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SHORT COMMUNICATIONS

Effect of estrogenic and antiestrogenic triphenylethylene derivatives on progesterone and estrogen receptors levels of MCF-7 cells

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Estrogenic and antiestrogenic activity of triphenylethylene (TPE) analogues and derivatives has been widely reported in the endocrinological literature [1]. Of special interest has been the discovery that some of these compounds, especially those bearing a dialkylaminoethoxy side-chain, were able to produce regression of breast cancers. To identify the chemical group(s) of these molecules responsible for this property, the antiproliferative effects of several compounds were systematically analysed. Cultures of MCF-7 breast cancer cells were often selected for such investigations. Unfortunately, the potential influence of the compounds on the levels of progesterone (PgR) and estrogen (ER) receptor was only marginally taken into account leading to incomplete structure–activity relationships. The induction of PgR being an excellent test of estrogenicity we decided to submit to this test a series of TPE derivatives of which the influence on growth had been already established. Most of these compounds were structurally related to the well-known antiestrogen, tamoxifen. Our study revealed that induction of PgR is almost always associated with a growth increase while the near absence of induction is usually a requirement for a growth decrease.

Materials and Methods

Compounds. [^{3}H]ORG-2058 (50 Ci/mmol), [^{3}H]-estradiol (100 Ci/mmol) and [^{3}H]hydroxy-tamoxifen (90 Ci/mmol) were purchased from Amersham International (Amersham, U.K.). Unlabeled estradiol was obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and triphenylethylene from Aldrich Benelux (Brussels, Belgium). Tamoxifen and hydroxytamoxifen (*trans* and *cis* isomers) were kindly provided by ICI (Macclesfield, U.K.). All hydroxytamoxifen analogues were synthetized at the Institute of Cancer Research, Sutton [2–5].

Cell culture materials. Earle's based minimal essential medium (MEM), fetal calf serum, L-glutamine and antibiotics (penicillin, streptomycin and gentamycin) were purchased from Gibco (Gent, Belgium); plastic flasks and dishes were from Falcon (Becton Dickinson).

MCF-7 cell growth conditions. MCF-7 cells maintained in monolayer culture at 37°, in T-75 flasks were removed by trypsinization (growth medium: MEM supplemented with L-glutamine, antibiotics and 10% heat-inactivated serum). Harvested cells were then cultured in serum depleted of unconjugated endogenous steroids (DCC treatment) for at least 3 days before assessing the effect of the TPE derivatives on PgR and ER levels. For that purpose cells were incubated for 3 days in the presence of increasing amounts of a given compound (range = 10^{-10} to 10^{-6} M); control cells were maintained in culture with or without estradiol (E_2) at 10^{-8} M to establish the optimal level in PgR induction/ER decrease ("processing") [6] (ratio of receptor levels respectively in absence or presence of E_2). Possible contaminating estrogen sulfates not removed by the DCC treatment of the serum [7] would not mask the effect of a given compound since we found they were unable to hamper the inhibitory effect of antiestrogens on cell growth under similar experimental conditions [8].

Receptors measurements. Control and hormone-treated cells were detached from the T-175 flasks with 1 mM EDTA in Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}. The cellular suspension was then centrifuged at 800 *g* and the pellet washed once with HBSS and twice with the homogenization buffer. Cytosol was obtained by homogenization of the final pellet in 10 mM phosphate buffer, pH 7.4, containing 1.5 mM EDTA and 1 mM monothioglycerol by means of a teflon–glass homogenizer and centrifugation at 130,000 *g* for 1 hr. The protein content of the cytosol ranged from 1 to 3.5 mg/mL. Nuclear extract assessed in a control experiment was obtained by 1-hr exposure of the ultracentrifugation pellet to a 500 mM KCl solution in the homogenization buffer at 4° followed by a second ultracentrifugation.

ER and PgR concentrations were measured in the cytosol fraction by Scatchard plot analysis according to the DCC procedure established by the EORTC Receptor Group [9] using, respectively, [^{3}H]E_2 and [^{3}H]ORG-2058 as labeled ligand.

In all experiments, variations of receptors concentrations (PgR induction/ER decrease) were expressed in percentage of the optimal variations produced by 10^{-8} M E_2.

Results and Discussion

Analysis of PgR induction patterns by E_2 and triphenylbutene (a simple TPE), revealed a 10,000-fold lower efficiency for the latter (Fig. 1). Efficiency of this molecule was increased 10–100 times by hydroxylation in position 4 of its phenyl ring, that is in the *cis*-vicinal relationship to the ethyl group (compound **A**); hydroxylation of its phenyl ring in the *trans*-relationship has also a stimulatory effect but with a largely lower efficiency (compound **B**). Assessment of non-isomerable analogues of these two phenolic isomers of TPE (hydroxybenzocycloheptenes; compounds **C** and **D**) led to a similar conclusion. Interestingly, hydroxylation of both phenyl rings did not further increase the efficiency as shown by the behavior of bisphenol of which the potency to induce PgR was between that of compounds **A** and **B**.

All these PgR induction patterns were associated with a corresponding decrease of ER concentration (processing) confirming the existence of a relationship between both phenomena [6, 10]. Interestingly, the extent of PgR induction occurred systematically at a slightly lower concentration of compound than the corresponding ER processing.

Grafting of a dimethylaminoethoxy side-chain to such phenolic TPE derivatives led to an important reduction of their ability to increase PgR synthesis (Fig. 2). This property was especially marked for the *trans*-isomers (according to the tamoxifen configuration) for which the induction potency was almost totally lost. *Cis*-isomers were still effective although they seem to be subjected to an autoinhibitory phenomenon at high concentrations (see 2′-Me, 4-OH tamoxifen). Remarkably, the inability of these compounds to significantly induce PgR was not associated with a loss in the capacity to decrease the ER concentration: the range of concentrations at which they regulate ER levels is similar to that of the TPE derivatives without side-

chain (i.e. compounds **A** and **B**). Hence, one may consider that they behaved as total (*trans*-isomers) or partial (*cis*-isomers) *defective agonists* without stimulatory properties (i.e. PgR synthesis) able to produce an antagonistic effect in reducing the sensitivity of the cells to a further estrogenic stimulus by decreasing their estrogen binding capacity.

As shown in Table 1, this reduction of sensitivity would be associated with a concomitant decrease of response to potent triphenylethylenic antagonists. Thus, in E_2 and OH-Tam treated cells, a parallel measurement of ER concentrations with [^{3}H]E_2 or [^{3}H]OH-Tam gave similar residual values; this property holds with the total receptor content of the cell (cytosolic and nuclear ER).

Table 2 shows that the compounds producing a significant

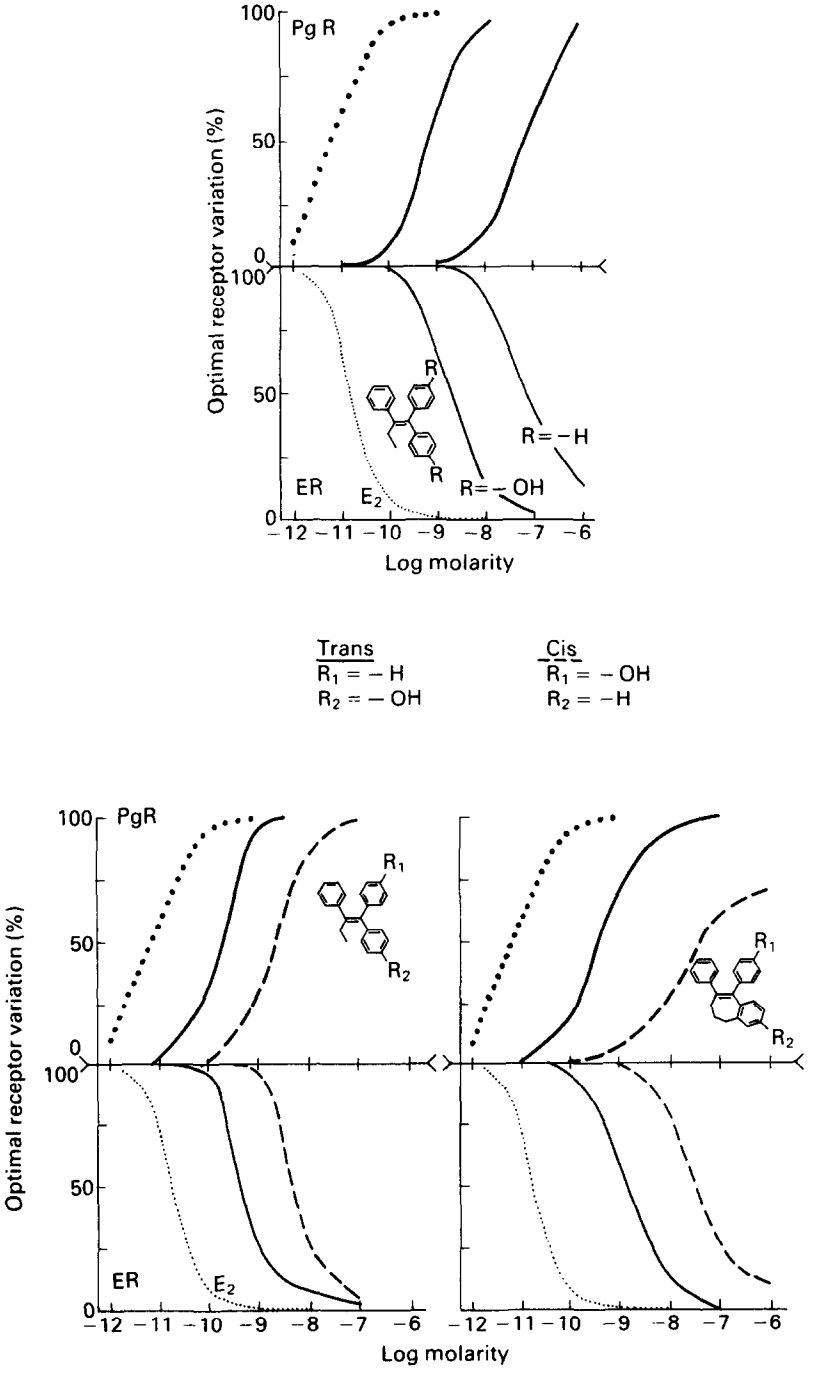

Fig. 1. Variation of ER and PgR concentrations of MCF-7 cells by triphenylethylene derivatives. Data were expressed in percentage of the optimal variations produced by E_2. Upper graph: effect of triphenylbutene and bisphenol; lower graph: effect of hydroxylated derivatives (compounds **A** and **B**, **C** and **D**). Curves were established from experimental data determined at the concentrations indicated on the log scale.

Trans	Cis
$R_1 = OCH_2CH_2NMe_2$	$R_1 = -OH$
$R_2 = -OH$	$R_2 = -OCH_2CH_2NMe_2$

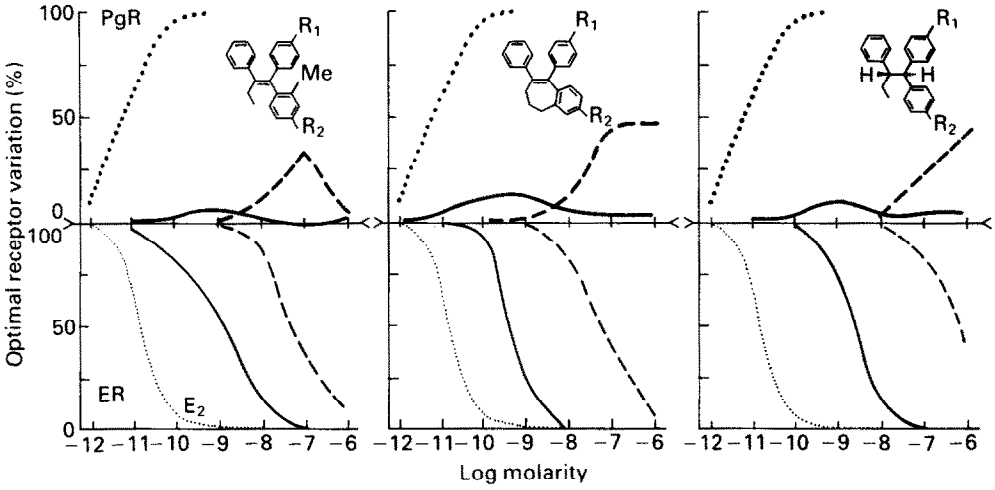

Fig. 2. Variation of ER and PgR concentrations of MCF-7 cells by non-isomerable analogues of *trans*- and *cis*-4-hydroxytamoxifen. Data were expressed as percentage of the optimal effect produced by E_2. Curves were established from the experimental data determined at the concentrations indicated on the log scale.

PgR induction almost always also increased the cell proliferation. Absence of induction as well as autoinhibition observed at a high concentration of *cis*-isomers were most often associated with a growth inhibition, suggesting a repressing activity of the potential agonistic activity of these isomers. This autoinhibition may be derived from the interaction of these compounds with targets other than ER involved in growth control and differentiation. TPE derivatives are, indeed, known to modulate various enzymatic activities playing such a role (i.e. prostaglandin synthetase, protein kinase. . ., [11–13]).

According to the present data, the assessment of PgR induction would be an effective test to evaluate the potential antitumor activity of TPE derivatives; the absence of significant PgR induction on a large range of concentrations of compounds offers indeed a valuable criteria for a primary selection. Identification of compounds with potential therapeutic activity could be subsequently performed by assessing their growth inhibitory potency in ER^+ and ER^- negative cell lines. The use of both positive and negative lines would help to discriminate between specific (ER-mediated) antiproliferative activity and systemic toxicity. On the other hand, the present data confirms that *all* TPE derivatives even those bearing an aminoethoxy side-chain were able to decrease the estrogen binding capacity of the cells [10]. The reason why the decrease produced by the latter derivatives is not associated with a significant stimulation of PgR induction or growth is an intriguing question. This property may result from the absence of blockade of ER mRNA synthesis by these compounds as usually found with estrogens; decrease of cellular estrogen binding capacity under antiestrogen treatment seems indeed to derive from a post-transcriptional regulation of the ER peptide leading to its accumulation in a non-binding form [14, 15]. In this regard, it should be stressed that the low, almost non-significant, induction of PgR measured here in the presence of OH-Tam corresponds to the level of PgR mRNA produced by this compound (~10% of E_2) [16] indicating that this receptor is not subject to such a post-transcriptional regulation. Phosphorylation of the newly

Table 1. Reduction by estradiol or hydroxytamoxifen of binding capacity of MCF-7 cells for [^{3}H]estradiol or [^{3}H]hydroxytamoxifen

	Binding capacity			
	[^{3}H]E_2		[^{3}H]OH Tam	
MCF-7 treatment	Cytosol	Nuclear extract	Cytosol	Nuclear extract
None	331*(100)†	270 (100)	328 (100)	224 (100)
10^{-11} M E_2	197 (59)	227 (84)	194 (59)	190 (85)
10^{-10} M E_2	57 (17)	92 (34)	100 (30)	83 (37)
10^{-7} M OH Tam	0 (0)	16 (6)	55 (17)	0 (0)

* fmol/mg protein.
† percentage.

Table 2. Relation between PgR induction and growth regulation of MCF-7 cells by triphenylethylene derivatives

	Structural features in 4-position		PgR induction/growth		
	Ring *cis* to alkyl on ethylene	Ring *trans* to alkyl on ethylene	10^{-8} M	10^{-7} M	10^{-6} M
1. Hydroxytriphenylethylene A	—OH	—H	+/+		
2. Hydroxytriphenylethylene B	—H	—OH	+/+	+/+	
3. Hydroxybenzocycloheptene C	—OH	—H	+/+	+/+	
4. Hydroxybenzocycloheptene D	—H	—OH	+/+	+/+	+/+
5. *trans*-4-Hydroxymethyltamoxifen	$—CH_2OH$	$—OCH_2CH_2NMe_2$	+/+	+/+	+/+
6. *cis*-Tamoxifen	$—OCH_2CH_2NMe_2$	—H	0/+	+/+	+/+
7. *cis*-4-Iodotamoxifen	$—OCH_2CH_2NMe_2$	—I	0/0	+/+	+/+
8. 1,1,2-Triphenyl-1-butene	—H	—H	0/0	+/0	+/+
9. *cis*-Hydroxybenzocycloheptene	$—OCH_2CH_2NMe_2$	—OH	0/+	+/0	+/−
10. *cis*-2-Methyl-4-hydroxytamoxifen	$—OCH_2CH_2NMe_2$	—OH	0/−	+/=	0/=
11. *cis*-4-Hydroxydihydrotamoxifen	$—OCH_2CH_2NMe_2$	—OH	0/+	+/+	+/0
12. *trans*-4-Thiotamoxifen	—SH	$—OCH_2CH_2NMe_2$	0/0	+/0	0/+
13. *trans*-Tamoxifen	—H	$—OCH_2CH_2NMe_2$	0/0	0/0	0/−
14. *trans*-4-Iodotamoxifen	—I	$—OCH_2CH_2NMe_2$	0/0	0/−	0/=
15. *trans*-4-Formyltamoxifen	—CHO	$—OCH_2CH_2NMe_2$	0/−	0/−	−/=
16. *cis*-4-Hydroxytamoxifen*	$—OCH_2CH_2NMe_2$	—OH	0/−	0/=	0/=
17. *trans*-2-Methyl-4-hydroxytamoxifen	—OH	$—OCH_2CH_2NMe_2$	0/−	0/=	0/=
18. *trans*-Hydroxybenzocycloheptene	—OH	$—OCH_2CH_2NMe_2$	0/−	0/=	0/=
19. *trans*-4-Hydroxydehydrotamoxifen	—OH	$—OCH_2CH_2NMe_2$	0/−	0/=	0/=
20. *trans*-4-Hydroxytamoxifen*	—OH	$—OCH_2CH_2NMe_2$	0/−	0/=	0/=

Growth data were taken from reported studies (Refs 2–5); + defines an increase, 0 no marked change, − a decrease, = a large decrease.

* Compound subjected to isomerism.

synthetized ER peptide has been reported to confer its estrogen binding capacity [17]. Known interference of tamoxifen and derivatives in the phosphorylation of peptides including ER [12, 13, 18] may, therefore, be at the origin of this accumulation of defective ER for which we suspect a competitive potency towards the mature (active) form of the receptor for binding to estrogen-responsive elements of the DNA or related transcription factors. In this respect it is noteworthy that ER phosphorylation has been shown to be calmodulin-dependent and thereby under the control of tamoxifen which is a strong calmodulin inhibitor [13, 18]. However, the potent hydroxylation derivatives are relatively weak calmodulin inhibitors [18] suggesting that while this inhibition may contribute, there is another mechanism also. Studies are now being undertaken to investigate the interaction of the binding and non-binding forms of ER with calmodulin [19] with the hope to elucidate the questions raised.

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Lauric acid hydroxylase activity and cytochrome P450 IV family proteins in human liver microsomes

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Cytochrome P450 enzymes are important in the metabolism of many endogenous substrates, as well as a large variety of drugs, chemical carcinogens and environmental pollutants. Ten families of cytochrome P450 enzymes have been identified so far in mammals [1]. Six of these are small families of one or two members and are involved in pathways of steroidogenesis or bile acid synthesis in highly specialized tissues [2]. One family, cytochrome P450 IV, consists of enzymes that are involved in hydroxylating fatty acids [2]. The remaining three families encode xenobiotic metabolizing enzymes. In these latter four families, it appears that a tremendous variability exists between species in the number of P450 genes and the substrate specificities of individual P450 forms [2]. It is therefore difficult to extrapolate toxicology and carcinogenicity studies from rodents to man. These species differences emphasize the necessity to study human cytochrome P450 enzymes.

One of the most active cytochrome P450 IV fatty acid-metabolizing enzymes is P450 IVA1, formerly termed cytochrome P452 [3–5]. The ω-hydroxylase activity towards lauric acid is suggested to be an indicator of cytochrome P450 IV activity [2, 3, 5]. After treatment of rats with hypolipidaemic, peroxisome proliferating compounds like clofibrate, nafenopin or di(2-ethylhexyl)phthalate (DEHP) both the ω-hydroxylase activity towards lauric acid, and cytochrome P450 IVA1 protein content are substantially induced in liver and kidney, whereas the (ω-1)-hydroxylase activity is much less induced [5–8].

Since the hypolipidaemic, peroxisome proliferating compounds are classified as epigenetic hepatocarcinogens [9, 10], attention has been focussed to elucidate the possible mechanism(s) of carcinogenesis. It has been suggested that the carcinogenicity might result from an increased hydrogen peroxide production generated via the peroxisomal fatty acid β-oxidation system in the liver [10]. Sharma *et al.* [6] proposed that the enhanced peroxisomal β-oxidation is a result of the perturbation of lipid metabolism, which in turn might be the result of an enhanced ω-hydroxylation of fatty acids by cytochrome P450 IVA1.

Species differences in the induction of peroxisome proliferation have been reported. Non-rodent species are reported to be less sensitive to peroxisome proliferators than rodents [11–13]. Since peroxisome proliferation is causally linked to hepatocarcinogenicity there is considerable debate about the significance of peroxisome proliferators as hepatocarcinogens in humans. More information regarding the cause of the interspecies differences of peroxisome proliferation is needed.

It seems possible that species differences in peroxisome proliferation are the result of differences in the presence or inducibility of cytochrome P450 IVA1 [11–13]. In this paper we have studied the presence of cytochrome P450 IV family enzymes in hepatic microsomes derived from human samples, as a preliminary step in determining the potential human risk posed by peroxisome proliferators.

Materials and Methods

Chemicals and reagents. Nafenopin was a gift of Ciba Geigy (Basle, Switzerland). Lauric acid, ω-hydroxylauric acid and NADPH were obtained from the Sigma Chemical