Pergamon

Biochemical Pharmacology, Vol. 47, No. 11, pp. 1927–1937, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)E0032-G

COMMENTARY

SPECULATION ON THE MECHANISM OF ACTION OF TRIPHENYLETHYLENE ANTIOESTROGENS

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Key words: tamoxifen; antioestrogen binding site (AEBS); hormonal regulation; coronary heart disease; bone disorders; prolactin

In the past decades, the triphenylethylene compound tamoxifen has been a major subject of investigation because of its breast-cancer-suppressing qualities. It is currently the antihormonal treatment of choice in all stages of breast cancer, and is also favourable because of its low incidence of side-effects. Recently, new trials have been started on its potential use as a tumour-preventing agent in women at higher risk for breast cancer [1, 2].

Therapy with tamoxifen increases the disease-free survival of patients [3]; its main mechanism of action is thought to be through competition with oestradiol for the oestrogen receptor (ER§), thereby preventing the growth-stimulating action of oestradiol [1]. The benefit of tamoxifen treatment, however, is not confined solely to patients with ER-positive tumours; 10–15% of ER-negative breast cancer patients also respond to tamoxifen therapy [4, 5].

The focus of the investigations has shifted in recent years to other characteristics of triphenylethylenic compounds: tamoxifen-treated women were found to have a reduced risk of coronary heart disease, and oestrogen-like effects on bone were found [6, 7]. Evidence has accumulated that tamoxifen can have ER-independent effects and also oestrogen agonistic activities. The major problem in the elucidation of the mechanisms of action of triphenylethylenes is that the biological response to antioestrogens differs, depending on animal species, organ or individual gene response studied [8]. Some of the effects can be reversed by oestradiol, some can be partially reversed, and others cannot be reversed at all.

A useful tool in the elucidation of oestradiol/tamoxifen-regulated mechanisms is the availability of pure antioestrogens. This new class of ER-directed antioestrogens probably either prevents the dimerization of the receptor, which is crucial in the induction of ER-mediated transcription, or causes an inactivation of the transcriptional activation function of the DNA-bound receptor complex [9, 10]. If the addition of this pure antioestrogen prevents an effect of either oestradiol or tamoxifen, one can be certain that this effect was indeed ER mediated.

In this commentary we will compare studies on *in vivo* and *in vitro* effects of tamoxifen and related triphenylethylenic compounds and on effects of endogenous or exogenous oestradiol in order to identify mechanisms by which these compounds could act.

Effects in breast cancer patients

Metabolism of tamoxifen. Steady-state levels of tamoxifen and some of its metabolites during long-term therapy of postmenopausal patients are given in Table 1 [1, 11]. Tamoxifen has a long plasma half-life of 7 days at steady state. 4-Hydroxytamoxifen has been proposed as the metabolite representing antioestrogenic activity; it has a 20–30 times higher affinity for the ER than tamoxifen. Tamoxifen, however, is available in at least a 25 times higher concentration than 4-hydroxytamoxifen. Unfortunately, serum levels are unrelated to response rates; 99% of the drug is present in peripheral compartments, suggesting extensive tissue binding. Tissue levels are 10–60 times higher than serum levels. The relative quantities of metabolites in serum and tissue, including malignant tumours, are roughly comparable (Table 1); tamoxifen and 4-hydroxytamoxifen are present in a ratio of approximately 100:1 [12].

Hormones and growth factors. Oestrogens are important in the growth stimulation of breast cancer *in vivo*; they have direct effects on cell proliferation and influence the secretion of growth factors at distant sites [13].

In ten postmenopausal women with ER-positive/

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§ Abbreviations: ER, oestrogen receptor; PR, progesterone receptor; HRT, hormone replacement therapy; LH, luteinizing hormone; FSH, follicle-stimulating hormone; GH, growth hormone; GHRH, GH-releasing hormone; IGF-I, insulin-like growth factor I; tHRT, transdermal HRT; CVD, cardiovascular disease; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; Lp(a), lipoprotein (a); AT-III, antithrombin III; PKC, protein kinase C; DMBA, dimethylbenzanthracene; HBD, hormone binding domain; TAF, transcription activation functions; and AEBS, antioestrogen binding sites.

Table 1. Serum and tissue levels of tamoxifen and its metabolites [1, 11]

Metabolite	Serum			Relative tissue level
	Range (ng/mL)	Mean (ng/mL)	Mean (nM)	
Tamoxifen	8–574	148	400	1
N-Desmethyl	35–709	290	800	1.2
4-Hydroxy	1–5	3	15	0.01
Metabolite Y	10–16	13	35	
N-Didesmethyl				0.2
4-OH-*N*-desmethyl				0.05

PR-positive breast cancer, receiving tamoxifen treatment for 8 days, mean ER and PR (progesterone receptor) levels in fine needle aspiration biopsies were increased significantly by 201 and 163%, respectively. On an individual basis, however, two women had lower ER levels and four had lower PR levels. The fact that tamoxifen up-regulates the ER in oestrogen-deprived MCF7 cells shows that the up-regulation is mediated not only by blocking the oestradiol-induced down-regulation of ER but also by other means [14, 15].

The large rise in circulating oestradiol (and also in cortisol and testosterone) that occurs in tamoxifen-treated premenopausal women is thought to be a result of direct stimulation of ovarian activity by tamoxifen without intermediary gonadotropin stimulation. There is no feedback by an ER-induced factor to the hypothalamus or gonadotropes [16].

In postmenopausal women, hormone replacement therapy (HRT) and tamoxifen produce very similar decreases of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels [17]. Circulating oestradiol and progesterone levels remain low during tamoxifen treatment [18, 19]. Tamoxifen may be acting as an oestrogen agonist on the hypothalamus-pituitary axis in older women.

The importance in breast cancer of prolactin, which plays a prominent role in normal breast differentiation and in rodent breast cancer, is still uncertain [13]. Prolactin levels are found to decrease or be unaffected by tamoxifen; the response of prolactin secretion to thyrotropin-releasing hormone or oestrogens is attenuated. Normally oestradiol potentiates the secretion of prolactin; it has been suggested that tamoxifen causes a blockade of ER on hypothalamic pituitary levels with regard to prolactin release [18, 20].

Growth hormone (GH) release from the pituitary is controlled by hypothalamic GH-releasing hormone (GHRH) and somatostatin, and is depressed by insulin-like growth factor I (IGF-I). GH stimulates the secretion of IGF-I by the liver [21]. In adults, there is a strong positive correlation between oestradiol levels and GH secretion. Oral HRT causes reduced circulating IGF-I levels and increased basal and stimulated GH levels [22, 23], possibly as a result of the lower IGF-I level. Transdermal HRT (tHRT), however, inhibited stimulation of GH secretion by GHRH with unchanged IGF-I levels in one study [24], while increased circulating IGF-I levels with unchanged GH were found in another [25]. Circulating IGF-I levels are reduced in tamoxifen-treated women; this may be the result of a suppression of the amplitude of GH secretion caused by increased somatostatin, as was found in rats [26], or be caused by decreased basal and GHRH-induced GH release, as was found in sheep [27], but pituitary-independent actions of tamoxifen are also suggested [17, 28–30]. Somatostatin analogues inhibit mammary tumour growth in animal models [31]; a rise in endogenous somatostatin may contribute to the tumour-preventing potency of tamoxifen. The lack of knowledge of the precise effects of oestradiol on the GH–IGF axis and of the biological role and regulation of binding proteins for these hormones prevents a definitive interpretation of the effects of tamoxifen on GH and IGF-I [21, 32].

Lipids. After menopause, ovarian failure or oophorectomy, the incidence of cardiovascular disease (CVD) increases; levels of low density lipoprotein cholesterol (LDL-C) and total cholesterol increase, but no significant change or a small decrease in high density lipoprotein cholesterol (HDL-C) is found [33, 34].

Both HRT and tamoxifen treatment protect against CVD. The changes in various CVD risk factors during HRT and their role in the beneficial effect of HRT on CVD have been discussed by Nabulsi *et al.* [35] and Lobo [34]. Changes in the lipid profile are shown in Table 2 [6, 18, 33–41].

Levels of LDL-C decrease with long-term tamoxifen therapy and also with oestradiol treatment in men and women [42, 43]. Oestrogens decrease LDL-C by increasing LDL-receptors and -liver uptake and degradative metabolism [34]; GH and IGF-I increase macrophage uptake and degradation [44]. These actions normally play a protective role in the removal of LDL from blood, but macrophage uptake can turn into a CVD risk-increasing factor when very high circulating levels of LDL are present, causing conversion of macrophages into foam cells. Tamoxifen lowers GH/IGF-I; this can decrease the macrophage uptake of LDL and thereby prevent foam cell formation in patients with very high circulating levels of LDL. This does not explain the lowering of LDL-C with tamoxifen treatment, but perhaps the reduced macrophage LDL degradation

Table 2. Comparison of the effects of oestrogenic hormone replacement therapy (HRT) and tamoxifen treatment with that of natural menopause on the serum lipid profile in women

	HRT		Tamoxifen		Effects of post-menopause
	E	E + P	Premenopause	Postmenopause	
HDL-C	↑ abcd*	↑ ade	↑ f/− ↓ g	−ghi/↑ fg/↓ NSj	small ↓/−j
apoA$_1$	↑ abd	↑ ad		↑ j	
LDL-C	↓ abc	↓ ae	↓ fg	↓ fgijk	↑ k
apoB	↓ ab	↓ a		↓ j	
Lp(a)	↓ a	↓ ae			
Total triglycerides	↑ abc	− ac	−f	↓ NSk/−f/↑ i	
Total cholesterol	↓ b	↓ e	−fg/↓ g	↓ ghij/↑ NSk/−f	↑ j

* References are denoted by letters as follows: a = 35; b = 34; c = 39; d = 40; e = 38; f = 37; g = 18; h = 6; i = 41; j = 33; and k = 36.

can explain why tamoxifen treatment results in a smaller reduction of LDL-C than HRT does.

Tamoxifen treatment has no clear effect on HDL-C, total triglycerides or total cholesterol (Table 2). The pattern of changes differs between tamoxifen and oestradiol; oestradiol gives significant HDL increases. According to Love *et al.* [33], tamoxifen does not change the HDL-C concentration but causes a shift in the HDL_2/HDL_3 ratio. Bruning *et al.* [37], however, found significant increases of HDL-C in all breast cancer patients treated with tamoxifen. In responders to tamoxifen therapy, the change in HDL was already significant after 2 months of treatment.

The predominant effects of oral oestrogens on lipoproteins are mediated via a first passage hepatic effect. Non-oral oestrogens also increase HDL-C and/or decrease LDL-C, but to a smaller extent, and do not affect hepatic parameters such as globulins [34].

Gylling *et al.* [45] describe the inhibition of cholesterol biosynthesis by tamoxifen as a cause for reduced serum cholesterol levels. The data are suggestive of an inhibition of Δ^8-isomerase [45]. Since an extensive analysis was done in only one woman and key sterol metabolites were measured in four others, further research is needed.

High lipoprotein(a) (Lp(a)) levels represent a high risk for atherosclerotic diseases. The decrease in Lp(a) levels with HRT is thought to be very important in reducing the risk of CVD; unfortunately, no data are available on the effect of tamoxifen treatment on Lp(a) [46, 47].

It has been suggested recently that the benefit of tamoxifen in CVD is a result of its anti-oxidant properties. However, the IC_{50} values of tamoxifen and 4-hydroxytamoxifen for the *in vitro* inhibition of lipid peroxidation were, respectively, 75 and 200 times higher than the average concentrations found in serum of women treated with tamoxifen (Table 1) [48]. Therefore, we have to wait for *in vivo* evidence to accept that this effect is of biological importance.

Changes in plasma proteins. Tamoxifen appears to produce oestrogen-like effects, such as an increase in sex hormone- (SHBG), cortisol- (CBG) and thyroxine-binding globulins (TBG), in all women [17, 18, 37, 40, 49, 50]. HRT causes a decrease in antithrombin III (AT-III) concentrations and fibrinogen levels in plasma; no significant differences or changes after 2 years of tamoxifen treatment were found in some studies, but others described lower levels [18, 35, 41, 43, 49, 51].

The parallel effects of HRT and tamoxifen on lipid profiles in serum and on liver-related synthesis of proteins, such as AT-III and hormone-binding globulins, shows that tamoxifen has oestrogen-like effects on liver. It remains to be investigated whether these effects are mediated by agonistic effects through liver ERs or by changes in other messenger pathways.

Bone. Oral HRT with oestrogens is associated with decreased bone resorption. No change or a slight decrease in bone formation is observed. The efficacy of oestrogens in maintaining bone mass and in decreasing the incidence of osteoporotic fractures has been firmly established [52–54], but the mechanism of action is still unknown [32]. ERs have been demonstrated in cartilage of long bones and vertebrae in fetuses of 10 weeks or older [55]. Tamoxifen is suggested to behave as an oestrogen agonist on bone metabolism [7, 50, 56, 57]; possibly, tamoxifen acts directly on bone cells through an ER-mediated mechanism. Other options could be that the influence of tamoxifen on bone is related to the changes in the GH/IGF-I axis or in other growth factors, such as TGF-β. GH secretory responses decrease with increasing tHRT doses. This makes it unlikely that the known effects of tHRT on bone are mediated through increases in basal levels of circulating GH and IGF-I, but does not preclude the possibility of tHRT-induced increases in the biological activity or paracrine action of IGF-I [22, 24].

Animals

In rodents, triphenylethylene antioestrogens have both oestradiol-agonistic and -antagonistic activities on uterus and bone [17, 58–60]. Pure antioestrogens (ICI 164.384, ICI 182.780) prevent binding of oestradiol to the ER and ER-mediated effects on gene transcription. In intact female rats, tamoxifen reduced uterine weight, growth rate of the animals, and serum LH levels. ICI 164.384 also reduced

uterine weight but had no effect on growth rate and serum LH. Ovariectomy decreases uterine weight and increases growth rate and serum LH concentrations. Tamoxifen can prevent the decrease in uterine weight in ovariectomized rats and causes a decrease in overall growth rate. Oestradiol treatment of ovariectomized rats produces a dose-dependent increase in uterine weight and decrease in growth rate.

The absence of an increase in growth rate and serum LH in ICI 164.384-treated rats is surprising. The authors suggest that the doses used were too low to block pituitary/hypothalamic ER or that ICI 164.384 is unable to reach the hypothalamus/pituitary region [61]. Unfortunately, there is no information available on the effect of this pure antioestrogen on other hypothalamic/pituitary-related factors, and we are unable to conclude if this implies that the effects on LH and growth rate are secondary or directly mediated through pituitary ER.

Oestradiol alters rat pituitary functions by significantly increasing both soluble and particulate protein kinase C (PKC) levels in all pituitary cell types; the oestradiol-induced changes in pituitary function include selective effects on PKC activity involved at different levels in receptor-coupling mechanisms, causing selective changes in the sensitivity of the pituitary and shifts in pituitary output [62].

Mammary tumour growth induced by dimethylbenzanthracene (DMBA) is reduced by tamoxifen and ICI 164.384. In rats carrying DMBA-induced mammary tumours, tamoxifen causes a reduction in circulating oestradiol and prolactin [17]. In breast cancer patients, however, increases in circulating oestradiol are found. Therefore, special care should be taken when effects in rodent tumour models are compared with *in vivo* effects in patients.

An unexplained element in the mode of action of antioestrogens is the effect of tamoxifen on MCF7-tumour growth in oestrogen-withdrawn ovariectomized athymic mice. After prolonged tamoxifen treatment, the tumour growth is stimulated by tamoxifen. Withdrawal of tamoxifen stops tumour growth. These tumours have high ER levels and measurable PR levels that are not stimulated by tamoxifen. Oestradiol also stimulates tumour growth, but the pure antioestrogen ICI 164.384 inhibits both oestradiol- and tamoxifen-stimulated growth [1, 63].

The absence/presence of circulating oestradiol could play a role in the maintenance of the ER in a specific state in different tissues in intact or ovariectomized animals; the effect of oestradiol is tissue dependent and causes the "shaping" of its own receptor, possibly through selective phosphorylation processes.

Oestrogen receptor models

The ER belongs to a superfamily of transcription factors that include receptors for steroids, thyroid hormones and retinoids. Binding of oestradiol to the ER results in the alteration of the expression of target genes. Antagonists can interfere in several ways with the ERs in their mechanism of action.

Heat shock proteins [64]. In the absence of hormones, steroid receptors are noncovalently associated through their hormone binding domain (HBD) with a heterocomplex of heat shock proteins (hsp 56, hsp 70 and a dimer of hsp 90), and are unable to bind to DNA [65]. A heterocomplex of these three heat shock proteins exists in the cytosol independent of steroid receptors and is thought to have a function in protein folding and cellular transport. Binding of the specific ligands causes release of this complex and transforms the receptor into a DNA-binding form. An androgen antagonist, for example, was found that was unable to induce the dissociation of the receptor from the heat shock protein complex [66, 67]. ER are predominantly located in the nucleus, however, even in the absence of oestradiol, and it is not certain whether heat shock proteins have a biological role in ER-(in)activation in intact cells [68].

Dimerization. Hormone binding causes receptor dimerization, necessary for high-affinity binding to DNA. A number of the amino acid residues, essential for oestrogen binding, are also involved in receptor dimerization, suggesting that the hormone binding pocket is at or near the dimer interface [68]. Parker *et al.* found that pure antioestrogens increase receptor turnover in comparison with oestradiol and proposed that this was caused by inhibition of receptor dimerization and of nuclear uptake resulting in enhanced cytoplasmic degradation.

Transcriptional activation functions. A working mechanism for the differences in antagonistic/agonistic "antioestrogen" action at the ER at different genes/tissues has been described, introducing tissue and target gene-dependent regulation of expression through gene and promoter specific transcription activation functions (TAF) on the receptor [10, 68, 69]. The ER contains TAF1 located in the N-terminal region of the receptor and TAF2 in the HBD, which is also ligand specific. Binding of the ligand–receptor complex to an oestrogen response element activates gene expression through either TAF1 or TAF2 or the two of them working together; this depends on cell type and target gene. Type 1 antagonists of oestradiol action can only inhibit that portion of the activation of gene expression that is induced by TAF2. If gene expression is partly or completely induced by TAF1, the binding of the "antagonist" to the receptor can induce the same effects as oestradiol [68].

Phosphorylation. The ER has been shown to become phosphorylated upon oestradiol binding. IGF-I (20 ng/mL) and agents that raise cAMP levels can stimulate ER phosphorylation and ER-mediated gene transcription in transfection systems in the absence of ligand [70, 71]. This might be consistent with a role for phosphorylation in receptor activation, but the fact that binding of pure and partial antagonists of oestradiol to ER can also induce phosphorylation without activating transcription raises questions on the role of this process. Dopamine (100 μM) also appears to give rise to ligand-independent activation of the ER, which can be inhibited by pure antioestrogens; the involvement of phosphorylation in this action has not been investigated [71]. Glucocorticoid antagonists can become (promoter dependent) agonists by the

addition of protein kinase A activators [72]. Information on the specific sites of ER phosphorylation *in vivo* is not available [73].

Perhaps the phosphorylation state determines which gene is a target gene for the ER. It is unclear whether a nonphosphorylated ER can alter transcription processes, but the *in vitro* synthesized human ER binds oestradiol with high affinity and low efficiency and is converted into a high-efficiency binding state by tyrosine phosphorylation in the HBD [74], suggesting that at least this phosphorylation is necessary for ligand activation of the receptor.

In vitro *effects of tamoxifen and derivatives*

According to *in vitro* information, tamoxifen is suggested to have effects not mediated by ER. The *in vivo* value of these in tamoxifen-treated breast cancer patients remains, for the most part, putative [75]. A problem is the fact that, at least *in vitro*, tamoxifen can bind to or influence (purified) proteins and enzymes besides the ER. Tamoxifen was also found to inhibit the proliferation of ER-negative cell lines [76, 77]. The most extensively described effects of triphenylethylenic compounds are the inhibition of PKC and Ca^{2+}/calmodulin-dependent enzymes, and binding to antioestrogen binding sites (AEBS).

Effects in cell culture. Generally, antioestrogens inhibit oestradiol-stimulated processes such as the increase in plasminogen activator activity, the induction of the PR and increase in TGFα mRNA, and the production of several proteins and growth factors. The decrease in TGFβ caused by oestrogens is reversed by tamoxifen [13, 78].

The affinity of different antioestrogens for the ER correlates well with their potency in inhibiting breast tumour cell growth and the sensitivity of cell lines to growth suppression by tamoxifen correlates well with their ER content [78, 79]. Transfection of the ER into hormone-independent cells, however, results in cells that are growth-inhibited by oestradiol; pure antioestrogens reverse this inhibition [3]. Tamoxifen (50 nM) can inhibit ^{3}H-thymidine incorporation and cell proliferation of human malignant glioma cell lines that are completely insensitive to oestradiol and contain no ERs [80]. Some effects of tamoxifen can roughly be divided into oestrogen-reversible at up to micromolar tamoxifen concentrations and oestrogen-irreversible at higher concentrations. For instance, the accumulation in the G_0/G_1 phase of the cell cycle of MCF7 breast cancer cells can only be completely reversed by oestradiol at tamoxifen concentrations up to 5 μM [81].

The inhibition of oestradiol-stimulated prolactin synthesis in rat pituitary cells by antioestrogens is a measure of antioestrogenic potency. The dimethyl amino ethoxy side chain of triphenylethylenic compounds appeared important for activity. 4-Hydroxytamoxifen has high antioestrogenic potency; deletion of the side chain turns it into a full oestrogen agonist. Substitution for an allyl side chain or substitution with bulky aryl groups produces partial agonistic activity, but with the introduction of oxygen the antioestrogenic activity returns [17, 82].

ERs have a different conformation depending on the ligand bound [17]. Some ER-antibodies do not affect binding of 4-hydroxytamoxifen or oestradiol to ER, but decrease the association rate of tamoxifen to the ER. Another antibody inhibits oestradiol binding but not 4-hydroxytamoxifen binding. Other evidence suggests that antioestrogens may also interact with a region on the receptor that is distinct from the oestradiol-binding site [83].

In ER-positive breast cancer cells, 4-hydroxytamoxifen can block the activities of EGF and IGF-I in the absence of oestrogens. This action can be reversed by the addition of oestradiol. MCF7 cells treated for 5 days with 50 nM 4-hydroxytamoxifen had a decreased IGF-receptor content and an increased EGF-receptor content. The EGF receptors, however, appeared to have lost their "second-messenger" autophosphorylation ability. The result was that the mitogenic activity of both EGF and IGF-I was reduced [84]. Retinoic acid has the same effect as tamoxifen, indicating that this anti-GF effect is not restricted to ER ligands.

In breast cancer cell cultures, a monoclonal antibody to the IGF-I receptor causes growth inhibition that can be reversed by IGF-I. This inhibition is not seen in serum-free medium, but the antibodies block the stimulatory effects of exogenous IGF-I and IGF-II. The antibodies fail to block oestrogenic stimulation of cell growth, suggesting that IGF secretion into medium is not a primary mediator of oestradiol-stimulated growth. After prolonged prestimulation with oestradiol, the stimulation of cell growth by oestradiol and IGF-I was higher than additive; this was explained by the possibility of IGF-I secretion into the culture medium [85, 86]. It is not clear from these experiments, however, if the effects could also result from intracellular up-regulation of the IGF-I receptor second messenger pathway.

Enzymes. The growth inhibitory potency of triphenylethylenes may result from potent inhibition of PKC. *N*-Didesmethyltamoxifen and *N*-desmethyltamoxifen were most potent, but their IC_{50} values were far greater than 100 μM [87]. Tamoxifen also blocks voltage-dependent K^+ channels of neuroblastoma cells [88], $(Ca^{2+} + Mg^{2+})$-ATPase and Na^+/Ca^{2+} exchange of brain cortex membranes [89], and calmodulin-dependent cAMP phosphodiesterase [90, 91]. The inhibition of this last enzyme, in particular, is thought to be relevant since antioestrogenic effects on quail oviduct have been attributed to the regulation of cAMP levels. The inhibitory potency against cAMP-PDE increases with lipophilicity [91] and is highest for *N*-desmethyltamoxifen [92].

Many inhibitors of PKC are not specific, are lipophilic drugs binding non-specifically to the hydrophobic regulatory site of the enzyme, and are also Ca^{2+}/calmodulin antagonists. The most potent PKC inhibitor, taurosporine, can also inhibit protein tyrosine kinase and cAMP-dependent protein kinases. Drugs of this type are thought to be directed against cell membrane-associated signalling pathways [87, 93, 94].

The oestrogen-irreversible actions of tamoxifen at high concentrations might be explained by this "chemotherapeutic" activity on the cell membrane.

In vivo, the direct effects of tamoxifen on these enzymes remain questionable because of the high concentrations needed to achieve them. Changes in membrane fluidity caused by these agents are probably corrected by treated patients themselves. *In vivo* experiments using *Escherichia coli* have shown that the ratio of saturated to unsaturated fatty acids of phospholipids in the membranes decreases with decreasing temperature [95]. In the serum of the cockerel, tamoxifen was indeed found to cause a decrease in the ratio of C16/C18 fatty acids to C20/C22 polyunsaturated fatty acids in phosphatidylcholine species [96]. More research on the effect of tamoxifen treatment on fatty acid composition of membrane lipids and on membrane fluidity is needed.

AEBS. Triphenylethylene antioestrogens can also bind with high affinity and specificity to the AEBS, a site that cannot bind oestrogens [97]. The presence of AEBS in breast tumour tissue is not related to the presence of ER [78]. The biological functions of the AEBS and its (putative) endogenous ligand are still unknown. A wide range of functions [discussed in Ref. 98] has been proposed, but thus far no conclusive evidence on its nature (or that of the ligand) has been obtained. In our experiments, ligands for muscarinic and histaminic receptors could not inhibit binding of tamoxifen to the AEBS [99, 100].

Rat liver AEBS levels were found to increase with age, with increasing ambient temperature, and with fasting. Refeeding decreases the level [101]. The order of tissue distribution levels in tamoxifen-treated patients [12] (liver > pancreas > fat > brain > muscle > serum) parallels to a small extent the concentrations of AEBS in rats found in our laboratory: liver > brain > fat. Rat muscle and serum contained no measurable AEBS; pancreas showed high non-specific binding.

The binding of tamoxifen to AEBS is inhibited *in vitro* by unsaturated fatty acids by a non-competitive mechanism probably involving the lipid environment of the binding site [102]. Inhibition was also found using cholesterol metabolites. Relative binding affinity of 7-ketocholesterol, lathosterol and 7-ketocholestanol were, respectively, 0.5, 0.2 and 12% compared with tamoxifen [103, 104]. This last (oxy)-sterol could inhibit the proliferation of cells in culture. Polyoxygenated sterols inhibit the biosynthesis of cholesterol efficiently by depressing the activity of HMG-CoA-reductase; the inhibitory potency increases with increasing distance between C3 and the second oxygen [105]. Oxysterols were suggested as endogenous ligands for AEBS, regulating cell growth and cholesterol biosynthesis. In these experiments, however, ER-positive breast cancer cells were used, and in establishing a correlation between affinity for AEBS and antiproliferative capacity not only oxysterols were included but also some potent antioestrogens that could have blocked cell growth through ER [106]. Furthermore, oxysterols are negatively charged compounds at physiological pH, and the dialkyl amino side chain of triphenylethylenes, which is very important in binding to AEBS, is positively charged; this precludes binding to the same site on AEBS [100].

The affinity for the AEBS in other experiments did not parallel the inhibitory potency for ^{3}H-thymidine incorporation, and a very high concentration (10^{-4} M) of the best AEBS-ligand was needed to inhibit cholesterol biosynthesis [107]. Oestradiol stimulates cholesterol biosynthesis through an ER-mediated pathway (after 8 hr of incubation); 4-hydroxytamoxifen inhibits within 15 min, also in ER-negative cell lines. This is suggested to be mediated by the AEBS [108].

The major flaw in most of the proposed roles for the AEBS is the fact that the effective doses of tamoxifen do not match its affinity for the AEBS. One exception, described by Biswas and Vonderhaar, is the inhibitory effect of triphenylethylene antioestrogens on binding of lactogenic hormones to microsomes from the rat Nb2 lymphoma cell line that lacks ER [109] and from mouse mammary gland [110]. Growth stimulation of Nb2 cells by lactogenic hormones is effectively inhibited by triphenylethylenes at 10^{-10} M; the effects parallel the affinity for the AEBS, indicating a possible involvement in this antilactogenic effect [111].

Hypothesis

Prolactin has a role in many different biological activities: stimulation of cell growth, modulation of the immune system, and control of reproduction by means of lactotropic and antigonadotropic effects [112]. Shand and West [113] found that the initial stage in the stimulation of milk secretion involves a decrease in the activity of acyl-CoA: cholesterol acyltransferase and that the phosphorylation level of HMG-CoA-reductase is modulated by both prolactin and GH acting in opposition.

Prolactin is thought to influence macromolecular synthesis at the cell nucleus or Golgi complex after receptor-mediated endocytosis. Prolactin induces specific enzyme markers expressed early during the G_1-phase of the cell cycle. Prolactin is thought to exert its effects through the activation of PKC, and administration results in a rapid activation of hepatic PKC with a characteristic elevation of activity in the particulate fraction. Rat prolactin stimulates, at picomolar concentrations, PKC activity in purified rat liver nuclei. This activation can be inhibited by PKC inhibitors, by antiserum to prolactin and by monoclonal antibodies to the rat liver prolactin receptor [114]. Some authors have reported translocation of PKC activity to the nucleus after prolactin treatment; others found translocation of prolactin itself from medium to nucleus. Early events after binding of prolactin to its receptor include activation of Na^+/H^+ exchange and (within 20–100 sec) of Ca^{2+} uptake [115].

The fact that most of the effects of prolactin parallel many effects that have been reported to be inhibited by nanomolar concentrations of triphenylethylenes indicates that tamoxifen may exert its effects by interfering via AEBS with the prolactin second messenger pathway. No clear second messengers have been identified for GH and prolactin [115]. The activation of PKC and its concomitant translocation to the plasma membrane are essential steps in mitogenic signalling by a variety of growth factors. Most growth factors induce

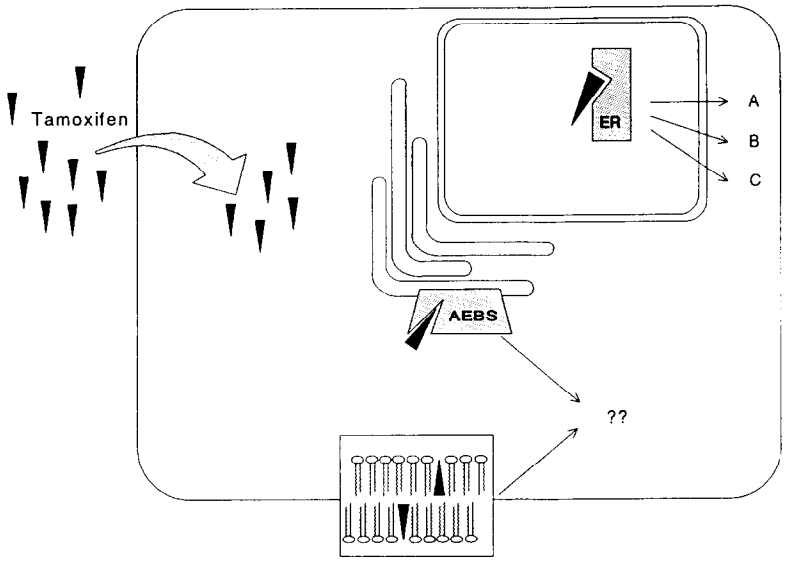

Fig. 1. Possible sites of action of tamoxifen. At the ER, tamoxifen can (A) block the stimulative effect of oestradiol, (B) have a stimulative effect where oestradiol causes a blockade (for example at the production of TGF-β), and (C) have oestrogen-like effects; it is not certain, however, that these effects are directly mediated by ER. The effects of tamoxifen resulting from its interaction with the AEBS and its possible presence in phospholipid bilayers are still being investigated.

phosphorylation processes inside cells but remain themselves outside. Receptors for prolactin have been reported in a number of subcellular organelles and may influence phosphorylation rates in the organelles where they are residing or by a subcellular redistribution of phosphorylation activities. AEBS may be an intracellular protein that can influence the activities of the microsomal prolactin receptor (or possibly of other growth factors, too) by interfering with the up-regulation/down-regulation of their second messenger pathways. AEBS or prolactin receptors may have a role in mediating the cross-talk between receptors [116, 117].

Unexplained

Figure 1 shows three different modes of action of triphenylethylenic compounds, i.e. through ER in the nucleus, through AEBS in the endoplasmatic reticulum, and through the membranes. An *in vivo* effect of binding to AEBS is still a matter of debate, since its contribution to growth inhibition is not proven and its role in resistance to therapy has been proposed [118, 119]. The presence of tamoxifen in cellular membranes as a cause for high peripheral levels and its effect on membrane fluidity and enzymes need further examination.

With the present ER models, some differences in the effects of antioestrogens can be explained, but not all. Both tamoxifen and oestradiol stimulate transplanted MCF7 cancer tumour growth after prolonged tamoxifen treatment in the absence of oestradiol. ICI 164.384 inhibits these effects. The presence/absence of oestrogens may shape the receptor and other proteins in the transcription complex to achieve (tissue-dependent) antagonistic or agonistic responses. Modifications of the ER complex can cause shifts from antagonist to agonist, and vice versa, of the same ligand. ER transfection into non-oestradiol target cells results in oestradiol inhibition of cell growth. Part of the ER-shaping machinery is missing or inactive in these cells. It could be interesting to investigate the effects of pretreatment with oestradiol.

There also may be differences in antagonistic and agonistic effects between postmenopausal and premenopausal tamoxifen-treated patients. It remains to be investigated whether these effects are dependent on age or on pituitary cell type. There may be need for a further division into hypothalamic-gonadatropic/somatotropic/lactotropic tissue axes.

In premenopausal women tamoxifen increases ovarian oestradiol production; the mechanism of action is unknown. Considering the amount of oestrogen-like effects that have been found in tamoxifen-treated patients, it remains to be concluded whether the name antioestrogen is still appropriate.

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