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MODE OF ACTION OF LN 1643 (A TRIPHENYLBROMOETHYLENE ANTIESTROGEN): PROBABLE MEDIATION BY THE ESTROGEN RECEPTOR AND HIGH AFFINITY METABOLITE

J. L. BORGNA, E. COEZY and H. ROCHEFORT

Unité d'Endocrinologie Cellulaire et Moléculaire, INSERM (U 148), 60, rue de Navacelles, 34100, Montpellier, France

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Abstract—After *in vivo* administration of [^{3}H]LN 1643, a triphenylbromoethylene antiestrogen, to immature female rats, polar metabolites were selectively accumulated in uterine nuclear fractions which contained most of the estrogen receptor. One metabolite comigrated with the 4-hydroxylated derivative (LN 2839) of LN 1643.* LN 1643 and LN 2839 inhibited competitively and reversibly the binding of estradiol to the estrogen receptor, and the affinity of LN 2839 for the estrogen receptor was about 150-fold higher than that of LN 1643. Both compounds prevented the growth of the MCF_7 human breast cancer cells and LN 2839 was about 10-fold more efficient than LN 1643. These results and previous data obtained with tamoxifen (a parent triphenylethylene antiestrogen) and its 4-hydroxylated metabolite, suggest that the antiestrogenic action of LN 1643 is mediated by the estrogen receptor as for the other synthetic antiestrogens, and that LN 1643 acts at least partly via its 4-hydroxy metabolite, LN 2839.

Synthetic triphenylethylene antiestrogens are currently used in human therapy: clomiphene to induce ovulation [1], tamoxifen to treat estrogen-dependent mammary cancer [2]. The structures of these classical antiestrogens are closely related (Table 1). They include a *para*-dialkylaminoethoxy substituent R_2 on the phenyl group which is in the *trans* position to the ethylenic substituent R_3. Considerable evidence suggests that these compounds act by competing with estrogens for the estrogen receptor. More recently, 4-hydroxytamoxifen (Table 1) and other hydroxylated metabolites were suggested to mediate *in vivo* the antiestrogenic activity of tamoxifen. 4-Hydroxytamoxifen was concentrated in estrogen target tissues after administration of [^{3}H]tamoxifen to rats and chickens [3], and displayed *in vitro* a much higher affinity for the estrogen receptor than tamoxifen [4] and a much higher antiestrogenic activity in the estrogen-sensitive MCF_7 human mammary cancer cell line [5, 6]. Similar results were found for Ci 628, a 4-methoxylated triphenylethylene antiestrogen which was also metabolized *in vivo* to give the 4-hydroxylated derivative [7].

LN 1643, a triphenylbromoethylene (Table 1), which has an ethyl group as R_2, behaved as a classical antiestrogen in the rat uterus, showing both partial estrogen agonist and antagonist activity [8]. This compound markedly inhibited the induction and the growth of mammary tumors in rodents [9, 10], as does tamoxifen [11, 12]. However, it was also considered as an antiprolactin agent since it decreased serum and pituitary prolactin levels [9, 10].

To specify the mode of action of LN 1643 and the role of the substituent R_2 in antiestrogenic activity [6], we have attempted to define the compounds (LN 1643 and/or metabolites) that accumulate in the rat uterus after administration of [^{3}H]LN 1643. We have also compared the relative efficiencies of LN 1643 and metabolites for binding *in vitro* to the estrogen receptor and for inhibiting the growth of MCF_7 cells in culture.

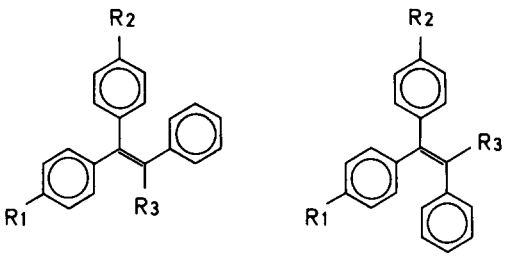
MATERIALS AND METHODS

Materials. [^{3}H]E_2 (sp. act. 50 Ci/mmole; radiochemical purity 97%) was purchased from CEA (Gif-sur-Yvette, France). [^{3}H]LN 1643 (sp. act. 1.3 Ci/mmole, radiochemical purity 95%) and unlabelled LN 1643, LN 2833 and LN 2839 were given by Dr. Dumery (Laroche–Navarron/Syntex Laboratories, Puteaux, France). Tamoxifen and 4-hydroxytamoxifen were given by Dr. Todd (ICI Laboratories, Macclesfield, U.K.).

Metabolism studies. [^{3}H]LN 1643 (0.9 mg/kg) was administered subcutaneously to immature female Wistar rats (18–20 days old) in 0.5 ml of 0.15 M NaCl solution containing 10% ethanol. Sixteen hours later, the radioactive compounds were extracted by ethyl acetate from plasma, liver homogenate and uterine subfractions (cytosol and KCl-nuclear extract) as described previously [3]. The extracts were dried under nitrogen, then dissolved in ethanol–ethyl acetate (50:50) containing unlabelled LN 1643, LN 2833 and LN 2839. The extracts were applied on Merck silica gel plates containing a 254 nm fluorescent indicator and were eluted with heptane–2-

* Abbreviations used: LN 1643, α-(4-ethyl) phenyl-α′-bromo *trans*-stilbene; LN 2833, α-(4-α-hydroxyethyl) phenyl-α′-bromo *trans*-stilbene; LN 2839, α-(4-ethyl) phenyl-4-hydroxy-α′-bromo *trans*-stilbene; E_2, estradiol; TE, Tris–HCl 10 mM, EDTA 1.5 mM, pH 7.4; DCC, dextran-coated charcoal, 0.5% charcoal Norit A, 0.05% dextran T 70 in TE; TLC, thin-layer chromatography.

Table 1. Structures and apparent affinities for the estrogen receptor of *trans* and *cis* triphenylethylene antiestrogens



trans cis

Compound	Isomer	R_1	R_2	R_3	Relative K_d
LN 1643	E, *trans*	H	C_2H_5	Br	2800
Clomiphene	E, *trans*	H	$O(CH_2)_2N(C_2H_5)_2$	Cl	70*
Tamoxifen	Z, *trans*	H	$O(CH_2)_2N(CH_3)_2$	C_2H_5	120†
LN 2299	Z, *cis*	H	C_2H_5	Br	
Clomiphene	Z, *cis*	H	$O(CH_2)_2N(C_2H_5)_2$	Cl	
Tamoxifen	E, *cis*	H	$O(CH_2)_2N(CH_3)_2$	C_2H_5	
LN 2839	E, *trans*	OH	C_2H_5	Br	19
4-Hydroxytamoxifen	Z, *trans*	OH	$O(CH_2)_2N(CH_3)_2$	C_2H_5	1.4†
LN 2833	E, *trans*	H	$CHOH-CH_3$	Br	2700

The values given for K_d are the relative dissociation constants for the estrogen receptor sites by taking K_d (E_2) = 1. These values were obtained, according to [24] and from competition experiments on calf uterine estrogen receptor (24 hr at 0°; except for clomiphene, 5 hr at 20°) as described in Fig. 2.

* Unpublished results.

† Calculated from Ref. 18.

butanone–ethanol (50:15:1). TLC fractions were counted for radioactivity.

Binding to the estrogen receptor. Cytosol (protein concentration 2 mg/ml) from calf uteri was prepared in TE buffer as described previously [13]. Aliquots of cytosol were incubated with [^{3}H]E_2 (final concentration 1 or 2 nM) alone or mixed with unlabelled E_2 (5–500 nM), 4-hydroxytamoxifen (5–500 nM), tamoxifen, LN 1643, LN 2833 and LN 2839 (50 nM to 5 μM) for 4 and 24 hr at 0°. Samples (0.3 ml) were then added with 0.3 ml of DCC suspension and the mixtures were agitated for 15 hr at 0°. Charcoal was then removed by a 5 min centrifugation and the radioactivity of the supernatants was measured.

Cell growth experiments. MCF_7 cells ($2.5–6 \times 10^4$) growing exponentially in Dulbecco's modified Eagle medium supplemented with 10% foetal calf serum were detached with trypsin (0.05%) and EDTA (0.02%). Cells were pelleted, then resuspended in growth medium and plated in triplicate in 35 mm plastic tissue culture wells, with medium containing 1% DCC-treated foetal calf serum. After cell attachment (~ 12–16 hr), the drugs were added to the medium in ethanol solution (final concentration 0.5%). The medium was changed every two days and cells were collected after detachment by trypsin–EDTA and neutralization with growth medium. They were passed through a 0.25 mm diameter gauge needle and counted in a model D Coulter counter (Coultronics, France) using the following settings: threshold 7, sensitivity 0.017, sample volume 0.5 ml. For each experiment, cell number was counted at least 3 times, and in triplicate wells. Results were checked by assaying total cellular DNA by ethidium bromide fluorescence [14] using a slight modification of the assay described by Karsten and Wollenberger [15].

Miscellaneous. Radioactivity was measured in a Kontron SL 30 scintillation spectrophotometer (Plaisir, France). Aliquots of sample (200 μl) and TLC fractions were counted in 3 ml ethanol and 10 ml PPO–POPOP–toluene scintillation mixture with 25–30% efficiency. Protein concentration was determined according to Layne [16].

RESULTS

Metabolism of LN 1643 in the rat

In order to specify which compounds were acting in uterus, [^{3}H]LN 1643 was injected to immature female rats and the radioactive compounds present in plasma and tissues were analysed 16 hr later, as described in Materials and Methods. The radioactive compounds extracted with ethyl acetate from plasma, liver homogenate and uterine subfractions represented, respectively, 15, 60 and 85% of the total radioactivity in these fractions. These compounds were analyzed by TLC (Fig. 1). In addition to the non-metabolized LN 1643 (TLC fraction No. 16), more polar metabolites were present in all samples. One of these radioactive metabolites co-migrated with LN 2839 (TLC fraction No. 8). It

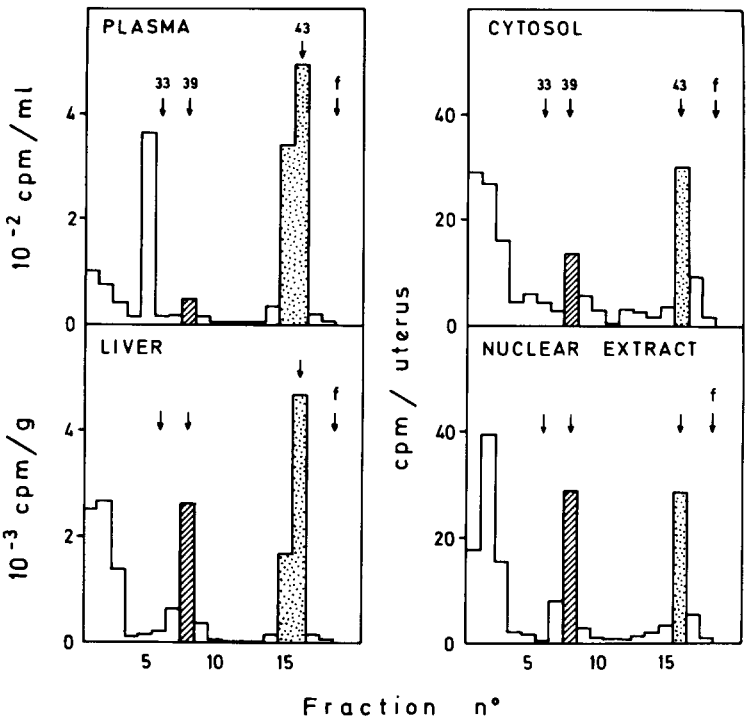


Fig. 1. Thin-layer chromatography of extracts from rat plasma, liver and uterus after injection of [^{3}H]-LN 1643. Groups of three immature (20-day-old) female rats were injected subcutaneously with 0.9 mg/kg [^{3}H]LN 1643 (1.3 Ci/mmole). Sixteen hours later, the animals were killed and tissue fractions prepared. The radioactivity extracted by ethyl acetate from plasma, liver homogenate and uterine cytosol and KCl-nuclear fraction was chromatographed with nonradioactive LN 1643, LN 2833 and LN 2839 on Merck F 254 silica gel plates with heptane–2-butanone–ethanol (50:15:1). After location of reference compounds under u.v. light, the plates were cut and counted for radioactivity. Fraction 1 corresponds to the deposit area, fractions 6, 8 and 16 correspond, respectively, to the migration of authentic LN 2833 (33), LN 2839 (39) and LN 1643 (43) and *f* indicates the front of migration of the eluant.

accounted for less than 3% of the radioactivity extracted from plasma, for about 10% of the uterine cytosol radioactivity and for about 20% of the radioactivity present in the liver homogenate and the KCl-extract of uterine nuclei.

More polar metabolites (TLC fraction Nos 1–3) were mainly present in liver (30%) and uterine subfractions (40%). The proportion of fraction 8 versus fraction 16 progressively increased from plasma to cytosol and to the KCl-nuclear fraction of the uterus, giving a final enrichment of over 10-fold. The comigration of fraction 8 with authentic unlabelled LN 2839 was also observed in another system of elution (heptane–ether, 70:30). However, the identity of this metabolite to LN 2839 was not demonstrated.

We conclude that LN 1643 was metabolized into several compounds. One metabolite, comigrating with LN 2839, was concentrated in the KCl-extract of uterine nuclei, suggesting a higher binding affinity for the estrogen receptor which is also located in this fraction after antiestrogen administration [13].

Relative binding affinity of LN 1643 *and derivatives for the calf uterine estrogen receptor*

We then studied the interaction of LN 1643 and derivatives with the uterine estrogen receptor. Attempts to demonstrate a direct binding of [^{3}H]LN 1643 to cytosol components were unsuccessful. We did not find any saturable and charcoal resistant binding in uterine cytosol, suggesting that the binding, if any, was of low affinity and rapidly dissociated from the receptor. However, non radioactive LN 1643 and derivatives markedly inhibited the saturable [^{3}H]E_2 binding. In competitive experiments (Fig. 2), LN 1643 and LN 2833 showed similar inhibition of [^{3}H]E_2 binding. This inhibition was much higher after 4 hr than after 24 hr incubation at 0° suggesting a rapid dissociation of the binding of these competitors as compared to the [^{3}H]E_2 binding [17]. LN 2839 was much more efficient than LN 1643. From these competition experiments, the apparent relative K_d of competitors were estimated and are presented in Table 1. The relative affinities of LN 1643 and LN 2839 are therefore approximately 10-fold lower than those found for tamoxifen and 4-hydroxytamoxifen in similar conditions [18]. However, for both types of antiestrogens, the hydroxylated metabolite had a $\simeq$100 fold higher affinity for the estrogen receptor than the parent compound.

Growth inhibition of MCF$_7$ human breast cancer cells in culture

The MCF$_7$ cells contain estrogen receptors, their

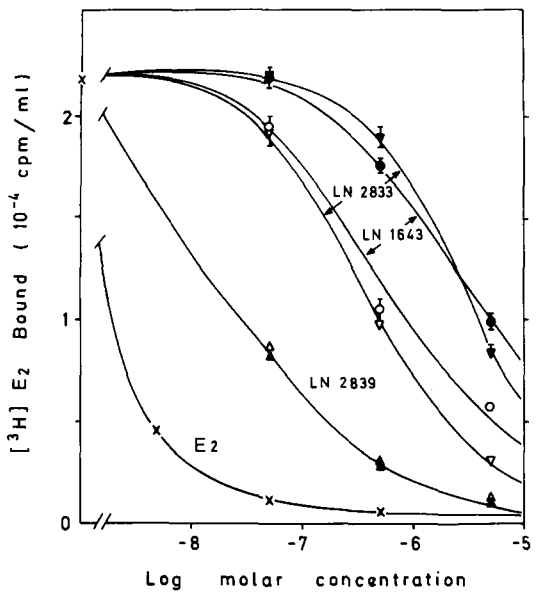


Fig. 2. Competitive binding of LN 1643 and derivatives on the estrogen receptor. Calf uterine cytosol (2 mg protein/ml) was incubated for 4 hr (×, ○, ▽, △) or 24 hr (×, ●, ▼, ▲) at 0° with 1 nM [^{3}H]E_2 and various concentrations of unlabelled E_2, LN 1643, LN 2833, LN 2839. The [^{3}H]E_2 binding, assayed after 15 hr of charcoal assay at 0°, is plotted against the concentration of the unlabelled competitor. E_2 (×); LN 1643 (○ ●); LN 2833 (▽ ▲); LN 2839 (△ ▲).

growth is specifically inhibited by the antiestrogen tamoxifen [19, 20] and some of its metabolites [6], this inhibition being relieved by adding estrogens to the culture medium [19]. Since these cells are unable to metabolize tamoxifen or its hydroxylated derivative [6], they provide an excellent *in vitro* system to determine the relative potencies of antiestrogens and metabolites at the target cell level even though we have not checked the metabolism of LN 1643 and LN 2839 in these cells. We have therefore evaluted the effect of LN 1643 and LN 2839 on the growth of MCF_7 cells in the absence of added estrogens as described previously [6]. Figure 3 shows that both LN 1643 and LN 2839 inhibited the growth of MCF_7 cells. The efficiency of LN 2839 was similar to that of tamoxifen and roughly 10-fold higher than of LN 1643. A similar increase in efficiency after hydroxylation was found for tamoxifen [6].

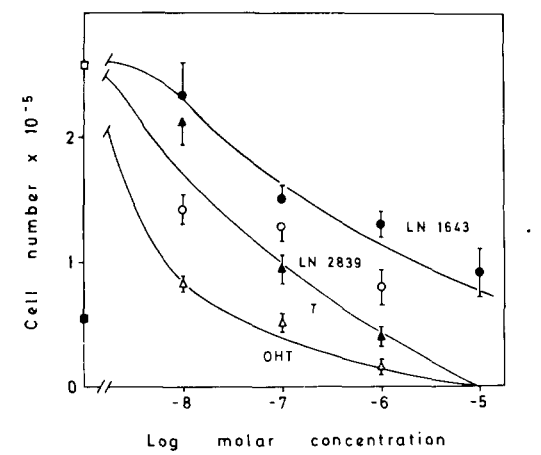


Fig. 3. Inhibition of MCF_7 growth by LN 1643, tamoxifen and their 4-hydroxylated derivatives. MCF_7 cells were plated in tissure culture wells with medium containing 1% DCC-treated foetal calf serum at day 0 (■). After cell attachment, various concentrations of LN 1643 (●), LN 2839 (○), tamoxifen (T, ▲) and 4-hydroxytamoxifen (OHT, △) were added to the medium. Control cells (□) were not treated with antiestrogen. The medium was changed every day. After 8 days of culture, the cells were collected and counted in a Coulter counter.

DISCUSSION

Our results suggest that the antiestrogenic activity of LN 1643 and its hydroxylated metabolite LN 2839 are mediated via their interaction with the estrogen receptor in estrogen target tissues. LN 1643 appears to behave as other classical antiestrogens, such as tamoxifen and Ci 628, as far as metabolism [3, 7] binding to the estrogen receptor [4, 7] and cell growth inhibition of estrogen responsive cells [6] are concerned. (1) Polar metabolites of LN 1643, including metabolite comigrating with LN 2839, are markedly concentrated in the uterine fractions enriched in estrogen receptor. (2) The affinity of LN 2839 for the estrogen receptor appears to be more than 100-fold higher than that of LN 1643. (3) To inhibit the growth of MCF_7 cells, LN 2839 is about 10-fold more efficient than LN 1643. Very similar results were observed for tamoxifen and 4-hydroxytamoxifen which are the respective homologues of LN 1643 and LN 2839. The present results and the recent identification of 4-hydroxytamoxifen as one of the major compounds mediating *in vivo* the antiestrogenic activity of tamoxifen strongly suggests that the *in vivo* antiestrogenic effects of LN 1643 result from the interaction of LN 2839 and other polar metabolites with the estrogen receptor. The affinity of the triphenylbromoethylene LN 1643 and derivatives for the specific antiestrogen binding sites recently described [21] has not been determined. For the triphenyl ethyl-ethylene and triphenylchloroethylene series, it appears that (a) compounds in which R_2 is constituted by the short and low polarity substituent $O–CH_3$, similar to the R_2 substituent $CH_2–CH_3$ of LN 1643 and LN 2839, have practically no affinity for these sites [22]. Therefore, we anticipate that the affinity of LN 1643 and LN 2839 for these specific antiestrogen binding sites would be much lower than that found for the estrogen receptor. (b) 4-Hydroxylation of a compound does not improve its affinity for these antiestrogen sites [23]. (c) the *cis* isomers, which are usually very weak estrogens and poor antiestrogens possess an affinity for these sites similar to that of their powerful antiestrogenic *trans* isomer [22, 23]. It appears, therefore, that the *in vitro* relative antiestrogenic activities of 4-hydroxylated *trans* derivatives, of the parent *trans* isomers and of the *cis* isomers are correlated with the affinity of these compounds for the estrogen receptor but not with their affinity for the specific antiestrogen binding site. Therefore, these antiestrogen binding sites do not seem to play an important role in the antiestrogenic activity. The nature of the

R_2 substituent appears important for the antiestrogenic activity, as tamoxifen or clomiphene and 4-hydroxytamoxifen which have a bulky lateral chain (R_2) display *in vitro* a higher affinity for the estrogen receptor [18, 24, 25] and a better efficiency to prevent the growth of the MCF_7 [6] than LN 1643 and LN 2839, respectively. *In vivo*, to inhibit the effect of estrogen on the uterine weight, tamoxifen [26] appears more efficient than LN 1643 [8]. It is difficult to compare the relative antitumoral activities of tamoxifen [11, 12] and LN 1643 [9, 10] from experiments performed by different laboratories. Both compounds efficiently prevented the growth of dimethylbenz[*a*]anthracene-induced rat mammary tumours. A decrease of prolactin levels has been described for LN 1643 [9, 10] but is more controversial for tamoxifen [27, 28]. Without eliminating the possibility that LN 1643 may also act by other mechanisms to prevent tumor growth, our results strongly suggest that its antitumoral activity is at least partly mediated by the estrogen receptor.

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