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Disruption of Spermatogenesis and Sertoli Cell Structure and Function by the Indenopyridine CDB-4022 in Rats[1]

Sheri Ann Hild,[2,4] Jerry R. Reel,[4] Janice M. Larner,[3,4] and Richard P. Blye[5]

BIOQUAL, Inc.,[4] Rockville, Maryland 20850
Contraception and Reproductive Health Branch,[5] National Institute Child Health and Human Development, Rockville, Maryland 20852

ABSTRACT

The present studies were undertaken to determine the testicular cell type(s) affected by the antispermatogenic indenopyridine CDB-4022. At the oral threshold dose (2.5 mg/kg), CDB-4022 induced infertility in all males. CDB-4022 did not alter ($P > 0.05$) Leydig cell function as assessed by circulating testosterone, seminal vesicle, and ventral prostate weights or body weight gain compared to controls. Conversely, CDB-4022 reduced ($P < 0.05$) testicular weight, spermatid head counts, and percentage of seminiferous tubules undergoing spermatogenesis. In a second study, adult male rats received a maximally effective oral dose of CDB-4022 (12.5 mg/kg), dipentylphthalate (DPP; 2200 mg/kg; a Sertoli cell toxicant), or vehicle and were necropsied 3, 6, or 12 h after dosing to determine acute effects. Serum inhibin B levels were suppressed ($P < 0.05$) by 6 h after CDB-4022 or DPP treatment, but epididymal androgen-binding protein (ABP) levels were not altered ($P > 0.05$), compared to controls. CDB-4022 and DPP increased ($P < 0.05$) the percentage of tubules with apoptotic germ cells, particularly differentiating spermatogonia and spermatocytes, by 12 h after dosing. Microscopic examination of the testis indicated a greater degree of vacuolation in Sertoli cells and initial signs of apical germ cell sloughing/shedding by 3 or 12 h after CDB-4022 or DPP treatment, respectively. In a third study, prepubertal male rats were treated with vehicle, 12.5 mg/kg of CDB-4022, or 2200 mg/kg of DPP, and the efferent ducts of the right testis were ligated 23 h before necropsy. Seminiferous tubule fluid secretion (difference in weight of testes), serum inhibin B levels, and ABP levels in the unligated epididymis were reduced ($P < 0.05$) at 24 and 48 h after dosing in CDB-4022- and DPP-treated rats compared to controls. Collectively, these data suggest that CDB-4022 disrupts spermatogenesis by inducing apoptosis in early stage germ cells via a direct action on the Sertoli cell.

inhibin, Leydig cells, male reproductive tract, Sertoli cells, spermatogenesis

INTRODUCTION

Indenopyridines have demonstrated antispermatogenic activity in various species, including mice, rats, and dogs [1–5]. In these studies, the indenopyridine under investigation resulted in either reversible infertility or caused damage to the testis that resulted in irreversible infertility in the majority of the animals. The mechanism by which indenopyridines induce depopulation of the seminiferous epithelium is not known. A preliminary study of the indenopyridine RTI-056L ([4aRS,5RS,9bRS]-2-ethyl-7-methyl-2,3,4,4a,5,9b-hexahydro-5-[4-carbomethoxyphenyl]-1H-indeno[1,2-c]pyridine hydrochloride) indicated that this compound induced apoptosis of germ cells, resulting in loss of fertility [6]. However, the results of this study did not indicate whether the indenopyridine acted directly on the germ cells or indirectly via effects on other testicular cell types, such as Sertoli and/or Leydig cells.

Determination of the mechanism of CDB-4022 action on Sertoli, Leydig, and germ cells will assist in understanding the irreversible and reversible effects of this indenopyridine. Sertoli cell toxicants typically result in irreversible testicular atrophy, even though putative stem cells are present after treatment [7]. In contrast, the effects of germ cell toxicants, which do not deplete the stem cell mass, are often reversible, because the seminiferous tubules are able to repopulate the germinal epithelium following termination of treatment [7]. Leydig cell toxicants, as exemplified by ethane dimethyl sulfonate, result in infertility that is typically reversible following cessation of drug/compound exposure [8, 9]. Inhibition of testosterone production by Leydig cells caused stage-specific germ cell degeneration and infertility [9]. These effects were reversible once normal intratesticular testosterone levels were restored.

The present studies were undertaken to identify the testicular cell type(s) affected by the indenopyridine CDB-4022. The effects of CDB-4022 treatment on Leydig cell function were assessed by monitoring circulating testosterone levels and weights of the androgen-dependent accessory sex glands in adult male rats treated with the threshold oral dose of CDB-4022 that exhibited antispermatogenic/antifertility activity. The acute effects of CDB-4022 on germ and Sertoli cells were assessed in adult male rats by evaluation of testicular morphology, germ cell apoptosis, and production of the Sertoli cell proteins inhibin B and androgen-binding protein (ABP) [10–12]. Finally, a prepubertal male rat model was used to assess Sertoli cell function by measuring seminiferous tubule fluid (STF) secretion as well as inhibin B and ABP production. Taken together, the results of these studies suggest that CDB-4022 acts directly on the Sertoli cells to disrupt the early stages of spermatogenesis.

MATERIALS AND METHODS

Animals

Sprague-Dawley CD rats (Crl:CD(SD)IGS BR Stock) were purchased from Charles River Laboratories (Kingston, NY). All rats were housed in

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[2]Correspondence: Sheri Hild, BIOQUAL, Inc., 9600 Medical Center Dr., Rockville, MD 20850. FAX: 301 251 1260;
e-mail: sahild@compuserve.com
[3]Current address: Alexion Pharmaceuticals, Inc., 352 Knotter Dr., Cheshire, CT 06410.

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polycarbonate, solid-floor cages with Bed-o-Cob or Beta-Chip bedding (Andersons Industrial Products Group, Maumee, OH) and received Purina laboratory rodent diet (product no. 5001; Purina, Richmond, IN) and tap water ad libitum. The photoperiod was 14L:10D. Prepubertal males were group-housed, whereas adult males were individually housed, except during mating trials. For experiments that used proven fertile male rats, male fertility was assessed by in-house mating trials before study assignment [5]. The environmental conditions of the animal rooms were maintained as recommended in the National Research Council *Guide for the Care and Use of Laboratory Animals* [13] to the maximum extent possible. All study protocols were approved by BIOQUAL's institutional animal care and use committee.

Materials

CDB-4022 ([4aRS,5SR,9bRS]-2-ethyl-2,3,4,4a,5,9b-hexahydro-8-iodo-7-methyl-5-[4-carbomethoxyphenyl]-1H-indeno[1,2-c]pyridine-hydrochloride) was provided by Dr. C.E. Cook and colleagues (Research Triangle Institute, Research Triangle Park, NC). Estradiol-3-benzoate was purchased from Steraloids (Wilton, NH) and dipentylphthalate (DPP), a widely studied Sertoli cell toxicant, was purchased from ChemService (West Chester, PA). [1,2,4,5,6,7-^{3}H]-5α-Dihydrotestosterone (5α-DHT; 123 Ci/mmole) was purchased from New England Nuclear (NEN) Corporation (Boston, MA) and was used as the radioligand in the ABP assays. [1,2,6,7-^{3}H]Testosterone (85–105 Ci/mmole) was also purchased from NEN and was used for estimating 1-chlorobutane extraction efficiency in the testosterone RIA. Needles, syringes, anesthetic, and surgical supplies were purchased from A.J. Buck and Son, Inc. (Owings Mills, MD). High-purity 1-chlorobutane (high-performance liquid chromatography grade) was manufactured by Burdick & Jackson (Muskegon, MI) and purchased through VWR Scientific Products (Willard, OH). Reagent- or molecular biology-grade chemicals and enzymes were purchased from Sigma (St. Louis, MO), Supertechs (Rockville, MD), Boehringer Mannheim (Indianapolis, IN), or Quantum Chemical Co. (Tuscola, IL). Food-grade sesame oil (Hain, Uniondale, NY) was purchased from a local grocery store.

Immunoassays

Testosterone was extracted from serum samples using 1-chlorobutane. The extracts were taken to dryness in a Labconco vortex evaporator (Kansas City, MO), and the residues were reconstituted in a DPC zero calibrator and assayed for testosterone using a commercially available, coated-tube testosterone RIA (Coat-A-Count; Diagnostic Products Corp., Los Angeles, CA). The RIA data were analyzed using a four-parameter sigmoidal computer program (RiaSmart Data Reduction Program; Packard Instrument Co., Meriden, CT). The 1-chlorobutane extraction efficiency was determined from the recovery of [^{3}H]testosterone added to each serum sample. Serum testosterone values were corrected for extraction efficiency. The limit of detection in this assay was 0.1 ng/ml of testosterone, and the intra- and interassay variations were 7% (n = 3) and 16% (n = 6), respectively.

Inhibin B was determined in serum samples using an ultrasensitive inhibin B ELISA assay kit (Serotec, Oxford, UK). The assay has been described in detail previously [14] and has been used to determine inhibin B levels in rat serum [15]. Lyophilized human inhibin B extracted from follicular fluid (supplied with the kit) was reconstituted in castrated adult male rat serum, and a standard curve was generated by serial dilution of the human inhibin B with castrated adult male rat serum [15]. The standard curve was calculated using a linear fit program (EIACalc Dynatech MR-500, Chantilly, VA; Excel version 5.0 spreadsheet; Microsoft Corp., Seattle, WA). The limit of detection for the assay was 25 pg/ml of human inhibin, and the intra- and interassay variations were 2% (n = 3) and 20% (n = 16), respectively.

ABP Assay

The ABP content in the epididymal cytosol was determined by specific binding of [^{3}H]-5α-DHT and expressed as femtomoles bound per milligram of cytosolic protein [16]. Briefly, each frozen epididymis was thawed on ice, minced, and homogenized in four volumes of TE buffer (50 mM Tris, 1.5 mM EDTA) pH 7.2–7.4 at room temperature. The homogenate was centrifuged at 110 000 × *g* for 30 min at 4°C. Endogenous steroids were removed from the resultant supernatant by charcoal precipitation (DCC; 0.05% [w/v] dextran and 0.5% [w/v] charcoal in TE buffer). Total protein was measured in an aliquot of charcoal-stripped cytosol using a BCA protein assay (Pierce, Rockford, IL). Charcoal-stripped epididymal cytosol (40–50 μl) was incubated with 1 nM [^{3}H]-5α-DHT in the presence or absence of 500 nM unlabeled 5α-DHT for 2 h at 4°C. The DCC was added to separate bound from free radioligand. Optifluor (Packard) was added to the resultant supernatant and counted in a Beckman LS 1800 liquid scintillation counter (Fullerton, CA). The amount of rat ABP per milligram of cytosolic protein was calculated using an Excel spreadsheet. This assay had a limit of detection of 10 fmole ABP/mg protein and intra- and interassay variations of 31% (n = 3) and 26% (n = 6), respectively.

Determination of Apoptosis

The left testis was immersion-fixed in 4% paraformaldehyde/phosphate buffer at room temperature. The fixed tissues were embedded in paraffin and processed for detection of apoptotic cells [17]. As an internal control for the assay, tissue sections of mouse mammary gland obtained from dams after weaning were processed in parallel with the testicular sections. Following weaning, the involuting mouse mammary gland undergoes a natural apoptosis [18]. The tissue sections were incubated with proteinase K for 8 min at 37°C to increase signal intensity. The 3′ ends of fragmented DNA were labeled with digoxigenin-deoxyuridine-5′-triphosphase (digoxigenin-dUTP) using the enzyme terminal deoxynucleotidyl transferase (TdT). The digoxigenin-dUTP was visualized by incubation with a monoclonal antidigoxigenin-peroxidase antibody, followed by diaminobenzidine tetrahydrochloride (DAB) substrate and hydrogen peroxide. The DAB appeared as a brown, insoluble precipitate in the nucleus of apoptotic cells. Sections were counterstained with hematoxylin. Negative controls were treated identically, except that the incubation with TdT was omitted. These negative controls were used to establish background (i.e., nonspecific) immunostaining. The percentage of seminiferous tubules containing apoptotic cells was determined for the left testis of each animal by analyzing 200 tubules at 400× magnification. The identity of the apoptotic cells was defined based on location within the seminiferous tubule and general morphological criteria.

Experiment I: Effects on Leydig Cell Function and Fertility

Adult male rats of proven fertility received a single oral dose of control vehicle (10% ethanol/sesame oil, 5 ml/kg) or 2.5 mg of CDB-4022/kg on Day 0 (6 rats/group). This dose of CDB-4022 had been determined previously to be the minimally effective, or threshold, dose for inducing infertility in adult male rats [5]. The threshold dose was used in this study because adult male rats are considered to be more susceptible to the adverse effects of CDB-4022 than immature or peripubertal male rats [19]. In addition, a single oral dose of 2.5 mg/kg induced irreversible infertility in the majority of adult male rats [5]. Body weights were recorded, and blood was collected from the tail vein at Weeks 0, 1, 3, 5, and 8 of the study (the Week 0 sample was obtained immediately before treatment on Day 0). A mating trial was conducted by cohabitation during Week 9, and the female partners were necropsied approximately 17 days later to determine the number and condition of implantation sites. During Week 10, anesthetized male rats were killed by exsanguination via blood collection from the vena cava. Whole blood was allowed to clot for at least 4 h at 4°C, and serum was harvested by centrifugation (900 × *g*, 30 min, 2–6°C). Serum testosterone levels were determined by RIA. The ventral prostate, seminal vesicles, and testes were excised and weighed. Following removal of the tunica albuginea, the left testis was homogenized according to the method described by Meistrich and van Beek [20]. The number of spermatid heads was determined in the homogenate using a hemocytometer, and the data were expressed as the number of spermatid heads per testis [20]. The right testis was preserved in Bouin fixative, washed, dehydrated, and embedded in glycol methacrylate medium (GMA). Hematoxylin-stained cross-sections (thickness, 2 μm) of the testis were used to determine the tubule differentiation index (TDI), which was defined as the percentage of seminiferous tubules undergoing spermatogenesis beyond formation of B spermatogonia [20].

Experiment II: Acute Effects on Germ Cell Apoptosis and Sertoli Cell Function and Morphology

Proven fertile male rats (3 rats/group) received a single oral dose of either control vehicle (100% sesame oil, 5 ml/kg), CDB-4022 (12.5 mg/kg), or DPP (2200 mg/kg). The DPP was included as a known Sertoli cell toxicant. Doses of CDB-4022 and DPP that induced maximal testicular damage [5, 21, 22] were used to determine acute testicular effects. At 3, 6, or 12 h after oral dosing, the rats were injected i.p. with heparin (150 IU/kg) and anesthetized. Blood samples were collected from the tail vein, and the left testis and epididymis were excised. Serum was analyzed for inhibin B, the left testis was processed for detection and localization of apoptotic cells, and the left epididymis was processed for ABP content.

Rats were then perfused with 5% glutaraldehyde/cacodylate buffer (0.05 M, pH 7.4) according to the method described by Sprando [23]. The preserved right testis was embedded in GMA and processed for morphological evaluation. The percentage of seminiferous tubules exhibiting abnormal morphology was determined for the right testis by analyzing at least 100 tubules at 400× magnification. As a positive control for testicular germ cell apoptosis, an additional group of rats (n = 2) received estradiol-3-benzoate at 50 μg/day for 15 days via i.m. injection. The daily dose and dosing interval for estradiol benzoate was based on the results of a study that determined germ cell apoptosis in the testis of the male rat following steroid hormone treatments [24]. Rats were killed by CO_2 asphyxiation 24 h after the final dose of estradiol benzoate, and the testes were excised and processed for detection and localization of apoptotic cells.

Experiment III: Acute Effects on Sertoli Cell Function in Prepubertal Male Rats

Prepubertal male rats (age, 4–6 wk; 10 rats/group) received a single oral dose of control vehicle (100% sesame oil, 5 ml/kg), CDB-4022 (12.5 mg/kg), or DPP (2200 mg/kg). These doses of CDB-4022 and DPP had previously been determined to induce maximal testicular damage [5, 21, 22] and were used to observe acute effects. Rats were anesthetized and killed by exsanguination 24 or 48 h after dosing. Serum samples were analyzed for inhibin B. Twenty-three hours before necropsy, rats were anesthetized, and the efferent ducts of the right testis were ligated with 5-0 silk using aseptic technique. At necropsy, the left and right testis were excised and weighed individually. The difference in the unligated left and ligated right testes weights was calculated and used as an indicator of STF secretion, which is a fundamental function of Sertoli cells [7, 25]. The Sertoli cell STF secretion rate is linear for 24 h following efferent duct ligation [26]. The left epididymis was snap-frozen in liquid nitrogen and stored at −70°C until assayed for ABP content.

Statistical Analysis

To determine whether CDB-4022 treatment had a significant effect ($P < 0.05$) on body weight and circulating testosterone levels over the duration of the study in experiment I, a two-way ANOVA for repeated measures was performed (SigmaStat version 2.01; Jandel Scientific, San Rafael, CA). Significant differences ($P < 0.05$) between the two treatment groups were determined by Student *t*-test for testicular, ventral prostate, and seminal vesicles weights (SigmaStat). Because the TDI and spermatid head count data were not normally distributed, a nonparametric Mann-Whitney rank sum test was performed rather than a Student *t*-test (SigmaStat). Significant differences ($P < 0.05$) among the treatment groups were determined by one-way ANOVA followed by Student-Newman-Keuls (SNK) multiple-range test for the percentage of seminiferous tubules containing apoptotic germ cells and the percentage of seminiferous tubules exhibiting abnormal morphology (experiment II). Treatment effects on serum inhibin B levels and epididymal ABP levels were compared using a parametric or nonparametric one-way ANOVA (SigmaStat; experiments II and III). To perform the parametric ANOVA, the data had to meet the assumptions of normality and homogeneity of variance. A nonparametric test was used for data that did not meet these criteria. For a significant *F* value, a Dunn or SNK multiple-range test was performed. Treatment effects on STF secretion (experiment III) were determined by nonparametric one-way ANOVA on ranks, followed by the Dunn test for a significant *F* value (SigmaStat). Graphs were drawn using SigmaPlot for Windows version 4.0 (Jandel Scientific).

RESULTS

Experiment I: Effects on Leydig Cell Function and Fertility

A single oral CDB-4022 dose of 2.5 mg/kg, which was the minimally effective dose that induced infertility in adult males [5], did not significantly affect ($P > 0.05$) body weight (data not shown) or circulating testosterone levels (Fig. 1) throughout the 10-wk study interval compared to vehicle-treated rats. Serum testosterone levels did not fluctuate significantly ($P > 0.05$) over the study interval, with group means ranging from 3.1 to 4.8 ng/ml regardless of treatment. Likewise, CDB-4022 treatment did not affect the weights of androgen-dependent sex accessory glands, ventral prostate, or seminal vesicles (Table 1). In addition, the morphology of the Leydig cells appeared to be normal (data not shown). In contrast, CDB-4022 significantly decreased ($P < 0.05$) testis weight, spermatid head count, and number of seminiferous tubules containing differentiating germ cells at Week 10 (Table 1). All seminiferous tubules examined in vehicle-treated rats contained differentiating germ cells (TDI = 100%) (Table 1). CDB-4022 treatment rendered all male rats infertile, corresponding to depopulation of germ cells. Female rats that mated with the vehicle control-treated males had a typical complement of normal uterine implantation sites per pregnant rat (mean ± SEM, 15 ± 1 sites).

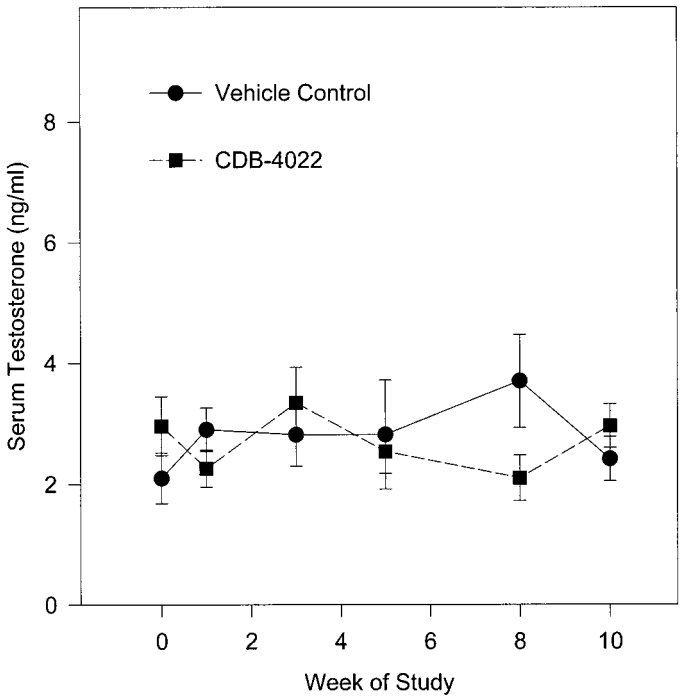

FIG. 1. Experiment I: serum testosterone levels in adult male rats throughout the study. Symbols represent mean ± SEM. No significant differences ($P > 0.05$) between the two treatment groups were found. Likewise, no significant time effect ($P > 0.05$) or treatment × time interaction ($P > 0.05$) was observed.

TABLE 1. Experiment I: effects of CDB-4022 treatment on accessory sex organ and testis weights, spermatogenesis, and fertility.

	Treatment group	
Parameter (mean ± SEM)	Vehicle control (5 ml/kg)[a]	CDB 4022 (2.5 mg/kg)[b,c]
Ventral prostate weight (g)	1.07 ± 0.08	1.10 ± 0.01
Seminal vesicle weight (g)	1.51 ± 0.10	1.35 ± 0.07
Right testis weight (g)	1.80 ± 0.07	1.01 ± 0.04[d]
Spermatid head count (cells/testis × 10^6)	113 ± 7.24	0.67 ± 0.18[d]
TDI (%)	100 ± 0	20.4 ± 7.1[d]
Male fertility index	6/6	0/5[d]

[a] n = 6.
[b] n = 5.
[c] One rat was killed before study termination as recommended by the attending veterinarian due to nontreatment-related swelling of the hindlimbs.
[d] Significantly different ($P < 0.05$) from vehicle control.

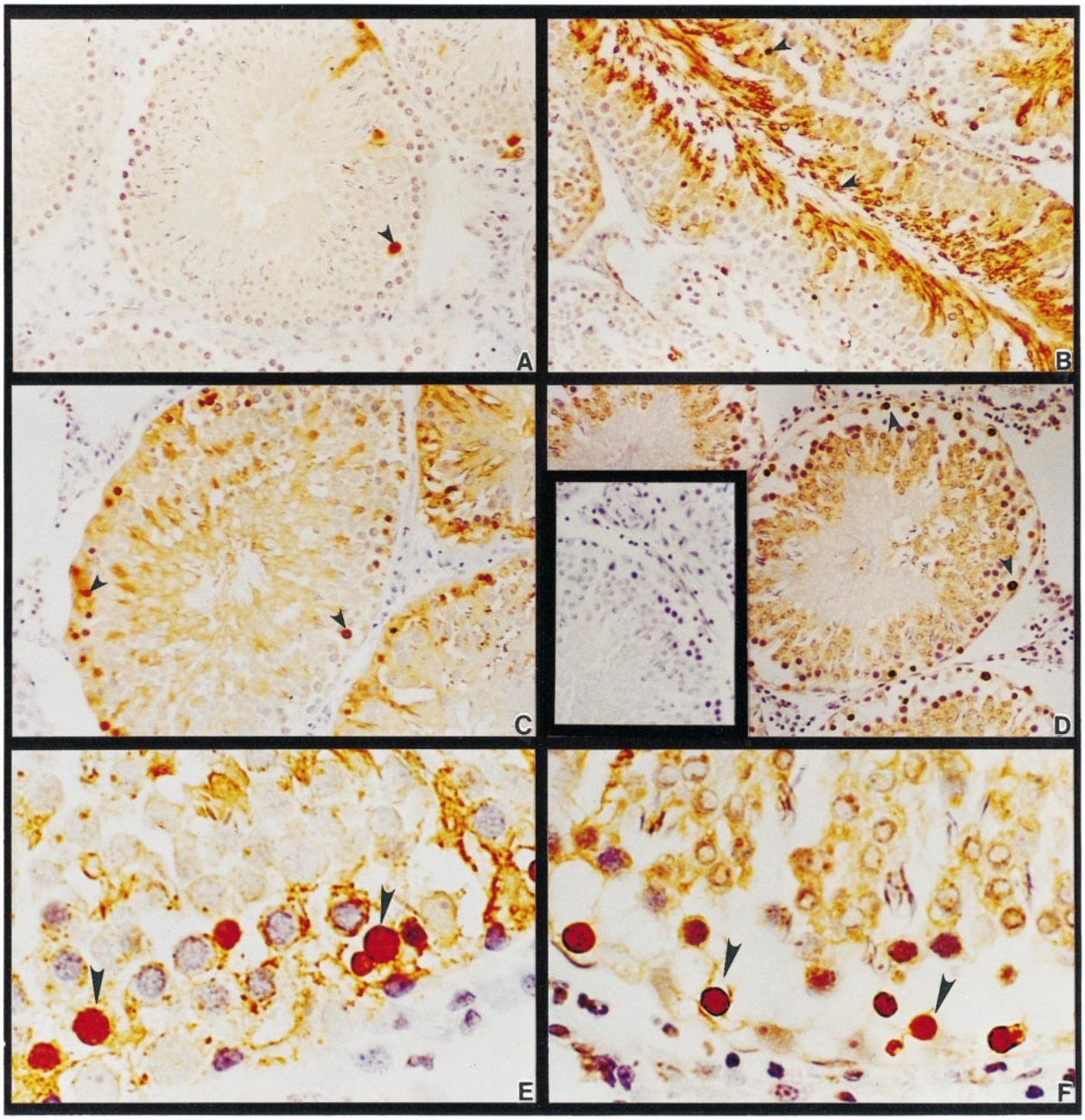

FIG. 2. Experiment II: representative testicular sections immunostained for apoptotic nuclei. **A**) Vehicle control-treated rat testis. **B**) Estradiol benzoate-treated rat testis. **C**) DPP-treated rat testis at 12 h after dosing. **D**) CDB-4022-treated rat testis at 12 h after dosing. Inset shows the negative control of the same rat testis in which TdT was omitted. **E**) Higher magnification of the tubule in **C**. **F**) Higher magnification of the tubule in **D**. Arrowheads indicate apoptotic nuclei; the DAB precipitate appears dark brown. Final magnifications ×120 (**A**–**D**) and ×450 (**E** and **F**).

Experiment II: Acute Effects on Germ Cell Apoptosis and Sertoli Cell Function and Morphology

A few seminiferous tubules in the testis of vehicle control-treated rats contained one or more apoptotic germ cells (Fig. 2A and Table 2). However, the majority of tubules in the vehicle control-treated rats did not contain apoptotic cells (mean, 77%), supporting the findings of other investigators [24, 27, 28]. The internal positive control (i.e., the involuting mouse mammary gland) demonstrated numerous apoptotic cells (data not shown). Estradiol benzoate treatment increased the percentage of tubules containing apoptotic germ cells, with more tubules containing multiple apoptotic cells (mean ± SD, 78 ± 11; n = 2). Estradiol benzoate appeared to increase apoptosis in spermatocytes and spermatids (Fig. 2B), as reported by Blanco-Rodriguez and Martinez-Garcia [24]. Both CDB-4022 and DPP increased the percentage of tubules containing one or more apoptotic germ cells per tubule (Fig. 2, C and D, and Table 2). This effect was significantly greater ($P < 0.05$) than in the vehicle control group at 12 h after dosing with CDB-4022 (Table 2). Significant increases ($P < 0.05$) in the percentage of seminiferous tubules containing apoptotic germ cells were apparent at 6 and 12 h after treatment with DPP (Table 2). The negative control, in which TdT was omitted from the reaction, did not demonstrate nuclear staining (Fig. 2D, inset). Both DPP and CDB-4022 tended to induce apoptosis in early spermatocytes and differentiating spermatogonia based on the location of the apoptotic cells in

TABLE 2. Experiment II: serum inhibin B, epididymal ABP, and percentage of seminiferous tubules with apoptotic germ cells or abnormal morphology following a single oral dose of CDB-4022, DPP, or vehicle to adult male rats at 3, 6, or 12 h before necropsy.

Treatment	Time after dosing (h)	Serum inhibin B (pg/ml)[a]	Epididymal ABP (fmol/mg)[a]	% Seminiferous tubules with apoptotic germ cells[a]	% Seminiferous tubules with abnormal morphology[a]
Vehicle	3	148.6 ± 9.0[b]	189 ± 39	23 ± 4[b]	8 ± 3[b]
CDB-4022	3	138.9 ± 20.0[b,c]	190 ± 18	21 ± 3[b]	43 ± 4[c,d]
CDB-4022	6	86.6 ± 8.4[c]	130 ± 23	40 ± 7[b,c]	44 ± 7[c,d]
CDB-4022	12	95.6 ± 19.0[c]	103 ± 3	59 ± 6[d]	70 ± 5[d]
DPP	3	120.4 ± 2.6[b,c]	160 ± 11	36 ± 3[b,c]	30 ± 2[b,c]
DPP	6	82.9 ± 9.6[c]	177 ± 15	48 ± 6[c,d]	28 ± 15[b,c]
DPP	12	61.9 ± 7.0[c]	156 ± 31	65 ± 3[d]	56 ± 3[c,d]

[a] Mean ± SEM, n = 3/group.
[b,c,d] Group means with different letters were significantly different ($P < 0.05$) from each other.

the periphery of the tubule and the general size and shape of the apoptotic cell (Fig. 2, C–F). An increase in apoptosis in late-stage spermatids (steps 7–19), in which a tail is present, was not observed in the testes of DPP- or CDB-4022-treated rats. Further identification of spermatogenic cell types undergoing apoptosis was not possible in these paraffin-embedded sections.

The testis of vehicle control-treated rats demonstrated normal morphology (Fig. 3A). In contrast, the testis of estradiol benzoate-treated rats demonstrated shrunken tubules and sparse interstitial tissue, with atrophy of Leydig cells and decreased spermatogenesis (data not shown). These findings are consistent with estrogen suppression of gonadotrophin and testosterone secretion in males [29, 30]. Vacuolization of Sertoli cells and detachment of germ cells from the seminiferous epithelium, which is an initial sign of apical germ cell sloughing/shedding, was apparent in both DPP-treated (Fig. 3B) and CDB-4022-treated rats (Fig. 3, C and D). A low percentage of seminiferous tubules with abnormal morphology was observed in vehicle-treated rats, and this may be an artifact of tissue processing (Table 2). However, significant increases in the percentage of seminiferous tubules with abnormal morphology, primarily vacuolization of Sertoli cells, was apparent in CDB-4022- and DPP-treated rats by 3 and 12 h after dosing, respectively.

In conjunction with the effects observed on germ cell apoptosis and Sertoli cell morphology, both CDB-4022 and DPP inhibited Sertoli cell function, as indicated by decreases in serum inhibin B levels (Table 2). Serum inhibin B levels were significantly reduced ($P < 0.05$) at 6 h after a single dose of DPP or CDB-4022 compared to vehicle control-treated rats, and these levels remained low at 12 h after dosing. Epididymal ABP content was not significantly decreased ($P > 0.05$) at these early times after a single dose of CDB-4022 or DPP (Table 2).

Experiment III: Acute Effects on Sertoli Cell Function in Prepubertal Male Rats

Both CDB-4022 and DPP at doses that induced maximal testicular damage [5, 21, 22] significantly suppressed ($P < 0.05$) STF secretion by Sertoli cells within 24 h after a single oral dose (Fig. 4). The STF remained suppressed 48 h after treatment with either 12.5 mg/kg of CDB-4022 or 2200 mg/kg of DPP. CDB-4022 also induced a significant decrease ($P < 0.05$) in serum inhibin B levels (Fig. 5) and epididymal ABP content (Fig. 6) at 24 and 48 h after a single oral dose (12.5 mg/kg). Inhibition of serum inhibin B levels (Fig. 5) and epididymal ABP content (Fig. 6) were also observed following a single oral dose of DPP (2200 mg/kg). Interestingly, CDB-4022 resulted in a significantly greater ($P < 0.05$) suppression of serum inhibin B levels at 24 h after dosing compared to DPP treatment, but by 48 h after dosing, the effect of both compounds was equivalent ($P > 0.05$).

DISCUSSION

Collectively, these data indicate that CDB-4022 interferes with early stages of spermatogenesis by disruption of Sertoli cell structure and function. Previously, Sertoli cell toxicants have been demonstrated to have acute and specific adverse effect(s) on Sertoli cells, which typically manifest morphologically by vacuolation of the Sertoli cell cytoplasm, apical germ cell sloughing or shedding, and germ cell death [7]. Functionally, decreased STF secretion and changes in secretion, including direction (i.e., apical vs. basal), of Sertoli cell-specific products have been observed [7]. CDB-4022 treatment induced vacuolization of Sertoli cells at comparable or earlier times than the widely studied Sertoli cell toxicants, DPP (present study; [21, 22]) and 2,5-hexanedione (2,5-HD) [7]. Additional studies are required to determine if the CDB-4022-induced Sertoli cell vacuolization observed at the light microscopic level reflects ultrastructural changes similar to those observed for other Sertoli cell toxicants (e.g., swelling and coalescence of intracellular membrane organelles) [7, 22]. Significant decreases in STF secretion were also apparent within 24 h after treatment with CDB-4022 that were comparable to those of other known Sertoli cell toxicants, such as DPP and related phthalates (present study, [31]) and 2,5-HD [25]. Likewise, production of the specific Sertoli cell proteins inhibin B and ABP [10–12] was significantly decreased at early time points following CDB-4022 treatment. The apparent delay in suppression of epididymal ABP compared to the decline in serum inhibin B levels may be due to an extended half-life of ABP in the epididymis. Preliminary data from our laboratory indicate that epididymal ABP content does not decline significantly until 48 or 72 h after efferent duct ligation or castration plus testosterone replacement, respectively (unpublished data). Decreases in serum inhibin B levels have been associated with depletion of specific germ cells, and measurement of serum inhibin B has been proposed as a noninvasive method of assessing fertility in men [12, 32]. The production and direction of inhibin B secretion by the Sertoli cells into either the interstitial fluid (i.e., basal) or seminiferous tubule fluid (i.e., apical) may be modulated by germ cells, particularly spermatocytes and spermatids [12]. Hence, long-term suppression of serum inhibin B levels in adult male rats treated with a single dose of CDB-4022 (unpublished data) may be attributable to a lasting effect on the Sertoli cell and/or

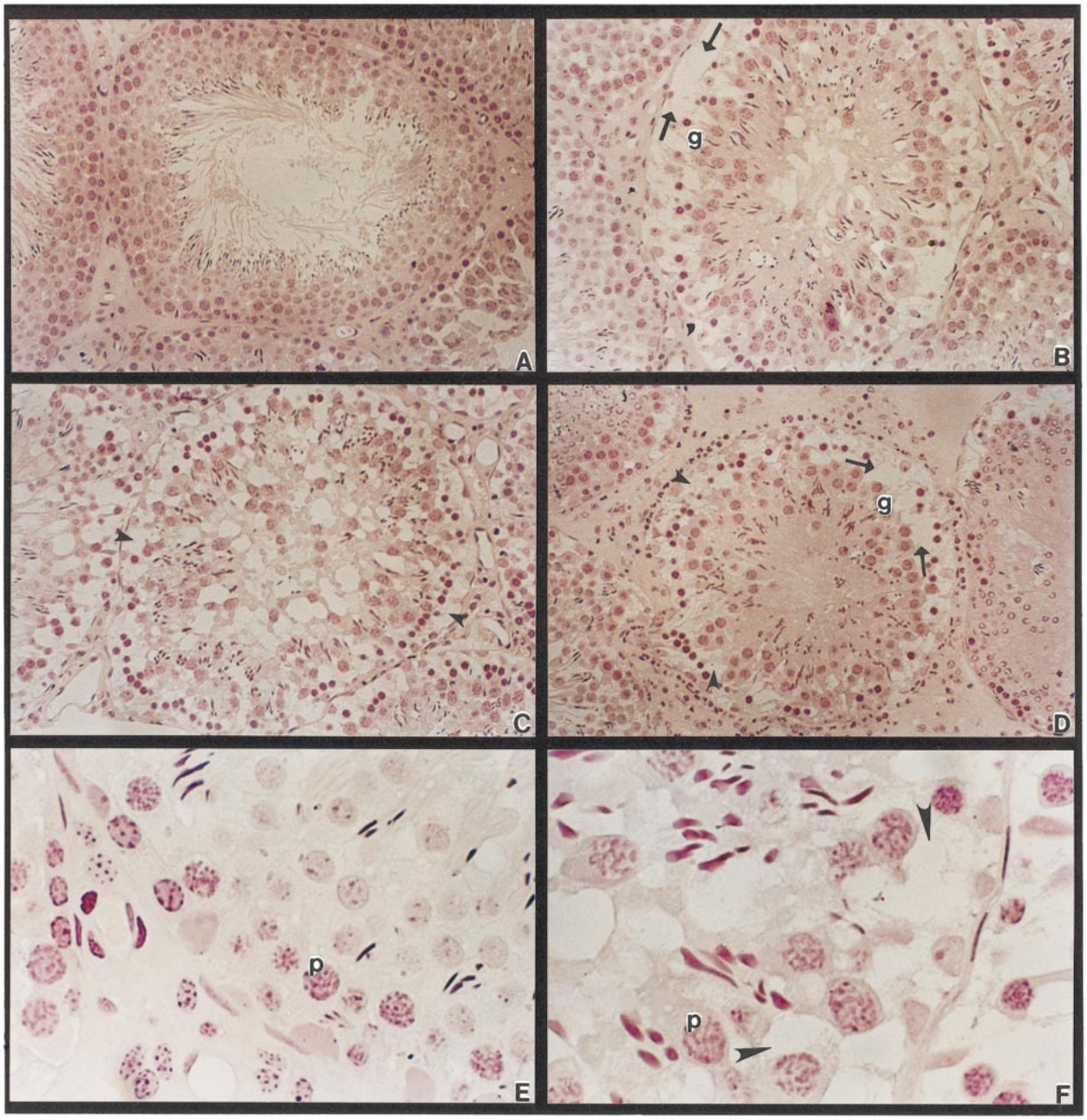

FIG. 3. Experiment II: representative testicular morphology. **A**) Vehicle control-treated rat testis. **B**) DPP-treated rat testis at 12 h after dosing. **C**) CDB-4022-treated rat testis at 6 h after dosing. **D**) CDB-4022-treated rat testis at 12 h after dosing. **E**) Higher magnification of the tubule in **A**. **F**) Higher magnification of the tubule in **C**. The increased nuclear size and ovoid shape of pachytene (p) spermatocytes in **F** relative to **E** were due to differences in the stage of the spermatogenic cycle (maturational changes) and were not considered to be treatment related. Arrowheads indicate vacuolization of Sertoli cells; arrows indicate detachment/cleavage of germ cells (g) from the seminiferous epithelium, an initial sign of apical sloughing/shedding. Final magnifications ×120 (**A**–**D**) and ×450 (**E** and **F**).

the lack of differentiating germ cells in the seminiferous tubules (present study; [5]). Importantly, the decrease in serum inhibin B levels preceded germ cell apoptosis and depopulation of the seminiferous epithelium, implying that CDB-4022 acted directly on the Sertoli cell; germ cell apoptosis was presumably a result of disruption of Sertoli cell structure and/or function. Although the primary target of CDB-4022 appears to be on the Sertoli cell, direct actions of CDB-4022 on specific germ cells cannot be ruled out at present.

In contrast to actions on Sertoli cells, CDB-4022 treatment did not adversely affect the morphology or function of the Leydig cell (present study; [5]). Serum levels of testosterone were not altered by CDB-4022 treatment. Furthermore, CDB-4022 did not suppress body weight gain or decrease the weights of accessory sex organs. These data support the supposition that CDB-4022 did not affect testosterone production, because atrophy of the accessory sex organs and decreased body weight are indicators of testosterone suppression [33, 34]. Libido was also not adversely affected by treatment with indenopyridines, because the treated males mated successfully with female rats or mice (present study; [3, 5]). In a previous study [35], testicular testosterone concentrations in adult rats were not significantly affected by treatment with the indenopyridine 20-438 at 24 and 48 h after oral dosing. These data support

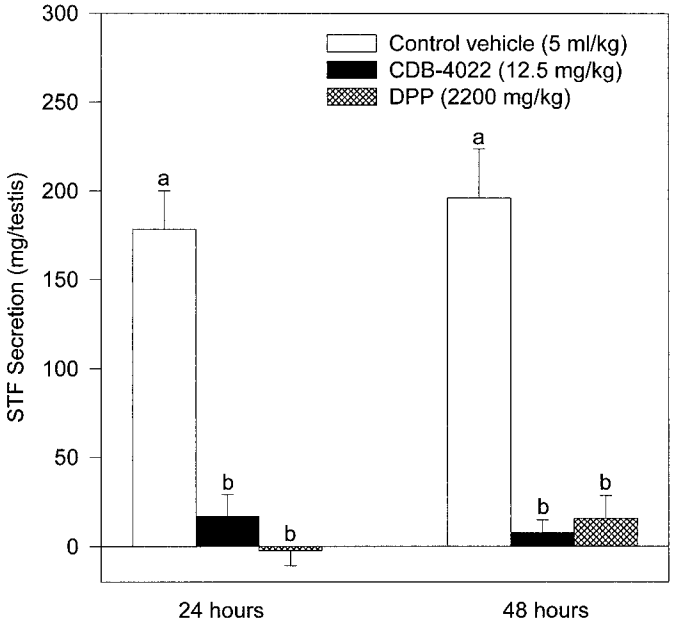

FIG. 4. Experiment III: STF secretion in prepubertal male rats following a single oral dose of vehicle, CDB-4022, or DPP. Bars represent mean ± SEM (n = 9–10/group). Bars with different letters are significantly different from each other ($P < 0.05$).

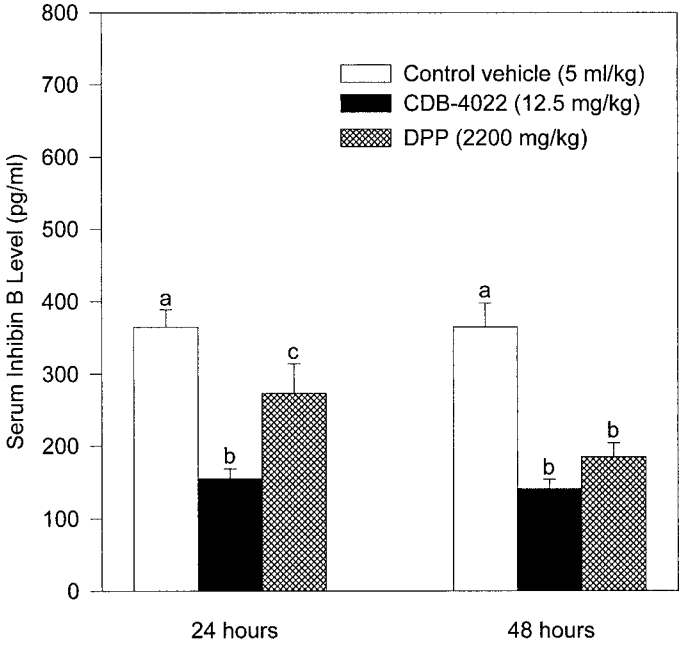

FIG. 5. Experiment III: serum inhibin B levels in prepubertal male rats following a single oral dose of vehicle, CDB-4022, or DPP. Bars represent mean ± SEM (n = 9–10/group). Serum inhibin B levels are expressed as human inhibin B equivalents. Bars with different letters are significantly different from each other ($P < 0.05$).

the lack of an effect of CDB-4022 on circulating testosterone levels in the present study. Furthermore, the data suggest that CDB-4022 induces germ cell apoptosis at early stages of spermatogenesis, in contrast to estradiol benzoate, which induces apoptosis at later stages (present study; [24]). The pattern of CDB-4022-induced testicular germ cell apoptosis was also distinctly different from that observed following gonadotropin/testosterone deprivation produced by GnRH antagonist treatment (Nal-Glu) [36]. Gonadotropin deprivation resulted in increased apoptosis at late stages of spermatogenesis, primarily pachytene spermatocytes and spermatids (steps 7 to 19). Moreover, an increase in testicular germ cell apoptosis was not observed until 5–7 days after daily GnRH antagonist treatment, whereas CDB-4022 resulted in increased germ cell apoptosis within 12 h after a single treatment. These data support the hypothesis that CDB-4022 does not act via a gonadotropin- or testosterone-mediated mechanism.

A related indenopyridine, RTI-056L, also increased apoptosis in spermatogenic cells, as determined by increased DNA strand breaks detected via TUNEL labeling, at 18 h after treatment of adult male mice [6]. Those authors did not attempt to directly identify the cell type undergoing apoptosis; however, a decrease in the relative percentage of haploid cells compared to an increase in diploid and tetraploid cells at 18 h after treatment led them to conclude that RTI-056L was affecting step 3 and 4 spermatids. The data from the present study do not rule out increased apoptosis of early spermatids, because these could not be distinguished accurately. However, the data suggest that the indenopyridine CDB-4022 affects spermatogenesis at stages before spermatid formation, particularly differentiating spermatogonia and spermatocytes. The Sertoli cell toxicant 2,5-HD has been proposed to induce apoptosis of type A_3 and A_4 spermatogonia [37]. Hence, CDB-4022-induced apoptosis of differentiating spermatogonia would be consistent with its classification as a Sertoli cell disrupter. CDB-4022-induced apoptosis at these early stages of spermatogenesis is also compatible with the time frame (two or three spermatogenic cycles) required to initiate infertility (3–5 wk) in the male rat following treatment with CDB-4022 [5].

The increase in germ cell apoptosis induced by CDB-4022 treatment could be mediated via a Sertoli cell paracrine factor or disruption of Sertoli cell structure. The potential mechanisms involved include disruption of the stem

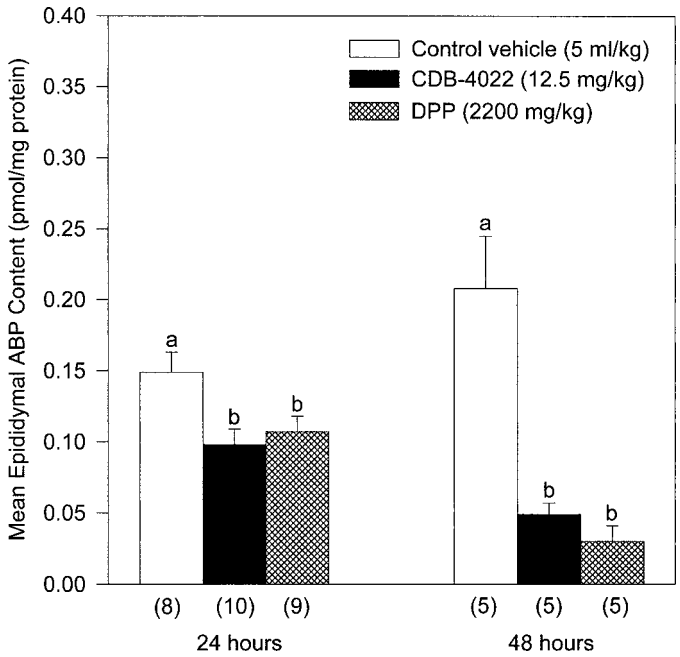

FIG. 6. Experiment III: epididymal ABP content in prepubertal male rats following a single oral dose of vehicle, CDB-4022, or DPP. Bars represent mean ± SEM (n is indicated in parentheses under each bar). Bars with different letters are significantly different from each other ($P < 0.05$).

cell factor (SCF)/c-*kit* system, modulation of the Fas system, modulation of the Bcl-2 family, and inhibition of microtubule/microfilament structure of the Sertoli cell. The SCF (c-*kit* ligand) is produced by the Sertoli cell and interacts with c-*kit,* a transmembrane tyrosine kinase receptor in differentiating spermatogonia, to promote survival and proliferation of differentiating spermatogonia [38]. Treatment of adult male rats with the Sertoli cell toxicant 2,5-HD resulted in an increase of the soluble as opposed to the transmembrane form of SCF and an increase in germ cell apoptosis [39]. The transmembrane form of SCF had previously been shown to be more effective than the soluble form at preventing germ cell apoptosis [40]. Infusion of SCF directly into the testis of 2,5-HD-treated rats was able to compensate, to some degree, for the Sertoli cell defect and led to an increase in survival of proliferating germ cells [39]. Recovery of spermatogenesis was accomplished in 2,5-HD-treated rats by posttreatment with Lupron Depot and was associated with re-establishment of normal SCF expression in Sertoli cells [41]. Taken together, these data indicate that SCF expression by Sertoli cells is one of the key mechanisms by which Sertoli cells regulate germ cell apoptosis. The Fas system is an additional paracrine signaling system by which Sertoli cells, which express Fas ligand, can initiate apoptosis of Fas-expressing germ cells [42]. An increase in Fas ligand expression in Sertoli cells was observed in rats treated with the Sertoli cell toxicants 2,5-HD and mono-(2-ethylhexyl)phthalate (MEHP), whereas germ cell injury induced by radiation increased germ cell expression of Fas [43]. Hence, compounds that result in Sertoli cell injury, such as CDB-4022, may increase germ cell apoptosis by upregulation of Fas ligand. Key regulators of apoptosis in several cell types, including testicular cells, are the relative levels of the various members of the Bcl-2 family that promote cell survival (e.g., Bcl-2, Bcl-X_L, Bcl-w) and those that antagonize it (e.g., Bax, Bak, Bad) [44]. Several of these factors are expressed in selective cell types, but the potential Bcl-2 family factors that may be affected by Sertoli cell toxicants remain unclear. In addition to these paracrine factors, germ cell apoptosis may be induced by detrimental effects on Sertoli cell microtubules and/or microfilaments. Alterations in the Sertoli cell cytoskeleton could affect germ cell attachment, disrupt the blood-testis barrier formed by Sertoli cell tight junctions, or result in defects in microtubule transport of products secreted into the STF [7]. Indeed, the Sertoli cell toxicant 2,5-HD resulted in disrupted tubulin expression [25], and MEHP adversely affected the vimentin filaments of Sertoli cell microtubules [45]. These data suggest that multiple Sertoli cell mechanisms could be involved in CDB-4022-induced germ cell apoptosis.

Because the majority of Sertoli cell toxicants cause irreversible infertility [7], the classification of CDB-4022 as a Sertoli cell disrupter may make it unsuitable as a male contraceptive. Reversibility of infertility would be essential for continued development of CDB-4022 as a contraceptive agent. Recently, we investigated treatments that may allow recovery of spermatogenesis in CDB-4022-treated males [5]. Treatment of adult male rats with the GnRH agonist, Lupron Depot, before and after administration of a single oral dose of CDB-4022, resulted in the return of spermatogenesis and fertility in the majority of treated rats. Lupron Depot treatment has also been used to restore spermatogenesis and fertility in male rats previously treated with 2,5-HD [41]. Furthermore, concurrent administration of the antioxidant vitamins C and E prevented the decrease in testicular weight, spermatogenesis, and spermatid production in di-(2-ethylhexyl)phthalate-treated rats [46]. These data indicate that the long-term antispermatogenic effects of Sertoli cell toxicants may be prevented, or even reversed, by various treatment regimens. The ability of GnRH antagonist treatment administered either before or after CDB-4022 to protect or restore spermatogenesis and fertility in adult male rats is under active investigation in our laboratory.

Data from the present studies indicate that CDB-4022 caused infertility by decreasing the number of seminiferous tubules undergoing complete spermatogenesis, thereby decreasing spermatid production. This antispermatogenic activity of CDB-4022 is most likely mediated via disruption of Sertoli cell structure and function, producing increased apoptosis of differentiating spermatogonia and spermatocytes. However, direct effects of CDB-4022 on germ cells cannot be eliminated from consideration at present. In contrast, CDB-4022 does not appear to have adverse effects on Leydig cells, because serum testosterone levels and libido were not affected (present study; [3, 5]). These observations, in combination with the lack of genetic or overt toxicity, and other adverse side effects of indenopyridines [1, 2, 47, 48], indicate that these compounds may have utility as male contraceptives. However, the ability to prevent or reverse the antispermatogenic effects of CDB-4022 will be critical for its development as a male contraceptive.

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