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4-AMINOANDROSTENEDIONE DERIVATIVES: A NOVEL CLASS OF IRREVERSIBLE AROMATASE INHIBITORS. COMPARISON WITH FCE 24304 AND 4-HYDROXYANDROSTENEDIONE

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Summary—FCE 24928 (4-aminoandrosta-1,4,6-triene-3,17-dione) was selected among a series of 4-aminoandrostenedione derivatives as a novel irreversible aromatase inhibitor. Its *in vitro* and *in vivo* properties have been studied and compared to FCE 24304 (6-methylenandrosta-1,4-diene-3,17-dione) and 4-OHA (4-hydroxyandrostenedione). FCE 24928 caused time-dependent inhibition of human placental aromatase with a $t_{1/2}$ of 4 min and K_i of 59 nM. Enzyme inactivation by FCE 24928 was faster than by FCE 24304 ($t_{1/2}$ 13.9 min). In PMSG-treated rats, microsomal ovarian aromatase activity was reduced 24 h after FCE 24928 dosing by both the s.c. (ED_{50} 1.2 mg/kg) and the oral (ED_{50} 14.1 mg/kg) routes. The compound was more potent than FCE 24304 and 4-OHA (ED_{50} 1.8 and 3.1 mg/kg s.c.). FCE 24928 did not show any interference with 5α-reductase and desmolase activity nor any significant binding affinity for androgen and estrogen receptors. Slight binding affinity for androgen receptor was observed with FCE 24304 and 4-OHA (0.21 and 0.25% of DHT). In immature, castrated rats, FCE 24928 did not show any intrinsic androgenic activity, up to 100 mg/kg/day s.c., in contrast to a slight androgenic activity observed with FCE 24304 at 10 mg/kg s.c.

INTRODUCTION

Inhibition of estrogen biosynthesis by means of selective aromatase inhibitors is receiving increasing interest in the treatment of estrogen-related pathological conditions, such as breast cancer. A number of irreversible aromatase inhibitors (i.e. suicide inhibitors) have been described, including 4-hydroxyandrost-4-ene-3,17-dione (4-OHA) [1, 2], 10-propargylestr-4-ene-3,17-dione (MDL 18962) [3, 4] and 1-methylandrosta-1,4-diene-3,17-dione (SH 489) [5, 6]. These compounds are now under clinical evaluation and their selectivity offers significant advantages in comparison with the presently available drug aminoglutethimide, a non-specific, reversible aromatase inhibitor [7]. 4-OHA is the most advanced steroidal derivative and is now in phase III clinical trials in postmenopausal breast cancer patients [8–10]. However, 4-OHA has been shown to be significantly inactivated through glucuronidation at the 4-hydroxy group [11] and has to be given parenterally.

We have previously described the aromatase inhibitory properties of 6-methylenandrosta-1,4-diene-3,17-dione (FCE 24304) an orally active irreversible inhibitor [12–14], endowed with some intrinsic androgenic activity [15] and which is now in phase I clinical evaluation. Its pharmacological properties are here reviewed and compared to 4-OHA.

In the present report we describe some *in vitro* and *in vivo* biological data of a novel irreversible aromatase inhibitor, 4-aminoandrosta-1,4,6-triene-3,17-dione (FCE 24928) (Fig. 1), an orally active compound without any intrinsic androgenic activity. The compound was selected among a series of 4-aminoandrost-4-ene-3,17-dione derivatives, with additional conjugations at C_1–C_2, at C_6–C_7 or at both positions (Fig. 1).

EXPERIMENTAL

Chemicals

FCE 24928 [16], its saturated analogues, 4-OHA and 4-MA (17β-*N*,*N*-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one) were synthesized at the Chemical Laboratory of the Oncology Line of Farmitalia Carlo Erba. For *in vitro* tests, stock solutions of the compounds were prepared in methanol and further diluted

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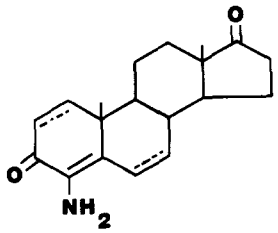

	FCE
$\Delta^{1,4,6}$	24928
$\Delta^{4,6}$	24210
$\Delta^{1,4}$	24661
Δ^{4}	24566

Fig. 1. Structures of 4-aminoandrostenedione derivatives.

with the appropriate buffer solution. The compounds were suspended in 0.5% methocel (A-4C Premium, Dow Chemical) containing 0.4% Tween 80 (Merck) when given orally or dissolved in benzylic alcohol and diluted in sesame oil when given subcutaneously. The other steroids and pregnant mare's serum gonadotropin (PMSG) were purchased from Sigma Chemical Co. Aminoglutethimide was purchased from Farmitalia Carlo Erba. [1β,2β-^{3}H]androst-4-ene-3,17-dione (40–60 Ci/mmol), [4-^{14}C]testosterone (52 mCi/mmol), [26-^{14}C]-cholesterol (55 mCi/mmol), [1,2,4,5,6,7-^{3}H]5α-dihydrotestosterone (DHT, 128 Ci/mmol) and [2,4,6,7-^{3}H]17β-estradiol (95 Ci/mmol) were purchased from New England Nuclear. NADPH tetrasodium salt was purchased from Boehringer Mannheim. Aluminium oxid 60G neutral type E (Alumina) and glycin were purchased from Merck.

Buffers

Aromatase phosphate buffer, pH 7.5, contained 10 mM potassium phosphate buffer, 100 mM KCl, 1 mM EDTA and 1 mM dithiothreitol. Reductase phosphate buffer, pH 6.5, contained 40 mM sodium phosphate buffer and 1 mM dithiothreitol. Desmolase phosphate buffer, pH 7.4, contained 10 mM potassium phosphate buffer, 20 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, 250 mM sucrose and 1 mM dithiothreitol. TED buffer, pH 7.4, contained 10 mM Tris–HCl, 1.5 mM EDTA and 1 mM dithiothreitol.

Animals

Immature male and adult male and female Sprague–Dawley rats were supplied by Charles River Italia.

Human placental aromatase inhibition

Microsomes were prepared from fresh human placenta according to Ryan [17]. Aromatase activity was tested in the assay of Thompson and Siiteri [18], which determines the rate of aromatization by measuring the release of ^3H_2O from [1β,2β-^{3}H]androstenedione. All incubations were carried out in a Dubnoff shaking incubator at 37°C in aromatase phosphate buffer. Time-dependent aromatase inhibition was determined by pre-incubating the enzyme preparation (100 μg of proteins) for appropriate periods with different concentrations of the inhibitor in the presence of 100 μM NADPH in 1 ml final volume. Each sample was assayed in duplicate. After the various pre-incubation times, [^{3}H]androstenedione was added to each tube to a 300 nM final concentration. After 10 min of incubation the assay was terminated by the addition of 4 ml $CHCl_3$. The radioactivity in the water phase was taken as an index of residual aromatase activity. Inactivation curves, corrected for the loss of activity in the pertinent pre-incubated controls, were analyzed according to Kitz and Wilson [19] and K_i and $t_{1/2}$ values were estimated. Each compound was tested in three assays.

In vivo aromatase inhibition

Adult female rats in diestrus were injected subcutaneously with 100 IU PMSG. The PMSG dose was repeated 96 h later (day 4). On day 7, s.c. or oral doses of the inhibitors were given to groups of 5 rats each. Animals were killed by decapitation 24 h later and ovaries excised, homogenized in 0.1 M phosphate buffer, pH 7.4, and microsomes isolated. Washed microsomal pellets were suspended with aromatase phosphate buffer. Ovarian aromatase activity was assayed by incubating in duplicate aliquots of microsomal suspensions, containing approx. 100 μg of microsomal proteins, with 100 nM [^{3}H]androstenedione and 100 μM NADPH in 1 ml final incubation volume for 30 min at 37°C. The radioactivity in the water phase was determined using the above procedure. Results were expressed as percentage of control aromatase activity. In dose–response studies at least 10 animals per dose were used.

In vitro 5α-reductase inhibition

Inhibition of the conversion of testosterone to 5α-dihydrotestosterone (DHT) was evaluated using rat prostate particulate fraction as the enzyme source, as described by Liang *et al.* [20]. The experiments were carried out in reductase phosphate buffer, 0.5 ml final incubation volume, containing 1 μM [^{14}C]testosterone, various concentrations of the inhibitors, 250 μM NADPH and 1 mg of protein of the enzyme

preparation. After 30 min incubation at 37°C the reaction was terminated by addition of 1.5 ml ethyl acetate. Testosterone metabolites in the concentrated extracts were separated in TLC on silica gel F 254 plates (Merck). Plates were scanned and the fractional 5α-reduction of testosterone was calculated by relating the ^{14}C-radioactivity in the DHT and 3α(β),17β-androstanediol regions to the total radioactivity of the plate.

Desmolase inhibition

Inhibition of desmolase (cholesterol side-chain cleavage enzyme) was evaluated by measuring the amount of [1-^{14}C]isocaproic acid formed during conversion of [26-^{14}C]cholesterol to pregnenolone, as described by Rabe *et al.* [21]. Mitochondrial preparations from adrenals of adult male rats were used as the enzyme source [22]. The experiments were carried out in desmolase phosphate buffer, 0.5 ml final volume, containing 10 μM [^{14}C]cholesterol, various concentrations of the tested compounds and 50 μg of mitochondrial proteins. A 20-min incubation at 37°C was started by addition of 100 μM D,L-isocitrate and 100 μM NADPH and terminated, in ice, by the addition of 1.5 ml of a 30% alumina suspension in 50 mM glycine, buffered to pH 9.5 with 50 mM K_2HPO_4 and 1 N KOH. After vortexing, to adsorb unmodified [^{14}C]cholesterol, the tubes were centrifuged at 3000 *g* for 20 min and radioactivity content in the supernatant, containing [^{14}C]isocaproic acid, was determined by liquid scintillation counting.

Androgen and estrogen receptor binding affinity

Binding of the compounds to cytoplasmic androgen (rat prostate) and estrogen (rat uterus) receptors was determined by standard dextran-coated charcoal adsorption techniques [23]. Uterine and prostatic tissue were obtained from rats 24 h after castration. Male rats were adrenalectomized 3 days before castration, to increase androgen receptor specific binding. Cytoplasmic receptors were prepared in TED buffer; competitive binding assays were done in 0.4 ml incubation volume containing 1 nM ^3H-labelled steroids (DHT or estradiol), various concentrations of the inhibitors, or the unlabelled ligands, and an aliquot of diluted cytosol. After 2 h incubation at 0°C, the bound and free ^3H-labelled steroids were separated and bound radioactivity was determined. The concentration of each compound required to reduce specific ^3H-steroid binding by 50% (IC_{50}) was determined.

Androgenic activity in the rat

Prepuberal 21-day-old male rats were castrated via scrotal incision under light ether anesthesia. Starting on the seventh day after orchiectomy, the rats were treated subcutaneously for 7 consecutive days either with testosterone propionate or with the tested compounds. Twenty-four hours after the last dose the rats were killed and ventral prostate removed and weighed.

Protein assay

Protein content in the various preparations was determined using the method of Lowry *et al.* [24] with bovine serum albumin as standard.

RESULTS AND DISCUSSION

FCE 24304

In co-incubation studies with the substrate (50 nM [^3H]androstenedione), FCE 24304 was reported to inhibit human placental aromatase with a potency (IC_{50}) similar to 4-OHA and about 40 times higher than aminoglutethimide (Table 1) [12, 14]. In pre-incubation studies with the enzyme and in the presence of NADPH, FCE 24304, like 4-OHA, has been shown to cause time-dependent enzyme inhibition (Table 1). Inactivation by 4-OHA was faster than that of FCE 24304, whereas no time-dependent aromatase inhibition was observed with aminoglutethimide. The K_i values of FCE 24304 and 4-OHA were similar. No inhibition of rat prostatic 5α-reductase [12] and of rat adrenal desmolase (E. di Salle *et al.*, unpublished results) was observed with FCE 24304 up to 30 and 100 μM respectively. FCE 24304 showed a weak binding affinity to the rat prostate androgen receptor (0.21% of DHT) [12]. No significant binding to the rat uterine estrogen receptor was observed (<0.005% of E_2) [12].

The *in vivo* inhibition of ovarian aromatase activity has been tested 24 h after a single administration in PMSG-stimulated adult rats

Table 1. Inhibition of human placental aromatase

Compound	IC_{50} (nM)[a]	$t_{1/2}$ (min)[b]	K_i (nM)[b]
FCE 24304	42.5 ± 4.3	13.9 ± 0.7	26.0 ± 1.4
4-OHA	43.7 ± 1.6	2.1 ± 0.2	29.0 ± 7.5
Aminoglutethimide	1754.0 ± 81.0	NTD	

Mean ± SE of at least 3 assays. Data from Ref. [14]. [a]Co-incubation studies with 50 nM [^3H]androstenedione. [b]Pre-incubation studies for 0–32 min with the enzyme in the presence of NADPH. NTD, no time-dependent inhibition.

[12, 14]. In dose–response studies, FCE 24304 has been shown to reduce ovarian aromatase with an ED_{50} of 1.8 mg/kg s.c. and 3.7 mg/kg orally. 4-OHA was effective by the s.c. route (ED_{50} 3.1 mg/kg) but orally it caused marginal enzyme inactivation, reaching 28% inhibition at 100 mg/kg [14]. When given s.c. for 7 days to male rats castrated 24 h previously, FCE 24304 was shown to have a slight androgenic effect, showing a potency of about 1% that of testosterone propionate [15]. Similar androgenic activity (1–4% of testosterone) has been reported for 4-OHA by Brodie *et al.* [1].

4-Aminoandrostenedione derivatives

Derivatives of 4-aminoandrost-4-ene-3,17-dione (FCE 24566) with additional conjugations at C_1–C_2 or at C_6–C_7 (Fig. 1) have been studied as a new class of aromatase inhibitors. In pre-incubation studies with human placental aromatase all the derivatives caused irreversible inhibition of the enzyme. The values of $t_{1/2}$ and K_i of each compound are reported in Table 2. FCE 24566 was the slowest aromatase inactivator, showing a $t_{1/2}$ of 31 min, compared to a $t_{1/2}$ of 2.1 min for the related compound 4-OHA (Table 1). The introduction of additional double bond at C_1–C_2 (FCE 24661) and particularly at C_6–C_7 (FCE 24210) or at both positions (FCE 24928) caused an increase of the inactivation rate, with $t_{1/2}$ values of 15, 4 and 4 min respectively. The new compounds showed an affinity for the enzyme (K_i) ranging from 37 to 68 nM, comparable to the apparent K_m value of 69 nM for androstenedione, evaluated in the same placental preparation.

The possible interference of the compounds with prostatic 5α-reductase was tested *in vitro* in comparison with 4-MA, a known 5α-reductase inhibitor [25]. Data summarized in Table 3 indicate that the compounds with a double bond in the C_1–C_2 position (FCE 24928 and FCE 24661) showed no modification of 5α-reductase activity up to 30 μM. The other two derivatives FCE 24210 and FCE 24566 inhibit 5α-reductase; however their potency (IC_{50} 11 and 3 μM respectively) was several times lower than that of the reference standard 4-MA (IC_{50} 0.03 μM).

Table 3. Inhibition of 5α-reductase and steroid receptor binding affinity

Compound	5α-Reductase inhibition [IC_{50} (μM)]	Steroid receptor binding affinity[a] Androgen (DHT = 100%)	Estrogen (E_2 = 100%)
FCE 24928	>30	<0.03	<0.005
FCE 24210	11	0.033	≈0.006
FCE 24661	>30	<0.03	<0.005
FCE 24566	3	0.057	<0.005
4-OHA	>30	0.25	<0.005
4-MA	0.03		

Results are the average of at least two experiments.
[a]Calculated by considering the potency (IC_{50}) of the specific ligand as 100%.

Adrenal desmolase inhibition by FCE 24210 and FCE 24928 was tested in comparison to aminoglutethimide. Up to 100 μM the two compounds did not affect the enzyme activity, whereas aminoglutethimide at 100 μM caused a 72% inhibition.

In steroid receptor binding studies all the new 4-aminoandrostenedione derivatives showed no or very low binding affinity for the androgen receptor (Table 3). The highest binding affinity was found for FCE 24566 (0.057% of DHT), which was lower than that found for the analogue 4-OHA (0.25%). No significant binding to the estrogen receptor was evident with any of the compounds (Table 3).

In order to test whether the new compounds inhibit aromatase in animal models *in vivo*, female rats with PMSG-stimulated ovarian aromatase were used. Twenty-four hours after a single subcutaneous dose of 30 mg/kg, FCE 24928 and FCE 24210 were the most effective inhibitors of the series, being even more effective than 4-OHA (Table 4). FCE 24661 and FCE 24566 were marginally effective or ineffective. In further dose–response studies by subcutaneous route, FCE 24928 and FCE 24210 showed ED_{50} values of 1.2 and 3.2 mg/kg respectively. Interestingly, the two compounds were also potent when given orally, their ED_{50} being 14.1 and 16.5 mg/kg respectively. As a comparison, the

Table 2. Time-dependent aromatase inhibition by 4-aminoandrostenedione derivatives. The compounds were pre-incubated for 0–32 min with human placental aromatase in the presence of NADPH

Compound	K_i (nM)	$t_{1/2}$ (min)
FCE 24928	59 ± 9.5	4.0 ± 0.1
FCE 24210	59 ± 7.3	4.0 ± 0.3
FCE 24661	68 ± 12.5	15.2 ± 1.3
FCE 24566	37 ± 5.1	31.3 ± 4.8

Mean ± SE of three experiments.

Table 4. Effect on ovarian aromatase activity in PMSG-stimulated adult rats

Compound (30 mg/kg s.c.)	Ovarian aromatase at 24 h (% of controls)
Vehicle	100.0 ± 12.1
FCE 24928	5.6 ± 2.7
FCE 24210	7.6 ± 0.5
FCE 24661	74.6 ± 10.5
FCE 24566	120.7 ± 11.2
4-OHA	16.0 ± 2.0

Mean ± SE of 5 animals per group.

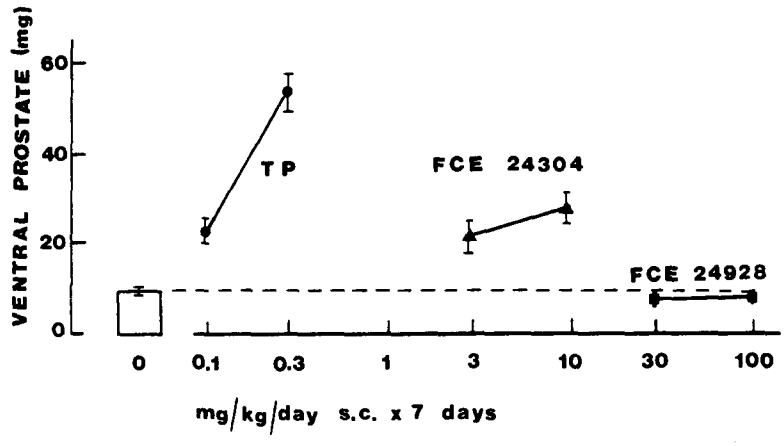

Fig. 2. Androgenic activity in immature castrated rats. Animals were treated on 7 consecutive days, starting on the 7th day after orchiectomy. Mean ± SE of 8 animals per group. TP = testosterone propionate.

ED_{50} values for 4-OHA were 3.1 mg/kg s.c. and > 100 mg/kg p.o. [14].

The possible androgenic activity of FCE 24928 was tested in immature castrated rats, in comparison with testosterone propionate (TP) and FCE 24304, which was previously shown to have some activity [15]. At subcutaneous doses of 30 and 100 mg/kg FCE 24928 did not induce any increase in the ventral prostate weight (Fig. 2). A slight androgenic activity was confirmed for FCE 24304 at doses of 3 and 10 mg/kg s.c.; however, its dose–response curve was flatter than that of TP at doses of 0.1 and 0.3 mg/kg s.c. No intrinsic androgenic activity was shown for FCE 24210 at doses of 100 mg/kg s.c.

The new class of 4-aminoandrostenedione derivatives has been developed to identify an orally effective compound without any intrinsic androgenic activity. From all the data here presented, FCE 24928 was selected. The compound was shown to be a faster aromatase inactivator, with a $t_{1/2}$ of 4 min and a K_i of 59 nM. *In vivo*, 24 h after a single administration, the compound was very effective subcutaneously (ED_{50} 1.2 mg/kg) and even orally (ED_{50} 14.1 mg/kg). No interference with 5α-reductase activity, with desmolase activity nor any significant binding affinity for androgen and estrogen receptor was found. In contrast to FCE 24304 [15] and 4-OHA [1], the new compound did not show any intrinsic androgenic activity even at the very high dose of 100 mg/kg s.c. Structural modification of FCE 24928, by introducing saturation at C_1–C_2, at C_6–C_7 or at both positions, caused various degrees of modification in the aromatase inactivation properties, the *in vivo* aromatase inhibition and the inhibition of 5α-reductase. The presence of the C_1–C_2 double bond in this series of compounds increases the aromatase inactivation rate, thus confirming our previous results with the FCE 24304 analogues [12] and the results obtained with the 4-OHA analogues [27] and the 7α-substituted androstenedione derivatives [28]. The presence of the C_6–C_7 double bond caused a further increase in the inactivation rate. The 4-aminoandrostenedione derivatives with the highest aromatase inactivation rate, FCE 24210 and FCE 24928, were found to be the most effective *in vivo* aromatase inhibitors, though their pharmacokinetic properties have also to be taken into account. The derivative with the C_4–C_5 double bond only, FCE 24566, was found to inhibit 5α-reductase. The further introduction of a double bond at C_1–C_2 was found to reduce this effect by more than 10 times, thus confirming our observations with FCE 24304 and its C_1–C_2 saturated analogue [12].

In conclusion, this study establishes FCE 24928 as a new potent and selective irreversible aromatase inhibitor. The compound is now under extensive preclinical evaluation.

REFERENCES

1. Brodie A. M. H., Schwarzel W. C., Shaikh A. A. and Brodie H. J.: The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on estrogen-dependent processes in reproduction and breast cancer. *Endocrinology* **100** (1977) 1684–1695.
2. Brodie A. M. H., Garrett W. M., Hendrickson J. R., Tsai-Morris C. H., Marcotte P. A. and Robinson C. H.: Inactivation of aromatase *in vitro* by 4-hydroxy-4-androstene-3,17-dione and 4-acetoxy-4-androstene-3,17-dione and sustained effects *in vivo*. *Steroids* **38** (1981) 693–702.

3. 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.
4. Johnston J. O.: Biological characterization of 10-(2-propynyl)estr-4-ene-3,17-dione (MDL 18962), an enzyme-activated inhibitor of aromatase. *Steroids* **50** (1987) 105–120.
5. Henderson D., Norbisrath G. and Kerb V.: 1-Methyl-1,4-androstadiene-3,17-dione (SH 489): characterization of an irreversible inhibitor of aromatase. *Endocrinology* **115** (1986) 776–785.
6. Henderson D., Habenicht U. F., Nishino Y., Kerb V. and El Etreby M. F.: Aromatase inhibitors and benign prostatic hyperplasia. *J. Steroid Biochem.* **25** (1986) 867–876.
7. Santen R. J. and Misbin R. I.: Aminoglutethimide: review of pharmacology and clinical use. *Pharmacotherapy* **1** (1981) 95–120.
8. Goss P. E., Powles T. J., Dowsett M., Hutchinson G., Brodie A. M. H., Gazet J. C. and Coombes R. C.: Treatment of advanced postmenopausal breast cancer with an aromatase inhibitor, 4-hydroxyandrostenedione: phase II report. *Cancer Res.* **47** (1986) 1957–1961.
9. Coombes R. C., Goss P. E., Dowsett M., Hutchinson G., Cunningham D., Jarman M. and Brodie A. M. H.: 4-Hydroxyandrostenedione treatment for postmenopausal patients with advanced breast cancer. *Steroids* **50** (1987) 245–252.
10. Dowsett M., Cunningham D. C., Stein R. C., Evans E., Dehenmin L., Hedley A. and Coombes R. C.: Dose-related endocrine effects and pharmacokinetics of oral and intramuscular 4-hydroxyandrostenedione in postmenopausal breast cancer patients. *Cancer Res.* **49** (1989) 1306–1312.
11. Goss P. E., Jarman M., Wilkinson J. R. and Coombes R. C.: Metabolism of the aromatase inhibitor 4-hydroxyandrostenedione *in vivo*. Identification of the glucuronide as a major urinary metabolite in patients and biliary metabolite in the rat. *J. Steroid Biochem.* **24** (1986) 619–622.
12. Giudici D., Ornati G., Briatico G., Buzzetti F., Lombardi P. and di Salle E.: 6-Methylenadrosta-1,4-diene-3,17-dione (FCE 24304): a new irreversible aromatase inhibitor. *J Steroid Biochem.* **30** (1988) 391–394.
13. Zaccheo T., Giudici D., Lombardi P. and di Salle E.: A new irreversible aromatase inhibitor, 6-methylenandrosta-1,4-diene-3,17-dione (FCE 24304): antitumor activity and endocrine effects in rats with DMBA-induced mammary tumors. *Cancer Chemother. Pharmac.* **23** (1989) 47–50.
14. Di Salle E., Giudici D., Briatico G. and Ornati G.: Novel irreversible aromatase inhibitors. In *Steroid Formation, Degradation and Action in Peripheral Normal and Neoplastic Tissues* (Edited by H. L. Bradlow). *Ann. NY Acad. Sci.*, **595** (1990) 357–367.
15. di Salle E., Giudici D., Ornati G., Briatico G. and Lombardi P.: Endocrine properties of 6-methylenandrosta-1,4-diene-3,17-dione (FCE 24304), a new irreversible aromatase inhibitor. *J. Steroid Biochem.* **28** (Suppl.) (1987) 146S.
16. D'Alessio R., Faustini F., Villa V., Di Salle E. and Lombardi P.: Synthesis and *in vitro* evaluation of novel aromatase inhibitors. *J. Steroid Biochem.* **25** (Suppl.) (1986) 46S.
17. Ryan K. J.: Biological aromatization of steroids. *J. Biol. Chem.* **234** (1972) 268–272.
18. Thompson E. A. Jr and Siiteri P. K.: Utilization of oxygen and reduced nicotinamide adenine dinucleotide phosphate by human placental microsomes during aromatization of androstenedione. *J. Biol. Chem.* **249** (1974) 5364–5372.
19. Kitz R. and Wilson I. B.: Esters of methanesulfonic acid as irreversible inhibitors of acetylcholinesterase. *J. Biol. Chem.* **237** (1962) 3245–3249.
20. Liang T., Heiss C. E., Ostrove S., Rasmusson G. H. and Cheung A.: Binding of a 4-methyl-4-aza-steroid to 5α-reductase of rat liver and prostate microsomes. *Endocrinology* **112** (1983) 1460–1468.
21. Rabe T., Rabe D., Bierwirth A. M. and Runnebaum B.: New assays for the enzymatic conversion of cholesterol to pregnenolone. *Steroids* **37** (1981) 555–571.
22. Simpson E. R., Jefcoate C. R., Brownie A. C. and Boyd G. S.: The effect of ether anaesthesia stress on cholesterol side-chain cleavage and cytochrome P-450 in rat adrenal mitochondria. *Eur. J. Biochem.* **28** (1972) 442–450.
23. Raynaud J. P., Bonne C., Bouton M. M., Mognilewsky M., Philibert D. and Azadian-Boulanger G.: Screening for anti-hormones by receptor studies. *J. Steroid Biochem.* **6** (1975) 615–622.
24. Lowry O. H., Rosenbrough N. J., Farr A. L. and Randall L. J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193** (1951) 265–275.
25. Brooks J. R., Baptista E. M., Berman C., Ham E. A., Hichens M., Johnston D. B. R., Primka R. L., Rasmusson G. H., Reynolds G. F., Schmitt S. M. and Arth G. E.: Response of rat ventral prostate to a new and novel 5α-reductase inhibitor. *Endocrinology* **109** (1981) 830–836.
26. Marsh D. A., Brodie H. J., Garrett W., Tsai-Morris C. H. and Brodie A. M. H.: Aromatase inhibitors. Synthesis and biological activity of androstenedione derivatives. *J. Med. Chem.* **28** (1985) 788–795.
27. Brueggemeir R. W., Li P. K., Snider C. E., Darbi M. V. and Katlic N. E.: 7α-substituted androstenedione as effective *in vitro* and *in vivo* inhibitors of aromatase. *Steroids* **50** (1987) 163–178.