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Novel Aromatase and 5α-Reductase Inhibitors

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Inhibitors of aromatase and 5α-reductase may be of use for the therapy of postmenopausal breast cancer and benign prostatic hyperplasia, respectively. FCE 27993 is a novel steroidal irreversible aromatase inhibitor structurally related to exemestane (FCE 24304). The compound was found to be a very potent competitive inhibitor of human placental aromatase, with a K_i of 7.2 nM (4.3 nM for exemestane). In preincubation studies with placental aromatase FCE 27993, like exemestane, was found to cause time-dependent inhibition with a higher rate of inactivation ($t_{1/2}$ 4.5 vs 15.1 min) and a similar $K_{i(inact)}$ (56 vs 66 nM). The compound was found to have a very low binding affinity to the androgen receptor (RBA 0.09% of dihydrotestosterone) and, in contrast to exemestane, no androgenic activity up to 100 mg/kg/day s.c. in immature castrated rats. Among a series of novel 4-azasteroids with fluoro-substituted-17β-amidic side chains, three compounds, namely FCE 28260, FCE 28175 and FCE 27837, were identified as potent *in vitro* and *in vivo* inhibitors of prostatic 5α-reductase. Their IC_{50} values were found to be 16, 38 and 51 nM for the inhibition of the human enzyme, and 15, 20 and 60 nM for the inhibition of the rat enzyme, respectively. When given orally for 7 days in castrated and testosterone (Silastic implants) supplemented rats, the new compounds were very effective in reducing prostate growth. At a dose of 0.3 mg/kg/day inhibitions of 42, 36 and 41% were caused by FCE 28260, FCE 28175 and FCE 27837, respectively.

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INTRODUCTION

Much interest has been displayed in recent years in the identification and development of novel aromatase and 5α-reductase inhibitors for the treatment of postmenopausal breast cancer and benign prostatic hyperplasia (BPH), respectively. In addition, a possible use of aromatase inhibitors in a subset of BPH patients has also been suggested [1].

Aminoglutethimide was the first aromatase inhibitor to be developed and marketed for the treatment of breast cancer. Several other nonsteroidal reversible aromatase inhibitors, more selective than aminoglutethimide, have also been described and are now in various stages of clinical evaluation, including fadrozole (CGS 16949A) [2], vorozole (R 76713) [3], rogletimide [4], CGS 20267 [5] and arimidex (ICI D1033) [6]. Attention has also been directed towards the clinical development of steroidal irreversible inhibitors of aromatase, including formestane (4-hydroxy-androstenedione) [7], recently introduced into the market as an intramuscular formulation [8], atamestane (SH 489) [9], exemestane (FCE 24304) [10] and minamestane (FCE 24928) [11]. In a continuous effort to identify more selective steroidal aromatase inhibitors devoid of intrinsic androgenicity, the present report describes some endocrinological properties of two analogs of exemestane and minamestane, namely the 4-amino-exemestane derivative FCE 27993 and the 4-amino-6-methylene bridged derivative FCE 27985. Their chemical structures are shown in Fig. 1.

The 5α-reductase enzyme catalyses the irreversible conversion of testosterone (T) to the tissue-specific androgen 5α-dihydrotestosterone (DHT). Finasteride (MK 906) [12], the prototype of 5α-reductase inhibitors, has recently been introduced into the market for the therapy of symptomatic BPH [13]. Other steroidal 5α-reductase inhibitors are undergoing clinical evaluation, including epristeride (SK&F 105657) [14], turosteride (FCE 26073) [15, 16] and MK 963 [17]. In a programme aimed at searching for novel potent and

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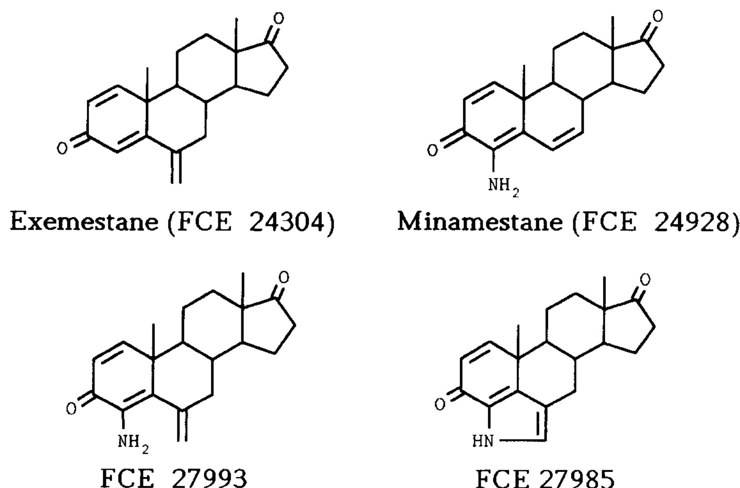

Fig. 1. Chemical structures of the aromatase inhibitors exemestane, minamestane, FCE 27993 and FCE 27985.

orally active 5α-reductase inhibitors, we synthesized various 4-azasteroids with fluoro-substituted-17β-amidic side chains. In the present study we describe the *in vitro* and *in vivo* activities of three such fluoro derivatives, i.e. FCE 27837, 28175 and 28260. The chemical structures of these derivatives are shown in Fig. 2.

EXPERIMENTAL

Chemicals

Formestane, exemestane, minamestane, finasteride and the new steroidal aromatase and 5α-reductase inhibitors were synthesized at the research laboratories of Pharmacia/Farmitalia Carlo Erba (Italy). Aminoglutethimide was obtained from Farmitalia Carlo Erba. [4-^{14}C]T (50 mCi/mmol), [1β-^{3}H]androst-4-ene-3,17-dione ([^{3}H]A) (28 Ci/mmol) and [1,2,4,5,6,7-^{3}H]DHT (128 Ci/mmol) were purchased from New England Nuclear. All the other unlabelled steroids and pregnant mare's serum gonadotropin (PMSG) were purchased from Sigma Chemical. NADPH tetrasodium salt, activated charcoal Norit A and dextran T-70 were purchased from Boehringer Mannheim, Serva and Pharmacia Biotech, respectively. The protease inhibitors aprotinin and leupeptin were obtained from Sigma Chemical.

Preparation of the solutions

For the *in vitro* tests, stock solutions of the compounds were prepared in methanol and diluted with the pertinent buffer. The amount of methanol in the final incubation volume was always $\leqslant 3\%$, and a similar amount of solvent was included in the control sample of each assay. For the *in vivo* studies 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.

Source of tissues and animals

Fresh human placenta at term and fresh surgical specimens from human benign hyperplastic prostate were provided by the Hospital of Rho (Italy). Prepuberal 22-day-old male Crl:CD^{R}(SD)BR rats and adult rats of both sexes were supplied by Charles River, Italy.

Assays with human placental aromatase

Microsomes were prepared from human placenta according to Ryan [18] and stored at −80°C. The rate

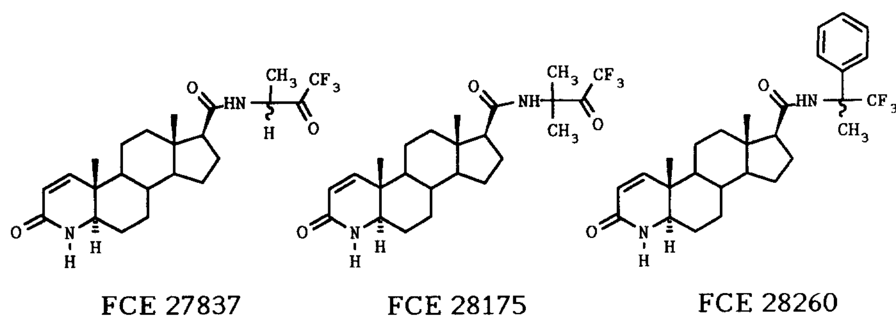

Fig. 2. Chemical structures of the 5α-reductase inhibitors FCE 27837, FCE 28175 and FCE 28260.

of aromatization was determined by measuring the tritiated water released from [1β-^{3}H]A. The assay was carried out in a final volume of 1 ml, in 10 mM phosphate buffer, pH 7.5, containing 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 100 μM NADPH, the enzyme preparation and the appropriate concentrations of the inhibitors (in duplicate) and the substrate. After a 10 min incubation at 37°C, the assay was terminated by the addition of 4 ml cold chloroform. The acqueous phase was treated with a charcoal suspension, the supernatant was removed and counted for radioactivity by liquid scintillation in Rialuma. For the determination of the IC_{50} values, various concentrations of inhibitors were incubated with 20 μg of microsomal protein, in the presence of a fixed amount (50 nM) of [^{3}H]A. Various substrate (5–105 nM) and inhibitor concentrations were incubated for 10 min with 2.5–10 μg of protein to determine the apparent inhibitory constant (K_i), derived from secondary replots (slope vs [I]) of Lineweaver–Burk plots (1/[S] vs 1/V). Aromatase inactivation was determined by preincubating the enzyme preparation at 37°C (100 μg of protein) for various periods of time with different concentrations of the inhibitors in the presence of 100 μM NADPH in a final volume of 1 ml. At each time a 100 μl aliquot (10 μg of protein) was removed and the remaining enzyme activity was determined during a further 10-min assay in a final incubation volume of 1 ml, as described above, in the presence of 300 nM of [^{3}H]A. The inactivation parameters, $K_{i(\text{inact})}$ and $t_{1/2}$, were derived from Kitz–Wilson plots [19].

In vitro inhibition of rat ovarian aromatase

Adult female rats were treated subcutaneously with 100 IU of PMSG twice, 4 days apart. Four days after the second treatment, the animals were sacrificed; ovaries were removed, homogenized in 0.1 M phosphate buffer, pH 7.4, and microsomes isolated and stored at −80°C. The assay for the IC_{50} determination was carried out as described above for placental microsomes, using various concentrations of the inhibitors in the presence of 50 nM [^{3}H]A and 200 μg of microsomal protein.

In vitro inhibition of 5α-reductase

Inhibition of the conversion of T to the 5α-reduced products DHT and 3α(β),17β-androstanediol was evaluated using the rat prostate and the human hyperplastic prostate as the enzyme sources. The particulate fraction (140,000 *g* pellet) from homogenate of adult rat prostates was obtained as described by Liang *et al.* [20]. The assay was performed in a final incubation volume of 0.5 ml, in 40 mM phosphate buffer, pH 6.5, containing 1 mM dithiothreitol, 0.5 mM NADPH and ≈700 μg of protein. The human prostatic tissue was homogenized in a w/v ratio of 1:3 with a 100 mM Tris–HCl buffer, pH 7.0, containing 20% glycerol, 100 mM sodium citrate, 100 mM KCl, 1 mM EDTA and 5 mM dithiothreitol (buffer *a*). To protect the enzyme during the preparation process, 0.5 mM NADPH, 1 μM T and 10 μg/ml of the protease inhibitors aprotinin and leupeptin were also added to buffer *a* [21]. The microsomes were then isolated, the pellet resuspended in buffer *a*, also containing the protease inhibitors, and stored at −80°C. Incubations were performed in a final incubation volume of 0.5 ml, in 100 mM Tris–HCl buffer, pH 5.5, containing the same ingredients as in buffer *a*, 0.5 mM NADPH and ≈300 μg of microsomal proteins. For both enzyme preparations, various concentrations of the azasteroids, in duplicate, were incubated with 1 μM [^{14}C]T for 30 min at 37°C. The samples were then processed as described previously [15].

Androgen receptor binding affinity

Binding of the compounds to cytoplasmic androgen receptor (AR) of rat prostate was determined by standard dextran-coated charcoal adsorption techniques, as described previously [15]. The assay was performed at 0°C for 2 h, in a final incubation volume of 0.4 ml containing an aliquot of cytosol, 1 nM of [^{3}H]DHT and various concentrations, in duplicate, of the tested compounds or unlabelled DHT.

Protein assay

Protein content in the various preparations was determined using the method of Lowry *et al.* [22] with bovine serum albumin as standard.

Androgenic activity

Prepuberal 22-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 the tested compounds or with T propionate (TP) (6–7 animals/group). Control animals received the vehicle (0.2 ml/kg of sesame oil). Twenty-four hours after the last dose the rats were sacrificed and the ventral prostate was removed and weighed.

In vivo inhibition of 5α-reductase

The standard test for the antiandrogenic effect in rats was used. Prepuberal 22-day-old male rats were castrated as described above. Seven days after orchiectomy, the rats were implanted subcutaneously with a 1-cm long Silastic® tubing (Dow Corning 602-265), filled with a mixture containing 25% T and 75% cholesterol. Starting from the day of implantation, the animals were treated daily for 7 consecutive days with various oral doses of the tested compounds (6–7 animals/group). Twenty-four hours after the last dose the rats were sacrificed and the ventral prostate was removed and weighed. Control animals (T-implanted controls) received the vehicle (0.5 ml/kg of Methocel/Tween 80). In each experiment one group of castrated rats was implanted with a Silastic tubing containing

100% cholesterol (castrated controls). The mean percentage of inhibition of the T-induced hypertrophic response was calculated according to the following formula: % inhibition = $100 \times (C_T - D)/(C_T - C_C)$, where C_T, C_C and D are the mean prostate weight of T-implanted control, castrated control and drug treated group, respectively.

RESULTS

Aromatase inhibitors

The aromatase inhibitory property on human placental and rat ovarian aromatase of the new steroidal compounds FCE 27993 and FCE 27985 were compared to those of the structurally related inhibitors exemestane and minamestane. In both enzyme preparations, the two compounds were found to be more potent than minamestane and less potent than exemestane (Table 1). FCE 27993 was approx. 4-fold more potent on the human than on the rat enzyme (IC_{50} 57 vs 224 nM), whereas FCE 27985 was similarly effective on both enzyme preparations (IC_{50} 126 and 117 nM). The kinetic analysis of aromatase inhibition was determined with placental aromatase. The placental preparation used for all the studies reported here showed a K_m for androstenedione of 11 nM and a V_{max} of 67 pmol estrogen/mg protein/min. From the Lineweaver–Burk plots, the inhibitors FCE 27993 and FCE 27985, like exemestane and minamestane, exhibited competitive inhibition, increasing the apparent K_m without affecting the V_{max} (data not shown). The apparent inhibitory constants (K_i values) are listed in Table 2. The new compounds FCE 27993 and FCE 27985 showed a very low K_i (7.2 and 10.1 nM), lower or similar to the K_m for androstenedione (11 nM). Exemestane was the most potent compound (K_i 4.3 nM), whereas minamestane was less active (45.7 nM). However, each of the four steroidal derivatives was several times (from 15 to 156) more potent than aminoglutethimide (K_i 671 nM). These compounds were also evaluated in time-dependent assays, where the enzyme was preincubated with the inhibitors in the presence of NADPH. Minamestane and exemestane were confirmed to cause enzyme inactivation, the first being a more rapid inactivator than exemestane ($t_{1/2}$ 3.6 vs 15.1 min), and to have similar $K_{i(inact)}$ values of 54.2 and 66.5 nM, respectively (Table 2). FCE 27993 was also found to be a rapid inactivator, with a $t_{1/2}$ of 4.5 min and a $K_{i(inact)}$ of 55.8 nM. FCE 27985 caused a time-dependent, biphasic loss of enzyme activity, however, the second linear phase between 10 and 40 min was found to be parallel to the normal loss in control sample (data not shown). FCE 27993, exemestane and minamestane were also evaluated in time-dependent assays with and without NADPH cofactor to determine whether the loss of enzyme activity was dependent on NADPH. No loss of enzyme activity was observed with any of the three compounds in the absence of NADPH, indicating that these compounds exhibited a mechanism-based inactivation property (data not shown).

In AR binding studies, FCE 27993 and FCE 27985 were found to have a very low binding affinity to the AR (IC_{50} 5.6 and 3.8 μM), lower than that of exemestane (0.9 μM) and higher than that of minamestane (48 μM) (Table 3). The possible *in vivo* androgenic activity of FCE 27993 was tested in immature castrated rats in comparison with exemestane, minamestane and TP. At subcutaneous doses of 30 and 100 mg/kg, FCE 27993, like minamestane, did not induce an increase in the ventral prostate weight (data not shown). A slight androgenic activity, at subcutaneous doses of 10 and 30 mg/kg, was found with exemestane, which, therefore, was approx. 2% as potent as TP.

Table 2. Competitive inhibition and time-dependent inactivation of human placental aromatase

Compound	K_i^a (nM)	$t_{1/2}^b$ (min)	$K_{i(inact)}^b$ (nM)
Exemestane	4.3	15.1	66.5
Minamestane	45.7	3.6	54.2
FCE 27993	7.2	4.5	55.8
FCE 27985	10.1	—	—
Aminoglutethimide	671.0	NTD[c]	—

Results are the mean of 2–3 separate assays.
[a]Apparent inhibitory constant derived from secondary replots (slope vs [I]) of Lineweaver–Burk plots (K_m for androstenedione = 11.1 nM); [b]inactivation parameters derived from Kitz–Wilson plots; [c]not time-dependent.

Table 1. In vitro inhibition of human placental and rat ovarian aromatase

Compound	IC_{50} (nM) Human	Rat
Exemestane	30	40
Minamestane	369	363
FCE 27993	57	224
FCE 27985	126	117
Formestane	85	78
Aminoglutethimide	3800	—

Incubations were performed in the presence of 50 nM [1β-^{3}H]A. Mean of 2–3 separate assays.

Table 3. Displacement of 1 nM [^{3}H]DHT from rat prostate AR

Compound	IC_{50} (μM)	RBA (%)
Exemestane	0.9	0.54
Minamestane	48.0	0.01
FCE 27993	5.6	0.09
FCE 27985	3.8	0.13
DHT	0.005	100.0

RBA = relative binding affinity (DHT = 100%).
Results are the mean of two separate assays.

Table 4. In vitro inhibition of human and rat prostatic 5α-reductase

Compound	IC_{50} (nM)	
	Human	Rat
FCE 28260	16	15
FCE 28175	38	20
FCE 27837	51	60
Finasteride	52	30

Results are the mean of 2–3 separate assays.
Incubations were performed in the presence of 1 μM [^{14}C]T.

5α-Reductase inhibitors

The abilities of the three novel fluorinated 4-azasteroids FCE 28260, FCE 28175 and FCE 27837 to inhibit the 5α-reduction of T from human and rat prostate are reported in Table 4. Their IC_{50} values were found to be 16, 38 and 51 nM for the inhibition of the human enzyme, and 15, 20 and 60 nM for the inhibition of the rat enzyme, respectively. In comparison, finasteride inhibited the human and rat enzymes with IC_{50} values of 52 and 30 nM, being 2–3 times less potent than FCE 28260 on both enzyme preparations.

In order to test the inhibitory effect of the new compounds on 5α-reductase in an animal model *in vivo*, immature castrated male rats were supplemented with a Silastic implant of T and then treated orally with the inhibitors for 7 consecutive days. The compounds FCE 28175 (0.3–10 mg/kg), FCE 27837 (0.03–10 mg/kg) and finasteride (0.3–10 mg/kg) were studied in 2, 3 and 4 experiments, respectively, and the results were combined. The T-implant caused approx. a 6-fold stimulation of prostate growth over the castrated control (50–60 vs 9 mg). The tested compounds caused a dose-related inhibition of T-stimulated prostate growth (data not shown). As an example, FCE 28175, FCE 27837 and finasteride at the highest tested dose of 10 mg/kg caused inhibitions of 62, 61 and 51%, and at the dose of 0.3 mg/kg of 36, 41 and 25%, respectively. The doses decreasing the prostate weight by 30% (ED_{30}) were found to be ≈ 0.1 mg/kg for FCE 27837, ≈ 0.2 mg/kg for FCE 28175 and ≈ 0.4 mg/kg for finasteride. In a preliminary experiment the effect of FCE 28260 was also tested. At oral doses of 0.3 and 1 mg/kg the compound caused a 42 and 44% inhibition of prostate growth.

DISCUSSION

The present study confirms the inhibitory effect of exemestane and minamestane on human placental aromatase [10, 11] and, for the first time, shows their inhibitory effects on rat ovarian aromatase. With both the rat and human enzyme preparations exemestane was more potent than minamestane. In addition, a kinetic analysis with the human enzyme showed that both compounds caused competitive aromatase inhibition with very low K_i values, i.e. 4.3 nM for exemestane and 45.7 nM for minamestane. Both compounds were also found to cause time-dependent inhibition of the human enzyme, thus confirming previous results [10, 11], and this property was found to be NADPH-dependent. These results clearly show that the two compounds are mechanism-based aromatase inactivators. In a continuous effort to identify novel irreversible steroidal aromatase inhibitors as potent as exemestane but devoid of intrinsic androgenicity [11], we have synthesized and characterized the 4-NH_2 derivative FCE 27993 and the 4-NH_2-6-methylene bridged compound FCE 27985. Both compounds were found to inhibit competitively the human placental aromatase, with K_i of 7.2 and 10.1 nM, thus being 6.3–4.5 times more potent than minamestane and 1.7–2.3 times less potent than exemestane. FCE 27993 was also characterized as a mechanism-based aromatase inhibitor, with an inactivation rate higher than that of exemestane ($t_{1/2}$ 4.5 vs 15.1 min) and a similar $K_{i(inact)}$ (56 vs 66 nM). The time-dependent loss of enzyme activity caused by FCE 27985 has to be characterized further, due to its biphasic inhibitory curve. Minamestane was previously reported to have a lower AR binding affinity than exemestane, and, in contrast to exemestane, to have no *in vivo* androgenic activity up to 100 mg/kg subcutaneously in rats [11]. These properties have been confirmed in the present study. In addition the new compounds, FCE 27985 and FCE 27993, were found to have a 4–6 times lower binding affinity than exemestane to the AR. Interestingly, FCE 27993, tested *in vivo* at the subcutaneous dose of 100 mg/kg, was found to be completely devoid of any intrinsic androgenicity, indicating greater selectivity than exemestane.

As to the three novel 5α-reductase inhibitors here described, they were found to be effective at nanomolar concentrations on either human and rat prostatic enzyme. The most effective compound was FCE 28260, which was 2–3 times more potent than finasteride on both enzyme preparations. The *in vivo* model of prostate growth in rats which we have used in the present work, though sensitive to both 5α-reductase inhibitors and AR antagonists, is considered to be useful for a preliminary evaluation of the antiprostatic effect of the inhibitors. However, we have noticed that such a model requires a very wide range of doses in order to characterize the dose–response curve of each compound, which was generally flat and which led to a maximal inhibition usually not greater than 50–60%. Therefore, the potency of the various compounds was tentatively evaluated by determining their oral ED_{30}. The following decreasing order of potencies was found: FCE 27837 > FCE 28175 ⩾ FCE 28260 ⩾ finasteride, with ED_{30}s ranging from ≈0.1 to ≈0.4 mg/kg/day. The *in vivo* antiprostatic effect of FCE 27837 is very

interesting, as the compound was found to be 2 times less potent *in vitro* than finasteride on the rat enzyme and equipotent to finasteride on the human enzyme.

Further studies are required to fully characterize the endocrinological properties of these new aromatase and 5α-reductase inhibitors.

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