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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

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Summary. The antitumor activity of the new irreversible aromatase inhibitor 6-methylenandrosta-1,4-diene-3,17-dione (FCE 24304) was studied in rats with 7,12-dimethylbenzanthracene (DMBA)-induced tumors; several endocrine parameters were evaluated in these animals. The compound was given s.c. and p.o. twice daily, 6 days/week, for 4 weeks. The control group showed 13% tumor regressions (0% complete remission, CR; 13% partial remission, PR). FCE 24304 given s.c. induced 44% (22+22) regressions at the dose of 3 mg/kg per day, 70% (40+30) at 10 mg/kg per day, 73% (27+46) at 30 mg/kg per day, and 70% (50+20) at 100 mg/kg per day. FCE 24304 given orally induced 25% (17+8) tumor regressions at 30 mg/kg per day and 50% (17+33) at 100 mg/kg per day. Rats were killed 4 h after the last dose and the aromatase activity of ovarian microsomes (OAA) was evaluated. OAA was reduced by 56% after s.c. treatment with 3 mg/kg per day FCE 24304; complete OAA suppression (≥96%) was obtained starting at 10 mg/kg per day s.c. Oral treatment slightly reduced OAA only at a dose of 100 mg/kg per day (36%). Body weight increased in all the groups s.c. treated with FCE 24304 but not in those treated orally. The weights of the pituitary, adrenals, and uterus were reduced in rats treated s.c. with 10 and 30 mg/kg per day; at 100 mg/kg per day, a decrease in ovarian weight was observed while uterus weight was similar to that of controls. Oral FCE 24304 increased ovarian weight at a dose of 30 mg/kg per day but not at 100 mg/kg per day. Serum prolactin (PRL) and luteinizing hormone (LH) levels did not change. In conclusion, FCE 24304 given s.c. proved highly effective against DMBA-induced tumors in rats but had less activity when given orally. Its intrinsic androgenic activity, higher after s.c. than after oral treatment, could contribute to the antitumor effect in the intact (premenopausal) rat model.

Introduction

Specific aromatase (estrogen synthetase) inhibitors are potentially useful therapeutic agents for estrogen-dependent tumors [1, 13]. Several irreversible aromatase inhibitors (i.e., suicide inhibitors) have been developed [3, 9, 10]; these compounds offer significant advantages in comparison with the presently available drug aminoglutethimide, a nonspecific, reversible aromatase inhibitor [8, 14]. 4-Hydroxyandrostenedione (4OH-A), the first-described irreversible aromatase inhibitor [2], has been clinically tested: a phase II study showed that 4OH-A can reduce plasma estradiol levels and produce tumor regression in postmenopausal patients with advanced breast cancer [7].

6-Methylenandrosta-1,4-diene-3,17-dione (FCE 24304) is a new irreversible aromatase inhibitor. In preincubation studies with human placental aromatase, FCE 24304 showed an apparent K_i of 26 n*M* and the enzyme inactivation half-life was 13.9 min. In pregnant mare's serum gonadotropin (PMSG)-pretreated rats, the compound reduced ovarian aromatase activity (OAA) 24 h after a single s.c. or oral dose, its ED_{50} being 1.8 or 3.7 mg/kg, respectively [4, 6].

In the present study we evaluated the antitumor activity of FCE 24304 in 7,12-dimethylbenzanthracene (DMBA)-induced mammary carcinoma in rats; in these chronically treated rats we also assessed the effect of the compound on endocrine organ weights, on OAA, and on serum prolactin (PRL) and luteinizing hormone (LH) levels. In this tumor model 4OH-A has been reported to be very effective in reducing tumor growth [17].

Materials and methods

Animals. Female CD Sprague-Dawley rats were supplied by Charles River, Italy. The animals were housed 4–5 per cage and maintained in an air-conditioned room with controlled temperature (23° C) and light (12 h light from 7:00 a.m. to 7:00 p.m.).

Mammary tumor model. At 50 days of age the rats were dosed intragastrically with 20 mg DMBA (Sigma Chemical Co., Saint Louis, Mo., USA) dissolved in sesame oil (1 ml/rat). Starting 40 days after DMBA treatment, the animals were examined weekly by palpation; when at least one tumor 1 cm in diameter was found the rats were placed sequentially into experimental groups. Animals without tumors by day 150 were discarded. The two perpendicular tumor axes were measured with calipers twice weekly during treatment. Tumor weight was calculated according to the formula $d^2 \times D/2$, where d is the minimal and D the maximal diameter [5]. Tumor response to the drug was designated as CR (complete remission, disappearance of the tumor), PR (partial remission, >50% reduction in tumor weight), NC (no change, <50% increase or decrease) or P (progression, >50% increase).

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Drug and treatment schedule. FCE 24304 (Farmitalia C. Erba, Milano, Italy) was suspended in 0.5% methocel when given orally or dissolved in benzyl alcohol and diluted in sesame oil when given s.c. Treatments were given twice daily (10:00 a.m. and 4:00 p.m.), 6 days/week, for 4 weeks. The compound was given orally in a volume of 5 ml/kg body wt. and s.c. in a volume of 2 ml/kg. Vehicle-treated rats served as controls. On the last experimental day rats were dosed at 10:00 a.m.; 4 h later they were killed by decapitation, and serum obtained after centrifugation was stored at −20° C for PRL and LH radioimmunoassay. The pituitary, ovaries, adrenals, and uterus were then removed and their wet weights recorded. Ovaries were stored at −20° C for aromatase assay.

Aromatase assay. Ovaries were thawed at ice temperature and homogenized in 6 ml 0.1 *M* phosphate buffer, pH 7.4. The homogenates were centrifuged in a Sorvall centrifuge at 10,000 *g* for 50 min at 4° C; the supernatants were transferred to ultracentrifuge tubes and centrifuged at 105,000 *g* for 1 h at 2°–4° C in a Beckman ultracentrifuge. The pellets were suspended in 0.15% KCl and ultracentrifuged as described above. Washed microsomal pellets were then suspended with incubation buffer (0.01 *M* phosphate buffer containing 0.1 *M* KCl, 0.001 *M* EDTA, and 0.001 *M* dithiothreitol, pH 7.5) at a concentration of 40 mg wet tissue/ml. OAA was assayed according to Thompson and Siiteri [15], by incubating in duplicate 0.6-ml aliquots of microsomal suspensions with 100 n*M* [1 β, 2 β −^{3}H]-androstenedione (specific activity, 40–60 Ci/mmol), supplied by N.E.N. (Boston, Mass., USA), and 0.1 m*M* NADPH in 1 ml final incubation volume for 1 h at 37° C in a Dubnoff shaking bath. At the end of incubation, steroids were extracted with 5 ml chloroform and the amount of ^3H_2O formed during the aromatization of androstenedione to estrone (E_1) was determined by liquid scintillation counting. OAA was expressed as the amount of E_1 formed per mg microsomal protein (pmol E_1/mg protein per h) or as the total amount formed by each pair of ovaries (pmol E_1/ovaries per h). The protein content of microsomal preparations was determined using the method of Lowry et al. [11], with bovine serum albumin as a standard.

PRL and LH radioimmunoassay. PRL and LH were assayed by a double antibody radioimmunoassay, using reagents supplied by the National Pituitary Agency (Baltimore, Md, USA). Results are expressed as ng/ml NIADDK-Rat PRL RP-3 or NIADDK-Rat LH RP-2. ^{125}I-PRL (specific activity, 20–50 μCi/mg) was supplied by N. E. N., and ^{125}I-LH was obtained by iodination with the chloramine T-method. The sensitivity of the assay was 0.6 ng/ml for PRL and 0.2 ng/ml for LH.

Results

Antitumor activity

The effect of FCE 24304 given s.c. or orally against DMBA-induced mammary tumors in rats is shwon in Table 1. The percentage of spontaneous tumor regressions (CR% + PR%) in the control group was 13% (0+13). FCE 24304 given s.c. induced 44% (22+22) tumor regressions at a dose of 3 mg/kg per day, 70% (40+30) at 10 mg/kg per day, 73% (27+46) at 30 mg/kg per day, and 70% (50+20) at 100 mg/kg per day. No progressions were observed at the highest dose. When the compound was given orally on the same treatment schedule, tumor regressions amounted to 25% (17+8) at a dose of 30 mg/kg per day and 50% (17+33) at 100 mg/kg per day. The number of new tumors was low in the control group and slightly lower in FCE 24304-treated rats (except in the group treated s.c. with 3 mg/kg per day). The average weight change indicated an anabolizing effect for the compound only after s.c. dosing.

Effect on endocrine organ weights

The pituitary, ovaries, adrenals, and uterus of tumor-bearing rats were weighed at the end of the 4-week treatment with FCE 24304. Relative organ weights are presented in Fig. 1. At s.c. doses of 10 and 30 mg/kg per day, FCE 24304 significantly lowered pituitary, adrenal, and uterine weights; at the highest dose, 100 mg/kg per day, the compound also reduced relative ovarian weight, but its effect on uterine weight was no longer evident. Oral FCE 24304 caused an increase in relative ovarian weight only at 30 mg/kg per day.

Table 1. Effect of FCE 24304 on DMBA-induced mammary tumors in rats

Route	Dose[a] (mg/kg per day)	No. of rats	No. of[b] tumors evaluated	Effect at the end of treatment					AWC[g] (g)
				No. (%) of				New tumors/rat	
				CR[c]	PR[d]	NC[e]	P[f]		
–	–	13	15	0 (0)	2 (13)	5 (33)	8 (54)	0.4	−1±6
s.c.	3	7	9	2 (22)	2 (22)	3 (34)	2 (22)	0.4	+29±6
	10	9	10	4 (40)	3 (30)	1 (10)	2 (20)	0.1	+34±5
	30	10	11	3 (27)	5 (46)	1 (9)	2 (18)	0.1	+44±3
	100	9	10	5 (50)	2 (20)	3 (30)	0 (0)	0.1	+45±8
p.o.	30	10	12	2 (17)	1 (8)	5 (42)	4 (33)	0.1	+3±2
	100	9	12	2 (17)	4 (33)	1 (8)	5 (42)	0.2	+7±4

[a] The compound was given twice a day, 6 days/week, for 4 weeks
[b] Tumors were induced by a single gastric intubation of 20 mg DMBA to 51-day-old female Sprague Dawley rats
[c] CR, complete remission
[d] PR, partial remission (reduction in tumor weight > 50% of initial tumor weight)
[e] NC, no change (increase or decrease in tumor weight < 50%)
[f] P, progression (increase in tumor weight > 50%)
[g] AWC, average body wt. change (difference between initial and final body wt.); mean ± SE

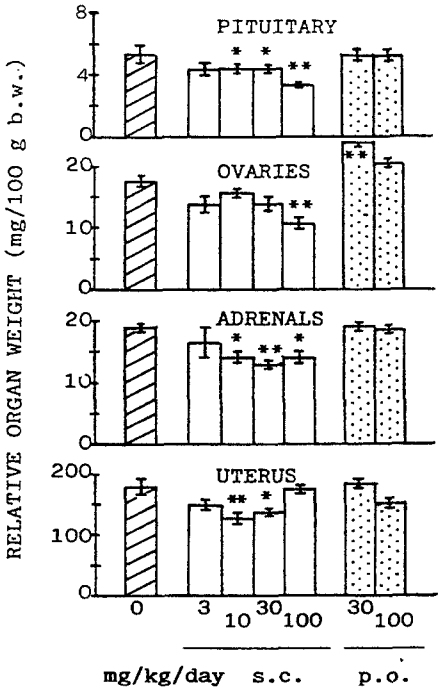

Fig. 1. Effect of FCE 24304 on relative organ weights of rats with DMBA-induced mammary tumors. The compound was given twice a day, 6 days/week, for 4 weeks. Bars represent mean ± SE (7–13 animals per group). * $P < 0.05$ and ** $P < 0.01$ vs controls, by Dunnett's test

Effect on OAA

Tumor-bearing rats were killed 4 h after the last dose and OAA was determined. The results, reported in Table 2, are expressed as the amount of E_1 produced per mg protein (specific activity) or per ovary (total activity). FCE 24304 given s.c. at a dose of 3 mg/kg per day reduced specific and total OAA by 56% and 70%, respectively; complete OAA suppression (≥96%) was obtained starting at a s.c. dose of 10 mg/kg per day. Oral FCE 24304 at a dose of 30 mg/kg per day had no effect on OAA; at 100 mg/kg per day p.o., the compound slightly reduced specific OAA (36%), which was completely suppressed in only 2/9 rats.

Table 3. Effect of FCE 24304 on serum PRL and LH levels in DMBA-induced tumors in rats

Route	Dose[a] (mg/kg per day)	No. of rats	PRL[b] (ng/ml)	LH[b] (ng/ml)
–	–	13	52.5 (2.9–>185)[c]	0.34 (0.20–19.5)[c]
s.c.	3	7	18.7 (8.6–173)	0.33 (0.20– 8.7)
	10	9	5.4 (3.5–30)	0.30 (0.20– 0.5)
	30	10	12.2 (3.3–>185)	0.35 (0.24–29.8)
	100	9	18.1 (7.0–47)	0.29 (0.17– 0.6)
p.o.	30	10	83.2 (5.1–>185)	0.80 (0.29–30.4)
	100	9	33.7 (4.1–184)	0.55 (0.25–24.4)

[a] The compound was given twice a day, 6 days/week, for 4 weeks
[b] Rats were killed 4 h after the last morning dose
[c] Median value (range)

Effect on serum PRL and LH levels

PRL and LH levels were assayed in rats killed 4 h after the last FCE 24304 dose, independently of the estrous cycle phase. This accounts for the wide variability of both hormonal parameters in all groups. Median values for PRL and LH are reported in Table 3. Serum PRL levels tended to be lower in the groups treated s.c.; in those treated orally they were similar to levels in controls. No change in serum LH levels was observed in any treated group.

Discussion

FCE 24304 proved to be effective against DMBA-induced mammary tumors in rats. When given s.c. the compound was effective starting at a dose of 3 mg/kg per day, inducing 44% tumor regressions. At 10, 30, and 100 mg/kg per day it showed similar antitumor activity (70%, 73%, and 70% tumor regressions, respectively), but at the highest dose no tumor progressions were observed. OAA was completely suppressed (≥96%) at all three doses. FCE 24304 was less effective in oral doses than by the s.c. route: at 100 mg/kg per day, tumor regressions amounted to 50% and OAA was reduced by 36%. These results do not confirm data obtained in PMSG-pretreated rats [6], where a

Table 2. Effect of FCE 24304 on OAA in DMBA-induced tumors in rats

Route	Dose[a] (mg/kg per day)	No. of rats[b]	OAA[c]	
			pmol E_1/mg protein per 60 min	pmol E_1/ovaries per 60 min
–	–	13	4.50[d] (2.47–18.90)	1.59[d] (0.76–5.73)
s.c.	3	7	1.97 (<0.20– 7.29)	0.47 (<0.05–1.81)
	10	9	0.20* (<0.20– 1.28)	0.05* (<0.05–0.56)
	30	10	<0.22* (<0.20– 1.46)	<0.05* (<0.05–0.36)
	100	9	<0.20* (<0.20– 2.49)	<0.05* (<0.05–1.38)
p.o.	30	10	3.82 (0.54–10.06)	1.66 (0.23–3.10)
	100	9	2.89 (<0.20–10.86)	1.30 (<0.05–5.73)

[a] The compound was given twice a day, 6 days/week, for 4 weeks
[b] Rats were killed 4 h after the last morning dose
[c] The amount of estrone (E_1) produced was calculated from the tritium released during aromatization of [1β, 2β-^{3}H] androstenedione and converted to ^3H_2O
[d] Median value (range)
* $P < 0.01$ vs controls (Dunn's test)

single dose of FCE 24304 was half as potent by the oral as by the s.c. route (ED_{50}, 3.7 mg/kg p.o. vs 1.8 mg/kg s.c.). A possible explanation for this difference in activity in this tumor model could lie in the compound's weak androgenic activity. FCE 24304 showed slight binding affinity for androgen (rat prostate) receptors (relative binding affinity, 0.2% vs dihydrotestosterone) [6] and, when given s.c. to castrated male rats, it had a slightly androgenic effect of about 1% that of testosterone propionate [4]. After oral dosing, the androgenic activity of FCE 24304 was several times lower (D. Giudici et al., unpublished results). The weak androgenic activity of FCE 24304 could contribute to the antitumor effect observed in DMBA-induced tumors in rats, which can be assumed as a model for the therapy of premenopausal cancer patients.

In fact, the inhibition of ovarian estrogen synthesis in the intact rat may lead to increased serum gonadotropin levels via feedback regulatory mechanisms. These rises may tend to stimulate ovarian aromatase neosynthesis and therefore counteract the effect of the aromatase inhibitor. Aminoglutethimide has been reported to be marginally effective against DMBA-induced tumors, possibly due to the extensive N-acetylation observed in rats [16] but also to the subsequent reflex increase in gonadotropins [17]. Conversely, 4OH-A, a weakly androgenic aromatase inhibitor, was reported to reduce LH levels in ovariectomized rats. This antigonadotropic effect may therefore contribute to its efficacy in causing the regression of DMBA-induced tumors [17]. Similarly, the androgenic effect of FCE 24304 could prevent increases in gonadotropins in tumor-bearing rats. In fact, in these rats FCE 24304 given s.c. reduced ovarian weight, and in preliminary experiments on castrated male rats the compound significantly lowered LH levels at doses ranging between 30 and 100 mg/kg per day s.c. (D. Giudici et al., unpublished results). The decrease in uterine weight at the lower s.c. doses might reflect reduced estrogen synthesis; this effect was no longer evident at 100 mg/kg per day s.c., possibly because it was counteracted by the compound's androgenic activity at this high dose.

In fact, high doses of androgens that cannot be aromatized, including dihydrotestosterone, have been shown to increase uterine weight in rats [12]. An increase in uterine weight has also been observed in 4OH-A-treated rats [17]. At all s.c. doses, FCE 24304 had an anabolizing effect. In the groups given the compound orally, no such effects were evident: body and uterine weights were unaffected by treatment, whereas ovarian weight was increased in the group treated at the lower dose. Median serum PRL levels were lower than those of controls in all the groups treated s.c. with FCE 24304; this could be related to aromatase inhibition.

In conclusion, FCE 24304 given s.c. is highly effective against DMBA-induced tumors, but it has less activity when given orally. The compound's intrinsic androgenic activity, higher after s.c. than oral treatment, makes a positive contribution to its antitumoral effect in this tumor model.

Clinical studies using 4OH-A have shown that its selective inhibition of estradiol synthesis could provide effective treatment for postmenopausal breast cancer [7], thus confirming that this new class of compounds may prove useful in the treatment of breast cancer and other estrogen-related diseases.

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