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Pharmacology of Novel Steroidal Inhibitors of Cytochrome $P450_{17\alpha}$ (17α-Hydroxylase/C17-20 Lyase)

S. E. Barrie,[1*] G. A. Potter,[1] P. M. Goddard,[1] B. P. Haynes,[1] M. Dowsett[2] and M. Jarman[1]

[1]*Drug Development Section, Institute of Cancer Research, Sutton, Surrey SM2 5NG and* [2]*Department of Academic Biochemistry, Royal Marsden Hospital, Fulham Road, London SW3 6JJ, England*

Medical or surgical castration for the treatment of prostatic cancers prevents androgen production by the testes, but not by the adrenals. Inhibition of the key enzyme for androgen biosynthesis, cytochrome $P450_{17\alpha}$, could prevent androgen production from both sources. The *in vivo* effects of 17-(3-pyridyl)androsta-5,16-dien-3β-ol (CB7598) and 17-(3-pyridyl)androsta-5,16-dien-3-one (CB7627), novel potent steroidal inhibitors of this enzyme, on WHT mice were compared with those of castration and two clinically active compounds, ketoconazole and flutamide. Flutamide and surgical castration caused significant reductions in the weights of the ventral prostate and seminal vesicles. CB7598, in its 3β-*O*-acetate form (CB7630), and CB7627 caused significant reductions in the weights of the ventral prostate, seminal vesicles, kidneys and testes when administered once daily for 2 weeks. Ketoconazole, given on the same schedule, caused no reductions. Plasma testosterone was reduced to ⩽0.1 nM by CB7630, despite a 3- to 4-fold increase in the plasma level of luteinizing hormone. Adrenal weights were unchanged following treatment with CB7630 or CB7627 but were markedly increased following ketoconazole, indicating no inhibition of corticosterone production by these steroidal compounds. These results indicate that CB7598, CB7630 or CB7627 may be useful in the treatment of hormone-dependent prostatic cancers.

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INTRODUCTION

Antiendocrine therapy with luteinizing hormone-releasing hormone (LHRH) agonists has become an established alternative to orchidectomy as a means of lowering circulating androgens in patients with cancer of the prostate. This treatment ablates the testicular production of androgens but leaves the adrenal supply of androgens and their precursors unaffected. The long standing premise that this adrenal supply is critical has been tested in several clinical trials, some of which showed a significant increase in survival as a result of combining LHRH agonists with antiandrogens when compared with the use of LHRH agonists alone [1]. An alternative to this combination would be an effective and selective inhibitor of the key enzyme in the androgen biosynthetic pathway, namely cytochrome $P450_{17\alpha}$ (17α-hydroxylase/ C17-20 lyase), which is present in both the testes and the adrenals. Ketoconazole, originally developed as an antifungal agent, potently inhibits this enzyme and has been used clinically in the treatment of hormone-dependent prostatic cancers [2]. Unfortunately ketoconazole also inhibits many other cytochrome *P*450 enzymes [3, 4] and was withdrawn from use because it caused liver damage [5, 6]. Several groups are seeking to develop more potent and selective inhibitors [7, 8]. Recently we reported briefly on the *in vitro* and *in vivo* results obtained with a novel steroidal compound, 17-(3-pyridyl)androsta-5,16-dien-3β-ol (CB7598) (Fig. 1), a potent inhibitor ($Ki_{app} < 1$ nM for the lyase activity) of the human cytochrome $P450_{17\alpha}$ [9]. In this paper the effects of CB7598 in its 3β-*O*-acetate form (CB7630), and an equally potent related compound 17-(3-pyridyl)androsta-5,16-dien-3-one (CB7627) (Fig. 1), on the circulating hormone levels and organ weights in mice are described, and compared with those of castration, ketoconazole and flutamide.

*Correspondence to S. E. Barrie.
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EXPERIMENTAL

CB7598, CB7630 and CB7627 were prepared from dehydroepiandrosterone where the key step involved a novel palladium catalysed cross-coupling of a steroidal enol triflate with a pyridyl borane [10, 11]. Ketoconazole was a gift from Janssen Pharmaceuticals (Beerse, Belgium) and flutamide was a gift from Kirby-Warwick Pharmaceuticals Ltd. (Bury St Edmunds, England) The WHT mice came from the Gray Lab (Mount Vernon Hospital, Mddx, England).

Adrenal cell assay

Guinea pig adrenal cells were obtained and assayed for the ACTH stimulated production of androstenedione and cortisol as described previously [12].

Radioimmunoassays

Testosterone was analysed using the DPC total testosterone coat-a-count kit. The detection limit was 0.1 nM and the within and between assay CVs were 8.0 and 12.9%, respectively at an assay value of 2.5 nM. The assay for luteinizing hormone (LH) used the Amersham International rat LH[^{125}I] assay system employing a magnetic separation system which cross-reacts with mouse LH. The detection limit was 0.4 ng/ml and the within and between assay CVs were 6.5 and 6.6%, respectively at an assay value of 4.5 ng/ml.

In vivo studies

Adult male WHT mice (12 weeks old) were housed under conditions of 12 h light–dark and were allowed free access to food (SDS expanded rodent diet) and water.

CB7598 was used in its 3β-*O*-acetate form (CB7630) as this was easier to formulate than the free 3β-hydroxy compound. CB7630, CB7627 and ketoconazole were prepared in 5% benzyl alcohol, 95% safflower oil. The preparations were given intraperitoneally, 5 ml/kg, to the mice once daily for 14 days. Flutamide was prepared in 5% dimethylsulphoxide, 95% arachis oil and was given subcutaneously, 5 ml/kg, following the same schedule. The doses given ranged from 0.02 to 0.5 mmol/kg/day. On day 15, the animals were anaesthetized with fluothane, the blood collected by cardiac puncture into heparinized tubes, and the organs of interest dissected out and weighed. After weighing, the organs from the animals treated with CB7627 were stored in modified methacarn for histological examination. The plasma was separated by centrifugation and stored at −20°C.

For the pharmacokinetic experiment, a single intraperitoneal dose of 0.5 mmol/kg CB7630 was given to the mice which were sacrificed at various times up to 24 h afterwards, and the blood taken by cardiac puncture as above. CB7598 and CB7630 plasma concentrations were determined by high pressure liquid chromatography. Plasma samples (250 μl) were mixed with acetonitrile (375 μl) and centrifuged. Aliquots (100–200 μl) of the supernatant so obtained were injected onto a 15 cm Spherisorb S3PC18 cartridge column (4.6 mm i.d.) with an eluent of acetonitrile–50 mM ammonium acetate buffer (60:40, v/v) flowing at 1.5 ml/min. Eluates were detected using a Perkin–Elmer LC-240 fluorescence detector set up with a excitation wavelength of 262 nm and an emission wavelength of 353 nm. The detection limit of the assay was 20 ng/ml (~50 nM) for both compounds.

Adult male WHT mice were castrated via the scrotal route while under pentobarbital anaesthesia. The sham operated group underwent the same surgical procedures as the castrates except that the testes were not

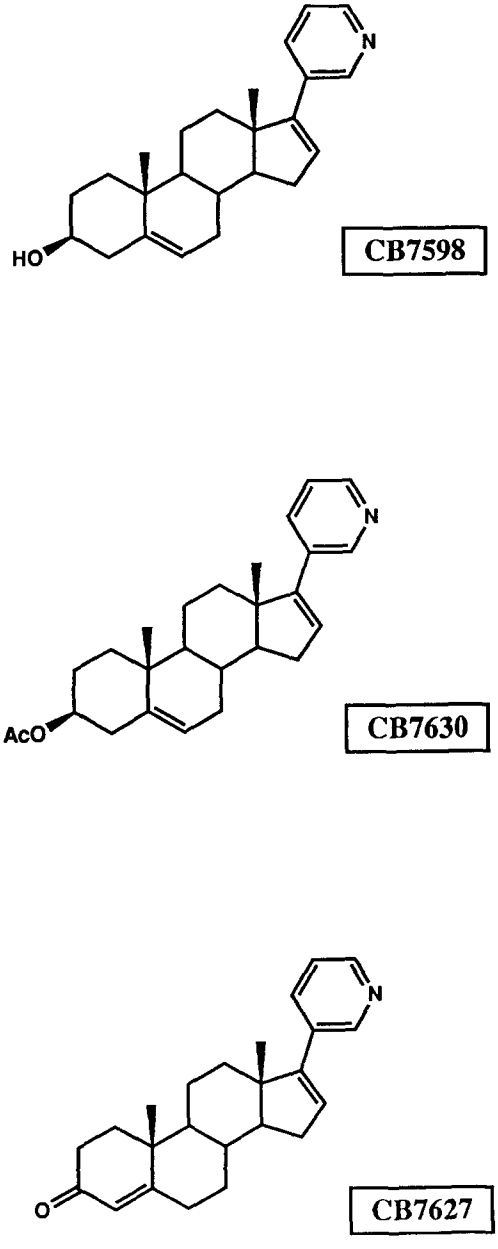

Fig. 1. Structures of the compounds CB7598, the 3-O-acetate form CB7630, and CB7627

Table 1. The effects of castration on the organ weights of WHT mice

Treatment/ time	Adrenals	Prostate	Seminal vesicles	Testes	Kidneys
Untreated	3.9 ± 0.1 (n = 21)	9.5 ± 0.4 (n = 21)	186 ± 6 (n = 21)	137 ± 2 (n = 21)	651 ± 26 (n = 8)
Sham					
1 wk (n = 7)	3.4 ± 0.1	11.1 ± 0.5	170 ± 10	124 ± 5	ND
2 wk (n = 8)	3.9 ± 0.1	9.5 ± 0.5	168 ± 6	126 ± 6	ND
4 wk (n = 8)	3.3 ± 0.1	7.1 ± 0.5	167 ± 8	137 ± 2	571 ± 23
Castrates					
1 wk (n = 8)	3.6 ± 0.1	4.2 ± 0.2*	27 ± 2*		ND
2 wk (n = 8)	3.9 ± 0.1	2.5 ± 0.2*	16 ± 1*		ND
4 wk	4.1 ± 0.1	1.9 ± 0.1*	15 ± 1*		413 ± 13*

The results are given in mg as means ± SE, with the number of animals in parentheses.
*$P < 0.01$ for the differences between the castrate groups and the sham groups; ND, not determined.

removed. After 1, 2, or 4 weeks the animals were sacrificed and the organs removed and weighed.

Statistical significance was assessed by analysis of variance, and subsequent multiple range testing used the least significant difference method using the Statgraphics 5 computer program.

RESULTS

ACTH stimulated the production of androstenedione and cortisol by guinea pig adrenal cells *in vitro* by 1703 and 38 nM, respectively after 3 h. CB7598, CB7627 and ketoconazole were able to inhibit this, achieving almost total suppression (< 10% stimulated production) at 10^{-5} M. This concentration was much higher than the K_is for the human cytochrome $P450_{17\alpha}$ enzyme as measured *in vitro*, and may reflect the effects of protein binding and membrane transport as well as differences in the enzyme structure and environment. A similar concentration of ketoconazole was required to inhibit corticosterone synthesis in rat adrenal cells [4].

The effect of castration on the organ weights of the WHT mice is shown in Table 1. There were marked decreases in the sizes of the ventral prostate, seminal vesicles and kidneys to 20, 8, and 63% of untreated controls, respectively. The effect on the seminal vesicles was greater than on the prostate at all times. The maximum effect was seen after approx. 2 weeks, and a 2-week dosing schedule with the various drugs was therefore followed.

The results of treating the mice with CB7630 and CB7627 are given in Figs 2 and 3 and the results obtained with ketoconazole and flutamide, agents that have been used clinically to treat hormone dependent prostatic cancer, are given in Figs 4 and 5. The mice did not lose weight or appear unwell with any of the treatments, and there were no drug related features seen at post-mortem except in the case of ketoconazole at the highest dose when the livers looked brown (no histology was carried out).

CB7630 and CB7627 caused a marked suppression in the weights of several androgen-dependent organs in a dose-dependent way. The reductions in the seminal vesicle weights (maximum reductions 87 and 85%, respectively) were more marked than that in the ventral prostate weights (maximum reductions 48 and 51%, respectively), as had been the case following castration. The kidneys were also reduced in weight (by 37 and 28%) as were the testes (by 62 and 58%). Flutamide, in contrast, produced equal reductions in the prostate and seminal vesicles (54 and 60%, respectively), and no change in the testicular weight (kidney weights were not measured). Ketoconazole did not produce any change in the weights of these organs.

After the experiment with CB7627, the testes were histologically examined. There was a thinning of the germinal epithelium with a reduced number of mature forms. There was no obvious reduction in Leydig cell numbers, although this was difficult to estimate in the presence of marked changes in the seminiferous tubules. There were no obvious toxic effects of the drug and all the changes were consistent with a reduction in testosterone.

Following treatment of the mice with ketoconazole, there was a marked increase in adrenal weight, while treatment with CB7630, CB7627, or flutamide caused no change in the weight of the adrenals.

The plasma testosterone and LH levels 24 h after the end of treatment are given in Table 2. The level of testosterone in control animals varied considerably, and there was a dramatic reduction in the levels in the vehicle controls probably related to stress. Comparing the vehicle controls with the drug treated groups, there was a decrease in circulating testosterone after keto-

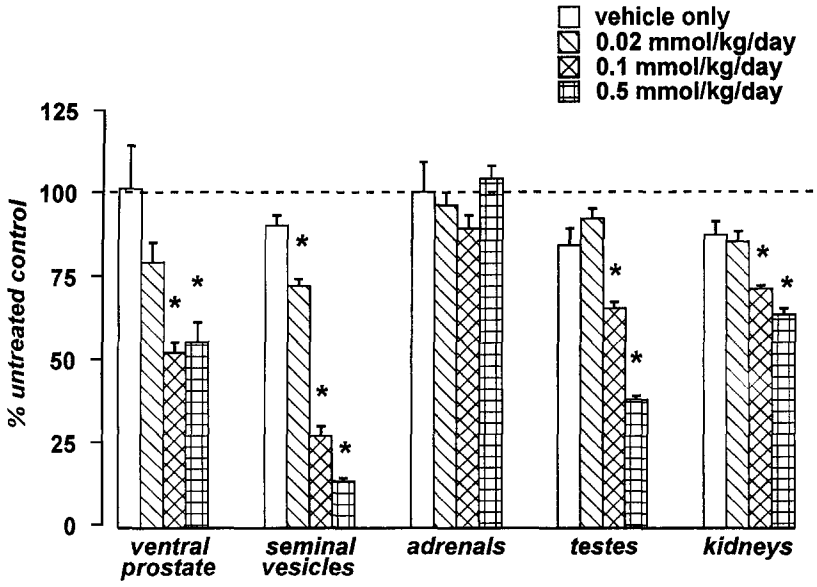

Fig. 2. Effects of 14 days treatment with CB7630 on the organ weights of WHT mice. Results are expressed as % of the untreated controls for which the values were ventral prostate 9.5 ± 0.3, seminal vesicles 182 ± 6, adrenals 4.2 ± 0.1, testes 144 ± 2, kidneys 677 ± 17 mg ($n = 20$). *$P < 0.01$ for the difference from the vehicle controls.

conazole, but this was not statistically significant, and was not associated with a rise in plasma LH. However, there was a marked dose-dependent decrease in testosterone to almost undetecteble levels following CB7630 and this was maintained despite the increased levels of LH which would normally stimulate the testicular production of the androgen. Following CB7627 there was also a dose-dependent decrease in plasma testosterone but not to such low levels as following CB7630. This was not statistically significant as the variability of the vehicle control group was so great.

The pharmacokinetics of CB7598 following the intraperitoneal administration of CB7630, the acetylated form of CB7598, are shown in Fig. 6. CB7630 was detected in only 2 of the 25 mice, indicating rapid de-acetylation to CB7598. The peak plasma concentration of CB7598 of approx. 400 ng/ml (1.0 μM) was seen after 6 h with the level dropping to 1/3 after 8 h. The concentration at 24 h was similar to that at 8 h, possibly due to a depot effect from the route of administration, and/or enterohepatic recirculation.

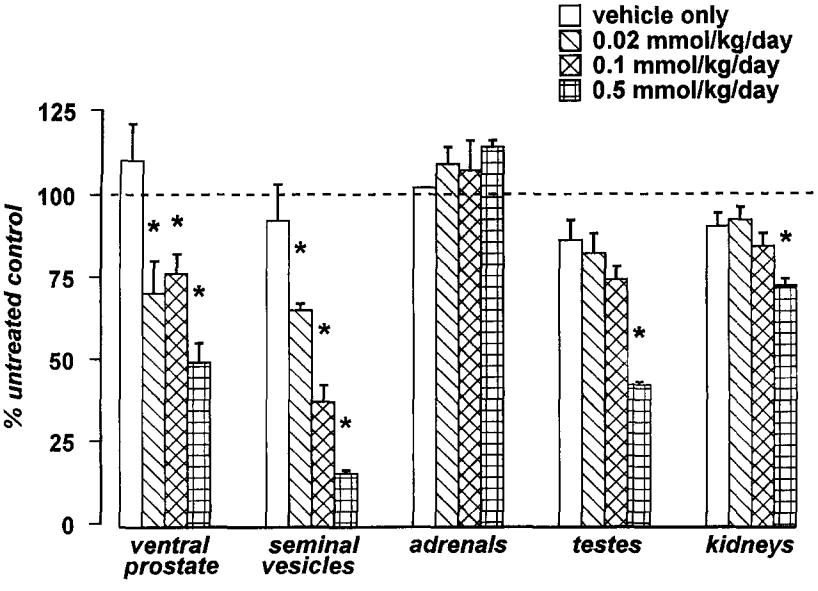

Fig. 3. Effects of 14 days treatment with CB7627 on the organ weights of WHT mice. Results are expressed as % untreated controls (values given in Fig. 2). *$P < 0.01$ for the difference from the vehicle controls.

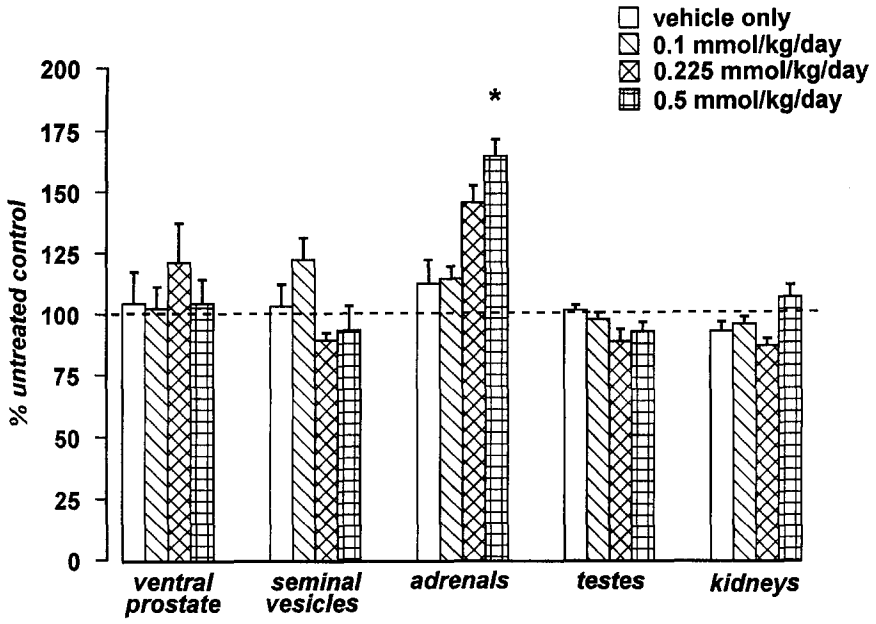

Fig. 4. Effects of 14 days treatment with ketoconazole on the organ weights of WHT mice. Results are expressed as % untreated controls (values given in Fig. 2). $^*P < 0.01$ for the difference from the vehicle controls.

DISCUSSION

Plasma levels of testosterone and LH are very variable in mice [13] and can be affected by stress of varying types [14], making the detection of significant drug induced changes difficult. Both the variability and the effect of stress are illustrated by the data here for the control and the vehicle control groups. Following ketoconazole, the level of circulating testosterone was reduced but this was not associated with an increase in plasma LH, nor with regression of the androgen-dependent organs. These results suggest that the testosterone suppression may have been of insufficient duration to cause any regression of the androgen-dependent organs and so might have been stress induced. On the other hand, following CB7630, acting as a prodrug form of CB7598, there was a marked drop in the circulating level of testosterone which was maintained despite the reflex rise in LH, which in turn would have stimulated testosterone biosynthesis. This indicates that CB7630 produced potent inhibition of testosterone production *in vivo*. The significant regression of several androgen-dependent organs could have resulted from this reduced level of circulating

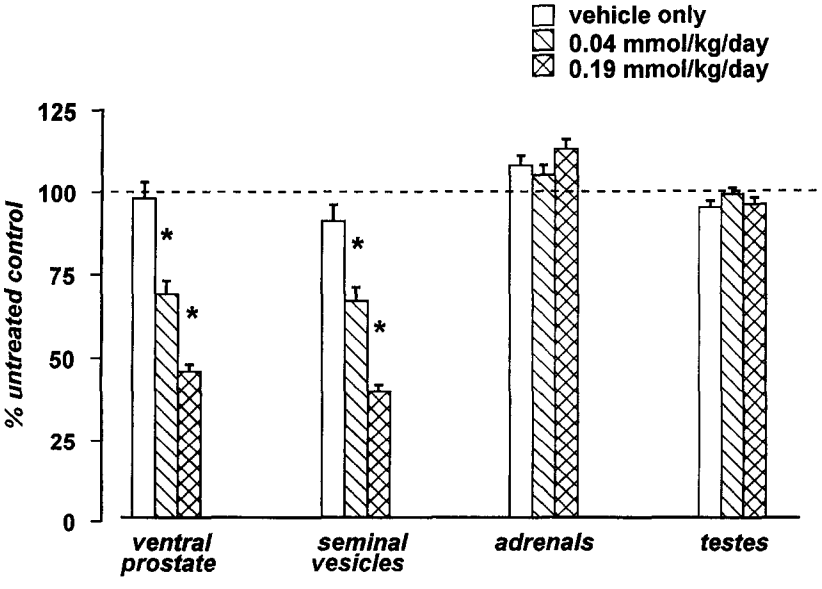

Fig. 5. Effects of 14 days treatment with flutamide on the organ weights of WHT mice. Results are expressed as % untreated controls (values given in Fig. 2). $^*P < 0.01$ for the difference from the vehicle controls.

Table 2. The levels of plasma testosterone and LH following drug treatments

	Testosterone (nM)	LH (ng/ml)
Ketoconazole		
Control	17.3 ± 7.1[a]	0.66 ± 0.05
Vehicle	1.3 ± 0.4	<0.4
0.1 mmol/kg/day	0.9 ± 0.2	<0.4
0.225 mmol/kg/day	0.7 ± 0.15	0.75 ± 0.02
0.5 mmol/kg/day	0.4 ± 0.1	0.76 ± 0.03
CB7630		
Control	9.8 ± 5.6	0.63 ± 0.16
Vehicle	2.5 ± 1.2	0.80 ± 0.09
0.02 mmol/kg/day	2.7 ± 0.5	3.4 ± 0.5*
0.1 mmol/kg/day	0.2 ± 0.1*	2.55 ± 0.45*
0.5 mmol/kg/day	0.1 ± 0.0*	2.25 ± 0.67*
CB7627		
Control	27.8 ± 11.4	ND
Vehicle	11.0 ± 5.6	ND
0.02 mmol/kg/day	4.5 ± 0.3	ND
0.1 mmol/kg/day	3.5 ± 1.1	ND
0.5 mmol/kg/day	0.4 ± 0.1	ND

The results are given as mean ± SE with 5 animals per group.

[a]Due to the large variation in the testosterone values in the untreated control group, the analysis of variance was carried out without including this group.

*$P < 0.05$ for difference from the vehicle only group; ND, not determined.

testosterone, although an additional direct effect of the compound cannot be excluded. CB7627 also induced a marked drop in plasma testosterone, although this was not to as low a level as CB7630. However, the effects on the target organs were significant indicating that this compound is also active by inhibiting testosterone biosynthesis and/or acting directly as an antiandrogen.

The reduction in testicular weight following treatment with CB7630 or CB7627 was surprising as no changes had been reported with ketoconazole, and antiandrogens like flutamide had no effect [15] as found here. Reductions in testicular weight have been documented following LHRH analogues [15, 16], but in these situations there was low LH in contrast to the situation following CB7630. The histology indicated that the changes were consistent with low tissue testosterone, which would result from the effective inhibition of the biosynthetic pathway by the test compounds.

The pharmacokinetic study showed that the deacetylated drug (CB7598) persisted at quite high levels (~0.3 μM) at 24 h. This may be related to the route of administration or may be the result of enterohepatic recirculation increasing the effective half-life considerably. This persistence of CB7598 would have been very important in maintaining adequate enzyme inhibition throughout the experiment. Ketoconazole has been shown to reduce circulating testosterone levels in rats and cause regression of the ventral prostate and Dunning R3327H tumours [17 18]. In those experiments it was given twice daily and this schedule difference may explain the lack of effect on the androgen-dependent organs observed here, despite a higher dose of the drug being given. However there were effects on the liver and adrenals indicating unwanted side-effects. Ketoconazole is known to inhibit other cytochrome *P*450s in addition to the $P450_{17\alpha}$ [3, 4] and was withdrawn from cancer therapy because of hepatotoxicity [5, 6] which was probably a result of these additional unwanted enzyme inhibitions.

17α-Hydroxylation is an essential step in the biosynthesis of androstenedione and cortisol, and inhibition of this step should inhibit the production of both steroids. This is borne out by the results with the guinea pig adrenal cells. In mice, corticosterone, and not cortisol, is the predominant glucocorticoid, and the biosynthesis of corticosterone does not require the cytochrome $P450_{17\alpha}$ enzyme. Therefore, a selective inhibitor should have no effect on adrenal function in mice. Inhibition of corticosterone production by, for example, aminoglutethimide inhibiting the cytochrome $P450_{scc}$ enzyme results in a marked increase in adrenal weight [19, 20]. Ketoconazole inhibits several steps in this pathway [3, 4] and, as shown here, also results in a marked increase in adrenal weight. The lack of effect on the mouse adrenals following CB7630 and CB7627 indicates that these compounds do not significantly inhibit other steps in the biosynthetic pathway, and therefore that they are more selective for the target enzyme.

For an inhibitor of cytochrome $P450_{17\alpha}$ (17α-hydroxylase/C17-20 lyase) to be effective in the treatment of hormone-dependent cancer of the prostate, it needs to be potent, selective and to have favourable pharmacokinetics and metabolic profile. The compound 17β-(cyclopropylamino)-androst-5-en-3βol has been shown to be an irreversible inhibitor of the target enzyme [7], but is considerably less potent than CB7598 or CB7627. No information was given as to its *in vivo*

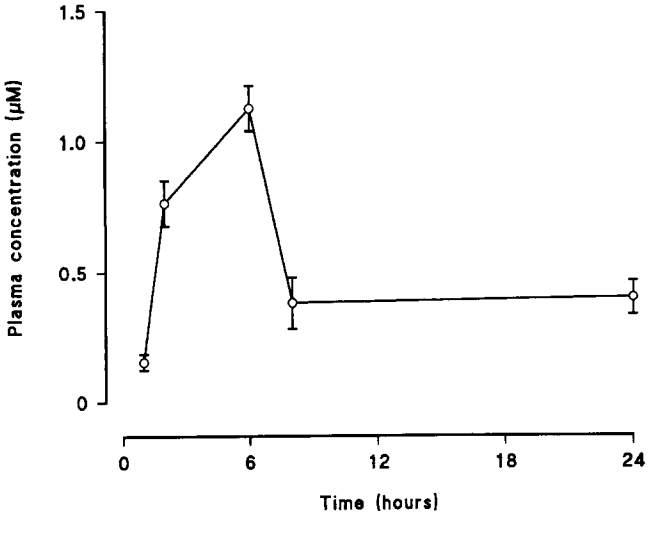

Fig. 6. Pharmacokinetics of CB7598 following intraperitoneal administration of 0.5 mmol/kg CB7630. The data from 5 mice per time point are expressed as mean ± SE.

activity. Another compound, 4-pregnen-3-one-20β-carboxaldehyde, has been developed to exploit this target in combination with 5α-reductase [8]. It was highly potent against the human 5α-reductase *in vitro* with $K_i = 16$ nM but in contrast poorly active against the rat cytochrome $P450_{17\alpha}$ enzyme giving K_i values in the μM range. *In vivo* repeated administration of the compound produced a very modest drop (50%) in plasma testosterone, and no details were given as to any effect on the androgen-dependent organs of the animals.

In conclusion, the steroidal compounds CB7630, acting as a prodrug form of CB7598, and CB7627 are potent inhibitors of the human target enzyme, while not inhibiting several other *P*450 enzymes, and show significant activity *in vivo*, making them worthy of further study as potential agents for the treatment of hormone-dependent prostate cancer.

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