. ICR male mice were intragastrically administered with a single dose of compounds **A**−**S** at 100 mg/kg, respectively. After 2 weeks post treatment, the mice were sacrificed and testes were collected for histological analysis. The results showed that only **R** (BHD) had an anti-spermatogenic effect among the compounds **A**−**S** on male mice (Figure 6). The other compounds did not have an anti-spermatogenic effect with a dose of 100 mg/kg treatment, which indicated that BHD might be a good choice for male contraception.

Then, we investigated the anti-spermatogenic effects of BHD with different doses. ICR male mice were divided into five groups (n = 10 for each group) and were intragastrically administered with a single dose of BHD at 50, 100, 200, and 500 mg/kg. We recorded the body weight changes of mice every 2 days during the experiment (Figure 7a). The mice were sacrificed, and testes were collected for histological analysis at 2 weeks post treatment. The results showed that the testis index was significantly decreased in BHD treatment in a dose-dependent manner (Figure 7b). The epididymis index showed no significant differences among all groups (Figure 7b). The morphology of the testes was significantly damaged in BHD

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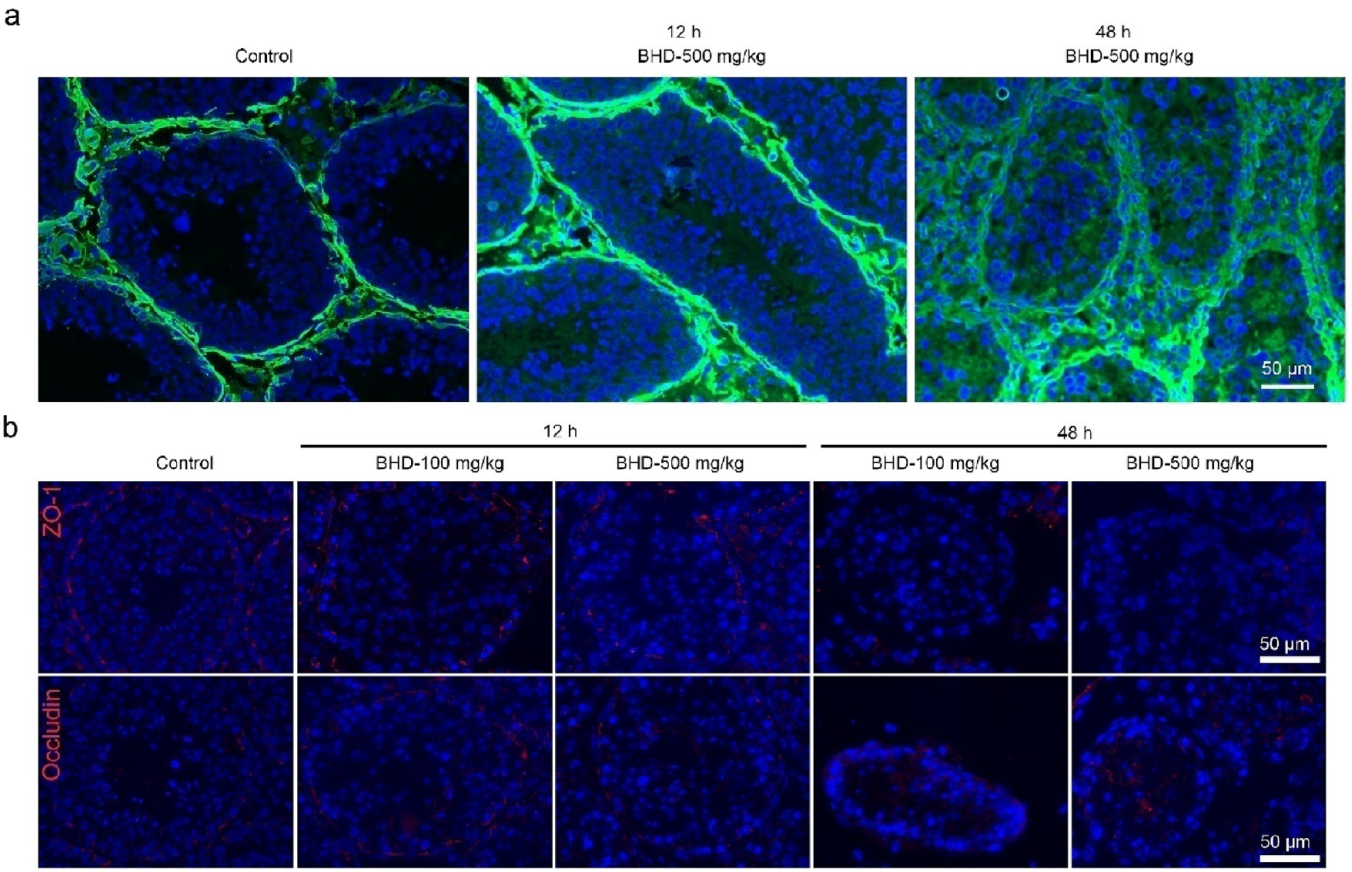

Figure 10. BHD destroyed the integrity of the blood−testis barrier. (a) Biotin-Alexa Fluor 488 (green) was intratesticularly injected into a mouse after a single oral dose of control and BHD-500 mg/kg after 12 and 48 h (n = 3 for each group). Localization of Biotin-Alexa Fluor 488 (green) in frozen sections of testes was used to assess the BTB integrity. (b) Immunofluorescence staining of ZO-1 and Occludin of testes in male mice with a single oral dose of control, BHD-100 mg/kg, and BHD-500 mg/kg after 12 and 48 h (n = 5 for each group). The nucleus was stained with Hoechst.

treatment, which showed a dose-dependent manner (Figure 7c). There were no sperms in epididymis with BHD treatment compared with the control group (Figure 7d). We also investigated the toxicity of BHD in male mice. The histology of the liver and kidney of male mice with BHD treatment showed that BHD did not induce significant toxicity in the liver and kidney (Supporting Information, Figure S1).

3.3. BHD Showed Reversible Contraception in Male Mice. The compound BHD had anti-spermatogenic effects in a dose-dependent manner. Here, we used a single oral dose of BHD at 100 and 500 mg/kg to investigate the male contraception effect. The results showed that the testes were damaged significantly at 2 weeks with BHD-100 mg/kg and BHD-500 mg/kg (Figure 8a,b). The damages of the testes were rescued at 6 weeks with BHD-100 mg/kg and BHD-500 mg/kg (Figure 8a,b). The fertility index showed that BHD had a 100% contraception effect on male mice at 2 weeks with BHD-100 mg/kg and BHD-500 mg/kg (Figure 8c). The fertility of mice was rescued to 90 and 50% at 6 weeks with BHD-100 mg/kg and BHD-500 mg/kg, respectively (Figure 8c). The average number of pups/pregnant female mice showed BHD-induced reversible male contraception from 2 to 6 weeks (Figure 8c). These results indicated that BHD was a reversible male contraception agent in mice.

3.4. The Mechanism of BHD-Induced Male Contraception in Mice. To explore the mechanism of male contraception of BHD in mice, the male mice were intragastrically administered with a single dose of BHD at 100 and 500 mg/kg, respectively. After 12 and 48 h post treatment, mice were sacrificed and testes were collected for histological analysis. We found that the spermatogenic cells were quickly shed in the seminiferous tubules due to germ cell exfoliation (Figure 9a). The abnormal seminiferous tubules were increased significantly with BHD-100 mg/kg and BHD-500 mg/kg treatments compared with the control group (Figure 9b). The TUNEL assay showed that the spermatogenic cells underwent apoptosis from 12 to 48 h in a BHD dose-dependent manner (Figure 9c). Also, the apoptosis marker that cleaved caspase-3 was increased with BHD treatment at 12 h (Figure 9d). These results indicated that BHD induced apoptosis of spermatogenic cells rapidly, conferring its male contraceptive activity.

BHD destroyed the histology of testes, which might impact the integrity of the blood−testis barrier (BTB). BTB was disrupted 48 h after BHD-500 mg/kg treatment. We demonstrated the presence of fluorescence signals of Biotin-Alexa Fluor 488 in the apical compartment of the epithelium beyond the BTB, which entered into the tubule lumen (Figure 10a). Next, we also investigated the BTB-associated proteins ZO-1 and Occludin, which constituted the tight junction in the BTB. Immunostaining results exhibited that ZO-1 proteins disappeared after 48 h at 100 and 500 mg/kg BHD treatments (Figure 10b). Occludin proteins were disordered in the testis sections after 48 h at 100 and 500 mg/kg BHD treatments (Figure 10b). These results indicated that the mechanism of

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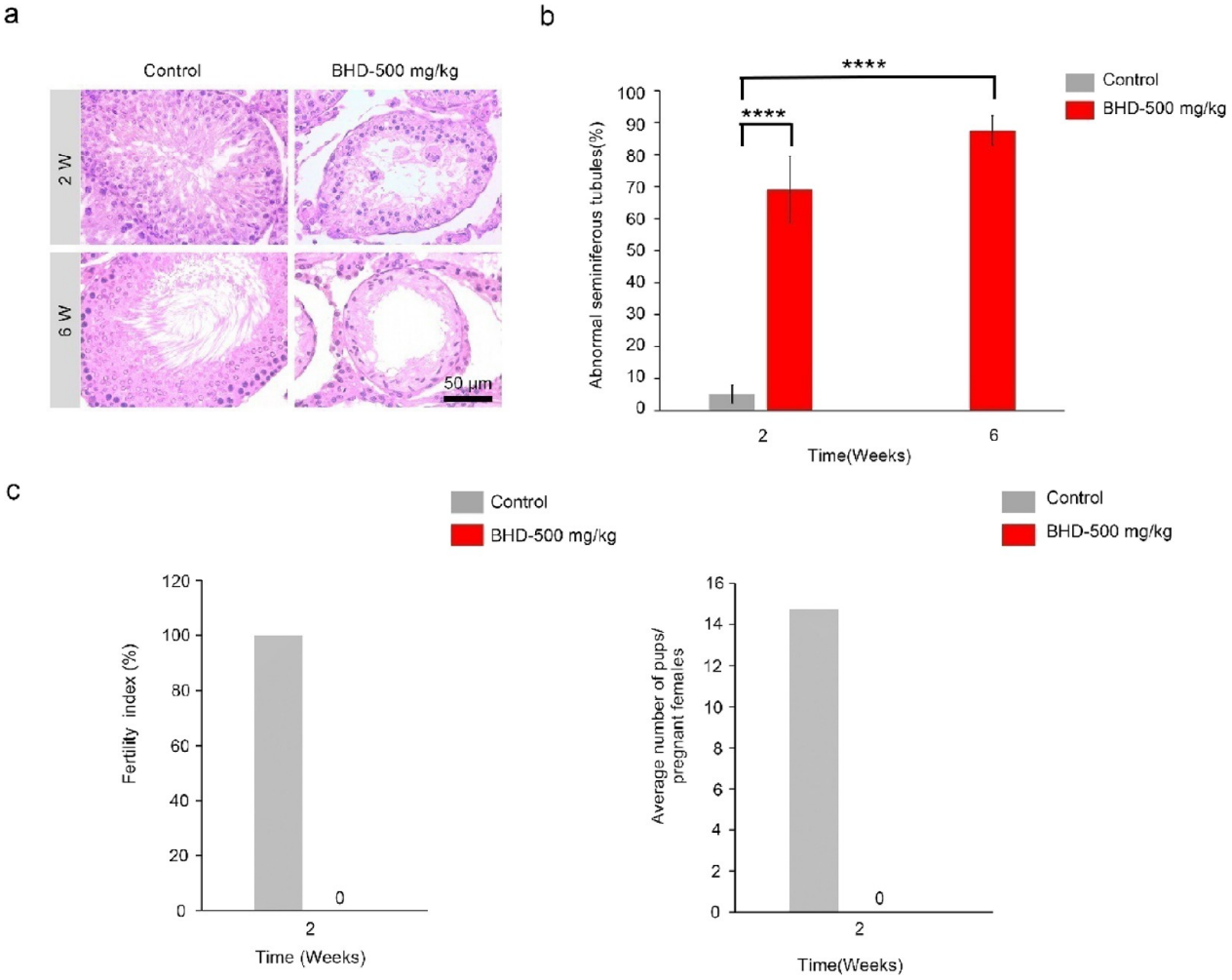

Figure 11. BHD induced male contraception in rats. (a) Cross sections of testes of male rats with a single oral dose of control and BHD-500 mg/kg after 2 and 6 weeks (n = 4 for each group). (b) Percentage of abnormal seminiferous tubules of male rats with a single oral dose of control and BHD-500 mg/kg after 2 and 6 weeks, which were counted from Figure 11a and showed that at least 200 seminiferous tubules were counted in a rat (n = 4 for each group). Statistical analysis was performed using Student's two-tailed t test. ****$p < 0.0001$. (c) Fertility index and average number of pups/pregnant female mice with a single oral dose of control and BHD-500 mg/kg after 2 weeks (n = 4 for each group).

BHD was that BHD induced the apoptosis of spermatogenic cells quickly and destroyed the blood−testis barrier.

3.5. BHD Showed Male Contraception in Rats. Finally, we investigated whether BHD has a male contraception effect on rats. SD male rats were intragastrically administered with a single dose of BHD at 500 mg/kg. The rats were sacrificed and testes were collected for histological analysis at 2 and 6 weeks post treatment. The morphology of the testes was significantly damaged in BHD treatment at 2 and 6 weeks post treatment (Figure 11a,b). The fertility index showed that BHD had a 100% contraception effect on male rats at 2 weeks with BHD-500 mg/kg (Figure 11c). The average number of pups/pregnant female rats showed BHD-induced male contraception at 2 weeks (Figure 11c). These results indicated that BHD could be used as a male contraceptive agent in rats.

4. CONCLUSIONS

In summary, developing a male contraceptive pill is urgent and important for men's health. Lonidamine and its derivative adjudin had failed to make them through the initial toxicity tests prior to clinical studies due to their unwanted side effects. Herein, we designed and synthesized new molecules derived from lonidamine. By designing a series of lonidamine derivatives, the molecule BHD shows effective and reversible contraceptive effects on male mice and rats. It may be a new potential male contraceptive agent in the future.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c01840.

Figure S1: histology of liver and kidney in the mouse by BHD treatment; structure data of compounds **A**−**S**; NMR spectra of compounds **A**−**S** (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Wenqing Li − *Institute of Reproductive Medicine, School of Medicine, Nantong University, Nantong, Jiangsu 226019, P. R. China*; orcid.org/0000-0002-2460-7945; Email: liwenqing505@ntu.edu.cn

Weiwei Yao − *Institute of Reproductive Medicine, School of Medicine, Nantong University, Nantong, Jiangsu 226019, P. R. China*; Email: yww@ntu.edu.cn

Fei Sun − *Institute of Reproductive Medicine, School of Medicine, Nantong University, Nantong, Jiangsu 226019, P. R. China*; Email: sunfei@ntu.edu.cn

Authors

Shengnan Gong − *Institute of Reproductive Medicine, School of Medicine, Nantong University, Nantong, Jiangsu 226019, P. R. China*

Shiyao Zhu − *Institute of Reproductive Medicine, School of Medicine, Nantong University, Nantong, Jiangsu 226019, P. R. China*

Peng Zhou − *Institute of Reproductive Medicine, School of Medicine, Nantong University, Nantong, Jiangsu 226019, P. R. China*

C. Yan Cheng − *Institute of Reproductive Medicine, School of Medicine, Nantong University, Nantong, Jiangsu 226019, P. R. China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c01840

Author Contributions

‡S.G. and S.Z. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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