THE INFLUENCE OF 17-OXO- AND 17-HYDROXY-16,17-SECOESTRATRIENE DERIVATIVES ON ESTROGEN RECEPTOR

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Since many of newly synthesised D-secoestratriene derivatives showed antiestrogenic effect, with almost a total loss of estrogenic activity, we studied the effects of some of these compounds on estrogen receptors (ER), the translocation of the estrogen–ER complexes formed in presence of competing substances into the nucleus, as well as the binding of these complexes to DNA. The results of uterotrophic effects of analysed derivatives are in agreement with the influence of these compounds on activity and binding parameters of estrogen receptors. Namely, compounds that show relatively high antiestrogenic activity predominantly increase K_d and inhibit translocation to nuclei of radioactive complexes formed in their presence. On the other hand, compounds that do not significantly change binding parameters of estrogen receptors do not show antiestrogenic effect in *in vivo* experiments.

Keywords: Steroids; Estrogens; Antiestrogens; D-secoestratriene derivatives; Estrogen receptor binding; Steroid–receptor interaction; Receptor activation; Nuclear translocation.

Among other factors, estrogens are well recognised to play a predominant role in breast cancer development and growth[1]. Since the first step in the action of estrogens in target tissues is binding to the estrogen receptors[2] (ER), a logical approach in the treatment of estrogen-sensitive breast cancer is the use of antiestrogens, or compounds that block the interaction of estrogens with their specific receptors.

The presence of estrogen receptors in about 66% of human breast cancers is now well established[3]. About 50–65% of patients with estrogen-receptor-positive breast tumours respond with remission to endocrine therapy[3a,4], while only 25% of patients with endometrial cancers[5] and very few with ovarian carcinomas[5a] respond to such a treatment.

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The current treatment of estrogen-dependent diseases involves application of therapeutics with mixed agonist/antagonist of estrogen action, thus limiting their therapeutic potential and possibly explaining the limited success obtained with these compounds[1a–1d,6]. Nowadays there are so-called "pure" antiestrogens[7], and chemists make efforts to synthesise new ones, in order to prepare therapeutics with a higher response rate.

Previously, we reported the synthesis of new D-secoestratriene derivatives[8]. Most of them showed a total loss of estrogenic activity, whereby some expressed moderate antiestrogenic action according to biological tests *in vivo*[9]. In order to explain the possible mechanisms of their *in vivo* action, the interaction of selected derivatives with isolated estrogen receptors was studied.

EXPERIMENTAL

Materials

[2,4,6,7-^{3}H]Estradiol (^{3}HEd), specific activity 72 Ci/mmol, was obtained from Amersham International. Standard unlabelled competitor diethylstilbestrol (DES), Tris and dithiotreitol (DTT) were purchased from Sigma, while activated charcoal (Norit A) and Dextran T-500 were obtained from Pharmacia-Fine Chemicals.

The substances tested were D-secoestratriene derivatives **1**–**4** (Fig. 1), synthesised in several synthetic steps, starting from estrone by the procedure described elsewhere[8b]. The compounds were dissolved in ethanol and the solutions were stored at 4 °C. Each solution was carefully examined for precipitate formation, immediately before the experiment.

All the tested compounds were analytical-pure substances, as well as other chemicals used in experiment.

Buffer A: 0.01 mol/dm^3 Tris HCl + 10% of glycerol, pH 8.0

Buffer B: 0.25 mol/dm^3 saccharose + 1 mmol/dm^3 EDTA + 0.5 mmol/dm^3 DTT + buffer A, pH 7.5–7.88 (DTT added immediately before experiment)

Buffer C: 1 mmol/dm^3 $MgCl_2$ + 0.05 mol/dm^3 Tris HCl, pH 7.4

Buffer D: 0.4 mol/dm^3 KCl + 0.01 mol/dm^3 Tris HCl, pH 7.4

Buffer E: 1 mmol/dm^3 $MgCl_2$ + 0.05 mol/dm^3 Tris HCl + 25% of glycerol, pH 7.4

Methods

The estrogen-receptor-binding assay was carried out using slightly modified known procedures[10]. Experiments were made with at least 10–15 female rats per group. Results are presented as mean values of three measurements.

Wistar female rats (2–2.5 months old and weighing ca. 200–220 g) were grown in standard laboratory conditions and were ovariectomised about ten days before experiment. Animals were decapitated, their uteri were removed and dissected free of adhering fat and connective tissue. The uterine tissue was cut into small pieces, and then homogenised in buffer B (1:5 w/v). Homogenisation was done for 3 × 30 s with a homogeniser Ika-Ultra Turrax, and homogenates were kept cool during the procedure. All further steps of the ex-

periments were performed at 4 °C, if not stated otherwise. Tissue homogenates were then filtered through a gauze cloth and centrifuged at 800 *g* for 10 min. The supernatant was centrifuged at 105.000 *g* for 1 h, to obtain cytoplasmic fraction (cytosol).

On the other hand, the resulting pellet, which contains nuclei and undestroyed cells, was used for preparing crude nuclear fraction. The pellet was suspended in buffer B and then centrifuged at 800 *g* for 5 min. Supernatant was removed and the washing procedure was repeated twice. The resulting pellet, taken as nuclear fraction, was suspended in buffer E, and stored at –10 °C for no longer than 18 h.

Determination of Binding Parameters

In order to analyse the protein-bound and -unbound ligand, cytosol was incubated with increasing concentrations of ^{3}HEd (in concentration range 0.247–80 nmol/dm^3), in the absence (total binding) or presence of 1000-fold higher concentration of DES as standard competitor (non-specific binding), or tested substances, in parallel probes, at 4 °C for 18 h.

After incubation, bound and unbound ligands were separated by charcoal adsorption (0.5% Norit A–0.05% Dextran T-70) at 4 °C for 10 min and after centrifugation at 12 000 *g*, portions of supernatant were removed, counted in a liquid scintillation counter and the molar concentrations of bound ligand were calculated[11]. The binding parameters, i.e., the apparent dissociation constant (K_d) and the binding capacity of the estrogen binding sites (N), were derived from the graphics formed from the ratio of specifically bound and unbound ligand vs specifically bound ligand using computer program[12].

Complex formation in competitive condition. To determine the ability of the tested substances to compete for the estrogen binding sites, tritiated estradiol (8 nmol/dm^3) was incubated with cytosol in the absence or presence of 1000-fold higher concentration of competing substance, DES, or the tested compounds. Final concentration of ^{3}HEd was 4–5 times higher than K_d for estradiol. Complexes were formed at 4 °C during 18 h. There were two parallel probes: the first one, after 18 h incubation at 4 °C, was activated by storing at 25 °C for 30 min, and the other was not thermally activated. Further analyses were carried out with thermally activated (25 °C) and non-activated complexes (4 °C).

Cytosol fractions containing the formed complex, after treating with dextran-coated charcoal (DCC) suspension (3.75% Norit A–0.375% Dextran T-500), were used for measurement of the bound ligand as well as for incubation them with nuclei. The value of specific radioactivity (SA) of bound estrogen is represented as disintegrations per minute per milligram of protein in cytosol.

Determination of ^{3}HEd–ER complex translocated to the nuclei. The nuclear fractions, after keeping them at –10 °C, were washed with buffer B, and incubated at 4 °C for 30 min with thermally activated or non-activated (non-heated up to 25 °C) ^{3}HEd–ER cytosolic complexes, formed in the absence or presence of analysed competitor. After incubation, samples were centrifuged at 800 *g* for 10 min and the nuclear pellets were washed three times with buffer C.

The washed nuclei were then lized with hypertonic buffer D at 4 °C during 30 min. The resulting suspension was centrifuged at 1200 *g* for 20 min. Radioactivity of the supernatant, referred to as the nucleoplasmic fraction, was measured and expressed as disintegrations per minute per milligram of proteins (dpm/mg of proteins). Radioactivity of the nucleoplasmic fraction corresponds to the ^{3}HEd–ER complex, translocated to the nucleus, but not bound to DNA.

The pellet obtained after separation of the nucleoplasmic fraction, was extracted overnight at 4 °C with absolute ethanol, then centrifuged at 1200 *g* for 20 min and the extracted radioactivity was measured and expressed as disintegrations per minute per milligram of pelleted DNA (dpm/mg of DNA).

The protein contents in cytosol and nucleoplasmic fraction were determined by the Lowry[13] method, while the DNA content in nuclear pellet was determined by the diphenylamine method[14].

RESULTS AND DISCUSSION

The uterotrophic and antiuterotrophic assay[9] (Table I) showed that the substances tested (**1**–**4**, Fig. 1) exhibited an almost total loss of estrogenic activity, while compounds **1** and **4** even prevented the action of endogenous estrogens. On the other hand, compounds **2**, **3** and **4** partially hindered the action of estradiol benzoate, behaving as moderate antagonists[8b]. The results of the uterotrophic assay are presented in Table I.

It is well known that the free 3-hydroxy function in estrogen molecule is crucial for receptor binding[15]. However, in *in vivo* experiments 3-benzyloxy-17-hydroxy-secoestratriene derivative **2** showed higher antiestrogenic activity than its 3-hydroxy analogue. This evidence prompted us to study ER binding of 3-benzyloxy derivatives (**1** and **2**), as well as of their hydroxy analogs (**3** and **4**).

The study of ER interaction with compounds **1**–**4** included analyses of their effects on the binding of radioactive estradiol to the receptors, i.e. the formation of compound–ER complexes in cytosol, the influence of the compound tested on the stability of the ^{3}HEd–ER complexes, as well as their translocation to the nuclei and binding to DNA.

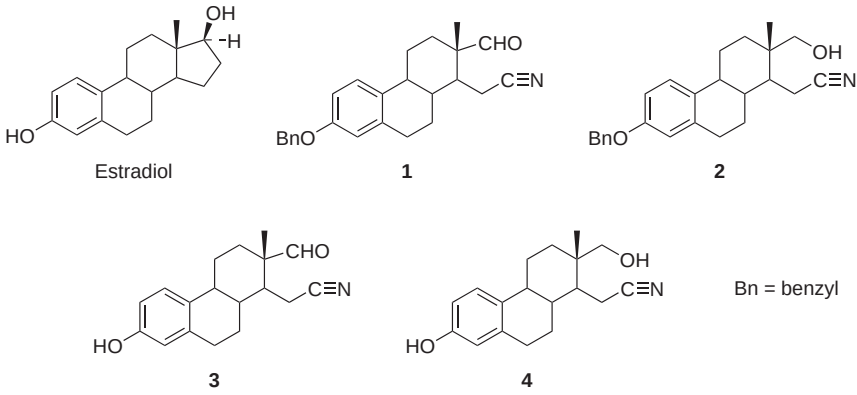


FIG. 1
The structures of estradiol and the tested compounds

Competitive binding of the tested compounds to ERs was analysed by determination of differences in the of ^{3}HEd–ER complex amount, formed in the absence or presence of the tested substances. DES was used as standard estradiol competitor in binding to receptor in order to determine non-specific binding (NSB). The difference between the total (T) and NSB represents the specific binding (SB).

The interaction of ER with ^{3}HEd in the presence of the tested substances was studied using the previously mentioned method[9]. Namely, it is well known that the compounds which increase the K_d value of the ^{3}HEd–ER complex compete with ^{3}HEd for the specific binding sites in uterine cytosol, i.e. decrease the binding of ^{3}HEd to ERs. The increase in K_d could be also caused by binding of the tested compound to ER, not to the hormone binding domain, but to an allosteric site, causing conformational changes of ER. As a consequence of these changes, the number of binding sites (N) could also decrease. The dissociation constant (K_d) and the binding capacity of the estrogen receptor (N), were determined by the method of Scatchard. K_d and N for the ^{3}HEd–ER complex, formed in the absence or presence of competitors are given in Figs 2 and 3, respectively. The mean value of K_d for ^{3}HEd–ER complex was 2.61 ± 0.74 nmol/dm^3; it is slightly higher than the same from the literature[16].

As it can be seen in Fig. 2, compounds **2**, **3** and **4** significantly increase the K_d of the ^{3}HEd–ER complex, while compound **1** only slightly decreases the receptor affinity. This shows that there is a weaker binding affinity of ^{3}HEd to ERs in the presence of these molecules. In the same time, compounds **3** and **4** decreased the number of binding sites on receptors in cytosol. The observed changes in K_d could be the reason for their higher influence on the binding affinity of estrogen to ER. On the contrary, compounds **1** and **2**, increased N compared with the control value (Fig. 3).

TABLE I
Agonistic and antagonistic effects of compounds **1**–**4** (at 5 mg/kg)

Compound	Agonistic effect, %	Antagonistic effect, %
1	–10.35	0.42
2	0.71	31.47
3	4.13	15.96
4	–2.06	21.07

The decrease in specific radioactivity of the ^{3}HEd bound to cytosol receptors in the presence of a 1000-fold higher concentration of potential competitors is proportional to the competitive ability of the tested substances. The percentages of SA in the non-activated (4 °C) vs thermally activated (25 °C) cytosolic ^{3}HEd–ER complexes formed in the presence of analysed compounds are shown in Fig. 4.

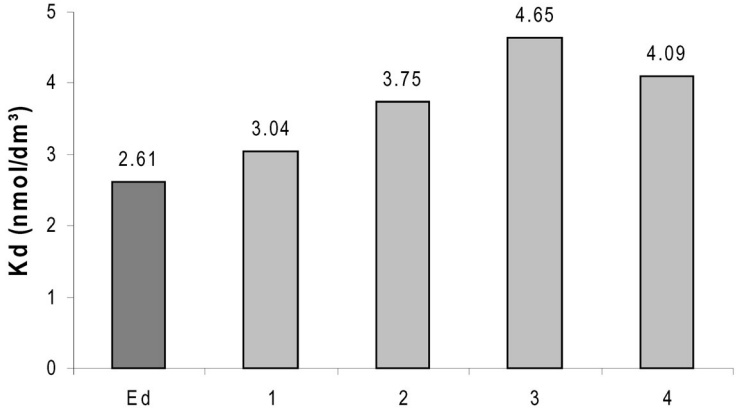


FIG. 2
The values of dissociation constant (K_d) in ^{3}HEd–ER complex, formed in the presence of the tested compounds

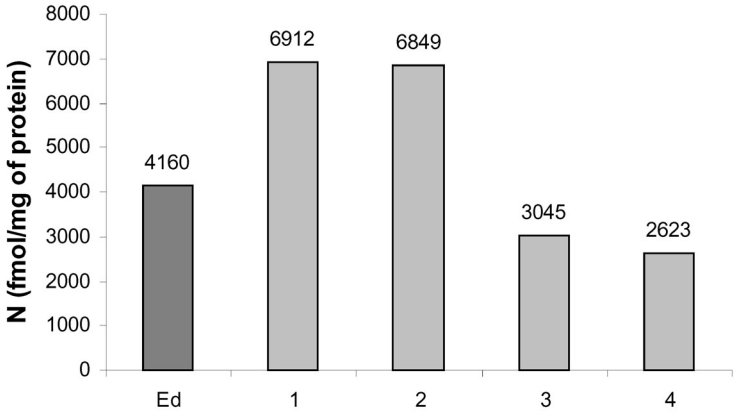
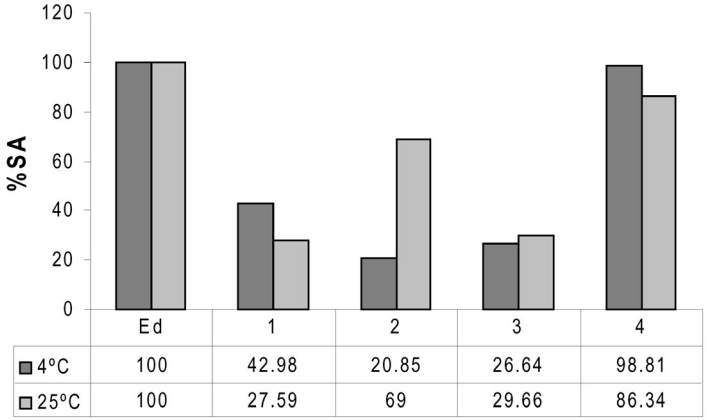


FIG. 3
The binding sites number (N) in ^{3}HEd–ER complex, formed in the presence of the tested compounds

It is obvious that compounds **1**, **2** and **3** significantly reduce the specific binding of ^{3}HEd to ERs at low temperature (4 °C). The same trend was observed in the case of thermally activated complexes (25 °C), although SA was higher than in the case of non-activated complexes formed in the presence of compound **2**. This evidence indicate different thermal stability of formed complexes under influence of analysed estrogen compounds. Interestingly, compound **4** did not influence significantly either binding capacity of ER or the stability of formed complexes, although in Scatchard analysis this compound showed quite a competitive property.

A certain amount of the ^{3}HEd–ER complex, formed in cytosol, translocates to nucleus and binds to DNA, influencing the synthesis of mRNA and corresponding proteins. The various amount of estradiol specifically bound to cytosol receptor, i.e. the changes in binding parameters of ER, that occur in the presence of certain analysed compounds, indicates different influence of the tested substances on ER.

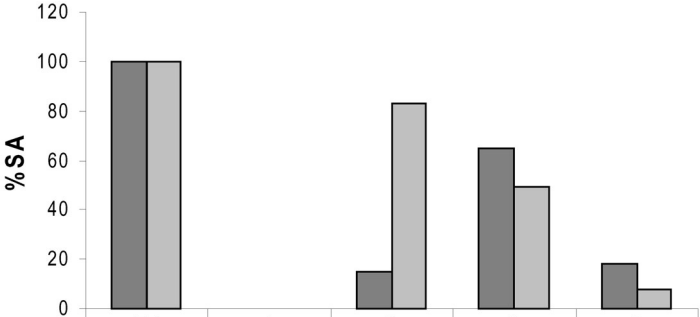
Except the formation of ER complex, the substances tested could potentially influence the nuclear translocation of the formed complexes or their binding to DNA. Figure 5 shows specific binding data of the complexes translocated to the nuclei, but not bound to DNA, while Fig. 6 shows SA data of the complexes bound to DNA, where specific binding is expressed in percentage of binding ^{3}HEd–ER complexes formed in the absence of analysed compounds.



	Ed	1	2	3	4
4°C	100	42.98	20.85	26.64	98.81
25°C	100	27.59	69	29.66	86.34

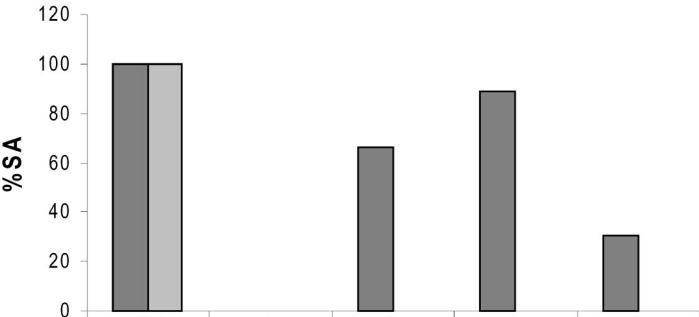
FIG. 4
Specific activity (SA) of the cytosolic ^{3}HEd–ER complex, formed in the absence (Ed) or presence of the tested compounds (**1**, **2**, **3** and **4**)

The results presented in Fig. 5 show that translocations to the nuclei of ^{3}HEd–ER complexes formed in the presence of compounds **2** and **4**, not exposed to 25 °C, were much lower than that of the control ^{3}HEd–ER complex, while in the case of compound **3** translocation of the formed complex was slightly decreased. On the other hand, the amount of non-activated complexes formed in the presence of compounds **3** and **4** was higher in the



	Ed	1	2	3	4
4°C	100	0	14.8	65.14	18.17
25°C	100	0	83.22	49.52	7.82

FIG. 5
Specific activity (SA) of the cytosolic ^{3}HEd–ER complex, formed in the absence (Ed) or presence of the tested compounds (**1**, **2**, **3** and **4**), translocated to nuclei, but not bound to DNA



	Ed	1	2	3	4
4°C	100	0	65.89	88.88	30.29
25°C	100	0	0	0	0

FIG. 6
Specific activity (SA) of the cytosolic ^{3}HEd–ER complex, formed in the absence (Ed) or presence of the tested compounds (**1**, **2**, **3** and **4**), translocated to nuclei and bound to DNA

nucleoplasmic fractions kept at 4 °C than in the case of complexes kept at 25 °C. However, in the case of compound **2**, the translocation of activated (i.e. heated) complex was much higher. These observations could be explained by different influences of analysed compounds on stability and activation of ER complexes. Translocation of activated or non-heated radioactive complexes, formed in the presence of compound **1** could not be observed (Fig. 5).

However, it is clear from Fig. 6 that the DNA binding of non-heated complexes formed in the presence of compound **4** is much lower, of **2** medium and of **3** only a little lower, compared with the complexes formed in the absence of competitor. DNA binding of both thermally activated complexes, as well as non-heated complex, formed in the presence of compound **1** could not be observed. The presented evidence is in agreement with results shown in Fig. 5, indicating a different influence of analysed compounds on functional properties of ER.

Data obtained by investigation of the ^{3}HEd interactions with cytosolic ERs, in the absence or presence of the studied estrogen derivatives, revealed the competing ability of the substances tested. All of analysed compounds more or less influence the interaction of estrogen with specific receptor. However, only compounds **3** and **4** decrease the binding capacity of ER, while in samples of the complexes formed in the presence of compounds **1** and **2**, binding capacity was even higher than that in the control ones.

The results of this study point out the fact that the compounds tested more or less bind to ERs as well as cause changes in the ^{3}HEd–ER complex stability and/or activity.

The effects of the tested compounds on binding of ^{3}HEd to ERs are confirmed by the data obtained by analysis of the formation, translocation and binding to DNA of ^{3}HEd–ER complexes, formed in the absence or presence of these compounds.

The results presented in Figs 4, 5 and 6 indicate that all the analysed compounds showed a competing ability, which manifests itself in different ways for different analysed structures. For example, compound **3** significantly decreases ^{3}HEd binding to receptors in cytosol, but does not effect significantly on nuclear translocation of the formed complexes. Compound **1**, a 3-benzyloxy analogue of compound **3**, decreases specific binding of estradiol in cytosol (Fig. 4), while the nuclear translocation of the ^{3}HEd–ER complexes formed in its presence could not be detected (Figs 5 and 6).

On the other hand, compound **4** did not significantly influence the ^{3}HEd–ER complex formation (Fig. 4), but reduced its nuclear translocation (Fig. 5) and binding to DNA (Fig. 6). Compound **2**, a 3-benzyloxy analogue

of compound **4**, inhibited the ^{3}HEd–ER complex formation in a great deal (Fig. 4), its nuclear translocation occurred after thermal activation (Fig. 5), as well as DNA binding, but this compound forms quite an unstable complex with ERs, which dissociates at higher temperatures, making ^{3}HEd binding to ERs possible.

Compound **2** inhibited the ^{3}HEd–ER complex formation at low temperature in a great deal (Fig. 4) and its nuclear translocation occurred especially after thermal activation (Fig. 5), as well as DNA binding (Fig. 6), but this compound forms quite unstable complex with ERs.

Results of the uterotrophic effects of analysed compounds are in agreement with their effects on binding capacity of ER. Compounds, which show higher antiestrogenic activity, predominantly increase K_d of ^{3}HEd–ER. However, compound **1**, which reduces significantly the formation of ^{3}HEd–ER complex (Fig. 4) shows no antiestrogenic effect in *in vivo* experiments.

Comparing binding data of 3-hydroxy and 3-benzyloxy analogues, it could be concluded that 3-hydroxy derivatives are more potent than the 3-benzyloxy derivatives. This fact could be explained by decreased activity of proteases, which are inhibited in *in vitro* experiments (proteases inhibitor was added during the experiment), so animal etherases could only slowly deprotect the OH function. Another explanation comprises the capability of the 3-benzyloxy function of binding to an allosteric site of ER via hydrophobic interaction.

Further, a comparison of biological effects of 17-aldehydes **1** and **3** with the biological response of the appropriate 17-hydroxy compounds **2** and **4**, indicates that hydroxy function causes higher effects on estrogen receptor under *in vitro* conditions, as well as biological response *in vivo*. Compounds **2** and **4** have OH group at position 17, as well as estradiol does, but in estradiol it is bound to the rigid ring system and β-oriented, while in compounds **2** and **4** it is bound to the steroid moiety over two flexible single bonds and the substituents have different orientation in comparison with the 17β-OH group of estradiol[8c]. This could be the reason for their different biological responses.

Further syntheses, directed towards obtaining compounds with higher antagonistic effect, as well as testing of their biological activity are in progress.

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