Cancer Chemother Pharmacol (1986) 17: 103–108

Cancer Chemotherapy and Pharmacology



# A new triphenylethylene compound, Fc-1157a

## I. Hormonal effects

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**Summary.** The basic pharmacological and biochemical properties of a new antiestrogen, Fc-1157a,[1] are described. Fc-1157a is bound specifically and with high affinity to estrogen receptors. The binding is competitive with estradiol. Fc-1157a treatment induces translocation of estrogen receptors from cytoplasm to nucleus. The turnover rate of nuclear estrogen receptors is markedly lower than with estradiol, but is more rapid than after tamoxifen.

Fc-1157a is an almost pure antiestrogen in rat uterus, but has intrinsic estrogenic activity in mouse uterus. In animal experiments Fc-1157a has shown antitumor properties, which are described in the companion paper.

### Introduction

The successful hormonal treatment of some hormone-dependent cancers has stimulated research towards new compounds, which inhibit tumor growth by specific hormone-mediated mechanisms. The breakthrough molecules in this area have been antiestrogens. They act by binding to estrogen receptors (ERs) and prevent cell proliferation [1, 30, 38] by a mechanism which is not yet wholly understood. Therefore, those breast tumors which contain measurable amount of estrogen receptors respond to antiestrogens [7, 27, 43]. The high predictive value of ER determination has inspired many laboratories to develop new antiestrogens, which appear to have few side effects even in comparison with other hormonal treatments [12, 14]. This is in marked contrast with the cytotoxic compounds, which have nonspecific actions against both benign and malignant cells. There are many features in favor of antitumor compounds that have a clearly defined specific activity against a specific type of tumor cells together with insignificant systemic toxicity and can be assayed by biochemical and pharmacological laboratory tests to predict the clinical efficacy.

About 350 new molecules have been synthesized since 1979 at the Farmos Group Research Center in the program aimed at antitumor compounds with biochemically and pharmacologically based mechanism of action. About half these compounds have a triphenylethylene structure and are antiestrogens. The compounds have been screened in a rational way with fairly simple laboratory tests consisting of an ER-binding assay, uterotrophic tests in immature mice and rats, and antitumour effects both in vitro against MCF-7 cells and in vivo against DMBA-induced rat mammary cancer. Preliminary toxicology and pharmacokinetic investigations have been performed with the most promising substances. One of the compounds, Fc-1157a 4-chloro-1,2-diphenyl-1-[4-[2-(*N,N*-dimethylamino)ethoxy]phenyl]-1-butene, has been a promising compound in all screening assays and has now reached clinical phase II studies as an antitumor agent against hormone-dependent breast cancers in women.

The present work, together with the companion paper, introduces Fc-1157a, a novel antiestrogen. In this first part the basic mechanism of action, i.e., the hormonal effects of Fc-1157a, are described.

### Materials and methods

*Animals*

Immature female NMRI mice weighing 8–10 g or immature female rats 18–20 days old were used in the uterotrophic tests. ER affinity studies were performed with uterine cytosol preparation obtained from 18 to 20-day-old female Sprague-Dawley rats. These rats were purchased from Alab, Stockholm, Sweden, at the age of 17–19 days. ER translocation studies were mostly carried out in ovariectomized rats, but immature 18–20 days old rats were also used. Ovariectomy was performed 10–14 days before the assays to rats about 50 day old under chloral hydrate anesthesia. All animals were purchased from Alab, Stockholm, Sweden, and kept in standardized conditions. They received standard chow (Anticimex, Stockholm, Sweden) and tapwater ad libitum.

The animals were killed by decapitationin in $CO_2$ anesthesia. The uteri were removed rapidly, prepared free of adhering fat and mesenterium on ice, weighed, and dipped into liquid nitrogen, and then transferred within 2 h to a deep freezer at −80 °C (So-Low, Environmental Equipment, Cincinnati, Ohio, USA). Uteri from about 50 rats were pooled for the ER affinity studies. In the translocation assay eight to ten uteri of ovariectomized rats or 20 uteri of immature rats were pooled for each analysis.

ER translocation kinetics were investigated after a single dose of estradiol valerate (Sigma, St. Louis, Mo,

[1] International nonproprietary name, toremifene

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USA (10 μg $kg^{-1}$ body weight), tamoxifen and Fc-1157a (both from Farmos Group Ltd, Medipolar, Oulu, Finland) (1 and 3 mg $kg^{-1}$ body weight). The animals were killed at 1, 4, 24, and 48 h after administration of the drug. Both cytosolic and nuclear ERs were determined at each time. The nuclear levels of ER and PR were also analyzed after tamoxifen and Fc-1157a five daily doses of 1 mg $kg^{-1}$ body weight and estradiol 10 μg $kg^{-1}$ body weight.

*Assays*

*Uterotrophic tests.* Uterotrophic tests with immature mice and rats were made according to Terenius [39]. The two control groups received vehicle (sesam oil) or 17-beta-estradiol valerate. Fc-1157a at the doses indicated in Fig. 1 was given alone and together with estradiol. For injection Fc-1157a was dissolved in ethanol ($10^{-2}$ mol $l^{-1}$). Saline was added to obtain the final dilutions, which were used immediately. The wet weight of the uteri was recorded on the 4th day and the ratio of uterine weight to animal weight was calculated.

*Preparation of samples for receptor assays.* ER and PR affinity studies were made with minor modifications of the method of Korenman and Dukes [22]. Briefly: The deep-frozen uteri were pulverized with a Mikro-Dismembrator II (Braun, Melsungen, FGR) using liquid nitrogen, and the powders were immediately transferred to weighed, cold glass tubes. All the procedures (unless otherwise stated) were carried out at +4 °C.

Cytosolic receptor fraction was prepared as follows: Phosphate buffer (pH 7.4), 1 ml, was added to 250 mg pulverized tissue. The mixture was gently homogenized with a Teflon rod. The homogenate was centrifuged at 800 *g* (Sorvall RC-5B) for 10 min at +4 °C. The pellet was washed twice with phosphate buffer and centrifuged at 800 *g* for 5 min. The three supernatants were combined and ultracentrifuged at 105000 *g* (Sorvall OTD-65) for 60 min at +4 °C to obtain the cytosolic fraction.

Nuclear ER extract was obtained from the washed nuclear myofibrillar pellet. It was extracted for 1 h with 0.6 mol $l^{-1}$ KCl containing TK-buffer (pH 8.5) [9] by vortexing every 10 min. Ultracentrifugation at 105000 *g* for 60 min yielded the nuclear extract.

*Receptor binding studies.* Cytoplasmic ER binding studies were as follows: 100 μl cytosol (protein concentration 1–2 mg $ml^{-1}$, adjusted according to Kute et al. [23], was incubated at +4 °C for 16–18 h with 100 μl (2,3,6,7-$^3$H)-17-beta estradiol (100 Ci $mmol^{-1}$, Amersham, England); the dilution series ranged from 0.6 n*M* to 10 n*M*. In the competitive affinity tests 2.5 n*M* $^3$H-estradiol was used in each tube. The concentrations of unlabelled competitive ligands were $10^{-4}$ to $10^{-10}$ mol $l^{-1}$. The cytoplasmic progesterone receptors were measured in a similar way to ER, except that $^3$H-ORG 2058 (45 Ci $mmol^{-1}$, Amersham, England) was used as the labelled ligand and the concentrations used were 0.3–5.0 n*M*. All the assays were performed in duplicate. The cytosol incubations were terminated by addition of 250 μl dextran-coated charcoal (DCC) suspension (dextran T-70, Pharmacia Fine Chemicals, Uppsala, Sweden; charcoal, Norit A, Amend, NJ USA). After 15 min the charcoal was centrifuged at 800 *g* for 10 min (Sorvall RC-5B) and the receptor-bound radioactivity of the supernatant was determined by liquid scintillation counter model 81000 (LKB Wallac, Turku, Finland). To correct for the low-affinity binding, cytosol samples were compared with the parallel samples with 200-fold excess of nonlabeled 17-beta estradiol (Sigma, St. Louis, Mo, USA).

The hydroxylapatite exchange assay (HAP assay) for nuclear receptors was carried out by the method of Garola and McGuire [9] with small modifications. Briefly: 200 μl nuclear extract was incubated first at +4 °C for 16–18 h and then at +30 °C for 60 min with $^3$H-estradiol (0.6–10.0 n*M*). Hydroxylapatite (DNA-grade Bio-Gel HTP, Bio Rad, Richmond, Calif, USA) was used to adsorb the $^3$H-estradiol-receptor complex after the incubation at +4 °C. The final share of HAP in the HAP slurry was 60%. HAP slurry 250 μl was added to each tube containing nuclear extract incubated as described above. The mixture was incubated for 30 min with vortexing every 10 min. The samples were centrifuged at 800 *g* for 5 min, and the pellets were washed three times with phosphate buffer. The washed pellets were extracted with absolute ethanol at room temperature by vortexing for 3 min. After centrifugation at 800 *g* for 10 min the radioactivity of the supernatant was counted.

The receptor binding characteristics were analyzed by the method of Scatchard [33]. The protein determinations in cytosol and nuclear extracts were carried out according to Lowry et al [24]. DNA measurements of nuclear pellets were made according to Burton [3] using diphenylamine (Sigma, St. Louis, Mo. USA) in colour production. Calf Thymus DNA and bovine serum albumin (both from Sigma, St. Louis, Mo, USA) were used as standards. All reagents were of analytical purity and were used without further purification.

Student's *t*-test was used in the statistical analyses.

## Results

*Uterotrophic test*

The results of the uterotrophic tests are summarized in Fig. 1 (rat) and 2 (mouse). The values are shown in relation to the effect of estradiol alone.

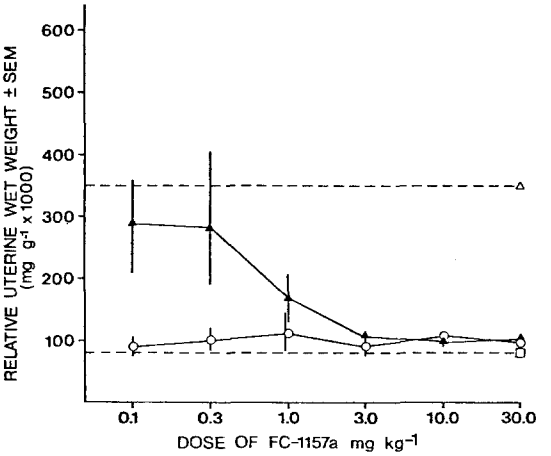



**Fig. 1.** Effect of Fc-1157a on the uterine wet weight of the immature rats. △, 17-beta estradiol; ○, Fc-1157a; △, Fc-1157a + estradiol; □, control level (vehicle only)

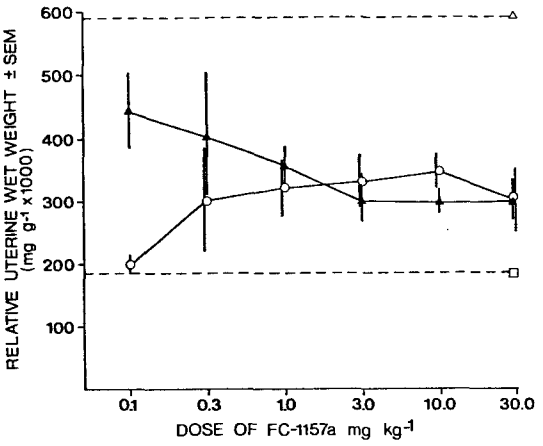



**Fig. 2.** Effect of Fc-1157a on the uterine wet weight of immature mice. (Symbols as in Fig. 1)

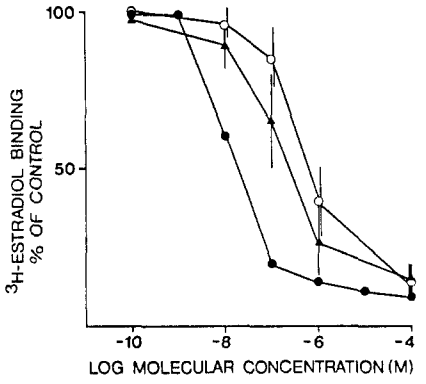
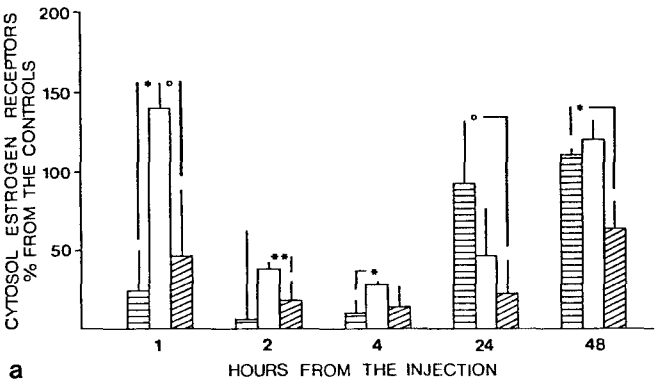



**Fig. 3.** The competition of Fc-1157a (O), with $^3$H-estradiol for binding onto cytosol estrogen receptors. Tamoxifen (▲) and 17-beta-estradiol (●) have been presented for comparison



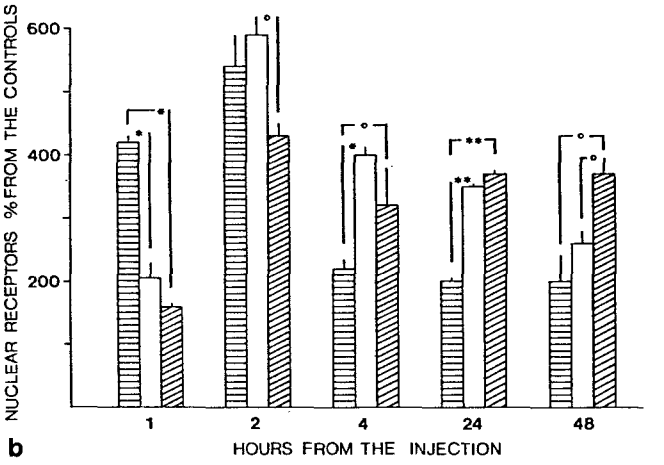




**Fig. 4a, b.** Estrogen receptor levels (means ±SD) in the soluble cytosol **(a)** and nuclear extracts **(b)** shown as percentages of the control values (vehicle-treated animals) after a single injection of 17-beta-estradiol (40 μg kg$^{-1}$ *(horizontal)*, Fc-1157a (1 mg kg$^{-1}$) *(open)*, and tamoxifen (1 mg kg$^{-1}$) *(oblique)*. Statistical significance: O, $P<0.05$; *$P<0.01$

Fc-1157a exerts both estrogenic and antiestrogenic actions in the mouse uterus. In rats its intrinsic estrogenic activity is very weak. The agonistic and antagonistic estrogen-like properties are therefore species-specific. The effects appeared at doses of about 0.3 mg kg$^{-1}$, and the effect reached its maximum with the daily dose of 1.0 mg/kg$^{-1}$ in mice and 3.0 mg kg$^{-1}$ in rats.

*Receptor affinity*

The binding of Fc-1157a to cytosol ERs of rat uteri showed that this new molecule is a competitive ligand of $^3$H-estradiol. Fc-1157a was bound to receptors with high affinity, IC-50 being $5 \times 10^{-7}$ mol l$^{-1}$ and $K_d$ about 1 nmol l$^{-1}$. There was no statistically significant difference between tamoxifen and Fc-1157a in this resepct (Fig. 3).

*Receptor translocation*

A single dose of Fc-1157a decreased the cytosol ER level to 30% of the control value in 2 h. The level remained low for at least 24 h (Fig. 4a). The effects of the higher dose, 3 mg kg$^{-1}$, were similar (results not shown). Figure 5a indicates the effects of five daily IP injections of Fc-1157a, 1 mg kg$^{-1}$, in immature and ovariectomized rats, respectively.

Figure 4b shows the ER accumulation into the nucleus after a single dose, 1 mg kg$^{-1}$, of Fc-1157a. These results are calculated as percentages of control values (same time, vehicle only). After Fc-1157a nuclear ER reached the maximum at 2 h, were substantially elevated at 24 h, and subsequently slowly declined towards the control level. Nuclear ER levels were significantly higher after Fc-1157a than after estradiol at 4 and 24 h ($P \leq 0.05$ and $P \leq 0.005$, respectively). After the administration of Fc-1157a daily for 5 days, nuclear ERs in immature rat uteri were at a high level (3608 ±611 fmol mg$^{-1}$ DNA$^{-1}$). This difference from the estradiol-treated group was statistically significant ($P<0.025$; Fig. 5a). In ovariectomized rats the difference was even more evident ($P<0.001$, Fig. 5b).

Progesterone receptors in cytosol were induced similarly by five daily doses of Fc-1157a, tamoxifen, and estradiol (mean values of 8–10 uteri were 423, 383, and 421 fmol mg$^{-1}$ prot$^{-1}$, respectively). The control value in the ovariectomized animals was 89 fmol mg$^{-1}$ prot$^{-1}$.

Average dissociation constants of nuclear ERs calculated from Scatchard plots were 0.89 n*M* for estradiol, 1.05 nM for Fc-1157a, and 1.29 n*M* for tamoxifen. The biological half-life of the nuclear receptor complex was about 3 days after a single dose, 1 mg kg$^{-1}$, of Fc-1157a

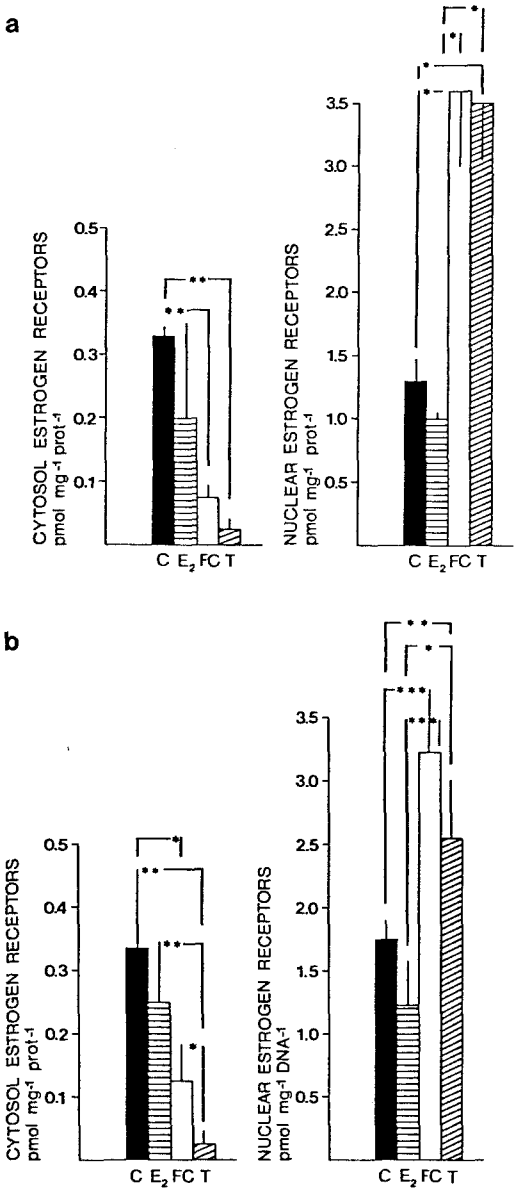



**Fig. 5a, b.** Uterine estradiol receptors (means ± SD) after five daily doses of Fc-1157a (1 mg $kg^{-1}$, FC), tamoxifen (1 mg $kg^{-1}$, T) and estradiol (10 μg $kg^{-1}$, $E_2$) in immature **(a)** and in ovariectomized **(b)** rats compared with controls (vehicle-treated, C). The samples were taken 1 day after the last dose. Statistical significance: * $P<0.05$, ** $P<0.01$; *** $P<0.001$

and longer (approximately 9 days) after the same dose of tamoxifen. This reflects differences in the biological elimination of these antiestrogens from the nuclear Ers.

## Discussion

It is well documented that the nonsteroidal triphenylethylene antiestrogens interact directly with cytoplasmic ERs in the steroid target cells and that the binding is competitive with estradiol [2, 6, 16, 17, 21, 31]. The competitive binding of a novel triphenylethylene compound, Fc-1157a, has been demonstrated in the present work (Fig. 3).

Affinity of a compound for ER does not indicate whether it has agonistic (estrogenic) or antagonistic (antiestrogenic) properties. Many well-known antiestrogens, e.g., tamoxifen, are pharmacologically partial agonists of estradiol [25]. The extent of intrinsic estrogenicity is both species- and organ-specific [40]. The different isomers of several triphenyl ethylene antiestrogens typically possess differences in intrinsic estrogenicity [11, 39]: *cis*-tamoxifen is fully estrogenic in rodents. As shown in Fig. 1 and 2, Fc-1157a is a partial estrogen agonist in the mouse uterus, but a very weak estrogen in the rat uterus. The *cis*-isomer of Fc-1157a is also estrogenic in the rat uterus (data not shown).

An essential part of the mechanism of action of the estrogens and the nonsteroidal antiestrogens is the translocation of the ligand-receptor complex from the cytoplasm to the nucleus [10, 15], where the receptor complex interacts with the chromatin [28, 35]. The role and even existence of cytoplasmic ERs has been recently questioned owing to the impossibility of detecting cytoplasmic ERs by using monoclonal antibodies to ER [20, 26, 34]. It has been stated, however, that the current monoclonals bind reliably only to nuclear ERs [26], and thus the classical receptor hypothesis still remains valid for research.

Clark et al. [4] suggested earlier that the failure to stimulate ER replenishment is the mechanism of antiestrogen action. Long-term retention of the antiestrogen-ER complex in the nuclear compartment has since been suggested to be the fundamental event in the antiestrogenic effect [8, 13]. The retention of ER in the nucleus correlates well with the hormonal responses [5]. A rapid turnover of ERs ("processing") from the nucleus, which occurs after estrogens, stimulates protein synthesis and cell mitosis (e.g., true, uterine growth). After antiestrogens the turnover is slow, and although some early estrogenic events, especially progesterone receptor synthesis, are induced, later events, e.g., cell mitosis, are inhibited [19, 41].

Fc-1157a has similar characteristics as other triphenylethylenes: it depletes cytosolic ER by complexing and transferring them to the nucleus [13]. The turnover of tamoxifen-ER complex from the nucleus is very slow: after an acute administration the half-life of the complex is approximately 9 days (Fig. 4b). The half-life of Fc-1157a-ER complex in the nucleus is slightly shorter (3 days), but anyway much longer than that of estradiol-ER complex (a few hours). This difference is very interesting, because in ovariectomized rats the ER levels both in cytosol and in nucleus are higher after Fc-1157a than after tamoxifen (Fig. 5b). We might now ask whether some of the binding sites for Fc-1157a and tamoxifen in the target tissue are different. It is unknown whether these possible sites are analogous to the highly specific cytoplasmic antiestrogen-binding sites (AEBS), other than ER, discovered by Sutherland et al [37], or some of the multiple binding sites, only some of which are common to estrogens and antiestrogens, as stated by Ruh et al. [32]. The function of these binding sites is partly unresolved, but it has been suggested that AEBS is of importance in antitumor effects of antiestrogens by exerting an unknown mechanism to mediate a non-ER-dependent cytolytic effect [36]. The interesting antitumor effects of Fc-1157a (see companion paper) are in agreement with Sutherland's hypothesis. It can be hypothesized that the slightly higher cytoplasmic and total ER levels after Fc-1157a than after tamoxifen could be partly due also to the release of estradiol-binding components from storages sites, such as cytoskeleton, microsomes, or

membranes [18, 29, 42]. Fc-1157a induces progresterone receptor synthesis in the rat uterus and thus differs from clomifen [13].

As a conclusion: we have described the pharmacological and basic biological properties of a novel, promising triphenyl ethylene antiestrogen, Fc-1157a. In many ways it resembles tamoxifen, but the differences in the turnover rate of the nuclear receptor complex and the possibly different binding sites within the target cells make Fc-1157a a very interesting and promising antiestrogen. The antitumor profile of Fc-1157a has been tested and is presented in the companion paper.

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Received June 6, 1985/Accepted December 5, 1985