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Androgen Receptor Plays a Vital Role in Benomyl- or Carbendazim-Induced Reproductive and Developmental Toxicity and Endocrine-Disrupting Activity in Rats

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<http://dx.doi.org/10.5772/intechopen.78276>

Abstract

Benomyl and its metabolite carbendazim were reported to induce reproductive and developmental toxicity and endocrine-disrupting activity in rats. The exactly underlying mechanism of reproductive and developmental toxicity and endocrine-disrupting activity still remain unclear. Based on our unpublished data it showed that the antiandrogen flutamide can completely recover the reproductive and developmental toxicity including embryoletality induced by benomyl and carbendazim in rats. This manuscript aimed to review and generalize the results based on our previous reports. Androgen receptor might play an important role in benomyl- and carbendazim-induced reproductive and developmental toxicity and endocrine-disrupting activity. The evidences were (1) androgen- and androgen receptor-dependent mechanisms are possibly involved in carbendazim-induced toxicity; (2) carbendazim exposure in utero displays a transient and weak androgenic effect and reduces flutamide antiandrogenicity in male rats; (3) antagonistic effect of flutamide on the carbendazim-androgenic effect on mRNA and protein levels; (4) benomyl and carbendazim exhibit an androgenic effect, leading to increase weight of ventral prostate and seminal vesicles and uterine fluid retention in young adult rats. The molecular underlying mechanism of reproductive and developmental toxicity and endocrine-disrupting activity induced by benomyl and carbendazim through androgen receptor need to be further investigated.

Keywords: benomyl, carbendazim, reproductive and developmental toxicity, endocrine-disrupting activity, rats

1. Introduction

As reported carbendazim (methyl-2-benzimidazole carbamate) is used to be a systemic fungicide [1]. Both carbendazim and its parent benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate] are exhibiting low acute toxicity [2]. In contrast, carbendazim and benzimidazole chemicals induced severe reproductive and developmental toxicity in rodents [3–6]. Reports showed that carbendazim and benomyl exhibited testicular toxicity such as sloughing of immature spermatids [7, 8], inhibition of microtubule assembly [9], seminiferous tubular atrophy [10], and testicular atrophy and infertility [11] in male rats. Both carbendazim and benomyl induced developmental toxicity in rodents. Prenatal treatment of carbendazim to rats during pregnancy exhibited embryonic death, growth retardation, and developmental abnormalities including exencephaly, microphthalmia and hydronephrosis in offspring [3, 12]. Treatment of benomyl to pregnant rats induced craniocerebral and systemic malformations such as cleft palate, hydrocephalus, and exencephaly in offspring of male and female rats [13]. In contrary to the more reports available on the reproductive and developmental toxicity of carbendazim and benomyl, studies for endocrine-disrupting activity or mode of action of the these two fungicides remains unclear. The earlier report on endocrine activity for carbendazim might be Rehnberg et al. (1989). They showed that administration of male rats with carbendazim raised testosterone concentration and the levels of androgen binding protein in the interstitial and seminiferous tubule fluid, meaning an association between endocrine disruption activity and carbendazim toxicity [14]. Recently Rama et al. (2014) reviewed and reported that carbendazim induced reproductive toxicity and possible hormonal effects in rats [15]. They reviewed the previous reports and generalized that carbendazim have androgenic effects acting directly in the androgen receptors and/or increasing the expression of androgen receptors. Some chemicals were reported to increase or decrease AR expression. Bisphenol A was reported to increase AR expression [16] while di-n-butyl phthalate (DBP) [17, 18] and sodium valproate [19] decrease it. We found out that it is common for estrogen receptor agonist and androgen receptor antagonist but not for androgen agonist in pesticides. Based on the chemical structure it seems to determine the AR agonist or antagonist. The degree of increase or decrease of AR expression might be depended on the chemical structure, which shared with nature ligand dihydrotestosterone. This manuscript would like to combine our unpublished data and previous studies to infer that androgen receptor plays an important role in benomyl- and carbendazim-induced reproductive and developmental toxicity and endocrine-disrupting activity in rats.

2. Previous studies of reproductive and developmental toxicity and endocrine-disrupting activity induced by benomyl or carbendazim

2.1. Endocrine-disrupting activity in carbendazim-induced reproductive and developmental toxicity in rats

2.1.1. Materials and methods

(1) Animals and related preparation

Both male and female SD rats, 3–4 week old, were obtained from the National Laboratory Animal Breeding and Research Center, Taipei, Taiwan. All rats were kept in specific-pathogen-free

animal facility in Taiwan Agricultural Chemicals and Toxic Substances Research Institute, Taichung, Taiwan. The animal rooms were kept on a 12-h light and dark cycle, $23 \pm 2^\circ\text{C}$, and $50 \pm 10\%$ relative humidity. When they were transported to animal room, the rats were quarantined for at least 1 week and opened on the bases of enough body weight gain and without clinical signs of disease of injury. Both carbendazim and benomyl with 99% pure were a gift from Sinon Co., Taichung, Taiwan. These two pesticides were suspended in polyethylene glycol 200 and treated to animals orally by gavage in a volume of 2 ml/kg body weight, once daily. In reproductive toxicity studies, male rats were administered with these two pesticides and/or flutamide for 28 days. In developmental toxicity studies, both male and female rats were administered with 200 mg/kg carbendazim or 100 mg/kg benomyl for 28 days. Then the female rats were mated with male within each treatment group for 14 days. No treatment was carried out during the mating period. Pregnant dams with plug detected were kept to deliver the offspring at term and conception rates were calculated. All rat offspring were weaned at 21 days postnatal and then fed up to 6 week old [20].

(2) Organ and tissue weight, morphology, and histopathological examination

Both testis and epididymis were weighed by right and left sides. The incidence of abnormal morphology was recorded. The half of testis or epididymis each was fixed in 10% neutral phosphate-buffered formalin solution for subsequent histopathological examinations. Tissues of testis and epididymis were processed by standard histopathological processes and stained using haematoxylin and eosin for light-microscopic examinations. Both testis and epididymis sections were stained with the Giemsa staining periodic acid-Schiff methods and then counterstained using haematoxylin as reported in Simoes and Schoning [21]. Both testis and epididymis histology were evaluated and histopathological findings were scored according to Oakberg [22] and Hess [23].

(3) In vitro androgen receptor binding assay

The ligand binding assay was processed to determine the concentration of androgen receptor in rat tissue according to Nonneman et al. [24]. The ligand [1, 2, 3, 5, 6, 7-3H(N)]-5 α -androstano-17 β -ol-3-one (dihydrotestosterone, 5 α -DHT) (110–150 Ci/mmol) was obtained from NEN Life Science Products, Inc., Boston. Nonlabelled 5 α -DHT was obtained from Sigma Chemical Company, St. Louis, MO, and recrystallized from ethanol prior to use. Both rat testis and epididymis were homogenized in ice-cold low-salt TEDG buffer, pH 7.4, consisting of 10 mM Tris, 1.5 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, and 1 mM each of dithiothreitol, phenylmethylsulfonyl fluoride, and sodium molybdate as described by Hardy et al. [25]. Tissue homogenates were centrifuged at $30,000 \times g$ for 1 h and the supernatant were processed to use as the low-salt extract. Before analysis, the endogenous steroids were removed from the low-salt extract by incubation with dextran-coated charcoal. The binding of [3H]-5 α -DHT to androgen receptor of testis and epididymis extract each was determined by competitive inhibition binding using nonlabelled 5 α -DHT. Charcoal-treated testicular and epididymal extract each was incubated with 1 nM [3H]-5 α -DHT at 4°C for 24 h. The nonspecific binding was carried out with incubating the extract with 100-fold excess nonlabelled 5 α -DHT. Unbinding [3H]-5 α -DHT was isolated from the binding steroid by adding the extract to packed hydroxyapatite in the low-salt TEDG buffer. Mixture were incubated for 30 min with several mixings and then centrifuged at $600 \times g$ for 3 min at 4°C . After that the supernatant was aspirated. An aliquot of the packed HAP was washed 4 times with ice-cold 50 mM Tris buffer, pH 7.3. In determination of total binding,

the binding [3H]-5 α -DHT was extracted from HAP with ethanol and counted for radioactivity using a Beckman model LS6000 TA liquid scintillator. For specific binding of testis and epididymis extracts they were determined by subtracting nonspecific binding from total binding and corrected for protein concentration. The protein concentration was determined according to Lowry et al. [26]. Analysis of effect of carbendazim on androgen receptor binding, specific binding of [3H]-5 α -DHT to testis extract was carried out with incubation of the charcoal-treated testis extract with [3H]-5 α -DHT in the presence of carbendazim at 4°C for 24 h. Incubation mixtures were carried out to the same procedures as before in the androgen receptor binding assay.

(4) Statistical analysis

All these data were expressed as mean \pm SE. All data were processed to analysis of variance followed by Student's t-test. The level of significance was set at $p < 0.05$.

2.1.2. Abstract

This study aimed to investigate the endocrine-disrupting activity of carbendazim-induced reproductive and developmental toxicity in rats. The male rats were co-treatment with 675 mg/kg carbendazim and 50 or 100 mg/kg flutamide, an androgen receptor antagonist, once daily for 28 days decreased testis weight induced by treatment with carbendazim alone. Co-treatment of carbendazim and flutamide blocked losses of spermatozoa and cell morphology and decrease of sperm concentration induced by carbendazim. An important evidence for endocrine disrupting activity induced by carbendazim and benomyl was that pre-mating treatment of male and female rats with 200 mg/kg carbendazim for 28 days resulted in androgenic effects including incomplete development of uterine horn, enlargement of urethra, absence of vagina, and induction of seminal vesicles in female offspring, without significant effects in male offspring. Also, pre-mating treatment with 100 mg/kg benomyl, the parent compound of carbendazim, produced incomplete development of uterine horn and absence of vagina in female offspring and induced testis and epididymis atrophy in male offspring. When male rats were treated with 25, 50, 100, 200, 400, and 800 mg/kg carbendazim for 56 days androgen receptor concentrations were increased in testis and epididymis with dose dependent. Furthermore, additions of 5, 50, and 500 M carbendazim to testis extract from untreated rats substituted binding of [3H]5-dihydrotestosterone to androgen receptor with concentration dependent. This study illustrated that reproductive toxicity exhibited by carbendazim is relieved by an androgen receptor antagonist flutamide in male rats and developmental toxicity of the fungicide shows androgenic properties in female offspring. The authors concluded that androgen- and androgen receptor-dependent mechanisms are quite possibly involved in carbendazim-induced toxicity.

2.2. Antagonistic and synergistic effects of carbendazim and flutamide exposures in utero on reproductive and developmental toxicity in rats

2.2.1. Materials and methods

(1) Animals and related preparation

Both male and female rats were obtained from the National Laboratory Animal Center, Taipei, Taiwan. All rats were kept in specific pathogen-free animal facility in Taiwan Agricultural

Chemicals and Toxic Substances Research Institute, Taichung, Taiwan. All animal rooms were kept under a 12-hour light and dark cycle, $23 \pm 2^\circ\text{C}$, and $50 \pm 10\%$ relative humidity. All animal had access *ad libitum* to reverse osmosis water and rodent chow (LabDiet[®] 5001, PMI Nutrition International, LLC, Brentwood, MO, USA). When the animals were transported to the animal room, all rats were quarantined for at least 1 week and opened on the basis of enough body weight and without clinical signs of disease or injury. Female rats were mated with male within each same treatment group for 14 days. Gestation day (GD 0) was defined as the day that sperm was detected in vagina of the mated female. Allocating animals to treatment groups was finished on the basis of body weight randomization to ensure unbiased weight distribution across groups. Dams and offspring were kept in polycarbonate cages on Laboratory Animal Bedding (TCP Chipsi Heimtier Steu, Germany) until weaning postnatal day 21 (PND 21), at which the test animals were housed, up to 5 per cage, by sex and treatment until necropsy on PND 56. All male and female offspring were euthanized by CO₂ asphyxiation and processed to subsequent postmortem examination [27].

(2) Treatment and dose design

Both carbendazim and benomyl with 99% pure were a gift from Sinon Co., Taichung, Taiwan. All other chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted. Carbendazim, benomyl, or flutamide each was suspended in corn oil and treated to animals orally by gavage in a volume of 2.5 mL/kg body weight, once daily. Groups of five rats from GD 0 to 20, were treated with carbendazim at 6.25, 12.5, and 25 mg/kg; benomyl at 25, 50, and 100 mg/kg; or flutamide at 0.6, 2.5, and 10 mg/kg. Also, rats were co-treated with 25 mg/kg carbendazim and 0.6, 2.5, and 10 mg/kg flutamide or co-treated with 100 mg/kg benomyl and 0.6, 2.5 or 10 mg/kg flutamide. Female rats were checked daily for clinical signs of toxicity. Female body weight and food consumption were measured daily throughout dosing and lactation period. All rat offspring were weaned at PND 21 and fed up to 8-week-old. All rat organ weights were determined on PND 21. Conception rate on GD 21 and 22, proportion of pups born alive on PND 1, proportion of pups surviving to weaning on PND 21, and sex ratio on PND 56 were measured and recorded.

(3) Determination of androgen-dependent reproductive development effects

What they determined for androgen-dependent reproductive end points were signs of clinical toxicity, anogenital distance (AGD), male and female pup weight, retention of areolae and/or nipples, malformations of external genitalia, testicular descent, preputial separation, vaginal opening, and organ weight and malformation on PND 56 [28, 29]. All pups were counted and examined for signs of clinical toxicity on PND 0 and were individually identified by tail-labeling on PND 21. All pups with AGD, and live male and female offspring weights were measured on PND 2, 22, and 42. Day of completion of preputial separation (PPS) and body weight in PPS of male offspring during PND 40 and 50 were also measured. Day of onset of vaginal opening (VO) and body weight in VO of female offspring during PND 30 and 45 were measured. End points of gross morphology of reproductive organs, nipple retention, abnormal testis and epididymis, hypospadias, underdevelopment of prostate or/and seminal vesicle, absent prostate or/and seminal vesicle, bladder stone, and underdevelopment of levator ani bulbocavernosus muscle in male offspring were determined on PND 56.

(4) Necropsy of rats

All pups were weaned on PND 21. Rats were euthanized by CO₂ asphyxiation. All body and organ/tissue weights including liver, kidneys, adrenals, uterus, ovaries, thyroids and number of implantation sites were measured on PND 21.

(5) Necropsy of F1 offspring

Both male and female offspring on PND 56 were euthanized by CO₂ asphyxiation and blood was collected via trunk. After blood collection, the ventral surface of offspring was shaved for counting the number of nipples. External genitalia, including the scrotum, prepuce, and penis of male offspring and vaginal of female offspring were visually inspected. End points of gross internal examination of the reproductive tract such as inspection of the testes, epididymides, prostate, seminal vesicles, levator ani bulbocavernosus muscle, and penis were measured. Also, the liver, kidneys, adrenal glands and thyroids were grossly examined and weighed. Body and organ weights such as testes, epididymides, prostate, seminal vesicles with fluid, levator ani bulbocavernosus muscle, and penis, liver, kidneys, adrenals and thyroids were collected. All examined tissues were fixed in 10% neutral buffered formalin, processed, sectioned, and stained with haematoxylin and eosin.

2.2.2. Abstract

Both carbendazim (methyl 2-benzimidazolecarbamate) and benomyl are reported to exhibit reproductive and developmental toxicity in male rats. This study was mainly to detect the ability of carbendazim exposure *in utero* to alter androgen-dependent development indicators in rat offspring and measure the effects of antiandrogen flutamide on the carbendazim-induced reproductive and developmental alterations. All pregnant female rats were administered with 6.25, 12.5 or 25 mg/kg carbendazim, 25, 50 or 100 mg/kg benomyl, and 0.6, 2.5 or 10 mg/kg flutamide by gavage once daily from gestational day 0 to 20. Also, group of female rats was co-treated with 25 mg/kg carbendazim or 100 mg/kg benomyl and 0.6, 2.5, and 10 mg/kg flutamide. The results showed that the various treatments decreased the survival rates of pups on PND 1 and 21. For male offspring, 12.5 and 25 mg/kg carbendazim increased AGD, an androgen-dependent indicator, on PND 2. Also, benomyl increased AGD of offspring. Co-treatment with 25 mg/kg carbendazim with 0.6, 2.5, and 10 mg/kg flutamide relieved the androgenic effect on AGD induced by carbendazim. The androgenic effects of AGD induced by carbendazim and benomyl on AGD were reversible on PND 22 and later. Carbendazim had no effects on other androgen-dependent indicators such as testis and epididymis malformations, hypospadias, nipple retention, and organ weights of seminal vesicle and levator ani bulbocavernosus muscle on PND 56. Quite surprisingly, carbendazim antagonized the antiandrogenic effects on these indicators induced by flutamide cotreatment. For female offspring, carbendazim exhibited synergistic effects on the flutamide cotreatment-mediated increases of organs weights in liver and kidney on PND 56. No significant effects on female reproductive organs were induced by carbendazim. These findings suggested that carbendazim exposure *in utero* exhibited a transient and weak androgenic effect and reduces flutamide antiandrogenicity in male rats. These two fungicides enhance flutamide-mediated increases of liver and kidney weight in female rats. The antagonistic and synergistic interactions between carbendazim and flutamide *in utero* need to be further investigated.

2.3. Carbendazim-induced androgen receptor expression antagonized by flutamide in male rats

2.3.1. Materials and methods

(1) Animals and related treatments

Male SD rats with three-week-old were obtained from the National Laboratory Animal Center, Taipei, Taiwan. All rats were kept in a specific-pathogen-free animal facility in the Taiwan Agricultural Chemicals and Toxic Substances Research Institute (TACTRI) in Taichung. The animal rooms were sustained at a 12-hour light and dark cycle, $23 \pm 2^\circ\text{C}$ and $50 \pm 10\%$ relative humidity. When the animals were transported to the room, the rats were quarantined for at least 1 week and were available for test only when they exhibited enough body weight gain and no clinical signs of disease or injury. Carbendazim with 99% pure was obtained from Sinon Co. (Taichung, Taiwan). Flutamide (FLU) and the other chemicals were obtained from Sigma (St. Louis, MO, USA), unless otherwise stated. The pesticide was suspended in corn oil and orally administered to five rats in each group once a day by gavage at a volume of 2.5 mL/kg body weight. Male rats (322 ± 15 g) were randomly assigned to each treatment group. In order to carry out the time- and dose-dependent tests, the protocol included two treatment-duration and dosages. The first one was as follows: The doses of carbendazim were 0, 25, 50, 100, 200, 400 and 800 mg/kg/day for 56 days. The doses of flutamide were 0, 6.25, 12.5, 25, 50 and 100 mg/kg/day for 28 days. In mixed doses, the rats were co-treated with 675 mg/kg/day of carbendazim and 0, 6.25, 12.5, 25, 50 and 100 mg/kg/day of flutamide for 28 days. The second one was as follows: The doses of carbendazim were 0, 6.25, 25, 100 and 400 mg/kg/day for 7 days, while the doses of flutamide were 0, 0.78, 3.13, 12.5 and 50 mg/kg/day for 7 days. The rats in the co-treatment group were given either 400 mg/kg of carbendazim and 0, 0.78, 3.13, 12.5 and 50 mg/kg/day of flutamide, or 50 mg/kg/day of flutamide and 0, 6.25, 25, 100 and 400 mg/kg/day of carbendazim for 7 days. All animal care and experimental procedures were approved by the Institution Animal Care and Use of Committee (IACUC) of TCATRI [30].

(2) Immunohistochemical (IHC) evaluation

The testes tissues of three groups of rats were tested: (1) 0, 25, 50, 100, 200, 400 and 800 mg/kg/day of carbendazim for 56 days; (2) 0, 6.25, 12.5, 25, 50 and 100 mg/kg/day of flutamide for 28 days; and (3) co-treatment with 675 mg/kg/day of carbendazim and 0, 6.25, 12.5, 25, 50 and 100 mg/kg/day of flutamide for 28 days. Testes from the following test groups were fixed in 10% neutral buffered formalin for 1 week. The tissues were then dehydrated with increasing concentrations of ethanol, cleared in toluene and embedded in paraffin. Sections were cut into 5-mm slices and deparaffinized, hydrated and treated with 0.3% H_2O_2 in PBS (pH 7.6) for 30 min to block endogenous peroxidase activity, and finally treated with a protein-blocking solution (5% goat serum diluted in phosphate-buffered saline). These steps were followed by heating the sections in a microwave oven for antigen retrieval using a 0.01 M citrate buffer solution (pH 5.5). Tissue sections were immunostained with rabbit anti-AR (N-20, Santa Cruz Biotechnology, Inc., CA, USA), which was diluted 1: 250 in phosphate-buffered saline and 0.25% bovine serum albumin and maintained at room temperature overnight. The tissue sections were then developed with a streptavidin-HRP kit (Chemicon IHC Select[®] CA, USA), using diaminobenzidine as the

chromogen, and were counterstained with haematoxylin. All images were optimized by using an inverted microscope (Leica, Wetzlar GmbH, Germany). To quantify the relative amount of AR protein in the IHC, 200 nucleus stained per field in a slide, 5 fields per slide, 5 slides per dose were counted. The intensity of AR protein stained in nucleus was graded as (0, negative), + (1, mild), ++ (2, moderate), +++ (3, intense), ++++ (4, more intense) or +++++ (5, very intense). The measurements were control group adjusted and the values were statistically analyzed.

(3) Reverse transcription-polymerase chain reaction (PCR)

Testes ($n = 5$) from the following treatment groups were stored at -80°C for 7 days. Total RNA was extracted with an RNeasy[®] Mini Kit (QIAGEN, TAIGEN Bioscience Corporation, Dusseldorf, Germany) according to the protocol provided by the manufacturer. For the reverse transcription (RT) reaction, 3 mL of total RNA was used from the individual rats of each group. The RT-PCR reactions in this study were carried out with SuperScript[™] III One-Step RT-PCR System with Platinum[®] Taq DNA polymerase kits from Invitrogen (Cat. No. 12574–026) in DNA Engine[®] & DNA Engine Tetrad[®] Peltier Thermal Cyclers (PTC-200, MJ Research, Incorporated, Massachusetts 02451 USA). For AR mRNA amplification, the primers were designed to amplify a 570-bp fragment (forward, 5'-TGCTGCCTTGTTATCTAGTCTCA-3'; reverse, 5'-ACCATATGGGACTTGATTAGCAG-3') (annealing temperature, 60°C ; the number of cycles, 24, 26 and 28; product size, 570 bp). PCR was subsequently performed using an optimized protocol of between 24 and 28 cycles. Each cycle consisted of the following: 94°C , 30 s; 60°C , 30 s and 72°C , 45 s. For b-actin mRNA amplification, the primers were designed to a 359-bp fragment (forward, 5'-CTGTGCCCATCTATGAGGGTTAC-3'; reverse, 5'-AATCCACACAGAGTACTTGCGCT-3') (annealing temperature, 60°C ; the number of cycles, 24, 26 and 28; product size, 359 bp). PCR was subsequently performed using an optimized protocol of between 24 and 28 cycles. Each cycle consisted of the following: 94°C , 30 s; 60°C , 30 s and 72°C , 45 s. PCR products were resolved in a 1.2% agarose gel and stained with ethidium bromide, and DNA bands from triplicate reactions were quantified using a FOTO/Analyst[®] Investigator System (Fotodyne Incorporated, Hartland, WI, USA). The PCR products for β -actin served as an internal standard.

(4) Western blot

A Polytron PT3100 homogenizer (Kinematica AG, Littau, Switzerland) was used to examine frozen testicular tissues from the following treatment groups. Tissues of testes from the first protocol were homogenized for a few seconds in an M-PER[®] Mammalian Protein Extraction Reagent (Cat. No. 78505, Pierce). The homogenates were then centrifuged at $105,000 \times g$ for 1 h at 4°C . The supernatants were aliquoted and stored at -86°C before use. Before western blotting, protein contents were measured by BCA protein assay (Cat. No. 23225, Pierce). Equal amounts of protein were loaded onto each polyacrylamide gel. The antibody dilutions were 1: 200 for the anti-AR antibody (N-20, Santa Cruz Co., CA) and 1: 5000 for the horseradish peroxidase conjugated goat anti-rabbit IgG (AP132P, Chemicon International). For each treatment group, five samples were analyzed in two separate blots. Total protein extracts from the testicular tissue were denatured and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 7.5% polyacrylamide. The proteins were transferred to nitrocellulose membranes. The membranes were then blocked for non-specific binding and incubated with polyclonal primary

antibodies for AR (N-20, Santa Cruz Co., CA) and β -actin (AP132P, Chemicon International). After incubation with primary antibody, the membranes were incubated with horseradish peroxidase-linked anti-goat IgG secondary antibody and visualized on film exposed to enhanced chemiluminescence (Visualizer™ Western Blot Detection Kit, Millipore, MA, USA). The relative amount of protein in the resulting immunoblot bands was estimated by measuring the optical densities of the bands on exposed films using a FOTO/Analyst® Investigator System (Fotodyne Incorporated, WI, USA). The measurements were background adjusted and the values were statistically analyzed. Protein for β -actin served as an internal standard.

(5) Hormone analysis

Serum luteinizing hormone (LH) (RPN 2562, Amersham, UK), follicular stimulating hormone (FSH) (RPN 2560, Amersham, UK; AE R004, Biocode, Belgium), 17 β -estradiol (E₂) (Cayman Chemical., Ann Arbor, MI, USA) and testosterone (T) (Cayman Chemical., Ann Arbor, MI, USA) levels were determined using the relevant EIA systems. The serum samples collected from rats treated with 0, 6.25, 25, 100 and 400 mg/kg/day of carbendazim for 7 days and 0, 0.78, 3.13, 12.5 and 50 mg/kg/day of flutamide for 7 days were directly applied to the well in the kit and measurements were taken according to the procedure described by the manufacturer.

(6) Statistical analysis

The values of AR in Western blot and RT-PCR were normalized against b-actin. All results were statistically analyzed with the t-test and $p < 0.05$ was considered statistically significant. The other data were expressed as mean \pm SE. Data were subjected to ANOVA followed by *t-test*. The level of significance was set at $p < 0.05$.

2.3.2. Abstract

Carbendazim was widely used as a fungicide, and it was reported to exhibit reproductive and developmental toxicity. This study aimed to detect the expression of androgen receptor caused by carbendazim and the antagonistic effect of flutamide. Groups of five rats were treated with carbendazim, flutamide or a combination of both to determine androgen receptor mRNA, immune activity and protein expression. Carbendazim increased androgen receptor mRNA with dose dependent, while flutamide, an androgen receptor antagonist, blocked it. When co-treatment with carbendazim and various flutamide doses it decreased the androgen receptor mRNA dose dependent. In contrast, co-treatment with flutamide and various carbendazim doses increased the androgen receptor mRNA with dose dependent. In the immunohistochemistry (IHC) and Western blot (WB) analyses it showed that carbendazim increased androgen receptor activity particular in rat testes with dose dependent, while flutamide decreased it. Moreover, treatment with carbendazim or flutamide for 7 days raised testosterone and follicular stimulating hormone concentrations in the serum of male rats with dose dependent, which might involve the disruption of the androgen receptor. Despite the fact that we need to examine the underlying mechanism of androgen receptor involved in the reproductive toxicity and endocrine-disrupting activity exhibited by carbendazim and its parent, benomyl, we should first discuss how to take advantage of flutamide antagonism on

carbendazim-produced reproductive and endocrine disrupting activity possibly in human. This study concluded that carbendazim exhibited androgen receptor expression in mRNA and protein levels, while flutamide antagonized it. As we know this is the first report on the antagonistic effect of flutamide on the carbendazim-androgenic effect on mRNA and protein levels. This study would give a light way to illuminate the mechanism of carbendazim- and chemical-produced developmental toxicity and endocrine disrupting activity.

2.4. Detecting benomyl and its metabolite carbendazim inducing androgenic activity in rats by using uterotrophic and Hershberger assays

2.4.1. Materials and methods

(1) Chemicals

The following materials were obtained: testosterone propionate (TP, purity $\geq 97\%$), Sigma-Aldrich Co. (Buchs, Switzerland); 17β -estradiol (E_2 , purity $\geq 98\%$), flutamide (Flu, purity $\geq 97\%$), and corn oil (0.9 g/mL), Sigma Chemical Co. (St. Louis, MO, USA); carbendazim (Mbc, purity $\geq 99\%$), and benomyl (Ben, purity $\geq 99\%$), Sino Co. (Taichung, Taiwan, ROC) [31].

(2) Animals, experimental conditions, castration (Cast) and ovariectomy (OVX) procedures

The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Taiwan Agricultural Chemicals and Toxic Substances Research Institute. Four-week-old male and female SD rats were purchased from the National Laboratory Animal Center, Taipei, Taiwan. All rats were accustomed at least to the laboratory environment for 3 weeks before test. During the experiment period, all rats were kept, two to three per cage, in suspended aluminum cages with stainless-steel wire-mesh front and floor under the controlled conditions, containing a temperature of $21 \pm 2^\circ\text{C}$, a relative humidity of 40–70%, a frequency of ventilation of more than 10 air exchanges per hour, and a 12-h light/dark cycle. Drinking water and pellet rodent diet were available *ad libitum*. Male rats at 7 weeks of age underwent Cast procedure. Under ether anesthesia an incision was made in the scrotum and both testes and epididymis were removed with ligation of blood vessels and seminal ducts. Chemical treatment was not initiated until 30 days later to allow for complete recovery from surgical stress. Ovariectomy (OVX) procedure for female rats was operated about 7 weeks of age under ether anesthesia by opening the dorsolateral abdominal wall at the midpoint between the costal inferior border and the iliac crest, a few millimeters from the two lateral margins of the lumbar muscle. On the abdominal cavity, the ovaries were located. On an aseptic field, the ovaries were physically removed from the abdominal cavity. A tie was operated between the ovary and uterus to control bleeding and the ovary was detached by incision above the tie at the junction of the oviduct and each uterine horn. Following the surgery, females were acclimated for 30 days to allow for recovery from operation stress and to monitor the estrus cycle to confirm the success of OVX. Only those animals in the diestrus or metestrus phase were used in the experiments.

(3) Study design and clinical examination

This study was designed according to standardized test guidelines, including OECD test 440 [32], USEPA OPPTS 890.1600 [33], OECD test 441 [34] and USEPA OPPTS 890.1400 [35], with

modification of endpoints. **Table 1** shows the treatment conditions for uterotrophic assay (estrogenic and antiestrogenic/estrogenic) and Hershberger assay (androgenic and anti-androgenic/androgenic) in young adult rats (275 ± 15 g). Each experimental group consisted of six animals. Test and reference substances were suspended or dissolved daily in vehicle (corn oil). Daily dosages of E_2 and TP were 2.5 ml/kg body weight (BW) administered via oral gavage and 0.5 ml/kg BW administered via subcutaneous injection. Oral gavage was selected because it is one of the potential exposure routes of test chemicals in humans. For all experiments, clinical signs, BW and weights of liver and kidneys were assessed as indices of systemic toxicity. Clinical signs including any abnormal behavior were recorded twice a day for each animal.

(4) Assessment of antiestrogenicity/estrogenicity in young adult rats

A 10-day uterotrophic assay using OVX rats was performed to determine if benomyl, carbendazim and flutamide interfere with estrogen receptor-mediated mechanisms. For assessment of antiestrogenicity/estrogenicity, 5 mg/kg/day E_2 was administered daily, as a reference estrogen, by subcutaneous injection on the dorsal surface, as previously described [32, 33] with modified dosage. Benomyl, carbendazim or flutamide was administered to OVX or E_2 -treated OVX rats by oral gavage for 10 days. A previous study has shown that AR antagonist flutamide blocks the androgenic effect induced by carbendazim [27]. To investigate the effects of AR agonists benomyl and carbendazim (50, 100, 200, 400, and 800 mg/kg/day) and antagonist flutamide (6.25, 12.5, 25, 50, and 100 mg/kg/day) on estrogenic activity in rats, these chemicals were administered to OVX rats by oral gavage for 10 days. The dose levels of benomyl, carbendazim or flutamide have been previously described [20]. One day after the final administration, rats were euthanized by blood withdrawal from the abdominal femoral artery under light ether anesthesia and exhaust ventilation to maintain the airborne concentrations of vapors below their respective threshold values. Uterus with fluid, vagina, thymus, thyroid, liver, lung, adrenal glands, kidneys and bladder were examined for gross lesions and then dissected and weighed after careful trimming to remove fat and other contiguous tissues in a uniform manner.

(5) Assessment of antiandrogenicity/androgenicity in young adult rats

A 10-day Hershberger assay using male rats was performed to determine if benomyl, carbendazim and flutamide interfere with AR-mediated mechanisms. For assessment of antiandrogenicity/androgenicity, 5 mg/kg/day testosterone propionate (TP) was administered daily, as a reference androgen, by subcutaneous injection on the dorsal surface as previously described [34–36] with modified dosage. Benomyl, carbendazim or flutamide was administered to Cast or TP-treated Cast rats by oral gavage for 10 days. Dosages of 50 and 100 mg/kg/day benomyl, carbendazim or flutamide [20] were administered as antagonist control for anti-androgenicity in young adult rats, as previously described. One day after the final administration, rats were euthanized by blood withdrawal from the abdominal femoral artery under light ether anesthesia and exhaust ventilation to maintain the airborne concentrations of vapors below their respective threshold values. The reproductive accessory glands/tissues (prostate, seminal vesicles with coagulating glands, levator ani plus bulbocavernosus muscles, and penis), as well as thymus, thyroid, lung, liver, adrenal glands, kidneys, bladder, and scrotum, were examined for gross lesions and dissected. All tissues were carefully trimmed to remove fat and weighed.

Dose (mg/kg/day)	Uterotrophic assay ⁶				Hershberger assay ⁶			
	Estrogenic		Anti-estrogenic/ estrogenic		Androgenic		Anti-androgenic/ androgenic	
Treatment group ¹	1	2	3	4	1	2	3	4
Control (intact)			+ ⁷				+	
Control (OVX) ²			+				- ⁸	
E ₂ (sc) ³	-	-	+	+	-	-	-	-
Control (Cast) ⁴			-				+	
TP (sc) ⁵	-	-	-	-	-	-	+	+
Corn oil (oral)	+	-	+	-	+	-	+	-
Benomyl (Ben)								
50	-	+	-	+	-	+	-	+
100	-	+	-	+	-	+	-	+
200	-	+	-	+	-	-	-	-
400	-	+	-	+	-	-	-	-
800	-	+	-	+	-	-	-	-
Carbendazim (Mbc)								
50	-	+	-	+	-	+	-	+
100	-	+	-	+	-	+	-	+
200	-	+	-	+	-	-	-	-
400	-	+	-	+	-	-	-	-
800	-	+	-	+	-	-	-	-
Flutamide (Flu)								
6.25	-	+	-	+	-	-	-	-
12.5	-	+	-	+	-	-	-	-
25	-	+	-	+	-	-	-	-
50	-	+	-	+	-	+	-	+
100	-	+	-	+	-	+	-	+

¹All treatment groups were treated with 6 male or 6 female rats.

²OVX: ovariectomy.

³E₂ (sc): 17β-Estradiol, 5 mg/kg/day (subcutaneous).

⁴Cast: castrated.

⁵TP (sc): testosterone propionate, 5 mg/kg/day (subcutaneous).

⁶Comparison pairs for uterotrophic and Hershberger assays are as follows, respectively: treatment group 1 vs. treatment group 2; treatment group 3 vs. treatment group 4.

⁷+: with.

⁸-: without.

Table 1. Study design for uterotrophic and Hershberger assays.

(6) Statistical analysis

Data are expressed as mean \pm SD. BW and organ weights were subjected to ANOVA followed by student's t-test. The level of significance was set at $p < 0.05$.

2.4.2. Abstract

The both benomyl and carbendazim are widely used systemic fungicides. It has been shown that benomyl and carbendazim induce endocrine-disrupting activity, resulting in reproductive and developmental toxicity, as well as androgen receptor (AR) gene expression in rats. The aim of this study was to link AR induction by benomyl and carbendazim, observed in our previous reports, with the results of Hershberger and uterotrophic assays. In an uterotrophic assay, neither benomyl nor carbendazim, except at 800 mg/kg/day, affected weight of uterus and vagina when compared to the ovariectomized control rats. Co-treatment with 17 β -estradiol (E₂) and 200 mg/kg/day benomyl or co-treatment with E₂ and 200, 800 mg/kg/day carbendazim significantly increased uterine weight when compared to treatment with E₂ alone in an uterotrophic assay. This uterotrophic activity might be mediated through AR. Treatment with flutamide alone or in combination with E₂ had no effect on uterine weight. In the Hershberger assay, treatment with 50 and 100 mg/kg/day benomyl increased weight of ventral prostate plus seminal vesicles. Carbendazim or flutamide alone exhibited no effect on reproductive accessory gland weight. Co-treatment with testosterone propionate (TP) and 50 or 100 mg/kg/day carbendazim, but not benomyl, significantly increased the weight of ventral prostate plus seminal vesicles. Co-treatment with TP and 50 or 100 mg/kg/day flutamide significantly decreased these reproductive accessory gland weights when compared with TP alone. Based on our previous report, carbendazim increases mRNA and protein expression of AR in testis, epididymis and prostate and antagonizes the reduced tissue weights of seminal vesicle and prostate of male offsprings induced by in utero exposure to flutamide in rats. This infers that benomyl and carbendazim increase the weight of ventral prostate plus seminal vesicles through induction of AR expression. Moreover, according to a previous report, TP, an AR agonist, induces fluid retention in uterus by exhibiting androgenic activity, similar to that of benomyl and carbendazim, in an uterotrophic assay. Based on these results, benomyl and carbendazim exhibit an androgenic effect, leading to increased weight of ventral prostate and seminal vesicles and uterine fluid retention in young adult rats. The exact mechanisms require further investigation.

3. Future work and recommendations

OECD takes much effort to promote adverse outcome pathways (AOP) methodology. Androgen receptor-mediated reproductive and developmental toxicity and endocrine disrupting activity would be a novel AOP. It is an approach to support the use of a mode (and/or mechanism) of action basis for understanding the adverse effects of chemicals and other stressors. AR mediated reproductive and developmental toxicity and endocrine disrupting activity would be a novel future application. Specific molecular signals of AR mediated effects would be the future work.

4. Diagram/schematic figure

In the respect of chemical structure, benomyl and carbendazim shared the same C and D ring structure with the natural ligand, dihydrotestosterone (**Figure 1**). We made a schematic labeling of the benomyl, carbendazim mimicking the main ligand interaction features of the natural ligand, dihydrotestosterone, with the androgen receptor referred to the previous report by Tamura et al. (2003) [37].

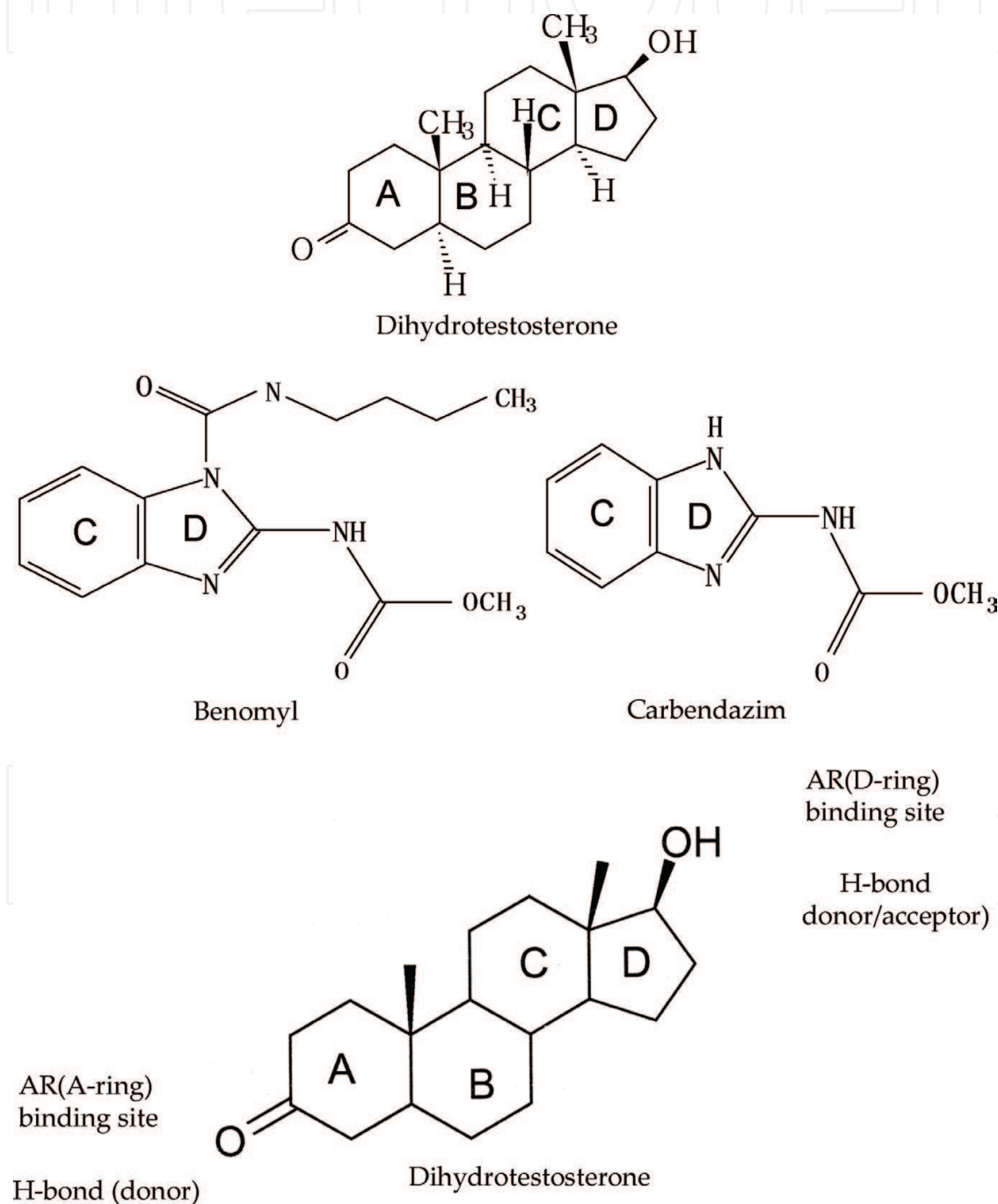


Figure 1. Schematic labeling of the benomyl, carbendazim mimicking the main ligand interaction features of the natural ligand, dihydrotestosterone, with the androgen receptor.

5. Conclusions

Based on the previous study firstly it proved that reproductive toxicity produced by carbendazim is relieved by an androgen receptor antagonist in male rats and developmental toxicity of the pesticide showed androgenic properties in female offspring. We concluded that androgen- and androgen receptor-dependent mechanisms are quite possibly complicated in carbendazim-produced toxicity. Secondly findings show that carbendazim exposure *in utero* displays a transient and weak androgenic effect and reduces flutamide antiandrogenicity in male rats. Thirdly we concluded that antagonistic effect of flutamide was on the carbendazim-androgenic effect on mRNA and protein levels. The results would help us to illustrate the mechanism of carbendazim- and chemical-induced developmental toxicity and endocrine-disrupting activity. Fourthly benomyl and carbendazim exhibit an androgenic effect, leading to increased weight of ventral prostate and seminal vesicles and uterine fluid retention in young adult rats.

Acknowledgements

The author would like to acknowledge the financial assistance of the Bureau of Animal and Plant Health Inspection and Quarantine, Council of Agriculture, Executive Yuan, Taipei, Taiwan, R.O.C. through the project 104AS-10.8.1-BQ-B1. The authors are grateful to Sinon Co., Taichung, Taiwan, for providing the benomyl and carbendazim standard material used in this study.

Notes/Thanks/Other declarations

No

Abbreviations

LD ₅₀	lethal dose with 50% mortality statistically
GD 0	gestation day, day of vaginal plug detected
PND	postnatal day
AGD	anogenital distance
PPS	preputial separation
VO	vaginal opening
AR	androgen receptor

OVX	ovariectomy
TP	testosterone propionate
OECD	Economic Co-operation and Development

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