Mechanism of Action of Estrogens and Xenoestrogens in a Human Breast Cancer Cell Model (MDA-MB 231)

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Presented by

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Doctoral Thesis Presented by

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Abstract

A mechanism by which the steroid hormone estrogen can exert its actions has been known for many years. This mechanism comprises binding of estrogen as a ligand to an intracellular receptor protein and association of this active complex with a specific DNA sequence in the genome, the estrogen response element (ERE). However, recent studies could show that this classical mechanism of estrogen action is unable to explain the majority of its effects. The investigation of regulation of gene expression on a genome-wide scale by micro-array hybridization led to the identification of a large number of genes, whose transcription is controlled by estrogen. However, in the promoter regions controlling the expression of these genes, estrogen response elements, known to be necessary for the classical mechanism of estrogen action, cannot be detected in most of the cases. These experiments led to the conclusion that transcriptional regulation of genes by estrogen uses a variety of different mechanisms.

The transcriptional regulation of the gene for the neuropeptide hormone oxytocin has been a central interest of research at the Institute of Hormone and Fertility Research for many years. Studies of the regulatory regions of the bovine gene led to the identification of a transcription factor binding site in the proximal oxytocin gene promoter specifically interacting with nuclear orphan receptors. In breast cancer cells a strong estrogen-dependent transcriptional up-regulation of the oxytocin gene promoter was observed, although it could be clearly demonstrated that ligand-activated estrogen receptors do not bind to this promoter. From these experiments it can be concluded that the transcriptional up-regulation of the oxytocin gene promoter by estrogens is not controlled by the classical mechanism of estrogen action. The transcription of this promoter is controlled by estrogens without direct binding of the ligand-activated estrogen receptor. The study presented here now aims at the elucidation of this non-classical mechanism controlling the oxytocin gene promoter by a detailed analysis of the components of the regulatory system.

For the experiments described in this study the breast cancer cell line MDA-MB 231 was used as a model system. Transient transfection experiments using indicator plasmid constructs containing the oxytocin gene promoter or an ERE-controlled promoter as well as expression plasmids for estrogen receptors were performed and the transcriptional effect of estrogenic compounds measured under a variety of experimental conditions. In addition to classical

estrogen agonists and antagonists, mixed agonists/antagonists (selective estrogen receptor modulators) and environmental chemicals with estrogenic properties (phytoestrogens, industrial xenoestrogens and metal ions) were investigated.

The selective estrogen receptor modulators 4OH-tamoxifen, raloxifen and the pure antagonist $ICI_{182,780}$ all showed clear agonistic effects on the oxytocin promoter, in sharp contrast to the established antagonistic effects on the ERE-controlled promoter. This finding demonstrates that transcriptional activation of the two promoters by estrogen receptor α is not achieved by the same mechanism of action. The subtype-selective activation of estrogen receptor α and β by the phytoestrogen naringenin and its substituted forms 8-prenyl-naringenin and 6-(1, 1-dimethylallyl) naringenin, could be confirmed on the ERE-controlled promoter as well as on the oxytocin gene promoter. In contrast to the activation of the ERE-controlled promoter, transcriptional up-regulation of the oxytocin gene promoter by the substituted naringenins was not dependent on binding of the activated estrogen receptor to the promoter, as shown by use of a mutant estrogen receptor α unable to bind to DNA. The phytoestrogens resveratrol and genistein showed agonistic effects on both promoters, whereas biochanin A did not show any effect. Using a panel of established xenoestrogens (2-hydroxy-biphenyl, methoxychlor, β-hexachlorhexane, chlordane, toxaphene, 1,1,1-trichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)ethane (o,p'-DDT) and 2-2-bis(4/chlorophenyl)-1-1-dichloroethyl (p,p'-DDE) no transcriptional activities of the two promoters could be detected in the MDA-MB 231 cell assay system. Two independent cell toxicity assays (Neutral Red Test and Plating Test) ruled out the possibility that this lack of activation is caused by cell-type-specific toxicity of these compounds. Furthermore, the established agonistic activity of o,p'-DDT in MCF-7 cells could be confirmed in control experiments. In addition to the studies of phytoestrogen and xenoestrogen effects, metal ions, which had been shown to exert estrogenic effects in the MCF-7 proliferation assay, were tested in the MDA-MB 231 assay system. Whereas most of the ions did not effect significant transcriptional activation, promoter-specific activation by some ions could be demonstrated, with copper and cadmium ions activating specifically the oxytocin gene promoter, and cobalt ions the ERE-controlled promoter.

The results of the study presented here demonstrate that many of the estrogenic activities of drugs used in hormone or anti-hormone therapy as well as the estrogenic effects of environmental compounds cannot be explained by the classical mechanism of estrogen action. Non-classical mechanisms, like the one described here, appear to control the estrogen-dependent expression of a large number of genes. Therefore the further elucidation of these mechanisms will be of great importance for the understanding of the role of estrogen in normal human development and clinically relevant diseases. Assay systems measuring the effects of estrogenic compounds mediated by non-classical mechanisms should be included into screening procedures for environmental estrogens or during drug development, in order to cover the whole diversity of mechanisms of estrogen action.

INTRODUCTION

1. Hormones in General

The production of hormones and their secretion is controlled by endocrine glands, e.g. the pituitary, the pineal gland, or the gonads. These tissues and the produced hormones form complex endocrine networks (Griffin, 2000; Henderson, 2005). The term HORMONE is derived from the Greek verb "horman" meaning "to excite" and hormones comprise a variety of biologically active molecules (Griffin, 2000). Hormones can be defined as: signalling molecules produced by specific endocrine glands of the body, dispersed into the bloodstream, and acting at specific sites in the body (Baulieu et al., 1990). The target cell possesses specific hormone receptors enabling the hormones to perform their function inside the target cell (Fig 1). In the case of membrane receptors, the activated receptors transport the extracellular hormone-intrinsic signal into the intracellular compartment and into the nucleus, where the expression of genes can be up-or -down regulated, leading to specific changes in the functions or differentiation status of the target tissues (Rozenzweig et al., 1999). As a whole, hormones can be classified by their chemical structure into four major classes (Henderson, 2005):

The first kind are protein hormones composed of amino acids, such as follicle-stimulating hormone (FSH), luteinizing hormone (LH), gonadotropin releasing hormone (GnRH), or insulin. The second types are called amine hormones, which are derived from a single amino acid such as thyroxin, epinephrine (adrenaline), nor-epinephrine (noradrenalin), and melatonin. The third class, the lipid and phospholipid hormones are derived from lipids such as linoleic acid and phospholipids such as arachidonic acid. The main class of these are the eicosanoids, which include the widely studied prostaglandins. The fourth class are the steroid hormones, composed of four interconnected rings of carbon atoms. The different types of steroid hormones are different in the number and kind of atoms attached to the basic ring structure. Important steroid hormones are the adrenal steroid hormones, glucocorticoids (e.g. cortisol) and mineralocorticoids (e.g. aldosterone), and the gonadal steroid hormones such as progestins (e.g. progesterone), androgens (e.g. testosterone) and estrogens (e.g. estradiol) (Henderson, 2005; Henriksen, 2005).

2. Steroid Hormones

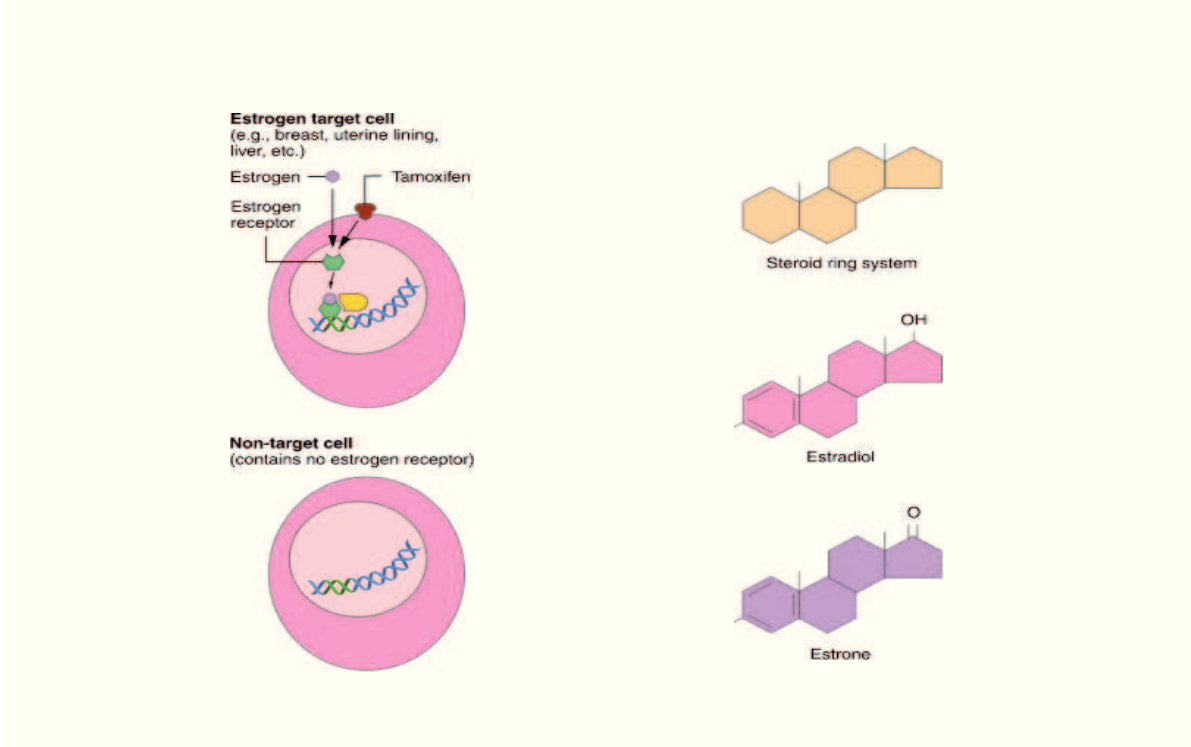
Steroid hormones comprise a large group of molecules all derived from the common sterol precursor cholesterol (Fig 2). Cholesterol is a lipid synthesized from acetate in many tissues of the body. It is a highly fat-soluble molecule with only limited solubility in water, and accordingly it localizes in the lipid regions of cell membranes (Baulieu, 1991). Cholesterol is converted by an enzyme to pregnenolone, another precursor steroid. Steroidogenesis within the gonads can then follow one of two pathways leading to the synthesis of the androgenic steroids, testosterone and androstendione. The enzyme aromatase expressed in growing ovarian follicles and in other tissues finally may convert testosterone to estradiol (Payne et al., 2004). Steroid hormones are composed of three six membered rings and one five membered ring. These organic compounds are easily identified from a visual standpoint by this "steroid nucleus", with the classical name cyclopentanophenanthrene (Carlstedt-Duke et al., 1989). All hormones have oxygen at C-3 and a varying substitute at C-17 (Fig 3). This substitute varies according to the different kind of steroid hormones and can be either in the alpha or beta configuration, depending on where it is situated relative to the plane of the molecule. There are six centres of asymmetry; as a result, there are 64 possible compounds (stereoisomers) with the same structure. The centers of asymmetry are located at C-5, C-8, C-9, C-10, C-13, and C-14. The rings prefer to adopt the puckered conformation over the boat form, because this conformation has the highest stability (Gower et al., 1989).

The endocrine glands responsible for the production of steroid hormones are the adrenal gland, the ovary, and the testis. Some examples of steroid hormones are 1: estrogens, 2: androgens (testosterone), 3: progesterone, 4: corticosteroids (Glucocorticoids and Mineralocorticoids) (Griffin et al., 1988; Gower et al., 1989). While all steroids contain the four-ring structure of the sterol nucleus and are remarkably similar in structure, they have enormous differences in their physiological effects. In vertebrates, steroid hormones function as genetic regulators, controlling the rate of synthesis of a particular protein. Steroid hormones are crucial for the induction of many enzymes reactions; the glucocorticoids trigger a variety of cellular responses including the synthesis of second messengers such as cAMP in the short term and the modulation of protein synthesis in the long term. The administration of the female sex hormone17β-estradiol (E_2) causes

chicken oviducts to increase their ovalbumin mRNA level from ~ 10 to ~ 50,000 molecules per cell. Similarly in insects, the steroid hormone ecdysone mediates several aspects of larval development (Voet et al., 1990). Steroid hormones, which are non-polar molecules, simply pass through the plasma membranes of their target cell to the cytosol where they bind to their respective receptors. The steroid hormone penetrates the cell membrane and moves through the cytoplasm towards the nucleus; it couples with the receptor protein, forming a hormone receptor complex. The steroid receptor complex, in turn, enters the nucleus where it can bind to specific chromosomal binding sites so that it can induce or repress the transcription of the associated gene (Weaver et al., 1989; Karavolas et al., 1990; Kato et al., 2005).

3. Estrogens

The basic hormonal functions of the female sex hormone estrogen were first realized in 1925, and its structure was described in 1931 by Adolph Butenandt (Diczfalusy, 1984; Davis et al., 2005). In his review in 1924 entitled "Untersuchungen über das weibliche Sexualhormon" (Investigations into the female sex hormone), Adolph Butenandt presented some biochemical data on estrogen. By using very basic methods of chemistry, he collected a large amount of information on the "progynon", as the female sex hormone was called at that time. Urine from pregnant women was the starting point of the investigations, and he was able to crystallize the hormone. He later observed that the "progynon" displayed specific properties with respect to solubility, and could easily be dissolved in alcohol, acetone, chloroform and benzol but hardly in water. For the chemist, this was already vital information (Corner, 1964; Karlson, 1995). He also found that the molecule did not contain nitrogen or sulphur and a first estimate on the chemical composition was made. The chemical formula of the progynon was believed to be either $C_{23}H_{28}O_3$ or $C_{24}H_{32}0_3$, which comes close to the actual formula of estradiol, which is $C_{18}H_{24}0_2$.



(A) **(B)**

Fig 1: Estrogens act on target tissue by binding to estrogen receptors. (Target and non-target cell) (A), Schematic structure of the steroid ring system estradiol, and estrone (B) (National Institute of Cancer, www.cancer.gov, 2003)

It was also recognized by that time that the hormone was neither a protein nor a typical carbohydrate, but displayed chemical similarities to the chemical compound family of the sterines and gallic acids (Corner, 1964).

The investigations of Jensen and Jacobson (1962) showed that estrogens act via specific receptors in the nucleus of the target cells, this action resulting in the production of specific proteins. The different actions of various estrogens (the natural hormone estradiol or other estrogenic compounds like phytoestrogens or industrial xenoestrogens or chemically synthesised estrogen receptor agonists or antagonists) are caused by the activity of binding to the same or to different estrogen receptors and by the stability of the steroid-receptor complex formed (Jensen et al., 1962; Jensen et al., 1973; Cuiper et al., 1998a; 1998b).

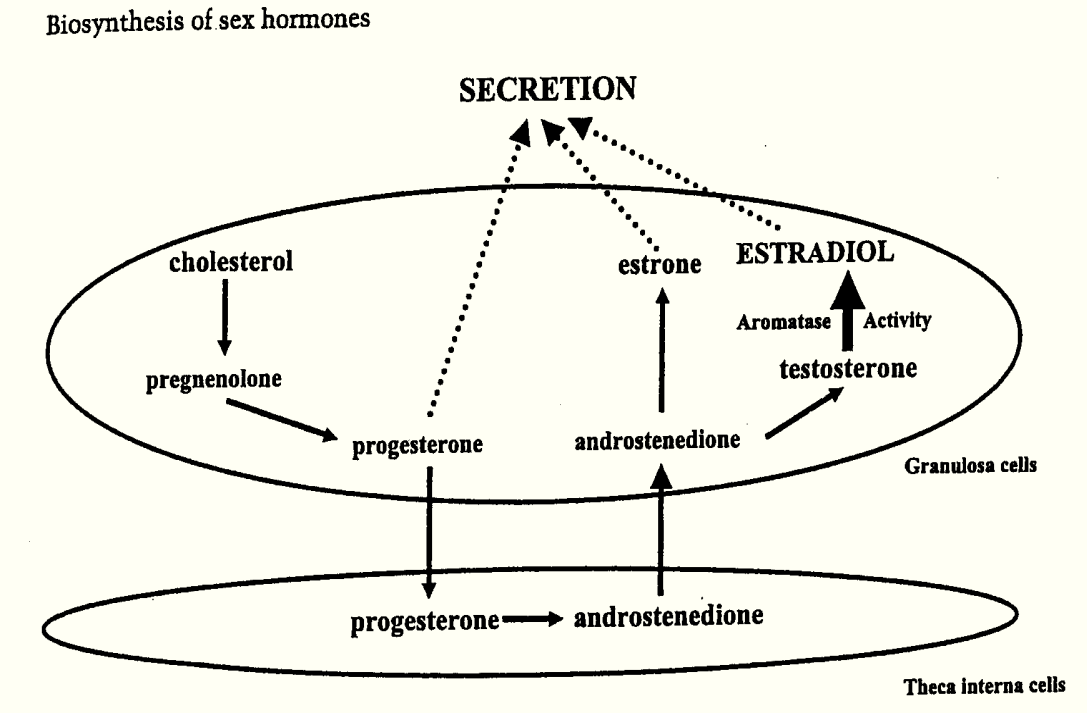


Fig 2: Compartmentalization of estrogens biosynthesis in the ovary (Oettel et al., 1999).

4. Estrogen Action

Estrogen hormones have effects at all levels of biological organisation and influence growth, differentiation, and function of the tissues of the female reproductive system, such as uterus, ovary, and breast, as well as of non-reproductive tissues such as bone (Turner et al., 1994) and the cardiovascular system (Sato et al., 1996; Yang et al., 1996), also in males. The effects of estrogens in tissues are mediated by two estrogen receptors (estrogen receptor α and estrogen receptor β) (Fig 4 and 6), which are expressed to varying extent in many organs, including uterus, ovary, breast, brain, lung, liver, gastrointestinal tract, bone, kidney, bladder, prostate and testis (Weusten et al., 1986; Barton et al., 1988; Vollmer et al., 1990; Enmark et al., 1997; Kuiper et al., 1997). Estrogen receptor α and estrogen receptor β are also expressed in malignant tissue, often in concentrations different from those found in the corresponding normal tissue (Vollmer et al., 1990; Grandien et al., 1995; Mosselman et al., 1996; Byres et al., 1997). Tree main mechanisms of estrogen action have been identified at the cellular level:

(1) Estrogen regulates the expression of target genes by binding to the nuclear estrogen receptor in the classical model of estrogen action, involving binding of the activated

receptor to an established binding site on the DNA.

(2) Ligand-activated estrogen receptor may interact specifically with transcription factors like AP-1 or Sp1 (Webb et al., 1995; Saville et al., 2000; Weatherman et al., 2001), thus acting at the genomic level, but using a non-classical mechanism.

(3) Estrogen modulates cell function through non-genomic pathways mediated by interaction with cell membrane-associated or cytoplasmic estrogen receptors in another non-classical action of estrogen (Aronica et al., 1994; Pappas et al., 1995).

Non-genomic actions of estrogens are frequently associated with the activation of various protein-kinase cascades (Losel et al., 2003). However, these actions may indirectly influence gene expression, through the activation of signal transduction pathways that eventually act on target transcription factors. The functions of many transcription factors, including AP-1, are regulated through protein kinase-mediated phosphorylation, and these transcription factors may thus be targets of nongenomic actions of estrogens. This signaling pathway can be referred to as nongenomic-to-genomic signaling, and it provides for a mechanism, distinct from protein-protein interactions in the nucleus, by which estrogen receptors can modulate the functions of transcription factors, and thus regulate the expression of genes that do not contain EREs (Bjornstrom et al., 2005).

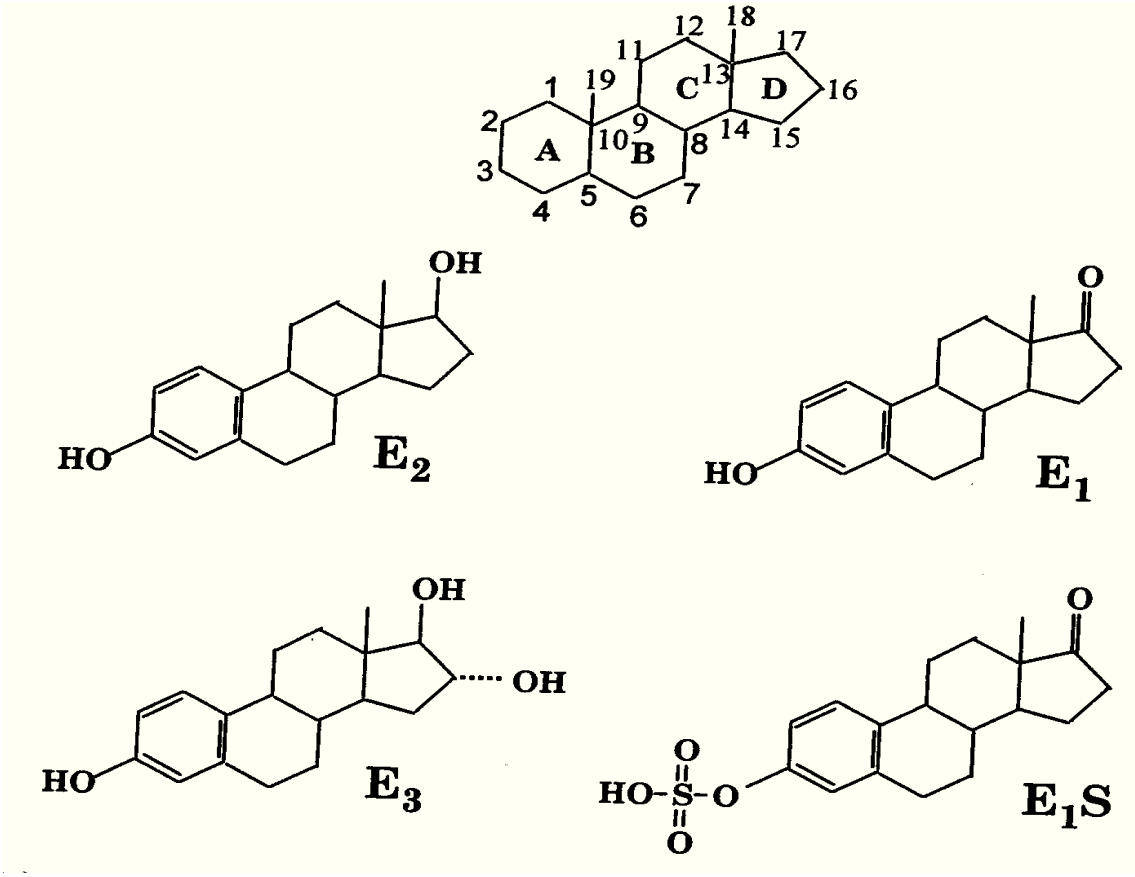


Fig 3: Estroid molecule with ring lettering and carbon numbering (Top) ,structure of 17β-estradiol (E_2),Estrone (E_1), Estriol (E_3), and Estrone sulphate (E_1S) (Parl, 2000)

5. Nuclear Receptors

The nuclear receptor (NR) super family comprises one of the largest classes of eukaryotic transcription factors (TFs). Over 180 nuclear receptor family members have been described and include transcription factors that are activated upon binding of steroid hormones, retinoids, thyroid hormones and vitamin D_3, as well as NRs that bind prostaglandin, fatty acid and bile acid ligands (Hart, 2000; Olefsky, 2001). In addition, other members of the nuclear receptor super family, whilst having similar structure, have no known ligand. These so-called orphan receptors may not require ligand binding, or may be activated by as yet unidentified ligands (Mangelsdorf et al., 1995; Olefsky, 2001; Hart, 2002). The ligand-induced nuclear receptors are activated by binding of their cognate ligands, which generally enter the cell by passive diffusion due to their lipophilic nature.

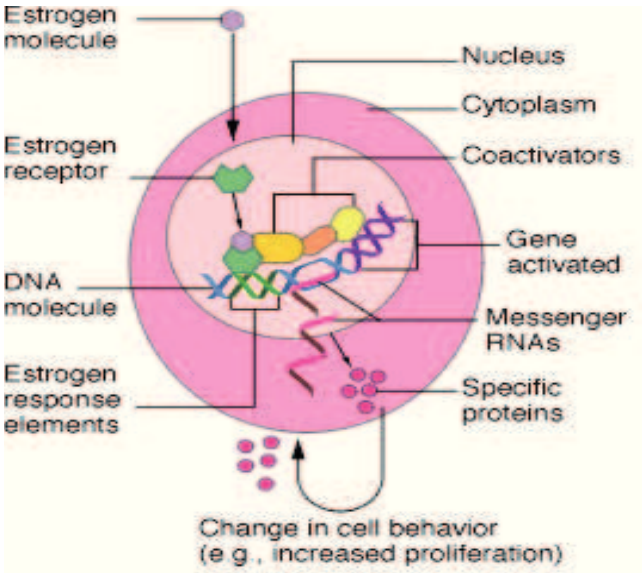


Fig 4: Schematic action of estrogen in a target cell (National Institute of Cancer, www.cancer.gov, 2003)

All members of the nuclear receptor super family have a common modular structure, possessing four independent but interacting functional modules. These are encoded in the A/B region, the DNA-binding domain (DBD), the hinge region and the ligand-binding domain (LBD) (Parker, 1993) (Fig 5). Despite their conserved structural features, the normal physiological functions of various nuclear receptors are quite diverse, from embryonic development to metabolic homeostasis, sex determination, development of sexual characteristics and fertility. The N-terminal (A/B) domain is the most variable region between NRs and comprises a ligand-independent activation function (AF-1) that allows constitutive activation of transcription (Jenster et al., 1995; Wilkinson, 1997; Towle, 1997). The highly conserved central DBD (region C) forms two zinc fingers and a carboxy-terminal extension (CTE) that together provide interfaces for binding to the DNA response element (RE) and protein binding (Perlmann et al., 1993; Zechel et al., 1994; Hart, 2002). Located C-terminal of the DBD is a flexible hinge region (region D) that allows rotation of the DBD to accommodate binding to REs, and which may be involved in coregulator binding, and interaction with HSP90 (Horlein et al., 1995; Chen et al., 1995; Couette et al., 1995). The C-terminal LBD (E/F) is well-conserved and contains the second activation function (AF-2), which is ligand-dependent. Analysis of the crystal structures of the LBDs of NRs reveals a common overall layered structure

consisting of 12 α-helices (Tanenbaum et al., 1998; Moras et al., 1998). The striking difference between the unliganded and ligand-bound receptors is the position of the C-terminal α-helix (helix 12), which contains the core AF-2 domain. In the unliganded form of the NR, helix 12 extends away from the hydrophobic ligand-binding pocket, however, on ligand binding helix 12 moves across the binding pocket like a lid (Driscoll et al., 1996; Shiau et al., 1998; Tagami et al., 1998; Nichols et al., 1998; Hart, 2002).

It has been shown that estrogen is binding as a ligand to a receptor molecule present in the target tissue (Jensen et al., 1962; Tom et al., 1966; Jensen et al., 1973, Shughrue et al., 2000). First it was believed that the receptor for the steroid hormones was just performing a simple transport function bringing the estrogen from the cytoplasm to the nucleus of the cell similar to a molecular shuttle (Jensen et al., 1973). Today we know that the idea that the estrogen receptor is just a simple transport protein was wrong. Estrogen receptors enter the cell's nucleus where the main function of the estrogen receptor (transcriptional control of estrogen regulated genes) is performed (Lin et al., 2004).

Estrogens have pivotal functions in both female and male physiology. In particular, 17β-estradiol (E_2) has a central role in the proliferation and differentiation of responsive cells, through changing the expression profile of target genes within responsive tissues (Metivier et al., 2003). The effect of E_2 as described before is mediated by proteins belonging to the nuclear receptor (NR) superfamily, the estrogen receptors alpha and beta (ERα) and (ERβ) (Katzenellenbogen, 1996; Bjornstorm et al., 2004; Katzenellenbogen et al., 2004).

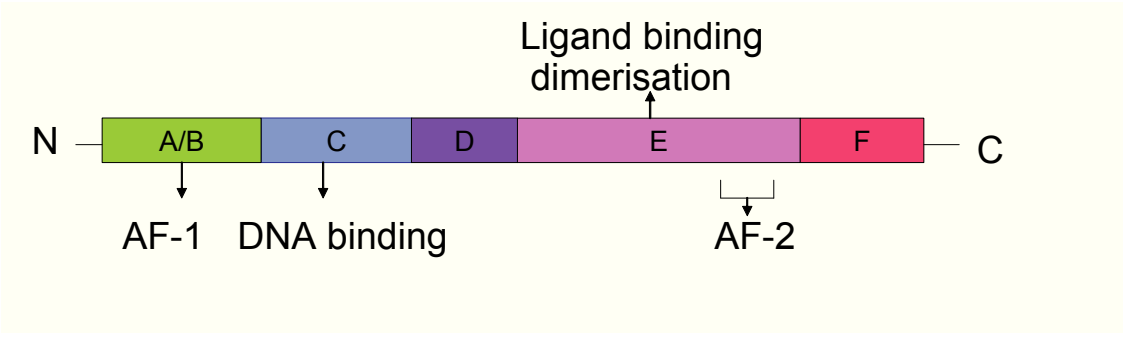


Fig 5: Schematic representation of a nuclear receptor. A typical nuclear receptor is composed of several functional domains. The variable NH_2-terminal region (A/B) contains the ligand-independent AF-1 Trans-activation domain. The conserved DNA binding domain (C).A variable linker region (D) connected to the conserved E/F region that contains the ligand-binding domain, the dimerisation surface and the ligand-dependent AF-2 trans-activation domain (Steffen, 2002).

The structure of the DBD and LBD are highly conserved across species, and are important in converting the hormonal “message” into transcriptional activity of a gene promoter. Recognition and binding of the ligand by the receptor generally results in binding to a specific nucleotide sequence known as a hormone response element (Parker, 1993).Nuclear receptors can be broadly divided into 4 classes according to their DNA-binding and dimerisation properties:

Class I: steroid hormone receptors function as ligand-induced homodimers and bind to DNA half-site motifs organized as palindromic repeats with nucleotide spacing of 3, e.g. estrogen receptors (ERs), glucocorticoid receptor (GR), progesterone receptor (PR), androgen receptor (AR), and mineral corticoid receptor (MR) (Beato, 1989).

Class II: nuclear receptors include all other known ligand-dependent nuclear receptors except those specified by steroids, e.g. thyroid hormone receptor (TRs), retinoic acid receptors (RARs), vitamin D receptor (VDR), and peroxisome proliferators activated receptor (PPARs). These receptors can form homodimers as well as heterodimers with retinoid-X-receptor (RXR), to bind to direct repeats (DR) of the half-site motif AGGTCA.

Since the nucleotide sequence of the half-sites are identical in all response elements recognized by these receptors, specificity of the receptor for the response element is defined by the number of nucleotides separating the half-sites, this '3-4-5' rule for DNA recognition by nuclear receptors is based on the nucleotide spacing between DRs (Umesono et al., 1991). Generally, these hormone receptors bind as dimers exclusively to their specified (DR2-5) response elements with the exception of RAR. RAR is less discriminating in terms of DNA-binding specificity; it can recognize DR1, DR2 and DR5 response elements as well as other direct repeats with lower affinity (Umesono et al., 1991; Durand et al., 1992).

Other members of the nuclear hormone receptor family resemble the classical receptors (steroid and other hormone receptors) in structure, but as yet lack known ligands. These nuclear receptors are therefore referred to as 'orphan receptors' and represent the remaining two classes.

Class III: orphan receptors are those that bind as homodimers or heterodimers with RXR to direct repeats of the half-site motif AGGTCA. This class of receptors include the

RXRs, chicken ovalbumin upstream promoter transcription factor (COUP-TF), ApoA1 regulatory protein (ARP), and hepatocyte nuclear factor 4 (HNF-4) (Cooney et al., 1992; Tsai et al., 1992).

Class IV: orphan receptors such as nerve growth factor-inducible gene B (NGFI-B), steroidogenic factor-1 (SF-1) as well as its homologs in other species, and germ cell nuclear factor (GCNF), bind as monomers to extended half-site motifs with AGGTCA as the core (Wilson et al., 1993; Ueda et al., 1992). Speculation as to the mode of action of the orphan receptors has led to two general concepts: orphan receptors are activated through phosphorylation, a result of cellular stimulation of a signal transduction pathway, or orphan receptors are activated by interacting with other nuclear receptors or by recruitment of cofactors (Enmark et al., 1996).

6. Structure of Estrogen Receptor α and Estrogen Receptor β

Estrogen receptors are members of the nuclear receptor subfamily comprising the receptors for steroids, thyroid hormones, and vitamin D (Wahli et al., 1991; Beato et al., 1995; Mangelsdorf et al., 1995; Kato et al., 2005).

The Estrogen receptor α was one of the first nuclear receptors to be cloned in the human (Greene et al., 1986; Wahli et al., 1991; Beato et al., 1995; Mangelsdorf et al., 1995; Kato et al., 2005) and has been identified in many species, such as mouse (White et al., 1987), rat (Knife et al., 1987) and Xenopus (Weiler et al., 1987). A new form of estrogen receptor, termed estrogen receptor β, was discovered in human (Mosselman et al., 1996), mouse (Tremblay et al., 1997) and rat (Kuiper et al., 1996). Both isoforms are encoded by single copy genes, localized on different chromosomes. The estrogen receptor α gene is located at the genomic position 6q25.1, it extends over more than 140 kb, contains eight exons, and encodes a protein of 595 amino acids with a predicted molecular weight of 66,182 Dalton (Menasce et al., 1993). Estrogen receptor β was identified and localized to position 14q 22-24, spanning 40 kb and encoding a protein of 485 amino acids (Kuiper et al., 1996; Mosselman et al., 1996; Katzenellenbogen et al., 1997).

The organization of estrogen receptor protein, like that of most of the other nuclear receptors, comprises the five regions A/B-F (Mangelsdorf et al., 1995).

6.1. The A/B Domain

This region is variable in length and in amino acid composition among different species (Greene et al., 1991). At first sight, no conserved structure could be deduced from this domain. However, there are regions of similarity, in particular some phosphorylation sites implicated in ligand-independent transactivation function (Nato et al., 1995; Guanin et al., 1996). This region is implicated in cross-talk with growth factors and in transcriptional activity of estrogen receptor α (Smith, 1998). Investigation of the cellular or intramolecular constraints modulating its three-dimensional structural properties will be necessary to understand better the structure-function relationships of the A/B domain.

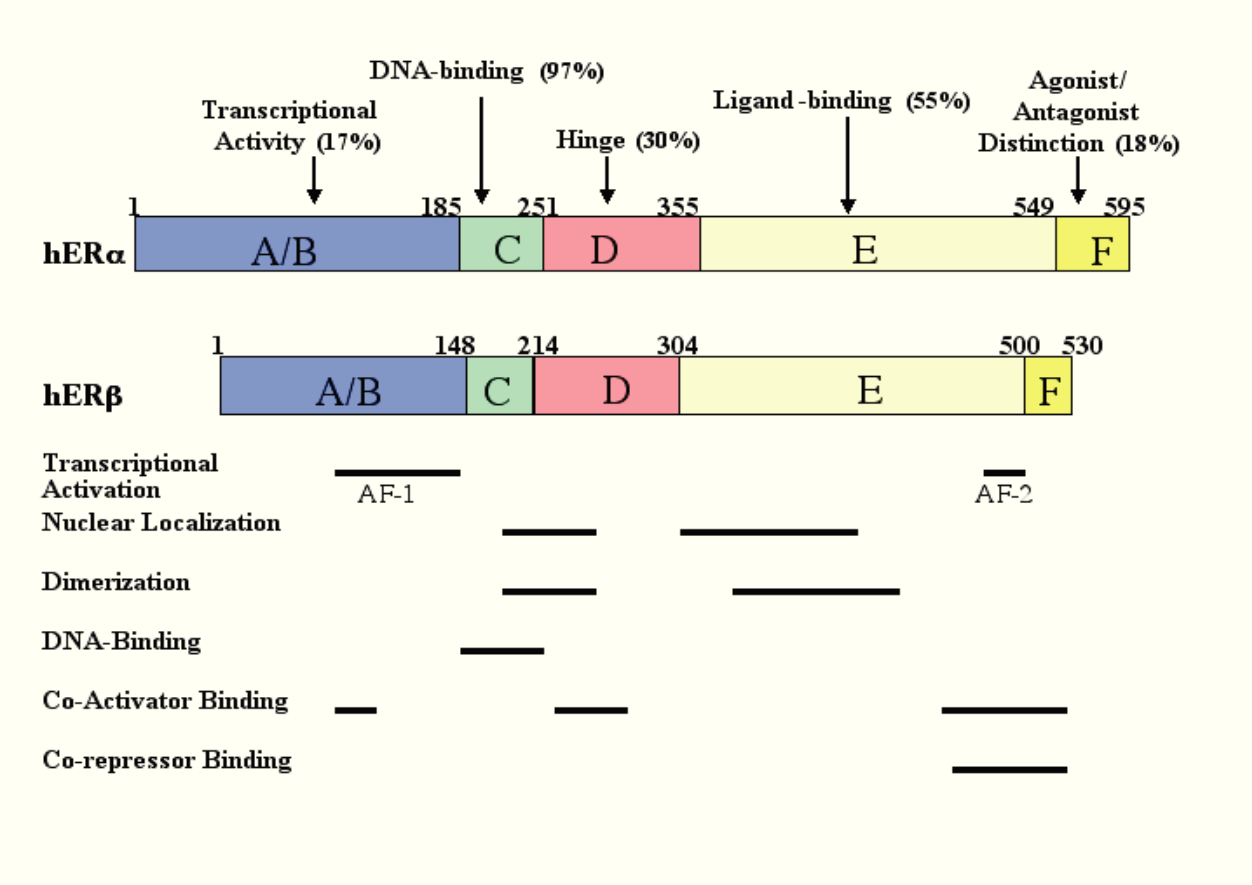


Fig 6: Domain structure of ERα and ERβ (values in % describe the degree of homology between ERα and ERβ) (Klinge, 2000)

6.2. The DNA-Binding Domain

The DNA-binding domain (DBD) is the most conserved domain between estrogen receptor α and other nuclear receptors. It has a core region of 66 amino acids, which folds into two "zinc fingers", a DNA-binding motif also found in other DNA binding proteins. Each zinc finger coordinates a zinc atom via two pairs of cysteine residues (Greene et al., 1991). For estrogen receptors, the so-called P-box determining the DNA-binding properties of the receptors is composed of the amino acids CEGCKA and mutations in this region alter estrogen receptor binding to DNA. The second zinc finger of the DBD encompasses a region called the D-box between the first two cysteines of this zinc finger. The D-box is important for the dimerization of the DBD moieties of estrogen receptor. This protein-protein interaction is important in maintaining the proper spacing of the two estrogen receptor molecules of a dimer on DNA (Greene et al., 1991).

6.3. The Ligand- Binding Domain

This so-called E/F domain is the region of the receptor which binds the ligand and provides some interacting surfaces on the receptor to allow interconnections with other cellular proteins. The crystal structures of the liganded estrogen receptor α and estrogen receptor β have been resolved (Brzozowski et al., 1997). The ligand binding domain (LBD) consists of an antiparallel α-helical sandwich containing 12 α-helices. This corresponds to the canonical structure for the LBD of nuclear receptors (Wurte et al., 1996). The LBD contains a binding pocket which is kept away from the external environment. This hormone cavity corresponds to an important part of the LBD hydrophobic core and its volume is twice that of estradiol, which may somehow explain the estrogen receptor's ability to bind different steroidal and non-steroidal ligands.

Ligand-dependent activity of nuclear receptors relies on a correct positioning of helix 12. In the estrogen receptor-E_2 complex, helix 12 "locks" the binding cavity and is positioned close to helices 3, 5/6 and 11. This folds the LBD into a transcriptionally competent AF-2 configuration, which is able to interact with co-factors and activate gene expression. The F domain of the estrogen receptor was not included in the X-ray studies and shows no real sequence similarities when estrogen receptor from different species is compared. Its length, however, is conserved within each estrogen receptor isotype, α or β, from

different species. This might reflect some important structural constraints for receptor activity (Nichols et al., 1998).

7. ER-DNA-Binding Properties

As a transcription factor, estrogen receptor modulates the transcriptional activity of target genes in a ligand-dependent manner or under the influence of growth factors. Estrogen receptor binds target promoters by recognizing a specific DNA segment called the estrogen response element (ERE), which it binds as a dimer. The consensus ERE contains a repeat of the AGGTCA motif organized as a palindrome with a three-nucleotide spacing between the half-sites (AGGTCANNNTGACCT) (Martinez et al., 1991). Estrogen response elements are found in estrogen receptor-regulated genes such as vitellogenin, ovalbumin, prolactin and c-fos (Walkea et al., 1984; Martinez et al., 1991). However, estrogen response elements have not been found in all estrogen-regulated genes. This finding probably reflects ERE-independent effects of estrogen receptor, not following the classical model of estrogen receptor action described above.

Within the nuclear receptor family, peroxisome proliferator-activated receptor (PPAR), a nuclear receptor for fatty acid and fatty-acid derivatives, heterodimerizes with retinoid-X receptor (RXR), the receptor for 9-cis-retinoic acid (Weinberger et al., 1996). The PPAR-RXR heterodimer is able to bind an estrogen response element, but less efficiently than an estrogen receptor, which can lead to a situation of competition for the response element. The PPAR-RXR complex does not stimulate transcription in the context of the vitellogenin promoter, which contains an estrogen response element, but inhibits estrogen receptor-mediated transactivation in transfection experiments (Keller et al., 1995). Genes influenced by estrogens and fatty acids could then be co-regulated by PPAR and estrogen receptor, most likely in a promoter-dependent manner. It is important to note that, also the estrogen related receptors (ERRs), bind to the estrogen response element and to the extended half-site, of which a subset can also be recognised by estrogen receptor (Giguere, 2002). These orphan receptors share significant homology with the estrogen receptors (ERs) but are not activated by natural estrogen. In contrast, the estrogen related receptors display constitutive transcriptional activity in the absence of exogenously added ligand.

8. ER Ligand-Independent Activity

While ligand binding triggers major conformational changes in estrogen receptors leading to activation, receptor activity is also possible in the absence of any hormone bound to the LBD (Cho et al., 1993). It is important to distinguish the latter situation from antagonist binding, which elicits an active inhibition of the estrogen receptor transactivation capacity. Ligand-independent activity of estrogen receptor α is supported by its N-terminal A/B domain. This domain contains a separable transcriptional function (AF-1) able to activate transcription, exhibiting cell-type and promoter-context specificity (Gronemeyer, 1991).

The ligand-independent activity of estrogen receptor can be triggered by growth factors such as epidermal growth factor (EGF). A decisive step in the understanding of the molecular mechanism of EGF's effect on estrogen receptor was accomplished with the unravelling of the pathway linking EGF, its membrane receptor activation, the mobilization of the mitogen-activated protein kinase (MAPK) cascade and direct estrogen receptor phosphorylation by MAPK. The phosphorylation site is located in the A/B domain and is conserved among species (Kato et al., 1995).

9. Ligand-Dependent Transcriptional Activity

In order to activate transcription, the DNA-bound estrogen receptor needs to be in a conformation that allows favourable interactions with the transcriptional machinery. Agonist ligands such as estrogen are capable of inducing such structural changes, which unmask a ligand-dependent activation function (AF-2) (Brozozowski et al., 1997). A careful analysis of AF-2 led to the identification of a conserved region responsible for this activity lying in the C-terminal part of the LBD. Mutations, in this region, have a negative impact on estrogen receptor transcriptional activity. This helped to define a core autonomous domain (AF-2 AD core) able to mediate gene transcription (Danielan et al., 1992). Another set of mutations, which had an effect only on transactivation, suggested that there might be some proteins interacting with the estrogen receptor that are necessary for transcriptional activation. Such co-factors were discovered using the yeast two-hybrid system (Onate et al., 1995) or protein-protein interaction assays (Halachmi et al., 1994). These investigations revealed two kinds of interacting molecules. The first category

consists of molecules that interact with estrogen receptor and other nuclear receptors in a ligand-independent way, but do not directly help estrogen receptor-driven transcription. An example is transcription intermediary factor 1 (TIF1), which is thought to mediate estrogen receptor interactions with those proteins implicated in chromatin remodelling that indirectly act on transcription (Glass et al., 1997).

The second category consists of real co-activators, which can be divided into three classes, namely steroid receptor co-activator-1, nuclear-receptor co-activator (SRCl/NCoAl) (Onate et al., 1995; Kalkhoven et al., 1998), transcription intermediary factor-2 glucocorticoid receptor interacting protein (TIF2/ GRIP1) (Voegel et al., 1996) and 3-cyclic AMP response element-binding protein and its p300 homologue (CBP/p300) (Chakravarti et al., 1996; Kamm et al, 1996).

10. Non-Classical Genomic Effects of Estrogen Receptor α and Estrogen Receptor β

Estrogen receptor α and estrogen receptor β regulate transcription by binding to a classical ERE, in the case of the insulin-like growth factor-1 (IGF-1) gene, for example the effects of estrogens are mediated through an AP-1 binding site (Umayahara et al., 1994). The estrogen receptor is thought to have protein-protein interactions with the DNA-bound AP-1 complex without necessarily having direct contacts with the DNA itself. In this model, the partial agonist tamoxifen was found to have agonistic effects in uterine cell lines but not in breast cell lines, which could explain some estrogen-like effects of tamoxifen in uterus (Webb et al., 1995). Surprisingly however, the ligand-binding activity profile of estrogen receptor β was reversed, compared with that of estrogen receptor α, when using another partial agonist, raloxifene, which had antagonistic effects on estrogen receptor β and agonistic effects on estrogen receptor α. Estradiol, however, was able to activate estrogen receptor α, but had no effect on estrogen receptor β-mediated transcription (Paech et al., 1997). This suggests that estrogens could be interpreted differently by the cell, depending on which of the two estrogen receptor isomers are active at complex promoters, such as those that are showing an AP-1 dependent activity.

11. Orphan Nuclear Receptors and their Role in Gene Regulation

Orphan receptors have been found to be expressed in most tissue and appear to constitute important components of the regulatory system controlling transcription (Walther et al., 1995). Two groups of orphan receptors can be defined by their manner of DNA binding. The first group, of which COUP-TF is an example, binds to DNA as a dimer and is able to form homodimers or heterodimers with other nuclear hormone receptors promoting binding to direct repeats of the AGGTCA motif (Cooney et al., 1992; Kliewer et al., 1992; Walther et al., 1995). COUP-TF is not only able to form heterodimers, but is also very versatile in its effects on transcription. Although having been described as a factor activating transcription of the chicken ovalbomin gene (Sagami, 1986), in most experimental systems the main effect of COUP-TF is the repression of transcriptional trans-activation. The tissue specific transcription factor SF-1 represents a member of a newly defined subgroup of the orphan receptors that bind to DNA as monomers (Wilson et al., 1993). These factors recognize related but distinct binding sites containing only one AGGTCA motif. SF-1 is tissue-specifically expressed in steroidogenic tissues as key regulator of steroidogenic enzyme expression (Rice et al., 1991; Lynch et al., 1993; Morohashi et al., 1993; Clemens et al., 1994). The developmental expression of SF-1 has been analyzed in detail showing an essential role of SF-1 in adrenal and gonadal development and sexual differentiation (Ikeda et al., 1994; Lou et al., 1994; Shen et al., 1994). Another monomeric orphan receptor, NGFI–B, has been shown to be induced by adrenocorticotropin hormone (ATCH), and binding of this factor confers ACTH inducibility to a heterologous core promoter (Wilson, 1993). This result clearly shows that binding of a monomeric orphan receptor may be sufficient to transduce an activating signal to the transcriptional machinery.

Although tissue-specific orphan receptors like SF-1 have an important function for the development and the function of the expressing tissue, their effects on transcription of genes containing binding sites for these factors in their regulatory region, when analyzed in transient transfection studies, are not as dramatic as those that can be seen with ligand - activated nuclear hormone receptors. In transfection experiments using a construct with a heterologous promoter only a two to three fold SF-1 dependent increase in transcription was found (Lynch et al., 1993; Walther et al., 1995). In order to facilitate analysis of orphan receptor function, multimers of the SF-1 binding site have been inserted next to a

basal promoter (Morohashi et al., 1993). In this arrangement the transcriptional effects of SF-1 are somewhat enhanced, but these effects do not correlate with the transcriptional activation by SF-1 in vivo.

12. Oxytocin

Oxytocin is a nine-amino-acid peptide that is synthesized in the Supraoptic (SON) and Paraventricular Nuclei (PVN) of the hypothalamus. The processed peptide is released from the posterior pituitary into the blood stream upon appropriate stimulation (Ivell, 1987). Oxytocin is also synthesized in small amounts in several peripheral organs, including the gonads and thymus. In ruminants, unlike in other mammals, the ovarian corpus luteum also produces high levels of oxytocin.

Hypothalamic oxytocin acts as a classical endocrine factor. In the female reproductive system, hypothalamic oxytocin has been identified as the neurohypophysial factor involved in induction of the milk ejection reflex (milk letdown) in nursing mothers (Ivell, 1987; Wakerly et al., 1988), and stimulation of uterine contractions during parturition (Higuchi et al., 1986; Walther et al., 1995).

13. Ovarian Oxytocin in Ruminants

By demonstrating that ovine corpora lutea (CL) contain large amounts of oxytocin, Wathes and Swann provided a fundamental stepping-stone in the understanding of ovarian oxytocin function (Wathes et al., 1982). Furthermore, the common precursor peptide for oxytocin as well as neurophysin could also be detected in bovine corpora lutea (Fields et al., 1983; Ivell et al., 1985; Camier et al., 1991). Further studies using immunohistochemical methods on ruminant ovarian tissue localized oxytocin and neurophysin to the secretory granules of large luteal cells (Guldenaar et al., 1984; Fields, 1986; Theodosis et al., 1986; Fehr et al., 1987; Fields et al., 1992). In ruminants, the expression pattern of oxytocin transcripts in the CL differs from that in other species; there is a dramatic up-regulation of the oxytocin transcripts in the developing CL at the time of ovulation (Ivell et al., 1984). Oxytocin transcript levels remain elevated in early CL, up to day 5 of the 21-day cycle, and then drop significantly (Ivell et al., 1985; Fehr et al., 1987; Jones et al., 1988; Ivell et al., 1990). The correlation between increased levels

of estrogen in the phase of follicle development and the rise in oxytocin mRNA levels in the bovine CL strongly suggests the estrogen-dependent up-regulation of this gene.

14. Oxytocin Genomic Structure and Transcriptional Regulation

The gene oxytocin is closely linked to the vasopressin gene on the same chromosome, separated by an intergenic region ranging from 3 to11 kilobasepairs (kb) depending on the species (Sausville et al., 1985; Hara et al., 1990; Schmitz et al., 1991).

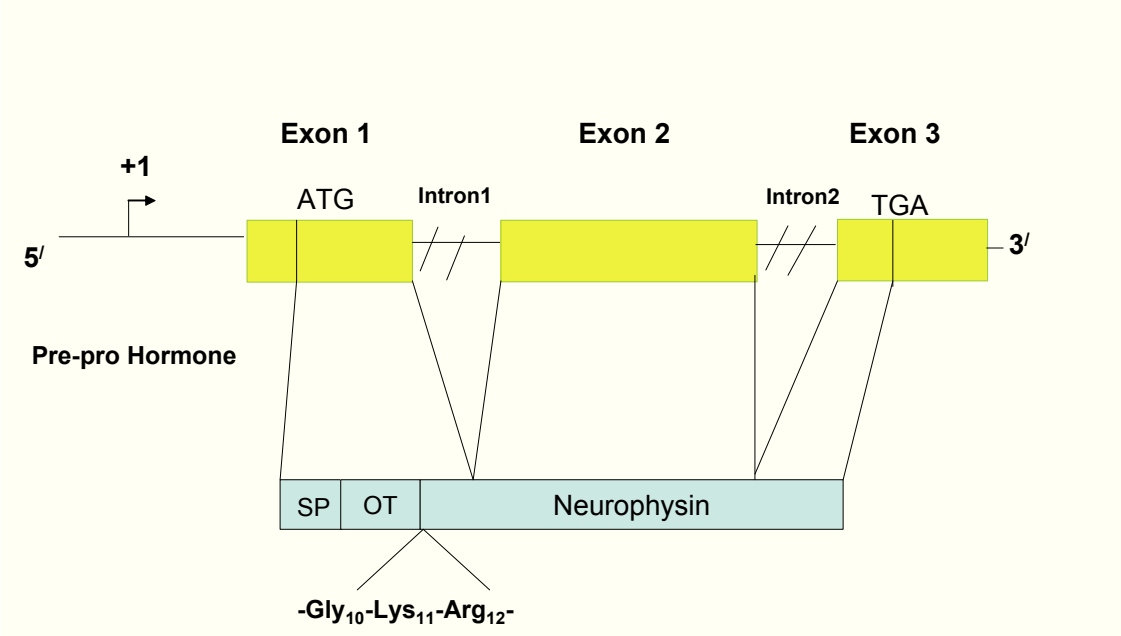


Fig 7: Structure of the oxytocin gene. The nucleotide sequence for oxytocin consists of three exons and two introns. (Stedronsky, 2000)

The oxytocin gene consists of three exons (Fig 7) encompassing less than 1 kb of genomic sequence; exon 1 encodes the signal peptide used to transport the precursor form into the endoplasmic reticulum for post-translational processing and the oxytocin peptide, as well as a portion of the neurophysin peptide sequence. Exons 2 and 3 encode the remaining peptide sequence of neurophysin. These structural arrangements of exons as well as the coding sequences within each exon have been shown to be conserved across species (Ivell et al., 1984; Ruppert et al., 1984; Sausville et al., 1985; Hara et al., 1990). Furthermore, the promoter nucleotide sequence also bears significant sequence homology across species, particularly within the first 200 base pairs (bp) upstream of the transcriptional start site.

Several basic regulatory elements, short nucleotide sequences that are recognized by transcription factors, have been identified within the oxytocin promoter. The promoter nucleotide sequence contains a modified TATA box, important for basal regulation of the gene via the assembly of the RNA polymerase II transcriptional complex, as well as a regulatory region upstream of the transcriptional start site.

Analysis of the proximal promoter identified an element located approximately -160 bp from the transcriptional start site. This element is highly conserved in all species studied so far and contains a direct repeat of the half-site motif AGGTCA as well as (in most species investigated) an imperfect palindromic estrogen-response element (ERE). Since this element is a composition of two distinct types of hormone receptor binding sequences, it is also referred to as a composite hormone response element (Burbach et al., 1990; Richard et al., 1990; Adan et al., 1991; Richard et al., 1991; Adan et al., 1992; Adan et al., 1993). In both the rat and human oxytocin gene promoters, this element contains a palindrome differing by one base from the sequence of the "classical" estrogen-response element, originally identified in the promoter of the Vitellogenin A_2 gene in Xenopus laevis (Klein-Hitpass et al., 1986). In the bovine in contrast, this palindromic sequence within the -160 element is not conserved (Walther et al., 1991).

15. Effect of Gonadal Steroids on the Oxytocin Gene Regulation

Clearly, the correlation between the up-regulation of hypothalamic or ovarian oxytocin gene expression and circulating steroid hormones, particularly estrogen, suggests that this hormone may be an important regulatory factor of the oxytocin gene in several aspects of reproduction (Stedronsky, 2002). Most research done on hypothalamic oxytocin gene regulation in vivo has utilized the rat as a model system. Just prior to the onset of parturition, the oxytocin gene appears to be up-regulated in the PVN and SON of the rat hypothalamus in preparation for birth and lactation, and has been correlated with the high estrogen environment of the uterus (Zingg et al., 1988). In the lactating rat, the oxytocin gene remains up-regulated in the hypothalamus in the first weeks of lactation, when estrogen levels, secreted by the regressing CL, are high (Crowley et al., 1993).

Studies addressing the issue of which factors regulate the oxytocin gene in vivo have used the bovine luteinizing granulosa cell model. In the bovine, DNA binding

experiments using nuclear extracts from bovine CL, as well as transfection studies, have clearly demonstrated that the bovine oxytocin promoter, particularly the -160 response element (Fig 8), does not bind estrogen receptors, nor does it convey estrogen responsiveness (Walther et al., 1991; Adan et al., 1992).

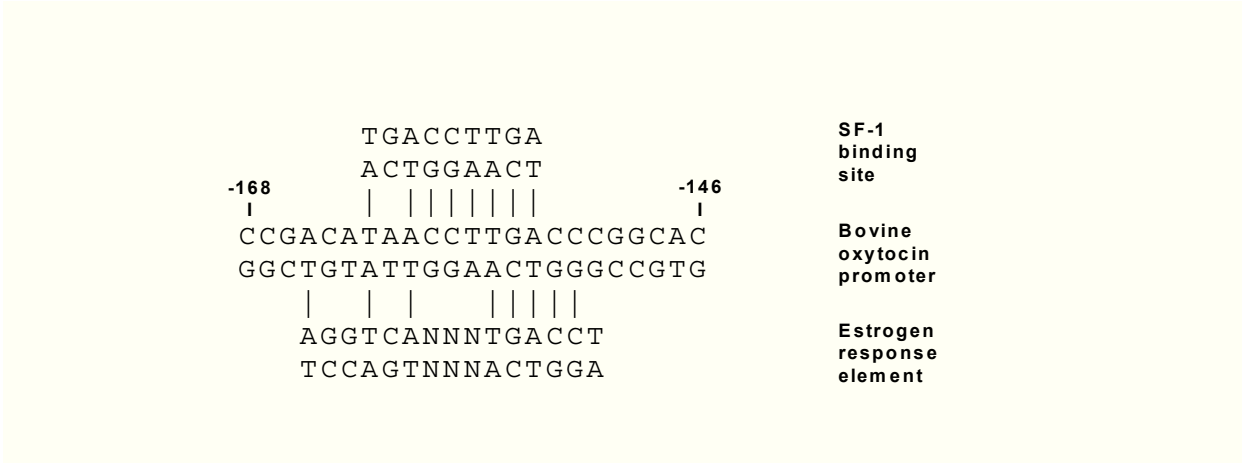


Fig 8: Conservation of a monomeric orphan receptor binding site in the promoter region of the bovine oxytocin gene. The regulation region of the bovine oxytocin promoter (-160 element) is compared to the established sequences for the steroidogenic factor-1 (SF-1) binding site and the estrogen response element; matches of basepairs are indicated by the vertical lines (Koohi et al., 2005).

In contrast, SF-1 and COUP-TFI (chicken ovalbumin upstream promoter-transcription factor) have been shown to interact with the -160 response element of the bovine oxytocin promoter, in both transfection assays and DNA-binding studies (Wehrenberg et al., 1994; Wehrenberg et al., 1994b). Under luteinizing conditions, SF-1 binds to the -160 response element and acts as an activator of oxytocin gene transcription. Under conditions favoring the down-regulation of oxytocin gene transcription, COUP-TF appears to be the main protein binding to the site. In conclusion SF-1 and COUP-TF appear to act by competing for binding to the -160 element, forming a molecular switch controlling the tissue-specific activation of the oxytocin gene.

16. Estrogen and Cancer

Paradoxically, estrogen can be both a beneficial and a harmful molecule. The main beneficial effects of estrogen include its roles in:

1- Programming the breast and uterus for sexual reproduction,

2- Controlling cholesterol production in ways that limit the build-up of plaque in the coronary arteries.

3- Preserving bone strength by helping to maintain the proper balance between bone build-up and breakdown.

Unfortunately however, estrogens are clearly carcinogenic in humans and rodents, but the molecular pathways by which these hormones induce cancer are only partially understood (Yager et al., 1990; Nandi et al., 1995; Okobia et al., 2005). In broad terms, two distinct mechanisms of estrogen carcinogenicity have been outlined. Stimulation of cell proliferation and gene expression by binding to the estrogen receptor is one important mechanism in hormonal carcinogenesis (Stoica et al., 2000). However, estrogenicity is not sufficient to explain the carcinogenic activity of all estrogens, because some estrogens are not carcinogenic (Nandi et al., 1995; Yager et al., 1996). Increasing evidence of a second mechanism of carcinogenicity has focused attention on catechol estrogen metabolites, which are less potent estrogens than estradiol, but can directly or indirectly damage DNA, proteins and lipids (Yager et al., 1996; Cavalieri et al., 2002). Estrogen carcinogenesis has been investigated in experimental animal models (Li et al., 1990; Yager et al., 1996). The best characterised of these is a kidney tumour induced in 80 to 100 percent of male Syrian hamster treated with estradiol (E_2) or diethylstilbestrol (DES) for at least 6 months (Baht et al., 1993).

17. The Importance of Estrogen for the Development of Breast Cancer

Breast cancer is the most common cancer in women world-wide (Parkin et al., 2001), with more than one million new cases being estimated every year and is the leading cause of death in women between the age of 35 and 47 (Stoica et al., 2000). Sex hormones have been implicated in various human cancers such as endometrial cancers (Key et al., 1992), breast and prostatic cancer (Kelsey et al., 1988; Sharma et al., 2000), colon cancer (English et al., 2001), gall-bladder cancer (Ray et al., 2001), and kidney cancer (Li et al., 1985; Yager et al., 2000). However, the association between estrogen and breast cancer assumes special significance, since breast cancer represents an enormous public health problem (Key et al., 1988; Bernstein et al., 1993; Park, 2000). Estrogen appears to hold the key to the understanding of breast cancer (Parkin et al., 2001). Estrogen deficiency

has been found to be associated with breast cancer and has been implicated as risk factor for breast cancer in postmenopausal women in multiple studies (Brinton et al., 1988; Hankinson et al., 1998; Clemons et al., 2001; Antony et al., 2001). The increase in risk appears to be related to the duration estrogen therapy. The development of an agent that selectively antagonizes this undesirable effect of estrogen in the breast has been the motivating force behind the development of selective estrogen receptor modulators (SERMs) (Goldits et al., 1995; Shairer et al., 2000).

18. Environmental Estrogens and Breast Cancer

The general agreement is that estrogens are involved in the etiology of breast cancer (Ray et al., 2001). Interestingly, various studies show that the environment may also contribute to the general estrogenic load to which men and women might be exposed. The estrogenic materials present in the environment are composed of widely diverse chemical substances (Ray et al., 2000). Numerous environmental chemicals have also been shown to possess estrogenic activity by virtue of their ability to bind to estrogen receptors (Lucier, 2000), and some of these environmental chemicals exert their estrogen-like effects by non-classical mechanisms (Steinmetz et al., 1996). Chemicals such as pesticides and herbicides have also been suggested to be able to interact with the endocrine system (Kristensen et al., 2001). Environmental factors probably play a prominent role in breast cancer etiology. Breast cancer incidence has been rising steadily in many countries and it has been suggested that part of the increase may be due to such unexplained environmental factors (Ray, 1997).

Association with breast cancer risk was demonstrated for some polychlorinated biphenyls measured in breast adipose tissue (Aronson et al., 2000; Stellman et al., 2000). Further it has been found that free-radical mediated oxidative stress is associated with some of the organochlorine pesticide residues in human breast tumours (Iscan et al., 2002). Straube et al. found changes in androgen concentration and lymphocytes in relation to pesticide exposure (Straube et al., 1999).

Unlike pesticides and related industrial estrogenic chemicals, vegetarian diets are rich in natural estrogenic substances (phytoestrogens) and have been shown to prevent or affect breast cancer incidence (Adlercreutz, 2002). The isoflavonoid glycosides and plant

lignans are two main groups of phytoestrogens, found in food like whole-grain rye products, soy beans, etc. Phytoestrogens may reduce breast cancer risk by affecting estrogen receptor, or, alternatively, these naturally occurring chemicals may alter the action /metabolism of steroid hormones (Horn-Ross, 1995).

19. Selective Estrogen Receptor Modulators (SERMs)

Prolonged stimulation of breast ductal epithelium by estrogen can contribute to the development and progression of breast cancer, and treatments designed to block estrogen effects are important options in the clinic (Riger et al., 2003). Tamoxifen and other similar drugs (Fig 10) are effective in breast cancer prevention and treatment, by inhibiting the proliferative effects of estrogen mediated through estrogen receptor (McDonnell et al., 1999; Jordan, 2001). However, these drugs also have many agonistic estrogenic effects depending on the tissue and target gene, and hence are more appropriately called selective estrogen receptor modulators (SERMs). SERMs bind estrogen receptor, alter receptor conformation, and facilitate binding of co-regulatory proteins that activate or repress transcriptional activation of estrogen target genes (Laine, 2002). Theoretically, SERMs could be synthesized that would exhibit nearly complete agonist activity on the one hand or pure antiestrogenic activity on the other (Herington, 2003). Depending on their functional activities, SERMs could then be developed for a variety of clinical uses, including prevention and treatment of osteoporosis, treatment and prevention of estrogen-regulated malignancies, and even for hormone replacement therapy. Tamoxifen is effective in patients with estrogen receptor-positive metastatic breast cancer and in the adjuvant setting. The promising role for tamoxifen in ductal carcinoma-in-situ or for breast cancer prevention is evolving, and its use can be considered in certain patient groups (Jordan, 2001). Other SERMs are in development, with the goal of reducing toxicity and/or improving efficacy, and future agents have the potential of providing a new paradigm for maintaining the health of women.

19.1. Tamoxifen

The nonsteroidal antiestrogen tamoxifen is well established as an effective treatment for patients with breast carcinoma, both for the treatment of metastatic disease and patients

with primary breast cancer (Howell et al., 2000). Tamoxifen is a selective estrogen receptor modulator that competes with estrogen for binding to the estrogen receptor (Pritchard et al., 2001). The drug tamoxifen was synthesized in 1962. Since it blocks the natural hormone estrogen, it was classified as a non-steroidal estrogen antagonist (Bedford et al., 1966; Kim et al., 2004). It has been used in the treatment of breast cancer for several years (Chang, 1998), although its efficacy has often been controversial (Pritchard, 1998). Articles in the Lancet in the spring and summer of 1998 respectively supported, and then refuted the value of tamoxifen with breast cancer (Anonymous, 1998; Powles et al., 1998). Later that year, the U.S. Food and Drug Administration (FDA) approved tamoxifen citrate for reducing the incidence of breast cancer in women at high risk for developing the disease. In addition to breast cancer, tamoxifen has been used to treat other cancers such as melanoma, hepatocellular carcinoma, ovarian cancer and prostate cancer (Flaherty et al., 1996; Bergan et al., 1999; Shy et al., 2000). The action of tamoxifen is now known to operate at several sites in the cell (e.g. as a channel blocker and to affect numerous genes), but early studies concentrated on its action as an antihormone, specifically an antiestrogen (Gradishar et al., 1997; Chesnoy-Marchais, 2005). Estrogens are known to promote the growth of breast cancer cells, so if an antiestrogen such as tamoxifen blocks those sites, the effect of estrogen is diminished. Experiments have shown that tamoxifen blocks the effect of estradiol in cultures of astrocytes, but in a manner related to the type of estrogen receptor (ER) present (Kuiper et al., 1997).

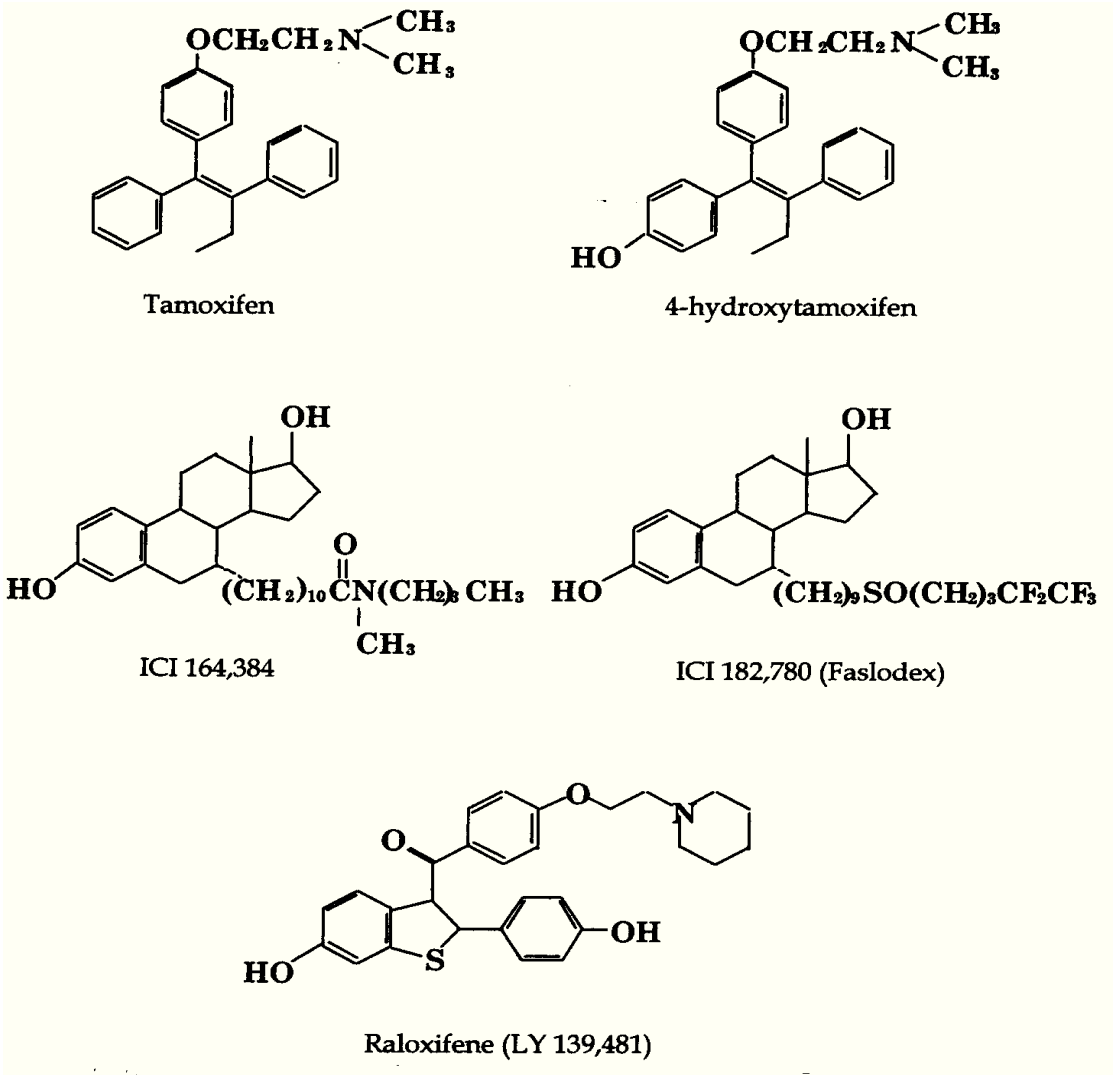


Fig9: The structure of some selective estrogen receptor modulators (Lindsay, 1997)

Although tamoxifen has been found to be effective in decreasing brain tumor proliferation, it remains controversial whether this is mediated via the estrogen receptor, despite evidence of such mechanisms in a glioblastoma cell line (Puchner et al., 2001). Other studies have shown a specific cell cycle action of tamoxifen, mediated by mechanisms other than estrogen inhibition. Also of importance is the interaction between the ER and an enzyme called protein kinase C, an enzyme involved in intracellular signal transduction (Engelke et al., 2002; Koh et al., 2003). In different tissues and in different

animal species, tamoxifen in this interaction like other (SERMs) may act as agonist or antagonist of estrogen (Ohmichi et al., 2005).

19.2. Raloxifen

Raloxifen is a SERM that has been shown to increase bone density in post-menopausal women and is currently indicated for the prevention and treatment of osteoporosis in this population (Delmas et al., 1997, Diehr et al., 2005). Raloxifen is of particular interest, because is appears, in early clinical and preclinical testing, to be less likely to cause endometrial stimulation and or to result in endometrial cancer (Cumming et al., 1999). Raloxifen has the ability to bind to and activate the estrogen receptor while exhibiting tissue-specific effects distinct from estradiol (Gradishal et al., 1997). As a result, raloxifen is the first of a benzothiophene series of antiestrogens to be labelled a SERM. Raloxifen was specifically developed to maintain beneficial estrogenic activity on bone and lipids and antiestrogenic activity on endometrial and breast tissue. In December 1997, the U.S. Food and Drug Administration (FDA) labelled raloxifen for the prevention of osteoporosis (Scott, 1999). Although the exact mechanism of action of raloxifen and other similar compounds has not yet been determined, it has been hypothesized that these agents work by inducing conformational changes in the estrogen receptor, resulting in differential expression of specific estrogen-regulated genes in different tissues (Mitalk et al., 1997; Ohmichi et al., 2005). Activation of the estrogen receptor by these compounds may involve multiple molecular pathways that may result in gene expression of ligand-, tissue- and/or gene-specific receptors.

19.3. $ICI_{182,780}$

$ICI_{182,780}$ is a pure estrogen receptor antagonist for both estrogen receptor α and estrogen receptor β (Bowler et al., 1989; Wakelly et al., 1991). This substance is now also known as fulvestrant (Faslodex, Astra-Zeneca), and has recently been approved for treatment of hormone-receptor-positive metastatic breast cancer in post-menopausal women with the disease progressing following anti-estrogen therapy (Wakelly et al., 1991; Fleming et al., 2004). Faslodex is an estrogen receptor down-regulator, and represents the first in a new class of breast cancer treatments. Due to its unique mechanism of action, Faslodex works

differently to other hormonal breast cancer drugs, in that it binds to the estrogen receptor in breast cancer cells, and this interaction results in degradation and loss of the estrogen receptor (Fleming et al., 2004). Currently, post-menopausal women with advanced breast cancer, whose tumours have been shown to be dependent on estrogen for growth and proliferation, are given drugs like tamoxifen that act by blocking estrogen receptors in the cancer cell, or aromatase inhibitors, such as Arimidex (anastrozole), that inhibit the production of estrogen in the body, cutting off the tumour's supply of the hormone (Howell, 2000; Howell et al., 1995). After a period of time, breast tumours can become resistant to these types of treatments and the cancer may progress. Faslodex represents a new way to attack cancer cells that have grown resistant to current hormonal treatment options and, unlike current therapies, is administered as a monthly intramuscular injection, an option which may offer compliance benefits (Fleming et al., 2004; Okubo et al., 2004).

20. Xenoestrogens

Xenoestrogens are defined as chemical substances entering the body from the external environment which may mimic or interfere with the action of endogenous estrogenic hormones. The biological effects of xenoestrogens depend largely on their availability and concentration within tissues and body fluids, their binding to and their effects on the estrogen receptor (Ignar-Trowbridge et al., 1993). Xenoestrogens derive from many sources and can be classified either as chemical and industrial compounds, which can be grouped together as industrial xenoestrogens, or as phytoestrogens, which are synthesized naturally by plants. In addition, there are metal ions with estrogenic potential, which can also be found in the environment.

20.1. Industrial Xenoestrogens

There has recently been considerable concern about the potential endocrine disrupting effect of chemicals released into the environment. Those chemical that have estrogenic activity are termed industrial xenoestrogens or endocrine disruptors. They are thought to mimic or disturb the function of estrogen and many are known to possess estrogen receptor binding activity (Danzo, 1997; Watanabe et al., 2004). Several synthetic

compounds, such as pesticides, herbicides, or industrial by-products display xenoestrogen activity. These compounds tend to accumulate in the environment and can also be found in food products. Thus they are considered as major environmental contaminants and it is assumed that these compounds, alone or in combination, may promote human disease (Auger et al., 1995; Coumoul et al., 2001).

Estrogen-like molecules are thought to function predominantly through estrogen mediated activation of transcription via estrogen responsive elements (ERE). Both estrogen receptor α and estrogen receptor β can interact with various cell cycle transcriptional factors (Frigo et al., 2002), but the molecular mechanisms of xenoestrogenic effects are not well understood. It is clear, however, that many of these chemicals function by binding estrogen receptors and blocking estrogen access (Lachlan., 1993; Hall et al., 2002). In addition to potential antagonistic activities, xenoestrogens can also induce activating conformational changes in estrogen receptors that enable the receptors to interact with co-activators and to activate target gene transcription (Routledge et al., 1993; Hall et al., 2002; Julie et al., 2002).

20.2. Phytoestrogens

Regulation of gene transcription is involved in the control of cell growth, proliferation, and differentiation (Hall et al., 2001; Muller et al., 2001; Muller et al., 2004). Due to the activation of estrogen receptor, some natural compounds produced by plants are referred to as phytoestrogens and have the potential to disrupt or modulate the endogenous estrogenic signalling. Phytoestrogens are compounds that are naturally presented in edible plant material. They are constituents of many human food stuffs such as beans, peas, sprouts cabbage, spinach, soybean, and hops. They include classes of compounds such as the isoflavones, lignanes, and comestans, such as comestrol (Price et al., 1985; Setchell et al., 1985). They are present to varying extents in the diet (Bennett et al., 1946; Kaldas et al., 1989). Some fifty years ago, researchers became aware that phytoestrogens in alfalfa and clovers could affect the fertility of livestock. More recently, multiple epidemiological studies have found a relationship between high dietary intake of isoflavones and lignans and lower rates of certain cancers, cardiovascular problems, and menopausal symptoms (MacMahon, 1973; Trichopoulos, 1984; Cornwell, 2004). As far

back as 1985, it was known that phytoestrogens could compete with estradiol for binding to intracellular estrogen receptors (Price, 1985). Although still inconclusive, scientific evidence is accumulating to suggest that phytoestrogens may have a role in preventing chronic disease. An especially strong body of evidence suggests that they may be effective in preventing and treating prostate cancer, due to their antiandrogenic properties (Adlercreutz, 2000). There is also concern that phytoestrogens in soy-based infant formulae might have adverse effects on the sexual development of infants (Sheehan, 1997). Phytoestrogens exert pleiotropic effects on cellular signalling and show some beneficial effects on estrogen-dependent disease (Muller et al., 2004). The phytoestrogens contained in the diet and in food supplements have the potential for both risks and benefits with respect to human health. Estrogenic chemicals consumed as constituent of plants (phytoestrogens) or fungi (mycoestrogens) exert a variety of adverse effects in animals and human including reduction in fertility in sheep grazing on phytoestrogen containing clover (Bennett et al., 1946; Kaldas et al., 1989), alteration of the uterus (Medlock et al., 1995; Branham et al., 2002), and behavioural development in rodents. Current research suggests that phytoestrogens may be natural "Selective Estrogen Receptor Modulators" (SERMs) (Setchell, 2001), which means that they can bind to certain estrogen receptors in some tissues, either activating or down-regulating cellular responses. Depending on concentrations of endogenous estrogens, as well as on the nature of the receptor complexes that are activated or down-regulated, SERMs can have either estrogenic or anti-estrogenic effects (National Cancer Institute, 2005). There has been tremendous interest in the possibility that dietary phytoestrogens may be an alternative to postmenopausal hormone therapy, because of concerns about side effects and long-term health consequences that prevent many women from using hormone therapy for amelioration of the discomforts and increased disease risk associated with the menopausal transition (Kurzer, 2003). Many postmenopausal women who have chosen not to take hormone therapy are currently using complementary and alternative therapies for menopause, and these include taking phytoestrogen supplements. Most of the supplements contain isoflavones derived from soybeans or red clover and some contain botanicals such as black cohosh (Kurzer, 1997; Anonymous, 2001; Kurzer, 2003).

20.3. Metal ions

Metals represent a new class of endocrine disruptors. Cadmium, selenite, copper, cobalt, nickel, chromium, lead, mercury, tin, vanadate and arsenite have been shown to activate estrogen receptor α by a mechanism involving the hormone-binding domain of the receptor (Garcia-Morales et al., 1994; Stoica et al., 2000 a; 2000 b; 2000 c; Martin et al., 2003).

Environmental contamination by toxic heavy metal ions such as cadmium, copper, zinc, and mercury from various sources (e.g., volcanic activity, weathering of rocks, and industrial, mineral mining, and agriculture activities) has been a problem for decades (Jensen et al., 1992), because these metals are not easily eliminated from the ecosystem. Cadmium enters the food chain through environmental contamination and concentrates within organisms because of its relatively long half-life. The main process for biological detoxification of heavy metals is binding to metallothioneins, which are a group of heavy-metal-binding proteins isolated from several tissues of many vertebrates (Olsson et al., 1993). Metal-containing proteins could be a major target of toxic heavy metals. One mechanism for such toxicity has been assumed to be the disturbance of proper metal – protein interaction that is essential for maintaining correct protein structure (Gong et al., 2000). Zinc finger structures are frequently found in transcription factors and DNA repair proteins, mediating DNA-protein and protein-protein binding. As low concentrations of transition metal compounds, including those of cadmium, nickel, and cobalt, have been shown to interfere with DNA transcription and repair, several studies have been conducted to elucidate potential interactions of toxic metal ions with zinc-binding protein domains. Various effects have been identified, including the displacement of zinc, *e.g.*, by cadmium or cobalt, the formation of mixed complexes, incomplete coordination of toxic metal ions, as well as the oxidation of cysteine residues within the metal-binding domain. Besides the number of cysteines and/or histidine ligands, unique structural features of the respective protein under investigation determine whether or not zinc finger structures are disrupted by one or more transition metals. As improper folding of zinc finger domains is mostly associated with the loss of correct protein function, disruption of zinc finger structures may result in interference with manifold cellular processes involved in gene expression, growth regulation, and maintenance of genomic integrity.

21. Aims of the Present Study

Although a mechanism by which estrogen exerts its actions - binding to its intracellular receptor and association of this complex with specific estrogen response elements in the genome - has been known for many years, this classical model is unable to explain the majority of estrogen effects. The regulation of estrogen-controlled genes nowadays can be investigated on a genome-wide scale by techniques such as microarray hybridization (Frasor et al., 2003; Hewitt et al., 2003). However, the promoter regions of most of these estrogen-stimulated genes apparently do not contain functional estrogen response elements. As described above, a number of non-classical mechanisms of estrogen action have already been characterized. These experiments led to the important conclusion that estrogen is able to regulate a large number of promoters by a variety of different mechanisms.

The regulation of the gene for the neuropeptide hormone oxytocin has been a central interest of research at the Institute of Hormone and Fertility (IHF) for many years. Other groups had tried to explain the estrogen-dependent up-regulation of the oxytocin promoter in transient transfection experiments (Richard et al., 1990; Burbach et al., 1990) by the classical mechanism of binding of the activated estrogen receptor to a partially conserved estrogen response element. The characterization of the regulatory regions of the bovine gene, where this palindromic sequence is not conserved, however, led to the identification of nuclear orphan receptors binding to this region in the proximal oxytocin promoter (Walther et al., 1991; Wehrenberg et al., 1992). A more detailed analysis of the proteins binding to this element revealed that, under conditions of active oxytocin expression in vivo, this binding site is occupied by the tissue-specific nuclear orphan receptor SF-1 (Wehrenberg et al., 1994a & 1994b). In a follow-up study aimed to resolve the question whether the partially conserved palindromic sequence in the human oxytocin promoter represents a functional estrogen response element, extensive quantitative in vitro binding studies were carried out (Stedronsky et al., 2002). The affinity of this human promoter element for both estrogen receptor isoforms was shown to be extremely low or non-existent, whereas the same sequence constitutes a high-affinity binding site for SF-1. From these experiments it can be concluded that the oxytocin promoter in all species investigated is not controlled directly by the activated estrogen receptor.

Transient transfection experiment using breast cancer cell lines performed in the course of this study nevertheless showed a dramatic up-regulation of transcription from the bovine oxytocin promoter in the presence of the ligand-activated estrogen receptor. Although the protein binding to the regulatory promoter element could not yet been identified, the up-regulation in this tissue culture system presumably is controlled by the same general mechanism, namely indirect transcriptional activation by interaction with a nuclear orphan receptor bound to the proximal oxytocin promoter. The study presented here now aims at the elucidation of this mechanism by a detailed analysis of the components of the regulatory system controlling the oxytocin promoter. Transient transfection experiments using the breast cancer cell line MDA-MB 231 were performed and are presented here. The result of this study will contribute to our understanding of the non-classical mechanisms of estrogen action that control the expression of a large number of genes important for normal human development as well as for clinically relevant diseases.

MATERIALS and METHODS

1. Plasmids and Cells:

Luciferase indicator plasmids containing the wild-type bovine oxytocin promoter (-183 to +17) (Figs 10 and 14), the bovine oxytocin promoter containing a mutant of the regulatory element around position -160 (Fig 10) or the bovine oxytocin promoter with the total substitution of the -160 element by a scrambled sequence had been constructed by cloning the respective sequences into the polylinker region of PGL3 Basic (Promega, Mannheim, Germany). As controls in transfection experiments, the empty vector PGL3 Basic carrying neither promoter nor transcriptional enhancer sequences (Fig 11) and the control vector PGL3 Control, a construct expressing high levels of luciferase under the control of promoter and enhancer from simian virus 40 (Fig 12) was used. For over-expression of estrogen receptor β, plasmid constructs expressing human and bovine estrogen receptor β cDNA sequences under the control of the cytomegalovirus immediated early promoter were used (Walther et al., 1999). A mutant of estrogen receptor α unable to bind specifically to DNA, had been constructed by substitutions of amino acids E230A and G204A, in analogy to the mouse estrogen receptor α P-box mutant (Jakasca et al., 2001) (Fig 13). These plasmid constructs were all kindly provided by Dr. Norbert Walther (Institute of Hormone and Fertility Research, (IHF) Hamburg, Germany).

A luciferase indicator construct containing the thymidine kinase promoter controlled by a vitellogenin estrogen response element (TK-ERE) (Lees et al., 1989), had been constructed by cloning these recombinant promoter sequences into the polylinker site of PGL3 Basic as described above (Stedronsky et al., 2002) (Fig 14). This plasmid construct, together with the β-galactosidase reporter construct pCMV lacZ, a vector expressing the Escherichia coli lacZ gene from the cytomegalovirus immediated early promoter, was kindly provided by Dr Ralph Telgmann (IHF). An expression vector containing the cDNA sequence of human estrogen receptor α (ERα) under the control of the cytomegalovirus immediated early promoter (Fig 6) (Walter et al., 1985; Green, 1986) was obtained from Dr. Katrin Stedronsky (IHF). The MDA-MB 231 breast cancer cell line was kindly provided by Professor Holger Kalthoff from Christian-Albrechts-

Universität, (Kiel, Germany), and the MCF-7 breast cancer cell line was a gift by Dr. Norbert Walther (IHF).

2. Estrogen, Xenoestrogens and Metals

The estrogen receptor agonists and antagonists: 17ß-estradiol, 4OH-tamoxifen, raloxifen, and $ICI_{182,780}$ all from Sigma-Aldrich were kindly provided by Dr. Norbert Walther (IHF). Naringenin, 6-(1, 1-dimethylallyl)-naringenin (6DMA-naringenin) and 8-prenylnaringenin (8p-naringenin) were a kind gift from Prof. Günter Vollmer (University of Dresden, Germany). Cupric chloride (Merck), zinc chloride (Fluka), lithium chloride (Merck), magnesium chloride (Merck), cupric sulphate (Sigma-Aldrich), cobalt chloride (Sigma-Aldrich), lead chloride (Sigma-Aldrich), mercury chloride (Sigma-Aldrich), zinc sulphate (Sigma-Aldrich), and cobalt chloride (Fluka) were kindly provided by Dr. Stefan Hartung (IHF). ß-HCH (1,2,3,4,5,6,-hexachloro-,(1alpha,2.beta,3.alpha,4.beta,5.alpha,6.beta) or (beta-benzenehexachloride) was purchased from Supleco, Bellefonte, USA and o,p'-DDT [1.1.1.-trichloro-2-(o-chlorophenyl)-2-p-chloriphenyl) ethane] from Chem Service, West Chester, USA. p,p'-DDE (2-2-bis(4/chlorophenyl)-1-1-dichloroethyl), Methoxychlor (1,1,1-trichloro-2-2-bis-(p-methoxy phenyl)ethane-2-2-bis(4-methoxyphenyl)1-1-1-trichloroethan), sodium metavanadate, toxaphen, diethylstilbestrol (DES), resveratrol (3,4,5-trihydroxy-transe-stilberne,5-(1E)-2-(4-hydroxyphenyl)-1-3-benzenediol), and Cadmium chloride were all purchased from Sigma-Aldrich.

3. Transformation

The E.coli strain DH5α (Invitrogen life technology, Carlsbad, CA, USA) was made competent via chemical modification as described by Inoue et al. (1990), and stored in aliquots at -80°C.

Competent DH5α cells were thawed on ice and aliquoted into microcentrifuge tubes (Eppendorf, Hamburg, Germany). 1µg of plasmid was added to the competent DH5α cells and incubated for 30 minutes on ice. The cells were heat-shocked at 42°C for 45 seconds, and cooled immediately on ice. 1 ml of LB (Luria Bernina) medium (10 g/l bacto-tryptone (Difco), 5 g/l Bacto yeast extract (Difco), 10 g/l NaCl (Merck), pH 7.5)

were added to each transformation reaction and the cells incubated at 37°C for 1 hour with shaking.

During this time the cells begin to divide, passing from stationary into logarithmic growth phase. After several generations, the bacterial cells possessing the desired recombinant vector will begin to express their antibiotic resistance. The standard cloning vectors used in this study all include the resistance gene bla coding for a periplasmic enzyme, ß-lactamase, which cleaves the ß-lactam ring of ampicillin and prevents it from impeding bacterial cell wall synthesis. Following incubation at 37°C for 1 hour, cells were plated on LB agar plates (LB medium and 15 g/l agarose) plus 100µg/ml ampicillin and incubated at 37°C overnight.

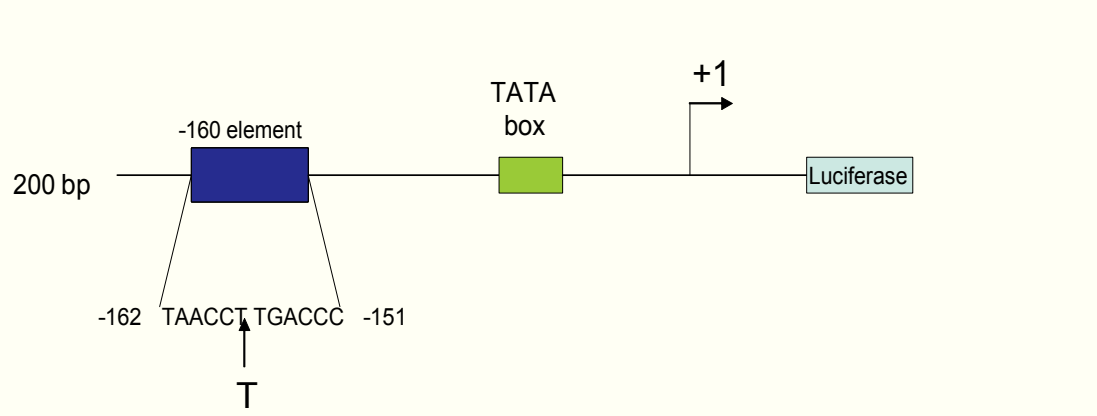


Fig 10: Mutation of the -160 element in the bovine oxytocin promoter: Insertion of a single T at position -157.

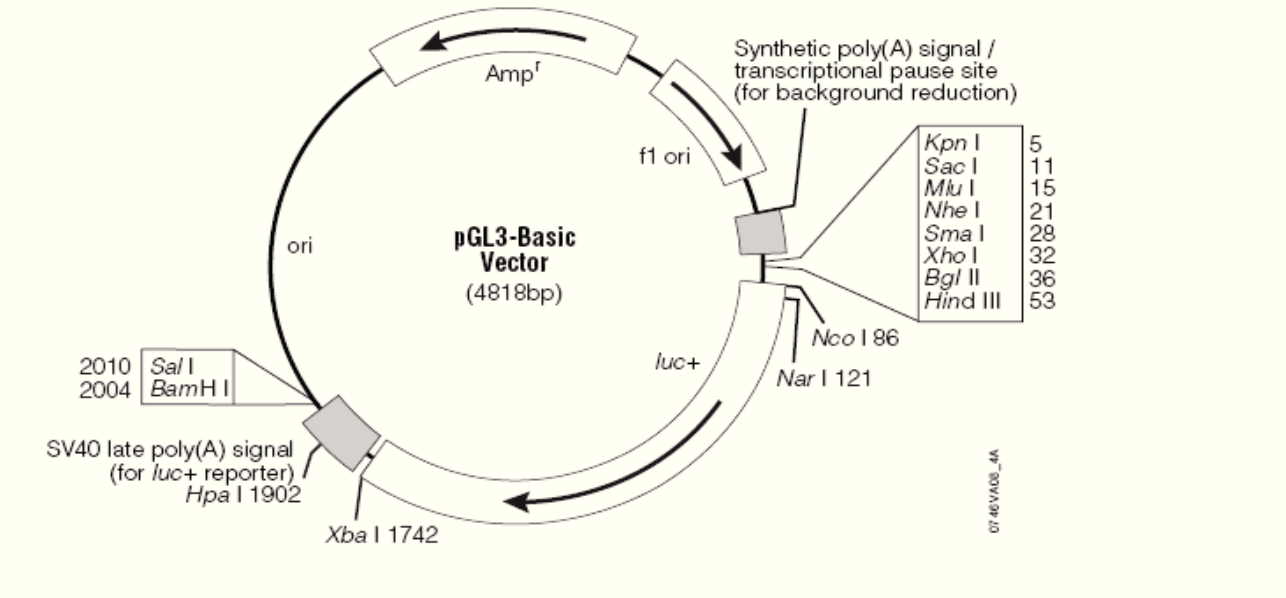


Fig 11: PGL3 basic vector circle map. Additional description: Luc^{+}, cDNA encoding the modified firefly luciferase; Amp^{r}, gene conferring ampicillin resistance in E.coli; f1 ori, origin of replication derived from filamentous phage; ori, origin of replication in E.coli. Arrows within luc+ and the Amp^{r} gene indicate the direction of transcription; the arrow in the f1 ori indicates the direction of ssDNA strand synthesis (Promega).

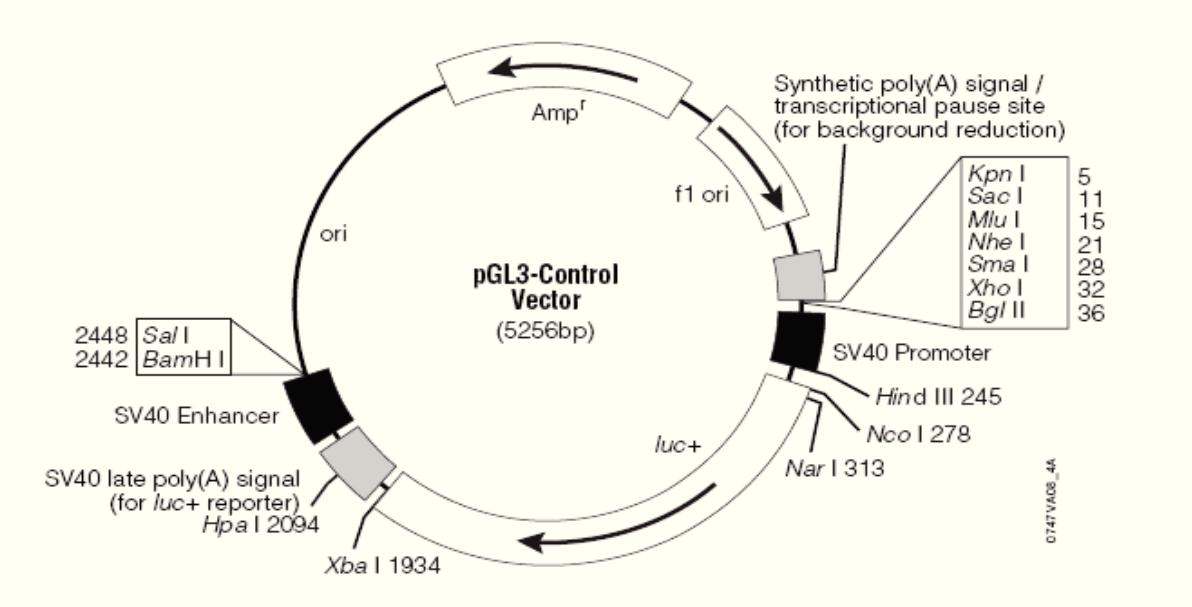


Fig 12: PGL3 control vector circle map. Additional description: luc+, cDNA coding the modified firefly luciferase Amp^{r}, gene conferring ampicillin resistance in E.coli; f1 ori, origin of replication derived from filamentous phage; ori, origin of replication in E.coli. Arrows within luc+ and the Amp^{r} gene indicate the direction of transcription; the arrow in the f1 ori indicates the direction of ssDNA strand synthesis (Promega).

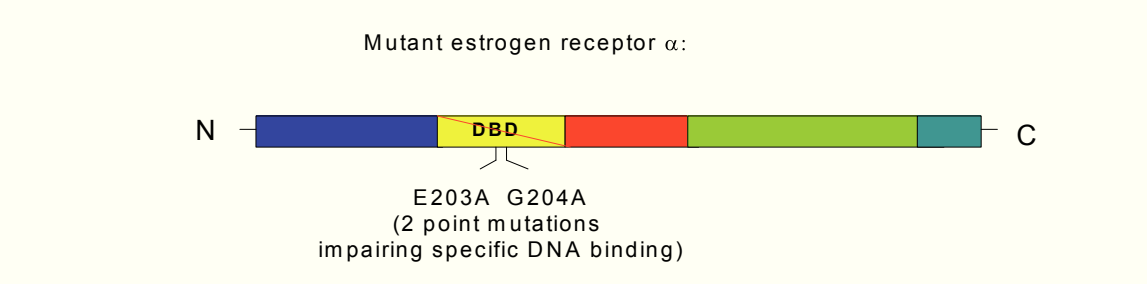


Fig 13: Structure of the mutant estrogen receptor α. Two point mutations impairing specific DNA binding of estrogen receptor α have been inserted into the P-box sequence within the DNA-binding domain (DBD).

4. Plasmid Preparation

One single antibiotic-resistant bacterial colony was used to inoculate 3 ml of LB medium in 15 ml conical tubes (Sarstedt), supplemented 100 µg/ml ampicillin. Cultures were incubated overnight at 37°C with shaking, and placed on ice for 5 min. Plasmid DNA was recovered using an alkaline lysis method for small scale plasmid preparation (Mini-prep) and resuspended in 50 µl of TE buffer (Le Gouill et al., 1994). This method generally yielded 2-3 µg of plasmid DNA. To confirm the presence of subcloned DNA, plasmid DNA was subjected to restriction endonuclease digestion, followed by analysis of the DNA fragments using agarose gel electrophoresis.

To obtain a larger quantity of the desired plasmid DNA (100-500 µg), aliquots of mini-prep cultures were used to inoculate a larger volume of medium (250 ml) supplemented with 100µg/ml ampicillin. After incubation overnight at 37°C with shaking, cells were harvested by centrifugation at 4°C for 10 minutes at 4000 rpm. Cultures were further processed using the Endofree Plasmid Maxi Kit (QIAGEN) for plasmid DNA preparation as described by the manufacturer. Purified DNA was stored at 4°C and DNA concentrations were determined spectrophotometrically (see below).

5. Spectrophotometric Determination of DNA Concentration

For quantification of DNA the optical density (OD) of the solution was spectrophotometrically measured at wavelengths 260 nm (OD260) and 280 nm (OD280). The ratio between OD260 and OD280 (OD260/OD280) estimates the purity of the nucleic acids; all preparations of DNA had an OD260/OD280 ratio of approximatly1.8,

indicating a lack of contaminating protein or other substances. The concentration of the plasmid DNA was calculated using the equation: One OD 260 unit is equivalent to a concentration of 50μg/μl. All plasmid preparations were adjusted with TE buffer to a final concentration of 1μg/μl. The plasmids were stored at 4°C for use in transfection experiment or at -20°C for long time storage.

6. Estrogen-free (charcoal-stripped) FCS (CCS) Preparation

Charcoal-stripped serum was prepared by a protocol proposed by Dr. Olaf Bartsch (IHF): Firstly, 1g of activated charcoal (Sigma) and 0.1 g dextran (Sigma) plus 200ml of water were mixed and shaken at 4°C overnight. The next day 500 ml of foetal calf serum (FCS) were mixed with 1000 units of sulfatase (Sigma) and incubated in a water bath at 37°C for 2hours. The activated charcoal was aliquotted into twenty 50 ml tubes (10 ml per tube) and the tubes were centrifuged at 3000-4000 rpm for 10 minutes. The supernatants were removed and the tubes were kept at 4°C. After 2 hour, the FCS was aliquotted into ten of the 50ml tubes containing the activated charcoal, which had previously been stored at 4°C. After mixing the tubes were placed in the 56°C water bath for 30 minutes and then on ice for 5 minutes. The tubes were centrifuged at 2000 rpm and 4°C for 15 minutes and the supernatants were transferred into the next 10 tubes containing activated charcoal. The tubes were centrifuged again as before, the supernatants were sterile filtered by 0.2 μm membrane filters and stored at -20°C.

7. Cell Culture

The MDA-MB 231 cell line was used because it had been shown to be devoid of functional estrogen receptor α and estrogen receptor β (Stedronsky et al., 2002). MDA-MB 231 cells were plated out in small 25 cm^2 culture flasks (Nunc, Wiesbaden, Germany) in phenol red free Dulbecco's modified Eagle's medium (DMEM) (Gibco, Karlsruhe, Germany) supplemented with 10% of steroid-free (charcoal-stripped) foetal calf serum (CCS) plus 1% of L-glutamine (Sigma, Taufkirchen, Germany) and 0.5% of penicillin/streptomycin (Sigma) and incubated at 37°C / 5% CO_2. Before reaching confluence, cells were detached from the bottom of the flask by the addition of 1.5ml 1x Viralex TM-EDTA solution (0,05% trypsin, 0,5 mM EDTA) followed by incubation at

37°C for 2 min. Cells were harvested by centrifugation for 5 minutes at 800 rpm and resuspended in culture medium. The resuspended cells were re-plated either into another flask (75cm^2) at 1/3 density or into 12- well plates (Nunc) at a density of 100000 cells per well for transfection.

8. Transient Transfection Assays– Principle

Transfection is a non-viral method of introducing DNA into eukaryotic cells. There are several transfection techniques which have been developed utilising either chemical (DEAE-dextran, calcium phosphate, artificial liposomes) or physical (direct microinjection, electroporation, biolistic particle delivery) manipulation of DNA into cells.

Generally a promoter or enhancer sequence is cloned 5' to the transcriptional start site of the coding sequence for the reporter gene. Once the reporter vector is introduced into the cell, synthesis of the reporter gene product is solely based on whether or not the cloned promoter or enhancer sequence can activate gene transcription. Cells can then be analysed for the presence of the reporter gene product following transfection by specific reporter assays.

All pGL3 vectors contain a modified firefly luciferase cDNA as a reporter gene. Therefore, transcriptional activity of a promoter or enhancer cloned into a pGL3 vector can be measured as a function of the enzymatic activity of firefly luciferase. The enzyme catalyses the oxidation of beetle luciferin through the intermediate luciferyl coenzyme A; this is a luminescent reaction since oxidation of beetle luciferin leads to photon production which can be measured in a luminometer at 560 nm.

8.1. Calcium Phosphate Co-precipitation Method

The calcium phosphate method for transfection involves mixing plasmid DNA with $CaCl_2$ in a phosphate buffer and incubating at room temperature. The calcium ions in solution are positively charged and will interact with negatively charged plasmid DNA to form a precipitate. The precipitate is distributed over cultured cells and is taken-up into the cells by endocytosis.

Cells were generally plated out at 100000 cells/well in 12-well plates (Nunc) the day before the transfection. Three hours prior to the transfection, the medium was replaced with fresh medium, and cells were incubated at 37°C / 5% CO_2 until transfection. Meanwhile the components of the Profection Mammalian Transfection System-Calcium Phosphate (Promega, Madison, USA) and the plasmids were equilibrated to room temperature.

For transfection, the DNA was added to 100.6 µl of nuclease free water in sterile Eppendorf tubes. For each transfection 3 wells were transfected in parallel with a total amount of 9µg of DNA (3µg/well) For standard transfection of 3 wells, 3µg of promoter–luciferase plasmid, and 4.5µg estrogen receptor expression vector, and 1.5µg pCMV lacZ (β galactosidase- plasmid) were employed. Together with 15.4µl of 2M $CaCl_2$ to a micro centrifuge tube and 125µl Hepes-buffered saline (2x HBS) was added to the other sterile 5 ml polystyrene round–bottom tubes (Falcon USA). Then, under constant shaking on a vortex mixer, the DNA-$CaCl_2$ solution was added slowly to the tube containing 2x HBS and the tube was left for 30 minutes at room temperature for the formation of the precipitate. After 30 minutes, 75µl of the resulting DNA-$CaCl_2$ precipitate were added to each of the parallel wells. The plates were incubated at 37°C / 5% CO_2 over-night.

9. Cell Stimulation

Sixteen hours after transfection, the medium was replaced by fresh medium and the cells were stimulated with the estrogen agonists, partial agonists, or antagonists, 17β-estradiol, 4OH-tamoxifen, raloxifen or $ICI_{182,780}$, or as indicated with industrial xenoestrogens, phytoestrogens, metals ions, or only vehicle. The compounds were dissolved in ethanol, DMSO or water, and stock solutions were stored at -20°C. For stimulation of cells, stock solutions were diluted with medium to a concentration 100 fold greater than the final concentration required, and 10µl of estrogen (ant)agonist dilutions were added per 1ml of medium per well. For the unstimulated controls, an equivalent amount of ethanol, DMSO, or water was added to the transfected cells. The plates were incubated again at 37°C / 5% CO_2. After 24hr, the cells were harvested for measuring the transfection efficiency and the transcriptional activity of the oxytocin promoter.

10. Preparation of Cell Lysates

24 hour after stimulation, cells were washed 2 times with phosphate-buffered saline (Sigma-Aldrich), then 100 µl of 1 x passive lysis buffer (Promega) was added and the plates incubated at room temperature for 5 minutes with slow shaking. Then the cell lysates were scraped with a cell scraper and transferred to Eppendorf tubes on ice. The tubes were centrifuged for 1 minute at 13000 rpm and the supernatants transferred to new tubes. The supernatant was either transferred immediately to -80°C or directly used for determination of the luciferase or the β-galactosidase activity.

11. Firefly Luciferase Assay

The firefly luciferase reporter gene assay system (Promega) was used according to the manufacturer's instructions to assess the transcriptional activity of the bovine oxytocin promoter or the thymidine kinase-ERE promoter. 20µl of lysate, prepared as described above, were added to 100 µl of Luciferase Assay Reagent (Promega) in luminometer tubes, and the luminiscence of the reaction was measured for 5 seconds with 2 seconds delay time in a luminometer (Berthold Sirius Luminometer).

12. β-Galactosidase Assay

pCMV-lacZ, co-transfected into mammalian cells, is a reporter vector expressing E.coli ß-galactosidase gene (LacZ). It is used in transfections as a positive control for monitoring the transfection efficiencies of mammalian cells. In all mammalian cells tested, the strong viral CMV promoter activates transcription of the LacZ gene. The Tropix Galacto-Light^{Tm} assay system (Applera Biosystem) was used to measure ß-galactosidase activity in the cell lysates. Apart from being able to cleave the molecule X-gal, ß-galactosidase is capable of deglycosylating the substrate Galacton^{TM} at a neutral pH. Following the protocol supplied with the kit, 5µl of lysate were incubated with 200 µl of Galacton substrate diluted 1:100 in reaction buffer (100 mM Na_2PO_4, 1 mM $MgC1_2$; pH 8.0) for 0.5- 1 hour at room temperature in luminometer tubes. Then 300 µl of Light Emission Accelerator Buffer was added to each reaction and light emission was measured for 10 seconds with 2 seconds delay time in a luminometer (Berthold Sirius Luminometer).

After measurement of luciferase and β-galactosidase activities of each single cell lysate, the transcriptional activity of the promoters under investigation were normalised by dividing the luciferase values by the β-galactosidase values to control for the transfection efficiency, resulting in arbitrary so-called "relative light units". The mean and standard deviation (± SD) were calculated for all stimulation experiments using the Graphpad Prism 3.0 software package (Graph Pad Software Inc., San Diego, USA). All transfections were performed at least in triplicate with identical results.

13. Cell Toxicity Assay

A cell toxicity assay had to be performed to ensure that the concentrations of the compounds used in transfection experiments were not toxic to the cells. Two different methods of cell toxicity assays were used:

13.1. Neutral Red Assay

MDA-MB 231 and MCF-7 cells were seeded into 75cm^2 flasks (Nunc) and the flasks were incubated at 37°C / 5% CO_2 at 90% humidity. After 24 hours the medium was removed from the flask and cells were washed 3 times with 5 ml of pre-warmed phosphate-buffered saline (PBS) (Sigma). Cells were detached with 1.5 ml of trypsin (Sigma), cells were then shaken from the flask, and 9ml of growth medium were added. The cell suspension was then put into a 15 ml centrifuge tube (Falcon) and centrifuged for 5 minutes at 1000 rpm. The supernatant was then discarded and 5 ml of PBS were added to the tube, and the cells were centrifuged again for 5 minutes at 1000 rpm. The supernatant was discarded again and cells resuspended in 10 ml of growth medium. Cells were counted in a Neubauer counting chamber, diluted to 10^5 cells /ml and then 200µl /well seeded in to 96 well plates (Costar-3595) (2.10^4 cells per well). Cells were incubated for 24 hrs at 37°C / 5% CO_2 at 90% humidity. On the second day, the samples to be tested for toxicity were prepared in different concentrations and diluted in medium. The control samples were also prepared (vehicle and control). The medium was removed from the cells and the controls and prepared samples were added to the wells (all 9 wells in the same row containing the same sample), (200µl per each well) and the plate incubated at 37°C / 5% CO_2 at 90% humidity for 24 hours. Meanwhile a neutral-red

(Sigma) solution was prepared at a concentration of 2mg/100ml of serum free Dulbecco's modified Eagle's medium (DMEM) (Gibco) and incubated at 37°C in an atmosphere containing 5% CO_2 at 90% humidity for 24 hours. On the third day the medium was removed from the wells and the cells were washed tree times with phosphate-buffered saline (PBS). Neutral red was sterilized by filtration through a membrane filter (0.2μm) and added to the wells (200 μl per well) and the cells were incubated for 3 hours at 37°C in an atmosphere containing 5% CO_2 at 90% humidity. After 3 hours the neutral red solution was removed from the wells and cells were washed 2 times with phosphate buffered saline (PBS). 1ml of 50% EtOH + 1% acetic acid solution was added to each well and the plate was used for photometric measurement at 540 nm in a Spectracount photometer (Packard), and the results used to prepare the graph of the range of cell toxicity.

13.2. Plating Test

The process of the experiment is as follows: Cells were seeded in 75cm^2 flask (Nunc) and after 24 hours medium was removed from the flask and cells were washed 2 times with 5 ml of pre-warmed phosphate buffered saline (PBS) (Sigma). Cells were detached with 1.5 ml of 1x trypsin (Sigma) and cells were scraped from the flask and 9 ml of growth medium were added, the cell suspension put in to a 15 ml centrifuge tube (Falcon) and centrifuged for 5 minutes and 1000 rpm. The supernatant was discarded and 5 ml of PBS were added to the tube and the cells centrifuged again for 5 minutes at 1000 rpm. The supernatant was discarded again and the cells resuspended in 10 ml of growth medium. Cells were counted in a Neubauer counting chamber. For the control and each concentration of compound, tree flasks(25ml- Nunc) were inoculated with 200 cells per flask and 4ml of medium (DMEM). Flasks were incubated for 24 hours at 37°C / 5% CO_2 at 90% humidity, on the second day the control and the different concentrations of compounds were prepared as described above. The medium of the cells was changed with fresh medium and 40 μl of control or prepared concentrations were added to the flasks (3 flasks for each concentration) and flasks were incubated again at 37°C / 5% CO_2 at 90% humidity again for 24 hours. On the third day the medium was changed with fresh medium and flasks were incubated as before. After a week the flasks were checked, the

medium was removed and the cells were washed 2 times with phosphate-buffered saline (PBS). 3 ml of methanol (Sigma) was added to the flasks for 20 minutes, then the methanol was removed and 4 ml of 10% Giemsa stain (Merck) was added to the flasks, after 20 minute the Giemsa stain was removed and the cells were washed with water. The flasks were put to dry and after drying the number of colonies in the flasks was counted. The average of colony number per flask dependent on the concentration of compounds or control was calculated and used to prepare the graph to show the concentration range of cell toxicity for the different concentrations of the compounds.

Results

1) Mechanism of Estrogen-Dependent Up-Regulation of the Oxytocin Promoter

In order to study the effects of estrogen and xenoestrogens on the transcription from promoters controlled by the classical or non-classical mechanism of estrogen action, the human breast cancer cell line MDA-MB 231 has been used as a model. The reason for selecting these cells is that they contain no estrogen receptor, allowing a detailed investigation of functions of wild-type and mutant forms of estrogen receptors, or different estrogen receptor subtypes in transient transfection studies. The luciferase indicator plasmid constructs used (Fig 14) contained either the thymidine kinase promoter, controlled by a single copy of the classical estrogen receptor binding site, the vitellogenin estrogen response element (TK ERE), or the wild type bovine oxytocin promoter (OTwt), shown to be unable to bind estrogen receptors efficiently (Stedronsky et al., 2002). These plasmids were transfected into MDA-MB 231 cells together with a plasmid expressing human estrogen receptor α (ERα), and a β-galactosidase expressing plasmid as an internal control for transfection efficiency. For comparison, the human MCF-7 breast cancer cell line was used as a control cell line in some of the experiments.

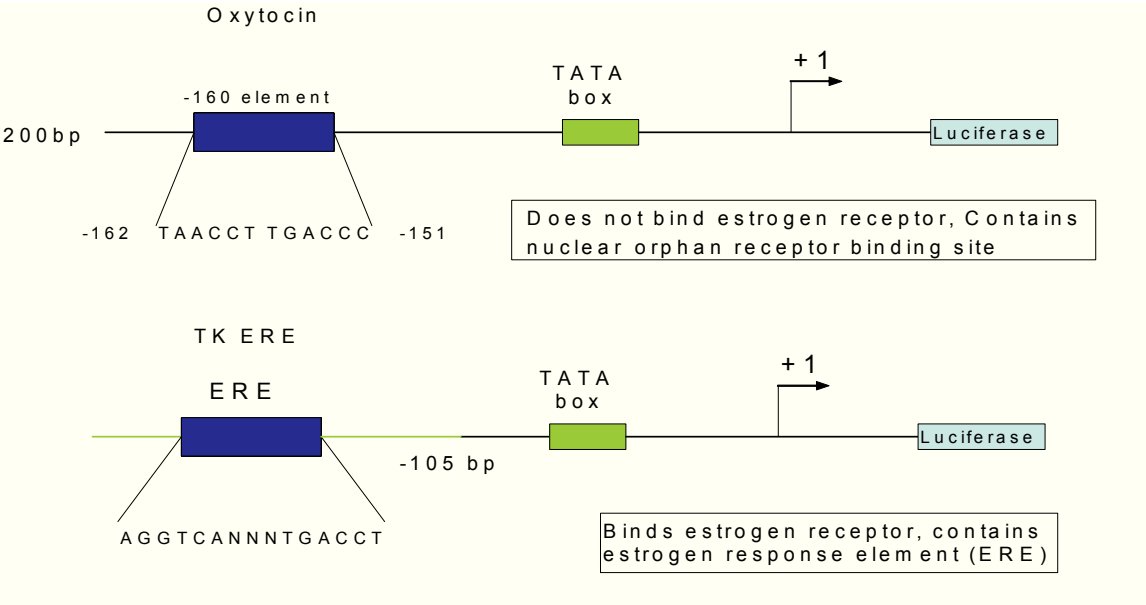


Fig 14: Structure of luciferase indicator plasmids containing the oxytocin, or the thymidine kinase ERE (TK ERE) promoter.

In preliminary experiments, the MDA-MB 231 and MCF-7 cells were transfected as described above and treated with increasing amounts of 17β-estradiol (E_2) (Fig 15). The results indicate that 17β-estradiol has dose-dependent and agonistic effects on the oxytocin promoter in MDA-MB 231 and MCF-7 cells in the presence of transfected estrogen receptor α. Half-maximal stimulation was achieved at a concentration around 10^{-10} M at the thymidine kinase-ERE promoter, whereas 10^{-9} to 10^{-8} M estradiol were necessary to achieve half-maximal stimulation of the wild type oxytocin promoter. Without co-transfection of estrogen receptor α, no estrogen-dependent up-regulation could be detected in MCF-7 cells indicating that the cells may have insufficient expression of the endogenous estrogen receptor for this promoter (data not shown).

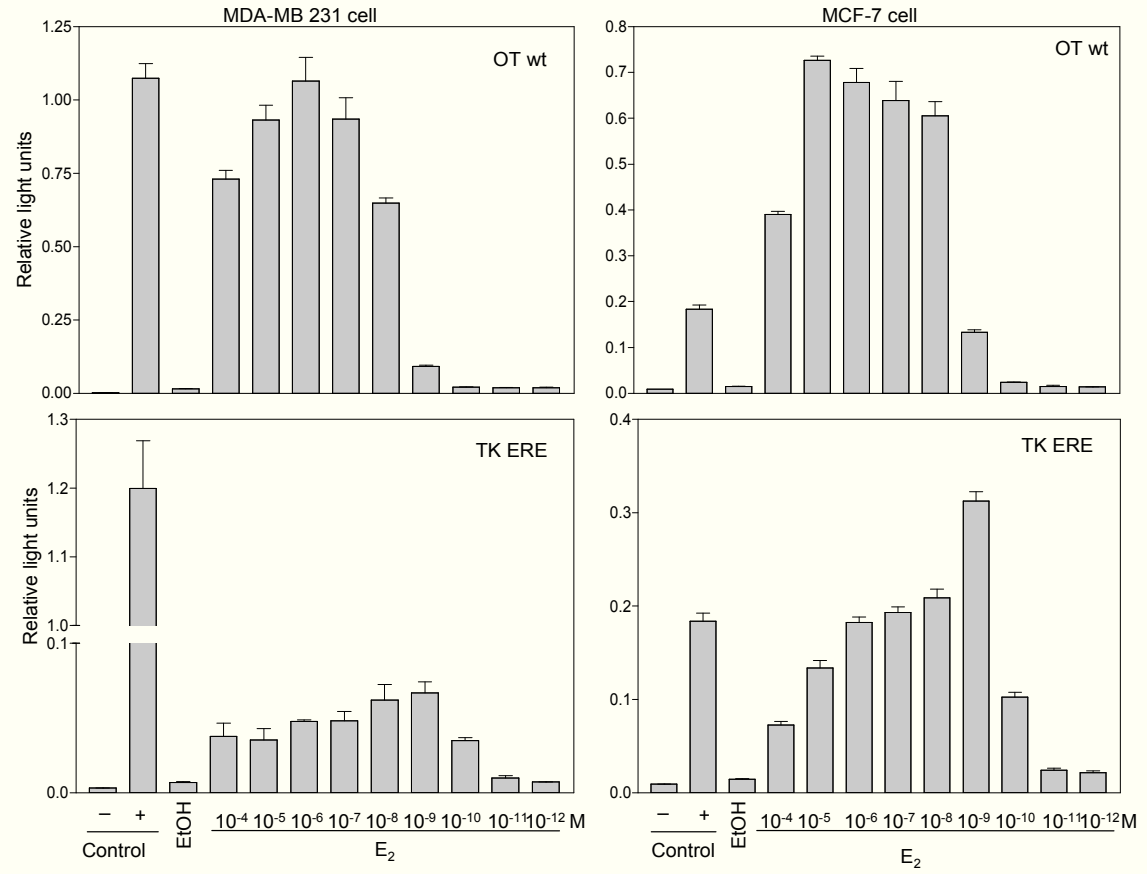


Fig 15: Activation of the oxytocin promoter (OTwt) and thymidine kinase–ERE promoter (TK ERE) by estrogen receptor α (ERα) bound by the ligand estradiol. MDA-MB 231 and MCF-7 cells were transfected with the OTwt construct or TK ERE construct and ERα and treated with different amounts of the estradiol (E_2).

The agonistic effects of E_2 are defined by comparison with the transcriptional activity of the respective promoter without any stimulation (only ethanol added). In further experiments the MDA-MB 231 cells were transfected with the luciferase indicator constructs containing the bovine oxytocin or the thymidine kinase-ERE-controlled promoter as well as the estrogen receptor α expression vector and treated with increasing amounts of 4OH-tamoxifen and raloxifen, as examples of SERMs (Fig 16, Fig 17). The results indicate that the two compounds show dose-dependent agonistic effects on the bovine oxytocin promoter, whereas no agonistic effects of the two compounds could be demonstrated at the thymidine kinase-ERE promoter. On the contrary, both compounds are able to suppress in a dose-dependent fashion the basal activity of the thymidine kinase-ERE promoter.

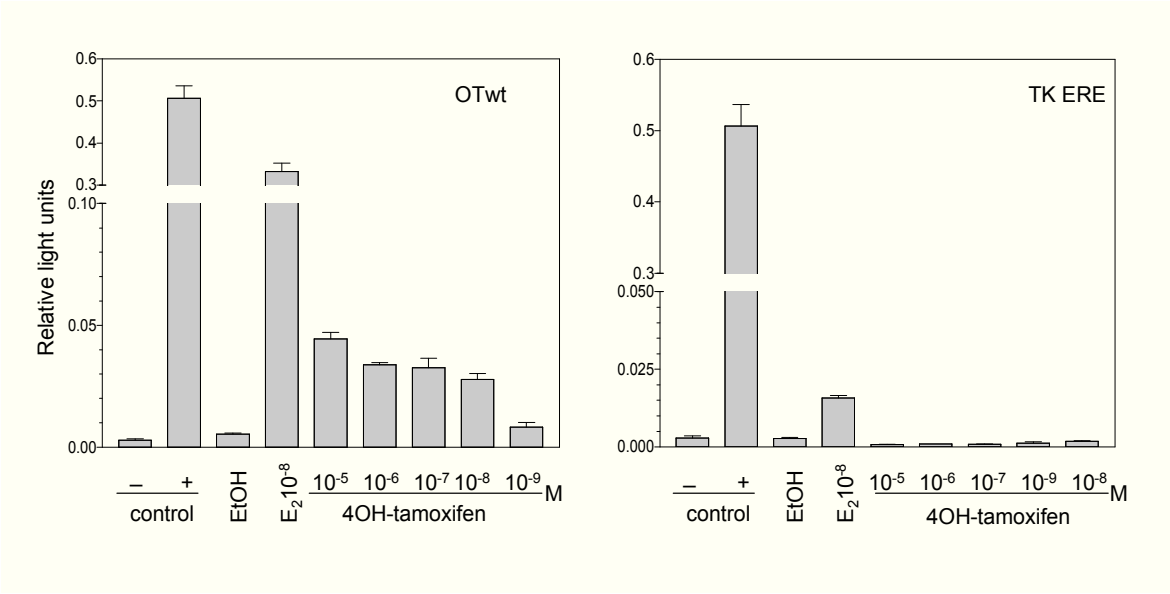


Fig 16: Activation of the oxytocin promoter (OTwt) and thymidine kinase–ERE promoter (TK ERE) by estrogen receptor α (ERα) bound by the ligand 4OH-tamoxifen. MDA-MB 231 cells were transfected with the OTwt construct or the TK ERE construct and ERα and treated with different amounts of 4OH-tamoxifen.

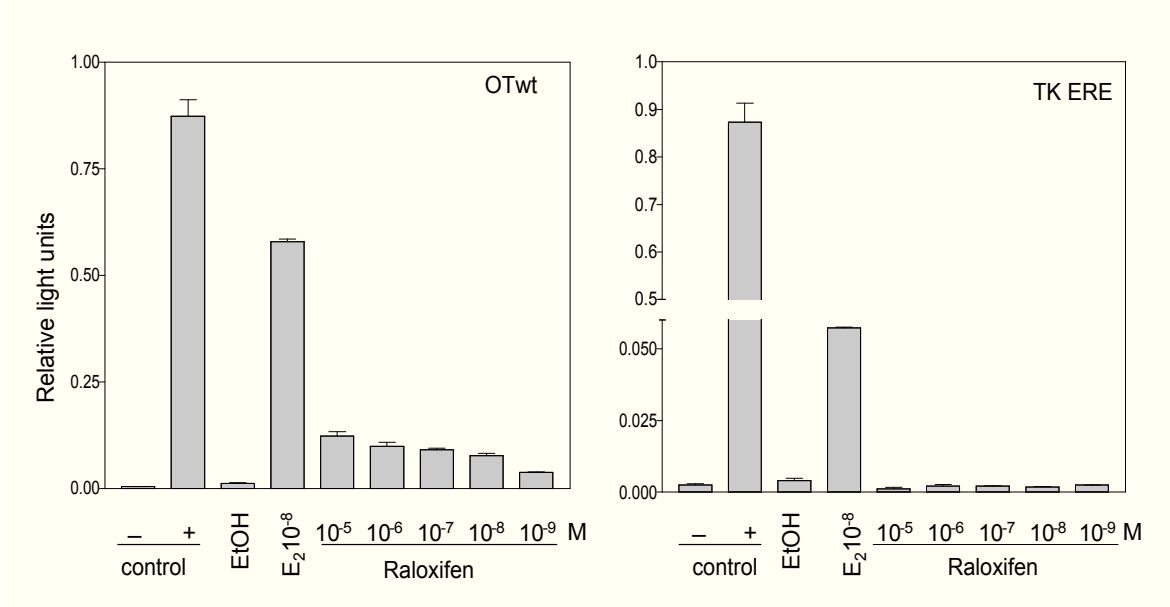


Fig 17: Activation of the oxytocin promoter (OTwt) or the thymidine kinase-ERE promoter (TK ERE) by estrogen receptor α (ERα) bound by the ligand raloxifen. MDA-MB 231 cells were transfected with the OTwt construct or the TK ERE construct and ERα and treated with different amounts of raloxifen.

For comparison of the results of the experiments using the partial estrogen agonists 4OH-tamoxifen and raloxifen with the effect of a pure estrogen antagonist, MDA-MB 231 cells were transfected as described above with the bovine oxytocin promoter or the thymidine kinase-ERE promoter and the estrogen receptor α expression vector. The transfected cells were then treated with increasing amounts of $ICI_{182,780}$ (ICI) (Fig 18).The results show that $ICI_{182,780}$, like estradiol, tamoxifen, and raloxifen, also has agonistic effects on the oxytocin promoter, whereas on the thymidine kinase-ERE promoter, as expected, this compound shows a complete lack of agonistic effects.

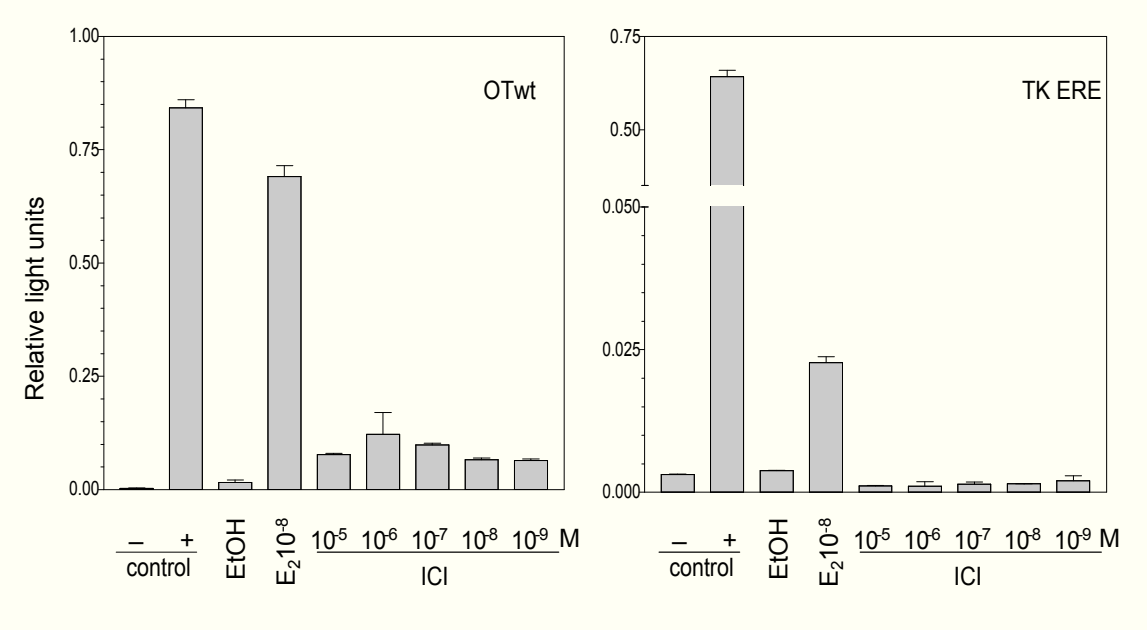


Fig 18: Activation of the oxytocin promoter or the thymidine kinase-ERE promoter by estrogen receptor α bound by the ligand $ICI_{182,780}$. MDA-MB 231 cells were transfected with the oxytocin promoter (OTwt) construct or the thymidine kinase-ERE promoter construct (TK ERE) and estrogen receptor α (ERα) and treated with different amounts of $ICI_{182,780}$ (ICI).

As a control experiment for the role of the estrogen receptor in estrogen – dependent up-regulation of the oxytocin promoter, MDA-MB 231 cells were transfected with the oxytocin promoter construct or the thymidine kinase-ERE promoter, but without co-transfection of estrogen receptor α expression vector. Then the cells were stimulated with selected concentrations of estradiol (10^{-8} M), 4OH-tamoxifen (10^{-6} M), raloxifen (10^{-6} M), or $ICI_{182,780}$ (10^{-6} M). This experiment (Fig 19) clearly demonstrates that the presence of the estrogen receptor α expression vector is essential for the stimulation of the estrogen-dependent transcriptional activity of the oxytocin promoter, just as it is absolutely necessary for the activation of the thymidine kinase-ERE promoter. This series of experiments suggests that in MDA-MB 231 cells the bovine oxytocin promoter is regulated by ligand activated estrogen receptor α. As estrogen receptors are not able to bind to the oxytocin promoter efficiently (Stedronsky at al., 2002), the regulation apparently uses a non-classical mechanism, substantially different from the classical ERE-mediated mechanism of promoter activation.

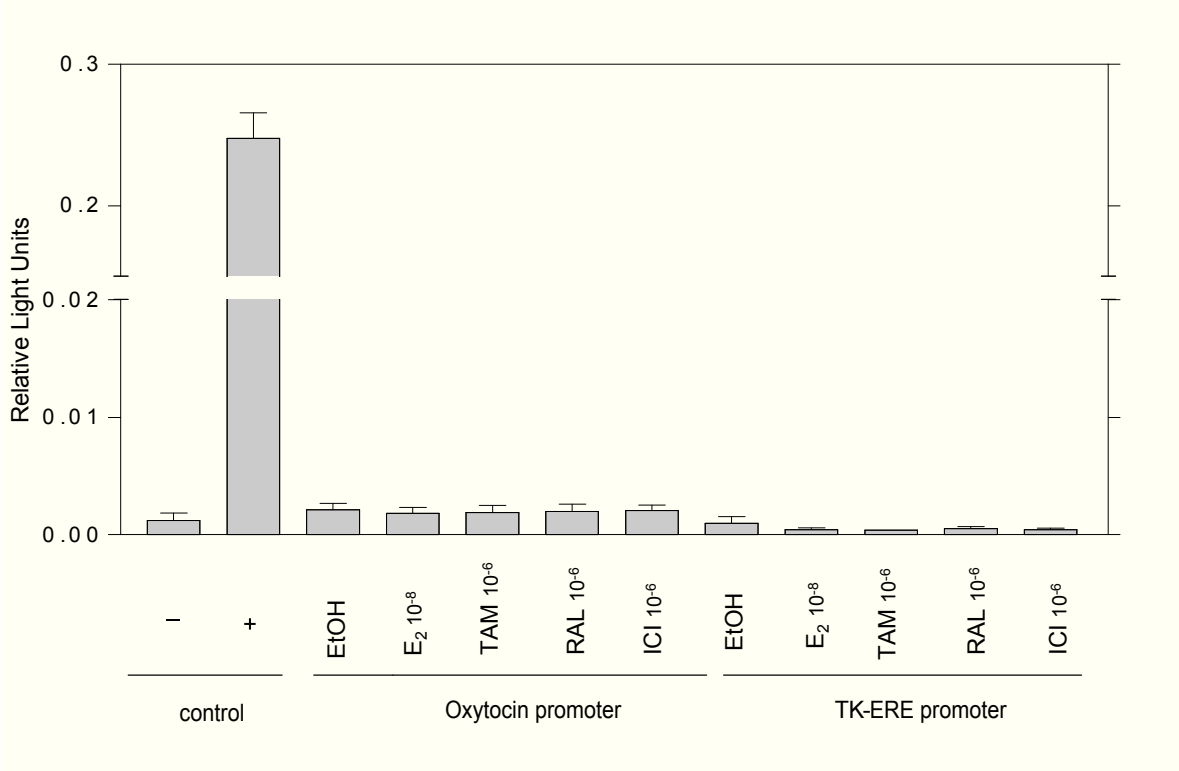


Fig 19: Lack of effects of different (ant) agonists in the absence of estrogen receptor α on the oxytocin and thymidine kinase-ERE promoter. MDA-MB 231 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter construct (TK ERE) without estrogen receptor α co-transfection and treated with selected concentration of estrogen receptor (ant) agonist estradiol (E_2), 4OH-tamoxifen (TAM), raloxifen (RAL), or $ICI_{182,780}$(ICI).

In order to measure the competition of the partial agonists and the pure antagonist with the natural ligand for binding to estrogen receptor α, the experiments described above (Figs 16-18) were repeated in the presence of the 10^{-8}M estradiol.

MDA-MB 231 cells were transfected with the estrogen receptor α expression vector and the oxytocin or the thymidine kinase-ERE promoter construct. The transfected cells were treated with estradiol (10^{-8}M), simultaneously with different concentrations of 4OH-tamoxifen (10^{-9} to 10^{-5}) (Fig 20). The results show that 4OH-tamoxifen at low concentration has no inhibitory effect on the estrogen-dependent stimulation of the oxytocin promoter, at higher concentrations of tamoxifen however, this agonistic action of estrogen is inhibited to approximately 60%, indicating a partial agonistic effect of 4OH-tamoxifen on the oxytocin promoter. At the thymidine kinase-ERE promoter, 4OH-tamoxifen at higher concentrations displays its antagonistic activity totally inhibiting the

estrogen dependent up-regulation of this promoter. The concentration necessary to achieve half-maximal inhibition on both promoters are comparable (10^{-7} M to 10^{-6} M), indicating that the different effects of 4OH-tamoxifen on the two promoters apparently are not the results of differences in binding to estrogen receptor α.

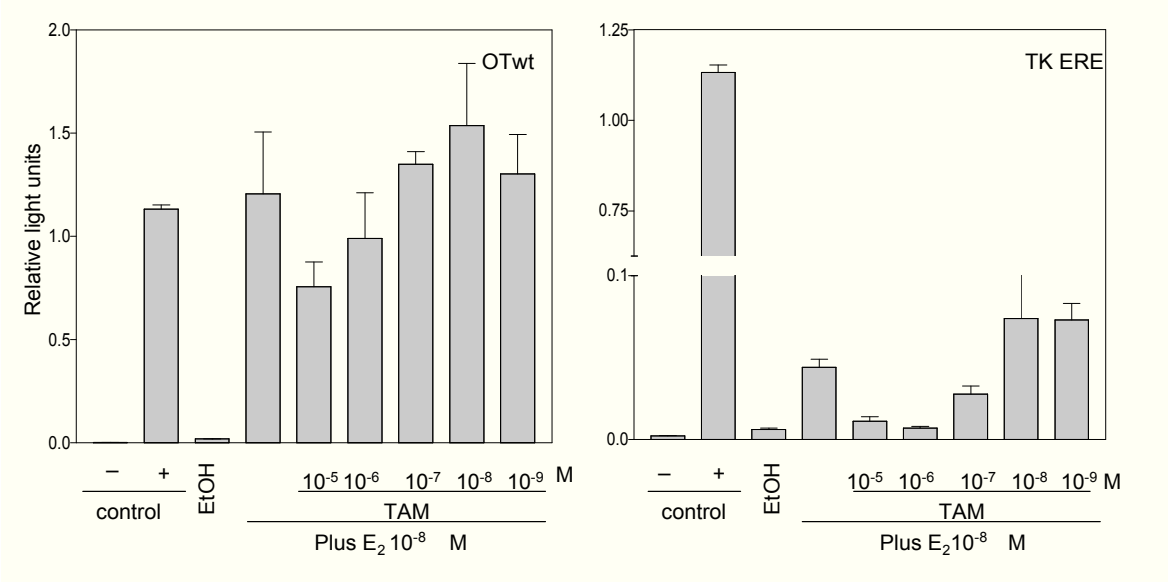


Fig 20: Effect of 4OH-tamoxifen on the transcriptional activity of estrogen receptor α bound by estradiol on the oxytocin and the thymidine kinase-ERE promoter. MDA-MB 231 cells were transfected with the oxytocin promoter construct (OT wt) or the thymidine kinase-ERE promoter (TK ERE) construct and treated with 10^{-8} M estradiol (E_2) simultaneously with different amounts (10^{-9} M to 10^{-5} M) of the partial estrogen receptor agonist 4OH-tamoxifen (TAM).

In a second set of transfections, the experiment was repeated using different concentrations of raloxifen (10^{-9}M to 10^{-5}M) (Fig 21). The results show partial agonistic effect on the OTwt and antagonistic effects on TK ERE promoter at higher concentrations of raloxifen. The partial agonistic effect of raloxifen at the oxytocin promoter appears to be lower than the corresponding activity of 4OH-tamoxifen.

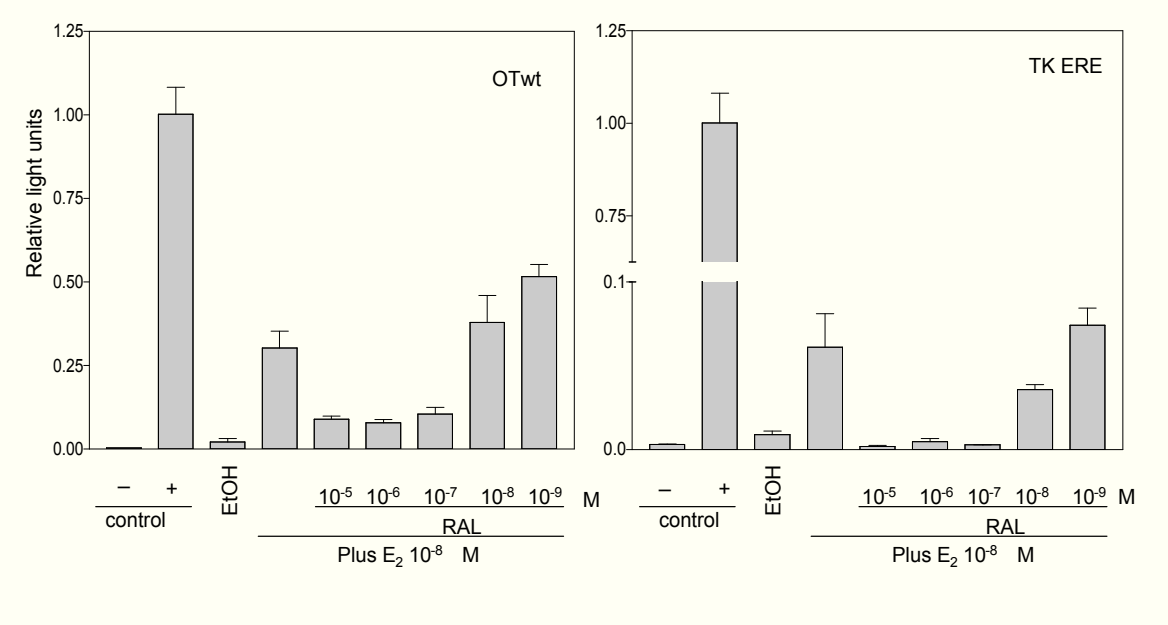


Fig 21: Effect of raloxifen on the transcriptional activity of estrogen receptor α bound by estradiol on the oxytocin and the thymidine kinase-ERE promoter. MDA-MB 231 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE (TK ERE) construct promoter and estrogen receptor α and treated with 10^{-8} M estradiol (E_2) simultaneously with different amounts (10^{-9} M to 10^{-5} M) of the partial estrogen receptor agonist raloxifen (RAL).

Finally, the same experiment was performed with the pure antagonist $ICI_{182,780}$ (Fig 22) treating the transfected cells with different concentrations of $ICI_{182,780}$ (10^{-9}M to 10^{-5} M) simultaneously with 10^{-8} M estradiol. As for the two partial agonists described above, $ICI_{182,780}$ in low concentrations has no effect, but a clearly antagonistic effect on the oxytocin promoter is observed at higher concentration of $ICI_{182,780}$. At the thymidine kinase-ERE promoter the compound displays its established activity as a highly efficient estrogen antagonist (Koohi et al., 2005).

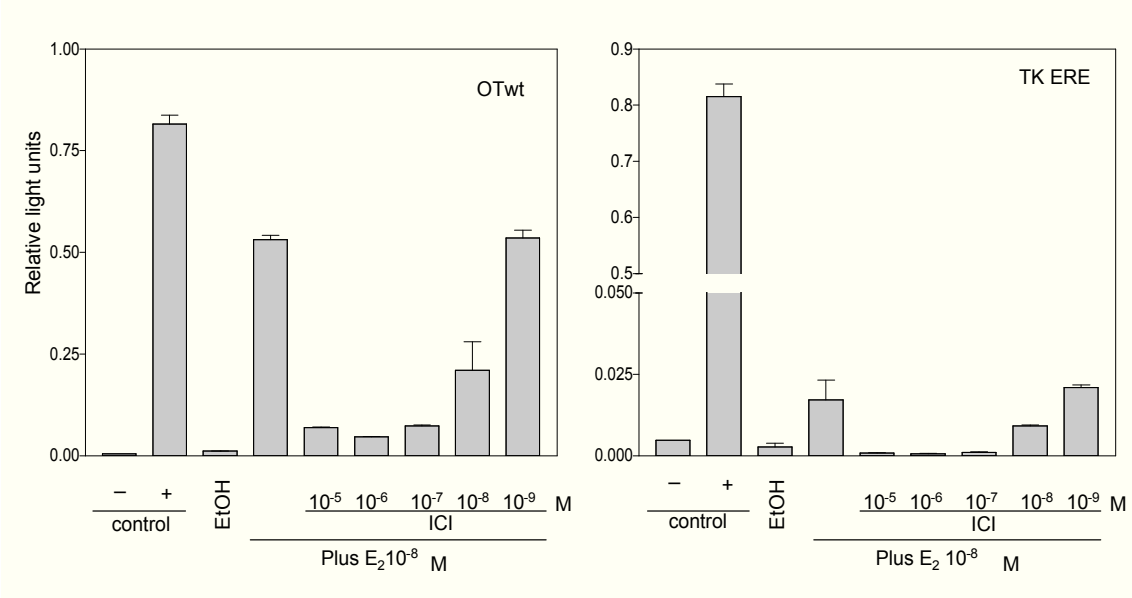


Fig 22: Effect of $ICI_{182,780}$ on the transcriptional activity of estrogen receptor α bound by estradiol on the oxytocin and the thymidine kinase-ERE promoter. MDA-MB 231 cells were transfected with the oxytocin promoter (OTwt) construct or the thymidine kinase-ERE (TK ERE) promoter construct and estrogen receptor α and treated with 10^{-8} M estradiol (E_2) simultaneously with different amounts (10^{-9} M to 10^{-5} M) of the estrogen receptor antagonist $ICI_{182,780}$ (ICI).

Promoter:	Oxytocin		TK ERE	
Effect / Compounds	agonistic	antagonistic	agonistic	antagonistic
E_2 (Natural agonist)	+	-	+	-
TAM (partial agonist)	+	-	-	+
RAL (partial agonist)	+	-	-	+
ICI (Antagonist)	+	-	-	+

Table 1: Effects of estrogen, agonists, partial agonists, and antagonists on estrogen-controlled promoters (oxytocin and thymidine kinase ERE) in MDA-MB 231 breast cancer cells (E2: estradiol, TAM: 4OH-tamoxifen, RAL: raloxifen, ICI: $ICI_{182,780}$)

This series of experiments clearly demonstrates that the three compounds tamoxifen, raloxifen as well as $ICI_{182,780}$ compete with estradiol for binding to estrogen receptor α and, bound to the receptor, function as partial agonists at the bovine oxytocin promoter

and as antagonists at the thymidine kinase-ERE promoter. In an attempt to elucidate the mechanism of action of the ligand-bound estrogen receptor α in the activation of the bovine oxytocin promoter, the estrogenic compound diethylstilbestrol was used. This compound is known to act as an agonist on estrogen receptor α, but as an antagonist on the nuclear orphan receptor estrogen related receptor α that has been shown to be expressed in MDA-MB 231 cells and may possibly bind to the bovine oxytocin promoter (Koohi et al., 2005). The transcriptional effects of diethylstilbestrol on the oxytocin promoter and the thymidine kinase-ERE promoter were measured (Fig 23).

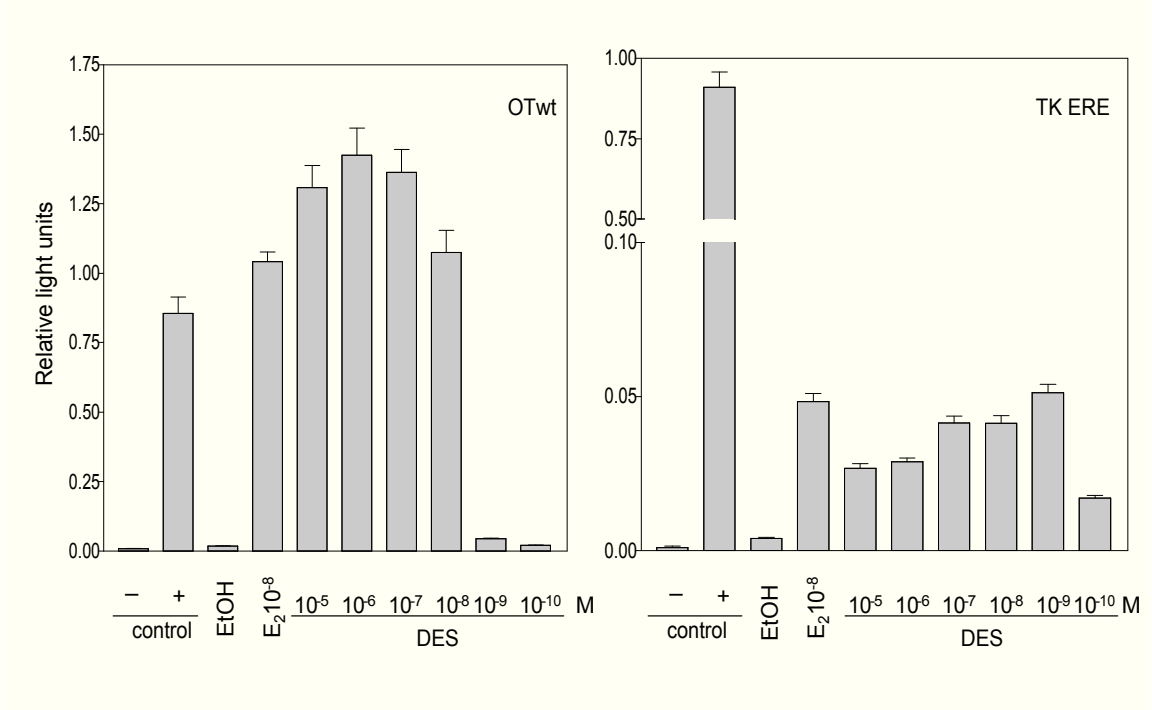


Fig 23: Transcriptional effects of diethylstilbestrol (DES) on the oxytocin promoter and the thymidine kinase-ERE promoter. MDA-MB 231 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter construct (TK ERE) and estrogen receptor α and treated with different amounts (10^{-10} to 10^{-5}) of diethylstilbestrol (DES).

The results show that diethylstilbestrol has agonistic activity at both the oxytocin and the thymidine kinase-ERE promoter, supporting its role as an estrogen receptor α-dependent agonist. However, the agonistic activity appears to be diminished at higher concentrations of DES, hinting to a possible involvement of estrogen related receptor α in transcriptional activation of both promoters. Further experiments will, however, have to be performed in

order to elucidate the involvement of this nuclear orphan receptor in the non-classical mechanism of estrogen action.

In order to discriminate clearly between the classical mechanism and non-classical mechanisms of estrogen action involving specific binding of activated estrogen receptor to its cognate response element, the transcriptional activities of estrogen receptor α and a P-box mutant with an impairment of specific DNA binding (Fig 13) were compared (Koohi et al., 2005). For these experiments MDA-MB 231 cells were transfected with the oxytocin or the thymidine kinase-ERE promoter and the wild type estrogen receptor α as described previously. In addition, the cells were also transfected with a mutant estrogen receptor α construct, in which the DBD had been mutated without influencing the ligand-binding properties of the receptor (Fig 24). Then cells were stimulated with estradiol (10^{-8} M), 4OH-tamoxifen (10^{-6} M) or $ICI_{182,780}$ (10^{-6} M), or 4OH-tamoxifen or $ICI_{182,780}$ in combination with estradiol. The results (Figs 24 and 25) clearly show that the mutant estrogen receptor α, which is unable to bind to DNA, exhibits full transcriptional activity at the oxytocin promoter, whereas at the thymidine kinase-ERE promoter no transcriptional activity whatsoever can be detected. These experiments demonstrate that binding of the activated estrogen receptor α to the promoter is not necessary for estrogen-dependent up-regulation of the oxytocin promoter, whereas the thymidine kinase-ERE promoter up-regulation, as expected, is totally depending on the classical binding of the activated estrogen receptor α to the estrogen response element.

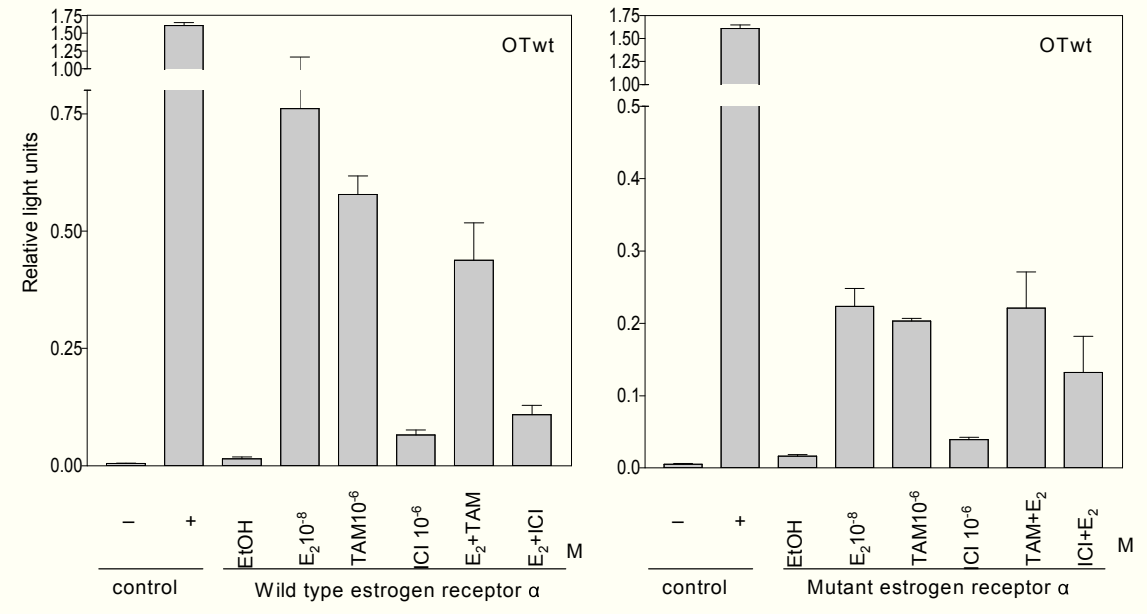


Fig 24: Comparison of the transcriptional activities of wild-type estrogen receptor α and a mutant unable to bind DNA on the oxytocin promoter. MDA-MB 231 cells were transfected with the oxytocin promoter construct (OTwt) and wild-type human estrogen receptor α or a P-box mutant unable to bind DNA. (Ant)agonists estradiol (E_2 10^{-8} M), 4OH-tamoxifen (TAM 10^{-6} M), or $ICI_{182,780}$ (ICI 10^{-6} M) were added alone or in combination with E_2 (10^{-8} M).

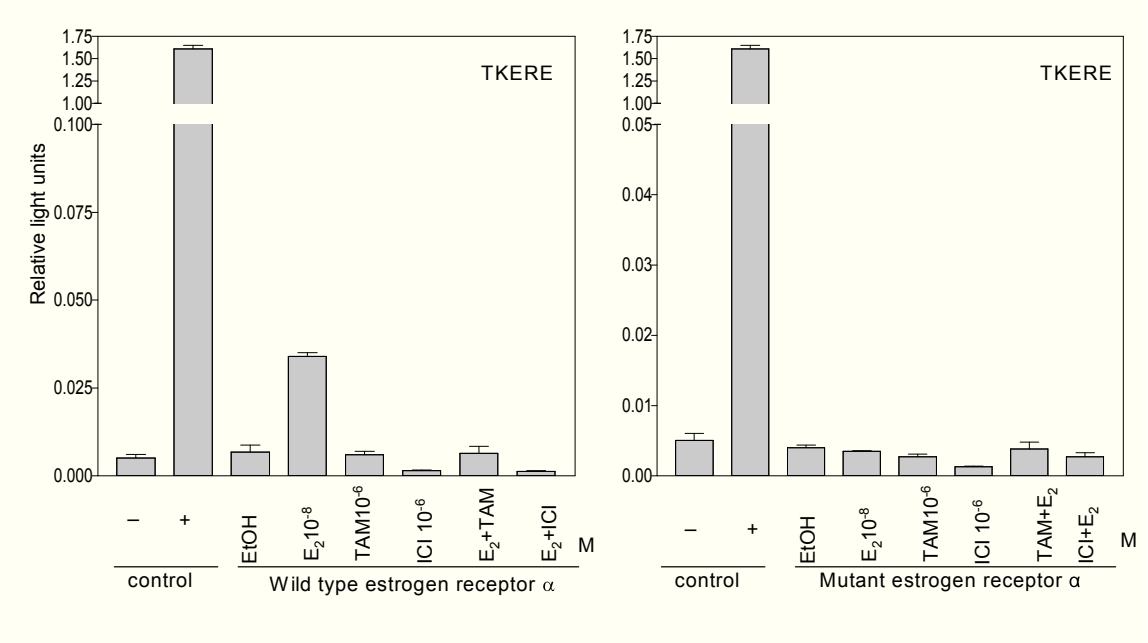


Fig 25: Comparison of the transcriptional activities of wild-type estrogen receptor α and a mutant unable to bind DNA on the thymidine kinase-ERE promoter. MDA-MB 231 cells were transfected with the thymidine kinase-ERE promoter construct (TK ERE) and wild-type human estrogen receptor α or a P-box mutant unable to bind DNA. (Ant)agonists estradiol (E_2 10^{-8} M), 4OH-tamoxifen (TAM 10^{-6} M), or $ICI_{182,780}$ (ICI 10^{-6} M) were added alone or in combination with E_2 (10^{-8} M).

The oxytocin promoter has been shown to be regulated by binding of nuclear orphan receptors to a conserved binding site around position -160 (Wehrenberg et al., 1994a; Wehrenberg at al., 1994b; Stedronsky at al., 2002). In order to define the regulatory elements involved in the estrogen-dependent up-regulation of this promoter, the role of the orphan receptor binding site in the bovine oxytocin promoter was investigated.

MDA-MB 231 cells were transfected with luciferase reporter constructs containing either the wild type oxytocin promoter or one of two mutants of the orphan receptor binding site, a one base pair insertion mutant, or a scrambled sequence (Fig 26).

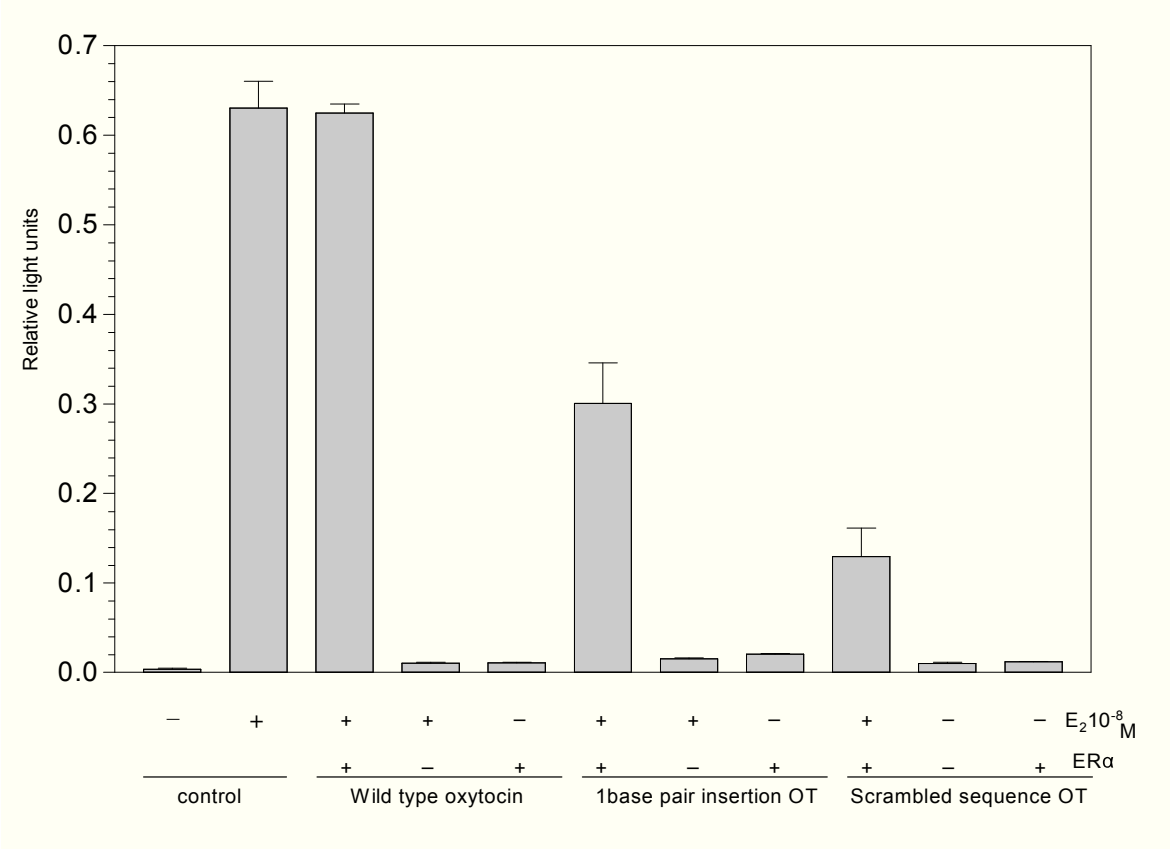


Fig 26: Activation of the oxytocin promoter by estrogen receptor α bound by estradiol is dependent on an intact orphan receptor binding site. MDA-MB 231 cells were transfected with the wild-type oxytocin promoter construct or one of two mutant promoters, together with or without estrogen receptor α (ERα) and treated or not with 10^{-8} M estradiol (E_2).

The transcriptional activity of the bovine oxytocin promoter is stimulated by estrogen-activated estrogen receptor α in the transfections with all three promoter constructs, but it is progressively lower in the two mutants, correlating with the degree of corruption of the

nuclear orphan receptor binding site. As expected, there is no transcriptional activity without addition of estradiol or estrogen receptor α co-transfection. This result shows that the stimulation of the transcriptional activity of the bovine oxytocin promoter depends mainly on the integrity of the orphan receptor binding site, suggesting an essential role for a monomeric nuclear orphan receptor in the estrogen-dependent up-regulation of this promoter in MDA-MB 231 cells.

2) Effects of Xenoestrogens on Transcription from the Oxytocin Promoter

In addition to the endogenous hormone estradiol, estrogen activity can be modulated by chemicals in the environment termed xenoestrogens, which include, industrial by-products (industrial xenoestrogens), natural plant compounds (phytoestrogens) and metal ions. In the past several decades, there has been increasing awareness and concern about the endocrine-disrupting effects of these chemicals and their impact on humans and wildlife. Although the molecular mechanisms of xenoestrogen action are not well understood, it is clear that many of these chemicals function by binding to estrogen receptors and blocking estrogen access. In addition to their potential antagonist activities, however, xenoestrogens can also induce activating conformational changes in the estrogen receptors that enable the receptors to interact with co-activators leading to activation of target gene transcription.

2.1. Effect of Industrial Xenoestrogens on Transcription from the Oxytocin Promoter

The investigation of the mechanisms by which industrial xenoestrogens act as estrogenic compounds will help to understand the toxic effects of these compounds. This investigation was focused on the effects of the organochlorine pesticide, 1,1,1-trichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)ethane (o,p'-DDT) (Steinmetz et al., 1996; Okoumassoun et al., 2002; Tollefsen et al., 2002; Diel et al., 2002), 2, 2-bis (4-chlorophenyl)-1, 1-dichloroethyl (p,p'-DDE) as a metabolite of DDT (Gaido et al., 1997; Larkin et al., 2002; Villa et al., 2004), toxaphene which is used as an insecticide (Jorgensen et al., 1997; Gaido et al., 1998; Stelzer et al., 1999; Yang et al., 1999; Arcaro et al., 2000), chlordane as a persistent organochlorine insecticide (Arnold et al., 1997;

Massaad et al., 1998; Yang et al., 1999; Cossete et al., 2002), the insecticide β-hexachlorhexane (Steinmetz et al., 1996; Hatakeyama et al., 2002; Tapiero et al., 2002; Rasmussen et al., 2003; Penza et al., 2004), the organochlorine pesticide methoxychlor (Bulger et al., 1978; Walthers et al., 1993; Cummings., 1995; 1997; Katchamart et al., 2002), and 2-hydroxybiphenyl which is used as a germicide/fungicide on food (Petit et al., 1997; Schultz et al., 2002; Cappelletti et al., 2003).

In order to check for effects exerted by the non-classical mechanism controlling the oxytocin promoter, MDA-MB 231 cells were transfected with the luciferase indicator construct containing the bovine oxytocin promoter or the thymidine kinase-ERE promoter construct as described above, and a vector expressing human estrogen receptor α. A ß-galactosidase expressing vector was used as an internal control for transfection efficiency, and the transfected cells were treated with increasing amounts of the xenoestrogen 1.1.1-trichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)ethane (o,p'-DDT) with or without the addition of 10^{-8} M estradiol. In the first experiment (Fig 27), neither with the oxytocin nor the thymidine kinase-ERE promoter any agonistic effect could be detected.

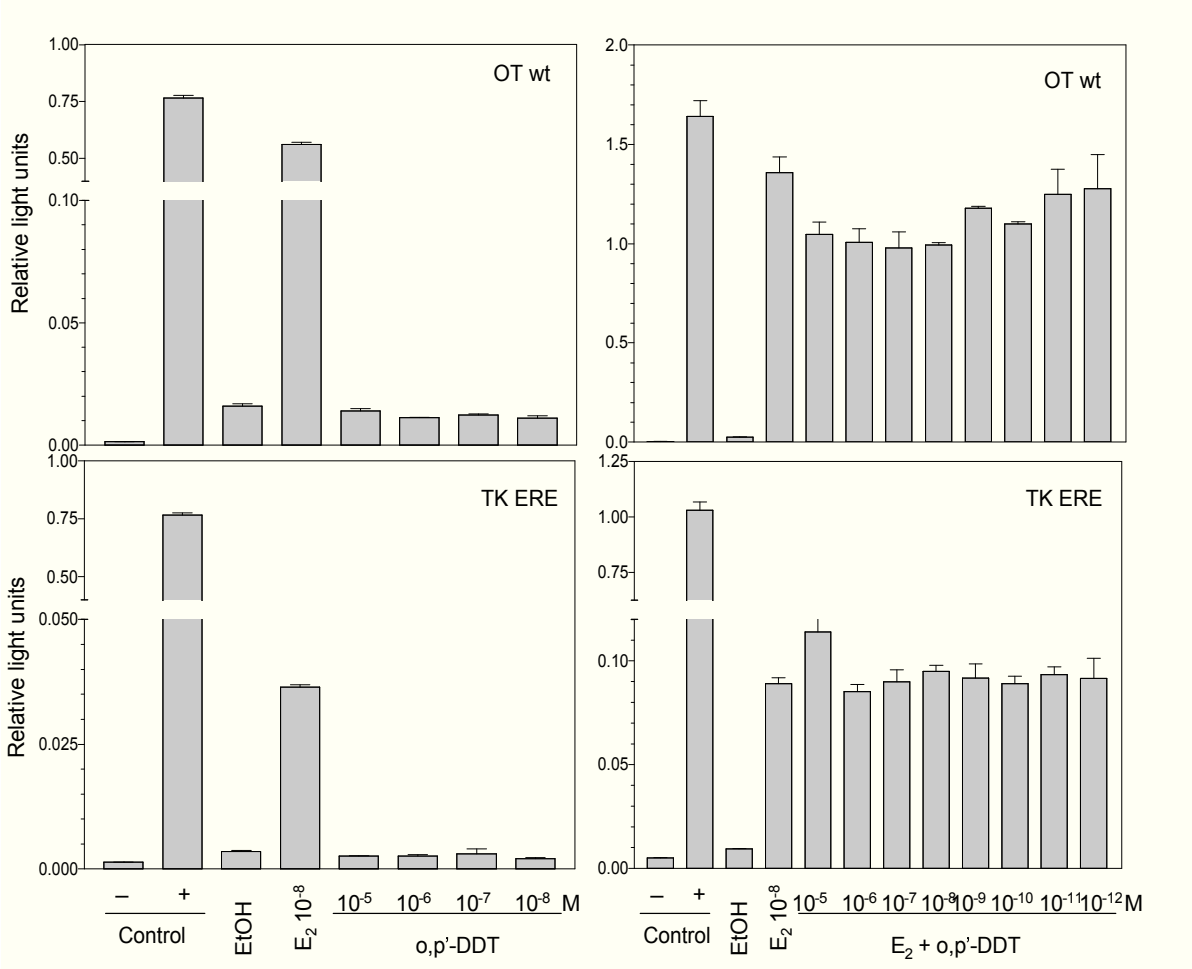


Fig 27: Transcriptional effects of, o,p'-DDT on the oxytocin and the thymidine kinase-ERE promoter by estrogen receptor α.MDA-MB 231 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter construct (TK ERE) and estrogen receptor α and treated with different amounts of o,p'-DDT alone or simultaneously with estradiol (E_2 10^{-8} M).

The detection of transcriptional activity of o,p'-DDT in this breast cancer cell system had been expected, as o,p'-DDT has been shown to exert estrogenic effects in MCF-7 breast cancer cells (Steinmetz et al., 1996). Therefore in a control experiment, the effect of o,p'-DDT in MCF-7 cells was measured. The MCF-7 cells were transfected as above with the oxytocin or the thymidine kinase-ERE promoter and estrogen receptor α as shown above and cells were stimulated with increasing amounts of o,p'-DDT (Fig 28). The result clearly indicates that o,p'-DDT has an agonistic effect on both the oxytocin and the thymidine kinase-ERE promoter in this cellular context, leading to a more than two-fold stimulation of transcription at 10^{-5} M o,p'-DDT. No inhibitory or synergetic effect could

be detected in this experiment on the simultaneous addition of o,p'-DDT and estradiol. These results clearly demonstrate that the agonistic effect of o,p'-DDT is cell specific, as no agonistic effect could be detected in MDA-MB 231 cells, whereas the established agonistic effect of o,p'-DDT in MCF-7 cells could be confirmed with this assay system.

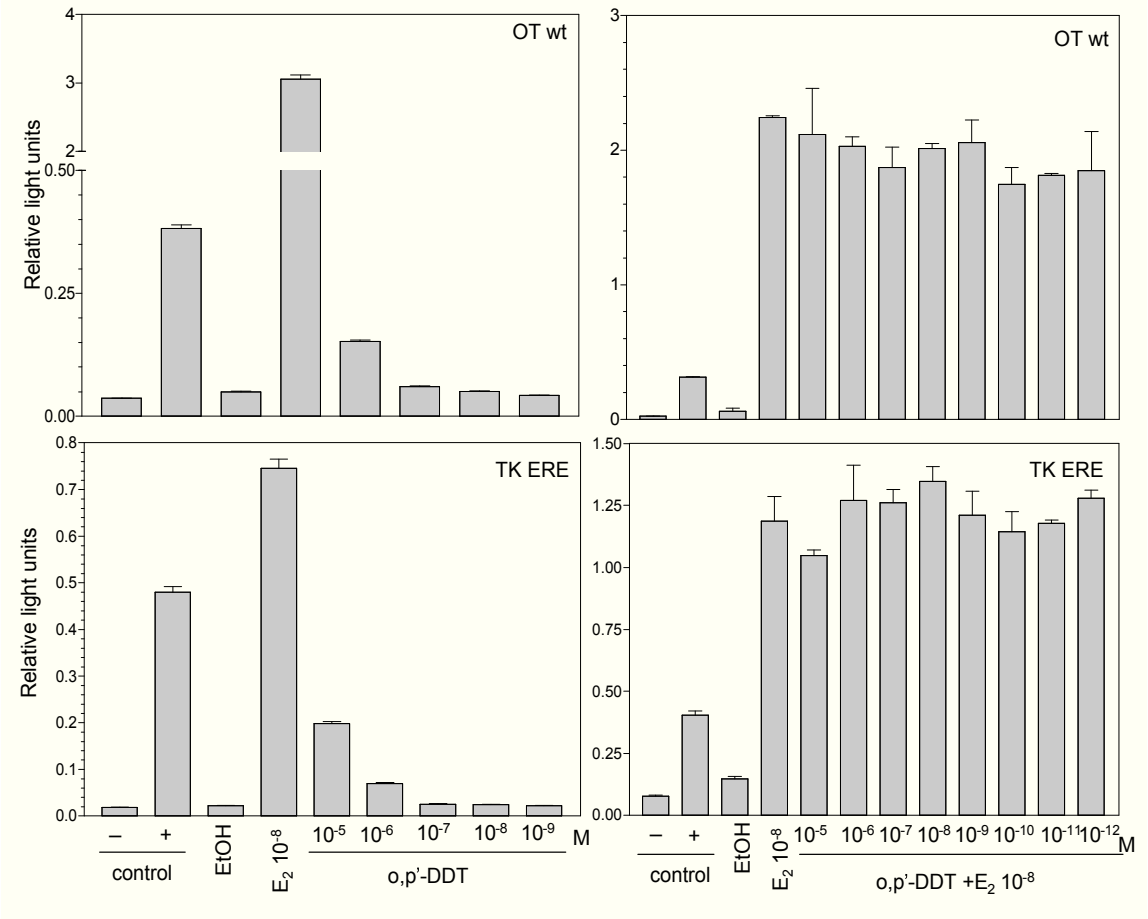


Fig 28: Transcriptional effects of o,p'-DDT on the oxytocin promoter and the thymidine kinase-ERE promoter by estrogen receptor α in the MCF-7 cells.MCF-7 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter construct (TK ERE) and estrogen receptor α and treated with different amounts of o,p'-DDT alone or simultaneously with estradiol (E_2 10^{-8} M).

In a further control experiment 2,2-bis (4-chlorophenyl)-1,1-dichloroethyl (p,p'-DDE) was used. MDA-MB 231 cells were transfected as described above with the oxytocin or thymidine kinase-ERE promoter, co-transfected with estrogen receptor α, and the transfected cells treated with increasing concentrations of p,p'-DDE (Fig 29). The results show that there is no agonistic effect with p,p'-DDE, neither on the oxytocin nor on the

thymidine kinase-ERE promoter. In parallel transfection containing in addition 10^{-8} M estradiol, neither an antagonistic nor a synergistic effect of p,p'-DDE could be detected.

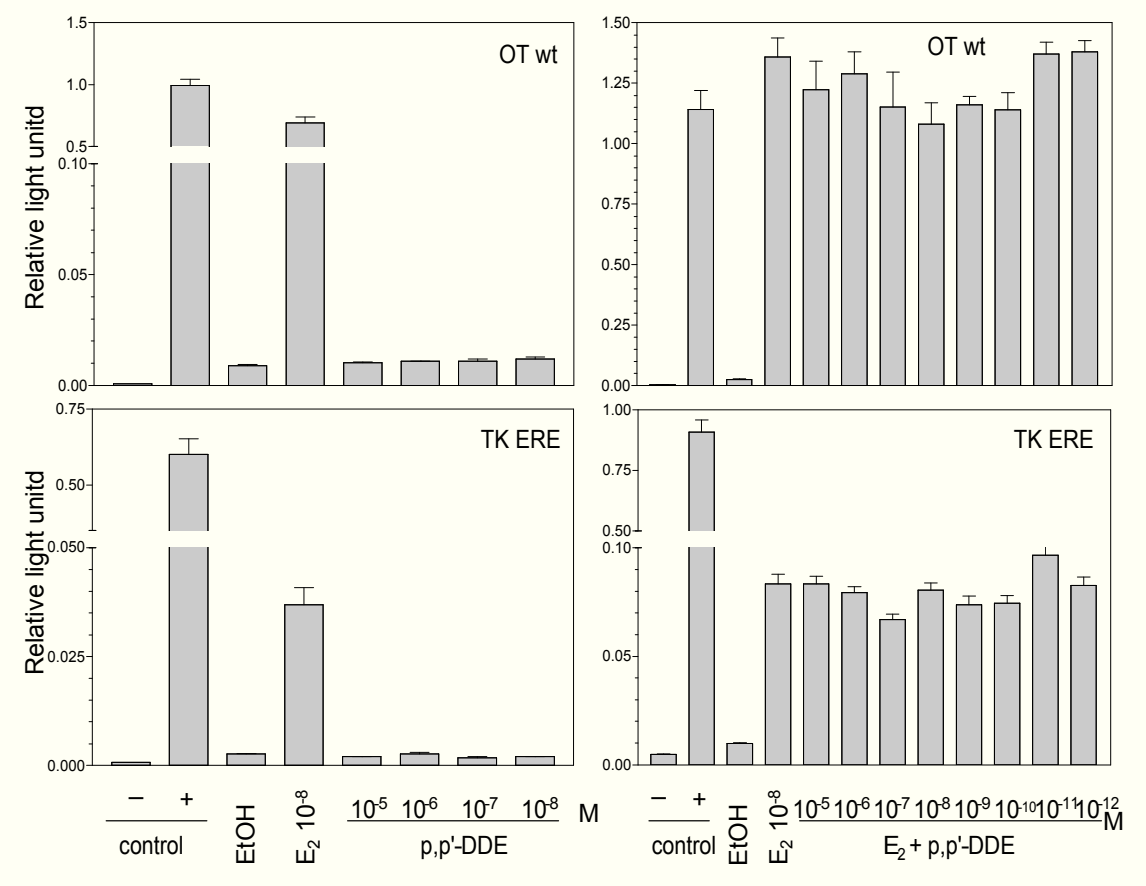


Fig 29: Transcriptional effects of p,p'-DDE on the oxytocin promoter and the thymidine kinase-ERE promoter by estrogen receptor α. MDA-MB 231 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter construct (TK ERE) and estrogen receptor α and treated with different amounts of p,p'-DDE alone or simultaneously with estradiol (E_2 10^{-8} M).

In order to complete the investigation of the cell-type-specific transcriptional effect of o,p'-DDT and related compounds, the effects of p,p'-DDE were also measured in MCF-7 cells. For this experiment MCF-7 cells were transfected as described above and the transfected cells were treated with increasing amounts of p,p'-DDE, (Fig 30). The results indicated that p,p'-DDE does not have any agonistic, synergistic or antagonistic activity in MCF-7 cells.

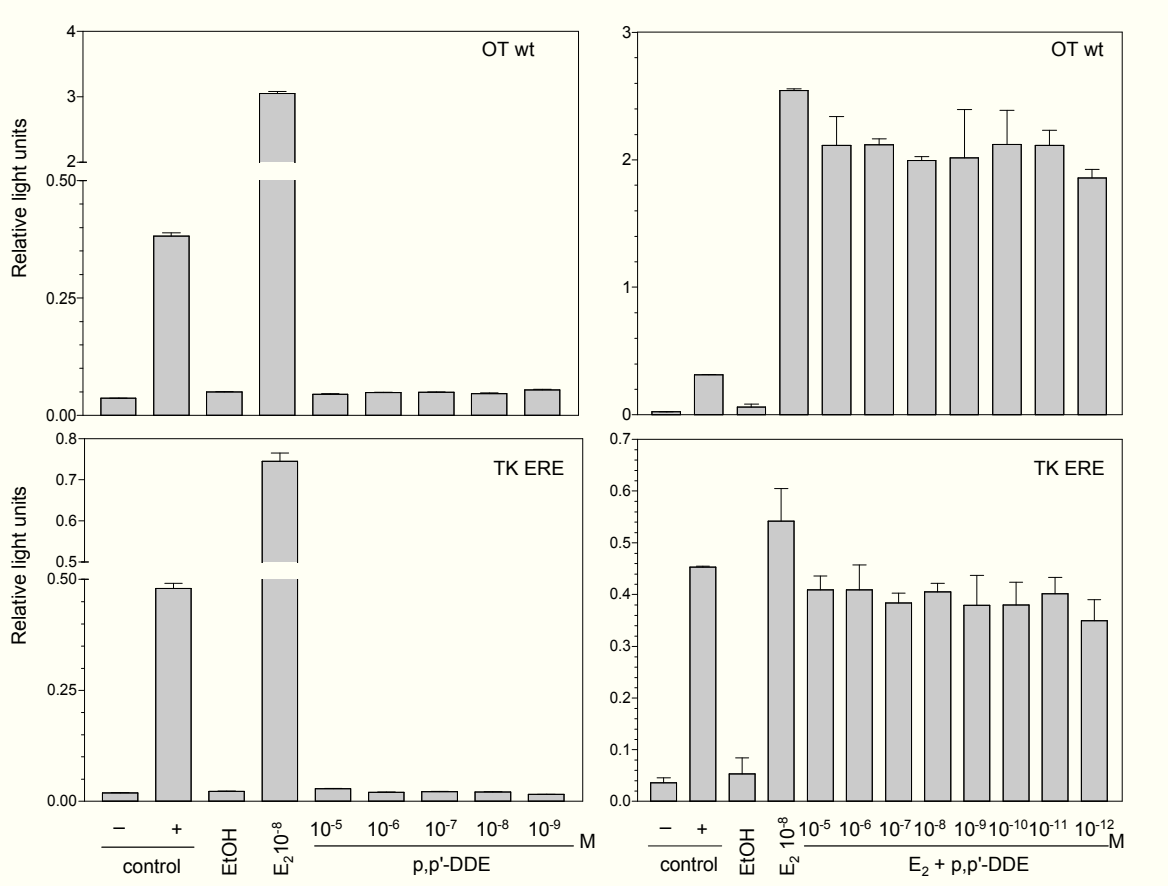


Fig 30: Transcriptional effects of p,p'-DDE on the oxytocin promoter and the thymidine kinase-ERE promoter by estrogen receptor α in the MCF-7 cells. MCF-7 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE (TK ERE) promoter construct and estrogen receptor α and treated with different concentration of p,p'DDE alone or simultaneously with estradiol (E_2 10^{-8}M).

Toxic effect of o,p'-DDT and p,p'-DDE might suppress a possible agonistic effect at the high concentrations needed to obtain a stimulation of transcription by o,p'-DDT in MCF-7 cells. In order to check for these effects, two independent cell toxicity tests, the neutral red and the plating test, were performed. For the neutral red test, the cells were plated in 96 well plates, on the second day the cells were treated with increasing concentrations of o,p'-DDT and on the third day, after adding the neutral red, the range of toxicity was measured as described in Materials and Methods (Fig 31). For the plating test, the cells were plated in a 25 ml flask and on the second day, cells were treated with increasing concentrations of o,p'-DDT as described in Materials and Methods. After a week the colonies grown from the plated cells were counted.

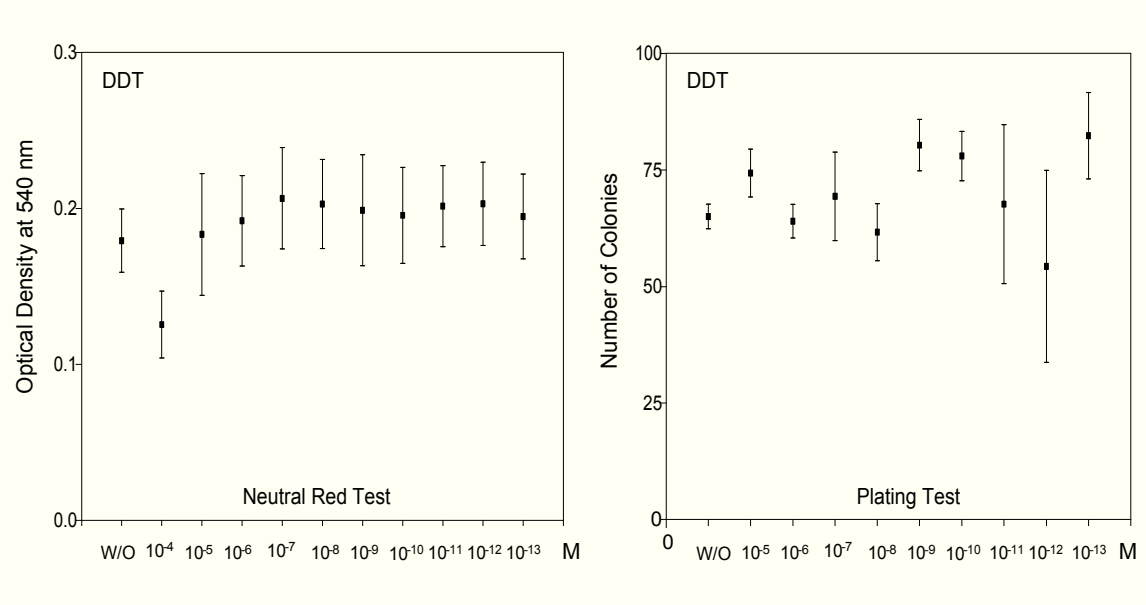


Fig 31: Cellular toxicity of o,p'-DDT in the MDA-MB 231 cells in the neutral red and the plating test. MDA-MB 231 cells were plated as described in Materials and Methods and treated with different concentrations of o,p'-DDT. (W/O: without sample)

The results of both tests indicate that o,p'-DDT has no toxic effect on MDA-MB 231 and MCF-7 cells in the concentrations used for the transfection assays. In a parallel experiment, p,p'-DDE was also tested for possible toxic effects (Fig 33). Cells were stimulated with increasing concentrations of p,p'-DDE and the neutral red test and the plating test were performed as described above. The results clearly show that, as with o,p'-DDT, there is not any toxic effect of p,p'-DDE in the concentration range used in the transfection assays. Low toxicity of p,p'-DDE was only detected at a concentration of 10^{-4} M, which was not used in the transfection assays.

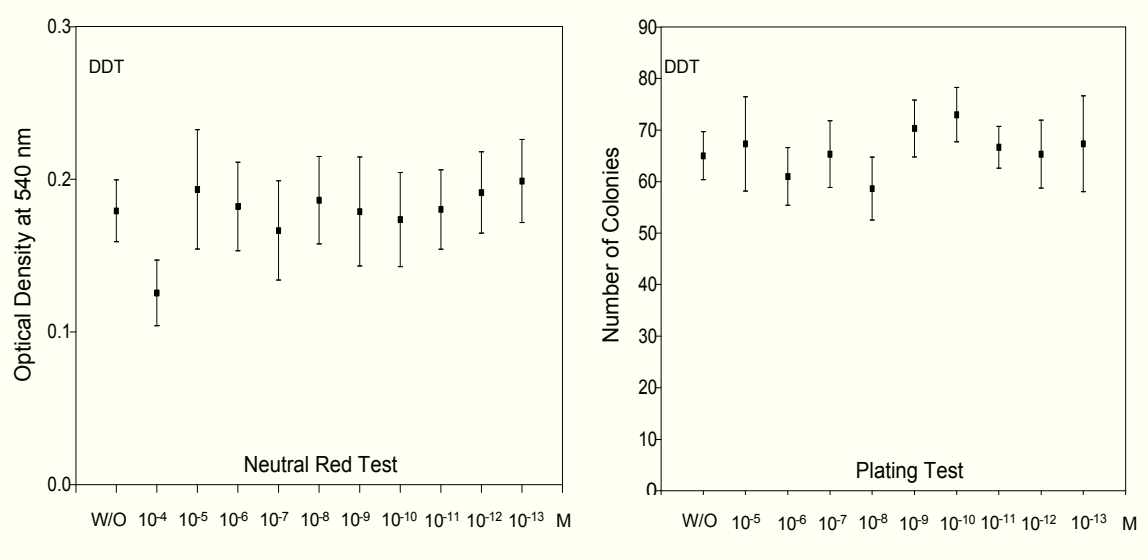


Fig 32: Cellular toxicity of o,p'-DDT in MCF-7 cells in the neutral red and the plating test. MCF-7 cells were plated as described in Materials and Methods and treated with different concentrations of o,p'-DDT. (W/O: without sample)

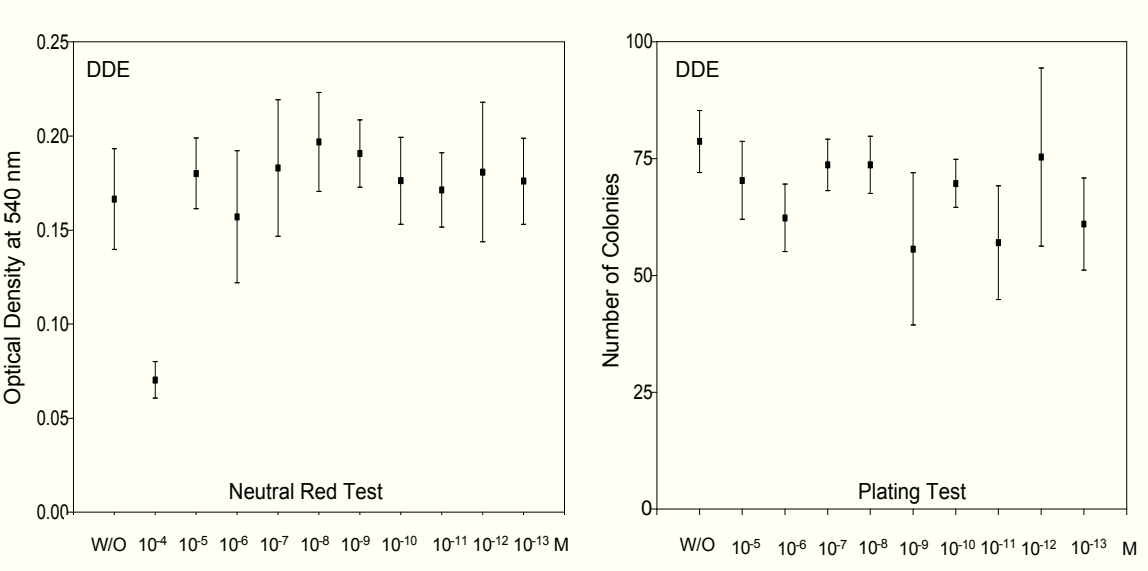


Fig 33: Cellular toxicity of p,p'-DDE in MDA-MB 231 cells in the neutral red and the plating test. MDA-MB 231 cells were plated as described in Materials and Methods and treated with different concentrations of p,p'-DDE. (W/O: without sample)

Having established the MDA-MB 231 cells as a robust assay system for measuring non-classical estrogenic effects (Koohi et al., 2005), further experiments were carried out to investigate the effects of various xenoestrogens. MDA-MB 231 cells were transfected

with the oxytocin or the thymidine kinase-ERE promoter as described above and the cells were treated with increasing concentrations of toxaphen (Fig 34) or chlordane (Fig 35) with or without the addition of estradiol. The results show that neither toxaphen nor chlordane has agonistic effects on the oxytocin or the thymidine kinase-ERE promoter. In the presence of estradiol, chlordane apparently exhibits a weak antagonistic effect at intermediate concentrations (10^{-9} M) both on the oxytocin and the thymidine kinase-ERE promoter, whereas toxaphen shows neither an antagonistic nor a synergistic effect on these promoters. The biphasic response curve of chlordane in the presence of estradiol (right panel) might hint to a displacement of estradiol from the estrogen receptor α by high concentrations of chlordane as a weaker agonist. However, an agonistic activity of chlordane could not be detected in the parallel experiments without addition of estradiol (left panel).

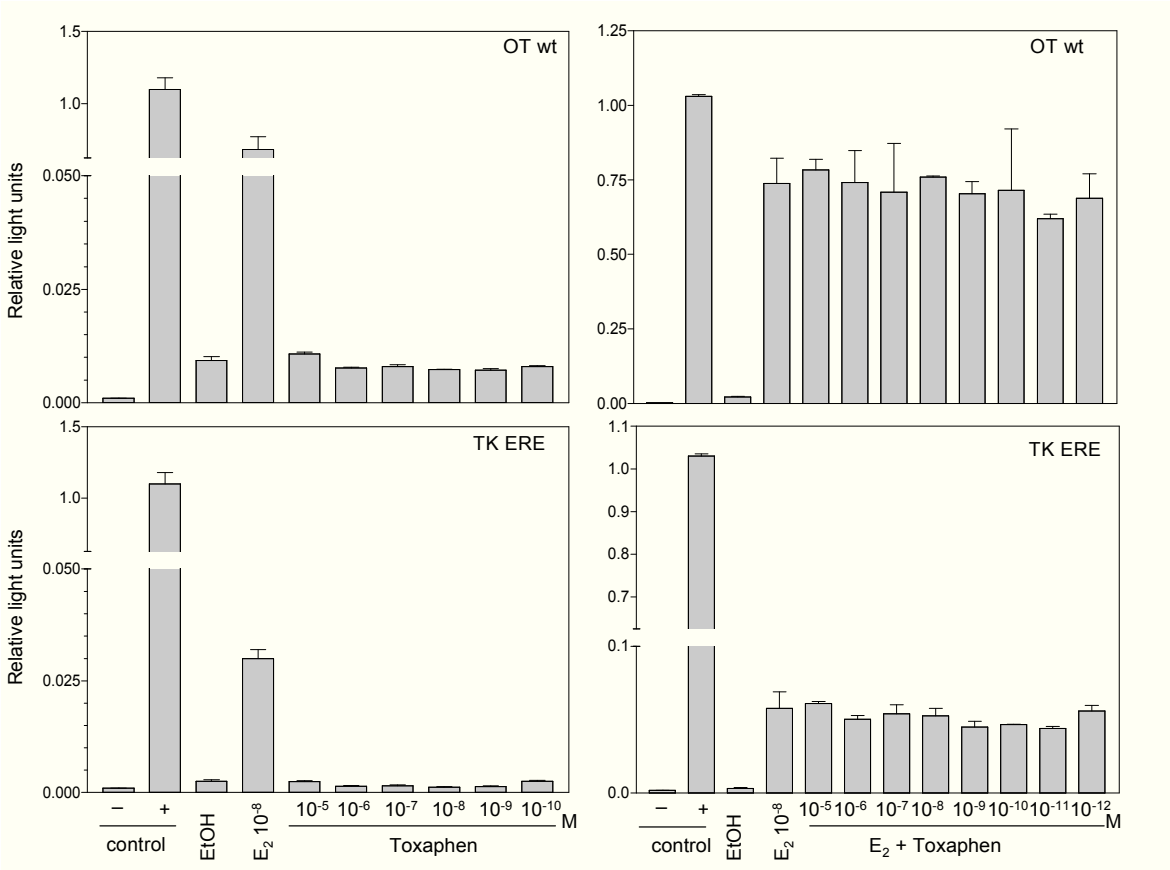


Fig 34: Transcriptional effects of toxaphen on the oxytocin promoter and the thymidine kinase-ERE promoter by estrogen receptor α in the MDA-MB 231 cells. MDA-MB 231 cells were transfected with the oxytocin promoter (OTwt) or the thymidine kinase-ERE promoter and estrogen receptor α and treated with different concentrations of toxaphen alone or simultaneously with estradiol (E_2 10^{-8}).

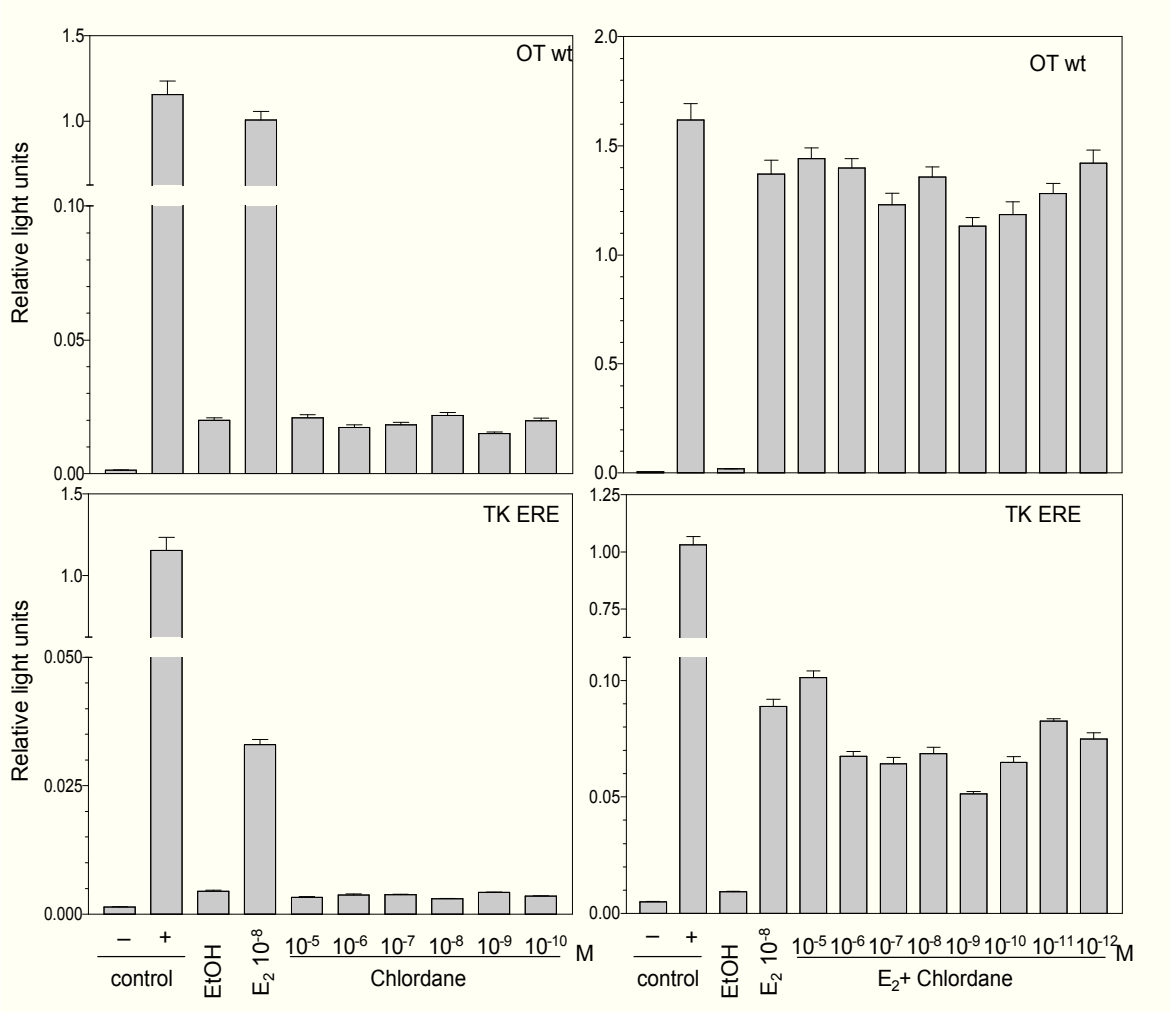


Fig 35: Transcriptional effects of chlordane on the oxytocin promoter and the thymidine kinase-ERE promoter by the estrogen receptor α in the MDA-MB 231 cells. MDA-MB 231 cells were transfected with the oxytocin promoter (OTwt) or the thymidine kinase-ERE (TK ERE) promoter and estrogen receptor α and treated with different concentrations of chlordane alone or simultaneously with estradiol (E_2 10^{-8} M).

In order to check for possible toxic effects of toxaphen and chlordane, cell toxicity assays were performed for these two compounds. The neutral red test and the plating test using MDA-MB 231 cells were performed as described above (Fig 36 and 37). No toxic effects of toxaphen and chlordane could be detected, ruling out the possibility that transcriptional effects of these compounds might be suppressed by their general toxicity.

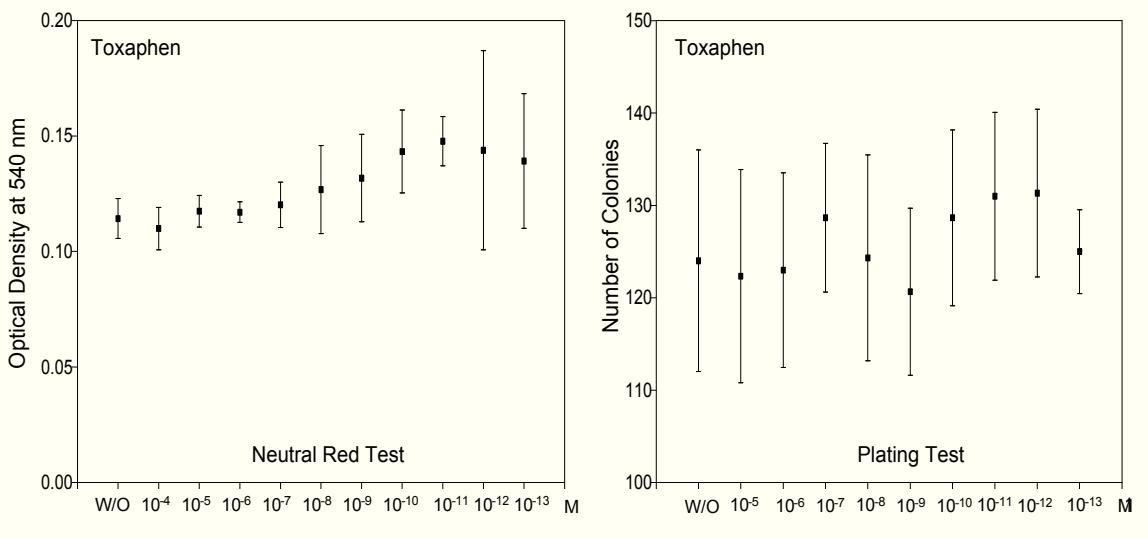


Fig 36: Cellular toxicity of toxaphen in MDA-MB 231 cells in the neutral red and the plating test. MDA-MB 231cells were plated as described in Materials and Methods and treated with different concentrations of toxaphen. (W/O: without sample)

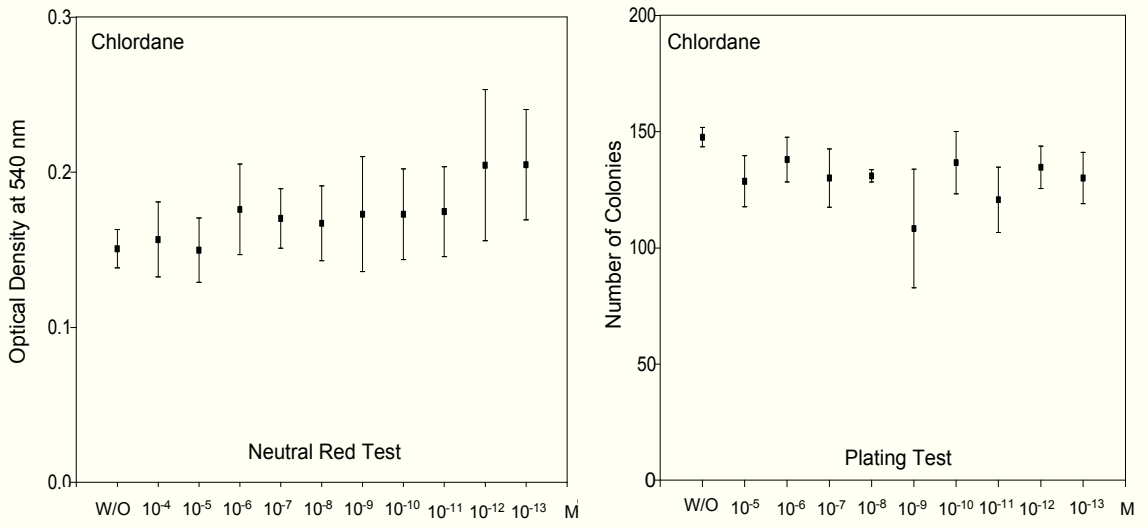


Fig 37: Cellular toxicity of chlordane in MDA-MB 231 cells in the neutral red and the plating test. MDA-MB 231cells were plated as described in materials and methods and treated with different concentrations of chlordane. (W/O: without sample)

As further industrial xenoestrogens, ß-HCH, methoxychlor and 2-hydroxybiphenyl were tested in the MDA-MB 231 system. MDA-MB 231 cells were transfected as described above with the oxytocin or the thymidine kinase-ERE promoter and co-transfected with

estrogen receptor α and the transfected cells were treated with increasing concentration of ß-HCH (Fig 38), methoxychlor (Fig 39) and 2-hydroxybiphenyl (Fig 40) with or without addition of the estradiol 10^{-8} M. The results show that none of these compounds exhibits agonistic activities on the oxytocin or the thymidine kinase–ERE-controlled promoter in this cell system.

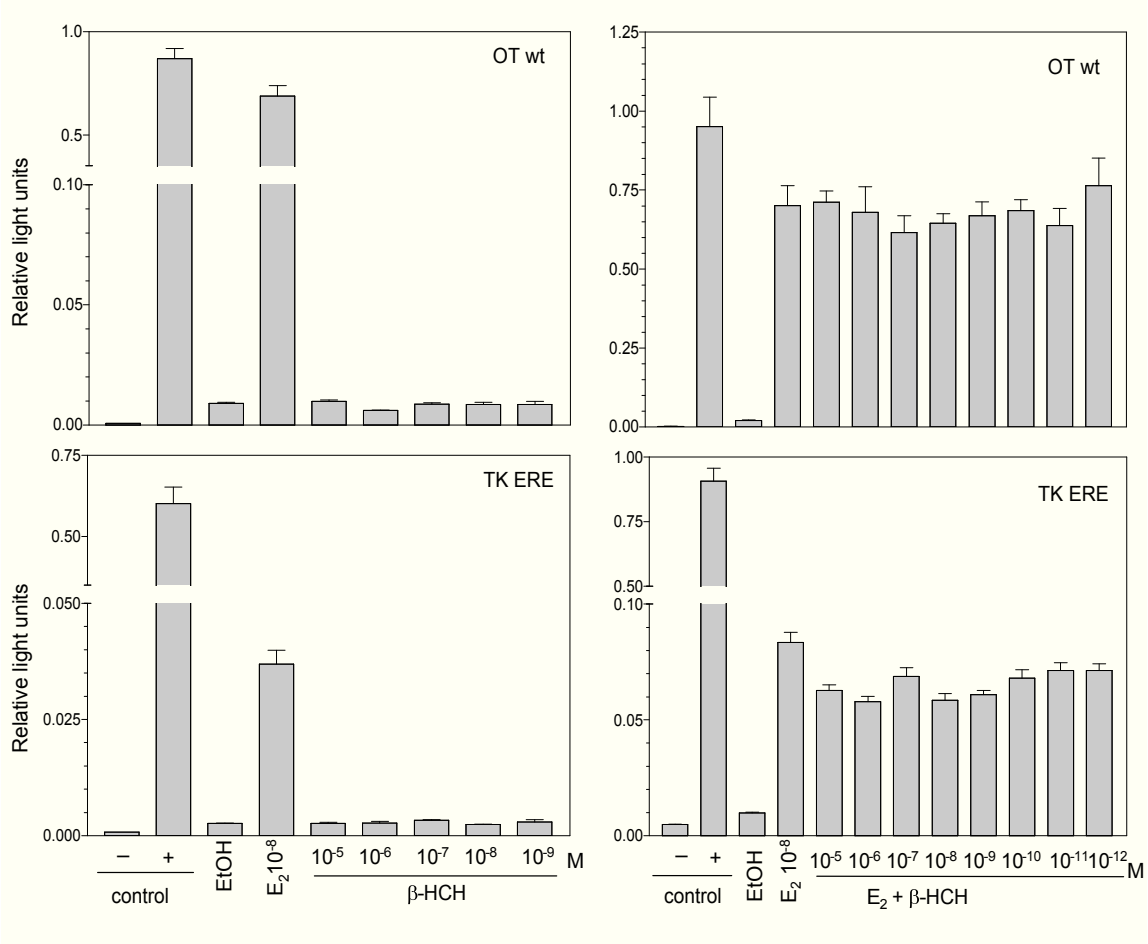


Fig 38: Transcriptional effects of ß-HCH on the oxytocin promoter and the thymidine kinase-ERE promoter by estrogen receptor α in the MDA-MB 231 cells. MDA-MB 231 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter construct (TK ERE) and estrogen receptor α and treated with different concentrations of ß-HCH alone or simultaneously with estradiol (E_2 10^{-8} M).

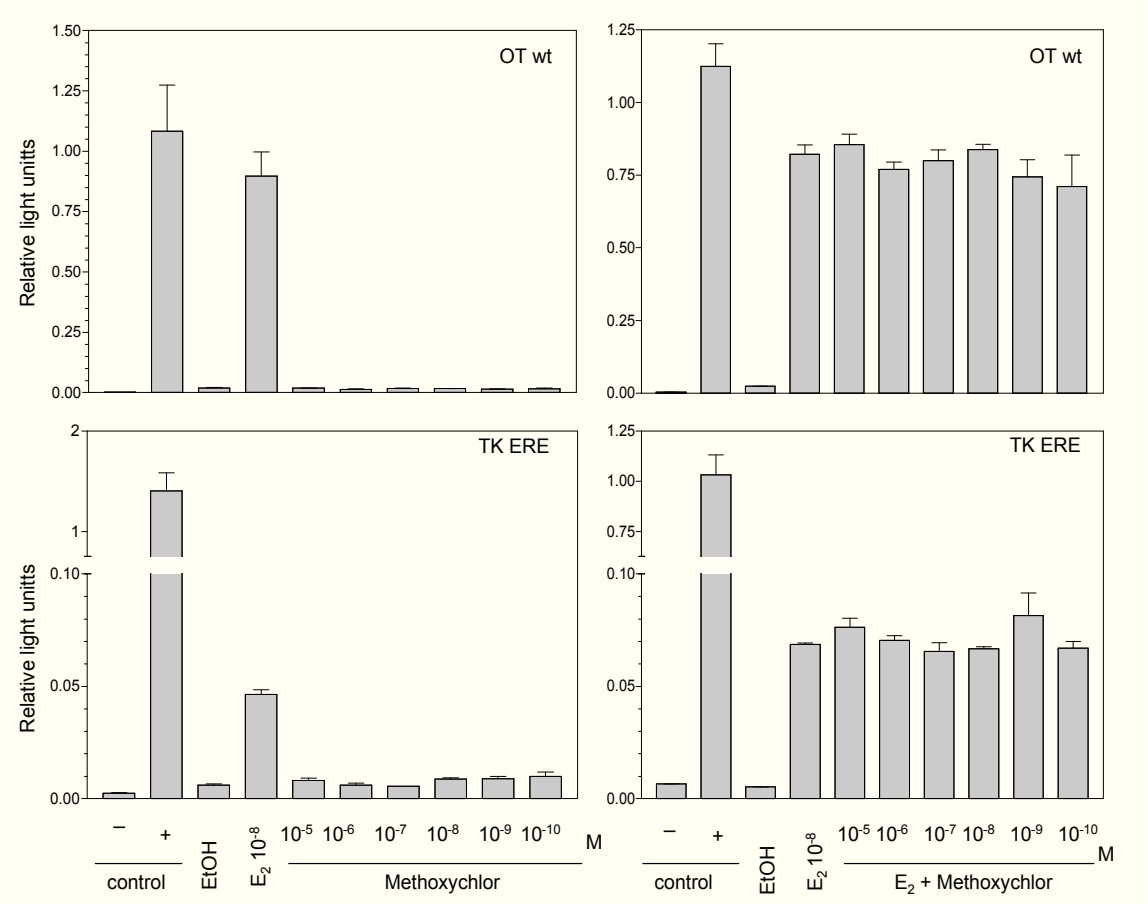


Fig 39: Transcriptional effects of methoxychlor on the oxytocin promoter and the thymidine kinase-ERE promoter by estrogen receptor α in the MDA-MB 231 cells. MDA-MB 231 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter (TK ERE) construct and estrogen receptor α and treated with different concentrations of methoxychlor alone or simultaneously with estradiol (E_2 10^{-8} M).

Whereas no antagonistic or synergistic effects could be detected with any of the compounds, in some cases a reduction in transcriptional activity could be observed in some concentration ranges of industrial xenoestrogens.

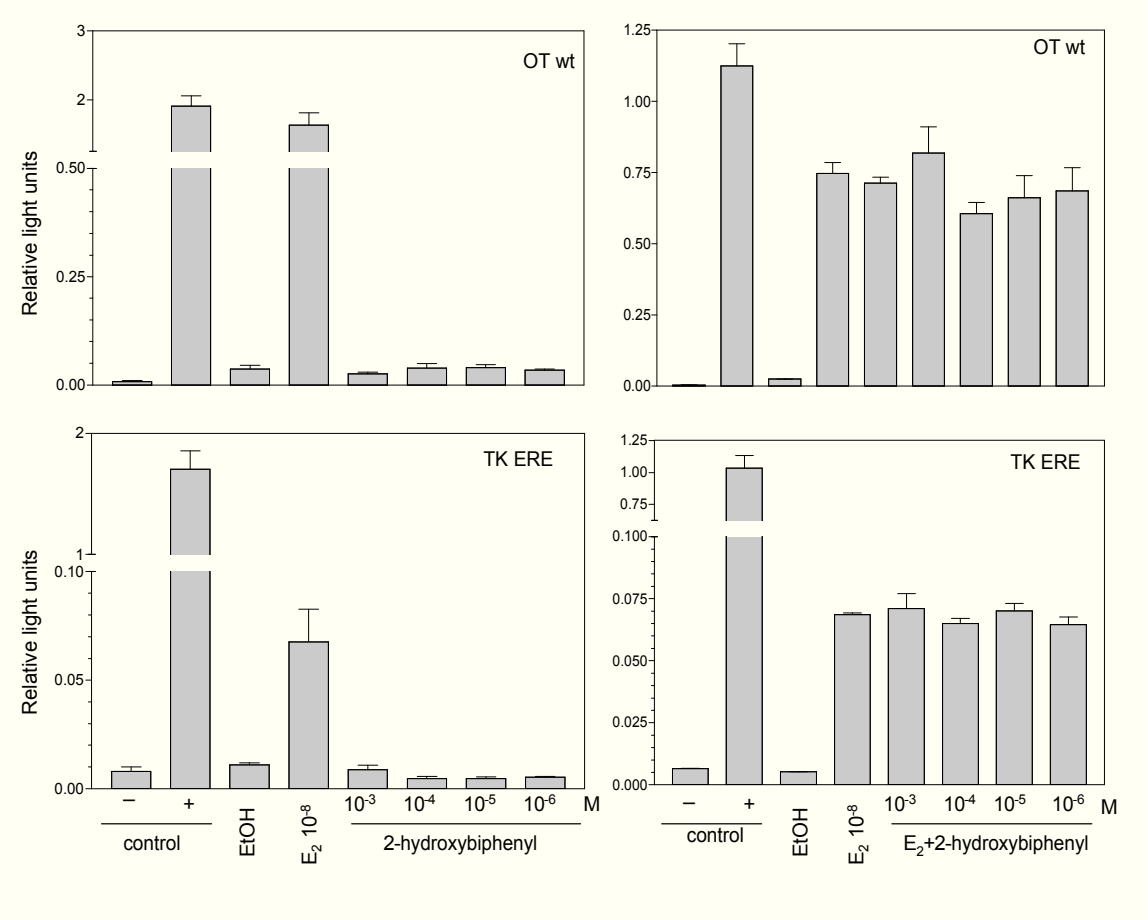


Fig 40: Transcriptional effects of 2-hydroxibiphenyl on the oxytocin promoter and the thymidine kinase-ERE promoter by estrogen receptor α in MDA-MB 231 cells. MDA-MB 231 cells were transfected with the oxytocin promoter (OTwt) or the thymidine kinase-ERE (TK ERE) promoter and estrogen receptor α and treated with different concentrations of 2-hydroxibiphenyl alone or simultaneously with (E_2 10^{-8} M).

However, these effects, like the weak inhibitory effect observed with β-HCH in the presence of estradiol apparently are not strictly dose–dependent.

2.2. Stimulation of the Oxytocin Promoter by Different Phytoestrogens

In this part of the study to investigate the effects of the influence of different phytoestrogens on transcription from the oxytocin and the thymidine kinase-ERE promoter was investigated in MDA-MB 231 cells. The investigation was focused on the effects of naringenin, and its chemical derivatives, 6-(1,1-dimethylallyl)naringenin (6DMA-naringenin) and 8-prenylnaringenin (8p-naringenin), as these compounds have been shown to exhibit considerable selectivity for one of the two subtypes of estrogen receptor (Vollmer et al., 2002; Zierau et al., 2002; Milligan et al., 2002; Jannette et al.,

2003). These substances have recently been isolated from hops and citrus. In addition, also the phytoestrogens resveratrol, a constituent of red wine grapes, genistein, mainly found in legumes, such as soybeans and chickpeas, and biochanin A, also found in legumes, were investigated. MDA-MB 231 cells were transfected with the oxytocin or the thymidine kinase-ERE promoter and co-transfected with expression vectors for different subtypes of estrogen receptors, human estrogen receptor α, human estrogen receptor ß, bovine estrogen receptor ß. A mutant estrogen receptor α unable to bind DNA was used to indicate non-classical estrogen receptor action, and the transfected cells were treated with increasing concentrations of naringenin (Fig 42). The results clearly show that naringenin in the presence of estrogen receptor α has no major agonistic effect at the concentrations used, either on the oxytocin or on the thymidine kinase-ERE promoter. Only an extremely weak agonistic activity on the oxytocin promoter could be detected at 10^{-5} M. In the presence of human estrogen receptor ß or bovine estrogen receptor ß, naringenin shows agonistic activity with half-maximal concentrations around 10^{-6} M. In the presence of the estrogen receptor α mutant unable to bind DNA, the lack of significant effects of naringenin corresponds to the results obtained with the wild type estrogen receptor α.

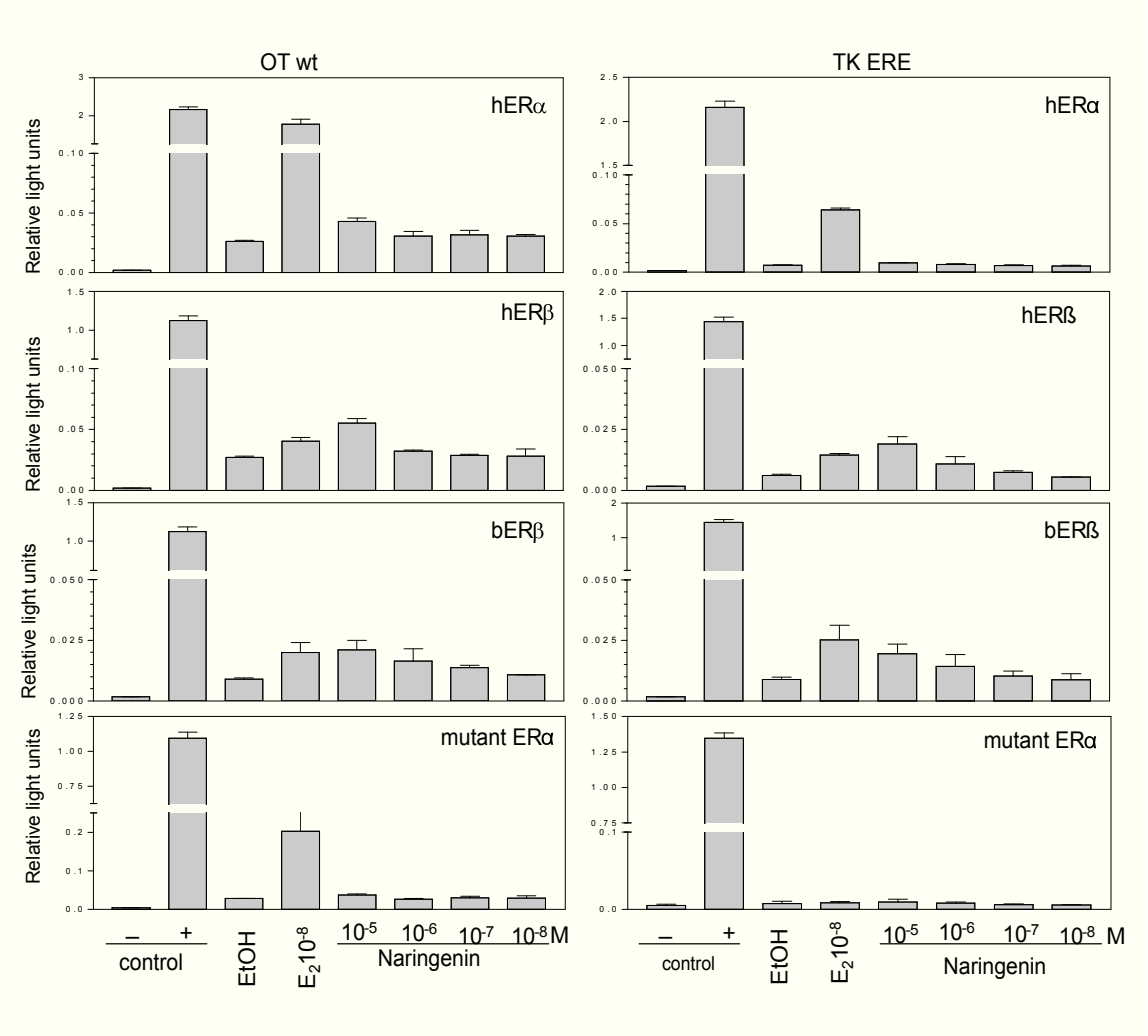


Fig 41: Transcriptional effects of naringenin on the oxytocin promoter and the thymidine kinase-ERE promoter by different estrogen receptors in the MDA-MB 231 cells. MDA-MB 231cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter construct (TK ERE) and human estrogen receptor α (hERα) human estrogen receptor ß (hERß), bovine estrogen receptor ß (bERß) or estrogen receptor α mutant unable to bind DNA (mutant ERα) and treated with different concentrations of naringenin.

In parallel experiments using 8p-naringenin which is found in hops, the MDA-MB 231 cells were transfected as described above with the thymidine kinase-ERE-promoter and co-transfected with the different subtypes of estrogen receptors as described above. The transfected cells were treated with increasing concentrations of 8p-naringenin (Fig 42). The results clearly show that 8p-naringenin has a high agonistic effect on the oxytocin promoter and the thymidine kinase-ERE promoter in the presence of human estrogen receptor α. Half-maximal stimulation was obtained at 10^{-7} M to 10^{-6} M on the oxytocin

promoter, in contrast to 10^{-8}M to 10^{-7}M on the thymidine kinase-ERE promoter. In the presence of human estrogen receptor β, 8p-naringenin has as agonistic effect on the oxytocin promoter, in the presence of bovine estrogen receptor ß, 8p-naringenin did not show any dose-dependent agonistic effect. The transcriptional stimulation of the thymidine kinase–ERE promoter observed in the presence of bovine estrogen receptor ß is not dependent on the dose of 8p-naringenin and could be also observed at concentration as low as 10^{-8} M. Apparently, there are significant functional differences between human and bovine estrogen receptor β. In order to discriminate between classical and non-classical estrogenic effects, the estrogen receptor α mutant unable to bind to DNA was used. The promoter-specific selective agonistic action clearly shows that on the oxytocin promoter, ligand-activated estrogen receptor α does not need to bind to DNA to stimulate the transcriptional activity.

In the other experiment following the same protocol, the effects of the other substituted naringenin, 6-(1, 1-dimethylallyl) naringenin (6DMA-naringenin), was investigated (Fig 43).

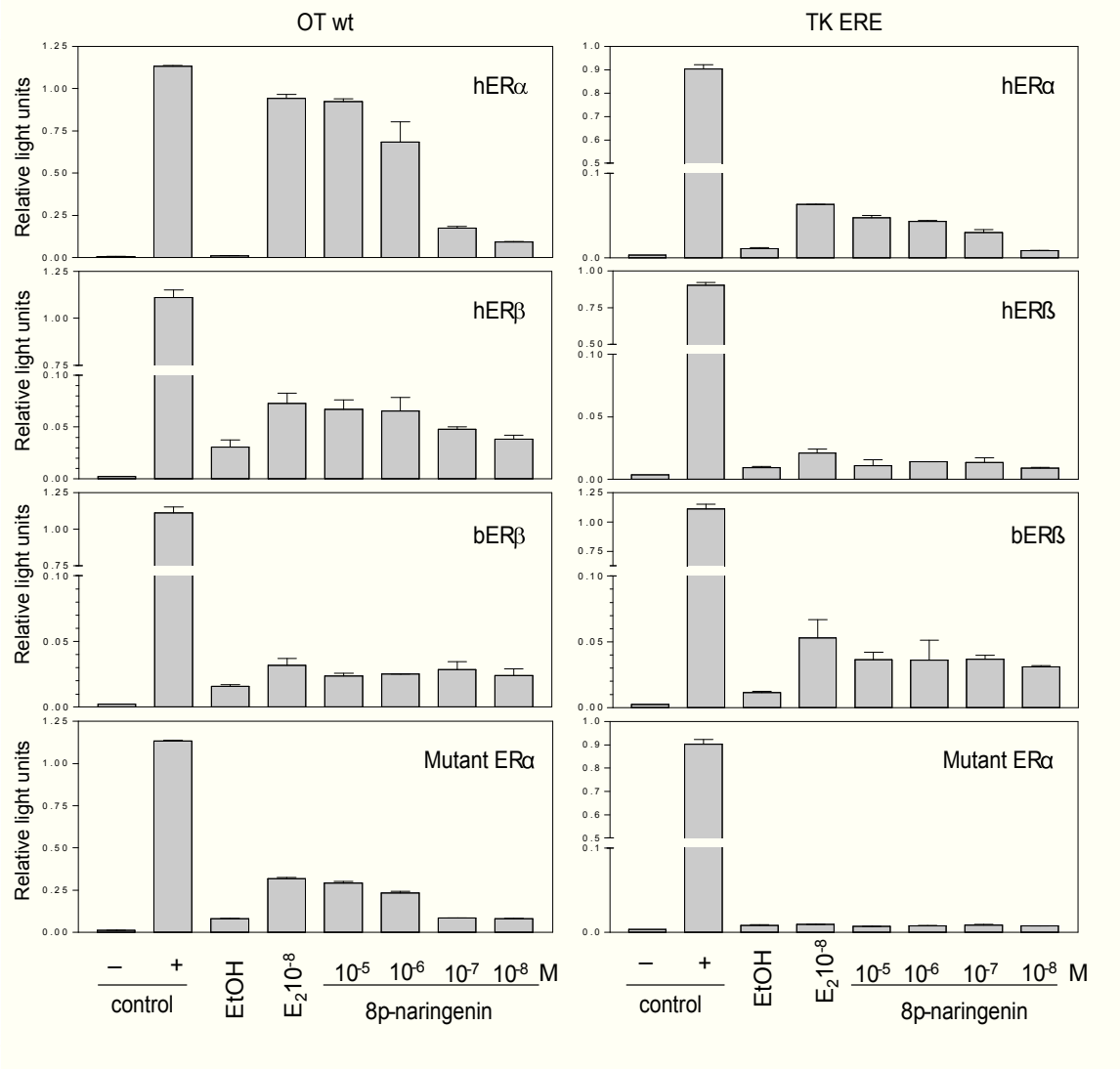


Fig 42: Transcriptional effects of 8p-naringenin on the oxytocin promoter and the thymidine kinase-ERE promoter by different subtype of estrogen receptors in MDA-MB 231cells. MDA-MB 231 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter construct (TK ERE) and human estrogen receptor α (hERα), human estrogen receptor ß (hERß), bovine estrogen receptor ß (bERß) or estrogen receptor α mutant unable to bind DNA (mutant ERα) and treated with different concentrations of 8p-naringenin.

The results show that 6DMA-naringenin has an agonistic effect on the oxytocin promoter and on the thymidine kinase-ERE promoter by human estrogen receptor α, with half – maximal stimulation in both cases reached at a concentration between 10^{-7} M and 10^{-6} M. In the presence of human or bovine estrogen receptor β, a clearly dose-dependent effect of 6-DMA-naringenin cannot be observed. A possible weak agonistic activity may be concealed by the apparent inhibition of transcriptional activation at high concentrations

of 6-DMA-naringenin, probably due to the toxicity of this compound. The agonistic effect of 6-DMA-naringenin in the presence of estrogen receptor α on the oxytocin promoter cannot be abolished by the mutation impairing the specific DNA binding of the receptor, underlining the non-classical nature of this activation, in contrast to the classical mechanism of activation at the thymidine kinase-ERE promoter.

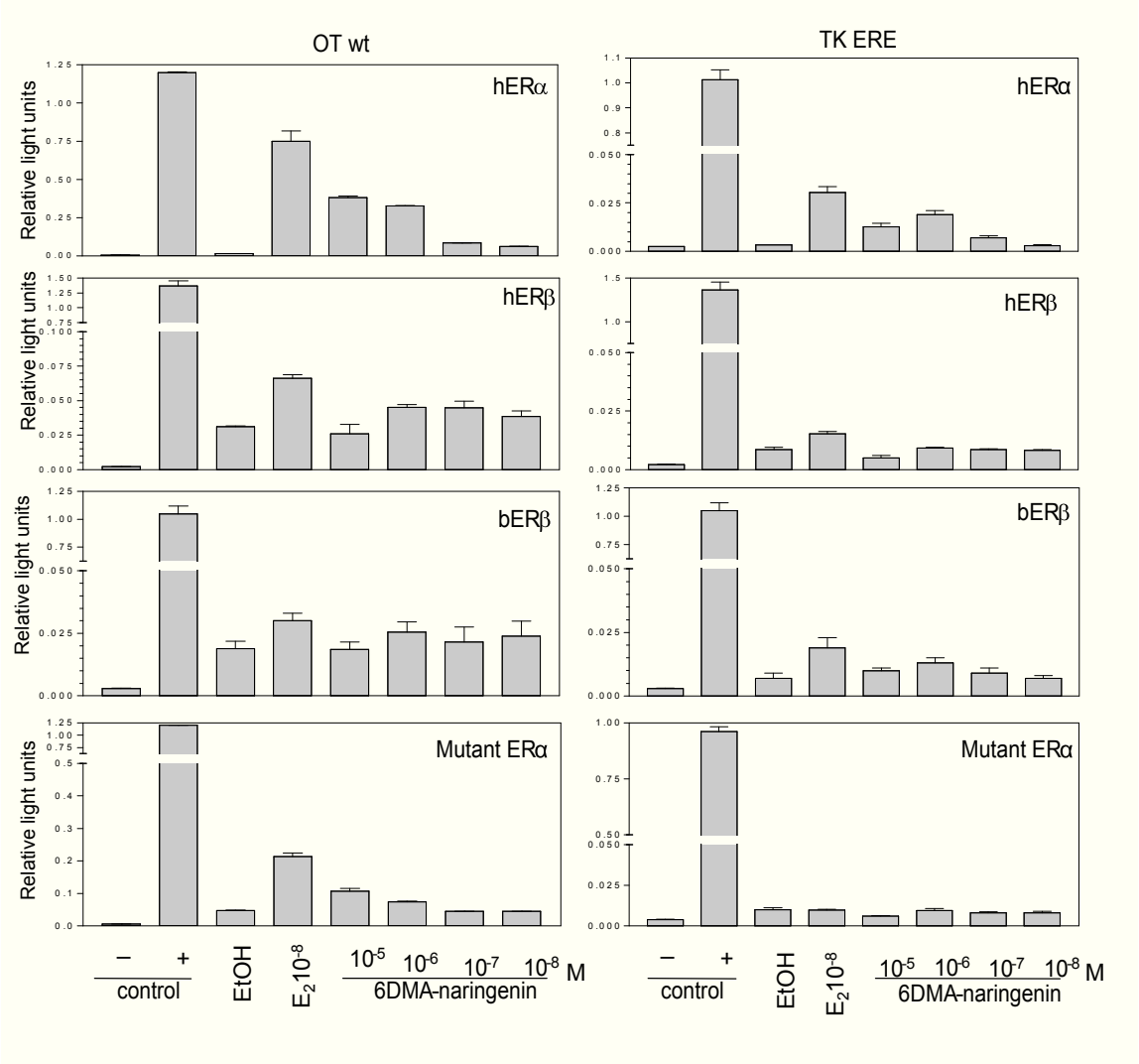


Fig 43: Transcriptional effects of 6DMA-naringenin on the oxytocin promoter and the thymidine kinase-ERE promoter by different subtype of estrogen receptors in MDA-MB 231cells. MDA-MB 231 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter construct (TK ERE) and human estrogen receptor α (hERα), human estrogen receptor ß (hERß), bovine estrogen receptor ß (bERß) or estrogen receptor α mutant unable to bind DNA (mutant ERα) and treated with different concentrations of 6DMA-naringenin.

Promoter:	Oxytocin				TK ERE			
Receptors: / Compounds	hERa	hERß	bERß	Mutant hERa	hERa	hERß	bERß	Mutant hERa
Naringenin	- *	+	+	-	-	+	+	-
8p-Naringenin	+	+ **	-	+	+	-	+	-
6DNA-Naringenin	+	-	-	+	+	-	-	-

Table 2: Transcriptional effects of phytoestrogens (naringenin, 6DMA-naringenin, and 8p-naringenin) on the oxytocin or the thymidine kinase ERE (TK ERE) promoter. (*: low stimulation at high concentration of naringenin, **: not dose-dependent)

In order to test a wider spectrum of phytoestrogens, the MDA-MB 231 transfection system was used to investigate the transcriptional effects of resveratrol. MDA-MB 231 cells were transfected as described above with the oxytocin or the thymidine kinase-ERE promoter and co-transfected with estrogen receptor α, and cells were treated with different concentrations of resveratrol (Fig 44). The results show that resveratrol has a weak agonistic effect on the oxytocin and the thymidine kinase-ERE promoter with half maximal stimulation reached at a concentration around 10^{-6} M. In contrast to all other substances tested so far, this effect was additive in respect of the stimulatory effects of 10^{-8} M estradiol, most clearly demonstrated at the oxytocin promoter.

In another experiment these results were compared with the transcriptional effects of the phytoestrogen genistein, present in soy and constituting an important alimentary factor in soy products. In this experiment, as before, MDA-MB 231 cells were transfected with oxytocin or the thymidine kinase-ERE promoter and estrogen receptor α and treated with increasing concentrations of genistein (Fig 45 A and B).

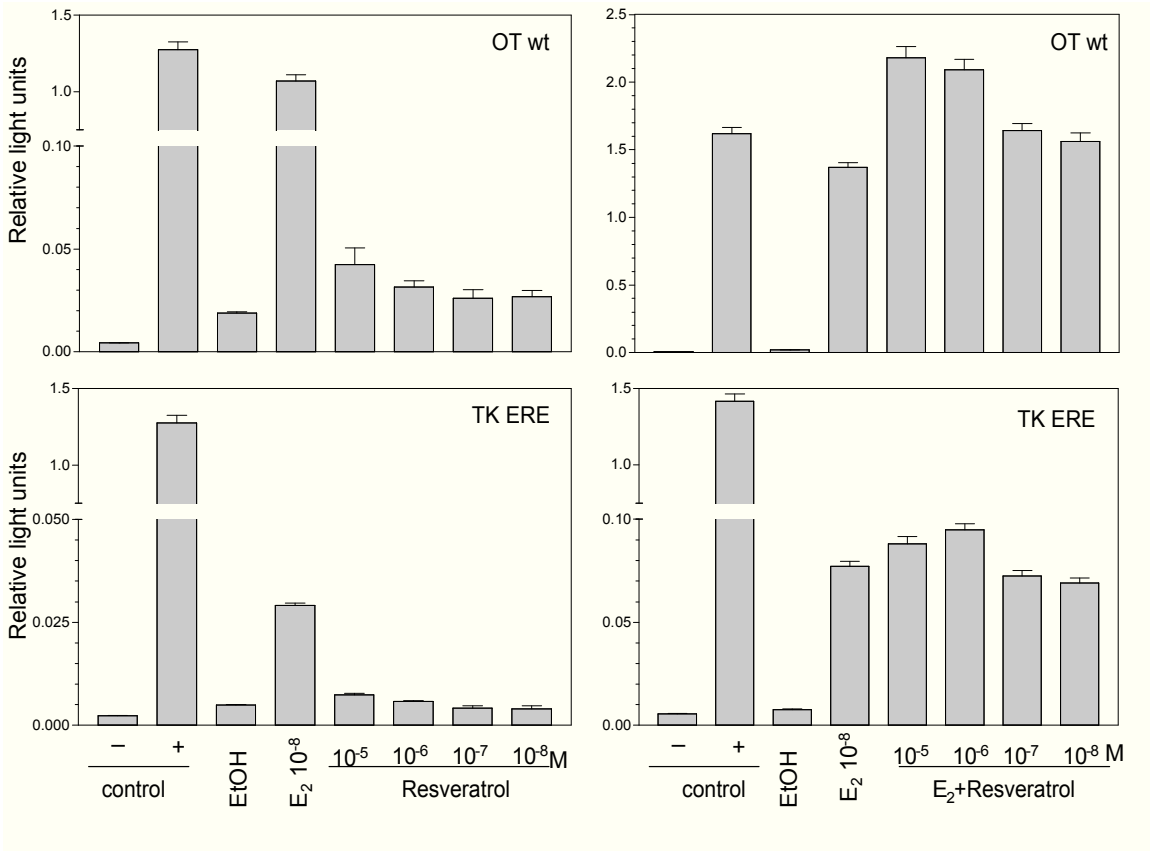
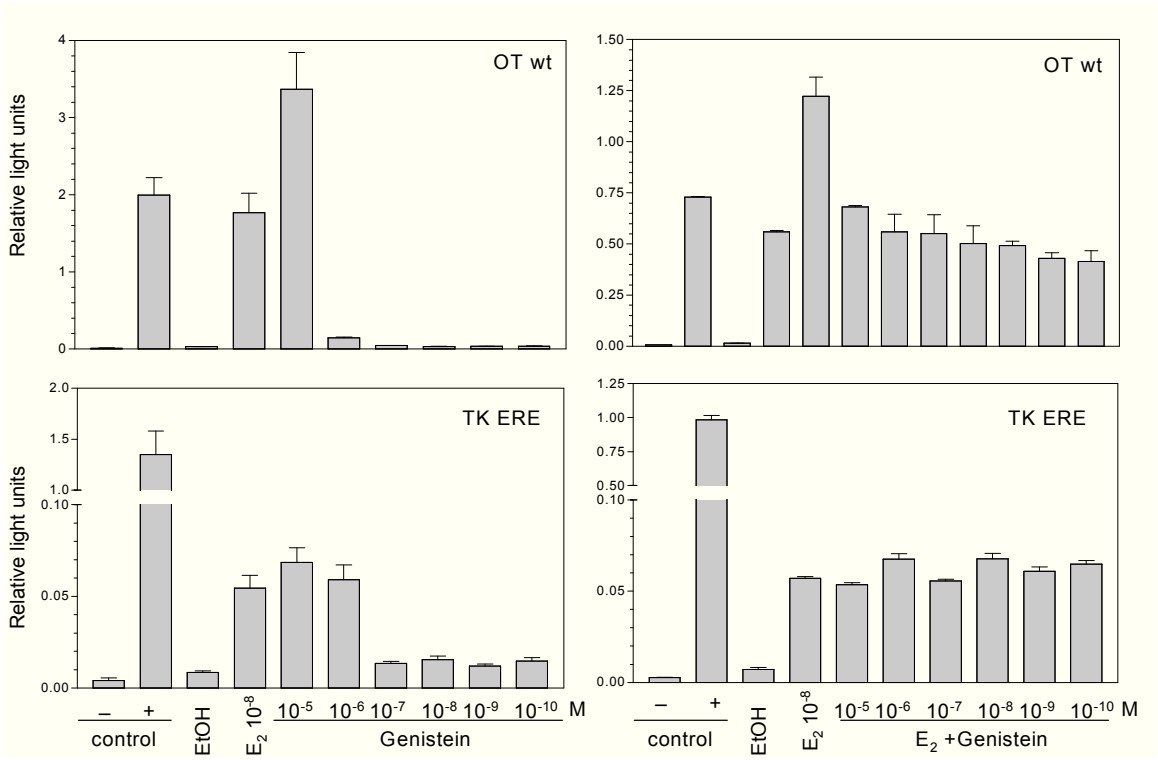


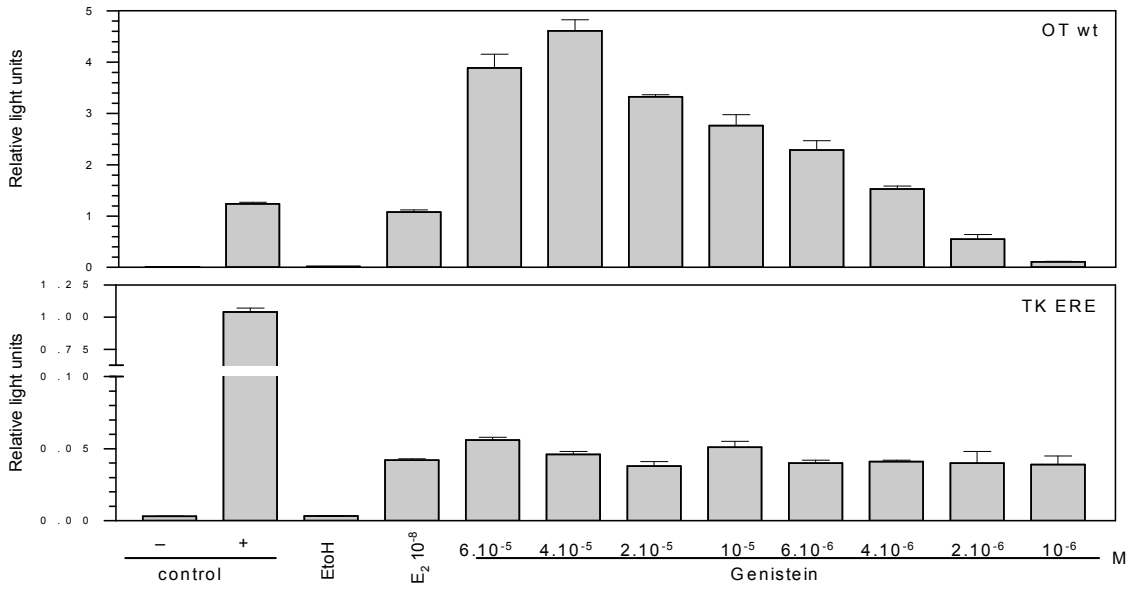
Fig 44: Transcriptional effects of resveratrol on the oxytocin promoter and the thymidine kinase-ERE promoter by estrogen receptor α in MDA-MB 231 cells. MDA-MB 231 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter construct (TK ERE) and estrogen receptor α and treated with different concentrations of resveratrol alone or simultaneously with estradiol (E_2 10^{-8} M).

The results indicate that high concentrations of genistein have a very strong agonistic effect on the oxytocin and the thymidine kinase-ERE promoter, with a half-maximal dose in the range of 10^{-6} M to 10^{-5} M in the case of the oxytocin promoter, compared to 10^{-7} M to 10^{-6} M on the thymidine kinase–ERE promoter. The maximal stimulation caused by high concentrations of genistein even exceeds the maximal stimulation by estradiol.

In order to determine the maximal transcriptional effect of genistein on the oxytocin promoter, the amounts of genistein added to transfected MDA-MB 231 cells were further increased (Fig 45 B).



(A)



(B)

Fig 45 (A and B): Transcriptional effects of genistein on the oxytocin promoter and the thymidine kinase-ERE promoter by estrogen receptor α in the MDA-MB 231 cells. MDA-MB 231 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter construct (TK ERE) and estrogen receptor α and treated with different concentrations of genistein alone or simultaneously with estradiol ($E_2\ 10^{-8}$ M).

Whereas the stimulation of the thymidine kinase-ERE promoter could not be further stimulated by genistein concentrations higher than 10^{-5} M, maximal stimulation of the oxytocin promoter was reached only at a concentration of 4×10^{-5} M, emphasizing again the different nature of the two mechanisms of promoter stimulation. In the last experiment using phytoestrogens, the effect of biochanin A was investigated in the MDA-MB 231 transfection system (Fig 46). Agonistic transcriptional effect caused by biochanin A could be detected, neither on the oxytocin nor on the thymidine kinase-ERE promoter. For the inhibitory action of biochanin A, surprisingly observed only at low concentration of the component, an explanation cannot be given on the basis of this experiment. That this inhibition is not dose-dependent might be indicative of another, indirect action of xenoestrogens, which cannot be reliably measured in this test system.

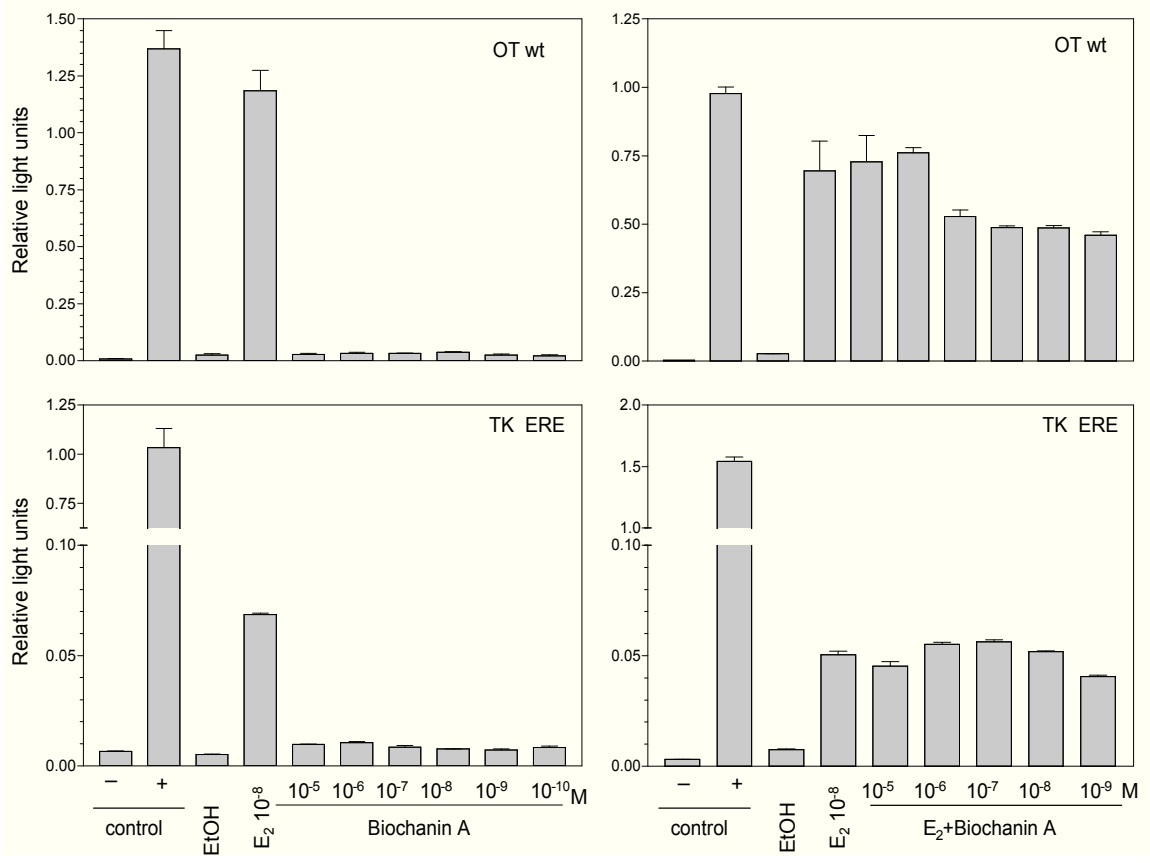


Fig 46: Transcriptional effects of biochanin A on the oxytocin promoter and the thymidine kinase-ERE promoter by estrogen receptor α in the MDA-MB 231 cells. MDA-MB 231 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter construct (TK ERE) and estrogen receptor α and treated with different concentrations of biochanin A alone or simultaneously with estradiol (E_2 10^{-8} M).

Promoter:	Oxytocin		TK ERE	
Effect / Compounds	Agonistic	Antagonistic	Agonistic	Antagonistic
Genistein	+	-	+	-
Resveratrol	+	-	+	-
Biochanin A	-	-	-	-

Table 3: Agonistic or antagonistic activity of phytoestrogens on the oxytocin or the thymidine kinase ERE (TK ERE) promoter in MDA-MB 231 cells.

2.3. Promoter-Specific Stimulation of Transcription by Metal Ions

In addition to the effects of industrial xenoestrogens and phytoestrogens, the MDA-MB 231 transfection system was used to determine whether "quasi-estrogenic" effects on transcription of metal ions can be detected using this assay system .

In the first experiment MDA-MB 231 cells were transfected with oxytocin or the thymidine kinase-ERE promoter and an expression vector containing the human estrogen receptor α. The transfected cells were treated with increasing concentration of metal ions, for magnesium and nickel ions (Fig 47) no agonistic effect on the two promoters could be shown.

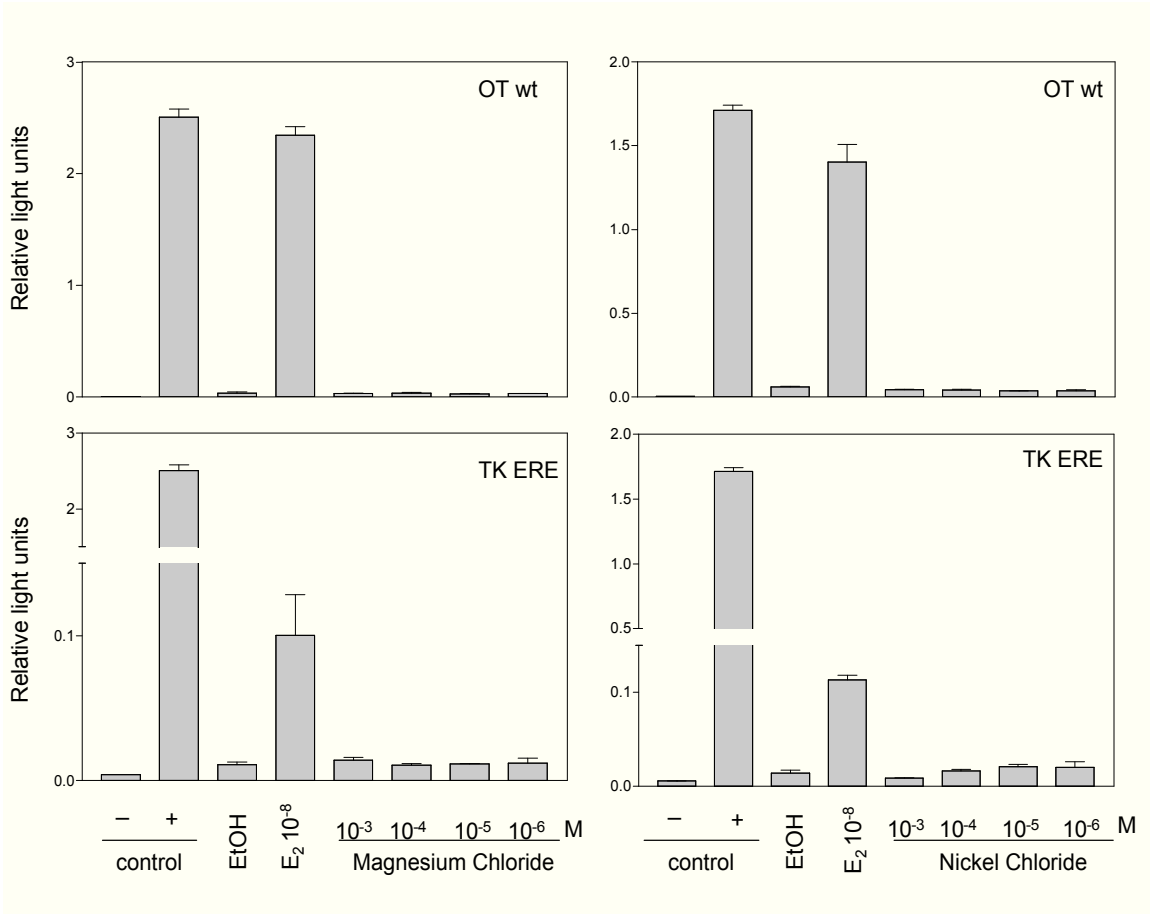


Fig 47: Transcriptional effects of magnesium chloride and nickel chloride on the oxytocin and the thymidine kinase-ERE promoter by estrogen receptor α in the MDA-MB 231 cells. MDA-MB 231 cells were transfected with the oxytocin promoter (OTwt) or the thymidine kinase-ERE promoter (TK ERE) and estrogen receptor α (ERα) and treated with different concentrations of magnesium chloride or nickel chloride.

In an other experiment MDA-MB 231 cells were transfected as described above with the oxytocin promoter or the thymidine kinase-ERE promoter and co-transfected with estrogen receptor α and the transfected cells were treated with different concentration of cupric chloride or cupric sulphate (Fig 48).

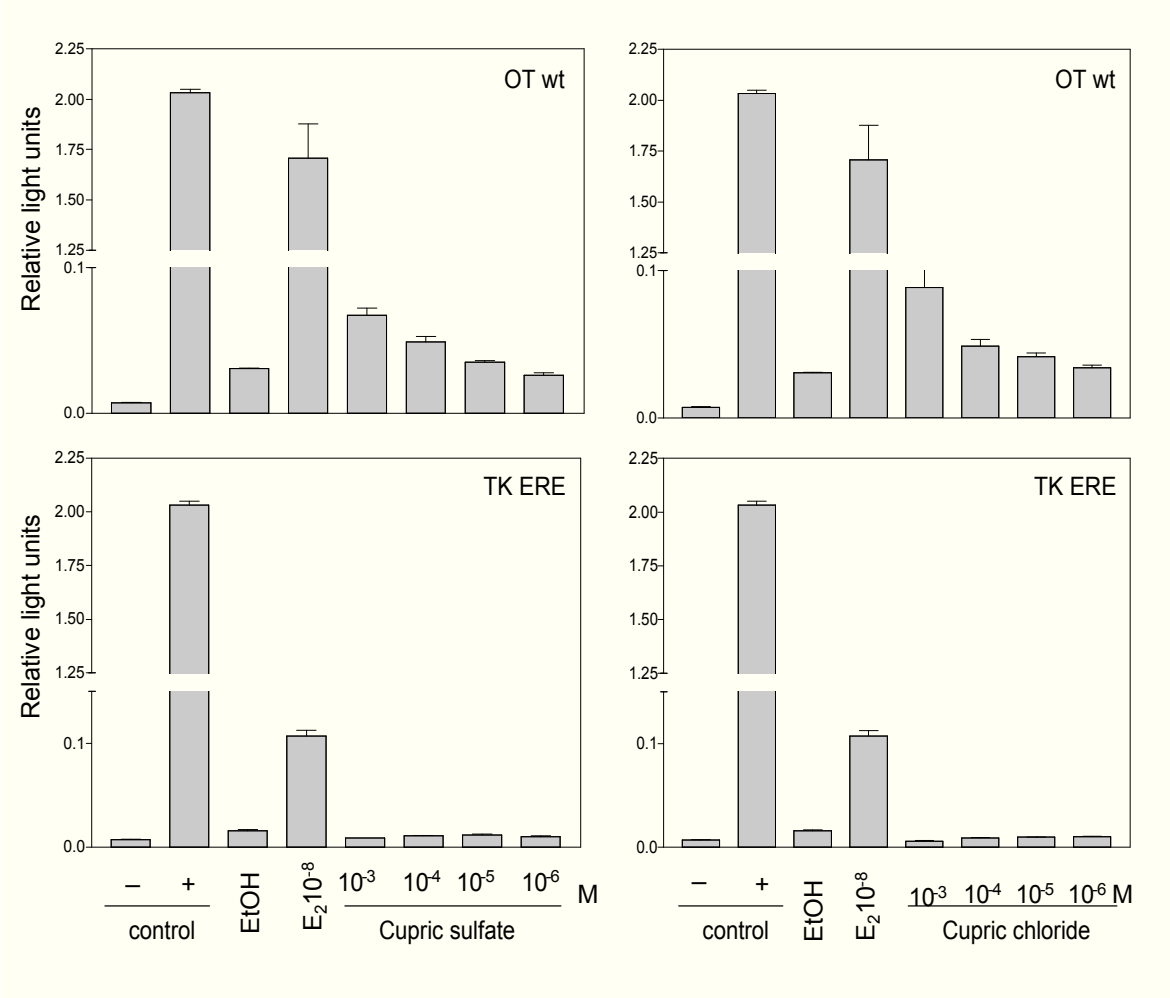


Fig 48: Transcriptional effects of cupric sulphate and cupric chloride on the oxytocin and the thymidine kinase-ERE promoter by estrogen receptor α in the MDA-MB 231 cells. MDA-MB 231 cells were transfected with the oxytocin promoter (OTwt) or the thymidine kinase-ERE promoter (TK ERE) and estrogen receptor α (ERα) and treated with different concentrations of cupric sulphate or cupric chloride.

These results demonstrate an agonistic activity of copper ions with a half-maximal stimulation at a concentration around 10^{-4}M specifically on the oxytocin promoter.

In another experiment, MDA-MB 231cells were transfected as described above and the transfected cells were treated with increasing concentrations of cobalt chloride or lithium chloride (Fig 49). Only a weak agonistic effect of cobalt ions could be detected that appears to be selective for the thymidine kinase-ERE promoter.

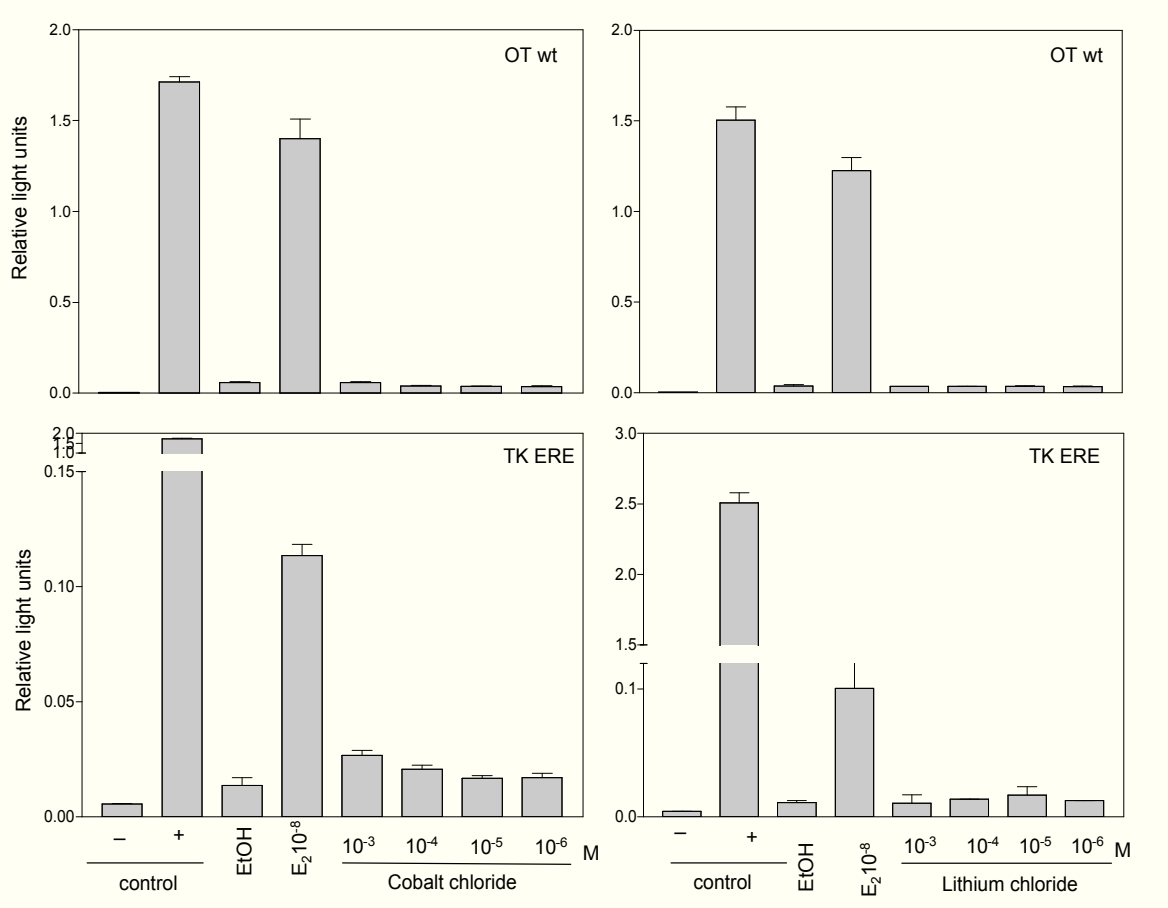


Fig 49: Transcriptional effects of cobalt chloride and lithium chloride on the oxytocin and the thymidine kinase-ERE promoter by estrogen receptor α in the MDA-MB 231 cells. MDA-MB 231 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter construct (TK ERE) and estrogen receptor α (ERα) and treated with different concentrations of cobalt chloride or lithium chloride.

In another experiment MDA-MB 231 cells as described above were transfected and the transfected cells were treated with increasing concentrations of mercury chloride or zinc sulphate (Fig 50). Only with mercury ion could a weak dose-dependent agonistic activity be detected on the thymidine kinase-ERE promoter. Unfortunately, it was not possible to employ higher concentration of mercury or zinc salts, like the ones used in the experiment with other metals described above, due to the high toxicity of mercury and zinc ions.

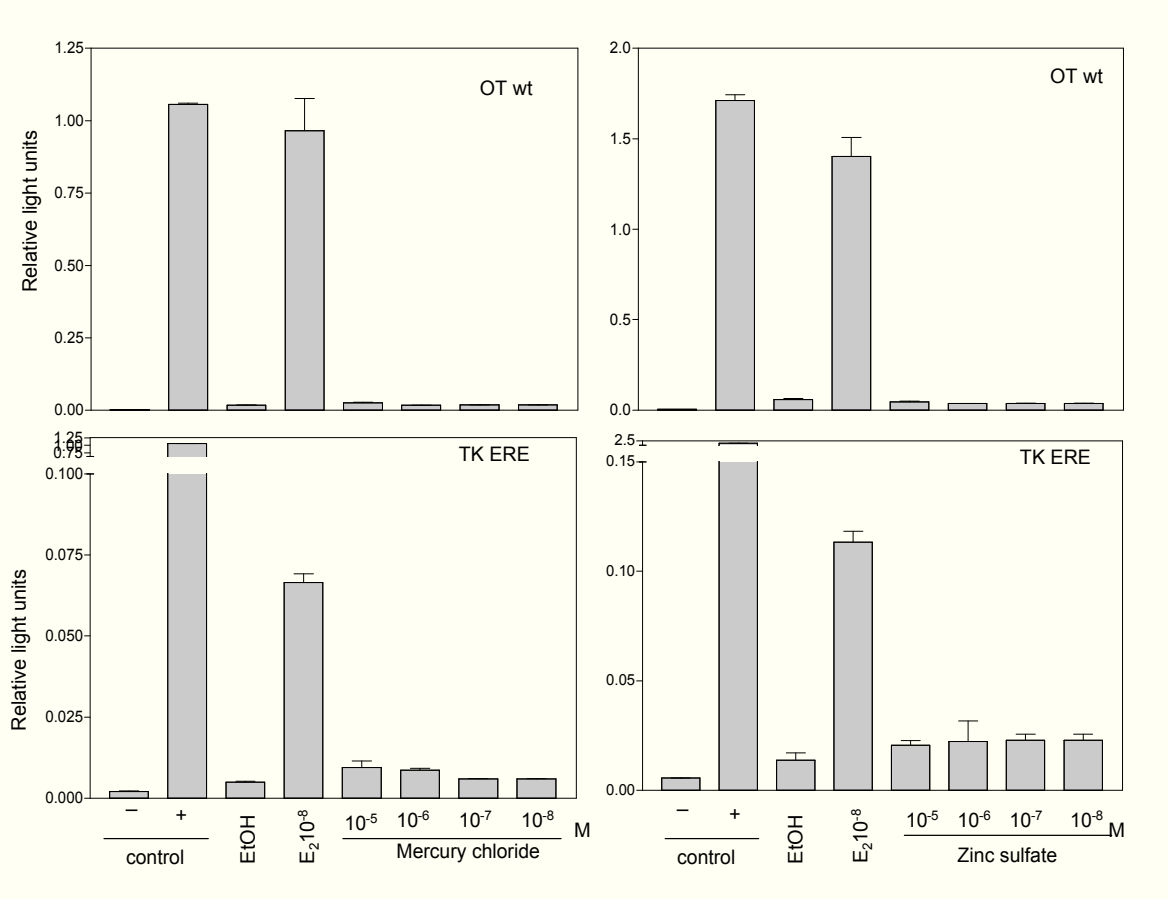


Fig 50: Transcriptional effects of mercury chloride and zinc sulphate on the oxytocin and the thymidine kinase-ERE promoter by estrogen receptor α in the MDA-MB 231 cells. MDA-MB 231 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter construct (TK ERE) and estrogen receptor α (ERα) and treated with different concentrations of mercury chloride or zinc sulphate.

In the last experiment investigating the effects of metal ions, MDA-MB 231 cells were transfected as described above and the transfected cells were treated with increasing concentrations of cadmium chloride (Fig 51). The results demonstrate that cadmium chloride has a significant agonistic effect on the oxytocin promoter with half-maximal stimulation reached at a concentration of approximately 10^{-5} M to 10^{-4} M. This effect, as the effect of copper ions, is totally selective for the oxytocin promoter.

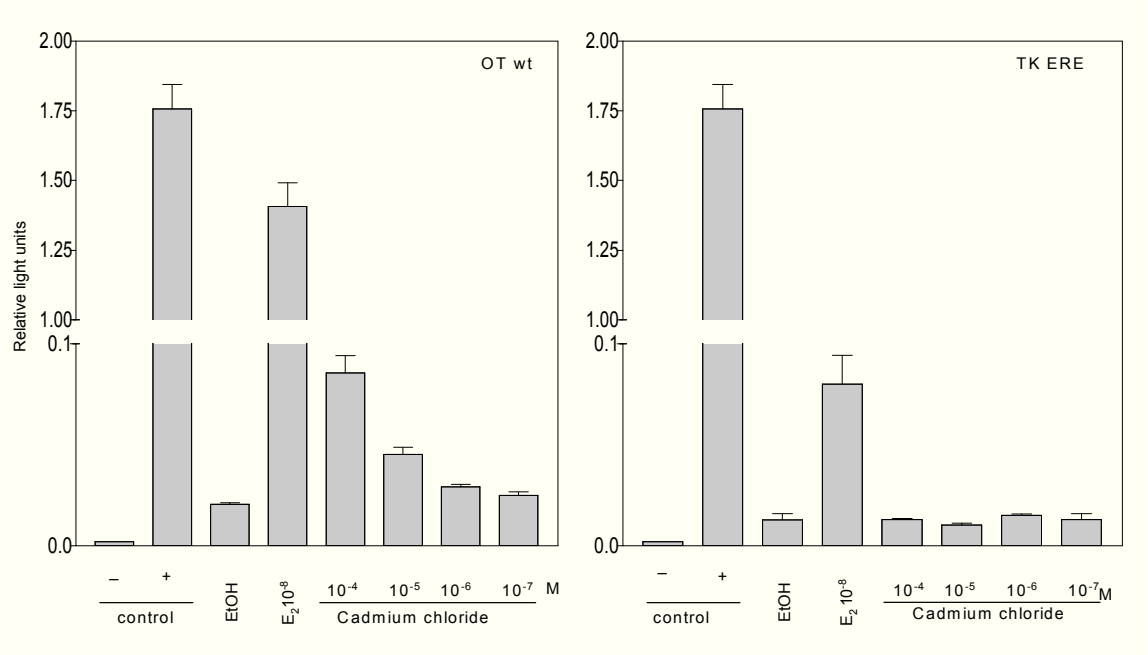


Fig 51: Transcriptional effects of cadmium chloride on the oxytocin and the thymidine kinase-ERE promoter by estrogen receptor α in the MDA-MB 231 cells. MDA-MB 231 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter construct (TK ERE) and estrogen receptor α (ERα) and treated with different concentrations of the cadmium chloride.

In summary, from the metal ions selected from the literature as having estrogen-like effects, several showed a total selectivity for promoters regulated by either the classical ERE-dependent mechanism (cobalt and, possibly, mercury), or the non-classical mechanism (copper and cadmium). Other metal ions, for which no transcriptional activity could be detected in this assay system, apparently exert their effects by still other mechanisms.

Promoters: / Compounds	Oxytocin	TK ERE
Magnasium chloride	-	-
Nickel chloride	-	-
Cupric chloride	+	-
Cobalt chloride	-	+
Lithium chloride	-	-
Mercury chloride	-	+
Zinc sulfate	-	-
Cadmium chloride	+	-

Table 4: Transcriptional activity of metal ions on the oxytocin or the thymidine kinase ERE (TK ERE) promoter in MDA-MB 231 cells.

DISCUSSION

Non-classical mechanisms of estrogen action provide a major contribution to the biological effects of this important gonadal steroid. The gene coding for the neuropeptide hormone oxytocin is one for which the molecular mechanisms by which estrogen controls gene activity has not yet be elucidated. The partially conserved estrogen response element present in the oxytocin promoter region in several species (Richard et al., 1990; Burbach et al., 1990) has been claimed to be the major cis-regulatory element controlling the effects of estrogen on the expression of this gene. However, the proposed mechanism of direct estrogen receptor binding cannot be of general importance, as the corresponding palindromic sequence is not conserved in the oxytocin promoter of ruminants, such as the bovine. In addition, it was shown in a previous study (Stedronsky et al., 2002) that the affinities of binding of both estrogen receptors to the human oxytocin promoter are not sufficient to confirm that this partially conserved palindromic sequence represents a functional estrogen response element. The discrepancy between the results presented by this study and the work of other authors apparently is caused by the different methods employed for the investigation of the oxytocin promoter region. Using mainly in vitro binding studies with nuclear extracts from tissues actively expressing the oxytocin gene, the alternative binding of two nuclear orphan receptors (SF-1 and COUP-TF I) to a common binding site overlapping the position of the partially conserved palindromic sequence could be shown (Walther et al., 1991; Wehrenberg et al., 1992; 1994a; 1994b). Other groups, on the other hand, have employed transient transfection studies in heterologous cell lines, mainly using an over-expression of various nuclear receptors by co-transfection. Under these conditions the oxytocin promoter could be regulated by estrogens (Richard et al., 1990; Burbach et al., 1990), retinoic acid (Richard et al., 1991), or thyroid hormone (Adan et al., 1992), suggesting the presence of a “multiple hormone response element” (Adan et al., 1993). Although it cannot formally be excluded that the expression of the oxytocin gene may be influenced by various hormones, the direct actions of activated hormone receptors on the oxytocin promoter presumably represent experimental artefacts caused by massive over-expression of nuclear hormone receptors in heterologous transfection experiments. Due to the high binding affinity of nuclear

orphan receptors SF-1 and COUP-TFI to the bovine as well as the human oxytocin promoter (Stedronsky et al., 2002), any attempt to explain the mechanism of hormonal regulation of the oxytocin promoter has to take into account that the regulatory site in the promoter is probably occupied by stimulatory or inhibitory nuclear orphan receptors contributing to the activity of oxytocin gene expression by an as yet uncharacterized mechanism.

The unexpected finding that the bovine oxytocin promoter was massively up-regulated by activated estrogen receptor α in breast cancer cell lines, although it does not encompass any sequence resembling a functional estrogen response element, prompted a detailed investigation of the mechanism of this regulation. In contrast to the studies criticized above, here the use of transient transfection experiments using co-transfection and over-expression of estrogen receptor α is appropriate, as the bovine oxytocin promoter used in this study does not contain a binding site for this receptor. The co-transfection system using the estrogen receptor α negative human breast cancer cell line MDA-MB 231, in addition, opens up the possibility to introduce estrogen receptor mutants and study in detail the transcriptional effects of isolated estrogen receptor functions. The interpretation of the most relevant findings of the extensive series of transfections in the first part of the experiments can best be summarized in the following statements:

Estrogen-dependent up-regulation of the oxytocin promoter is dependent on the integrity of the nuclear orphan receptor binding site.

In an experiment designed to identify the components essential for transcriptional activation, the dependence of the up-regulation of the bovine oxytocin promoter on the integrity of the nuclear orphan receptor binding site could be clearly demonstrated. For full activity of the oxytocin promoter the conservation of the binding sequence for a monomeric nuclear orphan receptor like SF-1 appears to be essential (Koohi et al., 2005). The insertion of a single base pair by site-directed mutagenesis destroys the binding site for monomeric orphan receptors, but improves the binding site for COUP-TFI by producing a direct repeat of the basic sequence motif separated by one base pair (DR1) (Tsai et al., 1987; Qiu et al., 1994). The promoter containing this mutated element exhibits a reduced activity, suggesting that either (1) the nuclear orphan receptor from

MDA-MB 231 cells binding to this site can also bind to the mutated site, or (2) another uncharacterized stimulatory factor binds, or (3) COUP-TF I binds, but has a stimulatory effect in this cellular context. Although the main manner of COUP-TF I action is the displacement of other transcription factors from their binding sites, thus exerting an inhibitory effect on transcription (Kliewer et al., 1992; Mietus-Snyder et al., 1992; Tran et al., 1992; Kimura et al., 1993; Cooney et al., 1992; Liu et al., 1993; Miyata et al., 1993; Burbach et al., 1994; Baes et al., 1995; Ben-shushan et al., 1995; Galson et al., 1995; Neuman et al., 1995; Klinge et al., 2000;Guo et al., 2001; Zahng et al., 2001; Lin et al., 2002; Xing et al., 2002), in many cases a stimulatory action of COUP-TF I can be demonstrated (Sagami et al., 1986; Kadowaki et al., 1995; Power et al., 1996; Ktistaki et al., 1997; Rohr et al., 1997; Petit et al., 1999; Pipaon et al., 1999; Sugiyama et al., 2000; Lin et al., 2000; Fernandez-Rachubinski et al., 2001;Metivier et al., 2002; Shibata et al 2003). The total scrambling of the sequence leading to the loss of the binding site results in a further reduction of promoter activity. The remaining low estrogen-dependent activity of this mutant indicates that the stimulation of the oxytocin promoter by estrogens is not totally dependent on the nuclear orphan receptor. Other transcription factors apparently contribute to this activity by synergistic actions, the nuclear orphan receptor playing, however, the most important role.

Estrogen-dependent stimulation of oxytocin expression uses a non-classical mechanism of transcriptional activation.

Different estrogen receptor partial agonists such as 4OH-tamoxifen and raloxifen and antagonists like ICI all show dose-dependent agonistic activities at the bovine oxytocin promoter, whereas, in all cases investigated, inhibition of the basal activity of an ERE-controlled promoter was observed (Koohi et al., 2005). These findings clearly demonstrate that the ligand-bound estrogen receptor up-regulates transcription from the bovine oxytocin promoter by a non-classical mechanism, possibly comparable to the interactions of activated estrogen receptor with general transcription factors like AP-1 (Webb et al., 1995; 1999) or Sp1 (Porter et al., 1997; Zou et al., 1999; Saville et al., 2000). Agonistic actions of estrogen receptor partial agonists and antagonists on AP-1 controlled promoters have been demonstrated in a variety of cell lines (Paech et al., 1997;

Webb et al., 1995, 1999; Jakacka et al., 2001; Weatherman et al., 2001). However, these effects could not be demonstrated in MCF-7 breast cancer cells (Webb et al., 1995; Jakacka et al., 2001). On the contrary, these compounds were able to activate transcription in MCF-7 and MDA-MB 231 cells from a Sp1-controlled promoter (Saville et al., 2000). Non-classical estrogen-dependent up-regulation induces the expression of numerous genes in MDA-MB 231 cells (Glidewell-Kenney et al., 2005).

As outlined in detail above, the up-regulation of the oxytocin promoter is dependent not on AP-1 or Sp1, but on the binding of a nuclear orphan receptor. Direct interactions between this factor and the ligand-bound estrogen receptor might play an important role in this transcriptional regulation. On the other hand, the transcriptional activity of nuclear orphan receptors has been found to be regulated by MAP kinase phosphorylation (Hammer et al., 1999; Desclozeaux et al., 2002). As estrogen receptor has been found to activate this signal transduction pathway by crosstalk (Wong et al., 2002), therefore the transcriptional effects of the ligand-bound estrogen receptor might very well be transmitted indirectly by this phosphorylation cascade, regulating the activity of the nuclear orphan receptor bound to the oxytocin promoter.

The stimulation of oxytocin gene expression by partial agonists and antagonists does not depend on changes in the affinities of binding of these substances to the estrogen receptor.

The addition of estrogen receptor partial agonists and antagonists simultaneously with the high-affinity ligand estradiol in a transfection assay allows estimation of the relative affinities of binding of these substances in relation to estradiol. The binding affinities are a direct indication of the conformation of the ligand binding domain of the receptor. In the experiments performed, at high concentrations all partial agonists and antagonists tested apparently dislocate estradiol from the estrogen receptor α binding site and exert their specific effects as weak agonists on the oxytocin promoter or as antagonists on the ERE-controlled promoter. These results show that the different effects of estrogen receptor α on both promoters are not the results of differences in the affinities of ligand binding to the estrogen receptor.

The stimulation of transcription from the oxytocin promoter is not dependent on binding of estrogen receptor to DNA.

As MDA-MB 231 cells do not express endogenous estrogen receptor α, the co-transfection of expression vectors for this receptor allows the use of mutants in order to elucidate the role of isolated functions of estrogen receptor in transcriptional activation of the oxytocin promoter. In analogy to the mutant described for mouse estrogen receptor α (Jackaka et al., 2001), a P-box mutant of human estrogen receptor α that does not bind to DNA was constructed by in vitro mutagenesis (Koohi et al., 2005) and used to compare the actions of this mutated receptor in classical and non-classical estrogen stimulation. At the oxytocin promoter, no significant difference in the stimulatory pattern was observable between wild-type and mutant estrogen receptor, whereas, at the ERE-controlled promoter, only the wild-type receptor was active. These results again emphasize the non-classical nature of the mechanism of estrogenic stimulation of transcription from the oxytocin promoter.

Estrogens have been shown to exert rapid, non-genomic effects on non-classical membrane receptors (Luconi et al., 2004; Toran-Allerand et al., 2004). In the MDA-MB 231 transfection system however, the transcriptional effect mediated by the co-transfected estrogen receptor is measured. Therefore the non-classical actions of this molecule by interaction with other signal transduction pathways have to be considered. As an important way of non-classical estrogen signalling, activation of the MAP kinase pathway by specific interaction of estrogen receptor α with the Src tyrosine kinase has been demonstrated (Wong et al., 2002).

Although the principal mechanism of hormonal regulation of the oxytocin promoter already can be characterized by the experiments presented in this research, the nuclear orphan receptor from MDA-MB 231 cells has not yet been identified. A possible candidate is the estrogen receptor-related receptor α (ERRα) that is expressed in breast cancer tissue and cell lines (Yang et al., 1998; Liu et al., 2003), and whose expression in the MDA-MB 231 cells has been confirmed in our laboratory (Koohi et al., 2005). Diethylstilbestrol, a synthetic estrogenic substance with agonistic effect on the estrogen receptor, but with antagonistic effect on ERRα (Lu et al., 2001) was used to determine

the contributions of both molecules to the transcriptional regulation of the oxytocin gene. In the experiments performed, a mainly agonistic effect of diethylstilbestrol was detected, showing that the action on the estrogen receptor α is dominant over the possible inhibition by ERRα. An inhibitory effect at higher concentrations cannot be excluded. This aspect, however, could not be clarified by this type of experiment due to the toxicity of the compound at higher concentrations.

The up-regulation of the oxytocin promoter by estrogen in MDA-MB 231 breast cancer cells could be shown to use a non-classical mechanism of estrogen action, encompassing stimulation of the MAP kinase pathway by ligand-activated estrogen receptor and binding of a nuclear orphan receptor, possibly estrogen-related receptor α (ERRα) to the oxytocin promoter (Koohi et al., 2005). In addition to the investigation of the regulatory mechanism, the transfection system was used for screening of different estrogenic substances for their potential to activate gene transcription by this non-classical mechanism of estrogen action.

In the second part of this study the transcriptional effect of xenoestrogens was measured in the MDA-MB 231 transfection system. Xenoestrogens are defined as chemical substances entering the body from the external environment, which may mimic or interfere with the action of endogenous estrogenic hormones. The biological effects of xenoestrogens depend largely on their availability and concentration within tissues and body fluids, their binding to and their effects on the estrogen receptor (Ignar-Trowbridge et al., 1993). Xenoestrogens derive from many sources and can be classified as chemical and industrial compounds, which can be classified as industrial xenoestrogens, phytoestrogens, which are synthesized by plants, and metal ions with estrogenic potential, which are found in the environment. The transcriptional effects of xenoestrogens from all three groups (industrial xenoestrogens phytoestrogens, and metal ions) were investigated. A panel of established industrial xenoestrogens, 2-hydroxy-biphenyl (Petit et al., 1997; Cappelletti et al., 2003; Schultz et al., 2002), methoxychlor (Bulger et al., 1978; Walther et al., 1993; Cummings., 1997; Katchamart et al., 2002) , ß-HCH (beta-benzenehexachloride) (Steinmetz et al., 1996; Hatakeyama et al., 2002; Tapiero et al., 2002; Rasmussen et al., 2003; Penza et al., 2004), chlordane (Arnold et al., 1997; Massaad et al., 1998; Yang et al., 1999; Cossete et al., 2002), toxaphen (Jorgensen

et al., 1997; Gaido et al., 1998; Stelzer et al., 1999; Yang et al., 1999; Arcaro et al., 2000), 1,1,1-trichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)ethane (o,p'-DDT) (Steinmetz et al., 1996; Okoumassoun et al., 2002; Tollefsen et al., 2002; Diel et al., 2002), and 2, 2-bis (4-chlorophenyl)-1, 1-dichloroethyl (p,p'-DDE) (Gaido et al., 1997; Larkin et al., 2002; Villa et al., 2004) were tested for transcriptional effects in the MDA-MB 231 transfection system. Surprisingly, no- transcriptional activities could be detected either on the oxytocin or on the thymidine kinase-ERE promoter. Cell toxicity assays were performed to rule out the possibility that this lack of activation is caused by cell-type-specific toxicity of these compounds. The total lack of any agonistic activity of the industrial xenoestrogens tested in the MDA-MB 231 transfection assay was not anticipated, as these compounds have been characterized as environmental estrogens by a variety of assays. In a control experiment, the well established xenoestrogen o, p'-DDT was tested in MCF-7 cells. In this cell line, widely accepted as a standard for testing of estrogenic substances, the established estrogenic activity of o,p'-DDT could clearly be demonstrated, ruling out the possibility that the lack of effects in MDA-MB 231 might be caused by inactivity of the used batch of the compound.

Phytoestrogens like resveratrol and genistein are able to exert estrogenic and/or anti-estrogenic effects. These compounds are able to reduce, but also stimulate estrogen-dependent tumour growth depending on dose and timing of the exposure (Allred et al., 2001; Cotroneo et al., 2002). Most phytoestrogens exert pleiotropic effects involving kinase inhibition, cell cycle regulation and antioxidative properties that are likely to contribute to their effects (Basly et al., 2000; Cappeletti, 2000). Therefore the role of estrogen receptor α and estrogen receptor β in phytoestrogen action was investigated. This study focused on the effects of naringenin, 6-(1,1-dimethylallyl)naringenin (6DMA-naringenin) and 8-prenylnaringenin (8p-naringenin), as these compounds have been shown to exhibit considerable selectivity for one of the two subtypes of estrogen receptor (Vollmer et al., 2002; Zierau et al., 2002; Milligan et al., 2002). In addition, resveratrol (Bhat et al., 2001; Delia et al., 2002; Gehm et al., 2004), genistein (Dees et al., 1997; Ross et al., 1997; Ueda et al., 2003), and biochanin A (Zand et al., 2000; Joung et al., 2003) were used for comparison.

A previous study using MCF-7 derived cells (Zierau et al., 2001) showed that 8-prenyl-naringenin and 6-(1, 1-dimethylallyl) naringenin at concentrations of 5.10^{-6} M and 10^{-6} M, respectively, stimulate transactivation to the same amount as estradiol at 10^{-8} M, whereas simple naringenin shows only very weak estrogenic activity. The results of the present study clearly demonstrate the subtype-selective activation of estrogen receptor α and β by the phytoestrogen naringenin (activating estrogen receptor β) and its substituted forms 8-prenyl-naringenin and 6-(1, 1-dimethylallyl) naringenin (activating estrogen receptor α), on the ERE-controlled promoter as well as on the oxytocin gene promoter. However, in contrast to the activation of the ERE-controlled promoter, transcriptional up-regulation of the oxytocin gene promoter by the substituted naringenins bound to estrogen receptor α was not dependent on binding of the activated estrogen receptor to the promoter, as shown by use of a mutant estrogen receptor α unable to bind to DNA.

The phytoestrogen resveratrol shows a weak agonistic effect on the oxytocin and the thymidine kinase-ERE promoter with half maximal stimulation reached at a concentration around 10^{-6} M. This effect appears to be additive in respect of the stimulatory effects of 10^{-8} M estradiol. This action was most clearly demonstrated at the oxytocin promoter.The case of genistein is of special interest because of its possible influence on mammalian reproductive tissues (Lee et al., 2004). This compound is thought to be the active ingredient in soy that possesses breast cancer preventive properties (Chen et al., 2003).The results of this part of the study show a very strong agonistic activity of genistein at the relatively high concentration of 10^{-6} - 10^{-5} M. On the oxytocin promoter this agonistic activity even exceeded the maximal stimulatory effect on estradiol by a factor of two. This strong agonistic activity at first sight appears to contradict the breast cancer preventive properties of this compound. However a recent study investigating the effects of genistein on the maturation of the rat mammary gland (Rowell et al., 2005) showed the enhancement of estrogen-dependent cell proliferation only during the development of this tissue. The results of this estrogenic stimulation are the more rapid maturation of the mammary gland, correlated with a reduced proliferation rate and hence a reduced susceptibility to breast cancer. All phytoestrogens used in this study act as agonists, showing at least a partial selectivity for one of the two estrogen receptor subtypes. This selectivity presumably is the basis for the specific effects of these

alimentary estrogenic substances that can be used for treatment of diseases or the improvement of reproductive health.

The last group of chemical compounds with estrogenic potential that were tested in this study were metal ions. The molecular mechanisms that form the basis for the cellular toxicity of metal ions are manifold. Many metal ions are able to react with functional groups in proteins, namely sulfhydryl-, hydroxyl-, amino-, and carboxyl-groups (Dekant et al., 2005). The major mechanisms of metal toxicity comprise the general induction of oxidative stress (Valko et al., 2005), as well as the specific exchange of the zinc stabilizing the conformation of "zinc finger" proteins (Predki et al., 1992). In addition, it could be shown that metal ions inhibit DNA repair processes, thus increasing the rate of mutations that can lead to carcinogenesis (Hartwig et al., 2002). Beside these general and specific toxic effects, metal ions can also exert estrogen-like activities. Divalent metal ions have been shown to activate estrogen receptor α (Stoica et al., 2000; Martin et al., 2003), thus directly exerting estrogenic effects.

Metal ions that had been shown to exert estrogenic effects in the MCF-7 proliferation assay (Garcia-Morales et al., 1994; Martin et al., 2003) were tested in the MDA-MB 231 transfection system. Whereas the majority of the ions did not affect transcriptional activation significantly, promoter-specific activation by some ions clearly could be demonstrated, with copper and cadmium ions activating specifically the oxytocin promoter, and cobalt ions specifically the ERE-controlled promoter. Whereas the estrogenic potential of these ions was expected from the results of other studies, the total selectivity for the oxytocin promoter, controlled by the described non-classical mechanism of estrogen action, or the classical ERE-controlled promoter was surprising. Even though the metal ions have been tested before (Garcia-Morales, 1994; Martin et al., 2003) for estrogenic effects on the pS2 promoter shown to contain an ERE (Barkhem et al., 2002) and the progesterone receptor promoter apparently controlled by AP-1 and Sp1 sites (Schultz et al., 2005), comparable differences in the estrogenic potentials could not be detected. The mechanism of metal ion action on estrogen receptor α (Stoica et al., 2000; Martin et al., 2003) appears to comprise non-competitive binding to the ligand binding domain and to require a functional AF-2 domain. However, even for cadmium, whose estrogenic potential was detected first (Garcia-Morales et al., 1994), the

mechanism of action could not yet be clarified in detail. Nevertheless, the importance of cadmium, as a common environmental toxic substance mimicking the effects of estrogen in vivo has been established (Johnson et al., 2003; Henson et al., 2004). Therefore the elucidation of the mechanisms controlling the hormone-like activities of metal ions will be helpful for the development of screening procedures assessing the biological effects of these environmental pollutants.

From the results of the study presented here, the conclusion can be drawn that estrogenic effects not only depend on the binding of a compound to an estrogen receptor and the induction of an active or non-active conformation, but also on the mechanism of estrogen receptor action, and the biochemical intracellular environment. The observed promoter-specific effects of estrogen (ant)agonists have to be classified primarily by the mechanism of estrogen receptor action. Even the classical genomic mechanism involving binding of the activated receptor to a palindromic estrogen response element apparently can exhibit a certain degree of promoter-selectivity, as different estrogen (ant)agonists show different activities depending on the context of the promoter (Hall et al., 2002). This classical mechanism can be discriminated from the non-classical, genomic interaction of estrogen receptor with transcription factors like AP-1 or Sp1, where a specific cell-type-specific pattern of (ant)agonist activities can be observed (Schultz et al., 2005). However, these two genomic mechanisms may act synergistically leading to novel promoter-specific estrogen effects by selective recruitment of co-activators (Barkhem et al., 2002). As described above, the importance of non-classical, non-genomic mechanisms of estrogen action has become evident; the mechanism of estrogen-dependent up-regulation of the oxytocin promoter presented here provides another example. Apparently, even these actions using cytoplasmic or membrane estrogen receptors can be mediated by co-activator recruitment (Zheng et al., 2005). The different activities of estrogen receptor (ant)agonists can be explained by different conformations of the ligand binding domain induced by binding of these compounds (Paige et al., 1999). Recent reports using peptide binding studies support this view, showing that tamoxifen binding induces a new AF-2 independent interaction site in the estrogen receptor ligand binding domain (Heldring et al., 2004; Kong et al., 2005). The exposure of this site leads to changes in the specificity of co-activator binding. These different specificities result in

cell-type-specific estrogenic potentials, depending on the availability of co-activators. The importance of non-classical estrogen effects for the progression of breast cancer cannot be underestimated, as a recent study (Glidewell-Kenney et al., 2005) could identify 268 non-classical estrogen receptor α target genes. Another aspect also has come into view over the last years, namely the dependence of estrogen receptor action on the cyclic proteasome-mediated degradation of the receptor bound to the promoter (Reid et al., 2003; Metivier et al., 2003); it could also be shown that binding of different (ant)agonists can have significant influence on the stability of the estrogen receptor protein (Wu et al., 2005). Therefore also the dynamics will have to be taken into account and investigated for the different mechanisms of estrogen receptor action.

Taken together, the effects of estrogen receptor (ant)agonists can be summarized as follows: Different estrogen receptor ligands induce different conformations of the ligand binding domain, leading to different affinities for cofactor binding. The different mechanisms of estrogen action (classical or non-classical, genomic or non-genomic) only can be functional in a given cell type, if all necessary molecular components are present and can interact to achieve transcriptional activation. Non-classical mechanisms of estrogen action make an important contribution to the total hormonal effects of estrogen. The results of the study presented here suggest a novel method for the prediction of tissue- and promoter-specific estrogenic effects in an in vitro tissue culture system.

These results will be a major advance in the prediction of tissue and promoter-specific estrogenic effects in an in vitro tissue culture system and could help to avoid unnecessary animal experiments and demonstrate that many of the estrogenic activities of drugs used in hormone or anti-hormone therapy as well as the estrogenic effects of environmental compounds cannot be explained by the classical mechanism of estrogen action. Non-classical mechanisms, like the one described here, appear to control the estrogen-dependent expression of a large number of genes. Therefore the further elucidation of these mechanisms will be of great importance for the understanding of the role of estrogen in normal human development and clinically relevant diseases. Assay systems measuring the effects of estrogenic compounds mediated by non-classical mechanisms should be included into screening procedures for environmental estrogens or during drug development, in order to cover the whole diversity of mechanisms of estrogen action.

Curriculum Vitae

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2000 Publication of book: *The Basics of Toxicology,* (in Persian); University Association Pub.; Tehran, Iran

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2005 Article : " Transcriptional activation of the oxytocin promoter by estrogens uses a novel non-classical mechanism of estrogen receptor action"; Journal of Neuroendocrinology 17: 197-207 (2005)

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