WS-9659 A AND B, NOVEL TESTOSTERONE 5α-REDUCTASE INHIBITORS ISOLATED FROM A *STREPTOMYCES*

III. BIOLOGICAL CHARACTERISTICS AND PHARMACOLOGICAL CHARACTERISTICS

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(Received for publication March 17, 1989)

WS-9659 A, a novel phenazine, produced by a *Streptomyces* sp., had testosterone 5α-reductase inhibition activity on rat, dog and human prostates. However, WS-9659 A did not show any inhibitory activities for aldose reductase on rabbit lenses and lactate dehydrogenase on pig hearts. WS-9659 A was a competitive inhibitor against testosterone 5α-reductase on rat prostates by use of testosterone as a substrate. Radio receptor binding assay of androgen receptor of rat prostates revealed that WS-9659 A had no affinity for this receptor.

WS-9659 A was tested subcutaneously in immature castrated rats to confirm its effect on the growth of the ventral prostates induced by testosterone propionate.

WS-9659 A was isolated as a novel enzyme inhibitor produced by a *Streptomyces* sp. It is well known that the development of prostatic hyperplasia is mediated *via* androgen metabolism. Among many reactions involved in testosterone metabolism, formation of 5α-dihydrotestosterone seems to be biologically important[1]. Also important in this process is the binding of 5α-dihydrotestosterone to the androgen receptor[2]. In this paper, we described the inhibition activity of testosterone 5α-reductase on rat, dog and human prostates, aldose reductase on rabbit lenses and lactate dehydrogenase on pig hearts. The data obtained from these experiment showed that WS-9659 A was a specific competitive inhibitor against rat testosterone 5α-reductase.

Radio-receptor binding assay of the androgen receptor of the rat prostate revealed that WS-9659 A had no affinity for this receptor.

Finally, we described the anti-prostatic activity of WS-9659 A given subcutaneously to immature castrated rats.

Experimental

Enzyme Inhibition Assay

The testosterone 5α-reductase inhibition assay was described in detail in a preceding paper.

Aldose reductase activity was measured according to the method described by NISHIKAWA *et al.*[3]. The following is a brief description; the reaction mixture contained 50 mM sodium phosphate buffer (pH 6.2), 0.125 mM NADPH, 400 mM lithium sulfate, enzyme solution and 3 mM *dl*-glyceraldehyde as a substrate in a total volume of 1 ml. The reaction was initiated by the addition of *dl*-glyceraldehyde and NADPH. The reaction-rate was measured for 2 minutes. The aldose reductase was prepared as described by NISHIKAWA *et al.*[3].

Lactate dehydrogenase which is purchased from Toyobo, is prepared from pig hearts and inhibition activity was measured using the kit from Wako Pure Chemical Industries, Ltd.

Androgen Receptor Assay

Mature Sprague-Dawley male rats were castrated for 24 to 40 hours before being killed. Ventral prostates were removed, minced and homogenized as described for the preparation of testosterone 5α-reductase[4], except that the buffer was replaced with ET buffer (EDTA 1 mM, Tris-HCl pH 7.4 10 mM, dithiothreitol 5 mM, Na_2MnO_4 10 mM).

The prostate homogenate was centrifuged at 100,000$\times g$ for 60 minutes. Aliquots of the resulting supernatant (20 mg of protein) were incubated in duplicate with 1 nM [^{3}H]testosterone (85~105 Ci/mmol, New England Nuclear) with or without competitors.

The incubation was at 0°C for 90 minutes in a final volume of 200 μl ET buffer. The solution was treated with dextran-coated charcoal (charcoal suspension 5 mg, dextran 0.5 mg). After incubation at 0°C for 10 minutes, the mixture was centrifuged at 1,500$\times g$ for 5 minutes to pellet the charcoal. The 100-μl of the supernatant was taken to determine the receptor bound radio activity in 10 ml of Aquazol-2 (New England Nuclear).

Effect of Drugs on Growth of the Ventral Prostate Induced by Testosterone Propionate in Immature Rats

Wister male rats (Nippon Crea) weighing approximately 90 g were castrated *via* a scrotal incision. The three days later, they received subcutaneously an injection of 0.2 ml vehicle (sesame oil containing 5% ethanol by volume) either alone or containing different doses of WS-9659 A. After 30 minutes they were given subcutaneously an injection of 0.2 ml vehicle alone or containing 30 μg of testosterone propionate. The administrations were repeated for 5 consecutive days once a day. Six hours after the fifth injection, the rats were sacrificed by decapitation, and then the ventral prostates and seminal vesicles were removed, cleaned of adherent tissue. Each gland was weighed individually.

Results

Biochemical Properties *In Vitro*

WS-9659 A and B inhibited testosterone 5α-reductase in a dose dependent manner. Fig. 1 shows the inhibition curves against testosterone 5α-reductase on rat prostates.

Moreover, WS-9659 A inhibited testosterone 5α-reductase against dog and human prostates (Table 1). The preparations of dog and human prostates are treated in the same method of that

Fig. 1. The inhibition curves of WS-9659 A and B for testosterone 5α-reductase on rat prostates.

● WS-9659 A, ○ WS-9659 B.

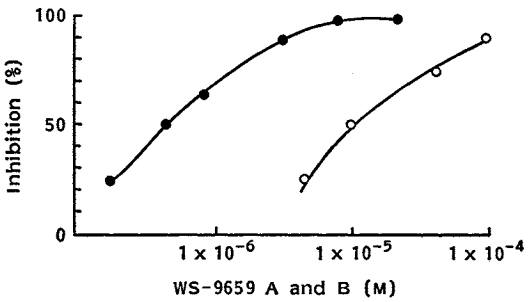


Table 1. Inhibition of testosterone 5α-reductase on rat, dog and human prostates by WS-9659 A.

Drug	IC_{50} (M)		
	Rat	Dog	Human
WS-9659 A	5.0×10^{-7}	7.2×10^{-7}	8.7×10^{-7}

Table 2. The comparison of IC_{50} values of WS-9659 A and 4-MA for rat testosterone 5α-reductase, rabbit aldose reductase, pig lactate dehydrogenase.

Drug	IC_{50} (M)		
	T5α-RI	ARI	LDHI
WS-9659 A	5.0×10^{-7}	$>1.0\times10^{-5}$	$>1.0\times10^{-4}$
4-MA	6.0×10^{-9}	$>1.0\times10^{-5}$	$>1.0\times10^{-4}$

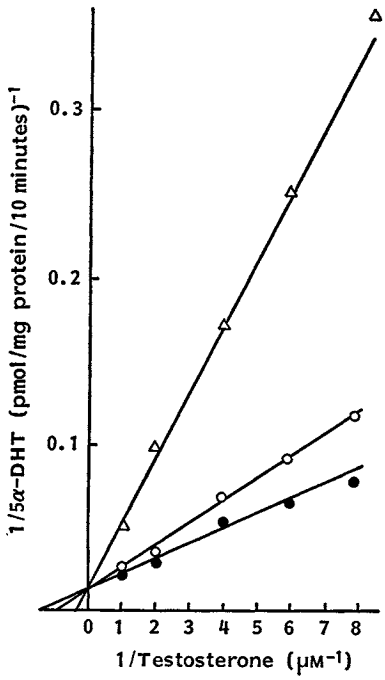
Abbreviations: T5α-RI, Testosterone 5α-reductase inhibition; ARI, aldose reductase inhibition; LDHI, lactate dehydrogenase inhibition.

of rat[4].

The IC_{50} values of WS-9659 A were 7.2×10^{-7} M for dog prostates and 8.7×10^{-7} M for human prostates. However, WS-9659 A did not inhibit aldose reductase and lactate dehydrogenase (Table 2). The IC_{50} of *N,N*-diethyl-4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxamide (4-MA) was calculated when assessed in the same experiment.

Fig. 2. Lineweaver-Burk plot for inhibition of testosterone 5α-reductase on rat prostates by WS-9659 A (37°C).

● Control, WS-9659 A concentration: ○ 0.5 μM, △ 1.5 μM.



The kinetic study of WS-9659 A was carried out using testosterone 5α-reductase from rat prostates.

Lineweaver-Burk plot and Dixon plot for testosterone 5α-reductase inhibition are shown in Figs. 2 and 3. The double reciprocal plots yielded patterns consistent with classical competitive inhibition. WS-9659 A had an apparent *Ki* value of 5.0×10^{-7} M (Fig. 3).

As shown in Table 3, WS-9659 A had no affinity for androgen receptor.

In Vivo Evaluation of WS-9659 A

In the *in vivo* evaluation experiment, WS-9659 A was administered subcutaneously for 5

Table 3. Inhibition of androgen receptor binding in rat prostates.

	WS-9659 A	4-MA	Chlormadinone acetate
IC_{50}	$>3.0\times10^{-4}$ (30%)	2.8×10^{-6}	1.0×10^{-8}

Fig. 3. Dixon plot for inhibition of testosterone 5α-reductase on rat prostates by WS-9659 A (37°C).

The abscissa represents the concentration of WS-9659 A. Testosterone concentrations: ■ 0.25 μM, ▲ 0.5 μM, ● 1 μM.

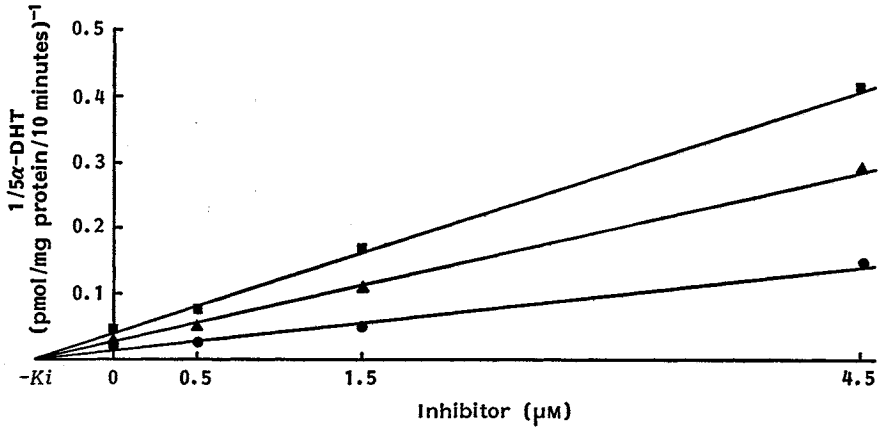


Table 4. *In vivo* effect of WS-9659 A on the weight of rat ventral prostates and seminal vesicles.

Drug	V.P. (mg/100 g B.W.)	Inhibition (%)	S.V. (mg/100 g B.W.)	Inhibition (%)
Testosterone propionate	29.3±0.7	0	31.7±2.1	0
Castration only	8.9±0.8	100	6.3±0.1	100
Testosterone propionate + WS-9659 A (10 mg/kg)	26.9±2.5	11.9	29.7±0.5	7.8
Testosterone propionate + WS-9659 A (100 mg/kg)	24.2±1.8	25.0*	29.9±0.1	6.9

Abbreviations: V.P., Ventral prostate; B.W., body weight; S.V., seminal vesicle.
* $P<0.05$.

There are 5 rats per group. In each experiment, for statistical purpose, the group given WS-9659 A and testosterone propionate were compared with the group given testosterone propionate alone.

Table 5. *In vivo* effect of 4-MA on the weight of rat ventral prostates and seminal vesicles.

Drug	V.P. (mg/100 g B.W.)	Inhibition (%)	S.V. (mg/100 g B.W.)	Inhibition (%)
Testosterone propionate	19.7±0.8	0	18.0±1.0	0
Castration only	6.0±0.8	100	6.0±0.6	100
Testosterone propionate + 4-MA (10 mg/kg)	15.9±0.8	27.8**	11.6±0.4	53.3**
Testosterone propionate + 4-MA (100 mg/kg)	10.3±1.4	68.6***	6.6±0.3	94.6***

Abbreviations: See footnote in Table 4.
** $P<0.01$, *** $P<0.001$.

There are 5 rats per group. In each experiment, for statistical purpose, the group given 4-MA and testosterone propionate were compared with the group given testosterone propionate alone.

days to castrated immature rats. This compound caused a mild decrease in the weight of the ventral prostate, but did not decrease the weight of the seminal vesicles compared with the vehicle-treated controls, while 4-MA caused a significant decrease in the both organs.

The results obtained in this *in vivo* test carried out with castrated immature male rats, are shown in Tables 4 and 5.

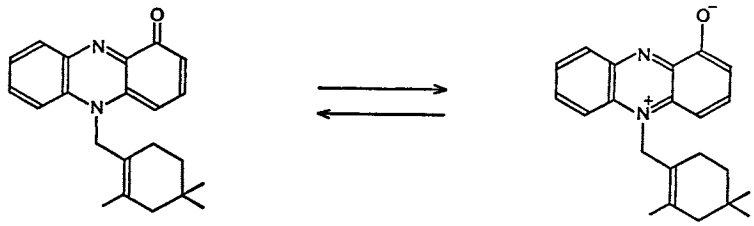
Discussion

The development of benign prostate hyperplasia is a almost universal phenomenon in aging man. The prostate gland weight only a few g at birth, at the time of puberty it undergoes androgen-induced growth and reaches the adult size of approximately 20 g by about 20 years of age[5]. The local accumulation of 5α-dihydrotestosterone may be involved in the pathogenesis of prostatic hyperplasia in man[6].

Continued high rate of conversion of testosterone to 5α-dihydrotestosterone within the gland may be involved in the pathogenesis hyperplasia. This evidence may be summarized as follows; first, after the administration of [^{3}H]testosterone to rats, 5α-dihydrotestosterone is the major radio active steroid bound to the prostatic nuclear chromatin[7]. Second, 5α-dihydrotestosterone is a potent stimulator of prostatic growth both *in vitro* and *in vivo* in the rat[8]. Third, early castration prevents the development of prostatic hyperplasia in man[9]. If one of these processes is blocked, prostatic growth will be inhibited or regression induced.

Therefore, we were interested in the process of conversion of testosterone to 5α-dihydrotestosterone and found active substances in a fermentation broth of *Streptomyces* sp. No. 9659. The main purified compound WS-9659 A had a novel phenazine structure and showed a highly potent activity in testosterone 5α-reductase inhibition assay.

Fig. 4. Tautomeric structures of WS-9659 A.



Moreover, WS-9659 A was shown to be pharmacologically active *in vivo*. Namely, the compound inhibited the growth of the ventral prostates induced by testosterone propionate in immature castrated rats.

The enzyme inhibition activity of WS-9659 A for testosterone 5α-reductase on rat, dog and human prostates was two or three orders of magnitude higher than those for aldose reductase on rabbit lenses and lactose dehydrogenase on pig hearts. The androgen receptor binding activity of WS-9659 A was very weak. These results indicate that WS-9659 A is a specific inhibitor for testosterone 5α-reductase. Furthermore, Table 1 shows that WS-9659 A showed highly potent inhibitory activity for testosterone 5α-reductase on dog and human prostates.

Urinary recovery of WS-9659 A, in *dd*Y mice (male, 8 weeks old) given intraperitoneal dosing of 30 mg/kg, was approximately 0.5%.

WS-9659 A and B are novel compounds belonging to simple phenazine. Although a vast number of phenazine antibiotics originating from microorganisms have been reported, a cyclohexyl methyl hydroxy substituent at the N-5 position is uncommon. The chromophore of WS-9659 A and B is the same of that of pyocyanine. These compounds have a zwitter ion as one of tautomeric structures and possibly exist as phenazium inner salts (Fig. 4).

Pyocyanine was also found to inhibit testosterone 5α-reductase (IC_{50}, 5.0×10^{-7} M), suggesting that it may be worthwhile investigating the chemical modification of WS-9659 A in order to increase the testosterone 5α-reductase inhibition activity or to study the structure-activity relationship of derivatives of this unique lead compound.

The decrease in the weight of the sex accessary gland which was shown to occur in rats treated with WS-9659 A is thought to have resulted from the inhibition of testosterone 5α-reductase. Pharmacological studies of WS-9659 A have as yet been preliminary and more precise pharmacological and toxicological studies will be required for the development of WS-9659 A as a clinical treatment of benign prostate hyperplasia.

In conclusion, we have found a novel compound, WS-9659 A, with a potent and specific testosterone 5α-reductase inhibition activity, from the culture broth of *Streptomyces* sp. No. 9659 utilizing radio isotopes. The use of the radio isotope technique will become a widely and efficiently applicable method of the screening of new pharmacologically active compounds of microbial origin.

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