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Endocrine Effects of Trilostane: *in vitro* and *in vivo* Studies

E. TUENI,* N. DEVLEESCHOUWER,* G. LECLERCQ,* M. NIJS,* A. COUNE,* A. VERMEULEN† and R. PARIDAENS*

**Service de Médicine et Laboratoire d'Investigation Clinique H.J. Tagnon, Institut Jules Bordet, Centre des Tumeurs de l'Université Libre de Bruxelles, 1, rue Héger Bordet, 1000 Brussels, Belgium and †Department of Endocrinology, Academic Hospital, State University of Ghent, De Pintelaan, 185, B-9000 Ghent, Belgium*

Abstract—*Trilostane (4-α-5-epoxy-17β-hydroxy-3-oxo-5-α-androstan-2-carbonitrile) is a modified steroidal molecule.* In vitro *and* in vivo *studies in rats have shown that it inhibits adrenal, ovarian and placental steroid synthesis. It seems to act by exerting a selective blockade on 3β-hydroxysteroid dehydrogenase. In this study, we investigated whether this molecule interacts with hormone receptors for estrogen, androgen or progesterone. We also tried to demonstrate the effect which Trilostane may have on cellular cultures of human mammary carcinoma (MCF-7 Evsa-T). We also studied hormonal modifications in a series of 12 patients treated with different doses of Trilostane, since this drug is supposed to inhibit the production by the adrenal glands of mineralocorticoids, of glucocorticoids and of the precursors of estrogens. Our results indicate that Trilostane does not react with any of the main hormonal sex steroid receptors, nor does it interfere with cultures of human mammary cancer cells either containing estrogen receptors and therefore allegedly hormone-dependent (MCF-7 line), or estrogen receptor-negative cells, presumably independent of hormonal manipulations (Evsa-T cell line). Finally, endocrine studies on postmenopausal women with advanced breast cancer show that Trilostane significantly reduces the plasma levels of estrone and of its major androgen precursor (androstenedione). However, the latter inhibition is no different from that exerted by hydrocortisone acetate administered alone at a dose of 40 mg/day. The results of clinical trials comparing hydrocortisone alone with hyrocortisone plus Trilostane are awaited.*

INTRODUCTION

TRILOSTANE (4-α-5-epoxy-17β-hydroxy-3-oxo-5-α-androstan-2-carbonitrile) is a modified steroidal molecule, synthesized by Neumann [1], and recently introduced for the treatment of advanced breast cancer. Like amino-glutethimide, but acting a different sites, this drug can block the synthesis of steroids and is therefore of potential interest for the treatment of hormone-dependent mammary cancers in postmenopausal women. Its main action, as demonstrated on rat adrenals, is the selective inhibition of 3β-hydroxysteroid dehydrogenase, a key enzyme necessary for the production of glucocorticoids, mineralocorticoids and of androgens, the latter of which are the direct precursors of estrogens. Trilostane has also been shown to inhibit both *in vitro* and *in vivo* ovarian and placental steroid syntheses in various species, including man [2–5].

As with aminoglutethimide, most clinicians using Trilostane have also given their patients parallel substitution doses of corticosteroids (dexamethasone or hydrocortisone acetate), for the purpose of avoiding the effects of cortisol deficiency and to limit the rise of ACTH concentration which might by increasing formation of Δ-5 steroids overcome the competitive enzymc block [6]. However, as far as humans are concerned, the effects of Trilostane on adrenal steroid synthesis and hormone-sensitive neoplastic mammary cells have not yet been completely explored.

In this study we characterized the *in vitro* affinity of Trilostane (Tr) and its main metabolite, 17-keto-Trilostane (17-kt), with intra-cellular receptors for steroid hormones, the latter being supposed to regulate the growth of hormone-dependent cancers [7]. Using a new microassay, we then studied the effect of Trilostane on the cellular growth of two human breast cancer cell lines cultured in our laboratory. Of these, one is hormone-sensitive and contains significant amounts of estrogen receptors (MCF-7 line, ER-positve), while the other is hormone-independent and totally devoided of detectable

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estrogen receptors (Evsa-T, ER-negative). Using plasma concentrations of sex hormones and several of their precursors in 12 postmenopausal women suffering from advanced breast cancer, we finally compared the effects of various dosages of Trilostane plus hydrocortisone with those of hydrocortisone administered alone.

MATERIALS AND METHODS

a. *Drugs for the* in vitro *study*

Trilostane and its 17-keto derivative were kindly provided by Sterling Winthrop. 17β-Estradiol and ethinylestradiol were purchased from Sigma. Hydroxytamoxifen was a gift from ICI, Macclesfield, U.K. ^{3}H-Labeled and unlabeled estradiol, and ORG-2058 dihydrotestosterone were purchased from Amersham, cyproterone acetate, corticosterone and estracyt from Steraloid, and methyltrienolone (R 1881) from New England Nuclear.

b. *Measurement of the affinity of Trilostane and 17-keto-Trilostane with hormone receptors*

Affinity with estrogen receptors (ER). The binding of Trilostane and 17-kt to ER was assayed by means of a standard method, in which the competitive inhibitory effect of drugs under test on the binding of [^{3}H]estradiol to an immature rat uterine cytosol preparation could be measured [8]. Briefly, cytosol was incubated at 18°C for 30 min with 5×10^{-9}M[^{3}H]estradiol. This operation was carried out both in the absence and in the presence of Trilostane and 17-kt at concentrations ranging from 10^{-8} to 10^{-5} M. After the removal of free (unbound) steroids by adsorption on dextran-coated charcoal, the remaining radioactivity representing the amount of [^{3}H]estradiol bound to the receptors (after correction for aspecific binding) was measured in a scintillation counter.

Affinity to progesterone receptor (PR). In order to induce PR synthesis immature rats were injected subcutaneously on a daily basis over 3 consecutive days with 250 μl of a saline solution containing ethinylestradiol (EE_2) at a concentration of 4×10^{-5} M. On the 4th day, the animals were sacrificed, their uteri were removed and were pounded in order to obtain a cytosol preparation. The binding ability of Trilostane and 17 kt to PR was assessed in the same manner as for ER, except that the cytosol was incubated at 4°C overnight with 5×10^{-9} M [^{3}H]ORG 2058.

Affinity to androgen receptor (AR). ARS were measured in benign human hypertrophic prostatic tissue (BPH) by means of electrophoresis on agar gel as described by Hawkins *et al.* [9]. The affinities of Trilostane and 17-kt as well as those of various other control steroids (DHT etc) were estimated by means of a competitive assay in which the binding of [^{3}H]dihydrotestosterone ([^{3}H]DHT, 1.4×10^{-9} M) was measured in the presence of increasing amounts of the competitors at concentrations ranging from 1.4×10^{-9} to 1.4×10^{-6} M. The binding inhibition was compared to that recorded in the presence of non-radioactive DHT at a 1.4×10^{-4} M concentration. The relative binding affinity (RBA) of a given compound is expressed as the ratio of DHT concentration achieving a 50% inhibition, and the concentration of this compound producing the same inhibition × 100.

c. *Effect of the compounds on* in vitro *cell cultures*

Cell lines. The MCF-7 and the Evsa-T cell lines were kindly provided by Dr. M. Rich (Michigan Cancer Foundation, Detroit) and by Dr. M.E. Lippman (National Cancer Institute, Bethesda, MD) respectively. Both lines were maintained in our laboratory as monolayers in closed T-25 Falcon flasks. The culture medium consisted of Eagle's minimum essential medium (MEM) supplemented with the usual doses of L-glutamine, antibiotics, and a 10% heat-inactivated fetal calf serum (Gibco, Glasgow, U.K.) [10].

Doubling time (DT) of the cells. The cells were removed from the monolayer by trypsinization (trypsin 0.05% and EDTA 0.025%). They were then suspended in MEM with 10% dextran-coated charcoal (DCC)-treated fetal bovine serum (dextran 0.005%, charcoal 0.5% in 1.5 ml MEM/1 ml serum left overnight at 4°C). Three different cell concentrations were plated in a series of 96 multiwell dishes (200 μl per well). The cultures were performed at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. Culture was pursued for a 2-week period during which the medium was regularly changed at 2-day intervals. As far as the MCF-7 and Evsa-T were concerned, one dish was removed on a daily basis and cell counts were made by the following two methods: (a) automated counts on a cell suspension injected in a Coulter counter; (b) the direct hematoxylin coloration of the cells in the wells after fixation with ethanol. The intensity of the coloration was spectrophotometrically evaluated at 540 nm, as recently described by Madeddu *et al.* [11].

Experimental scheme for compound testing. For the purpose of carrying out compound testing 24 h after plating, the incubation medium was removed and replaced by a fresh medium containing the compounds under study. Two days later, the medium with the compounds was renewed and incubation

was pursued for a further 3 days. After these 5 days of incubtion in the presence of drugs, the cells were counted by using the colorimetric method described above. Using this experimental scheme, we analyzed the effect of an estrogen, of 17β-estradiol, and of an antiestrogen (4-OH-tamoxifen), to check the hormone-dependency of our MCF-7 cells. We then tested the potential effect of Trilostane on the growth of this line.

d. *Study of endocrinal effects of Trilostane on postmenopausal women*

Twelve postmenopausal women with advanced breast cancer were treated with increasing amounts of Trilostane, associated with a substitute dose of hydrocortisone acetate (HC) 40 mg/day (10 mg at 8 a.m. and 5 p.m., and 20 mg at bedtime). Half of the patients were hospitalized for one reason or another (concomitant radiotherapy, weakness etc.). This group of patients (group A) received Trilostane at a starting dose of 240 mg/day, with a 240 mg/day escalation every three days so that, after 9 days, they reached a maximum dose of 960 mg/day associated with 40 mg/day of HC. The six other patients (group B) were treated on an outpatient basis, and the Trilostane was increased at 14 day intervals so that they reached their maximum dose of Trilostane after only 42 days.

Three and 14 days, respectively, after the patients in groups A and B had attained the maximum Trilostane dose, the administration of the drug was interrupted for a few days, during which time the administration of HC was continued.

A minimum of 20 ml of blood was collected in two different heparinized tubes before the start of treatment, at the time of each increase in the dose of trilostane, and at the time the trilostane was interrupted (i.e. when the patients were only taking HC). Within 2 h of collection, the plasma was separated after centrifugation (10 min at 1000 ***g***) and stored at −20°C.

In order to avoid interassay variations, all the samples were processed together, using the same kits. Cortisol and dehydroepiandrosterone (DHEA), together with its sulfate (DHEA-S) were measured by radioimmunoassay of the unextracted serum. Following ether extraction serum pregnenolone (P_5) and its 17-hydroxy derivative (17-OH-P_5), progesterone (P), androstenedione (AN), estrone (E_1) and estradiol (E_2) were measured by radioimmunoassay using antibodies raised in the laboratory. All methods used involve chromatographic steps, eliminating interfering substances as described [12–15]. Sensitivity and C.V. (%), respectively, were 0.2 ng/ml and 7.1% for androstenedione; for progesterone 0.05 ng/ml and 5.9%; for E_1 0.006 ng/ml and 10.2%, for E_2 0.006 ng/ml and 9.3%, for P_5 0.1 ng/ml and 7.6%; for 17-OH-P_5 0.2 ng/ml and 10.7%.

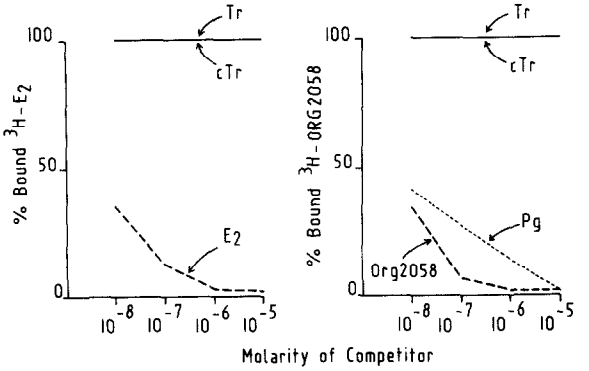

Fig. 1. The binding of tritiated estradiol ([^{3}H]E_2) to the estrogen receptor (ER) and of [^{3}H]ORG 2058 to the progesterone receptor (PR) are unaffected, even in the presence of high concentrations of Triolostane (T) or keto-Trilotane (17-kt). Unlabeled control steroids, i.e. estradiol (E_2) for ER and progesterone or cold ORG 2058, significantly compete for the binding to their own receptors.

RESULTS

a. *Affinity of Trilostane and 17-keto-Trilostane to hormone receptors*

Even at high concentrations (10^{-5} M), Trilostane and 17-keto-Trilostane did not produce any inhibition of the [^{3}H]estradiol binding to the ER of the immature rat uterine cytosol preparation. In the control test, unlabeled estradiol produced the expected reduction of [^{3}H]estradiol binding (Fig. 1). These compounds were also devoid of any inhibitory effect on the ORG-2058 binding to the uterine preparation taken from the estrogen-primed rats. In the control test, unlabeled ORG 2058 and progesterone produced the expected binding reduction (~ 75% at 10^{-8} M). This indicates that none of the test drugs has any binding affinity with the PgR.

Finally, Trilostane and 17-kt did not show any affinity with AR in any concentration (Fig. 2) since the pattern of the curve was unaffected, even in the presence of high concentrations of these drugs. By way of comparison, we show the inhibiting effect of the true comptitors in this system. Both cyproterone acetate (CA) with a RBA of 60, and estradiol (E_2) with a RBA of 25, lower the amount of [^{3}H]DHT linked to the receptor.

Corticosterone, which has a very low RBA (0.3), does not compete with [^{3}H]DHT except in very high concentrations.

b. *Calculation of the doubling time (DT) of the cell cultures, and the effect of Triolstane on the cell lines*

The new microassay used in these experiments makes good growth of the two cell lines (MCF-7 and Evsa-T) possible. We calculated the DT of the cells during their exponential growth phase.

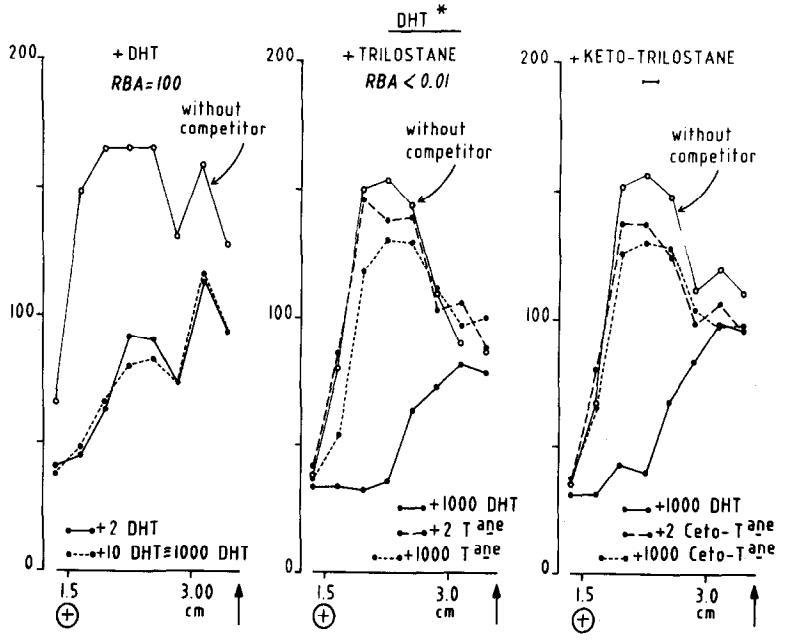

Fig. 2. The three panels show the binding of tritiated dihydrotestosterone ([^{3}H]DHT) to the androgen receptor (AR). The left-hand panel demonstrates that this binding is competitively inhibited by unlabelled dihydrotestosterone. No inhibitory effect of Trilostane (T) or keto-Trilostane (17 kt) could be observed (center and right-hand panels) on such a binding, even at very high concentrations.

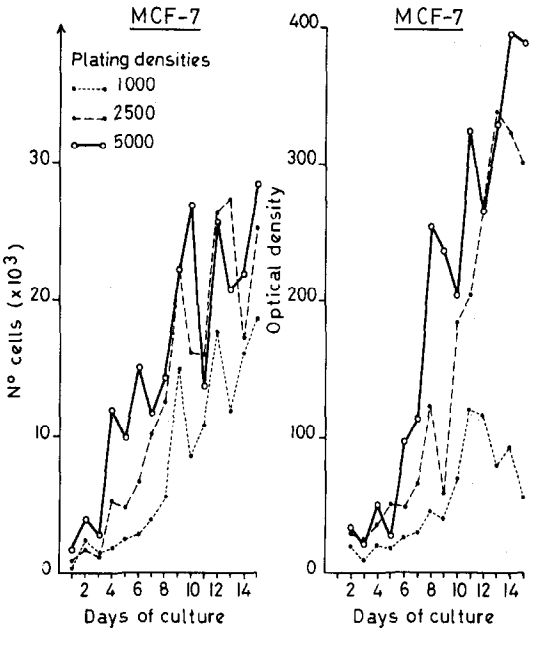

Fig. 3. (a) Left-hand panel: the growth of MCF-7 cells plated at low densities. The result is analyzed by Coulter counting. (b) Right-hand panel: a similar curve obtained by spectrophotometric counting.

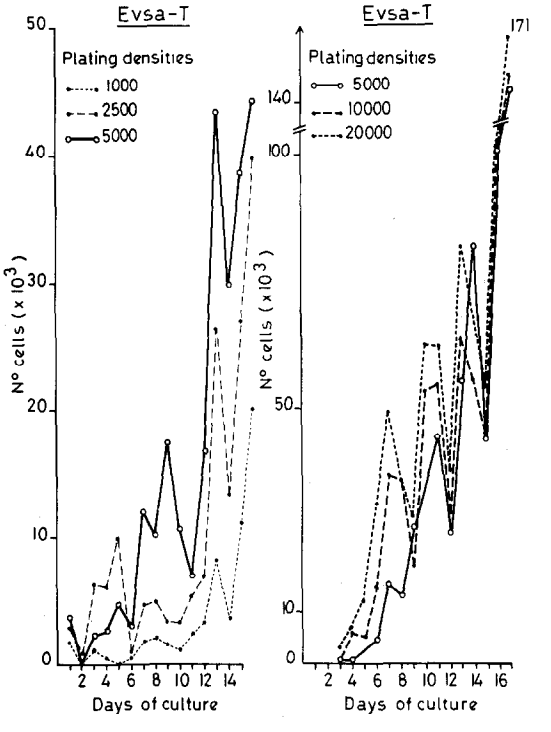

Fig. 4. (a) Left-hand panel: the growth of Evsa-T cells plated at low densities and counted with a Coulter counter. (b) Right-hand panel: the growth of Evsa-T cells at higher density plating.

With 1000–2500 and 5000 cells incubated per well at the plating time, the mean DT evaluated for MCF-7 cells was identical and ran to 65 h for both methods (colorimetric and direct counting, Fig. 3). For the Evsa-T cells, the mean DT evaluated by counting was 60 h at the lowest concentration of cells initially plated, i.e. 1000–2500 and 5000 cells per well, while at higher plating densities (5000, 10,000 and 20,000 cells/dish) the DT was 40 h (Fig. 4). It was also noticed that the adherence of the Evsa-T cells to the dishes was looser than that of the MCF-7 cells and some of the cells were probably detached and lost at each renewal of the medium. Moreover, in contrast to the MCF-7, and the spreading of the Evsa-T cells was bad and replicating cells easily formed clusters which hindered cell counting by colorimetry.

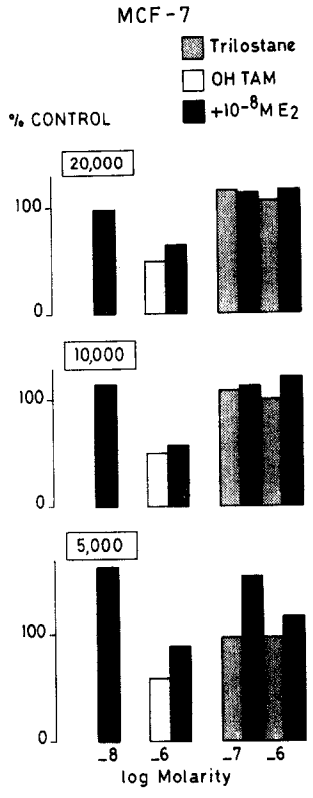

Fig. 5. Effect of hydroxytamoxifen (OH-TAM) and Trilostane (Tr) with or without 17β-estradiol (E_2) on MCF-7 cell growth as evaluated by spectrophotometric measurements. Each column corresponds to the mean value ± S.D. of four measurements.

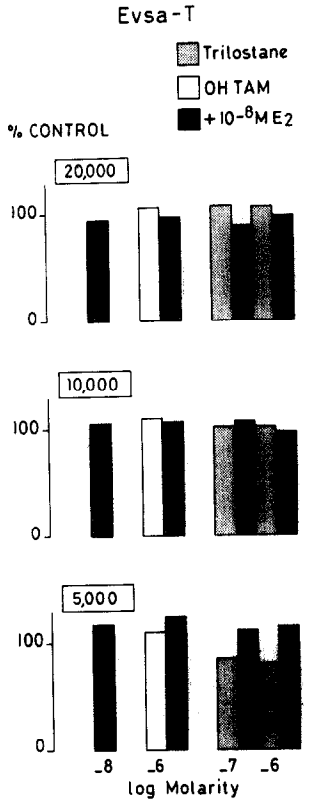

Fig. 6. Effect of hydroxytamoxifen (OH-TAM) and Trilostane (Tr) with or without 17β-estradiol (E_2) on the growth of Evsa-T cells evaluated by spectrophotometric measurements. Each column corresponds to the mean value ± S.D. of four measurements.

With regard to the growth experiments, we chose the best schedule providing exponential growth and good cell adhesion during drug exposure. On this schedule, the potent antiestrogen OH-tamoxifen at 10^{-6} M significantly slowed the growth ($P < 0.001$) of the MCF-7 cells at the two highest plating densities (10,000 and 20,000 cells per dish). This inhibitory effect of OH-tamoxifen was suppressed by estradiol at 10^{-8} M and this attested the hormone-dependency of the MCF-7 cells. At lower densities, this effect was weaker and was not statistically significant (Fig. 5). As expected, the antiestrogen was totally ineffective on the Evsa-T cells (Fig. 6). Trilostane at the two doses tested (i.e. 10^{-6} and 10^{-7} M) did not inhibit the growth of these cell lines. A slight (statistically significant ($P < 0.05$ one way variance analysis)) stimulatory effect at the highest plating density only on the MCF-7 cells (Fig. 5) was observed.

c. *Endocrine effects of hydrocortisone plus or minus Trilostane in postmenopausal women*

As shown in Table 1, various doses of Trilostane ranging from 240 to 960 mg/day were always administered in combination with HC (40 mg/day). During a brief period (3 days for group A and 14 days for group B), when Trilostane was interrupted, HC was administered alone. Under these conditions, the plasma concentrations of cortisol, of dehydroepiandrosterone (DHEA) and its sulfate (DHEAS), of estradiol (E_2) and of progesterone (P_4) did not undergo any significant modification in comparison with basal values. Trilostane and HC have a greater inhibitory effect on the synthesis of pregnenolone (P_5) and of its 17-hydroxy derivative (17-OH-P_5) than does HC administered alone ($P < 0.001$). Finally, both Trilostane plus HC, and HC alone, produce almost the same significant decrease in androstenedione (ADIONE) and estrone plasma levels (E_1).

DISCUSSION

The aim of this study is to obtain an insight into the way Trilostane acts upon breast cancer cells with reference to other well-known endocrine therapy modalities which function either directly at the cellular level by interaction with steroid receptors, or indirectly by modifying the hormonal environment. We investigated both possible modes of action of this compound by performing the following measurements:

1. The affinity of Trilostane and its derivative 17-kt with hormonal receptors.
2. The direct effect of Trilostane on the growth of two human breast cancer cell lines.

Table 1. *Endocrine effects of trilostane + hydrocortisone (12 postmenopausal patients)*

		BASAL	TRL240 HC40	TRL480 HC40	TRL720 HC40	TRL960 HC40	HC40
Cortisol	mean	13,823.0	10,816.7	12,172.7	11,033.3	9963.6	11,825.0
(ng/dl)	S.E.M.	1140.2	1882.1	2291.6	1818.7	1581.6	2019.3
	P (vs. BAS)		NS	NS	NS	NS	NS
DHEAS	mean	31,217.4	19,083.3	20,333.3	14,272.7	18,000.0	14,088.7
(nd/dl)	S.E.M.	5509.2	3491.0	5484.0	2709.7	5612.9	4422.4
	P (vs. BAS)		NS	NS	NS	NS	NS
DHEA	mean	104.5	80.5	68.0	90.3	95.2	73.4
(ng/dl)	S.E.M.	10.4	7.9	9.6	11.8	10.9	13.2
	P (vs. BAS)		NS	NS	NS	NS	NS
Adione	mean	70.5	32.8	29.7	37.4	32.3	37.4
(ng/dl)	S.E.M.	6.0	4.6	2.4	5.1	5.0	4.2
	P (vs. BAS)		***	***	**	***	**
E_1	mean	5.1	2.5	2.4	2.7	2.4	2.8
(ng/dl)	S.E.M.	0.6	0.3	0.2	0.3	0.3	0.3
	P (vs. BAS)		**	**	**	**	*
E_2	mean	0.5	0.3	0.5	0.3	0.4	0.4
(ng/dl)	S.E.M.	0.1	0.1	0.1	0.1	0.1	0.1
	P (vs. BAS)		NS	NS	NS	NS	NS
P_5	mean	55.9	19.6	11.0	18.0	15.0	27.3
(ng/dl)	S.E.M.	7.7	5.4	1.6	3.9	3.0	5.8
	P (vs. BAS)		**	***	**	**	NS
17-OH-P_5	mean	23.6	4.2	2.1	2.1	2.1	6.8
(ng/dl)	S.E.M.	2.5	1.7	0.6	0.9	0.9	1.8
	P (vs. BAS)		***	***	***	***	**
P_4	mean	0.4	0.3	0.3	0.1	0.2	0.3
(ng/dl)	S.E.M.	0.2	0.2	0.1	0.1	0.1	0.2
	P (vs. BAS)		NS	NS	NS	NS	NS

NS $P > 0.05$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Fischer variance analysis).

3. The effect of Trilostane on the plasma concentrations of sex steroids in postmenopausal women.

We showed that neither Trilostane nor 17-kt, its principal metabolite, display any binding affinity with intracellular major steroid hormone receptors (ER, PgR or AR).

After validating the new *in vitro* microassay, we tested Trilostane. These tests showed that Trilostane did not have any inhibitory effect on the growth of the ER-positive MCF-7 cells sensitive to hormonal maniplation, nor on the growth of Evsa-T cells lacking hormonal receptors and insensitive to such manipulation.

Finally, contrary to what has previously been found in rats treated with Trilostane, our study of circulating sex steroids in postmenopausal women with breast cancer shows that the association of Trilostane plus HC significantly diminishes the concentration of circulating pregnenolone, whereas Trilostane plus HC does not show any statistically significant effect on the circulating concentrations of cortisol, estradiol, progesterone, DHEA or its sulfate. This combination significantly lowers the levels of circulating estrone and of its androgenic precursor, i.e. androstenedione. These effects may explain the therapeutic action of Trilostane in some phase II preliminary studies [16–18]. However, this inhibition is not significantly different from that observed when HC is used alone.

This study is the first to show some evidence that the addition of HC to trilostane gives along way towards overcoming the blockade of 3β-hydroxysteroid deshydrogenase exerted by Trilostane alone [19], since the association of these two drugs decreases the blood concentrations of pregnolone and its 17-hydroxy derivative even better than HC alone. Our data do not concord with those of Beardwell *et al.* [20] who have recently shown that Trilostane administered alone increases the synthesis of 17-hydroxypregnenolone.

Although exerting significant endocrine effects when combined with corticoids, the exact action of Trilostane on hormone-dependent breast neoplasms remains to be clarified, especially in view of the data of Coombes *et al.* [21] who show that Trilostane alone is almost ineffective against

advanced breast cancer. In this respect, the Breast Group of the E.O.R.T.C. is conducting a randomized study which compares the clinical efficiency of Trilostane plus HC in opposition to hydrocortisone administered alone. The results of this study are eagerly awaited.

To sum up, we have demonstrated that in *in vitro* experiments, Trilostane does not bind to androgen, to estrogen or to progesterone receptors; that it does not inhibit the growth of MCF-7 cells; and that as far as its *in vivo* hormonal effects are concerned, the decrease in A and E_1 plasma levels observed during hydrocortisone treatment (40 mg/day) is not further accentuated by the addition of Trilostane.

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