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Functional genomics may allow accurate categorization of the benzimidazole fungicide benomyl: lack of ability to act via steroid-receptor-mediated mechanisms

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Abstract

Although benomyl and its metabolite carbendazim have been shown to adversely affect male reproduction, the mechanisms of action do not appear to involve the endocrine system. However, few studies have been conducted using currently proposed tests specifically focused on endocrine disruption. Here, potential estrogen- and androgen-mediated activity of benomyl was therefore investigated in vitro and in vivo. Benomyl and carbendazim proved negative for agonistic and antagonistic activity in reporter gene assays for the human estrogen receptor α and androgen receptor. In uterotrophic and Hershberger assays using Crj:CD(SD)IGS rats, benomyl (100, 300 or 1000 mg/kg/day, p.o., $N = 6$) did not exert agonistic effects. However, the highest dose decreased uterine weights in the uterotrophic assay, and decreased weights of some androgen-related tissues of castrated rats receiving a testosterone propionate (TP, 0.2 mg/kg) injection in the Hershberger assay; the effects were less severe than those with *p*,*p*′-DDE (100 mg/kg/day). When 4 mg/kg/day of TP was injected, decrease of organ weights due to benomyl was attenuated but still observed. Thus, its influence in some tissues was more potent than that of *p*,*p*′-DDE. Benomyl had no apparent effects on serum androgen levels. Microarray analysis of the gene expression profile in the ventral prostate of TP-injected castrated rats treated with benomyl indicated clear differences from the patterns observed with *p*,*p*′-DDE and flutamide. Taken together, these findings suggest the decreased organ weights observed in vivo to be caused by mechanisms that are not steroid-receptor-mediated, such as interfering with assembly of microtubules by benomyl. The study furthermore suggests that functional genomics may provide a reliable evidence for accurate categorization of test chemicals.

Keywords: Benomyl; Endocrine disruptor; Hershberger assay; Uterotrophic assay; Luciferase assay; DNA microarray

Introduction

Recent concern that chemicals in the environment are disrupting normal human endocrine function, leading to reproductive and developmental disorders, such as a reduction in male fertility or an increase in female breast cancer (National Research Council, 1999), has led to development of screening and testing programs focused on endocrine-activity. Early efforts to identify and characterize environmental endocrine-active chemicals were concentrated on xenoestrogens, which are chemicals capable of interfering with estrogen receptor function, but recent publications describing environmental chemicals with antiandrogenic activity have stimulated expansion of research efforts to include screening for chemicals capable of interfering with androgen receptor function (Gray et al., 2002).

Benomyl is a benzimidazole fungicide commonly used on a variety of food crops and ornamental plants whose fungicidal activity is based on the ability to interfere with the assembly of fungalmicrotubules (Davidse, 1986). Administration of benomyl or its metabolite, carbendazim,

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to rats is known to cause adverse effects on the reproductive system, including decreased epididymal and testicular weights, and reduced epididymal sperm counts and fertility (Barnes et al., 1983; Carter and Laskey, 1982; Correa et al., 2002; Hess et al., 1991; Linder et al., 1988; Nakai et al., 1992). Based on these findings, benomyl has been listed as a possible endocrine disruptor (Colborn et al., 1993). However, the California Environmental Protection Agency (California EPA, 1999) concluded that, "although benomyl and carbendazim have been shown to adversely affect male reproduction, the mechanism of action does not appear to involve the endocrine system. Consequently, neither benomyl nor carbendazim would be subject to the provisions of Food Quality Protection Act (FQPA) dealing with endocrine disrupters".

Recently, new protocols for screening for endocrine active chemicals have been proposed, which are currently being internationally validated for specificity, sensitivity and reproducibility. Benomyl has not been tested previously for its ability to directly interact with steroid hormone receptors using these screening methods. Therefore, to further assess the conclusion of the California EPA, we here investigated the interaction of benomyl with the estrogen and androgen receptors (ER and AR), using ERα and AR reporter gene assays for in vitro, and the uterotrophic and Hershberger assays in vivo. Recently developed genomics technology has the potential to improve our understanding of an organism's response to stressors (U.S. EPA, 2004). The gene expression profile for anti-androgenic activity was therefore determined by DNA microarray analysis in studies involving the Hershberger assay.

Methods

Test and reference chemicals. Chemicals and vehicles were obtained from the following manufacturers: testosterone propionate (TP, purity: ≥97%), 17β-estradiol (E2, purity > 97.0%) and methyltestosterone (MT, purity: ≥97%), carbendazim (purity: 98.9%), Wako Pure Chemical Industries (Osaka, Japan); 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene (*p*,*p*′-DDE, purity: 99%), Aldrich Chemical Company (Milwaukee, WI); ethynyl estradiol (EE, purity: 95%) and flutamide (purity: 99%), Sigma Chemical Company (St. Louis, MO); benomyl (purity: 98.0–99.44%), DuPont (Osaka, Japan); 5α-androstan-17β-ol-3-one (DHT, purity > 99.0%), Fulka (St. Luis, MO); 4-hydroxytamoxifen (HTM, purity > 98.0%), synthesized by Sumitomo Chemical Co. (Osaka, Japan).

Reporter gene assay. HeLa cells purchased from American Type Culture Collection (ATCC, Rockville, MD) were maintained in Eagle's MEM (Nissui Pharmaceutical Co., Tokyo, Japan) without phenol red and supplemented with 10% twice-dextran/charcoal-treated fetal calf serum (FCS, Hyclone, Logan, UT) plus 6 mL of 3% L-glutamine (Wako Pure Chemical Industries) and 9 mL of 10% sodium bicarbonate (Wako Pure Chemical Industries) at 37 °C in an atmosphere of 5% CO_2/95% air at saturated humidity.

The expression vectors, pRc/RSV-hERα and pRc/RSV-hAR, were generated by insertion of reverse transcription-polymerase chain reaction (RT-PCR) amplified full-length cDNAs of hERα and hAR with an efficient Kozak's translation initiator sequence (Kozak, 1984) into the blunting site of a pRc/RSV vector (Invitrogen, San Diego, CA). To construct the reporter plasmid for hER α, pGL3-TATA-EREx5, five copies of estrogen responsive elements (EREs) from the *Xenopus* vitellogenin gene were inserted into a pGL3 basic vector (Promega, Madison, WI) with a mouse metallothionein minimum TATA promoter. To construct the reporter plasmid for hAR, MMTV promoter sequences were removed from the pMSG vector (Amersham Pharmacia Biotech, Tokyo, Japan), converted into blunt ends and inserted into the blunting site of the pGL3 vector. The resultant plasmid was named pGL3-MMTV. To construct the control reporter plasmid, the herpes simplex virus thymidine kinase promoter sequences were removed from the pTK β vector (Clontech Laboratories, Palo Alto, CA), converted into blunt ends and inserted into the blunting site of the pGL3 vector. The resulting plasmid was named pGL3-tk.

For transfection experiments, 6 h before transfection, HeLa cells were plated at a density of 2×10^6 cells/dish and incubated. They were transfected using lipofectamine (GIBCO-BRL, Rockville, MD) in serum-free medium following the manufacturer's protocol. Cells were transfected with 3.5 μg of the expression vector and 3.5 μg of reporter plasmid per dish, incubated with liposome–DNA complexes at 37 °C for 16 h and then further incubated for 3 h after medium change. For the control assays, 7.0 μg of pGL3-TK was used for transfections. The cells were further treated with 0.25% trypsin–0.02% EDTA 3Na in phosphate buffered saline (−) (PBS(−)) and plated at a density of 2×10^4 cells/100 μL/well in 96-well multi-well plates using fresh medium. Dimethyl sulfoxide (DMSO, Kanto Chemical Co., Tokyo, Japan) solutions of chemicals (0.1% final DMSO concentration) were added to the cells and the total volume adjusted to 100 μL per well. For antagonist assays, 100 pM of E2 or DHT were incubated with the chemicals. Cells were cultured for an additional 40-h period, washed twice with PBS (−) (Nissui Pharmaceutical) after medium was removed and prepared for evaluation by treatment with PicaGene cell lysis buffer (Toyo Ink, Tokyo, Japan). After 30 min at room temperature, luciferase activity was assayed using a PicaGene kit (Toyo Ink) following the manufacturer's protocol. Luminescence of 15-μL samples and 50-μL PicaGene assay solution was measured with a Lumat LB 9501 luminometer from Berthold (Wildbad, Germany), over 1 s for each sample (N = 6). Luciferase values obtained for each well were adjusted into the relative values (the relative value = each luciferase value/the average of luciferase values of control ×