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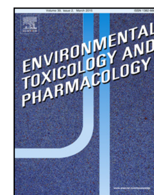

In vitro metabolism of methiocarb and carbaryl in rats, and its effect on their estrogenic and antiandrogenic activities

Satoko Tange[a], Nariaki Fujimoto[b], Naoto Uramaru[c], Fung Fuh Wong[d], Kazumi Sugihara[e], Shigeru Ohta[a], Shigeyuki Kitamura[c,*]

[a] Graduate School of Biomedical and Health Sciences, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8553, Japan
[b] Research Institute for Radiation Biology and Medicine, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8553, Japan
[c] Nihon Pharmaceutical University, Komuro 10281, Ina-machi, Kitaadachi-gun, Saitama 362-0806, Japan
[d] School of Pharmacy, China Medical University, No. 91, Hsueh-Shih Rd., Taichung, Taiwan
[e] Faculty of Pharmaceutical Science, Hiroshima International University, Hirokoshingai 5-1-1, Kure, Hiroshima Prefecture, 737-0112, Japan

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ABSTRACT

In this work, we examined the metabolism of the carbamate insecticides methiocarb and carbaryl by rat liver microsomes and plasma, and its effect on their endocrine-disrupting activities. Methiocarb and carbaryl were not enzymatically hydrolyzed by rat liver microsomes, but were hydrolyzed by rat plasma, mainly to methylthio-3,5-xylenol (MX) and 1-naphthol, respectively. When methiocarb was incubated with rat liver microsomes in the presence of NADPH, methiocarb sulfoxide was formed. The hydrolysis product, MX, was also oxidized to the sulfoxide, 3,5-dimethyl-4-(methylsulfinyl)phenol (SP), by rat liver microsomes in the presence of NADPH. These oxidase activities were catalyzed by cytochrome P450 and flavin-containing monooxygenase. Methiocarb and carbaryl both exhibited estrogen receptor α (ERα) and ERβ agonistic activity. MX and 1-naphthol showed similar activities, but methiocarb sulfoxide and SP showed markedly decreased activities. On the other hand, methiocarb and carbaryl exhibited potent antiandrogenic activity in the concentration range of 1×10^{-6}–3×10^{-5} M. Their hydrolysis products, MX, and 1-naphthol also showed high activity, equivalent to that of flutamide. However, methiocarb sulfoxide and SP showed relatively low activity. Thus, hydrolysis of methiocarb and carbaryl and oxidation of methiocarb to the sulfoxide markedly modified the estrogenic and antiandrogenic activities of methiocarb and carbaryl.

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1. Introduction

The carbamate insecticides methiocarb [3,5-dimethyl-4-(methylthio)phenol methylcarbamate] and carbaryl (1-naphthalenol methylcarbamate) are widely used throughout the world as broad-spectrum insecticides to protect a variety of crops as well as agricultural animals and pets. These insecticides act as acetylcholinesterase inhibitors, like organophosphate pesticides, but they vary with regard to water solubility, and are relatively nonpersistent in the environment (Plese et al., 2005). Methiocarb and carbaryl are considered to be safe because they are easily metabolized and degraded. However, carbaryl has been reported to accumulate in fish and invertebrates. Methiocarb and carbaryl may also be toxic to wildlife (Hill, 2003). For example, cardiac effects of carbaryl in zebrafish embryos were also reported (Lin et al., 2007). In catfish, carbaryl exposure suppressed serum thyroxin level (Sinha et al., 1991). On the other hand, some carbamate insecticides also influence steroidogenesis in animals and humans. Carbaryl also inhibited progesterone biosynthesis of primary human granulose–lutein cells (Cheng et al., 2006). In mice, there was a significant decrease in the number of estrous cycles and in the duration of the phases of each cycle, and a significant decrease in the number of healthy follicles (Baligar and Kaliwal, 2002). The carbamate insecticide carbofuran disrupts serum thyroid hormone levels in rodents (Goad et al., 2004). In SD rats fed aldicarb at 10 ppb for 6–16 weeks, serum thyroxin levels increased significantly (Porter et al., 1999). Carbofuran also exhibit neurotoxicity via inactivation of acetylcholinesterase and oxidative stress (Gupta et al., 2007). A neurotoxic effect of aldicarb on postnatal rats was demonstrated, and the potential effect in

* Corresponding author.
E-mail address: kitamura@nichiyaku.ac.jp (S. Kitamura).

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human infants was discussed (Vidair, 2004). There is potential human exposure to carbamate insecticides. Infants and children are potentially exposed to carbamate and organophosphates, because these insecticides persistently accumulate on residential surfaces and toys accessible to children after household application. Exposure may also occur via dust particles that have adsorbed these pesticides. Sperm shape abnormalities in carbaryl-exposed agricultural workers were also reported (Wyrobek et al., 1981; Xia et al., 2005; Corsini et al., 2005). The effect of pesticides, including carbamates, on human chronic disease has been reviewed (Mostafalou and Abdollahi, 2013).

As for endocrine-disrupting activity of methiocarb and carbaryl, there are conflicting reports (negative reports: Blair et al., 2000; Nishihara et al., 2000; Sonnenschein and Soto, 1998, and positive reports: Klotz et al., 1997; Andersen et al., 2002; Kojima et al., 2004). Specifically, carbaryl was reported to lack binding affinity for estrogen receptor from rat's uterus (Blair et al., 2000), and it did not show estrogenic activity in yeast two-hybrid assay and reporter gene assay (Nishihara et al., 2000; Sonnenschein and Soto, 1998). In contrast, Klotz et al. (1997) reported that carbaryl weakly activated estrogen- or progesterone-responsive reporter genes in breast MCF-7 and endometrial (Ishikawa) cancer cells at the concentration of 1×10^{-7} M, but at 1×10^{-9} M, these carbamate insecticides decreased estrogen- or progesterone-induced activity to approximately one-third. Furthermore, Andersen et al. (2002) reported that methiocarb exhibited estrogenic and antiandrogenic activities in cell proliferation assay and transactivation assay using MCF-7 human breast cancer cells. Methiocarb was estrogenic in the range of 1×10^{-5}–5×10^{-5} M in both assay systems. However, methiocarb acted as a very weak antiandrogen in transactivation assay using Chinese hamster ovary (CHO K1) cells. Kojima et al. (2004) examined the estrogenicity of 22 carbamate insecticides, and found that methiocarb activated estrogen receptor α (ERα) and ERβ. Interestingly, the activity toward ERβ was higher than that toward ERα. They also reported that methiocarb exhibited antiandrogenic activity toward CHO cells transfected with human androgen receptor (hAR) at 2.8×10^{-6} (IC_{20}). Birkhoj et al. (2004) also reported antiandrogenic activity of methiocarb (IC_{25}, 5.8×10^{-6} M), but found that it was negative in the *in vivo* Hershberger test using castrated rats. In contrast, Han et al. (2009) reported increased expression of testicular ERα in mice exposed to low doses of methiocarb. On the other hand, Hofmeister and Bonefeld-Jorgensen, 2004 reported that methiocarb had no effect on ERα mRNA level in MCF7-BUS cells, whereas ERβ mRNA level was increased.

Metabolic modification of the activities of endocrine disrupters is an important factor influencing the toxicity of these compounds. Some endocrine disrupters such as vinclozolin, polychlorinated biphenyls, methoxychlor, benzo[*a*]pyrene, and benzophenone-3 are proestrogens that require metabolic activation by liver microsomal mixed-function oxidase in animals (Kitamura et al., 2008; Watanabe et al., 2015). However, the influence of metabolic transformation of carbamate insecticides on their endocrine-disrupting activity has not been extensively studied. Here, we examined the estrogenic and antiandrogenic activities of methiocarb and carbaryl using reporter gene assays in MCF-7, CHO, and DT3 cells, and further examined the activities of their metabolites to establish whether metabolism of methiocarb and carbaryl influences these activities.

Thus, in this study, we examined the *in vitro* hydrolytic metabolism of methiocarb and carbaryl, the oxidative metabolism of methiocarb in rats, and the influence of metabolic modification on the estrogenic and antiandrogenic activities of methiocarb and carbaryl. We show that some metabolites of methiocarb and carbaryl exhibit altered (enhanced or decreased) estrogenic and antiandrogenic activities, compared with the parent compounds.

2. Materials and methods

2.1. Materials

Methiocarb, carbaryl, 1-naphthol, methiocarb sulfoxide, 4-methylthio-3,5-xyrenol (MX), 3,5-dimethyl-4-(methylsulfinyl) phenol (SP), estradiol (E2), dihydrotestosterone (DHT), flutamide, eserine, *p*-chloromercuribenzoic acid (PCMB), and (2-chloro-3′,4′-dimethoxybenzyl (CDMB) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Bis(4-nitrophenyl)phosphate (BNPP) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Male Sprague Dawley rats (Slc:SD, 180–210 g, Slc:Japan, Shizuoka, Japan) were used.

2.2. Tissue preparations

Rat liver was removed and homogenized in 4 volumes of 1.15% KCl. The homogenates were centrifuged for 20 min at 9000 *g*. The supernatant fractions were further separated into cytosol and microsomes by centrifugation for 60 min at 105,000 *g*. These microsomes were washed by resuspension in 2 volumes of the KCl solution and resedimentation for 60 min at 105,000 *g*. Rat plasma was obtained by centrifugation of blood.

2.3. Cell culture

MCF-7, CHO, and DT3 (a rat prostate cell line, Fujimoto et al., 2005) cell lines were maintained in minimum essential medium (MEM) (Sigma Chemical Co.) containing penicillin and streptomycin with 5% fetal bovine serum (Life Technologies, Rockville, MD).

2.4. Assay methods for methiocarb and MX oxidase activities

The incubation mixture consisted of 0.1 μmol of methiocarb or MX, 0.5 μmol of NADPH and liver microsomes in a final volume of 1 ml of 0.1 M K,Na-phosphate buffer (pH 7.4). The incubation was continued for 10 min at 37°C. After incubation, 10 nmol of benzophenone was added as an internal standard, and the mixture was extracted with 5 ml of ethyl acetate. The extract was evaporated to dryness and the residue was subjected to analysis by high-performance liquid chromatography (HPLC).

2.5. Assay method for hydrolase activity

The incubation mixture consisted of 100 nmol of methiocarb, methiocarb sulfoxide or carbaryl, and rat plasma or serum albumin in a final volume of 1 ml of 0.1 M K,Na-phosphate buffer (pH 7.4). The incubation was continued for 20 min at 37°C. After incubation of the mixture, 10 nmol of benzophenone was added as an internal standard, and the whole was extracted with 5 ml of ethyl acetate. The extract was evaporated to dryness and the residue was subjected to HPLC analysis.

2.6. HPLC

HPLC was performed in a Hitachi L-7110 high-performance liquid chromatograph (Tokyo, Japan) equipped with an ultraviolet absorption detector. The instrument was fitted with a 4.6×150 mm Inertsil ODS-3 column (GL Science, Tokyo, Japan). The mobile phase was acetonitrile–water (1:1, v/v). The chromatograph was operated at a flow rate of 0.4 ml/min and at a wavelength of 230 nm. Elution times of SP, methicarb sulfoxide, benzophenone (an internal standard), MX, methiocarb, carbaryl, and 1-naphthol were 4.9,

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