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Influence of the carbamate fungicide benomyl on the gene expression and activity of aromatase in the human breast carcinoma cell line MCF-7

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ABSTRACT

The carbamate fungicide benomyl reportedly inhibited the growth of the human breast cancer cell line MCF-7 by inducing apoptosis. However, influence of benomyl on the expression and activity of aromatase of MCF-7 cells remains to be examined, since benomyl was identified as an endocrine disruptor. We here confirmed through cell cycle analysis and immunofluorescence staining that benomyl damaged microtubules and caused apoptosis. We also found that benomyl inhibited histone deacetylase (HDAC) 1 and accumulated acetylated histone H3 in MCF-7 cells. Additionally, benomyl enhanced the levels of aromatase protein and mRNA, albeit at high concentrations. It is thus likely that benomyl enhanced the promoter activity of the aromatase gene *via* acetylation of histone H3 as does the HDAC inhibitor Vorinostat. In conclusion, benomyl remains to be a risk factor as an endocrine disruptor for breast cancer.

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1. Introduction

The carbamate fungicide benomyl is used for sterilizing seeds of vegetables as an agricultural chemical (Sassman et al., 2004; Yamada et al., 2005). Benomyl is metabolized to carbendazim by microorganisms in the soil (Yarden et al., 1985). Since carbendazim remains in the soil for a long time without decomposition, there has been concern that this compound might influence human health and cause diseases, such as congenital microphthalmia (Busby et al., 1998; Källén et al., 1996).

Recently, benomyl and carbendazim were reported as endocrine disruptors that increased both expression and activity of aromatase in the human ovarian granulose-like tumor cell line KGN (Morinaga et al., 2004; Ohno et al., 2004). Although aromatase was activated by the production of cAMP by forskolin, benomyl did not stimulate the production of cAMP and instead disrupted tubulin polymerization in KGN cells. Since the microtubule-interfering agent taxol

Abbreviations: HDAC, histone deacetylase; cAMP, cyclic adenosine monophosphate; RT-PCR, reverse transcription polymerase chain reaction; FBS, fetal bovine serum; PBS, phosphate-buffered saline; ER, estrogen receptor.

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increased an aromatase activity, it was deduced in KGN cells that disruption of the dynamic equilibrium between tubulin and microtubules may lead to increased aromatase activity (Morinaga et al., 2004). It was also reported that benomyl inhibited proliferation of the human breast cancer cell line MCF-7 *via* two different pathways (Rathinasamy and Panda, 2008). Exposure of a low concentration of benomyl to the cells causes acetylation of tubulin, which induces nuclear transfer of p53, leading to cell death by apoptosis, whereas incubation of a high concentration of benomyl disrupts tubulin polymerization. Carbendazim caused abnormal formation of nucleospindle at a low concentration, and induced multinucleated interphase cells at a high concentration in MCF-7 cells (Yenjerla et al., 2009). However, the influence of benomyl on the expression and activity of aromatase in MCF-7 has not yet been investigated.

In the present study, we confirmed the apoptosis and microtubule disrupting activity of benomyl and carbendazim on MCF-7 cells (Rathinasamy and Panda, 2008) by analyzing cell cycle and immunofluorescence staining. In addition, the influence of benomyl on the expression and activity of aromatase in MCF-7 cells were assessed by utilizing Western blot assay and reverse transcription polymerase chain reaction (RT-PCR) as well as indirect measurement of aromatase activity. Being compatible with the previous report (Rathinasamy and Panda, 2008), the cells of the G_2/M and sub-G_0/G_1 phases were accumulated in the cell cycle analysis, and abnormally assembled cytoskeletal microtubules were observed in the immunofluorescence staining. Furthermore, benomyl inhibited histone deacetylase (HDAC) 1 activity, led to the accumulation of acetylated histone H3 and increased the level of aromatase in MCF-7 cells, though at high concentrations. Additionally, since benomyl enhanced the level of mRNA in RT-PCR, it was assumed that benomyl stimulated the transcription of the aromatase gene *via* acetylation of histone H3. The indirect measurement of aromatase activity in MCF-7 cells indicated that the activity was not increased by exposure of benomyl at low concentrations.

2. Materials and methods

2.1. General

MCF-7 cells were purchased from American Type Culture Collection. RPMI 1640 medium was purchased from Sigma–Aldrich (St. Louis, MO, USA). The WST-8 cell counting kit was obtained from Dojindo Laboratories (Kumamoto, Japan). Benomyl and carbendazim were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Forskolin was supplied by Calbiochem (Fontenay sous Bois, France). Vincristine sulfate was obtained from Wako Chemicals (Osaka, Japan). Vorinostat (SAHA) was synthesized according to the reported method (Richon et al., 1996). Propidium iodide was obtained from Dojindo Laboratories (Kumamoto, Japan). Antibodies against acetyl α-tubulin, histone H3, acetyl-histone H3 (total) and aromatase were purchased from Sigma–Aldrich (St. Louis, MO, USA). ECL-plus reagent was obtained from GE Healthcare UK Ltd. (Buckingham, UK). The Cycletest Plus DNA reagent kit was purchased from Becton Dickinson and Company (NJ, USA). Results of flow cytometry were recorded with FACS™Cant II (Becton Dickinson and Company, USA). ISOGEN2 was obtained from Wako Chemicals (Osaka, Japan). ReverTra Ace® qPCR RT Kit was purchased from Toyobo (Osaka, Japan). *Taq* DNA porimerase with standard *Taq* buffer was obtained from New England Biolabs, Inc. (Ipswich, USA). dNTP mixture was purchased from Takara Bio Inc. (Shiga, Japan). SYBR® gold nucleic acid gel stain was obtained from Invitrogen™, Life Technologies Corporation (California, USA).

2.2. Cell culture

MCF-7 cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (20 U/mL) and streptomycin (20 μg/mL) (Gibco Life Technology, Gaithersburg, MD, USA) in a 5% CO_2 and 95% air atmosphere at 37 °C.

2.3. WST-8 assay of benomyl or carbendazim on MCF-7 cells

Cells were plated in 96-well plates at a density of 5000 cells/well. Twenty four hours later, benomyl and carbendazim were added at final concentrations of 1, 10, 25, 50 and 100 μM, and cells were cultured for 72 h. After the addition of WST-8 reagent, the cells were incubated for another 4 h. Absorbance was measured at 450 nm and 630 nm. The 50% growth inhibitory concentration (IC_{50}) is expressed as the concentration inhibiting 50% of the growth of drug-treated cells relative to the control (medium only).

2.4. DNA histograms analyzed by flow cytometry

MCF-7 cells were plated onto 60 mm-diameter dishes (1.0×10^6/dish). After incubation for 24 h, the medium was removed, and the cells were washed with medium (2× 1 mL) and incubated with benomyl, carbendazim (each 5 or 40 μM), vincristine (150 nM) or the control (0.4% DMSO) for another 24 h. The medium (about 5 mL) was then transferred to centrifuge tubes. The adhered cells, after being washed twice with cold phosphate-buffered saline (PBS) (each 1 mL), were treated with trypsin 0.25% (1×) (Invitrogen) (200 μL) for 3 min and transferred to the above tubes. The cells in the tubes were treated with the Cycle Test Plus DNA reagent kit according to the procedure recommended by Becton Dickinson and Company. DNA content was measured with a FACS™Cant II.

2.5. Immunofluorescence staining

Cells were seeded in a chamber slide at 1.0×10^5 cells/dish. After 24 h, they were treated with benomyl (5 or 40 μM), 0.5% DMSO or vorinostat (1 μM), and incubated for 24 h. After treatment, the medium was removed and the cells were fixed with 3% paraformaldehyde solution with PBS (200 μL) for 30 min at room temperature. Cells were washed twice with PBS and permeabilized with 0.05% TritonX-100 in PBS for 5 min. Then, the cells were washed twice with PBS and incubated with 3% skim milk in PBS for 1 h at room temperature. Acetyl α-tubulin was detected with anti-acetyl α-tubulin primary antibody diluted (1:100) with 3% skim milk in PBS overnight at 4 °C. Cells were washed twice with PBS and treated with FITC-conjugated

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