Androgen Receptor Antagonism by the Organophosphate Insecticide Fenitrothion

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Organophosphate insecticides represent one of the most widely used classes of pesticides with high potential for human exposure in both rural and residential environments. We investigated the interaction of the organophosphothioate pesticide fenitrothion (O,O-dimethyl O-(4-nitro-m-tolyl) phosphorothioate) with the human androgen receptor (AR). Fenitrothion blocked dihydrotestosterone-dependent AR activity in a concentration-dependent and competitive manner in HepG2 human hepatoma liver cells transiently transfected with human AR and an AR-dependent luciferase reporter gene. Schild regression analysis yielded an equilibrium dissociation constant value of 2.18 × 10⁻³ M. To determine the antiandrogenic potential of fenitrothion in vivo, 7-week-old castrated Sprague-Dawley rats were dosed once a day for 7 days. In contrast, significant decreases in the ventral prostate, seminal vesicle, and levator ani plus bulbocavernous muscles tissue weights. In contrast, blood acetylcholinesterase activity, a standard biomarker of organophosphate poisoning, was only inhibited at the higher dose of fenitrothion (30 mg/kg). Our results demonstrate that fenitrothion is a competitive AR antagonist, comparable in potency to the pharmaceutical antiandrogen flutamide and more potent, based on in vitro assays, than the known environmental antiandrogens linuron and p,p'-, 2,2-bis(p-hydroxyphenyl)-1,1-dichloroethylene (p,p'-DDE).

Key Words: androgen receptor; organophosphate pesticide; endocrine-active chemical; antiandrogen; HepG2 cells; Hershberger assay; transcriptional activation.

Organophosphate insecticides are widely used in both agricultural and landscape pest control and the potential for human exposure to this class of compounds is significant. A profile from 66 poison control centers in the United States in 1997 indicated that organophosphorous insecticides were involved in more poisonings than any other single class of pesticide (Litovitz et al., 1998). Individuals at greatest risk are those who most frequently handle these compounds, including formulators, applicators, farmers, and home gardeners. Organophosphate insecticides are also used extensively in the home environment, and, as a result, young children are at increased likelihood for exposure to these compounds (Eskenazi et al., 1999; Landrigan et al., 1999; National Research Council, 1993). The primary toxicity associated with acute exposure to organophosphate insecticides is cholinergic crisis resulting from acetylcholinesterase inhibition (Pope, 1999). However, these compounds have numerous other compound-specific chronic effects, including delayed polyneuropathy, immunotoxicity, carcinogenesis, and endocrine, developmental, and reproductive toxicity (Astroff et al., 1998; Cranmer et al., 1978; Reuber, 1985; Sultatos, 1994).

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 changes in the Food Quality Protection and Safe Drinking Water Acts to now require the United States Environmental Protection Agency to develop a screening and testing program for endocrine-active chemicals. Early efforts to identify and characterize environmental endocrine-active chemicals focused on xenoestrogens, which are chemicals capable of interfering with estrogen receptor function (Chen et al., 1997; Gaido et al., 1997; Krishnan et al., 1993; Soto et al., 1991; White et al., 1994). However, recent publications describing environmental chemicals with antiandrogenic activity has expanded the research effort to include screening for chemicals capable of interfering with androgen receptor function (Hosokawa et al., 1993; Kelce et al., 1995; Maness et al., 1998; Wong et al., 1995).
We investigated the interaction of the organophosphothioate pesticide fenitrothion [O,O-dimethyl O-(4-nitro-m-tolyl) phosphorothioate] with the human androgen receptor. We chose to investigate fenitrothion based on its structural similarities with the pharmaceutical antiandrogen flutamide and the environmental antiandrogenic herbicide linuron (Fig. 1). Fenitrothion’s androgen receptor activity was compared with its ability to inhibit acetylcholinesterase activity and alter motor activity, standard indicators of organophosphate insecticide toxicity. We demonstrate that fenitrothion competitively antagonizes androgen receptor (AR) activity in transfected cells and causes regression of androgen-dependent tissue weights in vivo. Inhibition of androgen receptor function in vivo occurred at a dose of fenitrothion that did not significantly alter blood acetylcholinesterase activity.

MATERIALS AND METHODS

Chemicals. Fenitrothion was obtained from Chem Service (West Chester, PA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). All chemicals were ≥ 97% pure.

Plating and transfection. Transfection experiments were performed as previously described (Gaido et al., 1999; Maness et al., 1998). HepG2 human hepatoma cells (ATCC, Rockville, MD) are used in our studies because of our extensive prior experience with this cell line, the utility of the cell line for the proposed study, the ability to compare results obtained with previously published studies, and because of their widespread use in toxicology and hormone receptor research. Under normal circumstances, HepG2 cells have limited metabolic capabilities. Our experiments indicate that very little metabolism of chemicals occurs over the 24-h treatment period of this assay (Gaido et al., unpublished observations).

HepG2 cells were plated in 24-well plates (Falcon Plastics, Oxnard, CA) at a density of 10^4 cells/well in complete medium consisting of phenol red-free Eagle’s Minimal Essential Medium (GIBCO/BRL, Grand Island, NY) supplemented with 10% charcoal-dextran-treated fetal bovine serum (Hyclone, Logan, UT), 2% L-glutamine, and 0.1% sodium pyruvate. Cells were transfected with 3 plasmids: receptor plasmid pRSAR at 10 ng/well, MMTV- luc reporter plasmid at 405 ng/well, and a constitutively expressed pCMVβ-gal plasmid (transfection control) at 10 ng/well (Maness et al., 1998). Transfected cells were rinsed with phosphate-buffered saline and dosed with various concentrations of test chemical and dimethyl sulfoxide (vehicle control) in complete medium. After the 24-h incubation, cells were rinsed with phosphate-buffered saline and lysed with 65 μl of lysing buffer (25 mM Tris-phosphate, pH 7.8, 2 mM 1,2-diaminocyclohexane-N,N′,N″-tetraacetic acid, 10% glycerol, 0.5% Triton X-100, 2 mM dithiothreitol). Lysate was divided into two 96-well plates for luciferase and β-galactosidase determination.

Luciferase activity was determined by adding 100 µl Luciferase Assay Reagent (Promega, Madison, WI) to 20 µl of lysate per well. Luminescence was determined immediately, using an ML3000 microtiter plate luminometer (Dynatech Laboratories, Chantilly, VA). β-Galactosidase activity was determined by adding 20 µl β-galactosidase assay reagent to 30 µl of lysate per well. β-Galactosidase assay reagent consisted of a 4-ml solution of chlorophenol red-β-D-galactopyranoside (CPRG) in 150 µl CPRG buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, 50 mM β-mercaptoethanol, pH 7.8). Absorbance at 570 nm was determined over a 30-min period, using a Vmax kinetic microplate reader (Molecular Devices, Menlo Park, CA). Luciferase values obtained for each transfected well were normalized by dividing by the associated β-galactosidase value for that well. This normalization corrects for any variation in transfection efficiency between wells and between experiments.

HepG2 cells lack detectable levels of endogenous steroid hormone receptors including AR, progesterone receptor, and glucocorticoid receptor. In the absence of transfected receptor, luciferase activity remains below the level of detection. Background activity following AR transfection averaged 5 ± 1 normalized luciferase units. Values presented in this study represent the means ± SE resulting from at least 3 separate experiments with triplicate wells for each treatment dose level. Dose-response data were analyzed using the sigmoidal dose-response function of the graphical and statistical program Prism (GraphPad, San Diego, CA).

Hershberger male rat assay. This study was conducted in accordance with Federal guidelines for the care and use of laboratory animals and was approved by the Institutional Animal Care and Use Committee at CIIT. Rats were housed in the CIIT animal care unit, a facility accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC) and were kept in a HEPA-filtered, mass air-displacement room with a 12-h light-dark cycle at 18–26°C and relative humidity of 30–70%. Rats had access ad libitum to deionized water and rodent chow (NIH-07, Zeigler Brothers, Gardner, PA).

Male Sprague-Dawley rats, castrated at 4 weeks of age, were purchased from Charles River Labs, Inc. (Raleigh, NC). Rats were weight-ranked, and a homogeneous population (mean weight ± 20%) of 32 male rats was selected for the study. Rats were treated once a day for 7 days, beginning at 7 weeks of age, with subcutaneous doses of testosterone propionate (50 µg/day in 0.2 ml corn oil) plus gavage doses of either corn oil vehicle or 15 or 30 mg/kg/day fenitrothion. While the acute toxicity of fenitrothion to mammals is considered low, it does cause typical signs of cholinergic stimulation such as muscle twitching, tremor, salivation, and diarrhea at a dose of 30 mg/kg/day following 14 days of exposure in rats (Kunimatsu et al., 1996). The dose of fenitrothion (30 mg/kg/day) selected for this study was chosen to ensure that an active concentration of fenitrothion was achieved, as determined by its ability to inhibit cholinesterase activity, that did not cause excessive toxicity (Kunimatsu et al., 1996). A second, lower dose (15 mg/kg/day) was selected to determine whether antiandrogenic activity could be detected at less toxic doses. An additional group of rats was treated with testosterone propionate plus flutamide (50 mg/kg/day) as an antiandrogen reference control. The dose level for flutamide was based on previously published studies (Ashby and Lefevre, 2000; Lambricht et al., 2000; O’Connor et al., 1999). Each of the 4 treatment groups consisted of 8 rats. Treatments were adjusted each day for body weight changes.

Neurotoxicity assessment. Two h after the last dose, animals were transferred to cages containing an automated photobeam activity system to monitor motor activity as an assessment of neurotoxicity (Dorman et al., 2000). Motor activity was measured during ten 6-min intervals for a total of 60 min, using an automated cage rack photobeam activity system (San Diego Instruments, San Diego, CA). The trial was initiated by the first activity of the rat. Total number of movements (beams broken) and number of ambulations (number of times that more than one beam is broken in succession) was recorded. A nested analysis of motor activity data was performed, using a repeated-measures
analysis with treatment as a grouping factor and interval as a within-subject factor (MANOVA).

Necropsy. On the day after the last treatment, rats were anesthetized with sodium pentobarbital. Cardiac puncture was performed to collect blood for an acetylcholinesterase assay, and rats were euthanized by exsanguination. Frontal cortex, hippocampus, and striatum were collected for the acetylcholinesterase assay. Liver, kidney, adrenals, ventral prostate, dorsolateral prostate, glans penis, seminal vesicle (with coagulating glands and fluid), and levator ani plus bulbocavernous muscles were collected and weighed. Organ weight data were analyzed using a regression analysis, the general-linear-models procedure on the statistical analysis system (SAS). Post-hoc tests were conducted when the overall analysis of variance was significant at the $p < 0.05$ level using the LSMEANS procedures available on SAS.

Acetylcholinesterase activities in striatum, hippocampus, frontal cortex, and whole blood were determined after solubilization of tissue with 1% Triton X-100 in 0.1 M phosphate buffer (pH 8.0) for 15 min at room temperature. Acetylthiocholine iodide (0.075 M) and 0.01 M 5,5′-dithio-bis(2-nitrobenzoic acid) were used as substrate, and analysis was performed on a Roche COBAS Fara II chemical analyzer. Protein was measured using a commercially available kit (Pierce, Rockford, IL). Acetylcholinesterase results were normalized to total protein and expressed as change in absorbance per min. Serum testosterone and corticosterone were measured by radioimmunoassay using commercially available kits (ICN Biomedicals, Inc., Costa Mesa, CA). Data were analyzed by one-way analysis of variance using JMP statistical analysis software (SAS Institute, Cary, NC).

RESULTS

We determined the in vitro interactions of fenitrothion with the androgen receptor (AR) by measuring AR-dependent luciferase activity in HepG2 human hepatoma liver cells transiently transfected with the human AR and an AR-dependent luciferase reporter gene. Transfected HepG2 cells were treated with fenitrothion over a concentration range from $10^{-8}$ to $10^{-5}$ M in the presence or absence of a maximally activating dose of dihydrotestosterone (DHT). Fenitrothion reduced DHT-dependent AR activity in a concentration-dependent manner, thus indicating that fenitrothion is an AR antagonist (Fig. 2). Slight AR agonist activity was detected at the highest concentration of fenitrothion used in this study ($10^{-5}$ M). Fenitrothion’s agonist activity was approximately 8% of the maximally inducible response with DHT.

The AR antagonist activity of fenitrothion was further characterized by determining the effect of set concentrations of fenitrothion over a complete DHT dose-response curve (Fig. 3). Fenitrothion caused a parallel displacement of the DHT dose-response curve to the right with no concomitant depression of maximal response. These results indicate that fenitrothion acts as a competitive AR antagonist (Kenakin, 1993). The equilibrium dissociation constant ($K_a$) for the antagonist-receptor complex was determined by Schild regression (Arunlakshana and Schild, 1959) to be $2.18 \times 10^{-8}$ M.

To determine the antiandrogenic potential of fenitrothion in vivo, we performed an assay in castrated male rats following the Hershberger protocol (Hershberger et al., 1953). This assay was developed as a short-term, in vivo assay for androgenic and antiandrogenic compounds, and has been recommended by the Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) as part of a Tier-1 screening battery for endocrine-active chemicals (U.S. EPA, 1998).

Rats dosed with 30 mg/kg/day fenitrothion weighed 17% less than controls at necropsy (Fig. 4A). Rats dosed with 15 mg/kg/day fenitrothion did not weigh significantly different from control rats at necropsy. Fenitrothion at 15 mg/kg/day and 30 mg/kg/day, respectively, caused a 30% and 92% decrease in body weight gain over the 7-day treatment period relative to control values (Fig. 4B). The effect of fenitrothion on body weight is a result of cholinergic stress. High-dose fenitrothion (30 mg/kg/day) caused a 22% decrease in absolute liver weight.
relative to controls, whereas flutamide treatment resulted in a 12% increase in absolute liver weight (Fig 4C). The effect of high-dose fenitrothion on liver weight was not significant when body weight was considered by covariance analysis (data not shown). The effect of flutamide on liver weight remained significant by covariance analysis with body weight. Thus, the reduced liver weight in fenitrothion-treated rats is likely due to the reduced animal weights, whereas, the increased liver weights in the flutamide rats is likely to a direct effect of flutamide on the liver. Kidney weight was not affected by any of the treatments (data not shown).

Both fenitrothion and the reference antiandrogen flutamide caused significant decreases in ventral prostate, seminal vesicle, and levator ani plus bulbocavernosus muscles tissue weights (Figs. 4D–4F). The reduction in androgen-dependent tissue weights remained statistically significant (p < 0.05) when analyzed by covariance analysis with terminal body weight, and thus were due to a direct effect of fenitrothion and

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**FIG. 4.** Effect of fenitrothion on androgen-dependent responses in the rat. Male Sprague-Dawley rats castrated at 4 weeks were treated once a day for 7 days beginning at 7 weeks of age with subcutaneous doses of testosterone propionate (50 μg/day in 0.2 ml corn oil) plus gavage doses of either corn oil vehicle, 15 or 30 mg/kg/day fenitrothion, or 50 mg/kg/day flutamide. Each treatment group consisted of 8 rats. C, Control; F15, fenitrothion 15 mg/kg; F30, fenitrothion 30 mg/kg; Flu, flutamide; *p < 0.05.
flutamide on these tissues rather than an indirect effect related to body weight (data not shown).

Serum testosterone concentrations were not altered by any of the treatments (Table 1). A trend for increased plasma corticosterone concentrations, an indication of cholinergic stress (Kunimatsu et al., 1996; Rattner et al., 1982), was evident only in the 30-mg/kg/day fenitrothion treatment group. However, this increase was not significantly different from control. Log transformation of the data did not enhance significance. Fenitrothion exposure was associated with other signs of cholinergic toxicity, including excess salivation, muscle tremors, and reduced motor activity (Fig. 5). Acetylcholinesterase activity was significantly reduced in whole blood and brain in rats dosed with 30-mg/kg/day fenitrothion dosage (Table 1). Brain acetylcholinesterase activity was also significantly reduced at 15-mg/kg/day fenitrothion. Blood acetylcholinesterase activity was not significantly reduced with 15-mg/kg/day fenitrothion (Table 1). Blood acetylcholinesterase was significantly elevated in the flutamide treatment group.

### DISCUSSION

Fenitrothion is registered in the U.S. for use in ant and roach baits. There are no approved domestic food or feed uses for fenitrothion, and exposure to fenitrothion in the U.S. is minimal. However, fenitrothion is used in other countries to control pests on crops, stored grains, and cotton. Fenitrothion is also used elsewhere in forest spraying and in public health campaigns. As a result, the human health effects associated with exposure to fenitrothion remain a concern, especially among pesticide workers and applicators whose acute exposure to organophosphate pesticides can sometimes occur at levels high enough to inhibit blood acetylcholinesterase activity (Nigg and Knaak, 2000; Ohayo-Mitoko et al., 2000; Satoh and Hosokawa, 2000). We demonstrate that fenitrothion is a competitive antagonist of the human AR and can inhibit androgen-dependent tissue growth in vivo.

Inhibition of androgen-dependent tissue growth in vivo occurred with a dose of fenitrothion (15 mg/kg) that was not associated with a significant decrease in blood acetylcholinesterase activity, which is often used as a biomarker for human exposure to organophosphate pesticides (Nigg and Knaak, 2000). Thus, antiandrogenic activity in the rat occurs at a dose level below that required for a significant reduction in blood acetylcholinesterase activity.

Fenitrothion was included as a negative control in a recently published study comparing the effect of antiandrogenic and estrogenic chemicals on preputial separation (Ashby and Lefevre, 2000), an androgen-dependent response that can be delayed by antiandrogen treatment (Monosson et al., 1999). Fenitrothion (15 mg/kg/day) failed to cause a significant delay in preputial separation (Ashby and Lefevre, 2000). However, the results were confounded by the negative effect of fenitrothion on body weight, which also delayed preputial separation (Ashby and Lefevre, 2000). As a result, the authors were unable to identify fenitrothion as an antiandrogen. These results suggest that preputial separation may not be the best method for detecting antiandrogens that also cause a decrease in body weight.

The in vitro potency of fenitrothion as a competitive AR

### TABLE 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Serum steroid (ng/ml)</th>
<th>Acetylcholinesterase (ΔA/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Testosterone</td>
<td>Corticosterone</td>
</tr>
<tr>
<td>Control</td>
<td>0.18 ± 0.03</td>
<td>72 ± 23</td>
</tr>
<tr>
<td>Fen 15</td>
<td>0.20 ± 0.03</td>
<td>109 ± 21</td>
</tr>
<tr>
<td>Fen 30</td>
<td>0.25 ± 0.02</td>
<td>223 ± 70</td>
</tr>
<tr>
<td>Flutamide</td>
<td>0.25 ± 0.02</td>
<td>66 ± 14</td>
</tr>
</tbody>
</table>

Note. Mean ± SE; *p < 0.05. Male Sprague-Dawley rats castrated at 4 weeks were treated once a day for 7 days, beginning at 7 weeks of age, with subcutaneous doses of testosterone propionate (50 µg/day in 0.2 ml corn oil) plus gavage doses of either corn oil vehicle, 15 or 30 mg/kg/day fenitrothion, or 50 mg/kg/day flutamide. Fen 15, fenitrothion 15 mg/kg; fen 30, fenitrothion 30 mg/kg.

FIG. 5. Total motor activity following repeated exposure to fenitrothion or flutamide. Motor activity was measured during ten 6-min intervals for a total of 60 min, using an automated cage rack photobeam activity system. Values represent mean ± SE; *p < 0.05.
**ANTIANDROGENIC ACTIVITY OF FENITROTHION**

Most sensitive periods for exposure to antiandrogens, and concentration tested ($10^{-8}$ M) did demonstrate slight agonist activity at the highest concentration tested ($10^{-5}$ M). We have previously reported on the ability of some AR antagonists to demonstrate agonist activity in vitro at high concentrations (Maness et al., 1998). The mechanism for this switch to agonist activity at high concentrations has not been determined.

The Hershberger assay used in this study, and proposed by the U.S. EPA as part of their endocrine disrupter screening program, was designed to identify agents that possess intrinsic antiandrogenic activity (U.S. EPA, 1998). This assay has been used for decades for screening chemicals for androgenic and antiandrogenic activity (Dorfman, 1962) and is extremely sensitive to antiandrogens, because the typical endocrine feedback loops have been eliminated. The Hershberger assay does, however, respond to several different mechanisms of action, so it is important to confirm the purported AR-activity with an in vitro assay as was done here. We use the Hershberger assay to demonstrate that fenitrothion can block androgen-dependent tissue growth. Fenitrothion’s effects on androgen-dependent tissue growth is administered during gestation, dosages as low as 12.5–25 mg/kg/day result in alterations of androgen-dependent tissues (Lambright et al., 1999). Future studies should determine the dose-responsive effect of fenitrothion on male reproductive tract development following in utero exposure, which will be more relevant for risk assessment.

Organophosphate insecticides have not been tested previously for their ability to directly interact with steroid hormone receptors. Structural similarities between fenitrothion and other organophosphorous compounds make it likely that additional organophosphate insecticides will have antiandrogenic activity. Indeed, the organophosphate pesticide parathion has been shown to inhibit DHT binding to the AR in the rat ventral prostate (Shain et al., 1977). Preliminary experiments conducted in our laboratory show that methyl parathion, as well as other structurally related organophosphates, demonstrate antiandrogenic activity in transiently transfected HepG2 cells (data not shown). The high potential for human exposure and the current concern for the effect of environmental antiandrogens on male reproductive development indicate the need for further study of this economically important class of compounds.

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Male reproductive tract development in utero is one of the most sensitive periods for exposure to antiandrogens, and chemicals that have been shown to display antiandrogenic activity in vitro and in the Hershberger in vivo assay induce malformations in male rat offspring at lower dosage levels following in utero exposure. Such toxicants include the herbicide linuron, vinclozolin, procymidone, flutamide and $p,p'$-DDE (Gray et al., 1999a,b; Lambright et al., 2000; McIntyre et al., 2000; Ostby et al., 1999; You et al., 1999). When linuron is administered during gestation, dosages as low as 12.5–25 mg/kg/day result in alterations of androgen-dependent tissues in male rats (Lambright et al., 2000; McIntyre et al., 2000). Similarly, administration of vinclozolin at 50 mg/kg/day during gestation causes hypospadias, while functional alterations (reduced AGD, areolas, retained nipples, reduced sex accessory gland size) are seen at lower dosage levels, ranging from 3 to 25 mg/kg/day (Gray et al., 1999a). When administered during sexual differentiation, procymidone induces hypospadias at 50 mg/kg/day, and similar to vinclozolin, functional alterations of androgen-dependent tissues are seen at lower dosage levels (Ostby et al., 1999). Future studies should determine the dose-responsive effect of fenitrothion on male reproductive tract development following in utero exposure, which will be more relevant for risk assessment.

Organophosphate insecticides have not been tested previously for their ability to directly interact with steroid hormone receptors. Structural similarities between fenitrothion and other organophosphorous compounds make it likely that additional organophosphate insecticides will have antiandrogenic activity. Indeed, the organophosphate pesticide parathion has been shown to inhibit DHT binding to the AR in the rat ventral prostate (Shain et al., 1977). Preliminary experiments conducted in our laboratory show that methyl parathion, as well as other structurally related organophosphates, demonstrate antiandrogenic activity in transiently transfected HepG2 cells (data not shown). The high potential for human exposure and the current concern for the effect of environmental antiandrogens on male reproductive development indicate the need for further study of this economically important class of compounds.

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