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SELECTION OF 19-(ETHYLDITHIO)-ANDROST-4-ENE-3,17-DIONE (ORG 30958): A POTENT AROMATASE INHIBITOR *IN VIVO*

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Summary—19-Mercaptoandrost-4-ene-3,17-dione (ORG 30365) has been reported to be both a competitive and irreversible inhibitor of aromatase. In comparison to the known aromatase inhibitors 4-hydroxy-androst-4-ene-3,17-dione (4OH-AD) and 1-methyl-1,4-androstadiene-3,17-dione (SH 489), ORG 30365 was found to be, respectively, about 16 and 8 times more active *in vitro* using human placental microsomes. Although the activity profile of ORG 30365 is very attractive, this compound was not selected for further development because it has limited pharmaceutical stability, which is probably due to its free —SH group and therefore a number of more stable dithio-derivatives of ORG 30365 have been synthesized. These derivatives are considered to be converted to ORG 30365 before they become active. The *in vivo* aromatase inhibiting activity of these derivatives was determined in hypophysectomized rats treated with the estrogen precursor dehydroepiandrosterone sulphate (DHEAS) using inhibition of cornification of vaginal epithelium as parameter. The 19-(ethyldithio)-derivative (ORG 30958) appeared to be the most active inhibitor in this series being twice as active as ORG 30365 and about 8 times as active as inhibitors like 4OH-AD and SH 489. Besides inhibition of cornification of vaginal epithelium ORG 30958 decreased ovarian aromatase and plasma E_2 levels in DHEAS-treated hypophysectomized rats. Plasma estradiol levels were also lowered by ORG 30958 in dogs which were treated with pregnant mare serum gonadotrophin in order to induce pro-estrus. ORG 30958 displayed much less than 1/400th of the androgenic activity of testosterone propionate in immature castrated rats and appeared to be devoid of estrogenic and anti-estrogenic activity in ovariectomized mature rats. A twice daily dose of 1.5 mg ORG 30958/kg postponed ovulation in mature female rats.

In conclusion: ORG 30958 is a potent aromatase inhibitor *in vivo*. It probably becomes active after cleavage of the —S—S— bond yielding ORG 30365 a potent irreversible aromatase inhibitor. ORG 30958 does not display other hormonal activities making it an attractive candidate for the treatment of estrogen-dependent diseases.

INTRODUCTION

Aromatase, an enzyme complex involving a cytochrome *P*-450 and an NADPH–cytochrome c reductase, catalyzes the conversion of androstenedione and testosterone to estrone and estradiol, respectively [1, 2]. Besides its physiological role, estradiol plays a negative role in some pathophysiological states such as breast cancer [3]. For this reason inhibition of estrogen production is considered to be of therapeutic value. For many years the methods for curbing estrogen production was surgical ablation of ovaries, pituitary gland or adrenals. However, a part of the estrogen synthetic capacity remained since aromatase is an ubiquitous enzyme system [2]. Alternatively estrogen levels can be lowered by inhibiting the aromatase by specific inhibitors and by this approach the peripheral estrogen production also may be blocked. The first clinically useful aromatase inhibitor aminoglutethimide appeared to be a non-specific cytochrome *P*-450 inhibitor affecting aromatase among many other enzymes [5, 6]. Because of this non-specific action of aminoglutethimide more specific aromatase inhibitors were sought. Nowadays a considerable number of more specific steroidal [7–11] and non-steroidal inhibitors [12, 13] have been synthesized. Clinical efficacy in lowering estrogen levels has been

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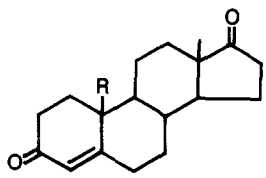

Compound	R	Additional substituents
ORG 30365	$-CH_2-SH$	
ORG 30958	$-CH_2-S-S-C_2H_5$	
4OH-AD	$-CH_3$	4-OH
SH 489	$-CH_3$	Δ^1,1-methyl

Fig. 1. Chemical structures of ORG 30365, ORG 30958 and reference compounds.

proven for some of them: including 4OH-AD [15] (Fig. 1) and the non-steroidal compound CGS 16949A [13].

Steroidal inhibitors may have some advantages above non-steroidal compounds, because of their competition with the natural substrate and their specificity. Non-steroidal inhibitors, which act by interference with the cytochrome *P*-450 moiety of the enzyme, may interfere with other cytochrome *P*-450 enzymes. This has recently been reported for CGS 16949 [14]. The steroidal inhibitor 4OH-AD has to be given in relatively large amounts (50 mg/i.m./wk, 250 mg/i.m./every 2 wk or 500 mg/o.r./day) before it becomes effective. Its major side effect was local irritation at the injection site due to the administration of these large quantities. Therefore more potent steroidal inhibitors are desirable. On the basis of the mechanism of aromatization it could be expected that substitution at C-19 of the natural substrate androst-4-ene-3,17-dione would yield interesting aromatase inhibitors.

Aromatase inhibiting activity of such compounds has been described [16–19], among them 19-mercapto-androst-4-ene-3,17-dione (ORG 30365) (Fig. 1), which was reported to be a potent irreversible inhibitor [18, 22]. However, ORG 30365 was not selected for further development because it has limited pharmaceutical stability which is probably due to its free —SH group. A number of dithio-derivatives have therefore been synthesized which are considered to be converted to ORG 30365 before they become active. In the present study the aromatase inhibiting activity of these dithio-derivatives of ORG 30365 was determined in the hypophysectomized mature female rat model. The finally selected 19-ethyl-dithio-derivative (ORG 30958, Fig. 1) was further tested in two species (rats and dogs). The endocrinological profile of ORG 30958 (i.e. androgenic, estrogenic, anti-estrogenic and anti-gonadotrophic, activity) was also assessed. In addition the *in vitro* aromatase inhibiting activity of ORG 30365, the active metabolite of ORG 30958, was determined and compared with that of reference compounds.

EXPERIMENTAL

Animals

Hsd/Cpb:ORGA rats were obtained from Harlan Sprague Dawley/Central Institute for the Breeding of Laboratory Animals of the Dutch Organization for Applied Scientific Research (HSD/Cpb), Zeist, The Netherlands. The rats were housed in light- and temperature-controlled rooms (14 h light–10 h dark; 21–23 or 25–26°C). Tap water and pelleted food (RMH-B, Hope Farms, Linschoten, The Netherlands) were given *ad libitum*. Pure-bred female Beagle dogs with a proven estrous cycle were supplied by CERM, Riom, France. The dogs were housed individually in kennels with central heating and with an outdoor run. They were fed daily with 300 g pelleted food (BD-1) supplied by Hope Farms, Linschoten, The Netherlands. Tap water was given *ad libitum*.

Reagents

Nicotinamide adenosine dinucleotide phosphate (NADP), glucose-6-phosphate (G-6-P) and glucose-6-phosphate dehydrogenase (G-6-P-DH) were purchased from Boehringer, Mannheim, F.R.G. [1β-^{3}H]Androst-4-ene-3,17-dione ([1β-^{3}H]AD) (NET 926) was supplied by New England Nuclear, Boston, Mass, U.S.A. Charcoal and Giemsa solution were delivered by Merck, Darmstadt, F.R.G. Dextran T70 and Sephadex LH20 were purchased from Pharmacia, Uppsala, Sweden. Pregnant mare serum gonadotrophin (PMSG) was supplied by Intervet, Boxmeer, The Netherlands. Bovine serum albumin (BSA) was obtained from Sigma Chemical Company, U.S.A.

Steroids

Dehydroepiandrosterone-3-sodium sulphate (DHEAS), estradiol-17β (E_2) and testosterone propionate (Tep) were supplied by Organon Int. BV, Oss, The Netherlands. All aromatase inhibitors were synthesized by the Organic Chemistry Research and Development Labs, Organon Scientific Development Group, Oss, The Netherlands. The chemical structures are given in Figs 1 and 2.

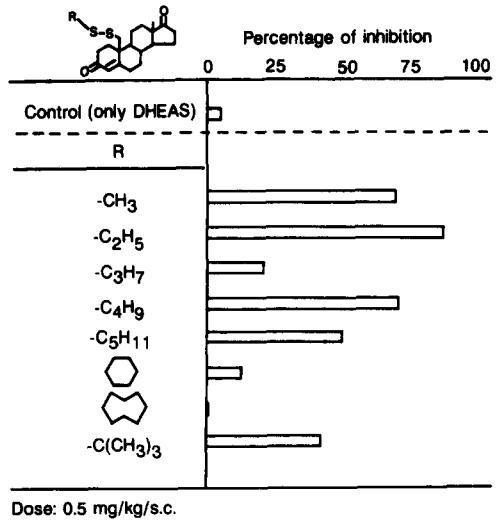

Fig. 2. Effect of various 19-dithio-derivatives of ORG 30365 on cornification of vaginal epithelium in Hypex rats treated with DHEAS (16 mg/kg/or). The derivatives were tested at a dose level of 0.5 mg/kg/s.c. The effects are given as a percentage inhibition of the control activity (without inhibitor).

Determination of aromatase inhibition in hypophysectomized (Hypex) mature female rats

The test was carried out as described by Deckers and Schuurs [21]. Briefly, mature female rats weighing 200–250 g were hypophysectomized. Seven days later the rats received an aqueous suspension of dehydroepiandrosterone sulphate (DHEAS) by gavage daily for 4 days at a dose level of 16 mg/kg. The suspension fluid was an aqueous solution of gelatine (5 mg/ml) and mannitol (50 mg/ml) (gel/man). The aromatase inhibitors were given subcutaneously (s.c.) as oily solutions immediately after DHEAS-treatment. Vaginal smears were taken, stained with Giemsa solution and evaluated daily for 6 days after the onset of administration. Vaginal smears were considered to be positive if they contained more than 50% nucleated or cornified epithelial cells. The results are expressed as the percentage inhibition: total number of smears – number of positive smears vs the total number of smears ($\times 100\%$).

When serum E_2 levels and ovarian aromatase activities were assessed in the experiments described above the rats were sacrificed under deep diethylether anaesthesia 2 h after the last treatment. Blood samples were collected from the abdominal aorta and ovaries dissected. Serum were prepared and stored at −20°C until determination of E_2 by radio-immunoassay (RIA). After weighing the ovaries were transferred to test tubes for determination of aromatase activity. The ovaries were minced with scissors. One millilitre of buffer (100 mmol potassium phosphate and 5 mmol K_3 EDTA/l, pH 7.4) was added to 5 mg of ovarian tissue. The tissue was homogenized with a Potter–Elvehjem at 0°C. The homogenate was centrifuged at 3000 N/kg for 10 min at 0°C. One millilitre of supernatant was added to a test tube containing 0.219 nmol [1β-^{3}H]AD. An NADPH generating system in buffer was added and an incubation was performed for 1 h at 37°C. Incubation was terminated by placing the tubes on ice for 15 min and this was followed by an extraction with 5 ml chloroform. After phase separation by centrifugation, the aqueous phase was diluted with an equal volume of dextran coated charcoal suspension containing 50 g charcoal and 5 g Dextran T70/l. Following centrifugation the ^3H_2O content was determined by liquid scintillation counting. The amount of ^3H_2O is a measure of the amount of estrogen produced.

The effect of ORG 30958 on plasma E_2 levels of mature Beagle dogs with PMSG-induced pro-estrus

Anestrus mature female Beagle dogs with proven estrous cycle were treated s.c. with PMSG (20 U/kg) daily for 10 days at the most. During this treatment vaginal smears were taken daily and the dogs were examined for vulval swelling and vaginal hemorrhagic discharge. Vaginal smears were stained with Shor's trichrome stain. The appearance of erythrocytes in the vaginal smears was considered as the onset of pro-estrus.

About 24 h after onset of pro-estrus the dogs were treated with a single s.c. dose of ORG 30958 in an oily vehicle or the vehicle only. Blood was taken just before treatment and at various times after treatment. Blood samples were collected from the jugular vein using evacuated blood collecting tubes (Venoject) with K_3 EDTA as anti-coagulant. Plasma was prepared by centrifugation of the blood by 15,000 N/kg during 10 min at 4°C. Plasma was stored at −20°C until determination of E_2 by RIA. In this model a compound is considered to be active if after its administration the initial fall of E_2 level is followed by a rise of E_2 level in order to exclude that the fall is due to the natural termination of pro-estrus.

E_2 determination by RIA

The serum samples were submitted to extraction with ethyl acetate and the extracts were subsequently evaporated to dryness. The E_2-

Table 1. Effect of ORG 30365, ORG 30958 and reference compounds on serum E_2 levels and ovarian aromatase activity in Hypex rats treated with DHEAS (16 mg/kg/or). Mean values ± SEM are given

Compound	Daily s.c. dose (mg/kg)	E_2 (pg/ml)	Aromatase activity (pmol estrogens/ mg protein/h)
Untreated	—	14 ± 3.9[a]	ND
Control	—	88 ± 21.6	26.6 ± 5.2
ORG 30365	4	15 ± 3.0[a]	6.7 ± 2.3[a]
ORG 30958	1	19 ± 2.8[a]	12.8 ± 1.7[a]
4OH-AD	4	34 ± 9.2[a]	4.6 ± 0.9[a]
SH 489	4	21 ± 4.7[a]	20.4 ± 6.3

[a]Statistically significant difference ($P < 0.05$) between treated group and control group.
ND = not determined.

Table 3. Effect of ORG 30958 and 4OH-AD on the weight of seminal vesicles (SV) and ventral prostate (VP) in castrated immature male rats. The test compounds were s.c. administered daily for 7 days

Compound	Dose (mg/kg)	Number of rats	Mean organ weights (mg ± SEM) SV	VP
Control	—	24	6.9 ± 0.3	10.1 ± 0.7
Tep	0.2	12	16.1 ± 0.9	30.2 ± 1.7[a]
	0.8	12	46.5 ± 2.1	63.4 ± 2.8[a]
ORG 30958	20	6	7.0 ± 0.3	9.2 ± 0.7
	80	6	7.4 ± 0.2	12.8 ± 2.0
4OH-AD	20	6	7.5 ± 0.4	34.4 ± 3.3[a]
	40	6	9.4 ± 0.3[a]	36.2 ± 6.7[a]
	80	6	13.4 ± 1.3[a]	57.7 ± 5.4[a]

[a]Statistically significant difference ($P < 0.05$) between treated group and control group.

containing fraction in the dissolved residues was isolated by LH20 Sephadex column chromatography. E_2 was determined by RIA as described by de Jong *et al.* [23].

Anti-estrogenic activity on vaginal epithelium in ovariectomized mature rats

The test was carried out according to van der Vies and de Visser [24]. In brief, 2 wk after ovariectomy mature rats weighing 230–375 g were treated s.c. with E_2 daily for 10 days at a dose level of 0.8 μg/kg. Immediately after E_2 treatment the aromatase inhibitors were administered s.c. Doses are given in the tables. E_2 was dissolved in arachis oil and the test compounds were suspended in a gel/man solution. Vaginal smears were taken daily during the treatment period and the number of positive smears was determined as described above.

Androgen activity of ORG 30958 and 4OH-AD in castrated immature male rats

The test was performed according to the procedure described by van der Vies and de Visser [24]. In brief, orchidectomized immature male rats weighing 55–65 g were treated s.c. with ORG 30958, testosterone propionate (Tep) or 4-OH-andros-4-ene-3,17-dione daily for 7 days. ORG 30958 and Tep were dissolved in arachis oil whereas 4OH-AD was suspended in gel/man solution. Doses are given in Table 3. At autopsy the animals were killed with CO_2 and the seminal vesicles and ventral prostate were dissected out and weighed. For statistical evaluation the analysis of variance was performed using the logarithms of the organ weights.

Table 2. Effect of ORG 30958 and 4OH-AD on E_2-induced cornification of vaginal epithelium in ovariectomized mature rats. The rats were s.c. treated with E_2 at a dose level of 0.8 μg/kg daily for 10 days immediately followed by administration of the test compound

Compound	Daily s.c. dose (mg/kg)	Number of rats	Percentage positive smears
E_2 ± vehicle	—	37	98
E_2 + ORG 30958	2	8	97
	8	8	99
E_2 + 4OH-AD	2	8	97
	4	8	92
	8	8	67

Estrogenic activity on vaginal epithelium in ovariectomized mature rats

The test was carried out according to van der Vies and de Visser [24]. In brief, 2 wk after ovariectomy mature rats weighing 230–375 g were primed with a single s.c. dose of E_2 (1 μg). Seven days after priming ORG 30958 was administered s.c. for 2 days at a dose level of 4 mg/kg. E_2 was dissolved in arachis oil and ORG 30958 was suspended in a gel/man solution. Vaginal smears were taken daily during the treatment period and the number of positive smears was determined as described above.

Ovulation inhibition of ORG 30958 and 4OH-AD in mature rats

The experiment was done as described by van der Vies and de Visser [24]. Mature female rats with a regular 4-day estrus cycle and weighing 265–400 g were used. A twice-daily s.c. treatment of the animals with the aromatase inhibitors was started at estrus and lasted 5 days. Doses are given in the table. ORG 30958 was given as a solution in arachis oil and 4OH-AD as a suspension in gel/man solution. On the morning of day 6 the animals were killed with CO_2 and the oviducts were dissected out and macroscopically examined for the absence or presence of ova (with or without granulosa cells).

Preparation of human placental microsomes

A freshly delivered human placenta was obtained from the local hospital. After washing with ice-cold 0.15 mol/l KCl solution the tissue was dissected free of adhering membranes and

large blood vessels. To each gram of tissue 1 ml of ice-cold 0.25 mol/l sucrose solution was added. The tissue was homogenized first with a Waring Blender and then with an Ultra Turrax, both carried out at 0°C. Microsomes were isolated by differential centrifugation as described by Ryan [20], resuspended in distilled water, lyophilized and stored at −20°C until use.

Aromatase inhibition in vitro

Inhibitors dissolved in ethanol were added to human microsomes suspended in assay buffer (composition: 10 mmol potassium phosphate, 100 mmol KCl and 1 mmol K_3 EDTA/l, pH 7.4). Final ethanol concentration was 0.5% (v/v). Microsomal protein content was determined by the Lowry method using bovine serum albumin as a standard [21] and was 0.13 g/l.

After mixing, these suspensions were added to tubes containing a fixed amount of [1β-^{3}H]AD. After a pre-incubation period of 5 min at 37°C an NADPH generating system dissolved in assay buffer was added to each tube. The final concentrations of the generating system in the incubate were 2.5 mmol/l NADP, 5 mmol/l G-6-P and 525 U/l G-6-P-DH. Samples of 1 ml were incubated for 15 min at 37°C. The incubation was determinated by adding 5 ml of chloroform and mixing thoroughly.

The ^3H_2O content of the incubate was assessed as described above.

RESULTS

In vivo aromatase inhibition

A number of stable dithio-derivatives of the potent inhibitor ORG 30365 were synthesized. The inhibiting effects of these derivatives on E_2-induced cornification of vaginal epithelium in Hypex rats, caused by a daily oral treatment of 16 mg/kg of the estrogen precursor DHEAS, are given in Fig. 2. At a s.c. dose of 0.5 mg/kg the 19-ethyl-dithio-derivative (ORG 30958) decreased induction of cornification of vaginal epithelium by about 87%. Substitution of the ethyl-group by a methyl-, propyl- or longer alkyl-groups as well as introduction of branched or cyclic substitutes gave less pronounced inhibiting effects than ORG 30958. No clear correlation was observed between the length or width of the substituent and the extent of aromatase inhibition. Being the most potent inhibitor in the series, ORG 30958 was selected for further development.

In order to estimate the inhibitory potency of

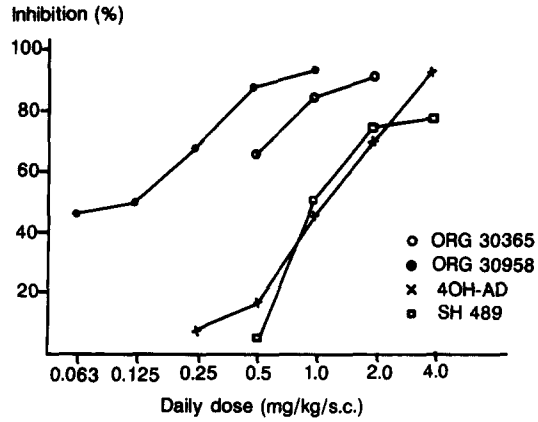

Fig. 3. Effect of ORG 30365, ORG 30958 and reference compounds on cornification of vaginal epithelium in Hypex rats treated with DHEAS (16 mg/kg/or). The effects are given as a percentage inhibition of the control activity.

ORG 30958 and ORG 30365, the assumed active metabolite of ORG 30958 vs the reference compounds 4OH-AD and SH 489 on vaginal cornification, various doses of these compounds were tested in Hypex female rats treated with DHEAS. Maximal aromatase inhibition (100%) is reached if none of the vaginal smears show cornification following administration of the inhibitor and DHEAS. From the log-dose curves in Fig. 3 a 50% inhibitory response was considered to be achieved for ORG 30958, 4OH-AD and SH 489 at doses of about 0.125, 1 and 1 mg/kg, respectively, whereas ORG 30365 displayed a 65% response at 0.5 mg/kg, indicating clearly that ORG 30958 is the most potent inhibitor in this model.

In a separate experiment the effects of the inhibitors on ovarian aromatase and plasma

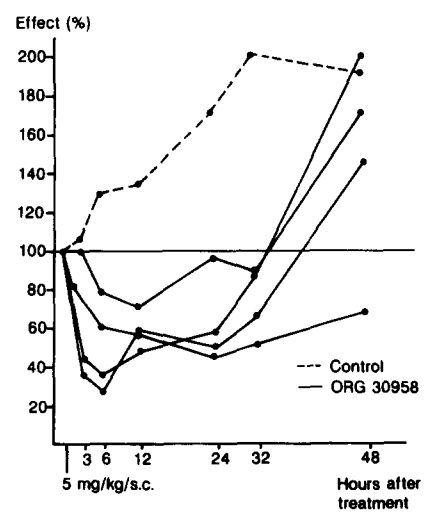

Fig. 4. Effect of ORG 30958 on plasma E_2 levels in Beagle dogs with PMSG-induced pro-estrus. ORG 30958 was tested s.c. at a dose level of 5 mg/kg. The E_2 values of individual dogs expressed as % of the E_2 levels at $T = 0$.

Table 4. Effect of ORG 30958 and 4OH-AD on ovulation in mature rats. The test compounds were s.c. administered twice daily for 5 days

Compound	Dose (mg/kg)	Number of rats with ovulation		
		Normal	Postponed	Inhibited
Controls	—	12	0	0
ORG 30958	0.75	6	0	0
	1.5	2	4	0
	3.0	1	5	0
4OH-AD	0.75	5	0	1
	1.5	0	6	0
	3.0	0	3	3

E_2 levels were also studied at the end of the treatment period in this rat model. ORG 30958 and ORG 30365 at a daily dose of 1 and 4 mg/kg, respectively, prevented the DHEAS-stimulated rise in E_2 levels. Table 1 shows that 4OH-AD and SH 489 were about 4 times less potent than ORG 30958. In contrast to the SH 489-treated group ovarian aromatase activity of the ORG 30958-, ORG 30365- and 4OH-AD-treated groups was significantly decreased in comparison to the control group, indicating that ORG 30958, ORG 30365 and 4-OH-AD act mainly as irreversible inhibitors whereas SH 489 acts mainly as a competitive inhibitor.

The aromatase inhibition of ORG 30958 was also determined in Beagle dogs with PMSG-induced pro-estrus. At a s.c. dose of 5 mg/kg ORG 30958 is active in all animals (Fig. 4).

Endocrinological profile

In order to assess whether ORG 30958 possesses additional hormonal activities, its endocrinological profile was determined.

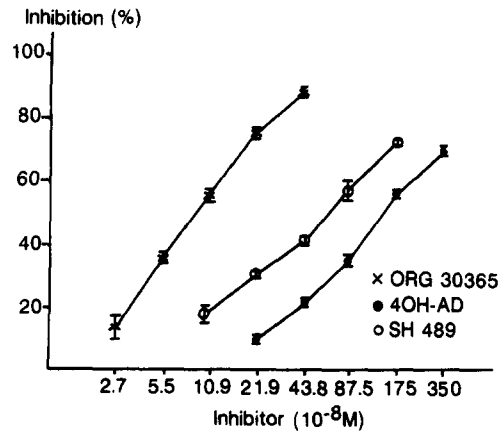

Fig. 5. Comparison of aromatase inhibition in human placental microsomes by ORG 30365 and reference compounds. The compounds were tested in the presence of 3.5×10^{-6} M [1β-^{3}H]androst-4-ene-3,17-dione and a NADPH generating system. Determinations were performed in triplicate. The amount of produced ^3H_2O is a measure for aromatase activity. The values are given as a percentage inhibition (±SD) of control activity (without inhibitor).

The anti-estrogenic activity of ORG 30958 and 4OH-AD in rats is given in Table 2. At a daily dose of 8 mg/kg ORG 30958 did not display any anti-estrogenic activity while 4OH-AD partially counteracted the E_2-induced vaginal cornification.

ORG 30958 at a daily dose of 80 mg/kg induced a very weak but statistically significant increase in the weight of the ventral prostate compared to the weight in untreated animals (Table 3). Its androgenic activity is, however, considerably less than 1/400th of the activity of Tep. The androgenic activity of 4OH-AD, based on ventral prostate weight increase, is approx. 1/100th of the activity of Tep.

At a daily s.c. dose of 4 mg/kg ORG 30958 displayed no estrogenic activity in ovariectomized rats.

The effects of ORG 30958 and 4OH-AD on ovulation in mature rats as a test for an effect on the pituitary (central effect) are shown in Table 4. At both doses tested (1.5 and 3 mg/kg/twice daily) ORG 30958 postponed ovulation in most animals. No inhibition of ovulation was seen. At a twice daily dose of 1.5 mg/kg, 4OH-AD postponed ovulation in all animals; while at a 3 mg/kg dose it inhibited ovulation in 50% of the rats.

In vitro aromatase inhibition

ORG 30958 itself appeared to be a very weak aromatase inhibitor *in vitro* as were several other dithio-derivatives. Since ORG 30365 is presumed to be the active metabolite of ORG 30958, its *in vitro* aromatase inhibiting activity was compared with that of two reference compound, viz. 4OH-AD and SH 489, using human placental microsomes, a fixed amount of [1β-^{3}H]AD and an NADPH generating system. Figure 5 shows that the IC_{50} values of ORG 30365, SH489 and 4OH-AD were 9, 70 and 140 $\times 10^{-8}$ mol, respectively, making ORG 30365 about 8 and 16 times more active than SH 489 and 4OH-AD, respectively.

DISCUSSION

The introduction of a thio function in the C-19 position of the natural substrate androst-4-ene-3,17-dione resulted in a compound (ORG 30365), which we have been shown to be a potent aromatase inhibitor, both *in vitro* and *in vivo* [18, 22], without additional hormonal activity. In the Hypex rat model ORG 30365 lowered both plasma E_2 and ovarian aromatase levels,

indicating an irreversible inactivation of aromatase similarly to 4OH-AD (Table 1). This is in agreement with the reported irreversible inhibitory activity of ORG 30365 [17] and 4OH-AD [30]. SH 489 reduced the aromatase activity only partly which means that it is predominantly a competitive inhibitor, confirming the results of Henderson *et al.* [26]. *In vitro* ORG 30365 was about 16 and 8 times as active as the known aromatase inhibitors 4OH-AD [24] and SH 489 [11], respectively, while *in vivo* in the Hypex rat model ORG 30365 was approx. 4 times as potent as these inhibitors. Although the activity profile of ORG 30365 was very attractive, this compound was not selected for further development because it has limited pharmaceutical stability which is probably due to its free —SH group. Looking for derivatives, which can easily be converted in biological systems to ORG 30365, compounds with an —S—S— bond were synthesized. Although these derivatives have very low intrinsic inhibiting activity *in vitro* they possess promising *in vivo* activity indicating a cleavage of the —S—S— bond. The 19-(ethyldithio)-derivative (ORG 30958) appeared to be the most active compound in this series being twice as active as ORG 30365 in the Hypex rat model when using cornification of vaginal epithelium as a response parameter. This increased activity of ORG 30958 compared to that of ORG 30365 can probably be explained by a better or more prolonged availability of the active compound in the target organs.

ORG 30958 displays in the Hypex rat model about 8 times the activity of both reference inhibitors 4OH-AD and SH 489. The similar potencies of 4OH-AD and SH 489 as found in the Hypex rat model are in accordance with the indentical effects of these two inhibitors on the reduction of E_2 levels in PMSG-treated juvenile rats [26]

In order to demonstrate aromatase inhibitory activity of ORG 30958 in another species the compound was administered to female Beagle dogs. In dogs plasma E_2 levels are very low during anestrus (< 10 pg/ml) but do increase rapidly during pro-estrus [27]. However, since it is rather impractical to use dogs with a natural pro-estrus which occurs only once or twice a year and whose onset of pro-estrus cannot be predicted because of considerable variations in the length of the estrus cycle [27], pro-estrus was induced in late-anestrus dogs by daily PMSG treatment. This induced pro-estrus is usually considerably shorter (3–5 days) than the natural pro-estrus period (8–10 days) [28] It was therefore decided to test the aromatase inhibitor 24 h after the onset of the PMSG-induced pro-estrus. ORG 30958 reduced E_2 levels in all dogs treated, thus proving the efficacy of the inhibitor in this species.

Additional intrinsic detrimental hormonal activities (i.e. estrogenic and androgenic) of aromatase inhibitors must preferably be avoided. However, anti-hormonal activities may even be additive. ORG 30958 displayed no estrogenic and only very weak androgenic activity. Its androgenic activity was much less than 1/400th of the activity of Tep, whereas the androgenic activity of 4OH-AD was about 1/100th that of Tep. Androgenic activity of 4OH-AD has also been reported by Brodie *et al.* [29, 30].

ORG 30958 proved to be devoid of anti-estrogenic activity (Table 2) which indicates that the absence of cornification of vaginal epithelium in DHEAS-treated Hypex rats is induced by the inhibition of the biosynthesis of estrogens and not by an anti-estrogenic activity. 4OH-AD appeared to have some anti-estrogenic activity which might be explained by its androgenic activity, since androgens have been shown to antagonize the effects of estrogens [31–33]. Although ORG 30958 was 8 times as potent as 4OH-AD with respect to the aromatase inhibiting activity, both ORG 30958 and 4OH-AD postponed ovulation in rats after a twice-daily s.c. dose of 1.5 mg/kg (Table 4). Doubling of the dose of 4OH-AD inhibited ovulation in 50% of the animals, whereas ORG 30958 did not show ovulation inhibition. This effect of 4OH-AD indicates a direct action of the compound on the pituitary-hypothalamus, which is in accordance with the results of Wing *et al.* in both normal cycling and ovariectomized rats [34].

In conclusion ORG 30958 is a potent aromatase inhibitor *in vivo*. It is very likely that it becomes active after cleavage of the —S—S— bond yielding ORG 30365 a known potent irreversible aromatase inhibitor. ORG 30958 has very low androgenic and no estrogenic or anti-gonadotrophic activity, making it an attractive candidate for the treatment of estrogen-dependent diseases.

REFERENCES

1. Ryan J. R.: Biochemistry of aromatase. Significance to female reproductive physiology. *Cancer Res.* **42** (1982) 3342–3344.
2. Brodie A. M. H.: Recent advances in studies on estrogen biosynthesis. *J. Endocr. Invest.* **2** (1979) 445–460.

3. Santen R. J.: Aromatase inhibitors for treatment of breast cancer, current concepts and new prospectives. *Breast Cancer Res. Treat.* (suppl.) **7** (1986) 23.
4. Kennedy B. J.: Hormonal therapies in breast cancer. In *Seminars in Oncology*, Vol. 2 (1974) pp. 119–130.
5. Cash R., Brough A. J., Cohen M. N. P. and Satoh P. S.: Aminoglutethimide (Elipten CIBA) as an inhibitor of adrenal steroidogenesis mechanism of action and therapeutic treatment. *J. Clin. Endocr. Metab* **27** (1967) 1239–1248.
6. Siitiri P. K. and Thompson E. A.: Studies of human placental aromatase. *J. Steroid Biochem.* **6** (1975) 317–322.
7. Schwarzel W. C., Kruggel W. G. and Brodie H. J.: Studies on the mechanism of estrogen biosynthesis. VIII. The development of inhibitors of the enzyme system in human placentae. *Endocrinology* **92** (1973) 866–880.
8. Schade K. and Schubert K.: Estrogen biosynthesis and its inhibition—a review. *Endokrinologie* **74** (1979) 90–99.
9. Brueggemeier R. W., Floyd E. E. and Counsell R. E.: Synthesis and biochemical evaluation of inhibitors of estrogen biosynthesis. *J. Med. Chem.* **21** (1978) 1007–1011.
10. Marcotte P. A. and Robinson C. H.: Design of mechanism-based inactivators of human placental aromatase. *Cancer Res.* (suppl.) **42** (1982) 3322–3326.
11. Henderson D., Norbisrath G. and Kerb U.: 1-Methyl-1,4-androstadiene-3,17-dione (SH 489): characterization of an irreversible inhibitor of estrogen biosynthesis. *J. Steroid Biochem.* **24** (1986) 303–306.
12. Wickings E. J., Middleton M. C. and Hillier S. G.: Non-steroidal inhibition of granulosa cell aromatase activity *in vitro*. *J. Steroid Biochem.* **26** (1987) 641–646.
13. Santen R. J., Demers L. M., Adlercreutz H., Harvey H., Santner S., Sanders S. and Lipton A.: Inhibition of aromatase with CGS 16949A in postmenopausal women. *J. Clin. Endocr. Metab.* **68** (1989) 99–106.
14. Dowsett M., Stein R. C., Mehta A. and Coombes R. C.: Potency and selectivity of the non-steroidal aromatase inhibitor CGS 16949 in postmenopausal breast cancer patients. *Clin. Endocr.* **32** (1990) 623–634.
15. Coombes R. C., Goss P. E., Dowsett M., Hutchinson G., Cunningham D. M., Jasman M. and Brodie A. M. H.: 4-Hydroxyandrostenedione: treatment for postmenopausal patients with advanced breast cancer. *Tumor Diagnos. Ther.* **8** (1987) 271–273.
16. Johnston J. O., Wright C. L. and Metcalf B. W.: Biochemical and endocrine properties of a mechanism based inhibitor of aromatase. *Endocrinology* **115** (1984) 776–785.
17. Terasawa T., Okada T., Kanda M., Kato G. and Suzuki T.: 6-Substituted and 19-substituted androst-4-ene-3,17-dione derivatives as potential aromatase inhibitors. *Steroids* **50** (1987) 603–604.
18. Bednarski P. J., Pormbeck D. J. and Nelson S. D.: Thiol-containing androgens as suicide substrates of aromatase. *J. Med. Chem.* **28** (1985) 775–779.
19. Loozen H. J. J. and van Luit P. J. N.: European Patent Application 0 149 499 (1985).
20. Ryan K. J.: Biological aromatisation of steroids. *J. Biol. Chem.* **234** (1959) 268–272.
21. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: Protein measurements with folin phenol reagent. *J. Biol. Chem.* **193** (1951) 265–275.
22. Deckers G. H. and Schuurs A. H. W. M.: Aromatase inhibition in hypophysectomized female rats: a novel animal model for *in vivo* screening. *J. Steroid Biochem.* **32** (1989) 625–631.
23. de Jong F. H., Hey A. H. and van der Molen H. J.: Effects of gonadotrophins on the secretion of estradiol and testosterone by the rat testis. *J. Endocr.* **57** (1973) 277–284.
24. van der Vies J. and de Visser J.: Endocrinological studies with Desogestrel. *Arzneim. Forsch.* **33** (1983) 231–236.
25. Brodie A. M. H., Marsh D. A., Wu J. T. and Brodie H. J.: Aromatase inhibitors and their use in controlling estrogen dependent processes. *J. Steroid Biochem.* **11** (1979) 107–112.
26. Henderson D., Habenicht U. F., Nishino Y., Kerb U. and El Eltreby: Aromatase inhibitors and benign prostatic hyperplasia. *J. Steroid Biochem.* **25** (1986) 867–876.
27. Concannon P. W., Hansel W. and Visek W. J.: The ovarian cycle of the bitch: plasma estrogen, LH and progestrone. *Biol. Reprod.* **13** (1975) 112–121.
28. Thun R., Watson P. and Jackson G. L.: Induction of estrus and ovulation in the bitch using exogenous gonadotrophins. *Am. J. Vet. Res.* **38** (1977) 483–486.
29. Brodie A. M. H., Garret W. M., Hendrickson J. R. and Tsai-Morris C. H.: Effects of aromatase inhibitor 4-hydroxy-androstenedione and other compounds in the 7,12-dimethylbenz(a)anthracene-induced breast carcinoma model. *Cancer Res.* **42** (1982) 3360s–3364s.
30. Brodie A. M. H.: Aromatase inhibition and its pharmacological implications. *Biochem. Pharmac.* **34** (1985) 3213–3219.
31. Edgren R. A.: Estrogen antagonisms: the effect of testosterone propionate and 17-ethyl-19-nortestosterone on oestrone-induced changes in vaginal cytology. *Acta Endocr.* **25** (1957) 365–370.
32. Edgren R. A. and Calhoun D. W.: Estrogen antagonisms: inhibition of estrone-induced uterine growth by testosterone propionate, progesterone and 17-ethyl-19-nortestosterone. *Proc. Soc. Exp. Biol. Med.* **94** (1957) 537–539.
33. Casey R. W. and Wilson J. D.: Anti-estrogenic action of dihydrotestosterone in mouse breast. Competition with estradiol for binding to the estrogen receptor. *J. Clin. Invest.* **74** (1984) 2272–2278.
34. Wing L. J., Garrett W. M. and Brodie A. M. H.: Effects of aromatase inhibitors, aminoglutethimide and 4-hydroxyandrostenedione on cyclic rats and rats with 7,12-dimethylbenz(a)anthracene-induced mammary tumors. *Cancer Res.* **45** (1985) 2425–2428.