Original Article

Anti-androgenic activity of procymidone and its metabolites

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Procymidone (Similex®) is a fungicide with a weak anti-androgenic activity. The anti-androgenic activity and androgen receptor (AR)-binding activity of procymidone and its six metabolites were evaluated. Anti-androgenic activity was determined in HeLa cells transiently transfected with an AREx3-luciferase reporter and full-length human or rat AR expression vectors (anti-androgen assay). AR-binding activity was determined by a competitive ligand-binding assay based on a fluorescence polarization method (AR-binding assay). It was clear that procymidone, its hydroxylated metabolite (PCM-CH₂OH), and their imide linkage-cleaved compounds (PCM-NH-COOH and PA-CH₂OH) have anti-androgenic activity and AR-binding activity, and procymidone has the highest activity among these compounds. There were no differences between rats and humans regarding the activities of procymidone and the metabolites. The activity of procymidone was 20–70 times lower than that of hydroxyflutamide, which is a potent anti-androgen. The results indicate that the anti-androgenic activities of procymidone and its metabolites are very weak. © Pesticide Science Society of Japan

Keywords: species differences, anti-androgenic activity, metabolites, metabolism, rat, human.

Electronic supplementary materials: The online version of this article contains supplementary materials (Supplemental Figures S1, S2 and S3), which are available at <http://www.jstage.jst.go.jp/browse/jpestics/>.

Introduction

Procymidone (Similex®) is a fungicide with both protective and curative potential used to control plant diseases such as fruit rots; gray mold on top fruits, vines, and vegetables; and Sclerotinia rot of kidney beans and vegetable crops.^{1–3)} Many toxicity studies were conducted to evaluate the safety of procymidone. When rats were dosed with procymidone at 125 mg/kg, hypospadias was observed in a vast majority of male pups and in all litters, the lowest teratogenic dose level being 37.5 mg/kg in rats. In contrast, there were no offspring with hypospadias in monkeys or rabbits, even at a dose of 125 mg/kg, the highest dose tested. These results suggested that rats are more sensitive to procymidone exposure than monkeys and rabbits.⁴⁾ Thus, a series of mechanistic studies related to developmental toxicity was conducted to determine whether there are species differences in anti-androgenic activity, metabolism, excretion, placental transfer, and protein binding among rats, rabbits, monkeys, and humans. In previous reports, findings on the metabolism of procymidone in mammals were documented, and it was clarified that the kinds of metabolites are nearly identical among species.^{5–7)}

The metabolic pathways of procymidone in mammals are shown in Fig. 1. Procymidone is hydroxylated at the methyl group of the imide ring. The hydroxylated metabolites (PCM-CH₂OH and PA-CH₂OH) are then metabolized in two ways: they are either oxidized at the hydroxyl group to form the carboxylated metabolites (PCM-COOH and PA-COOH) or transformed into glucuronide (PCM-CH₂OH-glucuronide and PA-CH₂OH-glucuronide). The carboxylated metabolites and glucuronide are more hydrophilic and thus more readily excreted. While the anti-androgenic activities of procymidone and its metabolites, PCM-CH₂OH and PCM-COOH, were investigated earlier,⁸⁾ imide linkage-cleaved metabolites, PA-CH₂OH and PA-COOH, and PCM-CH₂OH-glucuronide, which is a major metabolite in rabbits, were not included. Furthermore, in the previous study, a comparison of anti-androgenic activity between rats and humans was not conducted. The present report deals with the anti-androgenic activity of procymidone and its metabolites and a comparison of anti-androgenic activity between rats and humans. The results of other studies will be described elsewhere.

Materials and Methods

1. Compounds

Procymidone (C₁₃H₁₁Cl₂NO₂, purity: 99.6%), PCM-NH-COOH (C₁₃H₁₃Cl₂NO₃, purity: 98.6%), PCM-CH₂OH (C₁₃H₁₁Cl₂NO₃, purity: 99.9%), PCM-COOH (C₁₃H₉Cl₂NO₄, purity: 96.4%), PA-CH₂OH (C₁₃H₁₃Cl₂NO₄, purity: 98.8%), PA-COOH

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Fig. 1. Proposed main metabolic pathways of procymidone in mammals.

($C_{13}H_{11}Cl_2NO_5$, purity: 96.9%), and PCM-CH₂OH-glucuronide ($C_{19}H_{19}Cl_2NO_9$, purity: 99.5%) were synthesized by Sumitomo Chemical Co., Ltd. (Osaka, Japan) and used for anti-androgen assays and AR-binding assays. 5 α -Dihydrotestosterone (DHT, $C_{19}H_{30}O_2$, purity: $\geq 99.0\%$) and flutamide ($C_{11}H_{11}F_3N_2O_3$, purity: $\geq 98.0\%$) were purchased from Fluka Production GmbH (Buchs, Switzerland) and LKT Laboratories, Inc. (Minnesota, USA), respectively. Hydroxyflutamide (HFL, $C_{11}H_{11}F_3N_2O_4$, purity: $\geq 98.0\%$) was synthesized by Sumitomo Chemical Co., Ltd. DHT was used as an androgen for anti-androgen assays and as a positive control for AR-binding assays. Flutamide and HFL were used as further positive controls for both assays. All compounds were dissolved in dimethyl sulfoxide (DMSO) purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2. Anti-androgen assay

For the AR-mediated reporter gene assay, the expression vectors 'pRc/RSV-human AR' and 'pRc/RSV-rat AR' were generated by insertion of an RT-PCR-amplified full-length cDNA of human AR and rat AR with an efficient Kozak's translation initiator sequence into the blunting site of a pRc/RSV vector (Invitrogen, California, USA). The reporter plasmid for AR, 'pGL3-TATA-AREx3/luc', was generated by insertion of three copies of AREs derived from rat probasin into a pGL3 basic vector (Promega, Wisconsin, USA) with a mouse metallothionein minimum

TATA promoter. For the control assay, 'pGL3-TK' was generated by insertion of a herpes simplex virus thymidine kinase (HSV-TK) promoter into a pGL3 basic vector. Transient transfection was carried out in accordance with a method described previously.⁹ HeLa cells were routinely maintained in phenol red-free Dulbecco's modified eagle medium (DMEM) containing 10% charcoal-treated fetal calf serum on 10-cm plates. Twenty-four hours before transfection, 1.1×10^6 cells were seeded in the DMEM mixture per 10-cm plate. For transfection, the cells were incubated with 3 μ g/plate of 'pRc/RSV-human AR' or 'pRc/RSV-rat AR', 3 μ g/plate of 'pGL3-TATA-AREx3/luc' or 6 μ g/plate of 'pGL3-TK' (for control assay), and liposome-DNA complexes (lipofectamine, Invitrogen) at 37°C for 16 hr and then further incubated for 3 hr after medium change. The transfected cells were dissociated with 2.5 g/L trypsin/1 mM EDTA solution (Nacalai Tesque, Kyoto, Japan) and collected. The transfected cells were seeded into plates containing the DMEM mixture with each test chemical and DHT (50 pM) ($n=6$). The maximum concentrations of chemical were determined on the basis of solubility in medium or (anti-)androgen potential. After approximately 40 hr of incubation at 37°C, the cells were lysed by adding 50 μ L/plate of diluted ($\times 2$) Steady-Glo Luciferase Assay System (Promega), and after cooling at room temperature for 10 min luminous intensity was measured using a TopCount NXT microplate detection instrument (Packard BioScience Co., Connecticut, USA).

3. AR-binding assay

A competitive androgen receptor-binding assay was carried out by a fluorescence polarization method using a rat AR Competitor Assay Kit (Green, PanVera, Wisconsin, USA).¹⁰⁾ Changes in fluorescence polarization values with each test compound were measured using a multifunctional microplate detection system (ULTRA, Tecan Japan Co., Ltd., Kanagawa, Japan) with suitable 485 nm excitation and 535 nm emission filters.

4. Data analysis

In the anti-androgen assay, the activity in each concentration of each test compound was calculated using the following equation:

$$\begin{aligned} \text{Activity (\%)} \\ &= 100 \times \text{Luciferase activity by chemical with DHT} \\ &\quad (50 \text{ pM}) / \text{Luciferase activity by DHT (50 pM)}. \end{aligned}$$

The concentration of each test chemical at which the response in the anti-androgenic activity is 50% of the response induced by DHT (50 pM) only (IC_{50}) was calculated using GraphPad Prism statistical software (GraphPad Software Inc., California, USA).

In the AR-binding assay, the inhibition in each concentration of the test compounds was calculated as follows:

$$\begin{aligned} \text{Inhibition (\%)} \\ &= 100 \times \text{Changes of fluorescence polarization value} \\ &\quad \text{by chemical} / \text{Changes of fluorescence polarization} \\ &\quad \text{value by DHT (10 } \mu\text{M)}. \end{aligned}$$

The concentration of each test chemical at which the response in the AR-binding activity is 50% of the response induced by

DHT (10 μM) (IC_{50}) was calculated using GraphPad Prism statistical software.

The relative anti-androgenic activity and relative AR-binding activity of each test chemical were calculated using the following equations:

$$\begin{aligned} \text{Relative anti-androgenic activity} \\ &= IC_{50} \text{ of procymidone} / IC_{50} \text{ of chemical in} \\ &\quad \text{anti-androgen assay.} \end{aligned}$$

$$\begin{aligned} \text{Relative AR-binding activity} \\ &= IC_{50} \text{ of procymidone} / IC_{50} \text{ of chemical in} \\ &\quad \text{AR-binding assay.} \end{aligned}$$

Results

1. Anti-androgen assay with rat AR

Data are shown in Table 1 and Fig. 2. The IC_{50} values for two typical antagonists, HFL and flutamide, were 5.3 nM and 340 nM, respectively. Under the same conditions, procymidone showed clear anti-androgenic activity (IC_{50} = 310 nM), and PCM-CH₂OH, a metabolite of procymidone, showed weak activity (IC_{50} = 3.4 μM). However, no effects of PCM-COOH were observed at concentrations from 1 nM to 30 μM (Supplemental Fig. S1). PA-CH₂OH exhibited weak anti-androgenic activity (IC_{50} = 4.1 μM), and no effects of PA-COOH were observed at concentrations from 1 nM to 30 μM (Supplemental Fig. S1). PCM-NH-COOH showed weaker anti-androgenic activity than procymidone (IC_{50} = 1.4 μM). PCM-CH₂OH-glucuronide had no effects at concentrations from 1 nM to 30 μM (Supplemental Fig. S1). In the control assay, no significant effects were found on

Table 1. IC_{50} values and relative activities for DHT, HFL, flutamide, procymidone and its metabolites

Compounds		Anti-androgenic activity		AR-binding activity
		Rat	Human	
Procymidone	IC_{50} (μM)	0.31	0.29	12
	Relative activity	1.00	1.00	1.00
PCM-CH ₂ OH	IC_{50} (μM)	3.4	2.4	26
	Relative activity	0.09	0.12	0.46
PCM-COOH	IC_{50} (μM)	—	—	—
	Relative activity	—	—	—
PCM-NH-COOH	IC_{50} (μM)	1.4	1.2	100
	Relative activity	0.22	0.24	0.12
PA-CH ₂ OH	IC_{50} (μM)	4.1	3.5	227
	Relative activity	0.08	0.08	0.05
PA-COOH	IC_{50} (μM)	—	—	—
	Relative activity	—	—	—
PCM-CH ₂ OH-glucuronide	IC_{50} (μM)	—	—	—
	Relative activity	—	—	—
HFL (hydroxyflutamide)	IC_{50} (μM)	0.0053	0.0044	0.57
	Relative activity	58.49	65.91	21.05
Flutamide	IC_{50} (μM)	0.34	0.23	10.6
	Relative activity	0.91	1.25	1.13
DHT (Dihydrotestosterone)	IC_{50} (μM)	N.E.	N.E.	0.039
	Relative activity	—	—	307.69

Relative anti-androgenic activity and relative AR-binding activity of procymidone were defined as 1.00. N.E.: not examined, —: no activity.

Fig. 2. Anti-androgenic activity of HFL, flutamide, and procymidone and its metabolites (having activity) in the anti-androgen assay using the rat androgen receptor (mean $\pm$ S.D., $n=6$).

Fig. 3. Anti-androgenic activity of HFL, flutamide, and procymidone and its metabolites (having activity) in the anti-androgen assay using the human androgen receptor (mean $\pm$ S.D., $n=6$).

Fig. 4. AR-binding activity of HFL, flutamide, and procymidone and its metabolites (having activity) in the AR-binding assay (mean $\pm$ S.D., $n=3$).

treatment with procymidone or any of the tested metabolites at the concentrations of 1 nM to 30 μ M (Supplemental Fig. S1). In the assay, neither procymidone nor any of the tested metabolites caused any obvious cytotoxicity.

2. Anti-androgen assay with human AR

Data are shown in Table 1 and Fig. 3. The IC_{50} values for HFL and flutamide were 4.4 nM and 230 nM, respectively. Under the same conditions, procymidone showed clear anti-androgenic activity (IC_{50} =290 nM), and PCM-CH₂OH (IC_{50} =2.4 μ M), PCM-NH-COOH (IC_{50} =1.2 μ M), and PA-CH₂OH (IC_{50} =3.5 μ M) showed somewhat weak activity. PCM-COOH, PA-COOH, and PCM-CH₂OH-glucuronide had no effects at concentrations from 1 nM to 30 μ M (Supplemental Fig. S2). In the control assay, no significant effects were found on treatment with procymidone or any of the tested metabolites at concentrations of 1 nM to 30 μ M (Supplemental Fig. S2). Neither procymidone nor any of the tested metabolites showed any cytotoxicity.

3. AR-binding assay

Data are shown in Table 1 and Fig. 4. Significant dose-dependent changes in fluorescence polarization were detected with DHT, HFL, and flutamide, indicating that these chemicals can bind to AR with androgenic (DHT) or anti-androgenic (HFL and flutamide) effects. The IC_{50} values for DHT, HFL, and flutamide were 39 nM, 570 nM, and 10.6 μ M, respectively. Procymidone showed dose-dependent AR-binding activity (IC_{50} =12 μ M), and PCM-CH₂OH showed weak AR-binding activity (IC_{50} =26 μ M). PA-CH₂OH demonstrated weak but not negligible AR-binding activity at concentrations from 100 to 150 μ M (IC_{50} =227 μ M). PCM-NH-COOH showed weak AR-binding activity at concentrations from 10 to 150 μ M (IC_{50} =100 μ M). PCM-COOH, PA-COOH, and PCM-CH₂OH-glucuronide had no effects at concentrations from 100 nM to 150 μ M (Supplemental Fig. S3).

Discussion

As shown in Table 1, Fig. 2, and Fig. 3, the present evaluation of anti-androgenic activity of procymidone and its metabolites revealed no clear differences between human and rat AR anti-androgen assays. Therefore, it was considered that there are no differences between rats and humans regarding the anti-androgenic activity of procymidone and its metabolites. In the AR-binding assay, we used a fluorescence polarization method that is well established for high-throughput screening of nuclear receptor ligands, instead of conventional binding assays with radioactive ligands. In general, AR-binding assays based on fluorescence polarization are less sensitive than the conventional binding assays (data presented in the manufacturer's description), and significant differences were found between the IC_{50} values of AR-binding and anti-androgen assays in the present study (Table 1). In the anti-androgen assay, the relative anti-androgen activity of PCM-CH₂OH was approximately one-eighth that of procymidone. However, in the AR-binding assay,

the activity was about half. The AR-binding assay was conducted under cell-free conditions. On the other hand, the anti-androgen assay was performed with cells. Therefore, it is considered that the difference was caused by the low permeability of PCM-CH₂OH to cells, resulting in reduced activity in the anti-androgen assay. This indicates that the AR-binding results for PCM-CH₂OH might better reflect actual activity (behavior) inside the cell than the results of the anti-androgen assay. Furthermore, a reverse phenomenon was observed for imide linkage-cleaved metabolites (PCM-NH-COOH and PA-CH₂OH). Relative activities were weaker in the AR-binding assay than in the anti-androgen assay. Generally, it has been reported that pH values within cells (intracellular pH) are 0.6–0.7 units lower than those outside (in body fluid, such as blood) in all animal species, including humans, and intracellular pH is approximately 7.0 (the pH of blood is 7.4).^{11–13} In our previous study,¹⁴ it was demonstrated that the imide (cyclic) compounds (procymidone, PCM-CH₂OH, and PCM-COOH) and the corresponding imide linkage-cleaved metabolites (PCM-NH-COOH, PA-CH₂OH, and PA-COOH, respectively) are in equilibrium and, in cells of tissues, imide linkage-cleaved compounds are easily transformed into the corresponding cyclic compounds (from PA-CH₂OH to PCM-CH₂OH). Further, it has been demonstrated that under acidic conditions (less than pH 7.0, for instance: in tissue), imide (cyclic) compounds (procymidone, PCM-CH₂OH, and PCM-COOH) are stable, while under alkali conditions (more than pH 7.4, for instance: in blood), they are converted to imide linkage-cleaved metabolites (PCM-NH-COOH, PA-CH₂OH, and PA-COOH, respectively). In our anti-androgen assays, HeLa cells were used and incubated at 37°C for 48 hr. Therefore, the imide linkage-cleaved metabolites (PCM-NH-COOH, PA-CH₂OH, and PA-COOH) would have been transformed into the corresponding cyclic metabolites (procymidone, PCM-CH₂OH, and PCM-COOH, respectively) inside the cells. In contrast, a buffer of pH 7.4 was used as a sample solution (cell-free condition) for the AR-binding assay and incubated at room temperature. Therefore, in this reaction, it is considered that the above transformations would rarely occur. Accordingly, the activity of imide linkage-cleaved metabolites is considered to be higher in the anti-androgen assay than in the AR-binding assay. This indicates that the activity of imide linkage-cleaved metabolites in the anti-androgen assay might better reflect actual activity (behavior) inside the cell than the AR-binding assay did, although we must consider cell permeability of the compounds.

Therefore, as the most conservative scenario, to see the actual activity (behavior) of procymidone and its metabolites inside the cell (tissues) after penetration of the cellular membrane of the compounds, it is necessary to judge using a combination of anti-androgen and AR-binding assays. The relative binding affinity values were calculated using the equations below. In addition, AR-binding activity data (rat) were used as human data because there was no difference between human and rat AR anti-androgen assays.

Table 2. Calculated relative binding affinities of procymidone and its metabolites

Compounds	Relative binding affinity
Procymidone	1.00
PCM-CH ₂ OH	0.46 ^{a)}
PCM-COOH	—
PCM-NH-COOH	0.24 ^{b)}
PA-CH ₂ OH	0.31 ^{b)}
PA-COOH	—
PCM-CH ₂ OH-glucuronide	—

Relative binding affinity of procymidone was defined as 1.00. —: no binding affinity, *a)* data obtained from the AR-binding assay. *b)* data obtained from the equation (described in Discussion).

Relative binding affinity of the cyclic compound
(PCM-CH₂OH) = Relative AR-binding activity of
PCM-CH₂OH

Relative binding affinity of the imide linkage-cleaved
compound (PCM-NH-COOH and PA-CH₂OH)
= Relative anti-androgenic activity of the compound /
Permeability of the compound

However, because we could not assess compound permeability in this study, we used the permeability of the related cyclic compound as an expedient estimate.

Permeability of the imide linkage-cleaved compound
= Relative anti-androgenic activity of the related
cyclic compound / Relative AR-binding activity
of the related cyclic compound

Therefore,

Relative binding affinity of the imide linkage-cleaved
compound
= Relative anti-androgenic activity of the compound /
Permeability of the related cyclic compound
= Relative anti-androgenic activity of the compound /
(Relative anti-androgenic activity of the related
cyclic compound / Relative AR-binding activity
of the related cyclic compound)

The calculated relative binding affinity values based on the above definition are shown in Table 2. Relative binding affinity values for androgen receptors of PCM-NH-COOH, PCM-CH₂OH, and PA-CH₂OH in cells (tissues) were estimated to be 0.24, 0.46, and 0.31, respectively, with the binding affinity of procymidone defined as 1.00.

From our study, it was clear that procymidone, its hydroxylat-

ed metabolite (PCM-CH₂OH), and their imide linkage-cleaved compounds (PCM-NH-COOH and PA-CH₂OH) have anti-androgenic activity and AR-binding activity, and procymidone has the highest activity among these compounds. There were no differences between rats and humans regarding the activities of procymidone and the metabolites. The activities of procymidone were 20–70 times lower than those of hydroxyflutamide, which is a potent anti-androgen. The results indicate that the anti-androgenic activities of procymidone and its metabolites are very weak. From the above findings, it was concluded that species differences in developmental toxicity are not attributable to species differences in the affinity for AR. Further, it was speculated that the apparent toxic observations in rats were distinguished from the absence of toxicity in rabbits and monkeys by higher internal exposure of PCM and its metabolites, which have anti-androgenic characteristics.

In the future, we will conduct *in vivo* studies to determine whether there is a species difference for the production amounts of PCM and its metabolites having anti-androgenic and AR-binding activities.

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