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A new triphenylethylene compound, Fc-1157a

II. Antitumor effects

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Summary. The antitumor effects of a new antiestrogen, Fc-1157a[f] have been studied in vitro and in vivo. In vitro the effect of Fc-1157a was comparable to that of tamoxifen. The effect was dose-dependent, and at concentrations higher than 10^{-6} mol/l Fc-1157a induced real cell death of the MCF-7 cells. In DMBA-induced mammary cancer in rats Fc-1157a decreased the number of new tumors and inhibited the growth of existing tumors, these effects being statistically highly significant. The ratio of growing tumors to stable and regressing tumors was significantly decreased. Although these effects were slightly stronger with Fc-1157a than with tamoxifen, the difference between these two compounds was not statistically significant.

Murine uterine sarcoma, an estrogen receptor-negative tumor, was resistant to tamoxifen, but was statistically significantly inhibited by high doses (100 and 200 mg/kg^{-1} day^{-1} for 5 days) of Fc-1157a.

The antitumor effects of Fc-1157a are due mainly to the antiestrogenic activity. At high concentrations in vitro and at high doses in vivo Fc-1157a exerts antitumor effects some of which are different from those of tamoxifen and are directed even against estrogen receptor-negative tumors. The exact mechanism of the observed cytolytic effect at high doses is unknown.

Introduction

Comparable clinical antitumor effects, in breast cancer can be achieved with several hormonal treatments, including high-dose estrogen, high-dose progestin, antiestrogen, oophorectomy, and surgical or chemical adrenalectomy [3]. Many of these are very safe compared with classic cytotoxic treatments. However, the antiestrogen tamoxifen, although one of the safest antineoplastic drugs, has been reported to induce hyperplastic nodules in the rat liver at high doses [19]. In human patients liver damage (1) and exacerbation of several side effects have been described with doses higher than 40 mg daily, without any increase in the antitumor effect [17]. It is important that the antitumor efficacy of hormone treatments be substantially improved in many aspects.

The goal of our group has been to develop molecules with a better ratio of efficacy to side effects than the present antiestrogens. New Compounds have been synthesized and screened with rational biochemical and pharmacological tests. One of the compounds, Fc-1157a, chemically 4-chloro-1,2-diphenyl-1-{4-[2-(*N,N-dimethylamino)ethoxy*]-*phenyl*}-1-butene, was selected for further studies. In the companion paper the hormonal effects of this compound were documented. The present paper concentrates on in vitro and in vivo antitumor properties of Fc-1157a.

The estrogen receptor (ER)-positive human breast carcinoma cell line MCF-7 and dimethylbenzanthracene (DMBA)-induced rat mammary tumors have been extensively used in studies aimed at understanding the hormonal control mechanisms of human breast cancer [2, 6, 9, 11, 14]. The effects of synthetic nonsteroidal antiestrogens such as tamoxifen have been thoroughly studied in these models in past years [2, 6, 7, 9–12, 16, 18]. Transplantable murine uterine sarcoma is an ER-negative tumor. It is resistant to tamoxifen, but is sensitive to glucocorticoids. These three experimental tumor models were selected to elucidate the antitumor profile of the new compound, Fc-1157a. In addition, several primary tumor samples of ovarian origin were cultivated in vitro to compare the effects of tamoxifen and Fc-1157a. The number of living cells in vitro was estimated with a simple bioluminescence method [8].

Materials and methods

Drugs. Fc-1157a and tamoxifen, purity >98%, were synthesized by Farmos Group Research Center (Medipolar, Oulu, Finland). Both were used as citrate salts.

Cell lines. The MCF-7 cells (kindly provided by Dr Charles M. McGrath, Michigan Cancer Foundation) were grown in Eagle's minimum essential medium (MEM; Gibco Europe Limited, Renfrewshire, Scotland), supplemented with L-glutamine (292 mg/l; Fluka AG, FRG), gentamicin (10 μg/ml), inslulin (0.6 μg/ml; Collaborative Research, Inc., Lexington, Ma, USA), sodium pyruvate (111 mg/l; Merck, FRG), nonessential amino acids (Gibco Europe Ltd), Hepes buffer (2-hydroxyethyl-piperazine-*N*-2-ethanosulfonic acid, 25 m*M*; Sigma, St. Louis, Mo, USA) and 10% fetal calf serum (FCS, Gibco Europe Ltd). The cells were shown repeatedly to be free of mycoplasma contamination with DNA-specific fluorescent stain

[f] International nonproprietary name, toremifene

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Hoechst 33258 [4]. The cells were grown in a humidified incubator (95% air, 5% CO_2) at 37 °C.

Cell growth experiments. Ten days before the start of the experiment, MCF-7 cells were rinsed twice with phosphate-buffered saline, and the culture medium was replaced with the same medium containing 5% FCS treated with dextran-coated charcoal (FCS-DCC) to remove endogenous estrogens [12]. At this time insulin was depleted from the culture medium. On day 11 the cells were harvested with 0.05% trypsin. A suspension of 20000 cells/ml was prepared and then 0.02% EDTA solution. The cells were plated in 24-well or 96-well dishes (Nunc, Roskilde, Denmark; 1 ml or 100 µl, respectively). The antihormones to be tested (dissolved in ethanol) were added to the culture medium. The final ethanol concentration was 0.07%. The control wells also contained 0.07% of ethanol. At specific intervals the ATP content of the cells in the wells was measured.

The ATP content of the cells was measured by bioluminescence assay [8] using LKB Luminometer Model 1250 equipped with an LKB 2210 potentiometer recorder (LKB-Wallac, Turku, Finland). ATP is a basic energy source of living cells. The protocol of the ATP test was as follows: Trichloracetic acid (TCA) 100 µl, 1%, was added to the wells containing the MCF-7 cells (turns yellow in color) and mixed about 15 times with a Finnpipette, after which 100 µl of the solution was removed to cuvettes containing buffered ATP monitoring reagent (100 µl ATP monitoring reagent and 300 µl Tris-acetate buffer, 5 m*M* EDTA, pH 7.75), vortexed for 5 s, and read immediately in a luminometer. The ATP content of the cells has been shown to correlate with the amount of viable cells by the trypan blue exclusion test, electronic cell counting, ^{3}H-thymidine incorporation, and the stem cell assay [8].

Tumor samples were obtained at primary gynecological laparotomy in 33 patients. Several biopsies from areas of solid and active cancer tissue were taken for histology, receptor determinations, and tumor cell assay in vitro, as described by Grönroos et al. [5]. Samples for cell cultures were immediately transferred to sterile test tubes containing RPMI 1640 medium and minced with a scalpel within 1 h of excision. If a sufficient number of living cells was obtained by simple scraping the red blood cells present in the sample were lysed with NH_4Cl. Living cells were collected by centrifugation and used for culture. If necessary the tumor cells were detached from the matrix by overnight incubation with collagenase before lysis of the red cells. RPMI 1640 medium with 10% fetal calf serum, L-glutamine (292 mg/l), penicillin (100 i.u./ml), and streptomycin (100 µg/ml) was used as cell growth medium. The cells were incubated at 37 °C in 5% CO_2 for 1–5 days in microtiter plates (100 µl)/sample) or in plastic tubes (1–2 ml/sample). The drug effects were assessed as shown in MCF-7 cells.

Transplantable murine uterine sarcoma growing in female NMRI mice was kindly provided by Prof. H. Osswald, Deutsches Krebsforschungszentrum, Heidelberg, FRG). The solid tumor was gently homogenized in glass homogenizer, filtered through cheese cloth, and injected to mice IM in the left leg as a cell suspension (10^6 cells/mouse). The tumor size was estimated by measuring the diameter of the leg with a caliper; the diameter of the right leg was subtracted, and the difference was considered the tumor size. Mice with transplanted uterine sarcoma were treated with Fc-1157a or with tamoxifen, 100 and 200 mg/kg PO daily for 5 days, starting on day 1 after transplantation. The tumors were measured twice a week.

Mammary adenocarcinomas were induced in 50±2 days old female Sprague-Dawley rats by single PO doses of 12 mg 7,12-dimethylbenzanthracene (DMBA) (Sigma, St. Louis, Mo, USA) in 1.0 ml sesam oil. Induction was achieved in a special isolator (Metall & Plastic GmbH, Radolfzell, FRG). After about 6 weeks the tumors appeared. Treatments with the research compounds were started when the largest tumors were about 1 cm in diameter. The rats were palpated once a week. Number and size of the tumors were recorded individually up to at least 5 weeks. The sizes of the tumors were recorded following palpation, using two perpendicular diameters. The volume of the tumors was estimated from the equation:

$$\text{Size (cm}^3) = \pi\,(\text{width})^2 \times \text{length}/12.$$

As the DMBA-induced tumors are quite heterogenous, the tumors were classified in three groups: (1) growing tumors, which had increased ≥ 4 times in volume; (2) stable tumors with no change or the size increased <4 times in volume or decreased but was $>\frac{1}{4}$ of the original volume; (3) regressing tumors, with the volume decreased to $\frac{1}{4}$ or less of the original volume. Followup time for the evaluation was 5 weeks.

The rats were treated with antiestrogens daily (7 days/week). The doses (given PO) are indicated in Tables 2 and 3.

All animals were purchased from Alab, Stockholm, Sweden and received standard chow (Anticimex, Stockholm, Sweden) and tapwater ad libitum throughout the studies.

Students *t*-test and the χ^2-test were used in statistical analyses.

Results

The effects of a novel antiestrogen, Fc-1157a, on the growth of MCF-7 cells were tested in vitro. The results are shown in Fig. 1. Fc-1157a inhibited cell growth as a function of concentration, so that the concentration of 5×10^{-6} mol/l killed all cells in as little as 2 days.

Estradiol at a concentration of 10^{-8} mol/l did not stimulate the cell growth significantly in our experiments.

The inhibitory effect of Fc-1157a, 5×10^{-7} mol/l, on cell growth could be reversed by washing the cells and adding estradiol to the medium (Fig. 2).

Fc-1157a and tamoxifen were compared in vitro in 33 primary human ovarian cancers. As seen in Table 1, there were 2 of the 33 (patients 18 and 20) tumors that responded "completely" to these antiestrogens – almost no living cells were seen after 3 days. Other tumors responded slightly or not at all. There was no difference between the two antiestrogens in vitro.

Fc-1157a was not effective against leukemia P388. The increase in lifespan was <25% (data not shown). Fc-1157a inhibited the growth of murine uterine sarcoma significantly, as shown in Table 2, although tamoxifen had no effect.

The number of DMBA-induced mammary cancers, classified according to the growth properties as described in *Materials and Methods*, after treatment with different

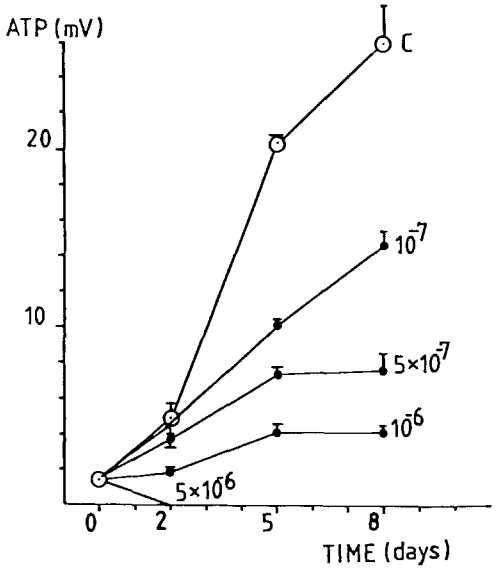

Fig. 1. Effects of Fc-1157a on growth of MCF-7 cells in vitro. Concentrations of Fc-1157a have been expressed as mol/l. ATP bioluminescence method (8) was used for determination of living cell number was used. *C*, control

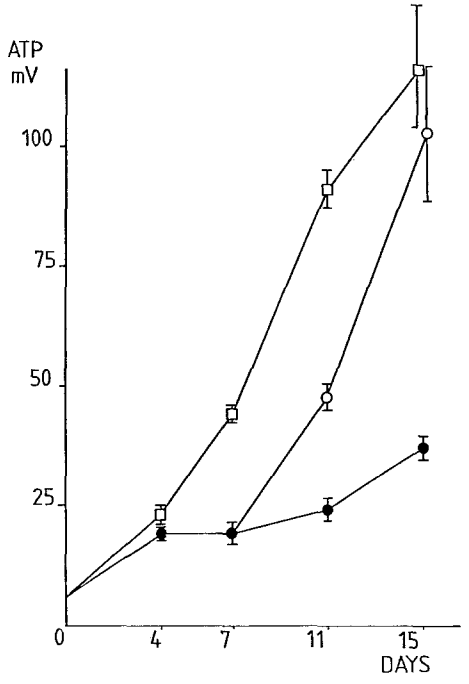

Fig. 2. Reversal of Fc-1157a growth inhibition by estradiol in MCF-7 cells. Cells were exposed to Fc-1157a, 5×10^{-7} mol/l, for 0, 4, 7, 11, and 15 days (-●-). On day 7 previously Fc-1157a-treated cells were washed twice with growth medium and then incubated with 10^{-8} mol/l estradiol (-○-). Controls (-□-) were cultivated without hormones. Means (±SD) or quadruplicate experiments. ATP bioluminescence method (8)

doses of Fc-1157a and tamoxifen have been presented in Table 3. The change in the number of tumors during the 5-week treatment has also been given in Table 3.

Preliminary toxicity studies indicate that Fc-1157a is a safe compound. It can be given to rats in higher doses than tamoxifen. No eye or liver changes were found in Fc-1157a-treated animals even at the highest dose level

Table 1. Effect of tamoxifen and Fc-1157a in vitro on human ovarian cancer cells obtained from primary surgical samples

Sample no.	Amount of living cells (percent of control)	
	Tamoxifen	Fc-1157 a
1	47	62
2	59	55
3	77	57
4	73	93
5	98	93
6	83	86
7	91	84
8	88	51
9	31	50
10	79	80
11	77	58
12	33	45
13	53	62
14	99	93
15	48	32
16	92	85
17	28	42
18	1	2
19	72	86
20	0	0
21	16	16
22	97	79
23	63	50
24	27	25
25	75	79
26	83	80
27	60	69
28	95	95
29	26	38
30	79	72
31	78	83
32	84	78
33	25	25
	61.7 ± 29.3	60.7 ± 26.9

The amounts of living cells, measured by ATP bioluminescence method, have been presented as percentages of numbers in control cultures without antihormones. Concentrations of tamoxifen and Fc-1157a in the culture medium were similar (10^{-6} mol/l). Growth time 3 days. Each value is the mean of triplicate cultures. Paired *t*-test: $t = 0.446$, $d.f. = 32$, not significant

(48 mg/kg daily for 26 weeks). Similar dose of tamoxifen induced hyperplastic nodules in the livers of all treated rats.

Fc-1157a was not found to be mutagenic in the Ames test. Fc-1157a had no alkylating activity demonstrable by the nitrobenzylpyridine test.

Discussion

The basic pharmacological mechanism of action of Fc-1157a resembles that of tamoxifen. Both compounds are bound to ERs and are antiestrogenic in inhibiting estradiol-induced uterotrophic effect in mice, rats, and humans. The receptor replenishment kinetics of the two compounds

Table 2. Effect of tamoxifen and Fc-1157a on the growth of mouse uterine sarcoma

Group	Dose (mg/kg) (p.o.)	Tumor growth (cm in diameter) within 2. weeks after the treatment		
Saline		1.22 ± 0.26	($n=15$)	
Tamoxifen	100	1.14 ± 0.40	($n=10$)	ns
Tamoxifen	200	1.16 ± 0.24	($n=7$)	ns
Fc-1157a	100	0.90 ± 0.16	($n=10$)	$P<0.01$
Fc-1157a	200	0.68 ± 0.43	($n=10$)	$P<0.001$

Both test groups were compared with the saline-treated control group with reference to Student's *t*-test

Table 3. Effects of Fc-1157a and tamoxifen on DMBA-induced mammary tumors in rats

Group		No. of animals	Classification[a]			P-value[b] test	No. of new tumors/animal	No. of tumors disappeared	Ratio class 1/class 2+3
			1	2	3				
Control (vehicle)		32	122	87	8	–	2.97 ± 2.63	4	1.28
Fc-1157a	0.3 mg/kg	8	19	20	5	NS	1.38 ± 1.18	4	0.76
	1.0 mg/kg	8	8	13	5	<0.0005	0.63 ± 0.74	2	0.44
	3.0 mg/kg	9	18	30	6	<0.005	0.67 ± 1.12	5	0.50
	7.5 mg/kg	8	13	22	5	<0.005	1.63 ± 2.00	1	0.48
	15.0 mg/kg	5	10	14	3	NS	1.80 ± 2.39	4	0.59
	30.0 mg/kg	10	10	20	9	<0.0005	0.60 ± 1.43	4	0.34
Fc-1157a total		48	78	119	33	<0.0005			
Tamoxifen	1.0 mg/kg	7	8	13	3	<0.05	2.00 ± 0.58	2	0.50
	3.0 mg/kg	15	38	59	9	<0.005	2.07 ± 1.44	0	0.56
	7.5 mg/kg	8	11	13	6	<0.001	0.25 ± 0.46	1	0.58
Tamoxifen total		30	57	85	18	<0.0005			

[a] 1, number of growing tumors; 2, static tumors; 3, regressing tumors. For further details of classification see *Methods*
[b] Obtained by comparison of each group with controls by X^2 test according to the number of tumors in each class

however, is slightly different (see companion paper). Comparison of the present work with earlier works reported by other groups with tamoxifen shows that Fc-1157a and tamoxifen have a similar antitumor effect against MCF-7 cells in vitro. Also, growth inhibition obtained at $<10^{-6}$ mol/l concentrations can be reversed with estradiol [9, 11]. In 33 primary human ovarian cancer samples the effects of tamoxifen and Fc-1157a were strikingly similar at equivalent concentrations (Table 1) in vitro. In the present work, cell growth in vitro was estimated by the ATP-bioluminescence method, which has been described recently [8]. This method is technically very simple and rapid, and has proved to be a useful tool for the measurement of cell growth and death. There was good agreement between the bioluminescence assay and other cell viability tests and between all these and the clonogenic stem cell assay [8].

In vivo the antitumor mechanism of antiestrogens is a more complex phenomenon than in vitro. It has been shown that antiestrogens, which are suggested to have a very low intrinsic estrogenic effect, like LY 117018 and 4-hydroxytamoxifen, are effective antitumor compounds in vitro but not in vivo [6, 7, 12]. It has been suggested that other binding sites than estrogen receptors are important in the onset of antitumor effect [15]. Metabolism and long biological half-life in the nucleus have also been thought to be of significance [6, 18]. Further, membrane changes induced by tamoxifen may be responsible for permeability changes and cell proliferation [13].

At high dose levels in vivo Fc-1157a seems to exert an antitumor effect which is only in part explained by antiestrogenic characteristics. Sutherland et al. [15] have suggested that binding to specific cytosolic antiestrogen binding sites (AEBS) is essential to this cytotoxic effect. Although there are no experimental data indicating Fc-1157a binding to AEBS, this hypothesis is in agreement with our results, which show a cytolytic effect of Fc-1157a both in vitro against MCF-7 at high concentrations and in vivo against murine uterine sarcoma at high doses. The uterine sarcoma is sensitive to glucocorticoids (data not shown) but resistant to tamoxifen. So, Fc-1157a seems to have antitumor or cytolytic effects, which appear at high doses and which may not be dependent on ERs. The mechanism remains obscure. It is not due to any alkylating properties, because of no alkylating activity of Fc-1157a was seen in nitrobenzylpyridine test.

In clinical phase I studies Fc-1157a proved to be safe antiestrogen in postmenopausal women. Fc-1157a has entered clinical phase II studies.

Conclusion

Fc-1157a is a new triphenylethylene antiestrogen. It has statistically highly significant and interesting antitumor properties, which are not wholly explained by the classical ER-mediated mechanism.

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