THE BINDING PROPERTIES OF PYRETHROIDS TO HUMAN SKIN FIBROBLAST ANDROGEN RECEPTORS AND TO SEX HORMONE BINDING GLOBULIN

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Summary—The pyrethroids are a class of natural and synthetic pesticides which were associated with an epidemic of gynecomastia in Haitian men in 1981. In the present study we tested several pyrethroids for their ability to interact with androgen binding sites in dispersed, intact human genital skin fibroblasts and in human plasma to sex hormone binding globulin (SHBG). All the pyrethroids tested inhibited fibroblast binding of [^{3}H]methyltrienolone (R1881) at 22°C with the following rank order of potency: pyrethrins > bioallethrin > fenvalerate > fenothrin > fluvalinate > permethrin > resmethrin. 50% displacement of [^{3}H]R1881 binding to fibroblast androgen receptors was achieved by 1.5–44×10^{-5} M concentrations of the competitors, respectively. Previous studies with cimetidine, a known inhibitor of androgen receptor binding, showed 50% competition at a concentration of 1.4×10^{-4} M in this system. Scatchard analysis of binding experiments performed with increasing concentrations of [^{3}H]R1881 in the presence of the pyrethroids indicated that the binding inhibition was competitive. On the other hand, of the pyrethroids examined only the pyrethrins (50% inhibition) and bioallethrin (43% inhibition) were able to displace [^{3}H]testosterone from SHBG when tested at a concentration of 10^{-4} M.

These data indicate that a novel class of non-steroidal compounds, the pyrethroids, can interact competitively with human androgen receptors and SHBG. These findings provide a mechanism by which chronic exposure of humans or animals to pesticides containing these compounds may result in disturbances in endocrine effects relating to androgen action.

INTRODUCTION

The pyrethroids are a class of natural and synthetic organic compounds which have been used commercially for many years because of their well known insecticide properties. These agents are still highly effective and are often included in many currently available household sprays [1]. Natural pyrethroids, derived from the pyrethrum plant *Chrysanthemum cinerariaefolium*, are only moderately toxic to warm-blooded animals when given orally, but can be highly toxic, primarily to the central nervous system, when given parenterally [1]. The difference between oral and parenteral toxicities is presumably due to rapid metabolic degradation of the active ingredients. The synthetic analogues of pyrethroids are much less acutely toxic to rats, and presumably to man, than are the natural pyrethroids. The synthetic pyrethroids (and most likely the natural products as well) are rapidly absorbed by the gastrointestinal tract and distributed through body tissues, including the

brain [1]. Neither the natural nor the synthetic pyrethroids, however, are believed to be absorbed through intact skin [1]. Although natural pyrethroids have been reported to be teratogenic on the genital tract of birds [2], as far as we are aware, there have been no clearly established endocrine side effects in men or women exposed to these agents that would be indicative of disturbances in gonadal function in humans.

We became aware of the pyrethroids following an epidemic of gynecomastia among Haitian refugees in 1981 [3]. Extensive epidemiologic, endocrine, and toxicological studies of this outbreak by the Centers for Disease Control implicated nutritional factors [3]. However, a subsequent study suggested that a component in an insecticide spray containing fenothrin (a synthetic pyrethroid), to which the refugees may have been exposed, had antiandrogenic properties [4]. The present studies were undertaken to evaluate fenothrin and a variety of other pyrethroids for their ability to interfere with androgen binding to human fibroblast androgen receptors and to human plasma sex hormone binding globulin (SHBG).

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EXPERIMENTAL

Reagents

Concanavallin A covalently linked to 4B-Sepharose (Con-A-Sepharose) was obtained from Pharmacia Fine Chemicals; [³H]testosterone, [17α-methyl-³H]-methyltrienolone [³H]R1881; 87 Ci/mmol, non-radio-active methyltrienolone and Aquasol were from New England Nuclear; and Tricine (N-Tris-(hydroxymethyl)methylglycine) and unlabelled testosterone were from Sigma Chemicals. Fetal calf serum (mycoplasma and virus screened), trypsin-EDTA, and penicillin-streptomycin were obtained from Grand Island Biological Co. (Grand Island, N.Y.). Gentamycin was obtained from Schering Pharmaceutical Corp. (Manati, P.R.). Tissue culture flasks (75 and 150 cm²) were purchased from Costar (Cambridge, Mass), Falcon Plastics (Oxnard, Calif.), and Corning Glass Works (Corning, N.Y.). Tissue culture medium (IMEMZO) was purchased from Associated Biomedic Systems (Buffalo, N.Y.) and PBS was obtained from Biofluids Inc. (Rockville, Md).

The radioinert pyrethroids tested were: pyrethrins, fenothrin, fenvalerate, fluvalinate, bioallethrin, permethrin, and resmethrin. They were obtained from Reed and Carnrick Pharmaceutical (Piscataway, N.J.), via the McLaughlin Gormley King Company (Minneapolis, Minn.). With the exception of the pyrethrins (a natural product of 20% purity containing several stereoisomers), the samples varied in purity from 80 to 100%, depending on the degree of synthesis reaction completeness and the amount of solvent in the final distillate. All samples were dissolved in absolute ethanol on a weight/volume basis. Molarity was estimated on the basis of the degree of purity provided by the manufacturer and the molecular weight.

Cell culture

Fibroblast cultures were established from genital skin specimens obtained from neonates at routine circumcision under an approved protocol as previously described [5, 6]. When colonies of fibroblasts had grown to approximately 50% confluence in the original culture flask (usually within 1–3 weeks), the cells were detached with 0.05% trypsin– 0.02% EDTA in PBS at 37°C and passed serially into larger flasks (75 and 150 cm²).

Whole cell [³H]R1881 binding assay

The binding of pyrethroids to the androgen receptor was determined by competitive displacement of [³H]R1881 from receptor sites on cultured human fibroblasts using a dispersed, intact cell system as previously described [5, 6], with the binding studies performed at 22°C to minimize metabolism of the radioligand and competitors, except that [³H]R1881 was substituted for [³H]DHT. Competition assays were performed with 0.5–1.0 nM [³H]R1881 and in-creasing amounts of the nonradioactive compounds. In some experiments, fixed amounts of competitor or vehicle (ethanol) were added to increasing amounts (0.1–1.0 nM) of [³H]R1881. Binding to low affinity sites was determined in the presence of 5×10^{-7} M R1881 and was subtracted from whole cell binding of [³H]R1881 obtained in the absence of any inhibitor to assess binding to high affinity sites (specific binding). All compounds were dissolved and diluted in ethanol and added in 10 μl aliquots to each tube. The ethanol was also added to separate tubes containing standard amounts of unlabelled R1881 (0.2, 0.5, and 1.0 nM), which were included in each assay as an internal control. The final incubation volume was 1.0 ml. The amount of R1881 required for 50% displacement of [³H]R1881 was 0.50 ± 0.08 (SE) nM (5 assays), identical to the K_d determined directly by Scatchard analysis with increasing amounts of [³H]R1881 [6]. Individual data points were determined in duplicate. The relative binding activity (RBA) of the test compound was calculated as the ratio of the concentration of unlabelled R1881 to the concentration of competitor required for 50% displacement of high affinity binding of [³H]R1881. The association constant of pyrethroid binding to the androgen receptor was evaluated from this RBA and the previously determined association constant of R1881 for binding to the androgen receptor in this system of 2×10^{9} M^{-1} [6].

SHBG binding assay

All studies were performed using pooled citrated plasma obtained from the Blood Bank from which the endogenous steroids had been removed by charcoal adsorption [7]. Binding to SHBG was determined at 37°C with [³H]testosterone using a solid phase method with Con-A-Sepharose as previously described [8]. Cortisol (2 μg) was also added to each tube in order to minimize the possible contribution of transcortin to the total [³H]testosterone binding of the absorbed glycoprotein fraction. Non-specific or low affinity binding was determined in the presence of 10^{-5} M unlabelled testosterone.

RESULTS

Binding to the androgen receptor

Binding of the pyrethroids to the human androgen receptor was assessed by determining the ability of each compound to displace [³H]R1881, a non-metabolizable synthetic androgen, from androgen binding sites in dispersed, intact cultured fibroblasts (Fig. 1). All of the pyrethroids tested were able to compete for androgen binding sites with slopes similar to the displacement curve for unlabelled R1881, albeit with 10^{-4} and 10^{-5} lower potencies. The natural pyrethrins were the most effective competitor, while resmethrin was the weakest. Fenothrin had an intermediate potency with 50% displacement of [³H]R1881 achieved at a concentration of
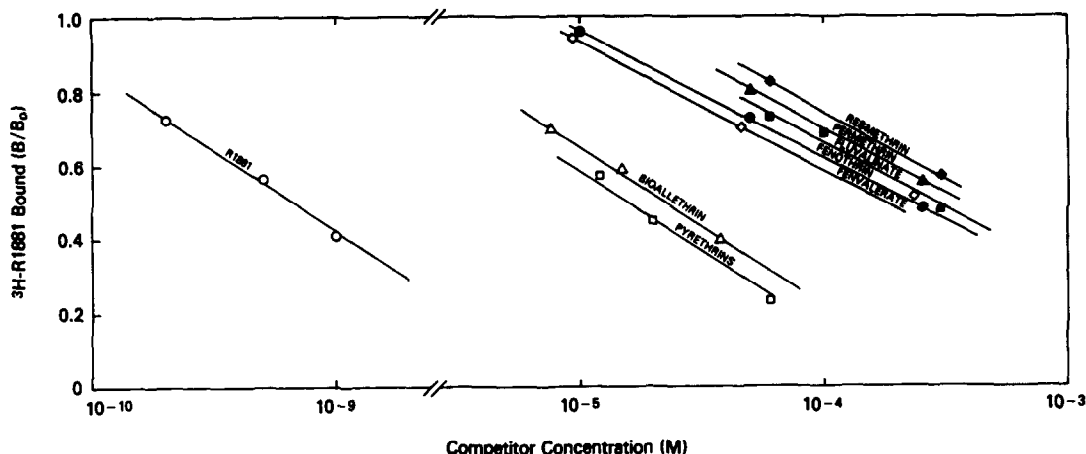
Fig. 1. Ability of various pyrethroids to compete for [³H]R1881 binding to androgen binding sites in dispersed intact human genital skin fibroblasts compared to R1881. Cells ($0.5-2.0 \times 10^6$ cells/tube) were incubated for 60 min at 22°C with $0.5-1.0 \times 10^{-9}$ M [³H]R1881 in the presence of increasing concentrations of the compounds shown. The values shown represent the average of duplicate determinations. $B/B_0 = 1$ represents the whole cell binding of [³H]R1881 in the absence of any inhibitor minus low affinity binding (plotted as $B/B_0 = 0$) of [³H]R1881 determined in the presence of 5×10^{-7} M R1881.

approximately 10^{-4} M. Initial androgen receptor binding studies with fenothrin, stated as being negative in the preliminary report [4], were erroneous, possibly due to incorrect reconstitution of the original samples. In that all the compounds tested, which were made separately and had varying degrees of purity, interacted with the androgen receptor with binding curves parallel to that for R1881, it is highly unlikely that the impurities accounted for the androgen receptor competition. Furthermore, fluvalinate, which was 100% pure, displaced [³H]R1881 nearly as well as or better than other compounds tested and with a parallel displacement curve. The effect of the addition of the insecticides on the solubility of the tracer was not determined. How-
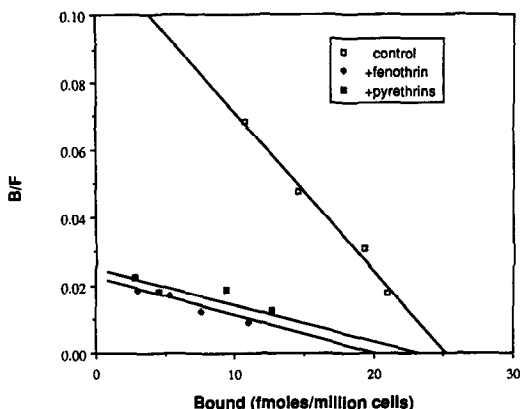
ever, all samples, regardless of the dilution, were added in a fixed volume of vehicle, and no precipitate became visible when the pyrethroid solutions were added.

When fixed concentrations of the pyrethrins or fenothrin were added to increasing amounts of [³H]R1881 in the binding reaction, competitive inhibition of androgen receptor binding was achieved as demonstrated by Scatchard plots of the binding data (Fig. 2). There was no effect of the pyrethrins on non-specific binding (data not shown).

Table 1 gives the relative binding affinities for the androgen receptor of each compound based on the composite results of several experiments. The mean K_i-values ranged from 1.5×10^{-5} to 4.4×10^{-4} M; in comparison, the K_i for cimetidine in this system is 1.4×10^{-4} M [6].

Lindane, a structurally unrelated insecticide, was also tested in this system and showed no inhibition of androgen receptor binding (data not shown), although it was difficult to test it at concentrations greater than 10^{-4} M because of limited solubility in aqueous solutions.

Fig. 2. Scatchard plot of specific binding of increasing concentrations of [³H]R1881 to androgen binding sites in dispersed, intact human genital skin fibroblasts. (□) Binding data obtained with the addition of vehicle (ethanol) alone; (■) binding data obtained with addition of 5×10^{-5} M pyrethrins; (◆) binding data obtained with the addition of 1×10^{-4} M fenothrin.

Table 1. RBAs of pyrethroids for human genital skin fibroblast androgen receptors

Compound	K_i(M)[a]	RBA ($\times 100$)[b]
R1881	$0.50 \pm 0.08 \times 10^{-9}$ (5)	100.0
Pyrethrins	1.5×10^{-5} (2)	0.00033
Bioallethrin	2.5×10^{-5} (2)	0.00020
Fenvalerate	1.8×10^{-4} (2)	0.000027
Fenothrin	2.3×10^{-4} (2)	0.000022
Fluvalinate	2.8×10^{-4} (2)	0.000018
Permethrin	3.3×10^{-4} (2)	0.000015
Resmethrin	4.4×10^{-4} (2)	0.000011

[a]Values are mean ± SEM of the concentration required for 50% inhibition of fibroblast [³H]R1881 binding. The number of determinations is in parentheses.
[b]RBA was calculated as the ratio of the K_i for R1881 to the K_i for the test compound.
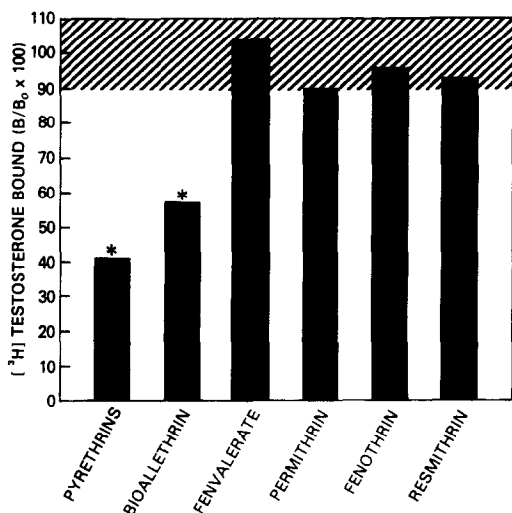
Fig. 3. Ability of pyrethroids $(10^{-4}\,M)$ to compete for [³H]testosterone binding to SHBG. The values above represent the average of duplicate determinations. Testosterone ED_{50} was $2 \times 10^{-9}\,M$. *Indicates $P < 0.01$. Shaded area indicates 95% confidence limit for zero dose.

Binding to SHBG

The binding of pyrethroids to human sex hormone binding globulin present in plasma was assessed by determining the ability of each compound to displace [³H]testosterone bound to SHBG. As can be seen in Fig. 3, only the pyrethrins and bioallethrin were able to compete for binding to SHBG when added at a concentration of 10^{-4} M. The pyrethrins inhibited the

binding of [³H]testosterone to SHBG by 60% while bioallethrin inhibited [³H]testosterone binding by 43%. None of the other pyrethroids competed significantly with [³H]testosterone for binding to SHBG.

DISCUSSION

Compounds which bind to hormone receptors can be either hormone agonists or antagonists. From the data presented in this study it is clear that the pyrethroids bind competitively to the human androgen receptor. However, there are insufficient *in vivo* data to indicate whether all the compounds in this class of drugs act as weak androgens, as inhibitors of androgen action, or whether there are some in each category. Preliminary studies performed with an' insecticide spray associated circumstantially with an epidemic of gynecomastia, suggested that a component of the spray, "multicide", containing the pyrethroid fenothrin (see Fig. 4 for chemical structure), acted as an anti-androgen when given intraperitoneally to androgen-treated prepubertal male rats [4]. Despite having two rings, structurally fenothrin appears to bear little resemblance to a steroid or to three other non-steroidal anti-androgens, cimetidine, flutamide, and ketoconazole, all associated clinically with gynecomastia [9–11]. Although gynecomastia often results from excess estrogen action, it is also believed to be a manifestation of inadequate androgen action or androgen antagonism in humans [12]. Thus, the data reported here are consistent with the possibility that fenothrin, at least,
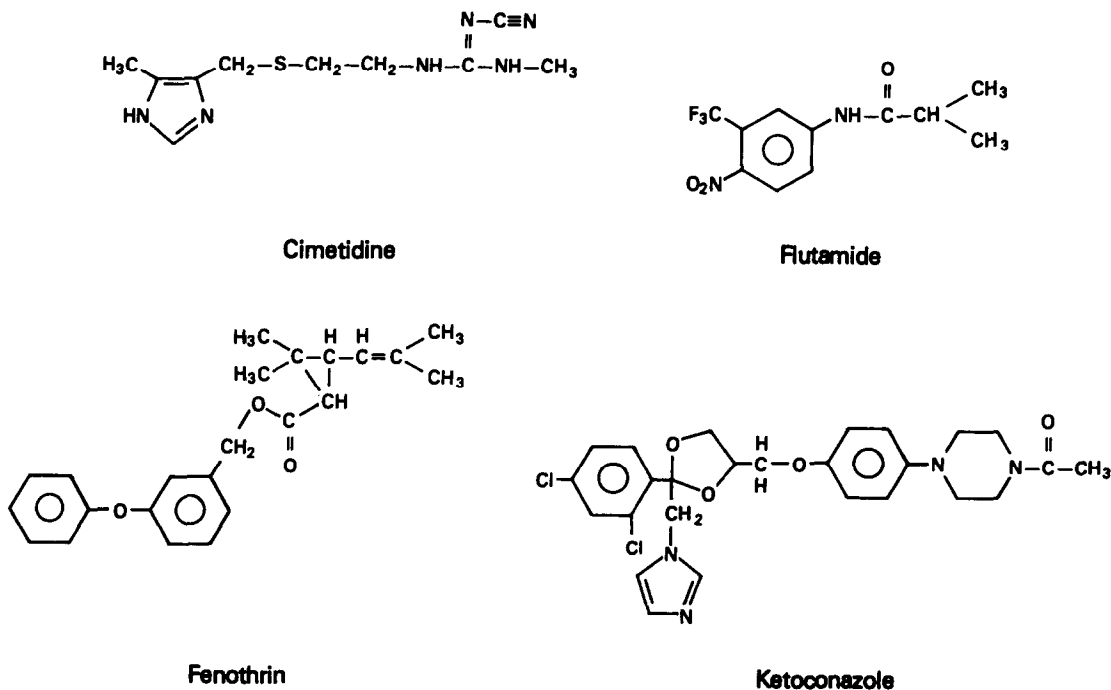

Fig. 4. Chemical structures of non-steroidal compounds capable of binding to androgen receptors and/or having anti-androgenic properties.

could conceivably produce gynecomastia by opposing androgen action in the human male breast.

Although the studies presented here indicate potential novel properties for the pyrethroids, it is not necessarily the case that these compounds exert anti-androgenic effects in humans or animals as currently used. First, humans or animals at risk to develop the features of impaired androgen action may not be exposed to amounts of the compounds that are active in this regard; the studies presented here indicate that these agents are relatively weak binders to the androgen receptor and huge amounts would probably be required to induce an effect. Second, *in vivo* metabolism of these compounds may lead to biotransformation to congeners with diminished or no affinity for the androgen receptor. Third, the pyrethroids are believed not to be absorbed through intact skin [1], and thus ingestion or inhalation would have to be the portal of entry. Finally, as to why anti-androgenic properties of these agents have not been noted before, it is possible that subtle androgen antagonism has, in fact, occurred from exposure to these agents but was not carefully looked for in early toxicity studies when this biologic property of the pyrethroids was not suspected.

This is not the first instance in which non-steroidal compounds have been shown to have affinity for the androgen receptor. Flutamide, an early non-steroidal agent used to treat prostate diseases that must be activated *in vivo* for full potency, has a reasonably high affinity for the androgen receptor and is an antagonist that has been used clinically [6, 9, 13, 14]. Two other structurally unrelated compounds, cimetidine, a histamine H_2 receptor antagonist, and ketoconazole, an imidazole cytochrome $P450$ inhibitor, have similar or lower affinities for the androgen receptor comparable to that of the pyrethroids and are both associated with gynecomastia in humans, presumably because they, too, are anti-androgens [6, 10, 11, 15]. The fact that some of the pyrethroids also bind to SHBG supports the notion of a structural similarity to natural androgens that may be the basis of the androgen receptor binding.

Until additional toxicological and endocrine studies can be conducted *in vivo* with the pyrethroids, it appears prudent to advise protection from any form of contact or ingestion of the pyrethroids in order to prevent any undesirable effects on the human reproductive system. Just as recent studies into the neurotoxicity of the pyrethroids have indicated interactions of these compounds with the GABA receptor and possibly another CNS stereo-specific receptor, the calmodulin-phosphodiesterase system, and sodium channels in the mammalian brain [16–18], the present studies may cast new light on the role of pyrethroids in androgen target tissues. Furthermore, now that the androgen receptor has been cloned [19–21] and its amino acid sequence deduced [22], including the hormone binding portion of the molecule, three-dimensional modelling with steroidal and non-steroidal compounds, like the pyrethroids, should provide insight into the structural requirements for the ligand binding site of the human androgen receptor protein.

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