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Differential Effects of Trilostane and Cyanoketone on the 3β-Hydroxysteroid Dehydrogenase-isomerase Reactions in Androgen and 16-androstene Biosynthetic Pathways in the Pig Testis

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3β-Hydroxysteroid dehydrogenase-isomerase (3β-HSD-I) activity in the pig testis is responsible for the conversion of dehydroepiandrosterone (DHA) to 4-androstenedione and also for the conversion of 5,16-androstadien-3β-ol (andien-β) to 4,16-androstadien-3-one (dienone). Therefore, 3β-HSD-I plays an essential role in the biosynthesis of hormonally and pheromonally active steroids. Previous studies from this laboratory have suggested that the 3β-HSD-I reactions in the androgen and 16-androstene biosynthetic pathways may be catalysed by different enzymes with selective substrate specificities [3, 4]. The aim of the present studies was to investigate the reactions further by examining the effects of two classical steroidal inhibitors of 3β-HSD-I, trilostane (WIN 24540) and cyanoketone (WIN 19578), on the kinetic parameters of the 3β-HSD-I reactions in immature (< 3 weeks) pig testis microsomes. In kinetic analyses of the conversion of DHA to 4-androstenedione, both trilostane and cyanoketone caused increases in the $K_{m(app)}$ for DHA which at the highest concentration used, were 15-fold the control $K_{m(app)}$ of 1.4 μmol/l. No effect on the $V_{max(app)}$ (6.55 ± 0.74 nmol/h/mg protein) was observed, demonstrating that competitive inhibition was evident. Slope and intercept replots confirmed the competitive nature of the inhibition and $K_{i(app)}$ values of 0.16 μmol/l for trilostane and 0.20 μmol/l for cyanoketone were respectively 9 and 7-fold lower than the $K_{m(app)}$ value. In contrast, trilostane and cyanoketone had no effect on the $K_{m(app)}$ for andien-β (0.26 μmol/l). The $V_{max(app)}$ (1.12 nmol/h/mg protein) was decreased by 40–50% only by trilostane at the highest concentration used, demonstrating a very low affinity for the andien-β active site. $K_{i(app)}$ values for trilostane and cyanoketone, obtained from slope and intercept replots were, respectively 1.1 and 1.6 μmol/l, which were 4 and 6-fold greater than the $K_{m(app)}$ for andien-β. Therefore, trilostane and cyanoketone were powerful competitive inhibitors of the conversion of DHA to 4-androstenedione but were weak non-competitive inhibitors of the conversion of andien-β to dienone. The selective effects of trilostane and cyanoketone on the 3β-HSD-Is involved in the androgen and 16-androstene biosynthetic pathways strongly suggest that the reactions are catalysed by separate enzymes, or at least separate, non-interacting active sites on a single enzyme.

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INTRODUCTION

The enzyme 3β-hydroxysteroid dehydrogenase-isomerase (3β-HSD-I, EC 1.1.1.51 and EC 5.3.3.1) converts 5-ene-3β-hydroxysteroids to the 4-ene-3-oxo configuration and, therefore, plays an essential role in the biosynthesis of hormonally active steroids such as androgens, estrogens and corticosteroids. In the pig testis, androgen biosynthesis from pregnenolone proceeds primarily through the action of 17-hydroxylase

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C-17,20 lyase (EC 1.14.99.9) which converts pregnenolone to dehydroepiandrosterone (DHA) which is then converted to 4-androstenedione by 3β-HSD-I [1]. An alternative fate for pregnenolone is the conversion to the odoriferous and pheromonally active 16-androstene steroids. This pathway involves the conversion of pregnenolone to 5,16-androstadien-3β-ol (andien-β) catalysed by a modified 17-hydroxylase C-17,20 lyase which removes the side chain of pregnenolone without the introduction of an oxygen function at position C17 of the steroid nucleus. Andien-β is then converted to 4,16-androstadien-3-one (dienone) by 3β-HSD-I. Further metabolism of dienone by the sequential actions of 5α-reductase and 3α- or 3β-HSD's leads to the formation of 5α-androstenone (5α-*A*), 5α-androsten-3α-ol (an-α) and 5α-androsten-3β-ol (an-β) [1]. 5α-*A* and an-α have pheromonal activities in pigs [2] and, therefore, 3β-HSD-I plays an essential role in the biosynthesis of these pheromones.

Evidence from previous studies [3, 4] suggests that the 3β-HSD-I in the androgen and 16-androstene pathways may be different enzymes; phosphatidylserine, when included in 3β-HSD-I assays, inhibited the conversion of andien-β to dienone but had no effect on the conversion of DHA to 4-androstenedione [3]. The possibility that there are substrate-specific 3β-HSD-Is in the pig testis was further investigated by enzyme kinetic analyses which demonstrated that andien-β and dienone did not inhibit the conversion of DHA to 4-androstenedione and that DHA and 4-androstenedione did not inhibit the conversion of andien-β to dienone [4]. To further investigate the possibility that distinct enzymes exist for these conversions, the effects of two classical steroidal competitive inhibitors of 3β-HSD-I, trilostane and cyanoketone, were investigated for their effects on 3β-HSD-I activities in androgen and 16-androstene biosynthesis. The results demonstrate that these inhibitors have different consequences for the 3β-HSD-I activities in the pig testis.

EXPERIMENTAL

Materials

Organic solvents were purchased from BDH (St Laurent, PQ). Unlabelled steroids were from Steraloids Inc. (Wilton, NH) and were recrystallized before use. [1,2,6,7-^{3}H]-DHA (100 Ci/mmol) and [4,7-^{3}H]-pregnenolone (15.6 Ci/mmol) were bought from Amersham (Oakville ON). [4,7-^{3}H]-Andien-β was prepared biosynthetically from [4,7-^{3}H]-pregnenolone (15.6 Ci/mmol) using piglet testis microsomal fraction as described previously [5]. All radioactive steroids were purified by thin layer chromatography prior to use. Nicotinamine adenine dinucleotide (NAD^+) was purchased from Sigma Chemical Co. (St Louis, MO). Trilostane (WIN 24540) and cyanoketone (WIN 19578) were generously donated by Drs Paul Juniewicz and Kenneth Thompson, Sterling Winthrop Inc., Collegeville, PA. All other chemicals were of reagent grade.

Animals, tissue preparation and enzyme assays

Testes from immature pigs (< 3 weeks old) were obtained from animals that had not been used in any experiments from the Centre for Food and Animal Research, Agriculture Canada, Ottawa, ON and were immediately frozen and stored at −80°C prior to use. Testes from several animals (6–10, testis weights ranged from 1 to 5g) were decapsulated and homogenized in 50 mmol/l Tris–HCl pH 7.5 containing 250 mmol/l sucrose, 25 mmol/l KCl, 5 mmol/l $MgCl_2$, 7 mmol/l mercaptoethanol and the microsomal fraction prepared exactly as described previously [4]. Protein determinations were done using the method of Lowry *et al.* [6].

3β-HSD-I activity was assayed by adding aliquots of microsomal fraction to Tris–HCl buffer pH 8.4 containing the steroid substrate (DHA or andien-β) and NAD^+ (0.25 mmol/l) at 37°C in a reciprocating water bath. Incubations were terminated by the addition of ethyl acetate (5 ml) containing carrier steroids. For the conversion of DHA to 4-androstenedione, the carrier steroids were DHA, 4-androstenedione, 5-androstenediol and testosterone (30 μg each). For the conversion of andien-β to dienone, the carrier steroids were andien-β, dienone, 5α-androstenone, an-α and an-β (15 μg each). Tubes were vortexed to extract the steroids, the organic phase separated by centrifugation (1000 rpm, IEC Centra 7, Needham Hts. MA), transferred to conical centrifuge tubes and evaporated using a Savant™ Speedvac Evaporator (Fisher Scientific, Ottawa, ON). The residue was applied to Whatman™ PE SIL-G plastic coated silica gel chromatography plates (Chromatographic Specialties, Montreal, PQ) which were developed in chloroform-acetone (9:1, v/v) for DHA metabolites or in hexane-ethyl acetate (5:3.5 v/v, run twice) for andien-β metabolites. Carrier steroids were located by UV illumination (254 nm) and exposure to iodine vapour. Steroid substrates and products were quantified by scintillation counting. The only product of DHA detected was 4-androstenedione and from andien-β, the only product was dienone.

Temporal linearity of 3β-HSD-I activity with respect to protein concentration, substrate concentration and limiting substrate conversion to less than 15% [7] were done as described previously [4]. Reactions were linear up to 90 min ($r > 0.99$) and substrate conversion was less than 15% when the following conditions were used: for DHA concentrations of 30, 60 and 100 nmol/l, protein concentrations were 1–2 μg/ml; for 300 and 600 nmol/l, protein concentrations were 4–6 μg/ml; for 1000 and 3000 nmol/l, protein concentrations were 13–18 μg/ml; and for 6000 and 10,000 nmol/l, protein concentrations were 60–80 μg/ml. Similarly, for andien-β concentrations of 30, 60, 100 and 300, protein concentrations were 4–6 μg/ml; for 600 and

1000 nmol/l, protein concentrations were 13–18 μg/ml; and for 3000, 6000 and 10,000, protein concentrations were 60–80 μg/ml. Having established appropriate conditions for enzyme kinetic analyses, the effects of trilostane and cyanoketone on the kinetic parameters of 3β-HSD-I were determined. The inhibitors were included in incubations at 0, 0.01, 0.1 and 1.0 μmol/l and for these studies, incubations were terminated after 1 h. Kinetic studies were done using four different microsomal preparations.

Data analysis

For each study, the $K_{m(app)}$ and $V_{max(app)}$ were determined using four different methods of analysis; the Direct Linear method uses each of the velocity (v) and substrate concentration [S] data points to draw lines from the substrate concentration value on the x-axis [S] through the corresponding velocity value on the y-axis (v). All the intersections from these lines are determined and the median values of the intersections represent the $K_{m(app)}$ and $V_{max(app)}$ [8]. The Wilkinson method is a non-linear least squares determination of a rectangular hyperbola [9]. The Hanes–Woolf [10] and Eadie–Hofstee [11] methods determine the $K_{m(app)}$ and $V_{max(app)}$ using linear regression analysis of, respectively, S/v vs S and v vs v/S plots. Slope and intercept replots were obtained from, respectively, $K_{m(app)}/V_{max(app)}$ vs [I] and $1/V_{max(app)}$ vs [I]. Linear regression analyses of the replots were done to determine the $K_{i(app)}$ values. Statistical comparisons of treatment effects on kinetic parameters were done using one-way ANOVA on log transformed $K_{m(app)}$ and $V_{max(app)}$ values from DHA studies and actual $K_{m(app)}$ and $V_{max(app)}$ values from andien-β studies (Normality and Equal Variance Tests passed with $P > 0.05$). Identification of treatments that were significantly different compared to controls, was done using Dunnet's multiple comparison procedure. (Sigmastat Programme version 1.0 for Windows, Jandel Scientific 1992–1994, San Rafael, CA).

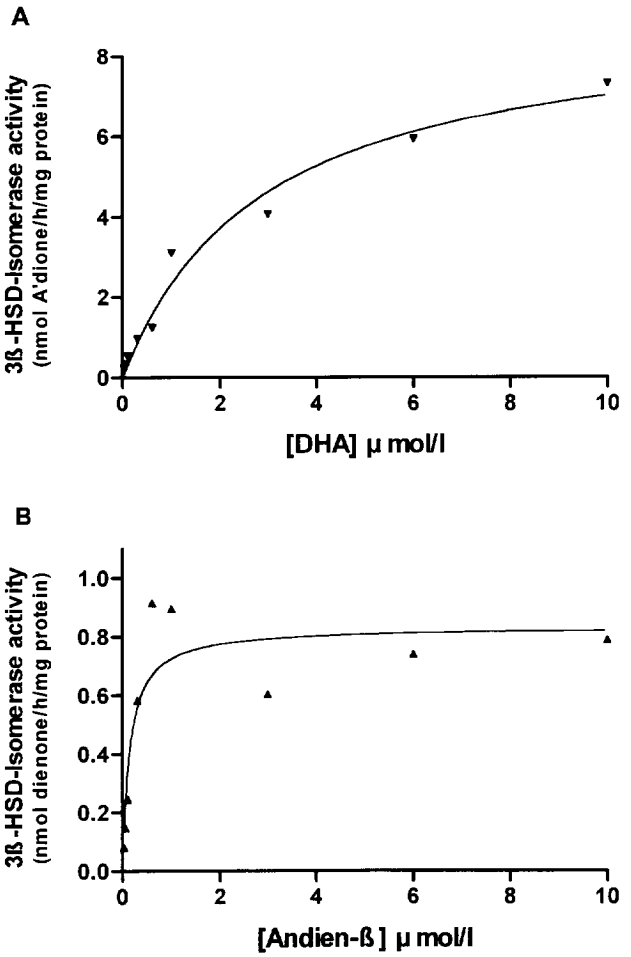

Fig. 1. Representatives of the four separate kinetic studies of 3β-HSD-I activities using; (A) DHA and (B) andien-β as substrate (0.03, 0.06, 0.1, 0.3, 0.6, 1.0, 3.0, 6.0 and 10 μmol/l, 40,000 cpm ^{3}H). Incubations were for 60 min. Other details were as described in Experimental and kinetic constants were determined as described in Data Analysis. In the examples above, the $K_{m(app)}$ and $V_{max(app)}$ in the DHA study were 2.47 μmol/l and 8.76 nmol/h/mg protein respectively, and for the andien-β study were 0.125 μmol/l and 0.834 nmol/h/mg protein respectively (Wilkinson analysis [9]).

RESULTS

Kinetic analyses of 3β-HSD-I activity for DHA and andien-β

Representatives of the four kinetic studies of 3β-HSD-I for DHA and for andien-β are shown in Fig. 1A and B respectively. It can be seen that saturation was not achieved for the DHA studies but was evident for andien-β. Mean values (± S.E.) for the $K_{m(app)}$ and $V_{max(app)}$ for the conversion of DHA to 4-androstenedione and for the conversion of andien-β to dienone are presented in Table 1 where it can be seen that, despite the different ways in which the four analyses determine the kinetic constants, there is good agreement between them (mean $K_{m(app)}$ values within the range 0.74–1.42 μmol/l, mean $V_{max(app)}$ values between 4.98 and 6.84 nmol/h/mg protein).

Effects of trilostane and cyanoketone on the kinetic parameters of 3β-HSD-I activity for DHA and andien-β

When DHA was the substrate, trilostane and cyanoketone at concentrations of 0.1 and 1.0 μmol/l, significantly increased the value of the $K_{m(app)}$ (Fig. 2A; $P < 0.05$) but had no significant effect on the $V_{max(app)}$ (Fig. 2B, Wilkinson analysis). The $K_{m(app)}$ value was increased approximately 15-fold by the highest concentration of inhibitor used. In contrast, when andien-β was the substrate, the same concentrations of these inhibitors had no effect on the $K_{m(app)}$ (Fig. 2C). Furthermore, cyanoketone had no effect on the $V_{max(app)}$ and trilostane, only at the highest dose, causing a 40% decrease in the $V_{max(app)}$ (Fig. 2D). Similar effects were seen when the data from the Direct–Linear, Hanes–Woolf and Eadie–Hofstee analyses were used (Table 1). For example, for DHA metabolism, the $K_{m(app)}$ was

Table 1. Kinetic constants for the effects of trilostane (Trilo) and cyanoketone (Cyano) on 3β-HSD-I activities (means + S.E. from 4 studies)

DHA as substrate

		Wilkinson		Eadie–Hofstee		Hanes–Woolf		Direct–Linear	
Inhibitor	μM	K_m(app) ± S.E.	V_{max}(app) ± S.E.	K_m(app) ± S.E.	V_{max}(app) ± S.E.	K_m(app) ± S.E.	V_{max}(app) ± S.E.	K_m(app) ± S.E.	V_{max}(app) ± S.E.
Control		1.42 ± 0.36	6.55 ± 0.74	0.74 ± 0.05	4.98 ± 0.25	1.37 ± 0.14	6.84 ± 0.54	0.99 ± 0.12	5.88 ± 0.59
Trilo	0.01	2.19 ± 0.47	6.93 ± 0.70	1.43 ± 0.28*	5.34 ± 0.63	2.25 ± 0.19*	7.32 ± 0.59	2.11 ± 0.26*	6.41 ± 0.65
Trilo	0.10	7.81 ± 1.49*	9.82 ± 1.77	4.09 ± 0.71*	5.33 ± 0.68	6.48 ± 0.29*	12.59 ± 3.27	11.38 ± 2.24*	11.64 ± 2.32*
Trilo	1.00	24.52 ± 9.94*	10.10 ± 3.92	4.31 ± 0.89*	2.53 ± 0.43*	5.16 ± 1.04*	5.78 ± 1.34	6.31 ± 0.74*	3.64 ± 0.44
Cyano	0.01	3.43 ± 1.23	8.16 ± 1.68	2.00 ± 0.54*	5.13 ± 0.73	3.34 ± 0.31*	9.32 ± 1.54	3.84 ± 0.28*	7.77 ± 0.41
Cyano	0.10	20.02 ± 7.18*	16.62 ± 5.68	4.72 ± 0.30*	4.97 ± 0.41	5.61 ± 1.06*	8.31 ± 1.58	11.96 ± 2.82*	10.53 ± 2.16
Cyano	1.00	19.50 ± 3.56*	7.84 ± 1.22	3.67 ± 1.01*	2.27 ± 0.51*	4.78 ± 1.02*	4.85 ± 0.89	5.33 ± 1.29*	3.34 ± 0.78*

Andien-ß as substrate.

		Wilkinson		Eadie–Hofstee		Hanes–Woolf		Direct–Linear	
		K_m(app) ± S.E.	V_{max}(app) ± S.E.	K_m(app) ± S.E.	V_{max}(app) ± S.E.	K_m(app) ± S.E.	V_{max}(app) ± S.E.	K_m(app) ± S.E.	V_{max}(app) ± S.E.
Control		0.259 ± 0.069	1.119 ± 0.122	0.342 ± 0.067	1.151 ± 0.128	0.288 ± 0.086	1.093 ± 0.150	0.406 ± 0.075	1.225 ± 0.165
Trilo	0.01	0.311 ± 0.055	1.131 ± 0.117	0.394 ± 0.047	1.127 ± 0.115	0.425 ± 0.089	1.151 ± 0.151	0.453 ± 0.037	1.207 ± 0.118
Trilo	0.10	0.177 ± 0.013	0.885 ± 0.048	0.274 ± 0.032	0.876 ± 0.056	0.231 ± 0.091	0.845 ± 0.093	0.405 ± 0.054	0.919 ± 0.085
Trilo	1.00	0.299 ± 0.073	0.624 ± 0.071*	0.377 ± 0.098	0.596 ± 0.092*	0.381 ± 0.126	0.656 ± 0.101	0.765 ± 0.128*	0.731 ± 0.128
Cyano	0.01	0.267 ± 0.041	0.963 ± 0.088	0.355 ± 0.033	0.970 ± 0.090	0.274 ± 0.096	0.923 ± 0.094	0.423 ± 0.032	0.978 ± 0.074
Cyano	0.10	0.251 ± 0.057	0.981 ± 0.114	0.348 ± 0.056	0.980 ± 0.128	0.374 ± 0.107	0.994 ± 0.092	0.421 ± 0.064	1.083 ± 0.122
Cyano	1.00	0.332 ± 0.059	0.857 ± 0.063	0.445 ± 0.087	0.826 ± 0.064	0.502 ± 0.049	0.865 ± 0.042	0.564 ± 0.108	0.895 ± 0.044

The Direct–Linear method of Eisenthal and Cornish–Bowden [8], and the methods of Wilkinson [9], Hanes–Woolf [10] and Eadie–Hofstee [11] were used to determine the Michaelis constant [$K_{m(app)}$; μmol/l] and the maximal velocity [$V_{max(app)}$; nmol/h/mg protein]. *Significantly different ($P < 0.05$) compared to control (for details see Data Analysis).

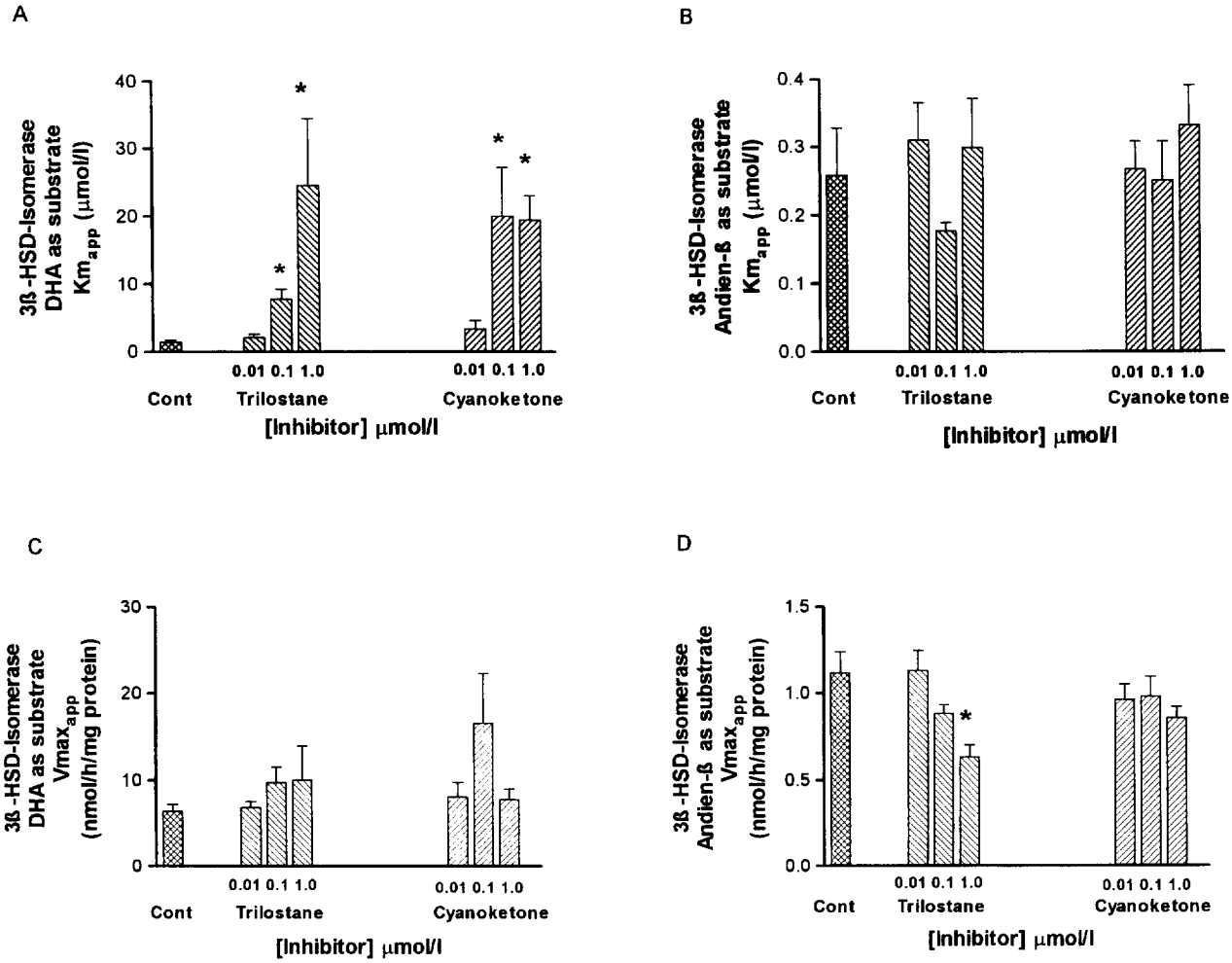

Fig. 2. Effects of trilostane and cyanoketone (0, 0.01, 0.1 and 1.0 µmol/l on the kinetic constants of 3β-HSD-I for the conversion of DHA to 4-androstenedione (A and C) and for the conversion of andien-β to dienone (B and D). Details were as described in Experimental. Bars represent the mean (± S.E.) of four separate studies (Wilkinson analysis [9]). *Statistically different ($P < 0.05$) compared to control.

increased by all concentrations of both inhibitors. Hanes–Woolf analysis showed no significant effects on the $V_{max(app)}$, the Direct–Linear and Eadie–Hofstee analyses showed significant decreases in the $V_{max(app)}$ (40–50%) with both trilostane and cyanoketone at 1.0 µmol/l ($P < 0.05$). With andien-β as substrate, cyanoketone was without effect in all analyses, and trilostane at 1.0 µmol/l caused a decrease (40–45%) in the $V_{max(app)}$ by the Wilkinson and Eadie–Hofstee analyses and a small increase (less than 2-fold) in the $K_{m(app)}$ by the Direct Linear method (Table 1).

Slope and intercept replots of the effects of trilostane and cyanoketone on the kinetic parameters of 3β-HSD-I activity for DHA and andien-β

When the data from the above studies were further analysed using slope and intercept replots, it was clear that both trilostane and cyanoketone had no effect on the value of the $V_{max(app)}$ with DHA as substrate but that both were inhibiting the reaction by increasing the $K_{m(app)}$ (Fig. 3A and B, the kinetic constants from Wilkinson analyses were used to generate the slope and intercept replots). The $K_{i(app)}$ for trilostane from the slope replot was 0.16 µmol/l and for cyanoketone was 0.20 µmol/l. Intercept replots were almost horizontal and estimates of the $K_{i(app)}$ were 50- and 440-fold greater than the estimate from the slope replot. Consequently, trilostane and cyanoketone were competitive inhibitors of DHA metabolism with $K_{i(app)}$ values that were 9- and 7-fold lower than the $K_{m(app)}$ value of 1.42 µmol/l. On the other hand, with andien-β as substrate, the slope and intercept replots demonstrated that any effect of trilostane or cyanoketone on the $K_{m(app)}$ for andien-β would be secondary to the effects on the $V_{max(app)}$ (Fig. 3C and D, the kinetic constants from Wilkinson analyses were used to generate the slope and intercept replots). Furthermore, the estimates for the $K_{i(app)}$ for trilostane and cyanoketone were respectively 1.1 and 1.6 µmol/l which were 4- and 6-fold greater than the $K_{m(app)}$ (0.26 µmol/l).

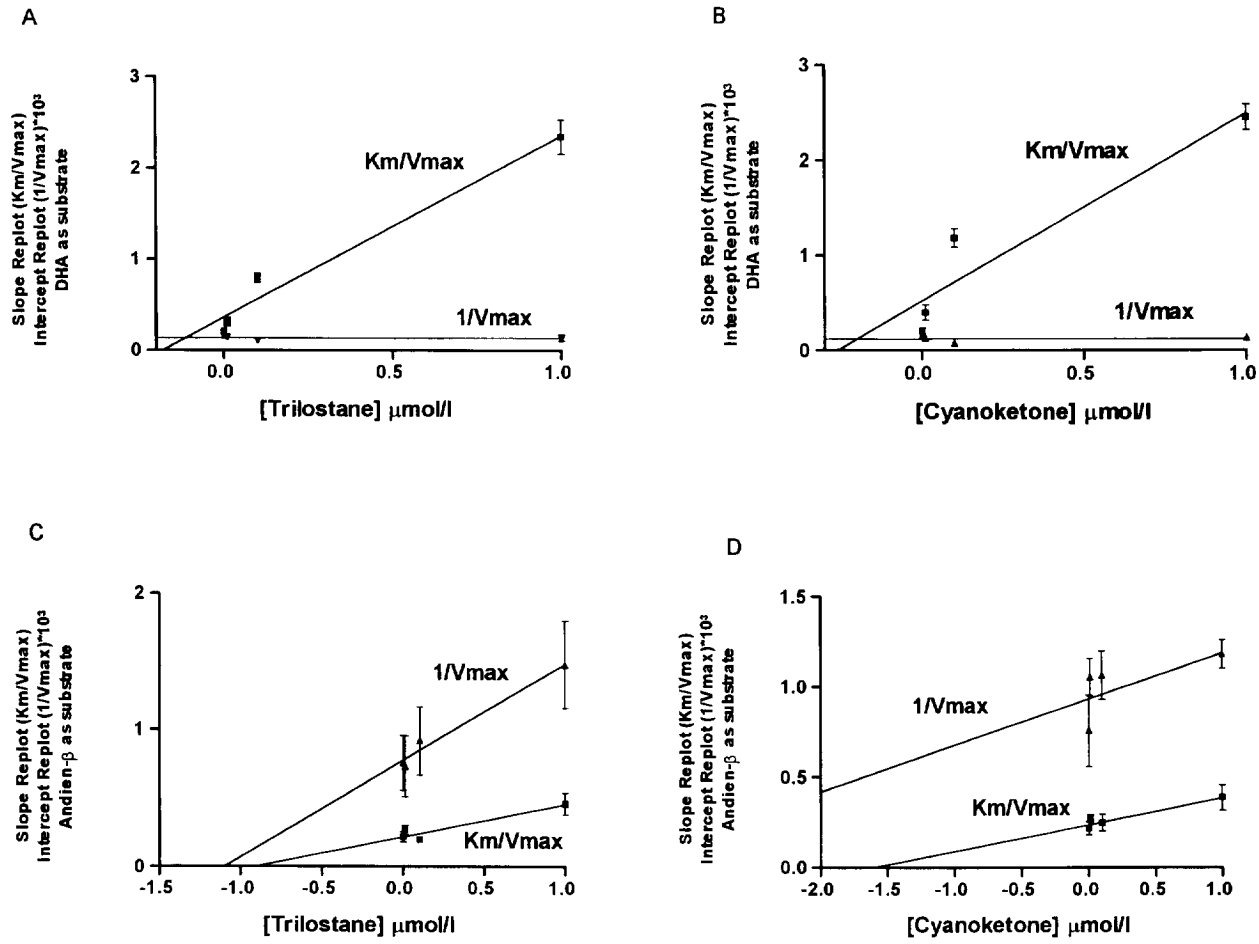

Fig. 3. Slope and intercept replots of the data depicted in Fig. 2. $K_{i(app)}$ values for the DHA studies were determined by linear regression analysis of the slope replots and for trilostane and cyanoketone were 0.26 μmol/l and 0.18 μmol/l respectively compared to the mean $K_{m(app)}$ value of 1.41 μmol/l. In the andien-β studies, the $K_{i(app)}$ values for trilostane and cyanoketone were respectively 1.1 and 1.6 μmol/l and the mean $K_{m(app)}$ value was 0.26 μmol/l.

DISCUSSION

Trilostane and cyanoketone have been shown to be competitive inhibitors of 3β-HSD-Is from several different sources [12–17]. The studies presented here demonstrate that in the pig testis, these compounds were also competitive inhibitors of the 3β-HSD-I reaction in androgen biosynthesis. Trilostane and cyanoketone increased the $K_{m(app)}$ value for DHA approximately 15-fold and had no significant effect on the $V_{max(app)}$. The $K_{i(app)}$ values were also 5–10-fold lower than the $K_{m(app)}$ demonstrating that they have a greater affinity for the active site than DHA. In marked contrast, trilostane and cyanoketone had little effect on the 3β-HSD-I reaction in 16-androstene biosynthesis. No effect on the $K_{m(app)}$ for andien-β was observed and only the highest doses had any effect on the $V_{max(app)}$. Slope and intercept replots confirmed the lack of effect on the $K_{m(app)}$ and the $K_{i(app)}$ values for trilostane and cyanoketone were, respectively, 4- and 6-fold greater than the $K_{m(app)}$ for andien-β. Therefore, for the 3β-HSD-I in the 16-androstene pathway, trilostane and cyanoketone could best be described as weak, non-competitive inhibitors. The selective effects of trilostane and cyanoketone on the 3β-HSD-Is involved in androgen and 16-androstene biosynthesis in the pig testis endorse earlier findings suggesting separate enzymes, or at least separate, non-interacting active sites on a single enzyme [3, 4].

Although it has not been shown previously that trilostane and cyanoketone are competitive inhibitors for the conversion of DHA to 4-androstenedione in the pig testis, it was anticipated that they would do so, thereby rendering them inappropriate as selective inhibitors of the synthesis of the odoriferous 16-androstene steroids. Rather, in these studies, they were used as tools to investigate their effects on the two 3β-HSD-I reactions in the pig testis. In this context, they were successful in demonstrating the markedly different patterns of inhibition for the 3β-HSD-I reactions. The importance of these findings lies in the development of future strategies to selectively inhibit the production of 16-androstene steroids in live pigs. The odour of the 16-androstenes causes the meat from

intact male pigs to be unpalatable [2]. Consequently, the normal method to alleviate the problem is to castrate male piglets within the first few days of life. This procedure, while extremely effective, has several disadvantages; the absence of androgens reduces food conversion efficiency and growth rate; the removal of testes reduces the gene pool for the selection of breeding boars; and such selections have to be made prior to any seminal analyses being possible. It is therefore of economic benefit to devise alternative means to inhibit 16-androstene biosynthesis without reducing androgen biosynthesis. Since androgen and 16-androstene biosynthesis have common mechanisms up to the point of pregnenolone production [18–20], it is necessary to consider means of selectively inhibiting enzymes in the 16-androstene pathway. Current ideas on the metabolism of pregnenolone propose that 17-hydroxylase C-17,20 lyase is involved in both DHA and andien-β biosynthesis [21–23]. Thus, the inhibition of this enzyme would be likely to decrease androgen and 16-androstene production, as has been shown with some steroidal inhibitors [24–26]. However, the second stage in the 16-androstene pathway may be catalysed by a 3β-HSD-I that is independent of that for androgen biosynthesis, thereby rendering this reaction a better target for selective inhibition. It should be possible to design inhibitors that will inhibit the 3β-HSD-I in the 16-androstene pathway without affecting androgen biosynthesis. Such compounds should possess kinetic characteristics that are similar to those exhibited by trilostane and cyanoketone for the 3β-HSD-I in the androgen biosynthetic pathway and, must be shown to be effective *in vivo*.

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