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Ontogeny and functions of the estrogen receptor subtypes in the pregnant rat uterus; the influence of stereoisomerism on ligand-receptor interactions

Ph.D. Thesis

by

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Szeged

2005

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Full papers and abstracts related to the Ph.D. thesis

I. P. Tapolcsányi, J. Wölfling, G. Falkay, Á. Márki, **R. Minorics**, Gy. Schneider: Synthesis and receptor binding examination of 16-hydroxymethyl-3,17-estradiol stereoisomers. Steroids 2002; 67:671-678.

II. J. Wölfling, E. Mernyák, É. Frank, G. Falkay, Á. Márki, **R. Minorics,** Gy. Schneider: Synthesis and receptor-binding examinations of the normal and 13-epi-D-homoestrones and their 3-methyl ethers. Steroids 2003; 68: 277-288.

III. **R. Minorics**, E. Ducza, Á. Márki, E. Páldy, G. Falkay: Investigation of estrogen receptor α and β mRNA expression in the pregnant rat uterus. Molecular Reproduction and Development 2004; 68: 463-468.

IV. **R. Minorics**, E. Ducza, Á. Márki, G. Falkay: Alteration of estrogen receptor subtypes of the pregnant myometrium in the rat. 3rd Congress of the Federation of European Physiological Societies, 28 June - 2 July, 2003, Nice, France.

V. **Minorics R.**, Ducza E., Páldy E., Márki Á., Falkay Gy.: Az ösztrogén receptor altípusok változásának vizsgálata terhes patkány uterusban. Ph.D. Tudományos napok 2004. Semmelweis Egyetem Doktori Iskola, Budapest, 2004. április 8-9.

VI. **Minorics R.**, Márki Á., Falkay Gy.: Módosított ösztradiol és ösztron izomerek farmakológiai vizsgálata. Magyar Tudományos Akadémia Szteroidkémiai Munkabizottság ülése, Szeged, 2004. június 28.

VII. **R. Minorics**, E. Ducza, G. Falkay: Comparison of the effects of tamoxifen, ICI 182,780 and PPT on estrogen receptor subtypes mRNA expression in the early pregnant rat uterus. 4th Congress of the Federation of European Pharmacological Societies, 14-17 July, 2004, Porto, Portugal.

VIII. **Minorics R.**, Ducza E.: Az ösztrogén receptor α és az α_{1A}-adrenerg receptor implantációban betöltött szerepének vizsgálata. VII. Clauder Ottó Emlékverseny, Visegrád, 2004. október 14-15.

LIST OF ABBREVIATIONS

α-MTG	α-monothioglycerol
AF-1	activation function-1
AF-2	activation function-2
AP-1	activation protein-1
AR	androgen receptor
Arg 752/766	arginine 752/766
Asn 705	asparagine 705
B_{max}	maximal number of binding sites
cDNA	complementary DNA
CNS	central nervous system
DHT	5α-androstan-17β-ol-3-one
	dihydrotestosterone
dNTP	deoxynucleotide mix
DTT	dithiothreitol
E_2	estra-1,3,5(10)-triene-3,17β-diol
	17β-estradiol
EDTA	ethylenediamineteraacetic acid
ER	estrogen receptor
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ERE	estrogen response element
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gln 711/725	glutamine 711/725
Glu 353	glutamic acid 353
Gly 521	glycine 521
His 524	histidine 524
HSP-70	heat shock protein-70
HSP-90	heat shock protein-90
Ile 424	isoleucine 424
K_i	inhibition constant
Leu 525	leucine 525
LBD	ligand binding domain

Met 909	methionine 909
M-MLV	moloney murine leukemia virus
mRNA	messenger RNA
N-CoR	nuclear receptor corepressor
ORG 2058	16-alpha-ethyl-21-hydroxy-19-norpregn-4-ene-3,20-dione
P_4	4-prgenene-3,20-dione
	progesterone
PPT	propyl-pyrazole-triol
PR	progesterone receptor
R1881	17β-hydroxy-17α-methyl-estra-4,9,11-trien-3-one
	methyltrienolone
RBA	relative binding affinity
RT-PCR	reverse transcription polymerase chain reaction
SERM	selective estrogen receptor modulator
SMRT	silencing mediator of retinoid and thyroid receptors
SR	selectivity ratio
SRC-1	steroid receptor coactivator-1
Thr 877	threonine 877
Tris-HCl	tris(hydroxymethyl)aminomethane
VEGF	vascular endothelial growth factor

1. INTRODUCTION

The sex steroids function as hormones to control or influence every aspect of reproduction. On the basis of their chemical structures and biological activities, there are three general classes of sex steroids: the androgens, estrogens and progestins. Physiologically, the estrogens, estrone and 17β-estradiol (E_2), are the most important of the female sex steroids synthesized by the ovaries. Among the steroids, estrogens stand out for their influence on a wide variety of tissues and biological targets [Saldanha and Schlinger, 1999]. Furthermore E_2 and its derivatives play appreciable roles in the treatment of breast cancer, postmenopausal symptoms, osteoporosis and other disorders related to the reproductive tract.

1.1. Structures of estrogen receptors α and β

More than 40 years ago, Jensen detected high-affinity, low-capacity estradiol-binding molecules in the rat uterus [Jensen, 1962], the estrogen receptor (ER) protein. Until 1996, this protein was thought to be the only binding site able to induce the transcription of the estrogen-dependent genes, but Kuiper and co-workers cloned a second, distinct ER from the rat prostate [1996]. This novel receptor was called ERβ, and the protein originally identified as "the ER" is now referred to as ERα.

Both ER subtypes belong in the nuclear receptor family, together with other sex steroid receptors (e.g. progesterone and androgen), certain vitamin receptors (e.g. vitamin D and retinoids) and the thyroid hormone receptor [Smith, 1999]. Like other members of the superfamily, the ER is a ligand-inducible transcription factor. Upon binding to estrogen agonists, such as endogenous E_2, it becomes activated and thereby able to positively regulate the expression of target genes.

The rat ERβ is a protein of 485 amino acids, i.e. it is smaller than the ERα, which consists of 595 amino acid residues [Kuiper et al., 1996] and both subtypes are composed of 6 functional domains, named A-F (**Figure 1**). The ligand-binding domain (E domain; LBD) is located within the carboxyl-terminal region of the ER and this region alone is sufficient for high-affinity estradiol binding. The degree of similarity of the amino acid sequences of the two subtypes is only 55% [Kuiper et al., 1996]. In 1997, a novel isoform of the ERβ was identified (β_2), in which 54 nucleotides encoding 18 amino acids are incorporated into the region of the cDNA encoding the LBD [Chu and Fuller, 1997].

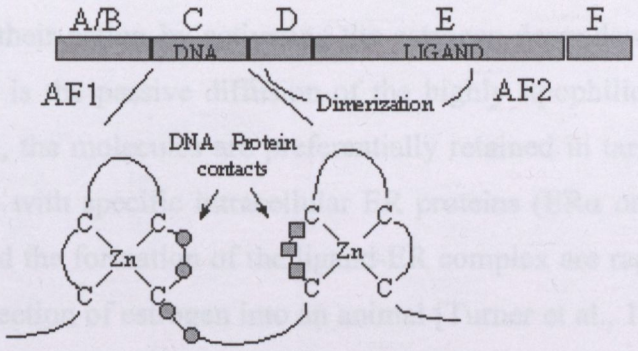


Figure 1. The functional domains of the ERα and ERβ and the structure of the zinc fingers in the DNA-binding region.

The most highly conserved region of the ERs is the DNA-binding domain (region C), showing 95% analogy [Kuiper et al., 1996]. This relatively cysteine-rich sequence forms a structural motif, proposed to form two type II zinc fingers. Each finger contains a peptide loop of 12 or 13 amino acids. The structures are stabilized by a coordinate binding between a single zinc atom and 4 cysteine residues. This region recognizes a specific DNA sequence referred to as an estrogen response element (ERE). The ER binds to EREs, the sequence of is composed of a palindromic repeat of GGTCA separated by 3 nucleotides, and these sequences within the DNA and LBDs mediate receptor-receptor interactions [Smith, 1999].

The DNA- and hormone-binding domains are connected by a hydrophilic stretch of ≈ 50 amino acids, called the hinge region (part D) [Ojasoo et al., 1991].

In their role as transcription factors, the ERs possess two activation domains. One is located in the A/B domain, called activation function-1 (AF-1) and is constitutively active. The second activation function (AF-2) is found in the carboxyl-terminal part of ER (domains E and F). It is notable that the A/B domain is very poorly conserved (<20% identity). The AF-2 overlaps the LBD, and its ability to activate target gene expression is dependent on estrogen binding [Smith, 1999].

The ERs are also phosphoproteins, and under basal conditions are phosphorylated to a relatively low extent. Five phosphorylation sites have been mapped in the ER, 4 in the amino terminus and 1 within the LBD. The presence of the 4 defined phosphorylation sites is essential for the transcriptional activity of the ER [Ing, 1999].

1.2. Mechanism of estrogen action

Estrogens exert their action by activating the estrogen-dependent signal transduction process. The first event is the passive diffusion of the highly lipophilic estrogen molecules into all cells. In general, the molecules are preferentially retained in target cells through the formation of complexes with specific intracellular ER proteins (ERα or β). The entrance of estrogen into the cell and the formation of the ligand-ER complex are rapid, occurring within minutes after the s.c. injection of estrogen into an animal [Turner et al., 1994]. The binding of estrogen evolves two different effects to transform the ER into its active form: the first is the dissociation of heat shock proteins, HSP-90 and HSP-70 [Segnitz and Gehring, 1995], with which the ER forms a complex immediately after the synthesizing of the new ER protein to ensure the proper protein folding [Brown, 1999]. The second is the conformational changes in the LBD [Allan et al., 1992; Beekman et al., 1993], facilitating the dimerization of the activated ER. In principle, the ERα and ERβ form homodimers [Cowley et al., 1997], but it has been reported that the ERα and ERβ can interact with each other to produce a heterodimer complex [Cowley et al., 1997; Kuiper et al., 1997].

The mode of binding of estrogen molecules to the LBD is of considerable importance in mediating the action of estrogen. From crystallographic studies, Tanenbaum et al. [1998] reported that estrogens adopt a well-defined orientation in the LBD of the ER. The 5-membered ring D is in contact with helix-11 via the hydrogen-bonded 17β-hydroxy group to His 524 (**Figure 2A**). The aromatic ring A is projected toward helix-3, its phenolic hydroxy group forms a water-mediated H-bond with Glu 353, and the molecule is fixed by van der Waals contacts around the binding pocket (**Figure 2B**).

New information about the proper functioning of the ER, as a transcription factor, has recently become known. Estrogens binding to the ERs are able to influence the transcription process in two, diverse ways. In the classical scheme, after dimerization, either ligand-ER complex is enabled to bind directly to the ERE, often located in the promoters of estrogen-responsive genes, and initiate the transcription of estrogen-dependent genes [Bulun and Simpson, 1999]. The ligand-activated, DNA-bound ER then recruits coactivators which serve as a bridge between the ER and the general transcription machinery [Lonard and Smith, 2002] (**Figure 3**).

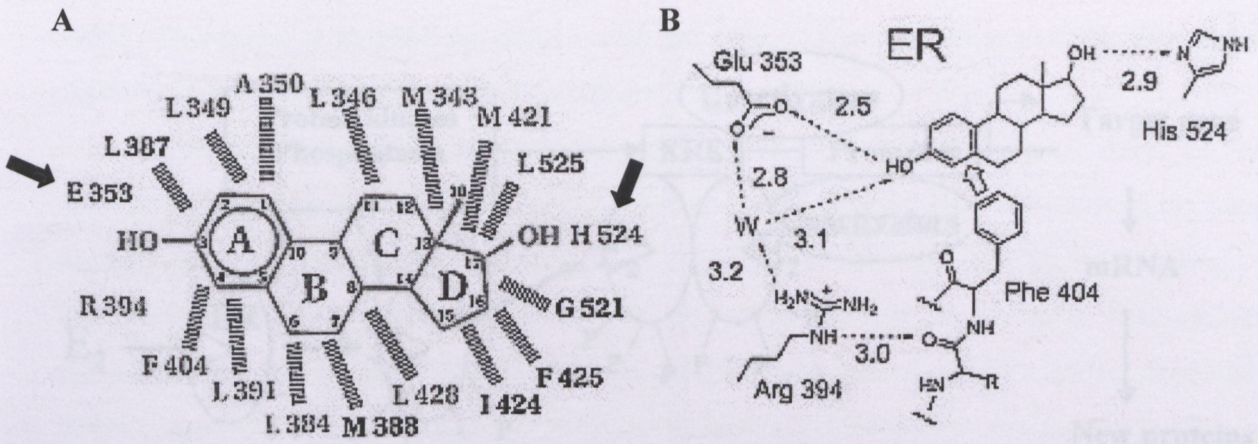


Figure 2. The mode of 17β-estradiol (E_2) binding to the estrogen receptor (ER) ligand-binding domain. **A.** Schematic illustration of E_2 binding in the ligand-binding pocket of the ER. The arrows show the amino acid residues bound directly to the hormone molecule; the dashed lines indicate hydrophobic van der Waals contacts with the hormone. **B.** The water-mediated H-bond network around the phenolic hydroxy group in ring A of the molecule.

Such coactivators possess more functions in stimulating target gene transcription: histone acetyltransferase activity, chromatin remodelling and transcription reinitiation activities [Jones and Kadonaga, 2001]. In 1995, the first characterized coactivator was the steroid receptor coactivator-1 (SRC-1) [Onate et al., 1995]; since then, many other coactivators have been identified [Leo and Chen, 2000; Westin et al., 2000]. If a selective estrogen receptor modulator (SERM) such as 4-hydroxytamoxifen binds to the ERα and acts as an antagonist, not coactivators but corepressors are activated [Lonard and Smith, 2002]. These proteins (N-CoR and SMRT) are associated with histone deacetylase activity, so the target gene expression will be blocked [Lavinsky et al., 1998; Shang et al., 2000].

Additionally, the ERα and ERβ have been shown to stimulate gene expression indirectly through binding to other transcription factors, such as activator protein-1 (AP-1) [Webb et al., 1995] and Sp1 [Porter et al., 1997], thereby considerably expanding the potential repertoire of the genes regulated by ERs.

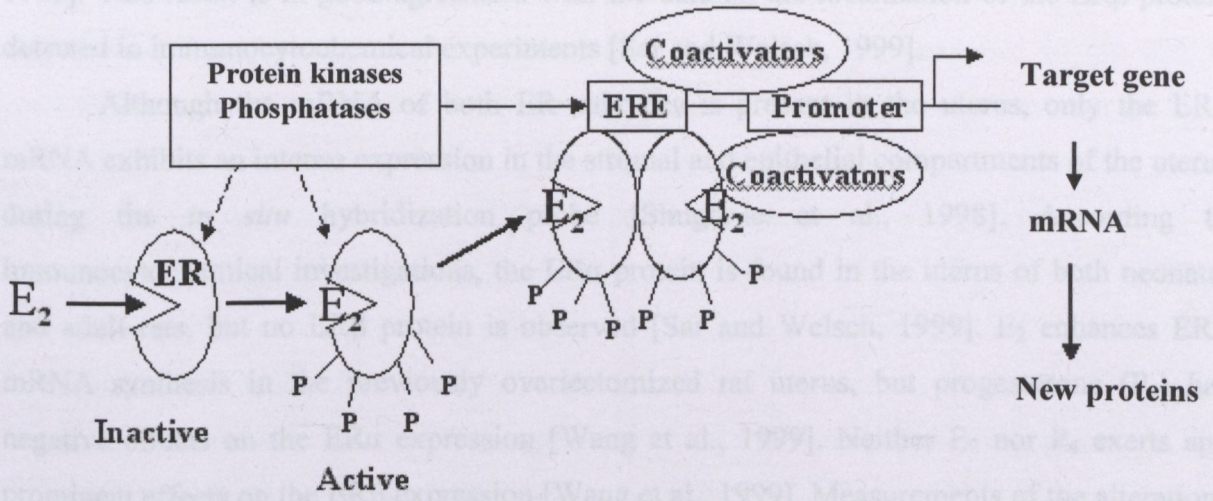


Figure 3. Model of the estrogen receptor (ER) activation process. Binding of the ligand 17β-estradiol (E_2) activates the ER (conformational change, phosphorylation and dimerization). The estrogen response element (ERE) recognizes the active ER, and after their interaction the transcription of the target gene is set off in its promoter region with the support of the specific coactivators.

1.3. Distribution and function of estrogen receptors α and β in the rat

After the identification of the ERβ, intensive investigations began to define the tissue distribution of the ERα and ERβ in different species and humans. This was shortly afterwards followed by determination of the functions of both ER subtypes in distinct organs. For many years, data have been published on the expressions and physiological roles of the ERα and ERβ in the rat.

The major target of estrogens is the female reproductive tract. The mRNA expressions of the ER subtypes were detected first by reverse transcription-polymerase chain reaction (RT-PCR) assay by Kuiper et al. [1997]. The ERα and ERβ mRNAs were visualized in the rat ovary and uterus, and the highest expression of the ERβ was found in the ovary. Later, Shughrue et al. [1998] determined the mRNA expressions of the ERα and ERβ in a more detailed way by an *in situ* hybridization technique. In the ovary, both ER mRNA transcripts were present, although their distributions were different. The ERα mRNA was characteristically seen in the stromal cells of the ovary. The ERα protein was localized in the thecal cells, interstitial gland cells and germinal epithelium [Sar and Welsch, 1999]. Similar findings were published after the examination of neonatal (on day 5-10 *post partum*) rat ovaries. The ERβ mRNA was primarily seen in the granulose cells of the developing follicles, and a weaker signal was observed in several cells of the new corpora lutea [Shughrue et al.,

1998]. This result is in good agreement with the data on the localization of the ERβ protein detected in immunocytochemical experiments [Sar and Welsch, 1999].

Although the mRNA of both ER subtypes is present in the uterus, only the ERα mRNA exhibits an intense expression in the stromal and epithelial compartments of the uterus during the *in situ* hybridization probe [Shughrue et al., 1998]. According to immunocytochemical investigations, the ERα protein is found in the uterus of both neonatal and adult rats, but no ERβ protein is observed [Sar and Welsch, 1999]. E_2 enhances ERα mRNA synthesis in the previously ovariectomized rat uterus, but progesterone (P_4) has negative effects on the ERα expression [Wang et al., 1999]. Neither E_2 nor P_4 exerts any prominent effects on the ERβ expression [Wang et al., 1999]. Measurements of the alterations in ERα and ERβ mRNA expression during the estrous cycle have revealed that the cyclic variations in ERβ mRNA expression are less evident than those in ERα [Wang et al., 2000]. It has been established that the ERα is the predominant ER not only in the rat uterus, but also in the oviduct and vagina/cervix. The ERβ is abundantly expressed in the ovary, weakly expressed in the uterus and vagina/cervix, and sparsely expressed in the oviduct [Wang et al., 2000].

Saji et al. [1999] reported that both ER subtypes are expressed in the rat mammary gland. The ERβ protein is more abundantly expressed at different stages of life than the ERα protein. The ERβ is always present in the cells, but the percentage of ERα-positive cells and the percentage of cells containing both receptors change during development and hormone responsiveness. The different stages as defined by the ER profile also represent the stages in breast development where E_2 elicits very different actions, such as rapid growth and maturation of the breast during pregnancy or inducing PR expression. During lactation however, the mammary gland is insensitive to estrogen effects.

Estrogens are also known to have important effects on the male reproductive system [Prins et al., 1998]. The ERβ was first cloned from the rat prostate [Kuiper et al., 1997]. Active ERβ mRNA synthesis has been demonstrated in several parts of the male urogenital tract (e.g. the epididymis, Sertoli cells and the smooth muscle of the bladder) [Nilsson et al., 1998]. The ERβ is presumably involved in testicular development and spermatogenesis in the rat [Saunders et al., 1998]. The prostatic expression of the ERα is very low [Prins et al., 1998], but it is highly expressed in the rete testis and Leydig cells [Fisher et al., 1997].

The presence of ERα and ERβ mRNA in the rat central nervous system (CNS) has been characterized extensively, showing a distinct expression pattern of the ERα and ERβ [Shughrue et al., 1997]. The ERα is weakly expressed in the CNS, but the absence of the ERα

in the ventromedial hypothalamic nucleus and arcuate nucleus negatively influences the sexual behaviour [Nilsson et al., 1998]. Both ER subtypes are expressed in the medial preoptic nucleus and are involved in mediating the relief of hot flushes by estradiol [Nilsson et al., 1998]. The localization of the ERβ in the neocortex, hippocampus and nuclei of the basal forebrain suggests that the ERβ is involved in mediating the beneficial effects of estrogen on learning and memory [Nilsson et al., 1998]. Thus, it is possible that the ERβ plays a role in improving the memory in patients with Alzheimer's disease [Nilsson et al., 1998]. The ERβ has also been localized to the paraventricular nucleus and supraoptic nucleus, where it has been suggested to synthesis the expression of oxytocin and arginine-vasopressin [Alves et al.; 1998, Hrabovszky et al., 1998]. Further possible CNS effects of ERβ may involve the regulation of coordination and balance, muscle movement, vision and seasonal rhythms [Shughrue et al., 1997].

Rat vascular tissues express functional ER [Knauthe et al., 1996]. It was also recently demonstrated that the ERβ expression is increased greatly, whereas the ERα expression is not changed significantly in vascular endothelial cells following balloon injury of the rat aorta [Lindner et al., 1998]. It is presumed that the ERβ may mediate some of the protective effects of estrogen in the healing of vascular injury. Experiments with other animal models revealed that estrogens have a positive effect in preventing the accumulation of cholesterol within the vascular cell wall, have vasodilatory effects on coronary arteries and regulate endothelial nitric oxide production [Nilsson et al., 1998].

Osteoblasts express both ERα and ERβ mRNAs [Onoe et al., 1997], but the ERβ is the predominant ER subtype. The expression pattern of ERβ mRNA is similar in male and female rats. The experimental results suggest a distinct mechanism of action of estrogen regulated by the ERβ in bone.

2. AIMS

In consequence of the steadily increasing therapeutic use of estrogens, investigation of the action of estrogens becomes one of the most exciting areas of research in medical science. This interest became even stronger after cloning of the new ER, ERβ. The estrogen functions are nowadays examined from two different aspects. The primary research tendency is determination of the distribution of the ER subtypes and their functions in various organs. The other aspect is the synthesis of new estrogen analogues to affect selectively the signal transduction through the classical ERα or ERβ and to acquire new knowledge about the ER-ligand interactions.

In accordance with this double consideration, the present thesis demonstrates new findings about the distribution and function of the ER subtypes in the pregnant rat uterus, and the importance of stereochemistry in ligand-ER binding and the *in vivo* efficacy of originally synthesized ER ligands.

The following aims were set:

1. Investigation of the ontogeny and functions of the ER subtypes in the pregnant rat uterus.
    a. Determination of the ER and progesterone receptor (PR) densities in the pregnant rat uterus during early pregnancy by radioligand saturation assay.
    b. Determination of the ERα and ERβ mRNA expressions in the rat uterus on days 4, 5, 6, 7, 8, 10, 15, 18, 20 and 22 of pregnancy and in the non-pregnant uterus by RT-PCR.
    c. Functional investigation of the ERα and ERβ during early pregnancy in the rat uterus by *in vivo* treatment with a selective ERα agonist, propyl-pyrazole-triol (PPT).

2. Pharmacological characterization of newly synthesized estrone and estradiol stereoisomers.
    a. Determination of the ER-binding ability and selectivity of 16 original estrone and estradiol derivatives by radioligand binding assay.

b. Determination of the *in vivo* estrogenic activity of the molecules by uterus weight measurement.

c. Study of the relationship between their chemical structure and pharmacological activity.

3. MATERIALS AND METHODS

Animal investigations were carried out with the approval of the Ethical Committee for Animal Research, University of Szeged (registration numbers: I-74-7/2002; I-74-8/2002).

3.1. Determination of estrogen receptor subtypes in the pregnant rat uterus

3.1.1. Animals and mating

Mature female (160-200 g) and male (240-260 g) Sprague-Dawley rats were mated in a special mating cage. A metal door, movable by a small electric engine, separated the rooms for the male and female animals. A timer controlled the function of the engine. Since rats are usually active at night, the separating door was opened before dawn. Within 4-5 h after the possibility of mating, vaginal smears were taken from the female rats, and a sperm search was performed under a microscope at a magnification of 1200 times. If the search proved positive, or when smear taking was impossible because of an existing vaginal sperm plug, the female rats were separated and were regarded as first-day pregnant animals.

The animals were given water and food *ad libitum* and were housed in temperature- (20-23 °C) and humidity-controlled (40-60%) rooms with a 12 h light/dark cycle.

3.1.2. Saturation experiments

The densities of the ER and PR were determined by radioligand saturation assay, using appropriate selective tritiated ligands and a cytosol preparation derived from the rat uterus on days 4-8 of pregnancy.

The uterine fractions were prepared as described by Ojasoo and Raynaud [1978]. Pregnant rats were anaesthetized with sodium pentobarbital (1 g/kg i.p.). The uterine tissues were rapidly removed and the embryos were dissected carefully. All subsequent steps were performed at 4 °C. The uteri were homogenized with an Ultra Turrax 25 homogenizer (IKA Labortechnik, Germany) in 6-10 volumes of 10 mM Tris-HCl buffer (pH 7.4) containing 0.25 M glucose and 15 mM Na_2MoO_4. The homogenate was centrifuged at 105 000 x g for 60 min and the supernatant was used for saturation experiments. The cytosol was stored at -70 °C until assayed. The protein concentration of the cytosol fraction was

measured by the method of Bradford [Bradford, 1976], with bovine serum albumin as standard.

[^{3}H]Estra-1,3,5(10)-triene-3,17β-diol ([^{3}H]E_2; 110 Ci mmol^{-1}) or [^{3}H]ORG 2058 (34 Ci mmol^{-1}) was used as radioligand at 6 different concentrations (1.0, 2.0, 5.0, 10.0, 20.0 and 30.0 nM) to saturate the ER or the PR, respectively. The tritiated ligands were purchased from Amersham, UK. Aliquots of 100 µL cytosol were incubated for 4 h at 4 °C with 100 µL homogenizing buffer for total binding and with 100 µL 10^{-5} M E_2 or ORG 2058 for non-specific binding at each concentration of the two radioligands. Unbound steroids were removed by treatment with dextran-coated charcoal (10% Norit-A and 0.1% dextran T 70 in 10 mM Tris-HCl buffer, pH 7.4). After incubation for 15 min at 4 °C, the samples were centrifuged at 1,500 x g for 10 min, and the radioactivity of the supernatant was determined in a Wallac 1409 liquid scintillation counter (Wallac, Finland). All assays were carried out at least three times in duplicate. The data were analysed with the computer program Prism 2.01 (GraphPad Software, CA). The maximum numbers of the ER and PR in the pregnant rat uterus (B_{max}) were calculated by using the Rosenthal plot [Rosenthal, 1967]. The concentration of the specific bound radioligand (B) was plotted against the ratio of the bound and free (B/F) radioligand concentrations. The resulting graph was a straight line; its x-intercept was equal to B_{max}.

3.1.3. RT-PCR studies

The changes in the expression of the mRNA of the ER subtypes in the non-pregnant and pregnant rat uterus were investigated by RT-PCR method.

Tissue isolation. Female Sprague-Dawley rats were anaesthetized with sodium pentobarbital (1 g/kg i.p.). Uterine tissues from non-pregnant animals and pregnant animals on gestational days 4-8, 10, 15, 18, 20 and 22 (n = 6 on each day) were rapidly removed, and the embryonic tissues were separated. The uteri were frozen in liquid nitrogen and then stored at -70 °C until total RNA extraction.

Total RNA preparation. Total cellular RNA was isolated by extraction with acid guanidinium thiocyanate - phenol - chloroform by the procedure of Chomczynski and Sacchi [1987]. After precipitation with isopropanol, the RNA was washed 3 times with ice-cold 75% ethanol and then dried. The pellet was resuspended in 100 µL DNase and RNase-free distilled water. The RNA concentrations of the samples were determined from their absorbances at 260 nm.

RT-PCR. The RNA (0.5 μg) was denatured at 70 °C for 5 min in a reaction mixture containing 20 μM oligo(dT) (Hybaid Corp., UK), 20 U RNase inhibitor (Hybaid Corp., UK), 200 μM dNTP (Sigma-Aldrich, USA) in 50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 5 mM $MgCl_2$ in a final reaction volume of 20 μL. After the mixture had been cooled to 4 °C, 20 U M-MLV reverse transcriptase (GIBCO, UK) and RNase H Minus (Promega, UK) were added, and the mixture was incubated at 37 °C for 60 min.

The PCR was carried out with 5 μL cDNA, 25 μL ReadyMix REDTaq PCR reaction mix (Sigma-Aldrich, USA), 2 μL 50 pM sense and antisense primer of the ERα or ERβ subtype and 16 μL DNase and RNase-free distilled water. The primer sequences used to amplify the ERα were 5'-TCC ACG ATC AAG TTC ACC-3' (for the forward) and 5'-GGA TGT GGT CCT TCT CTT-3' (for the reverse); these primers were anticipated to generate 311 bp PCR product. For the rat $ER\beta_1$ and $ER\beta_2$ cDNA, a 440 and a 494 bp PCR product resulted with forward primer 5'-GGA TCA TGG AAT TCA CAC AGA GAT AC-3' and reverse primer 5'-CGG TCT AGA GCA CCT TGA GTC CAG AGC-3' [Drummond et al., 1999]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as internal control in all samples [Tso et al., 1985]. The PCR was performed with a PCR Sprint thermal cycler (Hybaid Corp., UK) with the following cycle parameters: after initial denaturation at 95 °C for 5 min, the reactions were taken through 30 cycles (ERα) or 35 cycles (ERβ) of 30 s at 95 °C, 45 s at 55 °C and 40 s at 72° C, followed by lowering of the temperature to 4 °C. This PCR protocol represented optimized conditions and linear phase amplification for each of the primer sets employed. The optimum number of cycles for each set of primers was determined by performing kinetic analyses. When animal samples were used, amplification reactions were tested over a range of 4 cycles: 25, 30, 35 and 37 times. The RT-PCR products were separated on 2% agarose gels, stained with ethidium bromide and photographed under a UV transilluminator. Semiquantitative analysis was performed by densitometric scanning of the gel with Kodak EDAS290 (Csertex Ltd., Hungary). The log of the densitometric values was plotted against the number of cycles, and from the linear section of the kinetic plots, the number of PCR cycles was chosen for both receptor subtype genes.

3.1.4. Selective estrogen receptor α agonist treatment *in vivo*

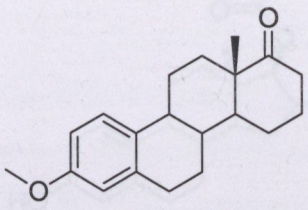
The effects of a modulated hormone profile on the ERα and ERβ mRNA expression patterns and the development of gestation were investigated in pregnant rats treated with the selective ERα agonist compound, PPT (Tocris, UK) *in vivo*.

From day 1, 2, 3 or 4 of pregnancy, the animals (n = 6) were treated once a day, s.c. with 250 µg PPT (Harris et al., 2002) dissolved in 0.1 ml olive oil for 3 days. The control animals (n = 8) were treated with 0.1 ml olive oil for 3 days. 24 h after the last dose, the animals were sacrificed. The uterine tissues were rapidly removed and the number of implantation sites was recorded. The tissues were frozen in liquid nitrogen and then stored at -70 °C until the RT-PCR assay. The ERα and ERβ mRNA expressions were determined in the samples from PPT-treated pregnant rats as described above.

3.2. Pharmacological characterization of newly synthesized estrone and estradiol stereoisomers

3.2.1. Newly synthesized estrone and estradiol analogues

In the present work, 4 newly synthesized estrone and 12 estradiol analogues were investigated to evaluate their estrogenic character *in vitro* and *in vivo* (**Figures 4** and **5**). The molecules possess modified substituents at positions C3, C13, C16 and C17 and can be divided into 4 subfamilies on the basis of the variety of the functional groups. Since each subfamily contains 2 epimer pairs, it seemed obvious to examine the influence of the stereochemistry on the receptor binding ability and the *in vivo* effectiveness.



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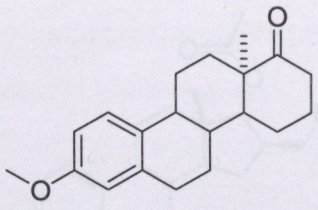

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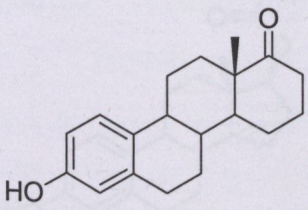

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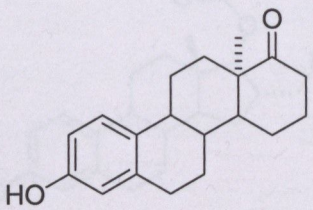

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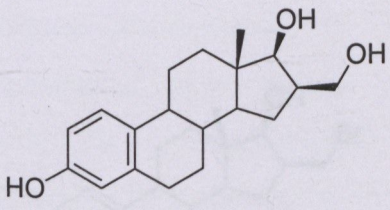

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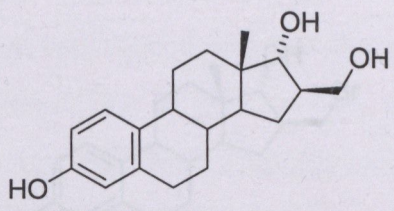

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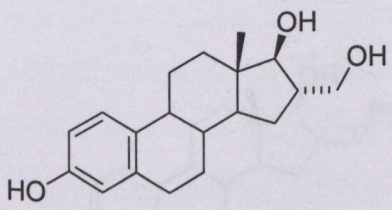

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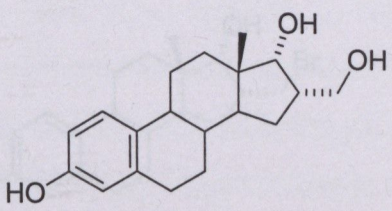

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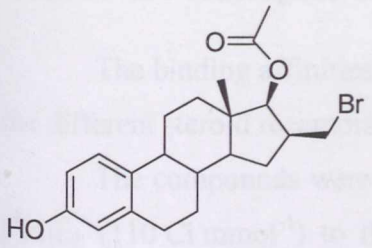
Figure 4. Chemical structures of the originally synthesized estrone and estradiol analogues. D-Homoestrone derivatives: 3-methoxy-D-homoestra-1,3,5(10)-triene-17a-one (**1**), 3-hydroxy-D-homoestra-1,3,5(10)-triene-17a-one (**2**), 3-methoxy-13α-D-homoestra-1,3,5(10)-triene-17a-one (**3**) and 3-hydroxy-13α-D-homoestra-1,3,5(10)-triene-17a-one (**4**). 16-Hydroxymethylestradiol derivatives: 16β-hydroxymethylestra-1,3,5,(10)-triene-3,17β-diol (**5**), 16α-hydroxymethylestra-1,3,5,(10)-triene-3,17β-diol (**6**), 16β-hydroxymethylestra-1,3,5,(10)-triene-3,17α-diol (**7**) and 16α-hydroxymethylestra-1,3,5,(10)-triene-3,17α-diol (**8**).



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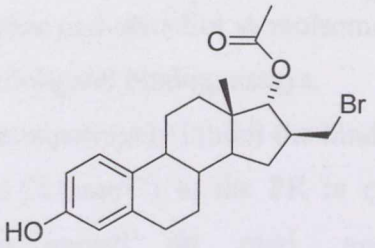

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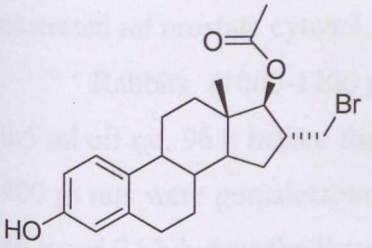

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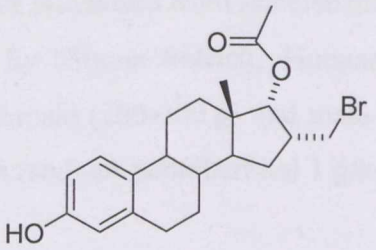

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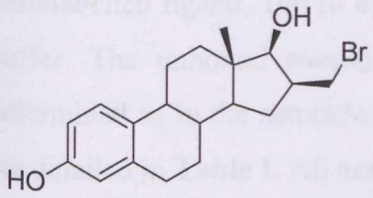

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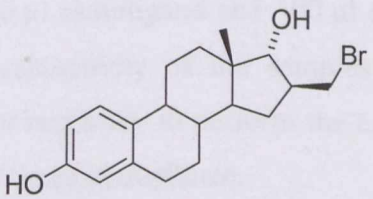

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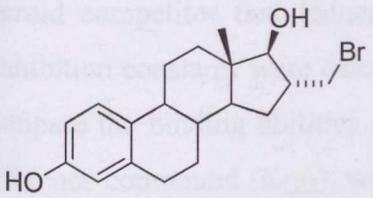

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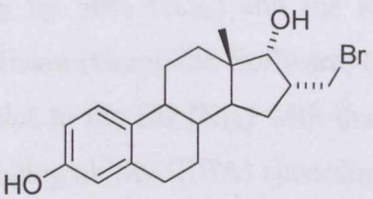

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Figure 5. Chemical structures of the originally synthesized estradiol analogues. 17-Acetate-estradiol derivatives: 16β-bromomethylestra-1,3,5(10)-triene-3,17-diol-17β-acetate (**9**), 16α-bromomethylestra-1,3,5(10)-triene-3,17-diol-17β-acetate (**10**), 16β-bromomethylestra-1,3,5(10)-triene-3,17-diol-17α-acetate (**11**) and 16α-bromomethylestra-1,3,5(10)-triene-3,17-diol-17α-acetate (12). 16-Bromomethylestradiol derivatives: 16β-bromomethylestra-1,3,5(10)-triene-3,17β-diol (**13**), 16α-bromomethylestra-1,3,5(10)-triene-3,17β-diol (**14**), 16β-bromomethylestra-1,3,5(10)-triene-3,17α-diol (**15**) and 16α-bromomethylestra-1,3,5(10)-triene-3,17α-diol (**16**).

3.2.2. *In vitro* radioligand binding assay

The binding affinities of the newly synthesized estrone and estradiol stereoisomers for the different steroid receptors were determined by using radioligand binding assays.

The compounds were evaluated for their ability to competitively inhibit the binding of [^{3}H]E_2 (110 Ci mmol^{-1}) to the ER, of [^{3}H]ORG2058 (34 Ci mmol^{-1}) to the PR in cytosol prepared from immature rabbit uteri or ovariectomized rat uteri, and of [^{3}H]dihydrotestosterone ([^{3}H]DHT; 110 Ci mmol^{-1}) to the androgen receptors (AR) in castrated rat prostate cytosol. All of the tritiated ligands were purchased from Amersham, UK.

Rabbits (1000-1200 g) were treated with 25 μg E_2 (Sigma-Aldrich, Hungary) in 0.5 ml oil s.c. 96 h before the uteri were excised. Mature female (200-300 g) and male (300-400 g) rats were gonadectomized under general anaesthesia (sodium pentobarbital 1 g/kg i.p.) 48 h and 24 h before the tissue preparation, respectively.

All subsequent steps were carried out at 4 °C. Aliquots of 100 μl cytosol were incubated with the appropriate radioligand in the presence of synthesized molecules in 11 different concentrations (10^{-12} M - 10^{-5} M). To determine the total bound amount of the radiolabelled ligand, 100 μl cytosol was incubated with 100 μl radioligand and 100 μl assay buffer. The unbound steroids were separated and the radioactivity of the samples was determined as in the saturation experiments. The parameters necessary to perform the assays are detailed in **Table I**. All assays were carried out at least 3 times in duplicate.

The percentage of the bound radioligand in the presence of the tested compounds was plotted against the concentration of unlabelled steroid. The molar concentration of the steroid competitor that reduced the radioligand binding by 50% (IC_{50}) and the K_i value (inhibition constant) were calculated with Prism 2.01 software (GraphPad Software, CA). To compare the binding abilities of the investigated molecules to the ER ($K_{i,x}$) with that of the reference compound ($K_{i,E2}$), we calculated the relative binding ability (RBA) according to the following equation: $RBA = (K_{i,E2} / K_{i,x}) \times 100$. To characterize the ER selectivity of each compound, we determined the selectivity ratio (SR), based on the following equation: $SR = K_{i,x}$ (AR or PR) / $K_{i,x}$ (ER).

Table I. The parameters of the preparation, incubation and radioligand separation for the estrogen receptor (ER), progesterone receptor (PR) and androgen receptor (AR). The following abbreviations are used: Tris-HCl – tris(hydroxymethyl)aminomethane, EDTA – ethylenediaminetetraacetic acid, α-MTG - α-monothioglycerol, DTT – dithiothreitol, [^{3}H]E_2 - [^{3}H]17β-estradiol, [^{3}H]DHT - [^{3}H]dihydrotestosterone. [a] Ojasoo and Raynaud [1978], [b] Martel et al. [1998], [c] Wilson et al. [1976]

Prepared tissue	Rabbit uterus[a]	Rat uterus[b]	Rat ventral prostate[c]
Steroid receptor	ER / PR	ER / PR	AR
Preparation buffer	10 mM Tris-HCl 0.25 M glucose 15 mM Na_2MoO_4	25 mM Tris-HCl 1.5 mM EDTA 10 mM α-MTG 10 % glycerol 10 mM Na_2MoO_4	20 mM Tris-HCl 1 mM EDTA 1 mM DTT 10 % glycerol
Radioligand	5 nM [^{3}H]E_2 4 nM [^{3}H]ORG2058	5 nM [^{3}H]E_2 4 nM [^{3}H]ORG2058	5 nM [^{3}H]DHT
Incubation time [h]	24	3 (ER) / 24 (PR)	4
Incubation temperature [°C]	4	25 (ER) / 4 (PR)	4
Separating buffer	0.5 % Norit-A 0.05 % dextran T70 1.5 mM EDTA 10 mM α-MTG 10 mM Tris-HCl	0.5 % Norit-A 0.05 % dextran T70 1.5 mM EDTA 10 mM α-MTG 10 mM Tris-HCl	1.25 % Norit-A 0.625 % dextran T70 10 mM Tris-HCl 1 mM EDTA

3.2.3. *In vivo* uterus weight measurement

The *in vivo* estrogenic effects of the *in vitro* tested compounds were established by measuring the uterus weight gain in gonadectomized female rats.

Mature female rats (180-200 g) were ovariectomized under general anaesthesia with sodium pentobarbital (1 g/kg i.p.). After laparotomy, the oviducts and the arteries supplying the ovaries were ligated on both sides, and the ovaries were excised. The abdomen and the skin were closed by surgical suture, and the animals were then observed until awakening. Three weeks after the operation, the animal groups (3-5 animals/group) received daily s.c. injections for 7 days. The tested molecules were dissolved in olive oil at a concentration of 10, 30 or 50 μg/0.1 ml. Pure olive oil and 10 μg/0.1 ml E_2 (Sigma-Aldrich, Hungary) were administered to the negative and positive control group, respectively. On day 8, the animals

were sacrificed and the uteri were removed. The weights of the uteri were determined wet, and they were then dried at 95-100 °C in a Heraeus T 5042 drying oven (Heraeus, Germany) for 24 h and weighed again.

The wet and the dry uterus weights per 100 g body weight were calculated from the measured data. Data were statistically analysed by one-way ANOVA with the Neuman-Keuls post-test, using Prism 2.01 software.

4. RESULTS

4.1. Estrogen receptor subtypes in the pregnant rat uterus

4.1.1. Determination of estrogen and progesterone receptors in the rat uterus on days 4-8 of pregnancy

The saturation experiments indicated that the ER protein expression in the rat uterus was highest on day 5 of pregnancy (B_{max} = 637.40 ± 76.10 fmol/mg). The amount of the ER was lower on other experimental days: B_{max} = 85.39 ± 34.30 fmol/mg (day 4), B_{max} = 302.05 ± 2.95 fmol/mg (day 6), B_{max} = 162.58 ± 63.07 fmol/mg (day 7) and B_{max} = 565.13 ± 114.68 fmol/mg (day 8). The PR protein expression did not undergo a significant change as compared with day 4 of pregnancy (B_{max} = 335.30 ± 45.62 fmol/mg on average) until day 8, but the amount of this protein was increased significantly on day 8 (B_{max} = 583.30 ± 98.70 fmol/mg) (**Figure 6**).

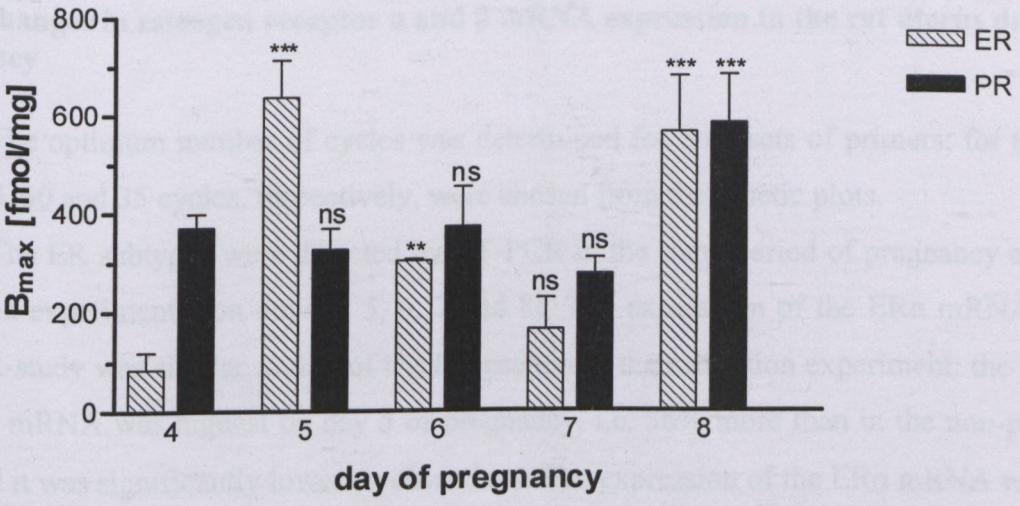

Figure 6. Changes in the maximum numbers (B_{max}) of the estrogen receptor (ER) and progesterone receptor (PR) in the rat uterus during early pregnancy (days 4-8), measured by radioligand saturation assay as described in the Materials and Methods. The B_{max} of the ER and PR were determined by using the Rosenthal plot. All assays were carried out at least 3 times in duplicate.
ns $p > 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared with the appropriate data on the previous day. Each bar represents the mean ± SD.

Nevertheless, the ratio of the ER and PR protein densities (ER/PR) was outstanding only on day 5 (ER/PR = 2.51 ± 0.36), indicating the predominance of the ER on that day

(**Figure 7**). The values of ER/PR on other days of pregnancy were as follows: 0.23 ± 0.08 (day 4), 0.72 ± 0.07 (day 6), 0.57 ± 0.11 (day 7) and 0.97 ± 0.10 (day 8).

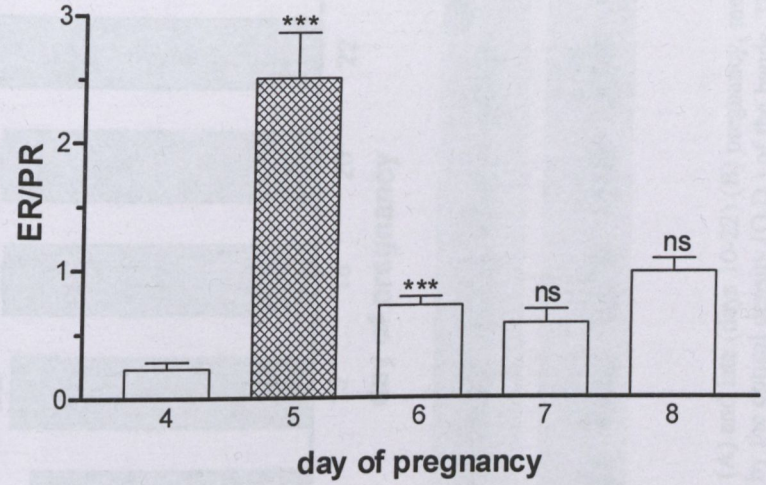

Figure 7. Changes in the ratio of the maximum numbers (B_{max}) of the estrogen receptor (ER) and progesterone receptor (PR) in the rat uterus during early pregnancy (days 4-8).
ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared with the data on the previous day. Each bar represents the mean ± SD.

4.1.2. Changes in estrogen receptor α and β mRNA expression in the rat uterus during pregnancy

The optimum number of cycles was determined for both sets of primers: for the ERα and ERβ, 30 and 35 cycles, respectively, were chosen from the kinetic plots.

The ER subtypes were detected by RT-PCR in the same period of pregnancy as in the saturation experiments (on days 4, 5, 6, 7 and 8). The expression of the ERα mRNA in the RT-PCR-study was similar to that of the ER protein in the saturation experiment: the level of the ERα mRNA was highest on day 5 of pregnancy, i.e. 36% more than in the non-pregnant uteri and it was significantly lower on other days. The expression of the ERα mRNA was 27% weaker on day 8 than on day 5; this was the minimum level of the ERα mRNA expression (**Figure 8A**). In the second half of pregnancy, the expression of the ERα mRNA increased gradually from day 10 to day 22, reaching its highest value on the day of labour (**Figure 8B**).

The ERβ mRNA expression was not detected by RT-PCR on any day in early pregnancy. The ERβ mRNA appeared on day 7, and its expression was enhanced by 11% on day 8 (**Figure 9A**). Furthermore, the ERβ mRNA expression could be detected between days 10 and 15. The ERβ mRNA expression ceased after day 15 (**Figure 9B**). The expression of the ERβ mRNA was highest on day 8, and it had decreased significantly by day 15.

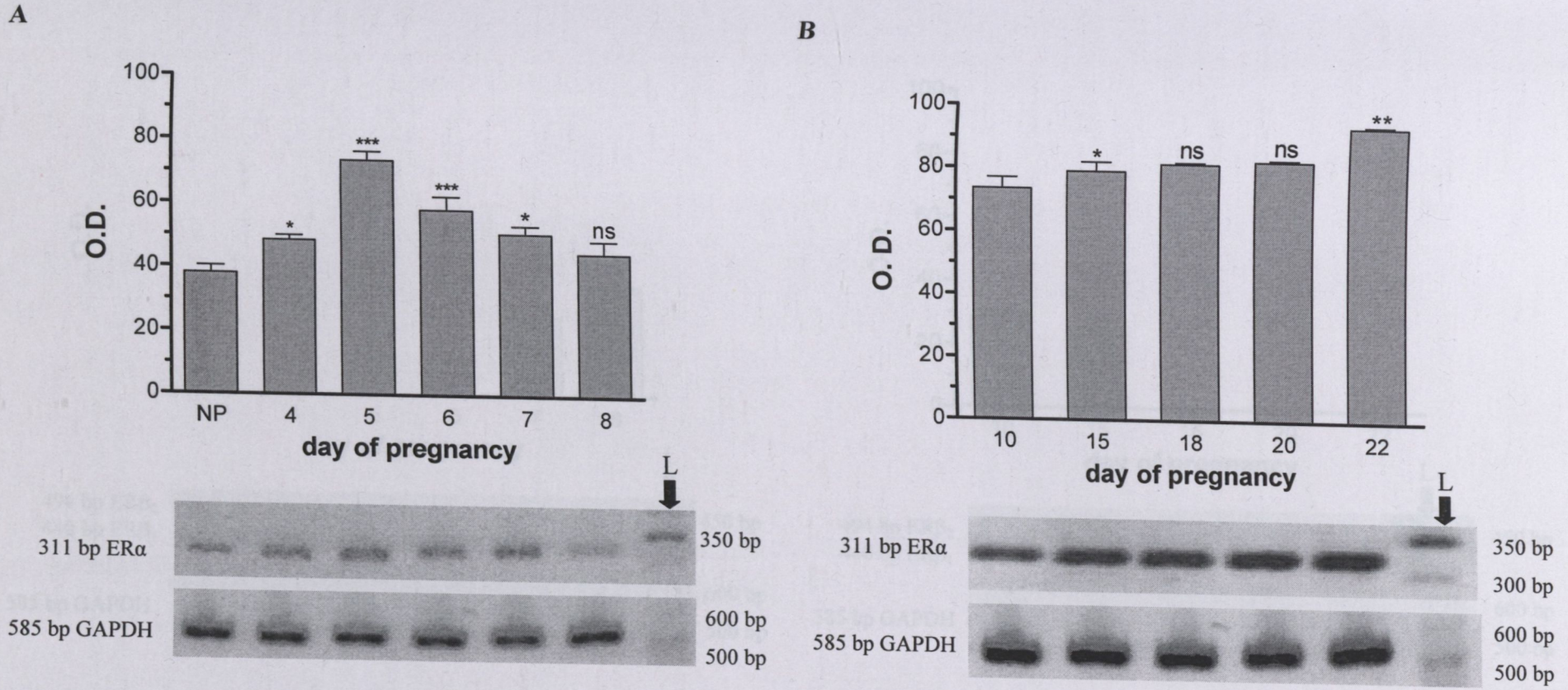

Figure 8. Changes in expression of the estrogen receptor (ER) α mRNA in the rat uterus during early (days 4-8) (**A**) and late (days 10-22) (**B**) pregnancy, measured by RT-PCR as described in the Materials and Methods. The relative amount of the receptor subtype mRNA is indicated by the optical density (O.D.) of the bands. The rat GAPDH shown in the second gel pictures was the internal control.
ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared with the data on the previous day. Each bar represents the mean ± SD, n = 6. L shows the appropriate part of the DNA ladder; the non-pregnant rat uterus is denoted by NP.

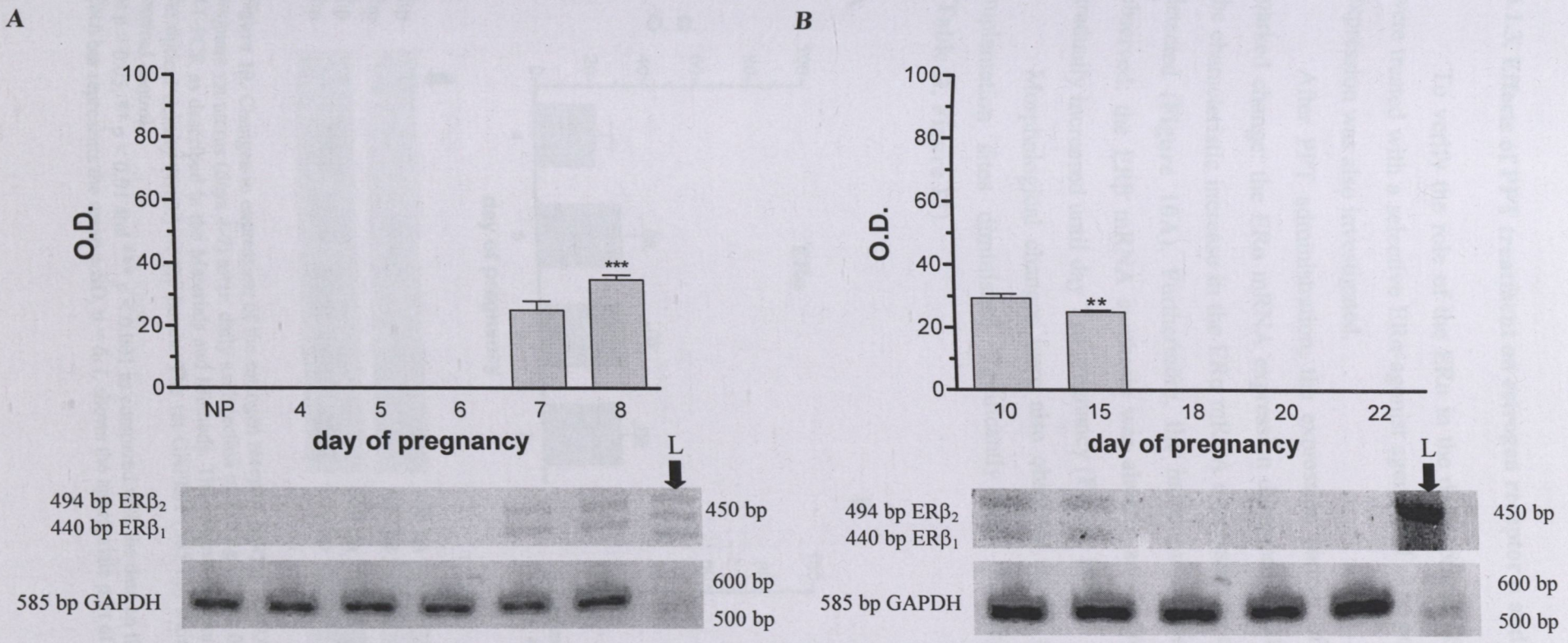

Figure 9. Changes in expression of the estrogen receptor (ER) β mRNA in the rat uterus during early (days 4-8) (**A**) and late (days 10-22) (**B**) pregnancy, measured by RT-PCR as described in the Materials and Methods. The relative amount of the receptor subtype mRNA is given by the optical density (O.D.) of the bands. The rat GAPDH shown in the second gel pictures was the internal control.

** $p < 0.01$, *** $p < 0.001$ as compared with the data on the previous day. Each bar represents the mean ± SD, $n = 6$. L shows the appropriate part of the DNA ladder; the non-pregnant rat uterus is denoted by NP.

4.1.3. Effects of PPT treatment on estrogen receptor α and β mRNA expression

To verify the role of the ERα in the development of the implantation, pregnant rats were treated with a selective ERα-agonist agent, PPT. The possible alteration in ERβ mRNA expression was also investigated.

After PPT administration, the expression pattern of the ERα mRNA underwent a marked change: the ERα mRNA expression decreased on all experimental days. Moreover, the characteristic increase in the ERα mRNA expression on day 5 of pregnancy could not be detected (**Figure 10A**). Furthermore, the induction of the ERβ mRNA expression was observed: the ERβ mRNA synthesis was already active from day 4 of pregnancy and it gradually increased until day 7 of pregnancy (**Figure 10B**).

Morphological changes were also observed in the uterine tissues. The number of implantation sites diminished significantly after the selective ERα-agonist treatment (**Table II, Figure 11**).

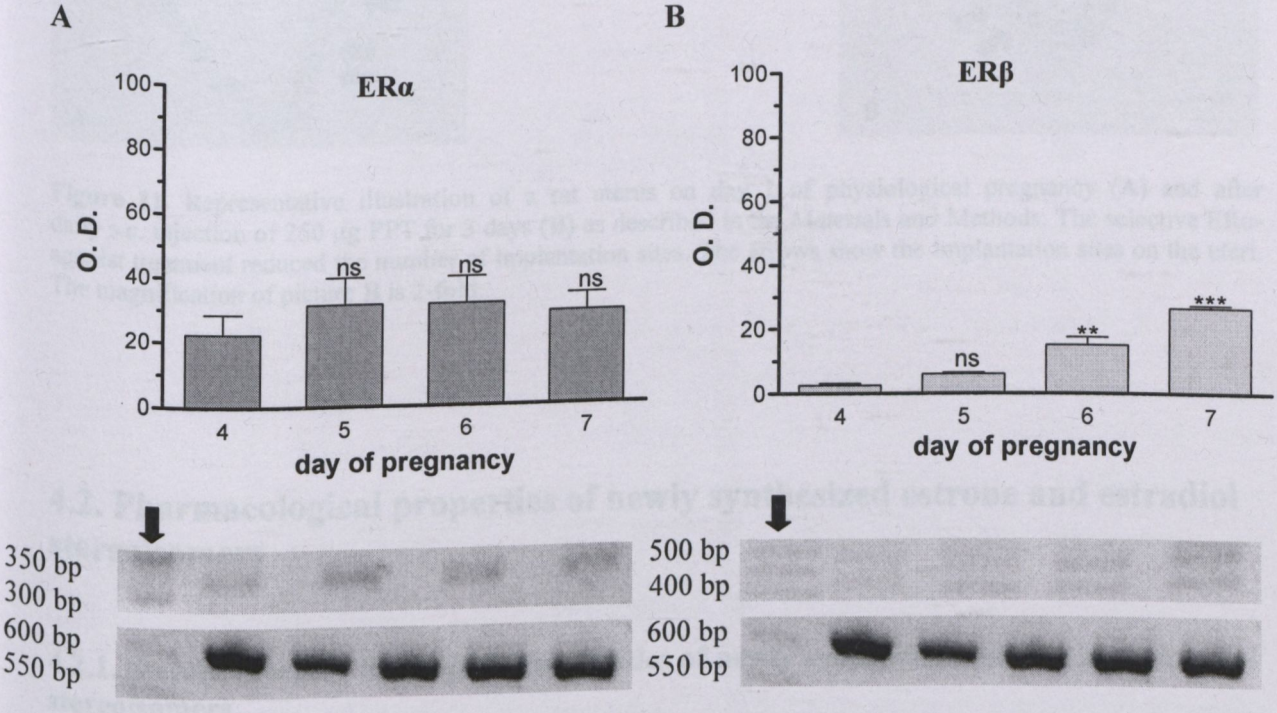

Figure 10. Changes in expression of the estrogen receptor (ER) α mRNA (**A**) and ERβ mRNA (**B**) in the early pregnant rat uterus (days 4-7) after. daily s.c injection of 250 μg PPT in 0.1 ml olive oil for 3 days, measured by RT-PCR as described in the Materials and Methods. The relative amount of the ER subtype mRNA is given by the optical density (O.D.) of the bands. The rat GAPDH (585 bp) shown in the second gel pictures was the internal control.
ns $p > 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as compared with the data on the previous day.
Each bar represents the mean ± SD, n = 6. L shows the appropriate part of the DNA ladder.

Table II. Effects of. daily s.c administration of 250 μg PPT for 3 days on the number of implantation sites in the pregnant rat uterus. The number of implantation sites was recorded on day 7 of pregnancy. All data are expressed as means ± SD; the level of significance is calculated in comparison with the normal pregnant rat uterus.

	Number of implantation sites [mean ± SD]	Significance
Normal pregnant rat uterus	15.5 ± 1.9 (n = 8)	
PPT-treated rat uterus	4.8 ± 0.8 (n = 6)	$p < 0.001$

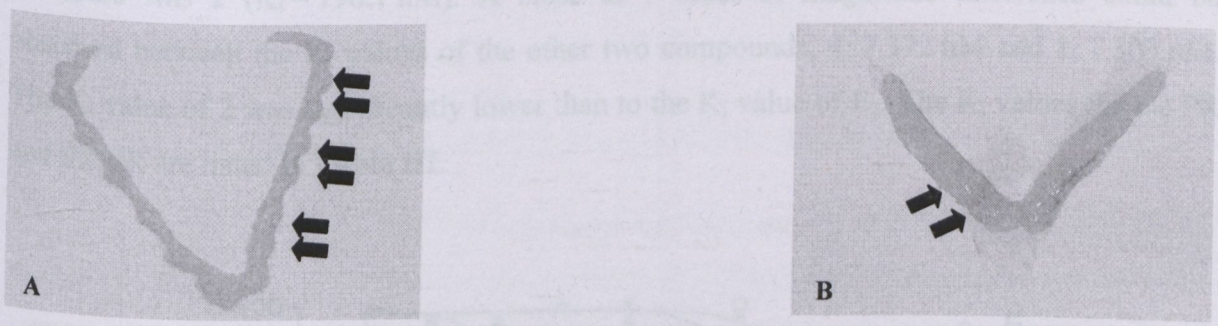

Figure 11. Representative illustration of a rat uterus on day 7 of physiological pregnancy (**A**) and after daily s.c. injection of 250 μg PPT for 3 days (**B**) as described in the Materials and Methods. The selective ERα-agonist treatment reduced the number of implantation sites. The arrows show the implantation sites on the uteri. The magnification of picture **B** is 2-fold.

4.2. Pharmacological properties of newly synthesized estrone and estradiol stereoisomers

4.2.1. Steroid receptor binding characteristics of newly synthesized estrone and estradiol stereoisomers

The binding affinities of the newly synthesized estrone (**1**, **2**, **3** and **4**) and estradiol stereoisomers (**5**, **6**, **7**, **8**, **9**, **10**, **11**, **12**, **13**, **14**, **15** and **16**) for the ER, PR and AR were determined by radioligand binding assay.

4.2.1.1. 3-Methoxy-D-homoestra-1,3,5(10)-triene-17a-one and its analogues

Specifically bound [^{3}H]E_2 was displaced from the immature rabbit uterine ER by all examined molecules, but their affinities were at least 3 orders of magnitude lower than the inhibition constant (K_i) value of the E_2, the endogenous ligand of the ER. The K_i and RBA values are listed in **Table III**. 3-Hydroxy-13α-D-homoestra-1,3,5(10)-triene-17a-one (**4**) was the most potent molecule in this series (**Figure 12**). 3-Hydroxy-D-homoestra-1,3,5(10)-triene-17a-one (**2**), the epimer of the previous compound, possessed a K_i value with the same order of magnitude. The remaining two derivatives, **3** and **1**, exhibited K_i values higher than 1 000 nM (1 131 nM and 3 394 nM, respectively).

The tested compounds bound to the PR labelled with [^{3}H]ORG2058 with different affinities. Compound **3** possessed the highest K_i value (25 640 nM), whereas the most potent molecule was **2** (K_i = 196.1 nM). A close to 1 order of magnitude difference could be observed between the K_i values of the other two compounds, **4**: 7 372 nM and **1**: 1 303 nM. The K_i value of **2** was significantly lower than to the K_i value of E_2. The K_i values for the PR and the SR are listed in **Table III**.

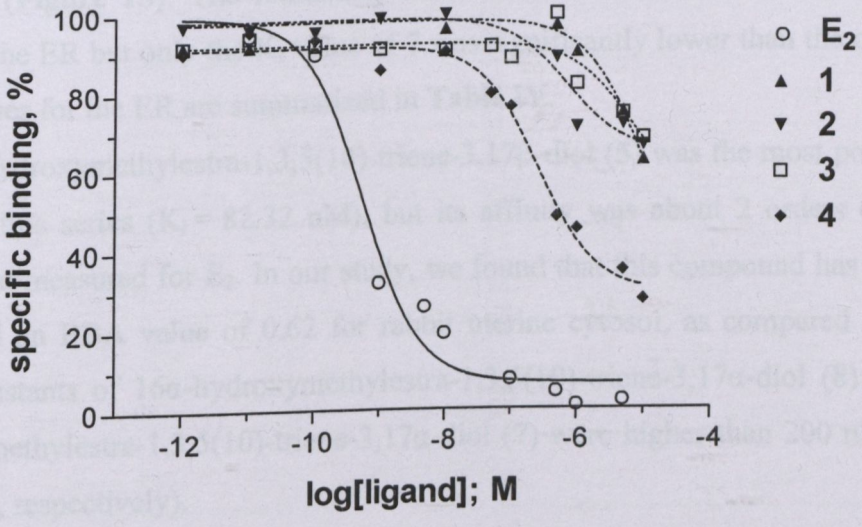

Figure 12. Competitive inhibition of [^{3}H]17β-estradiol in rabbit uterus cytosol by 3-methoxy-D-homoestra-1,3,5(10)-triene-17a-one and its analogues (**1**, **2**, **3** and **4**). The reference molecule is 17β-estradiol (E_2).

Table III. Steroid receptor binding properties of 3-methoxy-D-homoestra-1,3,5(10)-triene-17a-one and its derivatives (**1**, **2**, **3** and **4**), determined by radioligand binding assay, as described in the Materials and Methods. The inhibition constant (K_i) values were measured on rabbit uterine cytosol for the estrogen receptor (ER) and progesterone receptor (PR), and are displayed as means ± SD. The relative binding affinity (RBA) values describe the receptor-binding potency of the tested molecules to the ER as compared with the reference molecule, 17β-estradiol (E_2). The selectivity ratio (SR) data show the abilities of the tested compounds to discriminate between the ER and another steroid receptor.

compounds	ER		PR	
	K_i [nM]	RBA [%]	K_i [nM]	SR
E_2	1.19 ± 0.53	100	1 532± 102	1 287
1	3 394 ± 1131	0.04	1 303± 292	0.38
2	293.7 ± 167.5	0.41	196.1± 77.64	0.67
3	1 131 ± 17.68	0.11	25 640± 1612	22.67
4	1 68.8 ± 10.39	0.71	7 372± 3516	43.72

4.2.1.2. 16-Hydroxymethylestra-1,3,5(10)-triene-3,17-diol stereoisomers

Specifically bound [^{3}H]E_2 was displaced readily from the rabbit uterine ER by E_2 and compound **5** (**Figure 13**). The remaining three derivatives (**6**, **7** and **8**) possessed lower affinities for the ER but only the K_i value of **7** was significantly lower than the others. The K_i and RBA values for the ER are summarized in **Table IV**.

16β-Hydroxymethylestra-1,3,5(10)-triene-3,17β-diol (**5**) was the most potent estradiol derivative in this series (K_i = 82.32 nM), but its affinity was about 2 orders of magnitude lower than that measured for E_2. In our study, we found that this compound has a K_i value of 191.7 nM and an RBA value of 0.62 for rabbit uterine cytosol, as compared with E_2. The inhibition constants of 16α-hydroxymethylestra-1,3,5(10)-triene-3,17α-diol (**8**) and even of 16β-hydroxymethylestra-1,3,5(10)-triene-3,17α-diol (**7**) were higher than 200 nM (268.2 nM and 863.3 nM, respectively).

The tested compounds inhibited [^{3}H]ORG 2058 binding to the PR only in the micromolar range (**Table IV**). It is interesting to note that compounds **5** and **6** bound to the AR with affinities (K_i = 660.9 nM and 834.2 nM, respectively) more than 150 times higher those for the other compounds (K_i > 100 μM).

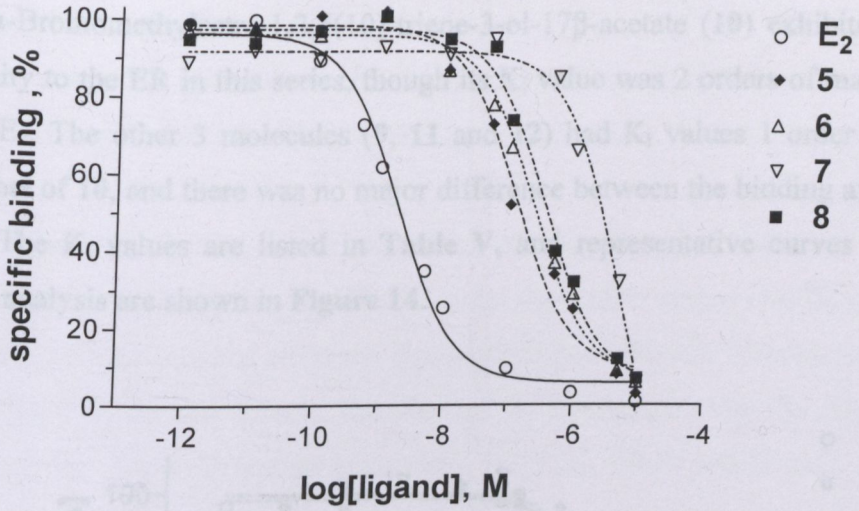

Figure 13. Competitive inhibition of [^{3}H]17β-estradiol in rabbit uterus cytosol by 16β-hydroxymethylestra-1,3,5(10)-triene-3,17β-diol (**5**) and its isomers (**6**, **7** and **8**). The reference molecule is 17β-estradiol (E_2).

Table IV. Steroid receptor binding properties of 16β-hydroxymethylestra-1,3,5(10)-triene-3,17β-diol (**5**) and its stereoisomers (**6**, **7** and **8**), determined by radioligand binding assay as described in the Materials and Methods. The inhibition constant (K_i) values were measured on rabbit uterine cytosol for the estrogen receptor (ER) and progesterone receptor (PR) or on rat prostate cytosol for the androgen receptor (AR), and are displayed as means ± SD. The relative binding affinity (RBA) values describe the receptor-binding potency of the tested molecules to the ER as compared with the reference molecule, 17β-estradiol (E_2). The selectivity ratio (SR) data show the ability of the tested compounds to discriminate between the ER and another steroid receptor.

Compounds	ER		PR		AR	
	K_i [nM]	RBA [%]	K_i [nM]	SR	K_i [nM]	SR
E_2	1.19 ± 0.53	100	1 532 ± 102	1 287	308.3 ± 91.58	259.08
5	82.32 ± 6.53	1.44	3 398 ± 249.5	41.28	660.9 ± 58.9	8.03
6	215.3 ± 47.6	0.55	>100 000	-	834.2 ± 127.3	3.88
7	814.4 ± 79.5	0.14	>100 000	-	>100 000	-
8	213.3 ± 35.7	0.56	6 242 ± 567	29.26	>100 000	-

4.2.1.3. 16-Bromomethylestra-1,3,5(10)-triene-3-ol-17-acetate and 16-bromomethylestra-1,3,5(10)-triene-3,17-diol stereoisomers

Our reference molecule for ovariectomized rat uterine ER was E_2, the inhibition constant of which under our experimental conditions (K_i = 2.31 ± 0.76 nM) was in good agreement with its K_i literature value (2.48 nM) [Martel et al., 1998].

Specifically bound [^{3}H]E_2 was displaced from the ER by **9** and its derivatives (**10**, **11** and **12**). 16α-Bromomethylestra-1,3,5(10)-triene-3-ol-17β-acetate (**10**) exhibited the highest binding affinity to the ER in this series, though its K_i value was 2 orders of magnitude lower than that of E_2. The other 3 molecules (**9**, **11** and **12**) had K_i values 1 order of magnitude higher than that of **10**, and there was no major difference between the binding affinities of the compounds. The K_i values are listed in **Table V,** and representative curves from the ER displacement analysis are shown in **Figure 14.**

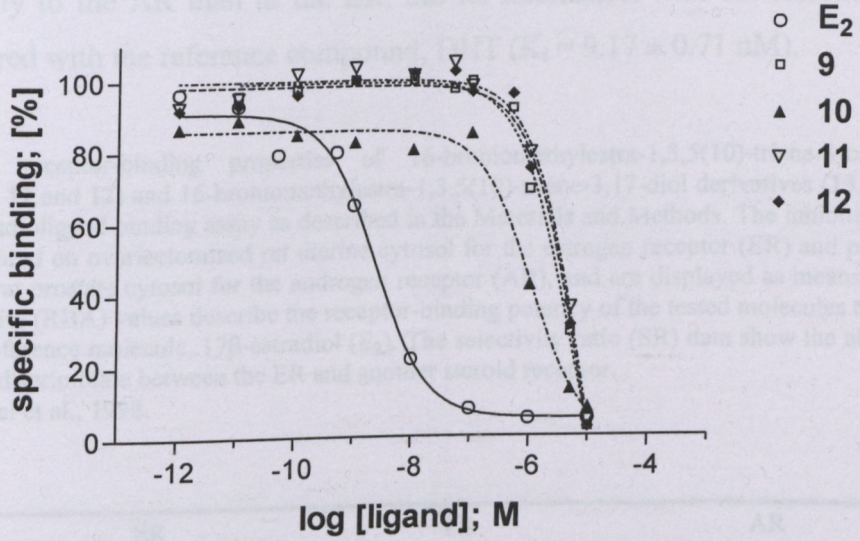

Figure 14. Competitive inhibition of [^{3}H]17β-estradiol in ovariectomized rat uterus cytosol by 16β-bromomethylestra-1,3,5(10)-triene-3,17β-diol-17-acetate (**9**) and its isomers (**10**, **11** and **12**). The reference molecule is 17β-estradiol (E_2).

The reference molecule for the PR was ORG 2058; its K_i value under our experimental circumstances was 7.40 ± 0.98 nM. All of the 17-acetate derivatives of estradiol bound weakly to the PR; their K_i values were higher than 3 000 nM, so the receptor did not recognize them.

Three compounds in this series showed higher affinity for the AR than for the ER. The most conspicuous difference was observed in the case of compound **12**, which bound 1 order of magnitude more strongly to the AR than to the ER. Its K_i value for the AR was the lowest in this group. The fourth isomer (**9**) could not displace the specifically bound [^{3}H]DHT from the castrated rat prostate AR.

The 16-bromomethylestra derivatives of E_2 recognized the ER well (**Figure 15**). Compound **14** exhibited the highest affinity for the ER (K_i = 2.55 ± 0.64 nM), binding to it as

strongly as E_2 does. The K_i values of the other molecules (**13**, **15** and **16**) were at least 200-fold higher than that of **14** (**Table V**).

14 also displayed the highest affinity for the PR, but this interaction was 200-fold weaker than that with the ER. The remaining 3 derivatives had K_i values higher than 3 000 nM.

The members of this chemical series displayed weak affinity for the AR ($K_i > 3\ 000$ nM); an exception was the 17α-estradiol derivative with a 16α-bromomethylestra substituent (**16**). This showed the strongest binding ability to the AR. This molecule bound 3-fold more strongly to the AR than to the ER, but its interaction with the AR was 23-fold weaker as compared with the reference compound, DHT ($K_i = 9.17 \pm 0.71$ nM).

Table V. Steroid receptor-binding properties of 16-bromomethylestra-1,3,5(10)-triene-3-ol-17-acetate stereoisomers (**9**, **10**, **11** and **12**) and 16-bromomethylestra-1,3,5(10)-triene-3,17-diol derivatives (**13**, **14**, **15** and **16**), determined by radioligand binding assay as described in the Materials and Methods. The inhibition constant (K_i) values are measured on ovariectomized rat uterine cytosol for the estrogen receptor (ER) and progesterone receptor (PR) or on rat prostate cytosol for the androgen receptor (AR), and are displayed as means ± SD. The relative binding affinity (RBA) values describe the receptor-binding potency of the tested molecules to the ER as compared with the reference molecule, 17β-estradiol (E_2). The selectivity ratio (SR) data show the ability of the tested compounds to discriminate between the ER and another steroid receptor.
[a] determined by Martel et al., 1998.
[b] not measurable

Compounds	ER		PR		AR	
	K_i [nM]	RBA [%]	K_i [nM]	SR	K_i [nM]	SR
E_2	2.31 ± 0.76	100	478.8 [a]	207.27 [a]	498 [a]	215.58 [a]
9	3 855 ± 2 350	0.06	15 201 ± 1 555	3.94	n. m. [b]	-
10	553.8 ± 198.3	0.42	6 414 ± 3 940	11.58	475.5 ± 7.28	0.86
11	5 254 ± 1 040	0.04	13 079 ± 109.6	2.49	2 963 ± 1 528	0.56
12	4 143 ± 2 269	0.09	12 921 ± 467.4	3.12	237.2 ± 23.76	0.06
13	461.4 ± 145.6	0.50	8 657 ± 407.7	18.76	3 512 ± 582.7	7.61
14	2.55 ± 0.64	90.59	525.2 ± 142.7	205.96	3 260 ± 334.4	1278
15	781.1 ± 149.1	0.29	5 283 ± 549.8	6.76	3 231 ± 509.2	4.14
16	684.4 ± 154.0	0.34	7 190 ± 311.7	10.51	214.7 ± 10.96	0.31

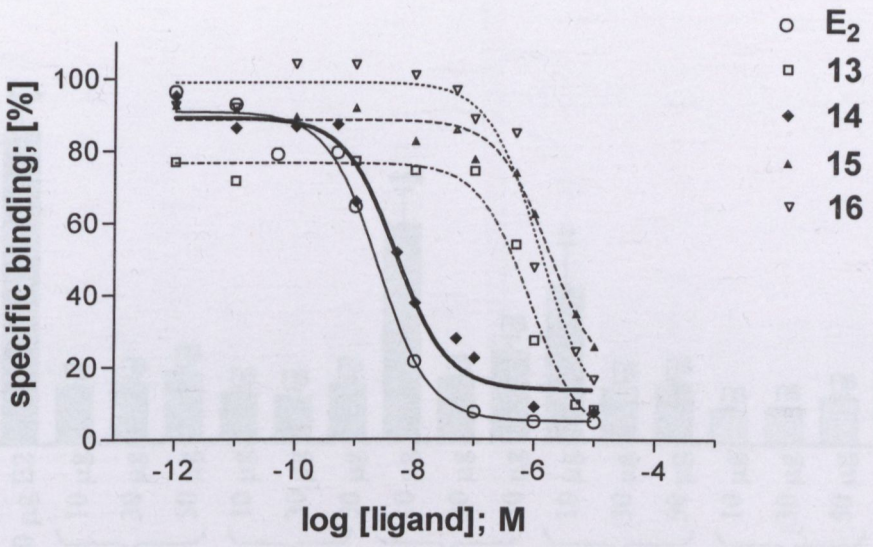

Figure 15. Competitive inhibition of [^{3}H]17β-estradiol in ovariectomized rat uterus cytosol by 16β-bromomethylestra-1,3,5(10)-triene-3,17β-diol (**13**) and its isomers (**14**, **15** and **16**). The reference molecule is 17β-estradiol (E_2).

4.2.2. Effects of newly synthesized estrone and estradiol stereoisomers on the uterus weight of ovariectomized rats *in vivo*

To perform the *in vivo* uterus weight measurement, the epimer pairs from each chemical series possessing the highest affinity for the ER were chosen: 3-hydroxy-13α-D-homoestra-1,3,5(10)-triene-17a-one (**4**) and 3-hydroxy-D-homoestra-1,3,5(10)-triene-17a-one-3 (**2**), 16β-hydroxymethylestra-1,3,5(10)-triene-3,17β-diol (**5**) with its isomer, 16α-hydroxymethylestra-1,3,5(10)-triene-3,17β-diol (**6**) and 2 molecules from among the eight 16-bromomethylestra derivatives: 16β-bromomethylestra-1,3,5(10)-triene-3,17β-diol (**13**) and 16α-bromomethylestra-1,3,5(10)-triene-3,17β-diol (**14**).

10 μg of E_2 as the dose of the positive control was determined in a preliminary test to develop the maximum uterus weight gain for ovariectomized rat uterine tissue under our experimental circumstances.

The effects of the 2 D-homoestrone isomers (**2** and **4**) did not differ significantly from the wet uterus weight gain evoked by olive oil (negative control) (**Figure 16**), although small-scale dose-response relationships could be observed in these cases. This finding did not alter after measuring the dry uterus weight (**Figure 17**).

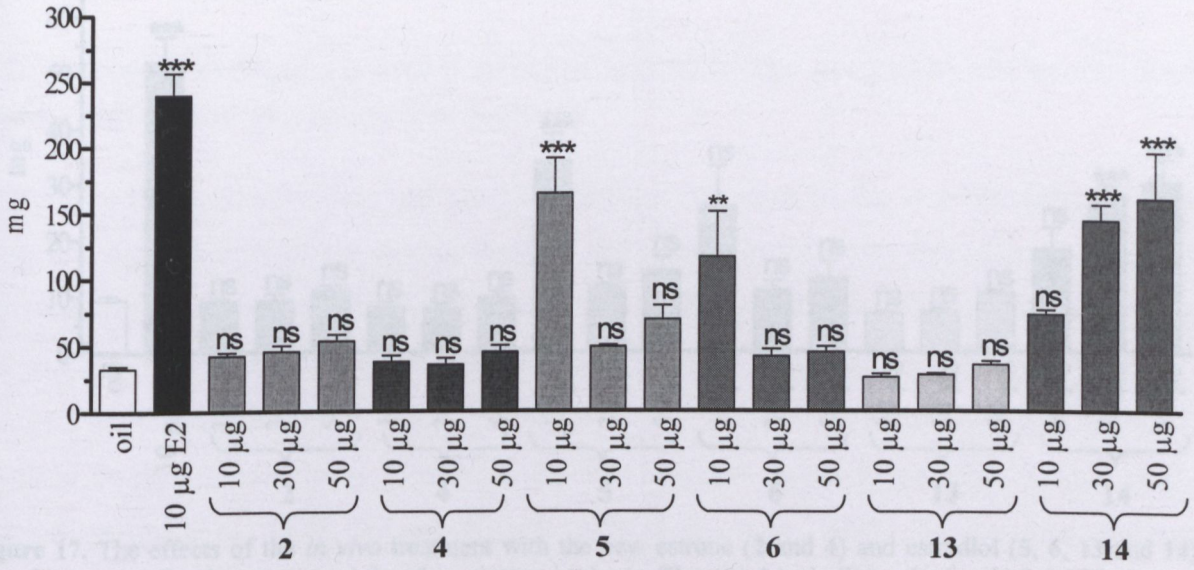

Figure 16. The effects of the *in vivo* treatment with the new estrone (**2** and **4**) and estradiol (**5**, **6**, **13** and **14**) stereoisomers on the wet uterus weight of ovariectomized rats. The asterisks indicate the level of significance of the difference from the olive oil-treated uteri (ns $p > 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

The treatment with both 16-hydroxymethylestradiol derivatives (**5** and **6**) resulted in significant increases in wet uterus weight (**Figure 16**). This alteration was the highest in the group that received 10 µg **5** or **6**. The estrogenic effect of **5** was stronger than that of **6**. In doses of 30 µg and 50 µg, neither molecule changed the wet uterus weight significantly. As regards, the dry uterus weight data, the same results were established (**Figure 17**).

Two members of the 16-bromomethylestradiol series (**13** and **14**) were tested for uterus weight gain. Compound **14** displayed a strong, dose-dependent estrogenic effect; the alteration caused by the dose of 30 µg of **14** already proved significant (**Figure 16**). The same outcome was detected during the evaluation of the dry uterus weight data (**Figure 17**). Its isomer (**13**) did not induce a significant increase in either wet uterus weight or the dry uterus weight (**Figures 16** and **17**).

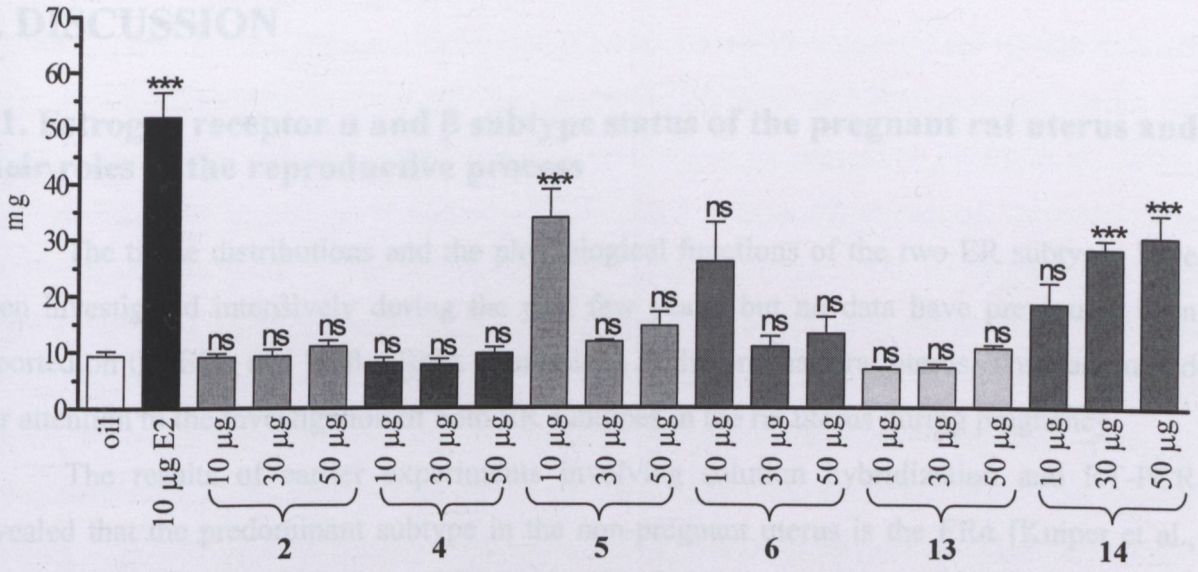

Figure 17. The effects of the *in vivo* treatment with the new estrone (**2** and **4**) and estradiol (**5**, **6**, **13** and **14**) stereoisomers on the dry uterus weight of ovariectomized rats. The asterisks indicate the level of significance of the difference from the olive oil-treated uteri (ns $p > 0.05$ and *** $p < 0.001$).

5. DISCUSSION

5.1. Estrogen receptor α and β subtype status of the pregnant rat uterus and their roles in the reproductive process

The tissue distributions and the physiological functions of the two ER subtypes have been investigated intensively during the past few years, but no data have previously been reported on the ERα and ERβ mRNA expressions in the pregnant rat uterus. This fact turned our attention to the investigation of both ER subtypes in the rat uterus during pregnancy.

The results of earlier experiments involving solution hybridization and RT-PCR revealed that the predominant subtype in the non-pregnant uterus is the ERα [Kuiper et al., 1997; Shughrue et al., 1998; Sar and Welsch, 1999; Wang et al., 1999]. We demonstrated that the ERα remained predominant in the rat uterus during pregnancy. The expression of the ERα mRNA varied during pregnancy, corresponding to the fluctuations in the E_2 level in the ovarian venous blood [Yoshinaga et al., 1969]. However, the pattern of the ERα mRNA expression in the uterus is in contrast with the ERα mRNA expression in the rat ovary during pregnancy [Telleria et al., 1998]: the ERα mRNA is expressed at low levels in early pregnancy; it then subsequently increases (day 12), and declines on the day of parturition.

Additionally, our experiments revealed a parallelism between the ER-binding site level and the ERα mRNA expression in the early pregnant uterus. During late pregnancy, the pattern of ERα mRNA expression corresponds with the alterations in ER density measured by Fang and co-workers [1996].

The elevated level of ERα mRNA on day 5 may be related to the blastocyst implantation, because estrogens are essential in the induction of implantation. It may be assumed that E_2 induces epithelial proliferation in the endometrium via the ERα, which is necessary for blastocyst attachment by the end of day 5. These findings are supported by a previous investigation which demonstrated that implantation was not observed in ERα knock-out mice [Hewitt et al., 2002].

It has already been reported that the ERβ mRNA is weakly expressed in the rat uterus, while it has a dense expression in the ovary [Kuiper et al., 1997; Shughrue et al., 1998; Sar and Welsch, 1999; Wang et al., 2000]. In contrast, we observed a characteristic expression of the ERβ mRNA in the uterus during gestation: the ERβ mRNA could be well detected only from day 7 to day 15. The elevation of the ER protein level on day 8 can be accounted for as a consequence of the maximum expression of ERβ mRNA on the same day. Although the ERβ

primer [Drummond et al., 1999] that we used distinguishes two isoforms of the ERβ (β_1 and β_2), we did not find a significant difference between the O. D. values of the isoforms. It has been reported that there is no significant alteration in ERβ mRNA expression during the estrous cycle, and thus the endogenous E_2 has no effect on its expression [Wang et al., 2000]. Similarly, there is no change in the ovary throughout pregnancy, except on the last 2 days before parturition, when its expression declines [Telleria et al., 1998]. In contrast, the ERβ mRNA displayed a maximum on day 8 and was significantly lower in the pregnant rat uterus on 3 other investigated days (days 7, 10 and 15). This period (days 7-15) when the ERβ mRNA is expressed in the rat uterus is coincident with the onset of decidualization, when the endometrial cells of the pregnant rat uterus are transformed into decidual tissue. This process requires E_2. Decidual ERβ mRNA expression was earlier demonstrated between days 9 and 13 of pseudopregnancy in the rat [Tessier et al., 2000]. The two results, considering the period of the ERβ mRNA expression, are in good agreement with each other. Otherwise, the decidua is dependent on P_4 for its maintenance and growth [Ogle, 2002]. It has already been proved that the PR expression is hormonally regulated, as it is induced by estrogens [Massart et al., 2003]. Since the PR expression displayed a significant elevation on day 8 after the beginning of the ERβ mRNA expression on day 7 of pregnancy, it may be presumed that endogenous E_2 may increase the PR expression, and therefore influence the development of the decidua via the ERβ signal route. The same conclusion was published by Kurita et al. [2001].

Wu and co-workers [2000] established that the ERβ mRNA expression ceased before the initiation of labour in the human myometrium, and thus the loss of inhibition of AP-1 (transcription factor) activity induces the function of connexin 43 gene and other labour-associated genes. As ERβ mRNA could not be detected in the samples derived from late-pregnant rats, it might be supposed that ERβ plays a similar role in the rat uterus.

5.2. Role of estrogen receptor α in the regulation of implantation

A crucial stage in pregnancy is the implantation of the embryo into the endometrium. In the rat, this event occurs at the end of day 5 of pregnancy [Maeda et al., 2000] and is regulated by several endogenous factors, e.g. steroid hormones [Albrecht and Pepe, 2003], cytokines [Krussel et al., 2003] and growth factors [Rabbani and Rogers, 2001; Rockwell et al., 2002].

As the results of the RT-PCR assays and ER saturation experiments revealed that both the ERα mRNA expression and the ER density were significantly higher on day 5 of pregnancy than on the other days, we hypothesized the regulatory role of ERα in the implantation process developing on day 5 of pregnancy.

An agonist effect on the ER was evoked by the PPT treatment of pregnant rats, which resulted in down-regulation of the transcription of the ERα mRNA, and thus the elevated mRNA expression on day 5 of pregnancy disappeared. The altered level of the ERα mRNA triggered a chain of events that finally resulted in a significant reduction in the number of implantation sites. This result confirmed the previous assumption that an estrogen signal mediating via the ERα pathway is necessary for the development of the implantation. The exact mechanism of this process has not been fully elucidated, but estrogens regulate several endogenous factors that are connected to blastocyst implantation. The lack of activation of connexins might be one of the regulatory routes [Winterhager et al., 1991]. Moreover, estrogen rapidly up-regulates vascular endothelial growth factor (VEGF) mRNA expression in the rodent uterus [Hyder et al., 2000], which enhances the microvascular permeability. Increased microvascular permeability also occurs in conjunction with implantation in the endometrium [Rockwell et al., 2002]. This regulatory process might also develop via the ERα. However, we do not have enough information about the other estrogen-dependent, endogenous factors participating in the development of implantation.

An unexpected alteration was observed in the ERβ mRNA expression after the ERα-selective agonist treatment: ERβ mRNA synthesis was already active from day 4. The binding affinity of PPT for the ERα is 2 orders of magnitude greater than that for the ERβ, and PPT can influence the gene transcription only via the ERα [Harris et al., 2002]. Hence, the earlier activation of the ERβ mRNA expression might be a result of cross-talk between the two ER subtypes, and the ERβ mRNA expression might be up-regulated by PPT via the ERα. The up-regulated ERβ is not able to fulfil its function in the rat uterus, because the implantation has failed.

On the basis of our experimental work, the elevated levels of ER-binding sites and ERα mRNA expression on day 5 of pregnancy are thought to be the initial steps in the development of the receptive endometrium before blastocyst implantation. In the future, it will be necessary to explore the exact roles of the other endogenous factors to clarify all the steps of the implantation-regulating system.

5.3. Pharmacological evaluation of originally synthesized estrone and estradiol stereoisomers

In this study, the influence of the stereoselectivity of the substituents at positions C3, C13, C16 and C17 in differently substituted estrone and estradiol derivatives on their steroid receptor binding abilities and *in vivo* effectiveness was investigated.

Examination of the binding of these molecules to the ER proved that the ER recognized them, although the differences between the K_i values reached 3 orders of magnitude. Despite the ERs being derived from distinct species (rat and rabbit), we can compare the results because the structure homology of the ligand-binding domain among the vertebrate ER proteins is 60-95% [Paolucci et al., 1999].

It is well known that the sterane skeleton is not essential for the synthesis of molecules binding strongly to ER. This recognition led to the discovery of diethylstilbestrol [Schneider et al., 1986], tamoxifen and its analogues [Wakeling et al., 1984] and the recently synthesized tetrahydrochrysene [Sun et al., 1999] and furan derivatives [Mortensen et al., 2001]. On the above basis, 4 original compounds containing a dodecahydrochrysene skeleton were prepared, which belong in one chemical series. These analogues showed the lowest affinity towards the ER (**1**, **2**, **3** and **4**). Compounds exhibiting high binding affinity for ER possess hydroxy functional groups at positions C3 and C17 to develop H-bonds with the ERLBD. Two of our 4 molecules (**2** and **4**) contain a 3-hydroxy group, and their K_i values on the ER proved to be higher than those of the other analogues with a 3-methoxy function (**1** and **3**). This 3-methoxy function in the remaining 2 derivatives weakens the power of the interaction in consequence of its lower H-donating ability.

Ring D of the tested compounds contains 6 C atoms, which can not influence the ER-binding ability significantly, because this part of the ER-binding pocket becomes wider, making place for the bulky molecules [Ekena et al., 1997].

The endogenous estrone possesses a 13-methyl group in the β position. Nevertheless, in our investigations the 2 D-homoestrone derivatives with a 13α-methyl group (**3** and **4**) exhibited K_i values 1 order of magnitude lower than those of their epimers (**1** and **2**).

Several research groups have investigated the effect of C16-substitution on the affinity of estradiol derivatives for the ER [Ponsold et al., 1977; Fevig et al., 1988]. They concluded that the ER tolerance for substituents at position 16α is mixed, although groups of moderate size and polarity are generally tolerated, e.g. halogens or alkyl halogens, but position 16β is sterically less permissive, even the smallest functional group (e.g. fluorine) decreasing the

binding ability substantially. Our experimental work with the 6 estradiol epimer pairs partially support these findings, because the tested 17β-derivatives with 16α-bromomethyl substituents (**10** and **14**) possessed K_i values at least 1 order of magnitude lower than those of the 16β-bromomethyl-substituted molecules (**9** and **13**), but the 16-hydroxymethyl analogues did not behave similarly. The 16β-hydroxymethyl-17β derivative (**5**) exhibited greater affinity for the ER than its 16α-hydroxymethyl counterpart (**6**). This difference presumably results from the different polarities of the bromomethyl and hydroxymethyl groups.

The RBA value of **14** is 90.95%, and this epimer exhibits a K_i value 2 orders of magnitude lower than that of its counterpart (**13**). Ring D of estradiol derivatives makes non-polar contacts with the Ile 424, Gly 521 and Leu 525 residues, and the Ile 424 residue can be found close to the C16 of the ligand in the ERLBD [Brzozowski et al., 1997]. Our results suggest that position 16β in the bromomethyl epimers is sterically unfavourable because of the side-chain of the Ile 424 residue that is close to the ligand.

The greatest difference in K_i values is observed between the members of the 17-acetate epimers (**9**, **10**, **11** and **12**) and the 3,17-diols (**13**, **14**, **15** and **16**), proving that a hydroxy group at position 17β of the estradiol ring is necessary for formation of a H-bond with the His 524 residue. Highly polar and bulky groups, such as acetate, are poorly tolerated by the ERLBD at positions C17α and β [Anstead et al., 1997].

The finding that the tested compounds bind to the AR and the PR with low affinity is an obvious indication that these compounds contain a 3-phenolic or 3-methoxy function and not a 3-keto function, which forms a H-bond with Gln 711 and Arg 752 in the case of the AR [Ekena et al., 1998, Sack et al., 2001] and with Gln 725 and Arg 766 in the case of the PR [Tanenbaum et al., 1998, Sack et al., 2001].

A relatively high PR affinity was observed during determination of the K_i values of the estrone derivatives (**1**, **2**, **3** and **4**). These molecules contain an 18-keto group, which might play a similar role in the ligand-PR binding procedure as the 20-keto group does in the P_4 molecule [Williams and Siegler, 1998]. The position of the 13-methyl group influences the PR-binding ability of the estrone analogues. The K_i values (on the PR) of the 2 epimers which show higher affinity for the ER (**2** and **4**) differed by 1 order of magnitude. These 2 compounds differ only in the position of the 13-methyl group, which is fixed by Met 909 of the PR-LBD through a van der Waals contact [Williams and Siegler, 1998]. If this group is in the β position, it is an unfavourable state according to our measurements, because the SR of the molecule decreases. A similar affinity difference can be found between the other 2 epimers (**1** and **3**), which supports our previous conclusion. The estrone derivatives were not

tested for AR binding because it was reported earlier that endogenous estrone could not displace the selective AR ligand, R1881 from the AR [Lamarre et al., 1985].

The investigated 16-substituted estradiol molecules are able to bind to the AR and PR, because the 3-hydroxy group can act not only as an H-donor but also as an H-acceptor [Anstead et al., 1997] and in the AR and PR a good H-donor glutamine residue can be found instead of the Glu 353 in the ER [Ekena et al., 1998; Tanenbaum et al., 1998]. Compounds **5** and **6** bind to the AR with more than 150 times higher affinity (K_i: 660.9 nM and 834.2 nM, respectively) than their counterparts (K_i > 100 μM), because the 17β-hydroxy group in E_2, **5** and **6** forms a H-bond with the side-chain of Asn 705 and Thr 877, while the 17α-hydroxy group in **7** and **8** does not. Moreover the 16-hydroxymethyl group in **5** and **6** can form another H-bond, which increases the affinity of these compounds toward the AR, whereas E_2, which lacks a 16-hydroxymethyl group, possesses much lower affinity than those of **5** and **6**, but significantly higher than those of **7** and **8**, which have a 17α-OH function. The 17-acetylestradiol isomers (**9**, **10**, **11** and **12**) can hardly distinguish between the steroid receptors, because ring A of the molecules is similar to that in E_2 with the phenolic hydroxy group on C3, and the acetylated ring D resembles those in P_4 and DHT.

On the basis of the results determined during the radioligand binding assay, 16α-bromomethylestra-1,3,5(10)-triene-3,17β-diol (**14**) is considered the best, originally synthesized ER ligand, with high ER selectivity.

The *in vivo* measurement of the pharmacological activity was designed with the molecules possessing the best ER-binding parameters and their isomers from the groups of differently substituted estrone and estradiol derivatives. By determining the wet and dry uterus weights, information is obtained about the capacities of the examined compounds to induce edema and cell proliferation in the uterus, respectively. When the growth of the uterus is the endpoint in an *in vivo* experiment, it is a measure of the estrogenic activity in the uterus, i.e. ERα action [Gustafsson, 2003]. Thus, our results show the potency of the tested molecules to evoke pharmacological effects through the ERα in the rat uterus.

The *in vitro* ER-binding affinity of the D-homoestrone derivatives is reflected in their *in vivo* estrogenic activity; there was no significant increase in the wet or dry uterus weight. 16α-Bromomethylestra-1,3,5(10)-triene-3,17β-diol (**14**) evoked a weaker, but dose-dependent uterotrophic effect than predicted by the result of the radioligand binding assay. Such discrepancies between *in vitro* and *in vivo* activities often result from biotransformation [Ferguson et al., 1977]. 16β-Hydroxymethylestra-1,3,5(10)-triene-3,17β-diol (**6**) caused significant increases in the wet and dry uterus weights, but the degree of the change induced

by the lowest dose (s.c. 10 μg/day/animal) was the highest. It might be presumed that this molecule in high doses possesses antiestrogenic characteristics on the uterine tissue. Long-term treatment with pure antiestrogens caused endometrial and myometrial atrophy, thereby reducing the uterine weight, similarly to our result. However, the chemical structures of our compounds differ from those of the steroid antiestrogens available for experimental work or medicinal practice [Hermenegildo and Cano, 2000]. It is currently thought that hydrophobic substitution at position 7α or 11β on the sterane skeleton is necessary to ensure the antiestrogenic activity of a new molecule.

Our results reflect the fact that stereoisomerism exerts a great influence on the ligand-receptor interaction, although comparisons can not be made routinely between the *in vitro* and *in vivo* results.

6. CONCLUSIONS

In this work, we characterized the distribution of the ERα and ERβ in the pregnant rat uterus, investigated the regulatory mechanism of the implantation process and determined the influence of the stereochemistry on the ligand-receptor binding and *in vivo* effectiveness. On the basis of our results, we reached the following conclusions:

1. ER subtypes in the pregnant rat uterus

   The mRNAs of the ERα and ERβ coexist in the rat uterus during pregnancy. The predominance of the ERα is noticeable during pregnancy. The expression of ERβ mRNA can be detected only in midpregnancy.

   As a characteristically increased level of ER-binding site number and ERα mRNA expression were observed on day 5 of pregnancy, we hypothesized that this signalling pathway mediates the initiating effect of endogenous E_2 in the implantation process. The *in vivo* investigation revealed that the ERα participates in the endogenous mechanism regulating the development of the receptive endometrium.

2. Chemical structure-pharmacological activity relationship

   The radioligand binding assay confirmed that the 3-hydroxy-group improves the ER-binding ability of the molecules.

   Better ER-binding properties were observed in the epi-D-homoestrone group, where the 13-methyl substituent is in the α position.

   The presence of the 17β-hydroxy group essentially influences the interaction between the ligand and the ER. Modification of the structure with an acetyl group on C17 results in weak AR-binding compounds.

   The ER-binding ability of the tested molecules depends on the character of the functional group on C16. 16α-Bromomethylestra-1,3,5(10)-triene-3,17β-diol is considered a good ER ligand, with excellent specificity.

   The *in vivo* efficacy of our compounds does not fully reflect their *in vitro* receptor binding abilities.

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ACKNOWLEDGEMENTS

I would like to express my thanks to my supervisor and the head of the Ph.D. Programme Pharmacodynamics, Biopharmacy and Clinical Pharmacy, **Prof. George Falkay**, for the possibility to take part in the Ph.D. studies and for his guidance, encouragement and management of my work.

I would like to offer my sincere thanks to **Prof. Gyula Schneider and his colleagues** for the pleasant cooperation and inspiring discussions.

I am grateful to my tutors, **Eszter Ducza Ph.D.** and **Árpád Márki Ph.D.** for their generous help and advice in the experimental work, and for critically reviewing the manuscript.

Thanks are due to all of my colleagues in the Department of Pharmacodynamics and Biopharmacy for the favourable athmosphere.

I am deeply grateful for my family and friends for their patience and love.