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Effects of novel 17α-hydroxylase/C17, 20-lyase (P450 17, CYP 17) inhibitors on androgen biosynthesis in vitro and in vivo

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Abstract

Aiming at the development of new drugs for the treatment of prostate cancer, the effects of steroidal compounds and one non-steroidal substance on androgen biosynthesis were evaluated in vitro and in vivo. **Sa 40** [17-(5-pyrimidyl)androsta-5,16-diene-3β-ol], its 3-acetyl derivate **Sa 41** and **BW 19** [3,4-dihydro-2-(4-imidazolylmethyl)-6-methoxy-1-methyl-naphthalene] are compounds from our group, which have been developed as inhibitors of CYP 17 (17α-hydroxylase-C17, 20-lyase, the key enzyme in androgen biosynthesis). They have been compared with **CB 7598** [abiraterone: 17-(3-pyridyl)androsta-5,16-diene-3β-ol], its 3-acetyl compound **CB 7630** and ketoconazole, compounds which already have been used clinically. The most potent compound toward human CYP 17 (testicular microsomes) was **Sa 40** (IC_{50} value of 24 nM), followed by **Sa 41**, **CB 7598**, **BW 19**, **CB 7630** and ketoconazole. **Sa 40** shows a type II difference spectrum and a non-competitive type of inhibition (K_i value of 16 nM). No recovery of enzyme activity was observed after preincubation of CYP 17 with **Sa 40** and subsequent charcoal treatment. In *Escherichia coli* cells coexpressing human CYP 17 and NADPH-P450 reductase, **Sa 40** was more active than **CB 7598** and **BW 19**, whereas the acetyl compounds were not active. The latter three compounds were equally active towards rat CYP 17. Male Sprague–Dawley (SD) rats were administered daily for 14 days **BW 19** and the acetyl derivatives **Sa 41** and **CB 7630** as prodrugs (0.1 mmol/kg intraperitoneally). The test compounds strongly reduced plasma testosterone concentration, as well as prostate and seminal vesicles weights. They showed moderate inhibitory effects on the weights of levator ani, bulbocavernosus and testes, whereas they led to an increase in adrenal and pituitary weights. The only exception was **BW 19** which did not change pituitary weights. Based on its superiority on the human enzyme, it was concluded that **Sa 40** in its 3β-acetate form (**Sa 41**) could be a promising candidate for clinical evaluation.

Keywords: 17α-Hydroxylase-C17; 20-Lyase (CYP 17); CYP 17 (P450 17) inhibitors; Prostate cancer; 5α-Reductase; Androgen biosynthesis

1. Introduction

Prostate cancer has displayed an increase in incidence unparalleled by any other tumor in the last two decades, with a steady, more gradual increase in mortality rate. It is the second leading cause of death from cancer and the most prevalent cancer amongst men in the western world [1]. Since approximately 80% of human prostatic tumors are androgen dependent, different endocrine strategies have been used for the treatment [2]. Besides antiandrogens different compounds interfering with the androgen production have been used. Estrogens unfold their activity on the hypothalamic level by reducing the release of gonadorelin. As a consequence, the reduced pituitary LH/FSH formation results in a decrease of the testicular androgen production. However, due to its considerable cardiovascular side effects, estrogen therapy became obsolete during the past couple of years [3]. Another method of chemical castration is widely used for the treatment of prostate cancer. The use of gonadorelin analogs also inhibits testicular androgen biosynthesis [3]. However, neither this strategy nor orchidectomy can reduce the production of adrenal androgens. Therefore, chemical or surgical castration has been combined with antiandrogen (flutamide, bicalutamide) treatment to reduce the stimulatory effects of the remaining androgens in order to achieve a longer survival period [4]. However, some investigators speculate that by using antiandrogens a mutation in the androgen receptor might occur leading to the recognition of antiandrogens as stimulators [1]. An in vitro study in hormone-independent cell lines was in accordance with this hypothesis [5]. A promising novel strategy for the treatment of prostate cancer might be inhibitors of CYP 17 (P450 17,

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17α-hydroxylase/C17-20-lyase). CYP 17 is the key enzyme in the biosynthesis of androgens [6]. This enzyme catalyses the 17α-hydroxylation of pregnenolone and progesterone and the subsequent cleavage of the C 20,21-acetyl group to yield the corresponding androgens [7]. The inhibition of this enzyme will decrease the production of the testicular as well as the adrenal androgens since it is responsible for the production of androgens in both organs. The use of one drug capable of inhibiting the androgen production everywhere is preferable and more effective than a combination therapy (two half lives have to be taken into consideration) or a compound which only inhibits testicular androgen production. The antimycotic ketoconazole, which is also an inhibitor of CYP 17, had been used clinically in the treatment of advanced prostate cancer [8–10]. Although this compound had shown antitumor activity, it was withdrawn from clinical use because of its short half life and its side effects due to its non-selectivity. This led us and others to look for more potent and selective steroidal [2,3,11–14] and non-steroidal [2,3,11,15–20] inhibitors of CYP 17. All these inhibitors contain a functional group—mostly a nitrogen bearing heterocycle—capable to form a coordinate bond with the heme iron of the enzyme. Newly abiraterone (**CB 7598**; 17-(3-pyridyl)androsta-5,16-diene-3β-ol (Fig. 1) [14]), a highly active and selective inhibitor of the title enzyme is in clinical trial. So far no significant side effects have been observed, especially the plasma levels of cortisol generally remained unchanged [21]. In fact, we also have developed a highly active non-steroidal inhibitor, **BW 19** (3,4-dihydro-2-(4-imidazolylmethyl)-6-methoxy-1-methylnaphthalin (Fig. 1) [22]), which is also in clinical study (personal communication). Recently [23], we reported briefly on the synthesis and the in vitro inhibition of a potent steroidal inhibitor **Sa 40** (17-(5-pyrimidyl)androsta-5,16-diene-3β-ol (Fig. 1). Since this inhibitor showed a better activity toward the human enzyme compared to the inhibitors mentioned above, we decided to study these compound in vitro and in vivo in more detail. Here, we report about the in vitro activity of **Sa 40** using human and rat enzymes as well as a cellular assay as a pre in vivo model. We also report on the enzyme kinetic profile of **Sa 40** and abiraterone. The effects of **Sa 41** which is the 3-acetyl derivative of **Sa 40** on the plasma testosterone (T) level and some organs weight in Sprague–Dawley (SD) rats are also described and compared with the effects of castration and **BW 19** and **CB 7630** (3-acetyl derivative of arbiraterone) treatment.

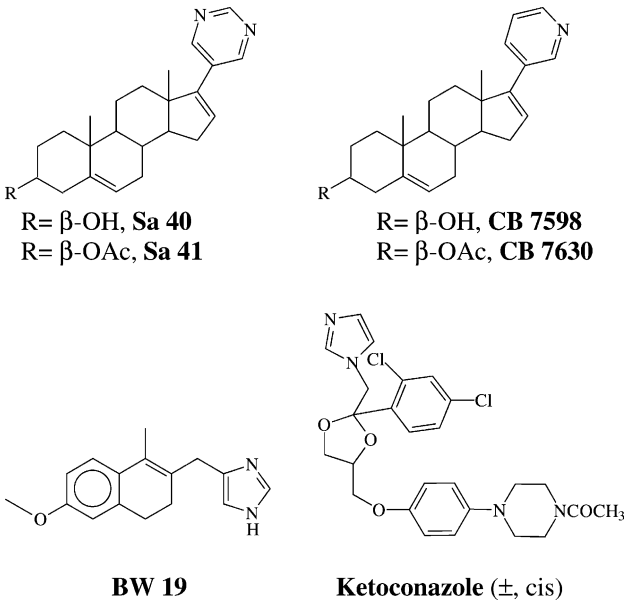

Fig. 1. Chemical structures of CYP 17 inhibitors.

2. Materials and methods

2.1. Inhibitors and reagents

The inhibitors were synthesized in our laboratory according to the procedures described by Haidar et al. for **Sa 40** and **Sa 41** [23], Potter et al. for **CB 7598** and **CB 7630** [14] and Hartmann et al. for **BW 19** [22]. Ketoconazole was obtained from Synopharm (Hamburg, Germany), progesterone from Caelo (Hilden, Germany) and 1,2-[^{3}H] progesterone (40–60 Ci/mmol) was obtained from NEN (Boston, MA, USA). The ^{125}I RIA kit for plasma testosterone quantification was purchased from ICN Biomedicals Inc. (CA, USA).

2.2. Preparation of microsomes and CYP 17 assay

The microsomal fractions (containing human or rat CYP 17) were prepared from human or rat testes as described by [17,24]. Microsomes were incubated with an excess of progesterone as substrate (25 μM) and NADPH (250 and 300 μM) for rat and human enzyme, respectively, and an appropriate concentration of inhibitor, in phosphate buffer (temperature: 32 and 37 °C for rat and human enzyme, respectively). Incubation was terminated after 20 min by addition of 1N HCl. The samples were submitted to HPLC (RP-18 column, CH_3OH:H_2O = 1:1, v/v) and measured using UV, 254 nm.

2.3. Enzyme kinetic study

K_i and K_m-values were determined according to Lee and Wilson [25]. Inhibitor concentrations were chosen between the IC_{40}- and IC_{70}-values of the compounds, substrate concentrations between 1.25 and 20 μM (Fig. 2). The incubation time was 15 min. All other parameters were identical to the regular CYP 17 assay.

2.4. E. coli (coexpressing human CYP 17 and NADPH-P450-reductase) assay

To test the inhibitory activity of compounds on human CYP 17 within cells, 0.1 M sodium phosphate, pH 7.4, was

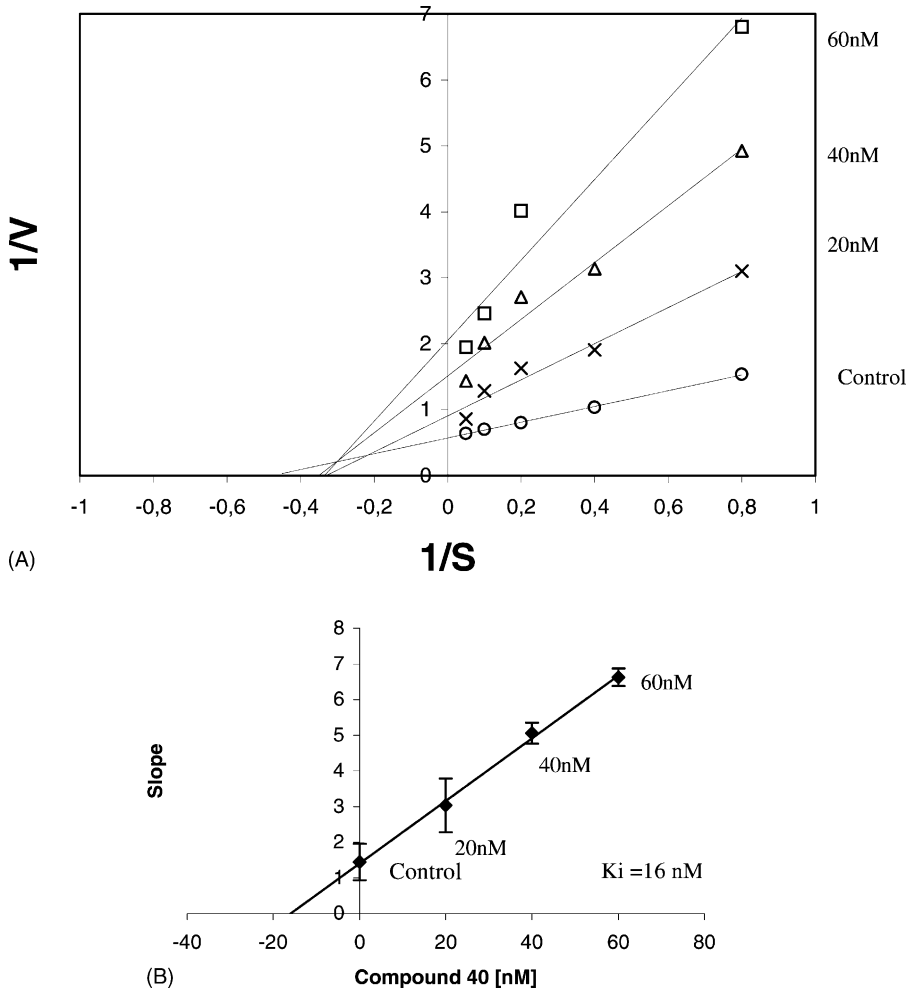

Fig. 2. Inhibition of human testicular microsomal CYP 17 by **Sa 40**. (A) Lee–Wilson plot of enzyme activities at various substrate and inhibitor concentrations. The K_m for progesterone was 2.85 μM. (B) Slope of each reciprocal plot against inhibitor concentration. The data from at least three experiments are expressed as mean ± S.E.

preincubated with 25 μM 1,2-[^{3}H] progesterone and an appropriate concentration of inhibitor at 37 °C for 10 min. The reaction was started by the addition of a suspension of recombinant *E. coli* XL1 pJL17/OR [24]. A_{578} was 3.0. After 45 min of vigorous shaking of the horizontally positioned cups at 37 °C, the reaction was stopped by heating at 95 °C for 5 min. Steroids were extracted with ethyl acetate by shaking for 5 min. The samples were evaporated, dissolved in methanol and analyzed by HPLC (RP-8 column, CH_3OH:H_2O = 1:1, v/v) as described before [26].

2.5. Irreversibility test

A preincubation of the enzyme with different concentrations of each inhibitor (0.5, 1, and 2 μM) was performed for 30 min using the same experimental procedure as in the regular test without substrate (Fig. 3). The unbound inhibitor was removed with charcoal: dextran treated charcoal, 2%, was added and the mixture was shaken for 10 min and centrifuged for 5 min at 6000 rpm. After incubation with the substrate the enzyme activity was determined at different time intervals [27].

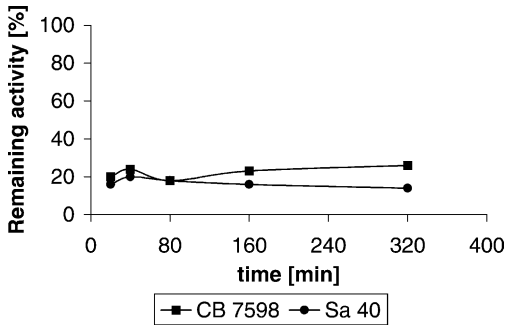

Fig. 3. Recovery of enzyme activity after preincubation with 1 μM of each inhibitor (preincubation 30 min, charcoal treatment for removal of unbound inhibitor, determination of enzyme activity after time intervals).

2.6. UV-Vis difference spectra

The UV-Vis difference spectra were recorded using a Perkin-Elmer Lambda 2 two-beam spectrophotometer with the corresponding computer software program PECSS. Rat testicular microsomes were suspended in 0.1 M sodium phosphate buffer containing 20% glycerol and 0.5% sodium cholate (pH 7.2) to a final concentration of 1 mg protein/ml. At 22 °C, the enzyme suspension was pipetted into two 1 cm pathlength cuvettes and a baseline was recorded from 300 to 500 nm. Difference spectra were then recorded at appropriate intervals following the addition of steroids dissolved in ethanol to the sample cuvette and an equivalent amount of ethanol added to the reference cuvette. The final concentration of ethanol in the cuvettes did not exceed 2% [27].

2.7. Test for selectivity

The selectivity of **Sa 40** was tested toward CYP 19 (aromatase) and CYP 11B2 (aldosterone synthase) according to the procedures described by [28,29].

2.8. In vivo studies

Male Sprague–Dawley rats weighing 250–300 g (2–3 months old) supplied by Harlan-Winkelmann, Germany, were housed under conditions of 12 h light dark cycle, temperature 20–22 °C and were allowed free access to food and water. One day after the arrival of the animals, one group was castrated under ether anesthesia. Rats (n = 6–8) were assigned to different treatment groups. The acetyl derivative of **Sa 40** (**Sa 41**) and the acetyl derivative of **CB 7598** (**CB 7630**) were used since they are better to formulate than the 3β-hydroxy compounds. Solutions of **Sa 41**, **CB 7630**, and **BW 19** were prepared in 100% olive oil. The preparations were given intraperitoneally (i.p.), 5 ml/kg, to the rats daily for 14 days. The dose of the inhibitor was 0.1 mmol/kg per day. The intact control and the castrated control groups were injected with vehicle for 14 days. Four hours after the last application, the animals were anaesthetized with ether and 1.5 ml of blood was collected by cardiac puncture in heparin (125 IU/ml of blood) containing tubes. The blood samples were centrifuged (3000 rpm, 4 °C, 10 min) to obtain the plasma which was stored at −70 °C. After decapitation, the organs of interest (ventral and complete prostate, seminal vesicles, levator ani, bulbocavernosus, testes, kidneys, adrenals, and pituitary) were dissected out and weighed after removal of adhering fat and connective tissue. A ^{125}I testosterone-RIA was used to determine the serum testosterone levels as described in the kit supplied by ICN Biomedicals Inc.

2.9. Data analysis and statistics

The inhibitory activity data are mean values of at least two experiments. The deviations were within ±10% (Tables 1 and 2). The organs weights were expressed as percentage of control weights. Values are mean ± standard error from six to eight rats. Comparisons between experimental groups were made using Dunnett's t-test. A P-value of less than 0.05 was considered as statistically significant (Fig. 4). Statistical analysis for the plasma T level was performed using Dunnett's t-test as well and the level of probability was 0.01 for statistical significance (Table 3).

Table 1
Inhibition of CYP 17 in human and rat testicular microsomes and in *E. coli* cells coexpressing human CYP 17 and NADPH-P450 reductase

Compound	IC_{50}[a] (nM) human[b]	IC_{50}[a] (nM) rat[c]	IC_{50}[a] (nM) *E. coli*[d]
Sa 40	24	220	30
Sa 41	38	1460	>2500
BW 19	110	210	110
CB 7598	73	220	54
CB 7630	110	1600	>2500
Ketoconazole	740	67000	>2500

[a] Concentration of inhibitor required to give 50% inhibition.
[b] Human testicular microsomes, concentration of progesterone (substrate) 25 μM.
[c] Rat testicular microsomes, concentration of progesterone 25 μM.
[d] Recombinant *E. coli* cells, concentration of progesterone 25 μM.
The given values are mean values of at least two experiments, deviations within ±10%.

Table 2
Effects of CYP 17 inhibitors on related P450 enzymes

Compound	% inhibition of CYP 19 (IC_{50}, μM)	% inhibition of CYP11B2 (IC_{50}, μM)
Sa 40	10[a]	n.i.[b]
BW 19	(1.2)	n.i.
CB 7598	(>20)[c]	n.d.

n.i.: no inhibition; n.d.: not determined.
[a] Concentration of inhibitor 2.5 μM.
[b] Concentration of inhibitor 36 μM.
[c] See [14].

Table 3
The effect of **Sa 41**, **CB 7630** and **BW 19** on the plasma testosterone level in adult SD rats after 14 days of treatment

Treatment group	Testosterone (ng/ml)
Intact control	2.215 ± 0.77
Castrated control	0.021 ± 0.016
Sa 41	0.061 ± 0.018
CB 7630	0.098 ± 0.062
BW 19	0.043 ± 0.0154

The results are given as mean ± S.E. with six to eight animals per group. Statistical analyses was performed using Dunnett's t-test. $P < 0.01$: intact controls were used as the reference control group. The concentrations were determined by RIA, as described in "Section 2".

3. Results

The inhibitory potencies of **Sa 40**, **Sa 41** and **BW 19** were determined for human and rat CYP 17 and compared to the

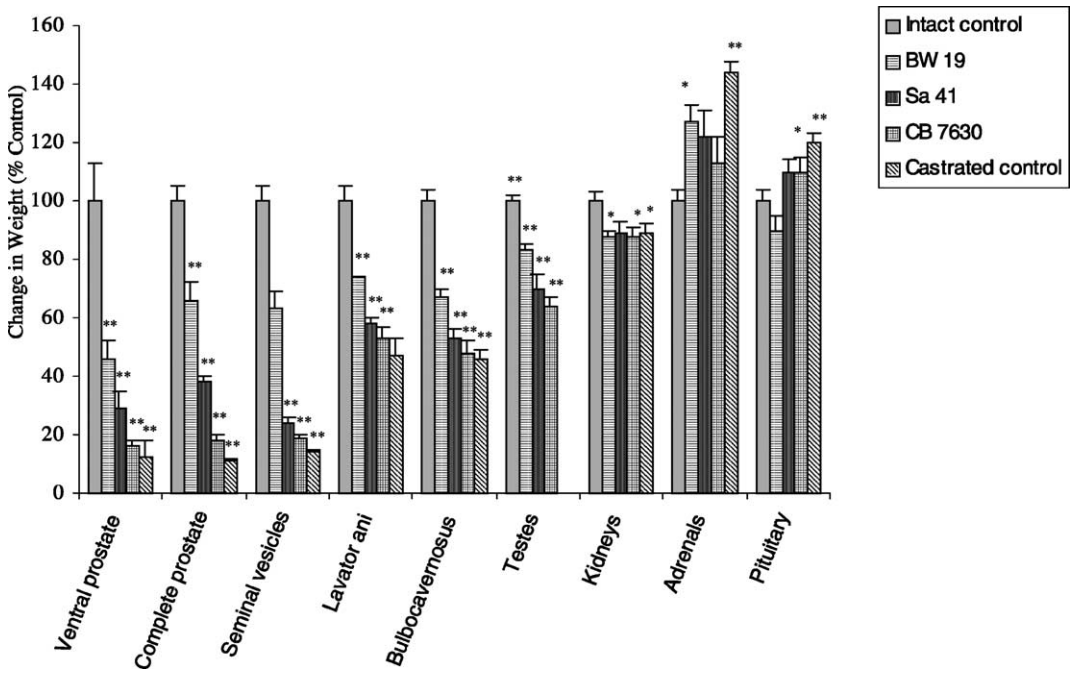

Fig. 4. Effects of 14 days treatments with **Sa 41**, **BW 19** and **CB 7630** on organs weight in adult SD rats. The animals were sacrificed 4 h after the last administration dose. The organs were removed and weighed. Values are expressed as the percentage of the control weights. Values are mean ± standard error from six to eight rats. (∗) $P < 0.05$, (∗∗) $P < 0.01$.

activities of **CB 7598** (abiraterone), **CB 7630** (abiraterone acetate) and ketoconazole (Table 1). **Sa 40**, **Sa 41** and **BW 19** showed very strong inhibition of human CYP 17 with IC_{50} values of 24, 38, and 110 nM, respectively, being more active or similarly active as **CB 7598** and **CB7630** (IC_{50} values of 73 and 110 nM, respectively). Ketoconazole was far less active than the other inhibitors with an IC_{50} value of 740 nM.

Having a closer look at the mode of inhibition, **Sa 40** showed a non-competitive type of inhibition (Fig. 2), while **BW 19** turned out to be a competitive inhibitor (data not shown) with corresponding K_i values of 16 and 56 nM, respectively (K_m of progesterone: 2.85 μM). Ketoconazole is known to be a competitive inhibitor of the target enzyme [1].

To study the mechanism of CYP 17 inhibition further, the chemical nature of the complexes formed between **Sa 40** and **CB 7598** and the rat enzyme were studied using microsomal preparations and UV-Vis difference spectroscopy following standard procedure. Each of the compounds induced a type II difference spectrum (trough: 390 nm, peak 422 nm for **Sa 40**; trough 388 nm, peak 418 nm for **CB 7598**) indicating coordination of the steroidal nitrogen to the heme iron of the CYP 17 enzyme, with formation of a low-spin iron (data not shown).

In order to find out whether the inhibitors are bound covalently to the enzyme, the reversibility of the inhibitor enzyme interaction was investigated. After a preincubation of **Sa 40** or **CB 7598** (1 μM) with the enzyme for 30 min and removal of the unbound inhibitor with charcoal, enzyme activity was determined after various time intervals (Fig. 3). There was no recovery of the enzyme activity within 320 min. Identical results were obtained using different inhibitor concentrations: 0.5 and 2 μM (data not shown). This indicates an irreversible inhibition of the enzyme by these compounds and is in accordance with observations of Jarman et al. for **CB 7598** [30].

The selectivity of **Sa 40** was determined toward CYP 19 (aromatase) and CYP 11B2 (aldosterone synthase) using the procedures as described by [28,29]. Taking into account that inhibition of these P450 enzymes in patients would subsequently cause severe side effects, it is very important to address to this question. High concentrations of **Sa 40** caused no inhibition of CYP 11B2 and only slight inhibition of CYP 19 (Table 2), indicating **Sa 40** to be a selective inhibitor of CYP 17.

A precondition for in vivo activity is the ability of a compound to permeate cell membranes. Therefore, the inhibitors were tested for inhibition of the target enzyme using a whole cell assay recently developed by [26]. Employing *E. coli* cells coexpressing human CYP 17 and NADPH-P450 reductase, active compounds have to inhibit the enzyme and must be able to permeate the cell membrane. Table 1 shows that **Sa 40** is more potent than **CB 7598** and **BW 19** with IC_{50} values of 30, 54, and 110 nM, respectively, while ketoconazole and the acetyl compounds **Sa 41** and **CB 7630** exhibited IC_{50} values >2500 nM.

For being tested in the rat, compounds have to be active in rat CYP 17 as well. In the assay with rat testicular microsomes **Sa 40**, **BW 19** and **CB 7598** exhibited very similar IC_{50} values around 200 nM. The acetyl compounds **Sa**

41 and **CB 7630** were less active with IC_{50} values of 1460 and 1600 nM, respectively, and ketoconazole only showed an IC_{50} of 67.000 nM (Table 1).

Based on the in vitro results described above, it was decided to study in vivo **BW 19**, **Sa 41** as a prodrug of **Sa 40** and **CB 7630** as a prodrug of **CB 7598**. Adult male Sprague–Dawley rats were assigned to different treatment groups to receive one of the compounds mentioned above (0.1 mmol/kg daily for 14 days) intraperitoneally. The animals were sacrificed at the end of the treatment, blood was collected and the tissues were removed and weighed. The results are summarized in Table 3 and Fig. 4. All tested compounds reduced the testosterone (T) levels strongly, almost reaching the level of the orchiectomy control. The T levels were reduced by all compounds for more than 95% compared to the control group. A strong activity of the tested compounds could also be observed in the organ weights. The weights of the ventral prostate and the seminal vesicles were strongly reduced by 54–84 and 37–81%, respectively ($P < 0.01$). The weights of levator ani and bulbocavernosus muscles were also remarkably reduced by 26–47 and 33–52%, respectively ($P < 0.01$). The testes weights were strongly reduced as well by 17–36% ($P < 0.01$). The kidneys weights of the castrated rats were reduced by around 10%. A similar reduction was also observed in all treatment groups. An increase in the weights of the adrenals was observed in all treatment groups as expected. Orchiectomy as well as treatment with **CB 7630** and **Sa 41** increased pituitary weights, while **BW 19** decreased this parameter (Fig. 4).

4. Discussion

CYP 17 is pivotal enzyme in the biosynthesis of androgens. Androgens have been shown to promote prostatic tumor growth. Compounds that reduce androgen formation, like gonadorelin analogs, have been shown to be useful in the treatment of androgen dependent diseases such as prostate cancer or benign prostatic hyperplasia (BPH). As outlined above inhibitors of CYP 17 could be more efficient therapeutics.

The data presented in this paper demonstrate that **Sa 40**, **Sa 41**, **BW 19**, **CB 7630** and **CB 7598** are highly potent inhibitors of CYP 17 in vitro and in vivo. In vitro **Sa 40** is the most active inhibitor for the human enzyme, showing a non-competitive type of inhibition. It binds strongly to the enzyme. This binding was unaffected after removing excess inhibitor by charcoal and incubating with high substrate concentrations indicating an irreversible type of inhibition. Applying these conditions on a reversible inhibitor, a recovery of enzyme activity within a short time can be observed [27]. It is important to note that Jarman et al. [30] demonstrated that the double bond in the 16, 17 position of the steroidal skeleton is essential for irreversible inhibition of steroidal CYP 17 inhibitors.

Sa 40 and **Sa 41** are three times more active than **CB 7598** and **CB 7630**, respectively. **Sa 40** is four times more active than **BW 19** and 30 times more active than ketoconazole, which had been used clinically for the treatment of prostate cancer. All tested compounds were highly active towards the rat enzyme except ketoconazole which showed a relatively poor inhibitory activity toward this enzyme. In the cellular assay, **Sa 40** was also better than all other tested compounds. The acetyl derivatives of **Sa 40** (**Sa 41**) and **CB 7598** (**CB 7630**) were not very active in this cellular test system probably because of the lack of esterase activity in *E. coli*. It is very likely that these acetyl compounds are not active or only slightly active as such and their activity observed in the testicular microsomes is due to their corresponding deacetylated metabolites. Further investigations to clarify this matter are presently performed by MS. In this context, however, it is important to note that a rapid deacetylation of **CB 7630** has been shown in vivo [31]. The poor inhibitory activity of ketoconazole in the cellular assay might be due to a poor permeability of this compound through the cellular membrane.

All tested compounds showed a potent inhibition of the plasma testosterone concentration in vivo. The significant regression of several androgen-dependent organs most likely is due to the reduced levels of circulating T. However, antiandrogenic effects cannot be excluded in case of the steroidal compounds. The tested substances were almost as effective as castration in reducing the plasma testosterone level. Higher doses might result in testosterone levels below orchiectomy values. However, in this study only one dose was used since it was our aim to determine the potency of **Sa 41** and compare it to other highly active inhibitors. Further investigations focusing on the question as to whether CYP 17 inhibitors are superior to surgical or medical castration are needed. All treatment groups reduced strongly the weights of prostates and seminal vesicles, castration showing a slightly better effect than **CB 7630**, followed by **Sa 41** and **BW 19**. In the orchiectomy group the weights of the kidneys were reduced while the weights of the adrenals and the pituitaries were increased. The same effect was observed using the three inhibitors except for **BW 19** where the pituitary weights remained unchanged. Our findings are in accordance with results of Barrie et al. who showed that **CB 7630** reduced the plasma T levels as well as the weights of androgen dependent organs in mice [31]. A further consequence of androgen blockade by surgical or medical castration is the suppressive effect on levator ani and bulbocavernosus weights. T produces its anabolic effect on muscles by androgen receptor stimulation [32] and therefore, orchiectomy leads to a suppression of the muscle mass. All tested compounds showed a significant reduction of these muscle weights similar to that observed after castration. It is interesting to mention that inhibitors of 5α-reductase (which converts T to the more active androgen DHT) showed completely different effects on these muscles. Häusler et al. reported that rats treated with finasteride or CGP 53153 (two highly active inhibitors of

5α-reductase) for 14 days did not show a decrease of the weights of these muscles, on the contrary, in some cases even an increase. This is due to the fact that 5α-reductase inhibition might lead to an enhancement of the T level [32].

The differences in the inhibitory activities of the compounds towards human and rat CYP 17 have been expected and are due to the fact that the enzymes have different amino acid sequences [3]. The fact that **CB 7630** is better in vivo than **Sa 41** although their deacetylated forms (**CB 7598** and **Sa 40**) show identical activity in vitro (rat), is certainly due to slightly better pharmacokinetic properties of **CB 7630** in the rat. Regarding activity in human patients, however, **Sa 41** should be superior to **CB 7630**, since the pyrimidyl compounds **Sa 40** and **Sa 41** are by a factor of three more potent towards human CYP 17 than their pyridyl analogs **CB 7598** and **CB 7630**. Therefore, **Sa 41** might be a promising candidate for clinical evaluation.

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