Benomyl- or Carbendazim-Induced Androgen Receptor Disrupting Might Lead to Spinal and Bulbar Muscular Atrophy in Multi-Generations of Rats

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Abstract

We have reported that benomyl and its metabolite carbendazim induced 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. This manuscript aimed to review and infer the conclusion based on some research, our unpublished data and previous reports. Androgen receptor plays 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(1) androgen- and androgen receptor-dependent mechanisms are possibly involved in carbendazim-induced toxicity; (2) Our unpublished data showed that the antiandrogen flutamide can completely recover the reproductive and developmental toxicity including embryolethality induced by in utero exposure to benomyl and carbendazim in rats. Based on the previous reports of relationship between androgen receptor expression and spinal and bulbar muscular atrophy (SBMA) or Kennedy's disease, our unpublished data supported this hypothesis by showing carbendazim-induced spinal and bulbar muscular atrophy in Wistar offspring rats. The molecular underlying mechanism of reproductive and developmental toxicity, endocrine-disrupting activity and spinal and bulbar muscular atrophy induced by benomyl and carbendazim through androgen receptor need to be further investigated.

Keywords: Benomyl; Carbendazim; Androgen receptor; Kennedy's disease; Spinal and bulbar muscular atrophy

Abbreviations: AR: Androgen Receptor; SBMA: Spinal And Bulbar Muscular Atrophy; DBP: Di-n-Butyl Phthalate; EDTA: Ethylenediaminetetraacetic Acid; AOP: Adverse Outcome Pathways.

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J Pharm Res

Introduction

Previous reports showed that co-localization of androgen receptor and nitric oxide synthase in the ventral premammillary nucleus of the newborn rat: an immunohistochemical study [1]. Vito, et al. reported androgen and estrogen receptors expressed in embryonic and neonatal rat brain [2]. The most important finding was that ontogeny of androgen receptor expression was found in spinal nucleus of the bulbocavernosus motoneurons and their target muscles in male mice [3]. Later, Scaramuzzino, et al. reported that protein arginine methyltransferase 6 enhances polyglutamine-expanded androgen receptor function and toxicity in spinal and bulbar muscular atrophy [4]. Based on the issue some researchers was tried to identify neuron selective androgen receptor inhibitors. To identify neuron-selective androgen receptor (AR) signaling inhibitors, which could be useful in the treatment of spinal and bulbar muscular atrophy (SBMA), or Kennedy's disease, a neuromuscular disorder in which deterioration of motor neurons leads to progressive muscle weakness [5]. Furthermore, researchers showed immunohistochemical analysis of huntingtin-associated protein 1 in adult rat spinal cord and its regional relationship with androgen receptor [6].

As reported carbendazim (methyl-2-benzimidazole carbamate) is used to be a systemic fungicide [7]. Both carbendazim and its parent benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate] are exhibiting low acute toxicity [8]. In contrast, carbendazim and benzimidazole chemicals induced severe reproductive and developmental toxicity in rodents [9-12]. Reports showed that carbendazim and benomyl exhibited testicular toxicity such as sloughing of immature spermatids [13,14], inhibition of microtubule assembly [15], seminiferous tubular atrophy [16], and testicular atrophy and infertility [17] 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 [9,18]. 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 [19]. 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 these two fungicides remains unclear. The earlier report on endocrine activity for carbendazim might be Rehnberg, et al. [20]. 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 [20]. Recently Rama, et al. reviewed and reported that carbendazim induced reproductive toxicity and possible hormonal effects in rats [21].

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 [22] while di-n-butyl phthalate (DBP) [23,24] and sodium valproate [25] 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, endocrine-disrupting activity and spinal and bulbar muscular atrophy (SBMA), or Kennedy's disease in rats.

Materials and Methods

Study I

Animals and related preparation

The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the Taiwan Agricultural Chemicals and Toxic Substances Research Institute. 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 ± 2°C, and 50 ± 10% relative humidity. When they were transported to animal room, the rats were quarantined for at least 1

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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 [26].

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 [27]. Both testis and epididymis histology were evaluated and histopathological findings were scored according to Oakberg [28] and Hess [29].

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. [30]. The ligand [1, 2, 3, 5, 6, 7-3H(N)]-5α-androstan-17β-ol-3-one (dihydrotestosterone, 5α-DHT) (110–150 Ci/mmol) was obtained from NEN Life Science Products, Inc., Boston. Nonlabelled 5α-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. [31]. Tissue homogenates were centrifuged at 30,000 × 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]-5α-DHT to androgen receptor of testis and epididymis extract each was determined by competitive inhibition binding using nonlabelled 5α-DHT. Charcoal-treated testicular and epididymal extract each was incubated with 1 nM [3H]-5α-DHT at 4°C for 24 h. The nonspecific binding was carried out with incubating the extract with 100-fold excess nonlabelled 5α-DHT. Unbinding [3H]-5α-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 × 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]-5α-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. [32]. Analysis of effect of carbendazim on androgen receptor binding, specific binding of [3H]-5α-DHT to testis extract was carried out with incubation of the charcoal-treated testis extract with [3H]-5α-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.

Statistical analysis

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

Results

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

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and benomyl was that premating 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, premating 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.

Study II

Animals and related preparation

The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the Taiwan Agricultural Chemicals and Toxic Substances Research Institute. Both male and female Wistar rats, 5-week old, were obtained from the BioLASCO (Taipei, Taiwan, ROC). 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 ± 2°C, and 50 ± 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 (doses: 0, 6.25, 12.5, 25 and 50 mg/kg/day) and benomyl (doses: 0, 6.25, 12.5, 25, and 50 mg/kg/day) with 99% pure were a gift from Sinon Co., Taichung, Taiwan. These two pesticides were suspended in corn oil and treated to animals orally by gavage in a volume of 2.5 ml/kg body weight, once daily. At 7-week old female rats were transferred into breeding cages in the evening and cohabited overnight with males at 1:1 ratio within treatment. The females were examined the

next morning for vaginal plugs and sperm in vaginal smears. Successful copulation was confirmed by the presence of vaginal plugs and /or sperm in vaginal smears. Gestation (GD) 0 was defined as the day that plugs or sperm was observed in the vaginal of the female following mating. The pregnant female rats were administered with 0, 6.25, 12.5, 25 and 50 mg/kg/day carbendazim or 0, 6.25, 12.5, 25, and 50 mg/kg/day benomyl for once a day from gestation days 0 through 20. Pregnant dams 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 8-week old. At 8-week old the F1 male and female rats were mated within treatment and different parents. During the pregnant period the F1 female were not treated as parents. F2 and F3 generations were produced as F1 without treatment. The study was continued to produce offspring until F3 generation is weaned at 21 days postnatal and fed up to 8-week old.

Results

In the first mating, male and female rat treatment with 6.25 mg/kg/day carbendazim within this dose induced 3/8 male and 1/5 female F3 offsprings with spinal and bulbar muscular atrophy (SBMA), or Kennedy's disease. In the second mating, female rats treatment with 6.25 mg/kg/day and male rat treatment with 50 mg/kg/day induced 2/11 male and 2/4 female F3 offsprings with spinal and bulbar muscular atrophy.

Future Work and Recommendations

As we know OECD takes much effort to promote adverse outcome pathways (AOP) methodology. Androgen receptor-mediated reproductive and developmental toxicity, endocrine disrupting activity and spinal and bulbar muscular atrophy (SBMA) 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.

Diagram/Schematic Figure

In the principle of AOP, functional group in the chemical structure plays an important role in inducing adverse outcome in mammals. 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. [33].
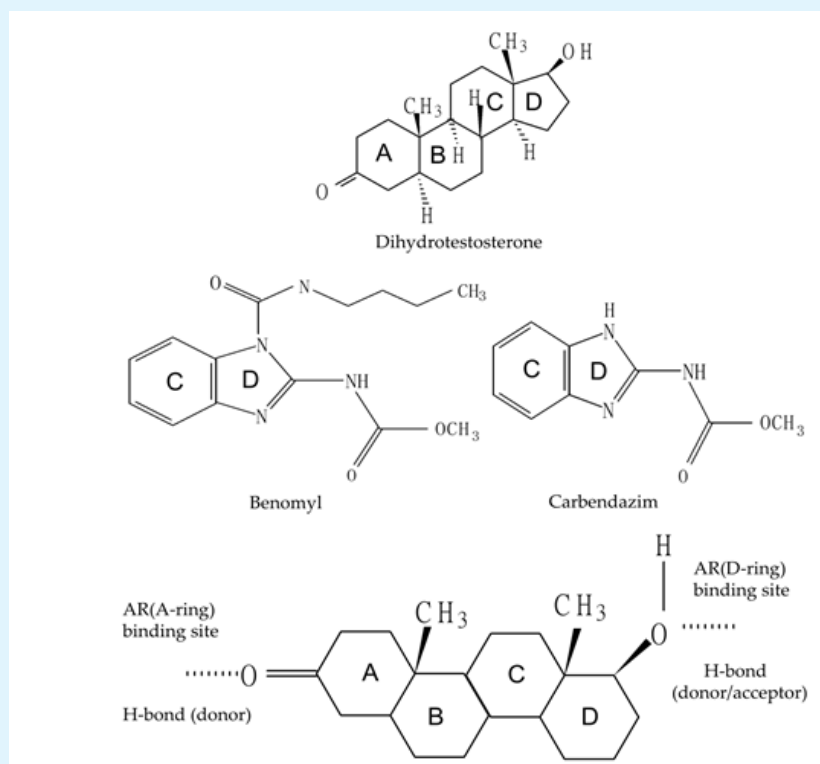
Figure 1: Schematic labeling of the benomyl, carbendazim mimic the main ligand interaction features of the natural ligand, dihydrotestosterone, with the androgen receptor.

Conclusion

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. Based on the previous reports of relationship between androgen receptor expression and spinal and bulbar muscular atrophy (SBMA) or Kennedy's disease, our unpublished data supported this hypothesis by showing carbendazim-induced spinal and bulbar muscular atrophy in Wistar offspring rats.

Acknowledgements

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