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EFFECTS OF DICYCLOHEXANE DERIVATIVES ON ANDROGEN METABOLISM IN THE RAT PROSTATE

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Summary—Dicyclohexane derivatives, which inhibit the binding of testosterone and dihydrotestosterone (DHT) to the androgen-binding protein (ABP) of rat epididymis without interfering with their binding to the androgen receptor, show a similar selectivity in their effects on androgen metabolism. Their ability to inhibit the aromatization of testosterone has been reported previously. This paper demonstrates that they are potent inhibitors of 3α(β)-hydroxysteroid:NAD(P)$^+$ oxidoreductase activity (3-HSD) in the particulate fraction from rat prostate gland; the values of K_i for their inhibition of this enzyme are similar to that of the K_m for DHT as substrate. The dicyclohexane derivatives are markedly less effective against the cytosolic NADPH-dependent 3-HSD, and they do not appear to inhibit testosterone 5α-reductase activity. These characteristics are likely to complicate the proposed use of the dicyclohexane derivatives as probes for the role of ABP *in vivo*. However, they may be of interest in the study of structure–activity relationships in androgen-metabolizing enzymes, particularly in the examination of the different forms of 3-HSD.

INTRODUCTION

Dicyclohexane compounds such as PRDX, PMDX and PRXL, derived from diethylstilboestrol (DES) or *meso*-hexoestrol [1, 2], are inhibitors of the binding of testosterone and dihydrotestosterone (DHT) to the androgen-binding protein (ABP) of rat testis and epididymis *in vitro*, but do not interfere with the binding of androgens to their intracellular receptor [3]. These characteristics suggest that such compounds might be useful in examining the putative role of ABP in spermatogenesis and sperm maturation *in vivo* without disrupting receptor-mediated androgen action.

However, androgen action can depend on steroid metabolism as well as on interaction with steroid-binding proteins; analogues of steroid hormones might influence androgen effects by alteration of steroid metabolism even in the absence of a direct action at the receptor level. We have already shown that the dicyclohexane compounds are competitive inhibitors of the aromatization of testosterone in human placental microsomes *in vitro* [4], and inhibit hormone-stimulated oestrogen production by primary cultures of Sertoli cells from immature rats [2]. In androgen target tissues, rather than those involved in oestrogen synthesis, the most important pathway for the metabolism of testosterone is 5α-reduction to DHT, the more potent androgen, and the reduction of DHT to the less active 5α-androstane-3α,17β-diol (3α-diol) and 5α-androstane-3β, 17β-diol (3β-diol) [5, 7]. We have therefore examined the effect of the dicyclohexanes on the activities of NADPH-dependent 4-ene-3-ketosteroid 5α-reductase (5α-reductase) and 3α(β)-hydroxysteroid:NAD(P)$^+$ oxidoreductase (3-HSD) in rat prostatic tissue fractions *in vitro*.

EXPERIMENTAL

Materials

[1,2,6,7-^{3}H]Testosterone (94–109 Ci/mmol) and [1,2,4,5,6,7-^{3}H]dihydrotestosterone (104–158 Ci/mmol) were obtained from Amersham International PLC. Nonradioactive steroids were supplied by the Sigma Chemical Company. Buffer constituents were purchased from Merck [Tris, Na_2HPO_4, NaH_2PO_4, sodium acetate, sucrose, dithiothreitol (DTT), acetic acid] or Boehringer [MES: 2-(*N*-morpholino)ethanesulphonic acid]. NADH, NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were also supplied by Boehringer. Solvents, of analytical or HPLC grades, were from Carlos Erba, UCB and Merck.

The synthesis of the dicyclohexane compounds used in this study has been described previously [1, 2]. Thin-layer chromatography (TLC) was carried out on silica 60 F_{254} pre-coated on plastic sheets (Merck). Male Wistar rats (150–200 *g*) were obtained from the Proefdierencentrum, Catholic University of Leuven.

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Systematic names for the dicyclohexane derivatives used in this study: PRDX, *d,l*-3,4-*bis*(4-oxocyclohexyl)-hexane; PMDX, *meso*-3,4-*bis*(4-oxocyclohexyl)-hexane; PRXL, *d,l*-3-(4-oxocyclohexyl)-4-(*cis*-4-hydroxycyclohexyl)-hexane; PRTL, *d,l*-3,4-*bis*(*trans*-4-hydroxycyclohexyl)-hexane.

Tissue fractionation

Animals were killed by cervical dislocation. Ventral prostate glands were removed, and frozen by immersion in liquid nitrogen before storage at −85°C. Tissue fractions were prepared as follows, based on the procedure of Liang and Heiss[8]. Tissue was chopped finely, thawed and homogenized (Ultraturrax, 3 × 10 s at 30 s intervals) in 3 vol 20 mM sodium phosphate buffer, pH 6.5, containing sucrose (0.32 M) and DTT (0.1 mM). The homogenate was fractionated by differential centrifugation at 10,500 rpm (g_{max} = 10,000) for 10 min and at 39,300 rpm (g_{max} = 140,000) for 1 h, using a Beckman 50 Ti II rotor in a Beckman L3-50 ultracentrifuge. The 140,000 *g* supernatant was retained as the cytosol fraction. The two pellets were washed once by suspension in 2–4 pellet volumes of buffer and recentrifugation, and then resuspended in buffer. Each fraction (cytosol, 10,000 *g* pellet, microsomes) was stored in small aliquots at −20°C until use.

5α-Reductase assay

The reaction mixture, final vol 0.5 ml, included DTT (1 mM), sodium phosphate buffer (0.04 M, pH 6.5), [^{3}H]testosterone (0.1 μCi), nonradioactive testosterone (2 μM, or as specified in figure legends) test compound if included (40 μM, or as specified in Figure legends), an NADPH-generating system consisting of glucose-6-phosphate (10 mM), NADP (1 mM) and glucose-6-phosphate dehydrogenase (5 U), and the enzyme preparation (combined 10,000 *g* and microsomal fractions, 0.2 mg protein). After incubation at 37°C for the specified period, tubes were chilled in ice-water and ethyl acetate (3 ml) was added. Tubes were vortexed (2 × 30 s), centrifuged (2500 rpm, 5 min), and the aqueous phases frozen in acetone–solid CO_2 prior to decantation and evaporation under nitrogen of the organic extracts. Residues were transferred with carrier steroids (testosterone, androstenedione and DHT) in ethyl acetate to TLC plates. After development in toluene–ethyl acetate, 5:3, v/v (2 × 15 cm) testosterone and androstenedione were located by fluorescence quenching under u.v. illumination, and DHT by prolonged exposure to I_2 vapour. Appropriate zones of the plate were cut, placed in vials with 5 ml Beckman HP/b scintillant, and the amount of tritium was measured. DHT formation, based on the proportion of recovered tritium located in the region of the carrier DHT, was expressed in terms of pmol·mg protein^{-1}.

3α(β)-Hydroxysteroid oxidoreductase assay

In initial studies the conditions for incubation, extraction and product isolation were identical to those used for the measurment of 5α-reductase activity, except that [^{3}H]DHT and nonradioactive DHT replaced [^{3}H]testosterone and nonradioactive testosterone as substrate. The carrier testosterone was used as a marker for 3α(β)-diol location on TLC. Enzyme activity was calculated from the proportion of recovered tritium migrating as 3α(β)-diol; no account was taken of possible further metabolism of the androstanediols.

Further experiments examined NADH- and NADPH-dependent activities. Incubations for the latter retained the NADPH-generating system for cofactor provision, but were carried out in 0.01 M Tris–HCl buffer, pH 7.4. NADH-dependent activity was examined in the presence of NADH (1 mM) in 0.05 M sodium acetate buffer, pH 4.6. The degradation of NADH is not negligible at this pH, and the cited concentration thus has a nominal value only.

A final series of experiments involved the separation of 3α- and 3β-diols by TLC in benzene (or toluene)–95% ethanol, 9:1, v/v (2 × 15 cm) [9]; the respective carrier steroids were located by I_2 vapour. Incubations were carried out in 0.01 M MES buffer, pH 6.5, whether NADPH (generating system) or NADH was used as cofactor.

Other methods

Tritium was measured at 30–35% efficiency in a Beckman LS 1800 liquid scintillation counter. Protein concentrations were determined by the method of Lowry[10].

Values for K_m and V_{max} were derived from saturation analysis experiments by graphical treatment of the data using direct linear plots [11].

RESULTS

Effect of dicyclohexane derivatives on DHT formation from testosterone

We first examined whether the dicyclohexane derivatives might inhibit the conversion of testosterone to DHT. In fact, DHT accumulation during the incubation of testosterone with pooled non-cytosol fractions of the rat prostate was increased in the presence of dicyclohexane derivatives. DHT production in control incubations was 6.0 pmol·min^{-1}·mg protein^{-1}; in the presence of PMDX, PRDX, PRXL or PRTL the rates were 12.6, 12.2, 10.6 and 7.8 pmol·min^{-1}·mg protein^{-1}, respectively. This effect was not seen with the precursors of these compounds, diethylstilboestrol or hexoestrol, which were weak inhibitors of DHT production (data not shown). Progesterone, an efficient substrate for 5α-reduction, and a potent competitive inhibitor of testosterone 5α-reductase activity, effectively decreased DHT formation, as expected [12] (data not shown).

The increase in DHT production provoked by PRDX was dose-dependent, 50% of the maximum increase being obtained at approx. 3–4 μM test compound (Fig. 1a). This effect resulted from an increase in apparent V_{max} for the reduction, with no change in apparent K_m value (Fig. 1b).

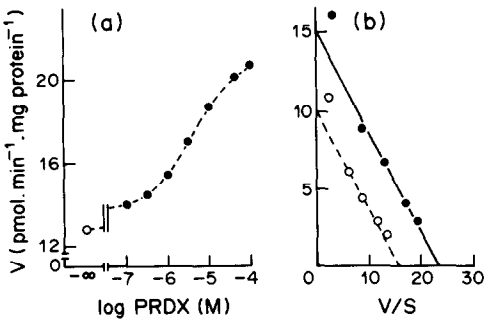

Fig. 1. Effect of PRDX on DHT formation from testosterone in rat prostate particulate fraction. In each experiment, incubation was for 10 min at 37°C with 0.2 mg protein. (a) testosterone (5 μM) alone or in the presence of the indicated concentrations of PRDX. (b) testosterone (0.15–5 μM) in the presence (●——●) or absence (○---○) of PRDX (40 μM). Results are displayed on an Eadie–Hofstee plot. Lines are obtained using values of K_m and V_{max} derived from direct linear plots.

Effect of dicyclohexane compounds on DHT metabolism

We examined the possibility that the increased production of DHT from testosterone in the presence of dicyclohexanes was due to an inhibition of the further metabolism of DHT. Preliminary experiments were carried out under conditions identical to those used for 5α-reductase measurement, except for the use of DHT as substrate. This work confirmed that such metabolism occurred, that it was inhibited by PRDX, and that the major metabolites co-migrated with carrier 5α-androstane-3α/β,17β-diols in the TLC system used (data not shown). Moreover, carrier testosterone was not fully resolved from 3α/β-diols in these conditions; metabolism of DHT had therefore not been detected in the 5α-reductase measurements.

Saturation analysis of this putative 3-HSD activity was carried out in the presence or absence of the dicyclohexanes using either cytosol or 10,000 *g* pellet as enzyme sources. The three compounds tested, PRDX, PMDX and PRXL, were either competitive inhibitors of 3-HSD activity or mixed type inhibitors with a predominant competitive component. Some results for PRDX and PMDX are shown in Fig. 2. The K_m values for DHT as substrate were 0.6 ± 0.1 μM ($n = 4$) and 2.0 ± 0.4 μM ($n = 7$) in cytosol and 10,000 *g* pellet fractions, respectively. K_i values measured for dicyclohexane compounds in single experiments were as follows (cytosol value precedes that for 10,000 *g* pellet): PMDX, 39 and 0.7 μM; PRXL, 136 and 1.1 μM; PRDX, 412 and 1.0 μM. It is noteworthy that for all three test compounds the K_i value for the inhibition of the cytosolic activity was much higher than that for the activity in the particulate fraction.

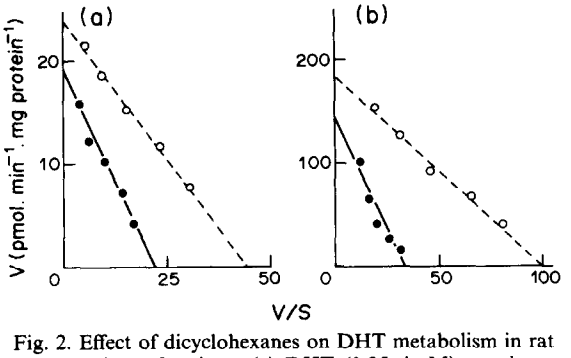

Fig. 2. Effect of dicyclohexanes on DHT metabolism in rat prostate tissue fractions. (a) DHT (0.25–4 μM) was incubated with cytosol (0.35 mg protein) for 10 min at 37°C in the presence (●——●) or absence (○---○) of PMDX (40 μM). (b) DHT (0.5–8 μM) was incubated with 10,000 *g* pellet fraction (0.13 mg protein) for 10 min at 37°C in the presence (●——●) or absence (○---○) of PRDX (2 μM). Mean values for duplicate incubations are shown on Eadie–Hofstee plots. Lines are obtained using values of K_m and V_{max} derived from direct linear plots.

Effect of PRDX on NADH- and NADPH-dependent 3-HSD activities in prostatic tissue fractions

The apparent ability of the dicyclohexane compounds to discriminate between cytosolic and particulate forms of 3-HSD led us to examine the three major categories of 3-HSD activity in androgen target tissues defined by Verhoeven *et al.*[13]: NADH-dependent activity in the particulate fraction (of which some is extracted into cytosol during tissue fractionation), NADPH-dependent activity in the particulate fraction, and a distinct NADPH-dependent activity in the cytosol. The relatively acid-stable NADH-dependent activity was measured in acetate buffer, pH 4.6, and the NADPH-dependent activities in Tris–HCl, pH 7.4. Representative results are shown in Fig. 3, and K_i values for competitive inhibition obtained in the various conditions are summarized in Table 1. In most cases the K_i for

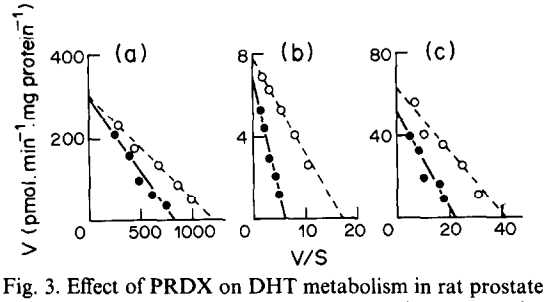

Fig. 3. Effect of PRDX on DHT metabolism in rat prostate tissue fractions. In all three examples incubation was for 10 min at 37°C. Mean values for duplicate incubations are displayed on Eadie–Hofstee plots. Lines are obtained using values of K_m and V_{max} derived from direct linear plots. (a) DHT (0.5–8 μM) was incubated with microsome fraction (5 μg protein) in sodium acetate buffer, pH 4.6 containing NADH (1 mM) in the presence (●——●) or absence (○---○) of PRDX (2 μM). (b) DHT (0.25–4 μM) was incubated with cytosol (0.4 mg protein) in Tris–HCl buffer, pH 7.4, containing an NADPH-generating system in the presence (●——●) or absence (○---○) of PRDX (288 μM). (c) DHT (0.5–8μM) was incubated with 10,000 *g* pellet fraction (0.1 mg protein) in Tris–HCl buffer, pH 7.4, containing an NADPH-generating system in the presence (●——●) or absence (○---○) of PRDX (3.2 μM).

Table 1. 3-HSD activity in rat prostate tissue fractions, and its inhibition by PRDX

Cofactor	pH	Fraction	K_m DHT (μM)	K_i PRDX (μM)
NADH	4.6	Cytosol	6.7 ± 3.0*	5.6 ± 3.0
		Microsomes	2.7 ± 1.4	3.6 ± 1.0
		10,000 *g* pellet	1.6 ± 0.3	2.4 ± 0.5
NADPH	7.4	Cytosol	0.6 ± 0.1	238 ± 71
		Microsomes†	(0.8, 2.3)	(0.6, 2.8)
		10,000 g pellet	1.5 ± 0.2	2.6 ± 1.1

*Mean ± SD ($n = 3$).
†Type of inhibition not reproducible between experiments.

PRDX was similar in magnitude to the K_m for DHT; the sole exception was the NADPH-dependent activity in cytosol, for which PRDX was a much less potent inhibitor. Thus, the pattern observed in the preliminary experiments was confirmed.

Effect of PRDX on the formation of 3α- and 3β-diols

The major product of DHT metabolism in all cases examined (NADH-dependent activity in microsomes, NADPH-dependent activities in cytosol and in 10,000 *g* pellet) migrated on TLC with 3α-diol carrier rather than with 3β-diol: the proportion of 3α-isomer was 87–99%. This distribution was not significantly altered by PRDX. The accumulation of 3β-diol was too low to reproducibly assess the effect of PRDX on 3β-HSD activity. When particulate fractions from rat liver instead of prostate were used as source of enzyme the products of NADPH-dependent DHT metabolism migrated with 3α-diol and 3β-diol carriers in approximately equal amounts. Preliminary experiments have not indicated any reproducible difference in PRDX effects on 3α- and 3β-HSD in these circumstances (data not shown).

DISCUSSION

Our initial studies of the effect of dicyclohexane derivatives on 5α-reductase activity in rat prostate particulate fraction were based on the assumption that metabolism of the product, DHT, to 3α(β)-diol was negligible. No formation of 3α(β)-diol was detected by Liang and Heiss[8] using incubation conditions which differed from those in our studies only by the use of NADPH rather than an NADPH-generating system, and of tissue from adult rather than pubertal rats. Our results, showing an apparent increase in the formation of DHT from testosterone in the presence of dicyclohexane compounds (Fig. 1), forced us to reconsider this aspect of the work. Preliminary checks, involving the incubation of DHT under identical conditions, confirmed that this substrate was extensively metabolised, and that the reaction was substantially inhibited by the concentrations of dicyclohexane compound used in the experiments on 5α-reductase activity. The major product(s) of DHT metabolism migrated with carrier 3α/β-diol on TLC: in our hands the diols were not fully resolved from testosterone either by the solvent system used in our work or that used by Liang and Heiss[8], and so would not have been detected in our testosterone 5α-reductase assays.

Saturation analysis experiments indicated that PRDX, PMDX and PRXL were potent competitive inhibitors of NADPH-dependent 3-HSD activity in the 10,000 *g* pellet fraction, while inhibition of the cytosol 3-HSD was much less pronounced (Fig. 2). More extensive examination of PRDX effects on 3-HSD activities in various tissue fractions, in conditions optimal for either NADH- or NADPH-dependent activities [13], confirmed that the dicyclohexane was a weak inhibitor of the cytosolic NADPH-dependent 3-HSD, but showed that for all other categories of the enzyme inhibition by PRDX was effective (Fig. 3, Table 1). The concentrations of dicyclohexanes required to inhibit cytosolic NADPH-dependent activity are high, and we cannot exclude the possibility of a general effect due to the lipophilic nature of the compounds; however, this might be expected to produce a more noncompetitive inhibition, which is not observed.

In the presence or absence of PRDX virtually all the product co-migrated with 3α-diol rather than 3β-diol on TLC in a solvent system which provided a degree of resolution of the isomers [9], although more rigorous product identification has not been carried out. The predominance of the 3α-isomer has been widely reported in the literature, but apparently reflects rapid further metabolism of the 3β-diol rather than an absence of 3β-HSD activity, at least for NADPH-dependent activity in particulate fractions [14]. Experiments on rat liver 10,000 *g* pellet fraction, in which 3β-diol is formed in appreciable quantities, showed that PRDX inhibits 3β- as well as 3α-HSD, although a complex pattern of inhibition for each reaction, perhaps due to multiple activities of differing sensitivities, made conclusions difficult.

These results suggest that the use of the dicyclohexanes to investigate the role of ABP and its binding of androgens in the testis and epididymis *in vivo* may be compromised by the potentially disruptive effect of these compounds on the normal metabolic regulation of DHT concentrations in androgen target tissues. The values of K_i reported here for the inhibition of particulate 3-HSD activity by PRDX are of the same order of magnitude as that of the K_m for DHT as substrate. In contrast, the value of K_i for the inhibition of DHT binding to ABP by PRDX (0.16 μM) [15] is two orders of magnitude greater than the K_d for the DHT–ABP interaction (2.3 nM) [16]. If these parameter values obtained *in vitro* are applicable to the appropriate interactions *in vivo*, then a treatment *in vivo* which leads to a concentration of PRDX in, say, the epididymis sufficient to inhibit the binding of DHT to ABP, and thus reduce the availability of the androgen at the luminar epithelium, would probably also lead to an inhibition of 3-HSD activity. This effect on DHT catabolism would tend to counteract

and obscure any consequences of the effect of PRDX on androgen binding to ABP. In our previous efforts to identify compounds of interest for the study of the role of ABP we have screened candidates on the basis of their ability to selectively inhibit the binding of DHT to ABP rather than to androgen receptor [3, 17]. The present report suggests that their ability to interfere with androgen metabolism should also be taken into consideration.

In more general terms, the dicyclohexane compounds, and possible derivatives, may be of interest in the study of the differing structure–activity relationships for inhibitors of the cytosolic and particulate 3-HSD activities. Verhoeven *et al.*[13] mentioned compounds, such as 5α-pregnane-3α,11β,17α,21-tetra-ol-20-one, which had properties similar to those of PRDX, that is, preferential inhibition of particulate rather than cytosolic 3-HSD. However, most inhibitors of 3-HSD reported in the literature, such as 5α-pregnane-3,20-dione [13], 6α-methylprednisolone [18] or medroxyprogesterone acetate [19, 20], show the inverse pattern, effectively inhibiting the cytosol enzyme but not that in the particulate fraction. As an example of apparently different active sites within one tissue catalyzing the same reaction, this system may repay more profound investigation.

In a still wider context, dicyclohexane compounds display a distinct selectivity in terms of their inhibition of some (3-HSD, aromatase, ABP-binding) but not all (5α-reductase, receptor-binding) processes involving testosterone and DHT. As such, they and potentially more specific derivatives promise to have further applications in the comparative investigation of the various different sites capable of interaction with common substrates, the androgens.

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