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Approaches for Searching of Modified Steroid Estrogen Analogues with Improved Biological Properties

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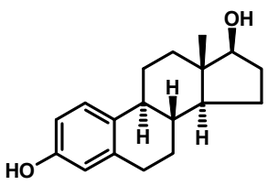
1. Introduction

Estrogens play a key role in multiple physiological functions in women. They have important actions on bone and lipid metabolism, cardiovascular function, and diffuse effects on other target organs. Estrogens have important roles in cognitive function and influence psychological well-being in women, in development and maintenance of the reproductive system in the female **(Gustafsson, 2003).** The most potent estrogens are 17β-estradiol **1,** estrone **2** and estriol **3** may have tissue-specific roles (**Gruber & Huber, 1999**).



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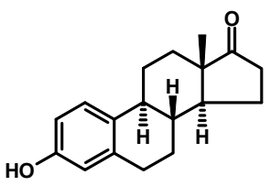

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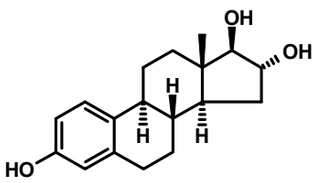

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In female body estrogens are formed in ovaries, and as result the menopause is associated with an increased risk of the development of cardiovascular diseases, osteoporosis and many other diseases. In postmenopausal women, many of these functions (positive effect on prevention of osteoporosis and improved serum profile enhanced by the use of estrogen replacement therapy) are achievement. The positive action of estrogens on prophylactic and treatment of osteoporosis has been proved undoubtedly, however other clinical data are considered as contradictory (**Davison & Davis, 2003**). Moreover, during the use of estrogens for HRT the number of strokes is increasing (**Bushnell, 2005**). It was absolutely unexpected because the adverse effect has been observed in experiments on animals. It was also found that during long-term using of estrogens the risk of some oncological diseases is increasing (**Beral, 2003; Beral et al., 2005**). Other side effects, which we discuss later, also indicate the necessity for the search of new safe estrogen analogues with the improved biological properties.

Firstly we consider the results in these directions, and then we discuss the main advantages of the creation of agents with directed biological action, which are perspective for HRT, for

the treatment of oncological estrogen-sensitive diseases, cardio-vascular system, osteoporosis, neuroendocrinal diseases. The division of agents on such groups has relative character, because the activity of steroid estrogens has multifunctional character and one compound may be effectively used in various fields. This is a main reason why modified steroid estrogens have the advantage in comparison with huge number of heterocyclic compounds, having more selective action.

Obviously that most perspective search of such compounds is done on the basis of knowledge about estrogen action mechanism, particularity of its structure and metabolism.

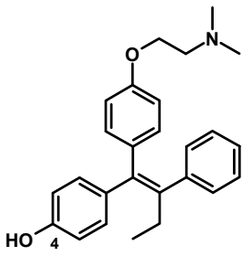
Several authors consider that main fast non genomic effects of estrogens are mediated by their membrane receptors (**Levin, 2002; Sak & Evaraus, 2004).** Experimental data about the identity of nuclear and membrane ERα in MCF7 cell are presented in the publication (**Pedram et al., 2009**). Obviously, ligand specificity may be significantly different. Mechanisms of membrane receptors action are still unclear, very often it is not proved that one or another effect is mediated by namely this group of receptors (**Warner & Gustafsson, 2006**). The absence of data about ligand specificity to membrane receptors does not allow to plan the synthesis of modified steroids with selective action, especially taking into account that their activity is realizing on many ways. During the consideration of osteoprotective action of estrogens and its influence on processes of cardio-vascular system we restrict ourselves to state the facts, because the decision concerning the synthesis of potential agents is usually reached on the analogy with known compounds.

2. Genomic actions of estrogens

The genomic effects of steroid estrogens are mediated through two subtypes of nuclear α- and β-receptors (ERα and ERβ). In mid 1980s. studies on the cloning of DNA encoding the steroid estrogen receptors were initiated (**Walter et al., 1986; Green, G.L. et al., 1986; Koike et al., 1987).** These studies led to the determination of their primary structure. Later they were assigned to ERα. In 1996, a number researchers discovered new members of the superfamily of nuclear estrogen receptors in rat prostate and ovaries (**Kuipper et al, 1996**) in human and mouse (**Mosselman et al., 1996**; **Tremblay et al., 1997)** and named it ERβ. Very soon the complete amino acid sequence of human ERβ (hERβ) was determinated (**Ogawa et al., 1998**). It was established that ERβ is significantly shorter than ERα. The comparison of amino acid sequences of these receptors and the investigation of affinity of various ligands to mutant forms of receptors allowed to establish that these receptors have 6 domains.

The N-terminal domains (A/B) have variable length and amino acid sequences. Usually, they exhibit a hormone-independent transactivation function (AF-1) that interacts with the elements of the transcription machinery and activate genes in target organs (**Tzukerman et al., 1994).** Domain C is responsible for the DNA recognition and receptor dimerization. This is a DNA-binding region, consisting approximately 50 amino acids. Domain E (HBD) contains approximately 250 amino acids and ensures the binding of hormones. This domain is the «hinge» - after the binding of ligand with receptors the following conformational rearrangement takes place with the participation of domain D. Clone 29 protein is highly homologous to rat ERα, particularly in DNA-binding domain (95%) and ligand-binding domain (55%) (**Kuipper et al., 1996**). High degree of conservation of DNA-binding domain (96%) and ligand-binding domain (58%) was registered by other authors (**Mosselman et al., 1996).**

Ligand-independent transactivational function AF1 is in zone A/B of both sub-types of receptors, zone E/F of α-receptor of estrogens has additional ligand-independent activation function (**Tora et al., 1989**). ERα has ligand-dependent trans-activation function AF-2.
Without ligand estrogen receptors are in complexes with heat shock proteins (Hsp90, Hsp70, Hsp56, Hsp60, Hsp48, Hsp23), this binding takes place in domain C. Probably, being in complexes with heat shock proteins estrogens are protected against the action of proteases. After binding its ligand conformational rearrangement of LBD of nuclear receptor and dissociation of complexes ER – heat shock proteins takes place. Except of the potential possibility to activate transcription, this rearrangement causes the change of topography of receptor regions, sensitive to proteolysis (**Ramsey & Klinge, 2001).** Phosphorylation of estrogen receptors is observed after their binding with hormone (**Kato et al., 1995)** that enhances the binding.
Transformed receptor forms dimers and in this form binds with DNA and may activate the transcription. Effective dimerization of ERα requires a weak constitutive activity of sequences in domain C (**Kumar & Chambon, 1988).** It is necessary to note, that AF1 and AF2 exhibit relatively weak activity, whereas the maximum of transcription induction is observed when they act together (**Tora et al., 1989; Pham et al., 1992)**. From other side, in some cases the activation of AF-1 is enough to activate the transcription. Thus, 4-hydroxytamoxifen **4** is unable to induce AF-2 activity, but it is a strong agonist in cellular and promoter context where AF-1 is effective transcriptional activator (**Berry et al., 1990**).



4-hydroxytamoxifen

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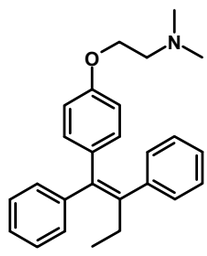

tamoxifen

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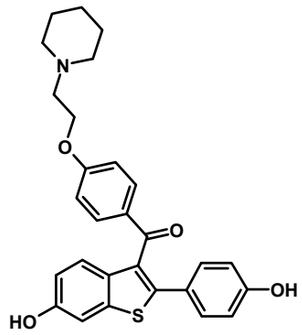


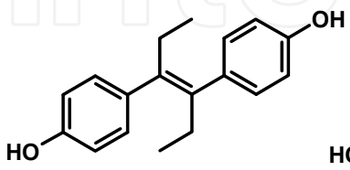
raloxifene

6

Transcriptional process is modulated by receptors' ligands and various co-regulators **(Cheskis et al., 1997; An et al., 1999; 2001; Tcherepanova et al., 2000; Wong et al., 2001; Liu, J. et al., 2003; Bai & Gugière, 2003; Xu & Li, 2003)**. Conformation of ER changes upon interaction with coactivator proteins **(Tamrazi et al., 2005)**, as result the activity of one ligand may change in depending on cell nature. Thus, tamoxifen **5** and raloxifene **6** are agonists of estradiol in cardiovascular system, whereas they show antagonistic properties in breast and endometrium **(Jordan, 2007)**. Many others SERMs have similar properties.
Nowadays more 10 estrogen receptor-β isoforms are known, which have been identified starting from 2000 (**Lu et al., 2000).**
To study the roles of each receptor *in vivo*, a series of the mice were generated lacking either a functional ERα and ERβ or both (αERKO, βERKO, αβERKO) (**Emmel & Korach, 2001).**
ERβ may modulate the functions of ERα, if these receptors are in the same cells (**Matthews et al., 2006**). It became crucial during the diagnostics and the treatment of oncological

diseases (**Leygue et al., 1998; Pujol et al., 1998; Hall & McDonnel, 1999; Lazennec et al., 2001; Monroe et al., 2005)**. Compounds having the preferential binding to ERβ have great perspectives to be used for the treatment of autoimmune diseases, prostate disease, depression and ovulation disorders (**Gustafsson, 2005**). The noted above is the evidence for actuality of the search for modulators with preferable affinity to ERα or ERβ.

Obviously, determination of complexes structure of estrogen receptors with various ligands with known biological properties was supposed to contribute to the development of models for mechanisms of estrogens action and understanding of the connection between structure and hormonal activity of ligands and thus to solve the abovementioned problem.



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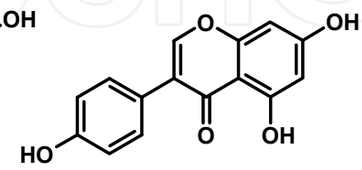

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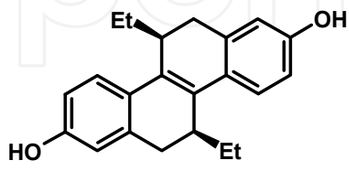

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X-rays data of ligand-binding domain of ERα with estradiol **1** and raloxifene **6** (**Brzozowski et al., 1997**), 4-hydroxytamoxifen **4** and diethylstilbestrole **7** (**Shiau et al., 1998**), and ligand-binding domain of ERβ with raloxifene **6** and genistein **8** (**Pike et al., 1999**) have been obtained. Estradiol **1** and diethylstilbestrol **7** are full agonists to ERα and ERβ, 4-hydroxytamoxifen **4,** raloxifene **6**, and genistein **8** are agonists/antagonists. Interestingly, synthetic compounds of type **9** bind with ERβ as effectively as genistein **8** (**Miller et al., 2003**). Removal of ligands **1, 4**, **6**, **7, 8** from its complexes in crystal with LBD of ERα and ERβ, docking of other potential ligands and the following optimization of its position in complex and binding energy by molecular modeling methods allow to evaluate the properties of new compounds independently from their belonging to steroid series. Some vagueness of evaluation of affinity to receptors is connected with the fact that the X-Ray data for complexes with different ligands are obtained only with LBD, but not with full-length receptors.

The last of utmost importance, because during the realization of transcriptional action the formation of complexes between the DNA binding domain of estrogen receptors and estrogen response element (ERE) of duplex DNA is necessary, which requires the exact disposition of these elements of structure. Earlier structure of the DNA-binding domain of the ERα has been investigated (**Schwabe et al., 1990**), however three-dimensional model of the DNA binding domain of ERα was proposed significantly later (**Deegan et al., 2010**). On account of the above mentioned the differences in RBA values of model compounds to full-length receptor and to its LBD could be significant. Thus, the affinity of (5S,11S)-5,11-diethyl-5,6,11,12-tetrahydrochrisene-2,8-diol **9** to full-length ERβ is in 10 times higher, than to its LBD (**Meyers et al., 1999**).

The results of quantum chemical calculations of ligand-receptor complexes allowed to synthesize a huge number of compounds having more then in 100-times higher effective binding to β-ER in comparison to α-ER (**Manas et al., 2004).**

There are other ways for evaluation of properties of new compounds. For example some of them are traditional QSAR methods (**Gantchev et al., 1994; Tong et. al., 1997; Wiese et. al, 1997; Azzaoui et al., 1998; Gao et al., 1999; Wurtz et al., 1998; Sippl, 2002; Klopman &**

Chakravarti, 2003; Wolohan & Reichert, 2004; Pasha et al., 2005). In spite of the fact that such approaches have moderate predicted force, they do not provide the deeper understanding of estrogen action mechanism. From our point of view, using the multimodal approach, based on the application of methods of comparative molecular field analysis and binding energy calculations is most perspective (**Wolohan & Reichert, 2004).**

The knowledge about conformational mobility of different groups of potential ligands of ERs has great importance (**Kym et al., 1993; Grese et al., 1998; Selivanov & Shavva, 2002).** These data have interest in connection with data about modeling of conformations of ligand-receptor complexes (**Kraichely et al., 2000; Egner et al., 2001**) and conformational dynamic of ERs (**Celic et al., 2007**).

Let's consider the examples of successful searching of modified estrogens which have selective binding affinity to hERα or hERβ (**Hillisch et al, 2004**).

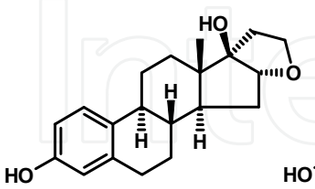
Structure analysis of LBD of these receptors shows that the nearest surrounding of bound estradiol in these receptors differs from each other on two amino acid residues: Leu384 and Met421 in ERα are substituted by Met336 and Ile373 in ERβ. Volumes of side chains of these amino acid residues are 85.9 Å (Met), 82.6 Å (Leu) and 82.3 Å (Ile). It was predicted that the increased flexibility of the linear Met side chain would allow larger substituent to be accommodated. Met336 ERβ is situated closely to position 8β, and the introduction of small lipophilic substituent into this position must lead to the increasing of RBA of modified analogue to ERβ. Met421 of ERα is at region of D-ring, and analogue with substituent at positions 16α and/or 17α will have the increased affinity to ERα.

Experiments have proved the stated predictions. As reference compounds steroids **10** and **12** have been used. First one has selectivity hERα/hERβ is 20, and for second one this value hERβ/hERα is 22.5. Modified analogues **11** and **13** have the mentioned ratios as 70 and 180 correspondingly.

Unfortunately, there is no correlation between RBA values for hERα and hERβ with values obtained in the experiments with the corresponding receptors of animals.

In some cases the ratio between these values are opposite (**Hillisch et al., 2004**).

The investigation of RBA for 71 compounds (mainly, metabolites of estrogens) to hERα and hERβ have shown that the differences in experimental values are not more then one order (**Zhu et al., 2006**).



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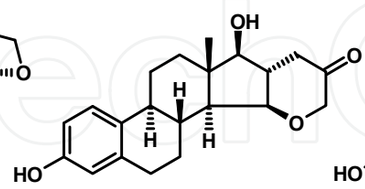

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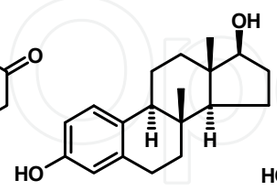

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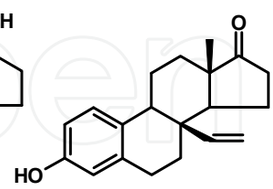

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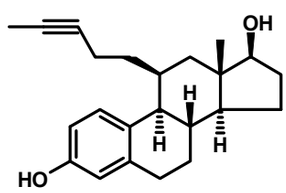

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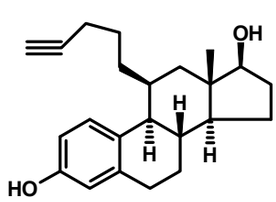

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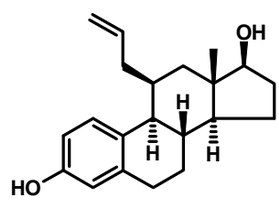
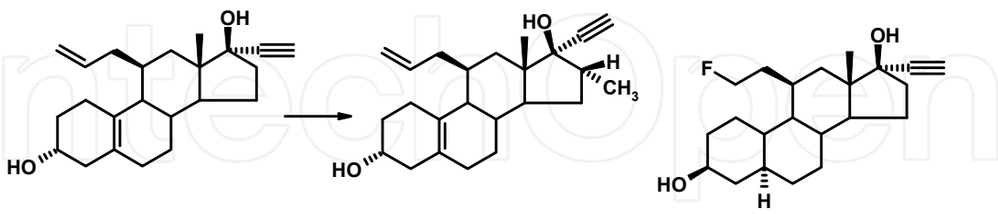


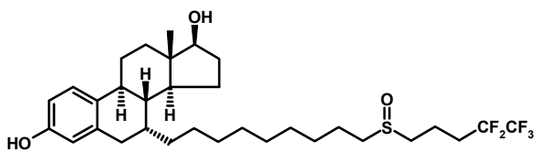
16

The introduction of relatively small linear substituents (4-5 carbon atoms) at position 11β of steroid skeleton may lead to the appearance of antagonistic activity to ERβ at agonistic properties to ERα (steroids **14** and **15**), whereas analogue **16** is agonist to both receptors (**Loosen, 1999**).



17 **18** **19**

Steroids with small substituents at position 11β without aromatic ring possess significantly higher affinity and transcriptional activity of ERα (**Loosen et al., 2000**), for example, analogues **17-19** have been found**.**



ICI 182,780 (fulvestrant)

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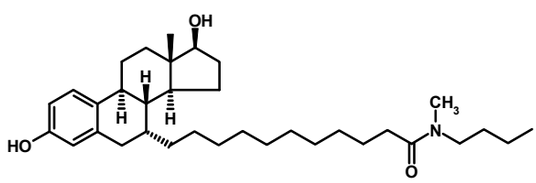

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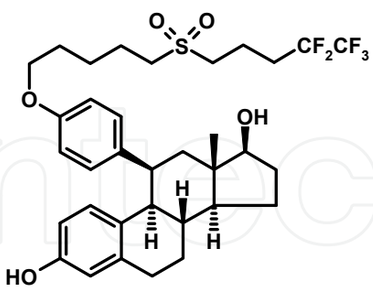

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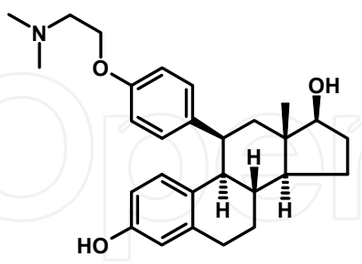


RU 39,411

23

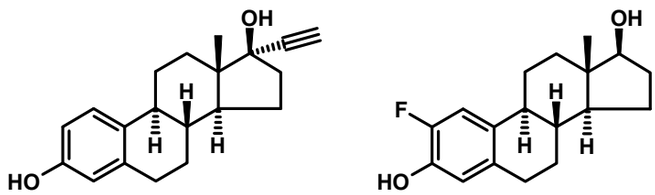
Finally, there are compounds, which fully block the binding of estradiol **1** with known ERs, for example steroids **20-23** (**Jordan, 2003**). A number of compounds with large substituents at C-7 (α) and C-11 (β) have been synthesized, such analogues are of great interest for the treatment of estrogen-dependent oncological diseases and hormones imbalance, caused by the increased formation of estrogens in the body.

3. Carcinogenicity causes of ER ligands

The establishment of at least major reasons of carcinogenicity of estrogens, their agonists/antagonists has significant importance, since this understandings lies in the fundament of the creation of medications with the improved properties.

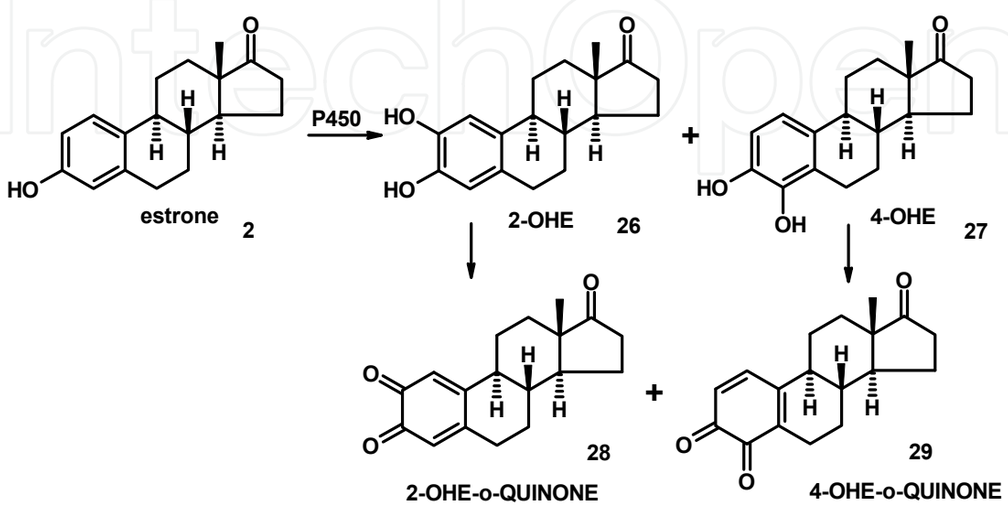
At the present time two main types of carcinogenesis are known - promotive and genotoxic (mutagenic), action of them may add up. Existence of the fist one is confirmed by well-known facts of tumor formation under the estrogen action (**Russo et al., 2002**) and promotion of tumor induction under the action of various agents, for example, nitroso-butylurea (**Sumi et al., 1984**). The same results have been obtained in other models. For example, 17α-ethynylestradiol **24** enhances the tumor formation in rat males under the action of diethylnitrosamine (**Yoshida & Fukunishi, 1981).** Estradiol increased dysplasia of breast on androgenized rats, induced by 7,12-dimethylbenz[a]anthracene.

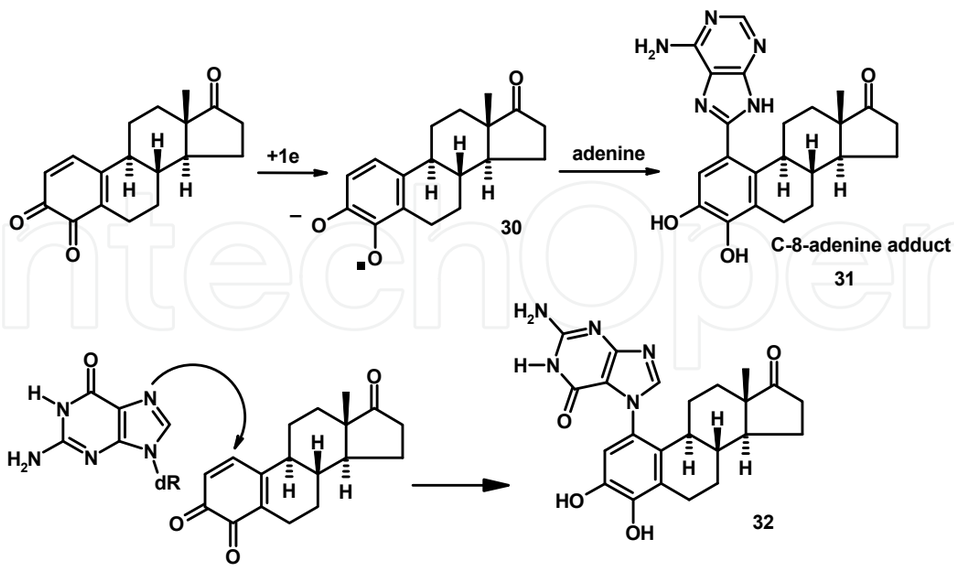
The explanation of this negative influence of estrogens is quite clear – when in active state the cells are vulnerable to attacks by reactive compounds.



24 **25**

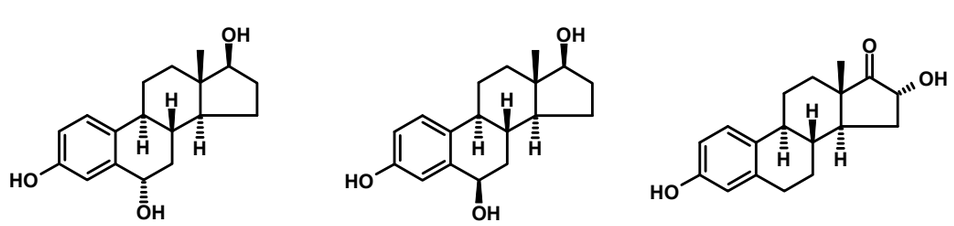
Lately using 2-fluoroestradiol **25** as an example it was shown that there is no correlation between carcinogenicity and hormonal action of estrogens (**Liehr, 1983**). It is important that 17α-ethynylestradiol **24** possesses high hormonal activity and lowered carcinogenicity (**Li, J.J. et al., 1995**). These investigations (and earlier ones) became the basis for searching of other mechanisms of estrogens' carcinogenicity.



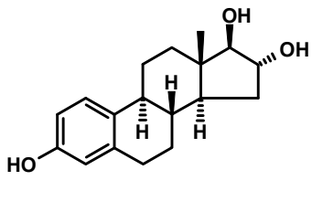


Specifically, it was established that estrogen metabolites may covalently bind with hamster microsomic proteins, and this process is inhibited by ascorbinic and cathehol-O-methytransferase action (**Haaf et al., 1987**). Therefore authors proposed that «active» metabolites are o-quinones, which possess heightened reactivity. In next 20 years it was clarified that the hydroxylation of estrogens in the body leads to the formation of 2- or 4-hydroxyestrogens of types **26** and **27**, which then converted to o-quinones of type **28** and **29** (**Liehr et al., 1995; 1996; Bolton et al., 1998; Bolton, 2002; Bolton & Thatcher, 2008; Zhang, F. et al., 1999; Liu, X. et al., 2002; Zhang, Q. et al., 2008).** Irreversible depurination of DNA that could not be regenerated by reparases may take place under the action of o-quinones. Compounds of types **31** and **32** are possible depurination products**.** Quinones of type **28** do not cause the damage of DNA. It is assumed that the reason of this is their heightened reactivity; therefore they may be deactivated before their migration into cell nucleus.

From our point of view one of possible variants for the decreasing of estrogens' carcinogenicity is the introduction of a substituent in the position 1, which blocks the interaction with DNA and thus prevents its depurination. In the case when compounds with substituent at position 1 will lead to decreasing of estrogenic activity, such modification (in dependence from the task) will be very perspective.



33 **34** **35**



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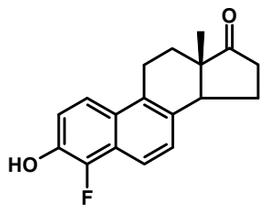

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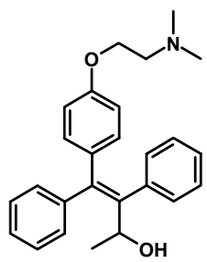
It is also known that metabolites with hydroxyl group at C-6 of types **33** and **34** may have carcinogenic properties (**Itoh et al., 1998**). Thus, compounds which can not be hydroxylated at position 6 are perspective potential candidates for synthesis and further investigations.

Another metabolite of estrogens - 16α-hydroxyestrone **35** is also considered as inductor of tumors' formation (**Lewis et al., 2001; Seeger et al., 2006).** Hydroxylation of estrone at position 16α is one of main directions of estrogen metabolism, which is elevated in women with high risk of breast cancer, as well with other oncological diseases. Estriol **3** content is informative as well: there is the correlation between breast tumors' development in mice (**Lewis et al., 2001**).

Products of hydroxylation of equilenin and equilin also possess carcinogenic properties (**Zhang, F. et al., 1999; Shen et al., 1997).** Corresponding quinones may damage DNA, and the investigation of this class of compounds with substituent at position 4 is of a great importance. Authors concluded that 4-fluoroequilenin derivatives **36** are promising alternatives to traditional estrogen replacement therapy due to their similar estrogenic properties with less overall toxicity (**Liu, X. et al., 2003**).



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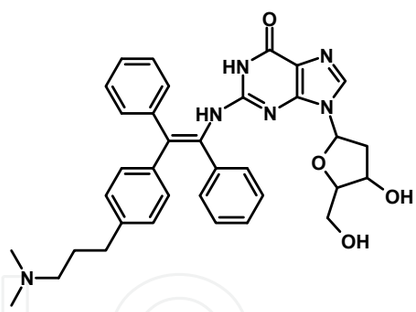


38

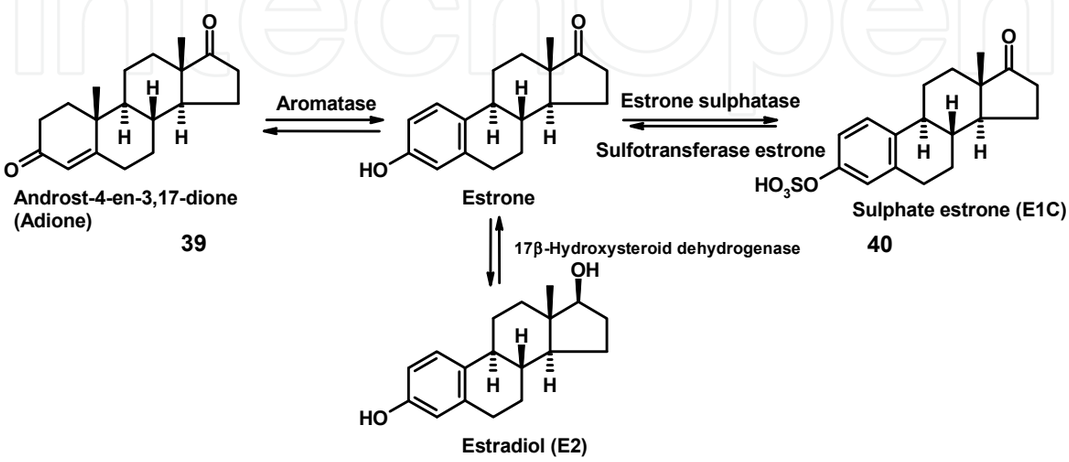
The hydroxylation of selective modulators of ERs and the following reaction products transformations may cause DNA depurination that leads to carcinogenicity of drugs. It was shown on the example of the formation of α-hydroxytamoxifen **37** and its possible transformation into compounds of type **38** (**Jordan, 2007**).

It is not surprising that during last years the efforts are directed to the methods of steroid estrogen level determination, because the exact value of their content in various tissues helps to diagnose differentially various hormone-sensitive diseases (**Arai et al., 2010; Blair et al., 2010**).

4. Inhibition of estrone formation in the body

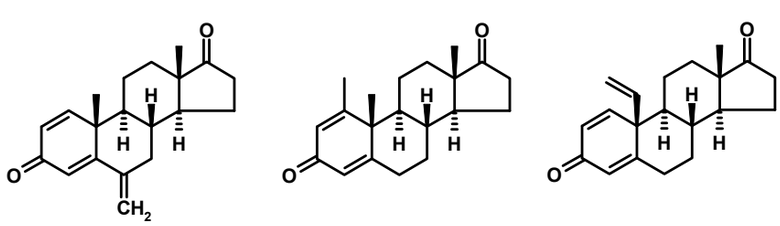
It is known that about two thirds of all the breast cancers occur in postmenopausal women, when estrogen is no longer synthesized in ovaries. However the estradiol content in

estrogen-dependent tumors is significantly higher than in blood plasma and in normal tissues on bourdes with tumors. Partly it can be explained by the ability of tumor to accept estradiol from the blood **(Pasqualini et al., 1996**), because estrogen synthesis may be enhanced in extragonadal organs. Furthermore, local synthesis of estrogens in tumor may be on high level (**Pasqualini et al., 199**7). In view of the aforesaid it is necessary to consider the ways of synthesis of these hormones and understand the influence on the formation of reactive estrogens in tumor (scheme).

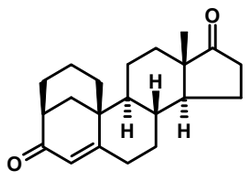
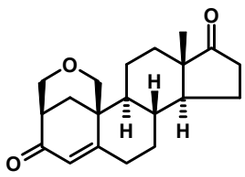
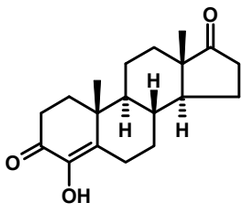


It is well-known, that estrogens are formed in the body from testosterone or androst-4-en-3,17-dione **39**. Aromatization is multistep process, proceeding under the action of enzymes of cytochrome P-450 system. The main steps of aromatization, key stage in estrogen formation are considered in many publications (**Jordan & Brodie, 2007** and citations herein). It is reasonable that the great attention is directed to the search of irreversible inhibitors of aromatase.

Most often the initial investigations are carrying out on human placenta cells, the criterion for the evaluation of effectiveness of investigated compounds is value of IC_{50} (concentration, at which the inhibition of enzyme activity is 50% from highest possible one under experimental conditions). Compounds having IC_{50} in the range of nanomolar concentrations have perspectives to be further investigated. Next step for the evaluation of inhibitory activity of perspective compounds is the investigation on animal models, and very often the preliminary evaluation of effectiveness is in contrast with data obtained in second series.



Exemestane **41** **42** **43**

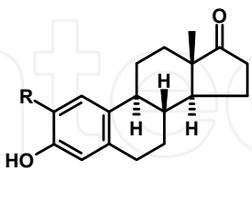
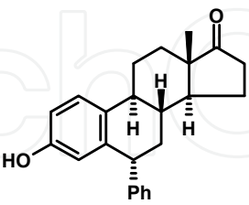
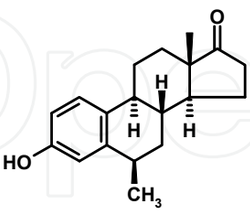




44 **45** **46**

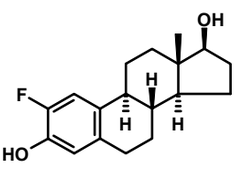
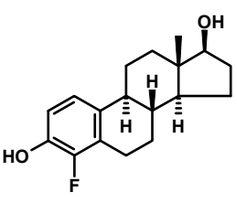
Steroid **41** and **42** properties investigation results could be used as an example (**diSalle & Robinson, 1990**). The IC_{50} values in the experiments on human placenta cells are approximately equal - 42.5±4.3 and 20.3±2.2 nM correspondingly. However, in the experiments on rats the values of ED_{50} (dose, at which the inhibition is 50% from highest possible one) are 3.7 and more 100 mg/kg under *per os* injection. Necessity of compounds investigation can be illustrated by huge number of examples in various animal models and by methods of steroids introduction into the body. In particular, compound **42** under subcutaneous introduction in doses 30 and 150 mg/kg blocks the growth of bearing DMBA-induced mammary tumors in female rats (**Nishino et al., 1989**) and after numerous investigations is clinically used with the trade name atamestane.

The main position for attack during demethylation of androst-4-en-3,17-dione is methyl group at C-19. Numerous derivatives at C-10 have been synthesized. Among them the most effective one is compound **43** with K_i 3 nM to human placenta enzyme (**Cole & Robinson, 1990).** It comes as no surprise, that this compound is clinically used (**Numazava et al., 2005**). Compounds **44** (**Greway et al., 1990**) and **45** (**Peet et al., 1992**) demonstrated good properties.

Detailed search of aromatase inhibitors led to the creation of medications of steroid nature for the treatment of oncological diseases in clinic, from them, exemestane **41** and formestane **46** are also widely known. However clinical application of aromatase inhibitors revealed the presence of a number of non-specific toxic side effects: asthenia, nausea, headache etc. Certain endocrinological side effects in postmenopausal women are notable, namely hot flashes and vaginal dryness (**Goss, 1999**). Therefore the search of aromatase inhibitors in steroid series must be done in series that is not belonging to androgens.

47 a) R=Cl, b) R=Br, c) R=CH_3 **48** **49**

25 **50**

Because estrone **2** or estradiol **1** are formed as result of aromatization process it was possible to assume that these compounds will be reversible inhibitors of aromatase. Indeed, later it was found that even estradiol is anti-aromatase agent in human breast cancer cells (**Pasqualini & Chetrite, 2006);** the same was found for estrone sulfatase inhibition **(Pasqualini & Chetrite, 2001).** Simultaneously, reasoning from the same assumption, the synthesis of modified estrogens with substituents at C-2, C-4 or C-6 was carried out (**Numazava et al., 2005**).

These analogues are competitive inhibitors of aromatase. 2-Substituted steroids **47 a,b,c,** have the best properties, they are significantly active in comparison with estrone **2**, K_i values are 0.130±0.017, 0.224±0.024, 0.360±0.019 and 2.50±0.22 μM correspondingly. Steroids **48** and **49** have similar activity - K_i values are 0.10±0.006 and 0.21±0.009 μM, however their synthesis is more complicated. Inhibitory activity of estradiol derivatives is significantly lower. From our point of view, its worth to pay attention to 2-fluoroestradiol **25,** in spite of the value of K_i 9.04±0.5 μM being higher in comparison to other compounds and the fact, that its synthesis is more complicated. It is more important that this analogue does not possess carcinogenic properties in the experiments on rats (**Liehr, 1983**). In the series of 4-substituted estrone derivatives – 4-fluoroanalogue **50** has the best properties (K_i = 1.28±0.07). All presented investigations had extensive character and it was impossible to take into account many details of biological properties. X-Rays analysis of complex of aromatase from human placenta with androst-4-en-3,17-dione **39** has been obtained (**Ghosh et al., 2009**), that opened the perspectives for the targeted search of aromatase inhibitors. Using the data obtained resulted in the docking of new reversible inhibitors in aromatase structure that allowed to explain their activity (**Yadav et al., 2011**).

Aromatase inhibitors are widely used for the treatment of estrogen-dependent oncological diseases; however they have a number of side-effects. Thus, the decreasing of estrogen content in the body automatically results in the increasing of cholesterol level in blood, and hence to the increased risk of cardiovascular diseases. This is the main reason for the creation of inhibitors on the basis of steroids in comparison with non-steroid ones. For example, exemestane has minimal negative effects on bone and lipid metabolism in animal and clinical studies (**Carpenter & Miller, 2005**). There is an opinion that exemestane may improve lipid profile (**Bundred, 2005**).

Molecular bases of interactions of various groups of ligands with aromatase have been considered relatively not long ago, that may lead to the creation of inhibitors with improved properties (**Hong et. al., 2008**).

Aromatase inhibitors are important, but not sufficient for blocking of growth of hormone-sensitive tumors. Point is that there is at least one more way for estrogens' formation in tumors.

It is known that estrone sulphate concentration in blood plasma of postmenopausal women is in 5-10 times higher in comparison with free hormone concentration. Assumption was made that estrogen sulphates may play a role of "source" of free estrogens in various organs, because its «half-life time» is longer than life-time of free estrogens (**Reed & Potter, 199**9). Estrogen sulphates may migrate into hormone-depended tumor and then converted to free hormones under estrone sulfatase action. It was found that estrone sulfatase activity in tumors is in 130-200 times higher in comparison with aromatase activity (**Chetrite et al., 2000**). This was the basis for initiation of investigations for the search for irreversible estrone sulfatase inhibitors.

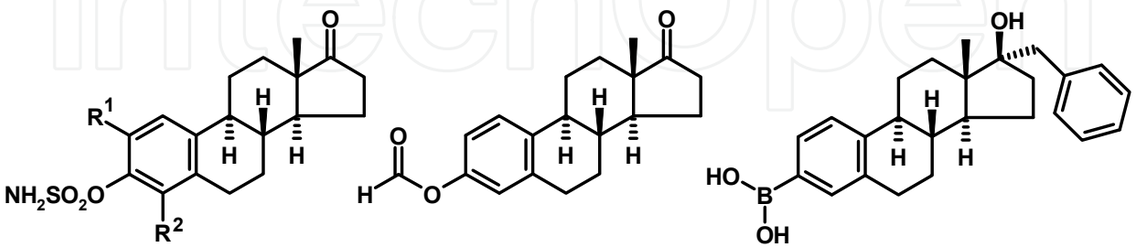
Estrone sulfatase from human placenta is suitable model object for searching for corresponding inhibitors. It is the transmembrane protein that consists of 583 amino acid residues (mainly it is hydrophobic amino acids).

On the basis of data about primary structure of this enzyme and taking into account its homology with human arylsulfatase A and human arylsulfatase B (X-Ray data of arylsulfatase were known), the structure of catalytic centre of estrone sulfatase was proposed (**Howard et al., 2002).** X-Ray data of estrone sulfatase has been obtained a little bit later and it was shown that its tertiary structure is formed by two domains – globular polar domain, containing the active center and transmembrane domain, formed by two antiparallel α-helixes. Polar domain consists of two sub-domains, one of them has the active centre of enzyme. Transmembrane domain opens the entrance into the active center of enzyme, which is situated deep under the "mashrooms hat", close to the membrane surface. Near the active center entrance a big amount of hydrophobic amino acids is concentrated, which are a part of TD (Phe178, 182, 187, 230, 233 and 237), and the polar domain (Phe104 and 557, Leu554). Side chains of these amino acids form a tunnel, which leads to the active center (**Hernandes-Guzman et al., 2003**). Catalytic area of active center of estrone sulfatase is highly homologous to active centers of arylsulfatase A and B. Nine from ten catalytically important amino acids Asp35, Asp36, FG75, Arg79, Lys134, His136, His290, Asp342 and Lys368 are in analogous positions to all three sulfatases. It is in quite correlation with the experimental data about the importance of hydrophobic interactions during the binding of enzyme with inhibitors of steroid series (**Ahmed S., 2001**).

Conceptions concerning estrogen sulfates hydrolysis mechanisms and ferment deactivation are presented in series of articles (**Reed et al., 2005**), where an important role is assigned to an unusual amino acid - formylglycine in 75 position.

Investigation of kinetic parameters of hydrolysis of estrone sulphate and dehydroepiandrosterone sulphate have shown that values of V_{max} for these compounds are correspondingly 9.95 and 1.89 μM/min, values of K_m are 72.75 and 9.59 μM (**Hernandez-Guzman et al., 2001**). These values could be used as standard for the evaluation of substrate specificity of estrone sulfatase of various natures.

In several investigations it was established that for the effective inhibition of estrone sulfatase molecules must have sulphamate group at position 3. Firstly the necessity of this group was demonstrated on the example of steroid **51a**, widely known as EMATE (**Purohit et al., 1995).** However this compound did not reach the clinical application as anti-tumor agent because its estrogenic activity under *per os* introduction is in 5 times higher in comparison with estrone **(Elger et al., 1995**).



51 **52** **53**

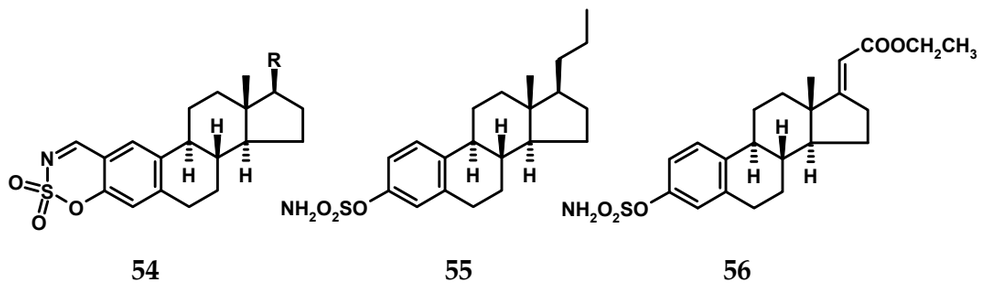
The investigations of Professor M. Reed' group are examples of the successful creation of estrone sulfatase inhibitors with lowered hormonal activity (**Purohit et al., 1998**), authors have synthesized compounds **51a-j**. The selection of modifications in EMATE structure was done on the basis of experimental facts about significant fall of uterotropic activity of

estrone derivatives when substituents like alkyl-, allyl- or nitro- groups were introduced at positions 2 and (or) 4 (**Purohit et al., 1999**). Some results on human placenta microsomes enzyme are presented in the Table 1.

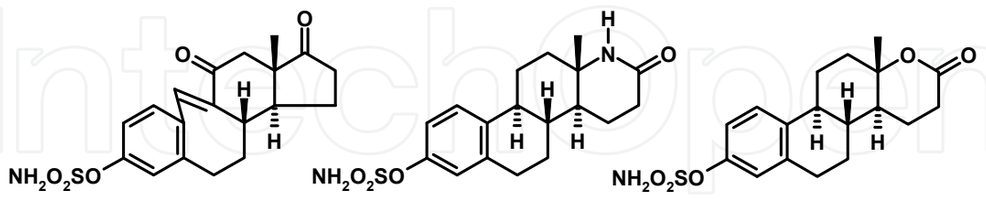
Steroid	R^1	R^2	IC_{50}, µM	Steroid	R^1	R^2	IC_{50}, µM
a	H	H	0.004	f	H	$CH_3(CH_2)_2$	> 100
b	OMe	H	0.03	g	$CH_3(CH_2)_2$	$CH_3(CH_2)_2$	> 100
c	NO_2	H	0.07	h	Allyl	H	2.5
d	H	NO_2	0.0008	i	H	Allyl	9
e	$CH_3(CH_2)_2$	H	29	j	Allyl	Allyl	> 100

Table 1.

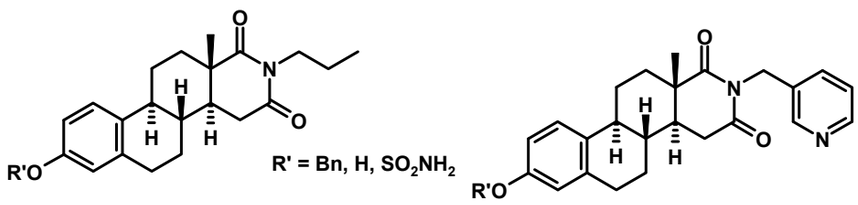
Irreversible E-ST inactivation has been observed in all cases. Most active inhibitor from this series is – 4-nitro derivative **51d** – in 5 times stronger than EMATE *in vitro* and had comparable activity with EMATE activity *in vivo*. The investigation of uterotropic activity has shown that this steroid increases uterus weight of ovariectomized rats, although to lesser degree than EMATE (158% in comparison with control, and 414% - EMATE). Monoallylic analogues are weaker inhibitors estrone sulfatase, but they are more active in comparison with propyl- ones. The presence of large substituents at C-2 and/or C-4 leads to decreasing of inhibitory activity, which may be caused by shielding of oxygen atom at C-3.
Estrone formiate **52** (**Schreiner & Billich, 2004**) and boronic acids **53** (**Ahmed, V., 2006**) have shown irreversible inhibitory activity, however these investigations had no further progress.



54 **55** **56**



57 **58** **59**



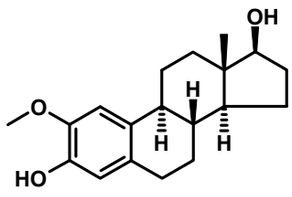
60 **61**

Compounds of type **54** were chosen as an example and authors used another analogue (oxatiazine ring, condensed with A ring) instead of sulphamate group at C-3 **(Peters et al., 2003)**. In the experiments *in vitro* in an intact MCF-7 human breast cancer cells model steroids have shown high inhibitory activity. *In vivo*, agent **54** showed moderate antitumor activity against MCF-7 breast cancer xenografts in BALB/c athimic nude mice. 3,′4′-Oxathiazine analogues showed weak inhibitory activity.

Compounds **55-57** effectively inhibit estrone sulfatase (**Tanabe et al, 1999**). It indicated specifically on the possibility for the creation of inhibitors in the series of D-homo analogues of estrogens that was confirmed using sulphamates **58** and **59** (**Reed & Potter, 1999**).

The development of this direction led to the creation of strong inhibitors **60** and **61** with values of IC_{50} - 1 nM (**Fischer et al., 2003**).

One more group of perspective inhibitors of estrone sulfatase was found – sulphamates of estrogens with methoxy- group at C-2 (**Raobaikady et al., 2003**). At the time of these investigations it was known that 2-methoxyestradiol **62** induces apoptosis in many various lines of tumor' cells, including prostate cancer cells, exhibits antiproliferative activity and blocks angiogenesis processes, which became a basis for the selection of model compounds.



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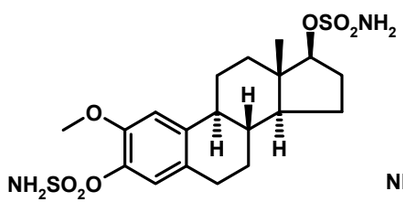

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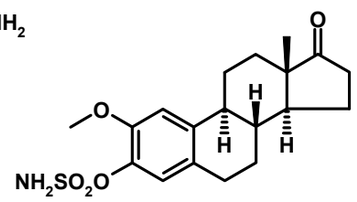

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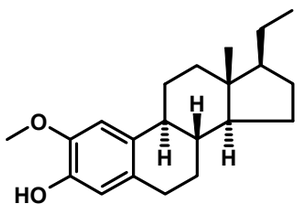

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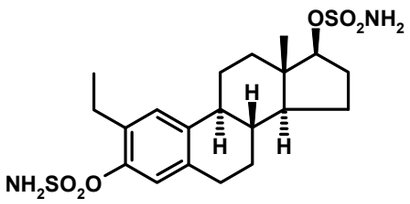

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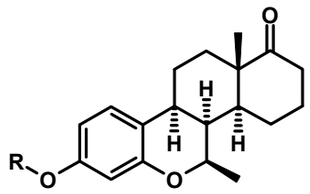


67 a) R = SO_2NH_2, b) R = H

The fact that bis-sulphamates of 2-methoxyestradiol **63** and 2-ethylestradiol **66** inhibit the growth of breast cancer cells, which are resistant to the action of many medications, has a crucial importance **(Suzuki et al., 2003).**

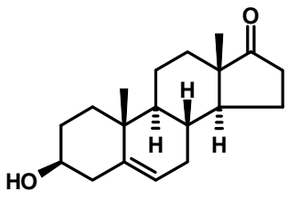
We have considered the ways for the search for modified estrogens for the inhibition of steroids metabolism enzymes which have been done in series of derivatives with natural rings junction. Obvious perspective direction is the investigation of analogues with unnatural rings junction since peculiarities of their structure have the number of important characteristics, comparable with ones in series of natural estrogens and comparable biological properties of both series.

It is known that high effectiveness of binding of estradiol **1** with ERα is a result of high hydrophobicity of steroid molecule and possibility to form hydrogen bonds of phenolic hydroxyl group at C-3 with Glu353, Arg394 and water molecule. Hydroxyl group at C-17 forms hydrogen bond with His524 (**Brzozowski et al, 1997**). Modified 8α-analogues of steroid estrogens have relative high affinity to ERα, and model explaining the binding with

ERα has been proposed **(Shavva et al., 2002** and citation herein). Metabolism of steroids with unnatural rings junction may be quite different from metabolism of natural steroids, which will allow to find the alternative solutions of many medicinal-biological tasks.

The perspectives for the creation of new inhibitors of steroid metabolism have been demonstrated using 6-oxa-D-homo-8α-analogues of estrogens **67** as an example (**Gluzdikov et al., 2007**). Authors accomplished the docking of different compounds of this series into ligand-binding domain of ERα. And after the analysis the assumption was made that this compound must have weak binding affinity to ERα or must have none in total. Sulphamate **67a** has been synthesized and the analogue showed a quite good inhibitory activity to estrone sulfatase. Steroid **67b** - potential product of hydrolysis has no uterotropic activity. It became the basis for the improvement of synthesis scheme of 7β-methyl-D-homo-8α-analogues of steroid estrogens (**Morozkina et al., 2009**) and investigation of peculiarities of their spatial structure (**Shavva et al., 2008**). Steroids of such structure may be used for the solution of other tasks (for example, as vectors for the transport of other classes of compounds into estrogen target-tissues).

Finally, 17β-hydroxysteroid dehydrogenase type 1 plays the important role in the induction and development breast cancer (**Vihko et al, 2002**). In ER^+ breast cancer cells under the action of this enzyme the dominate direction of the reaction is the transformation of estrone into more dangerous estradiol. In ER-positive breast cancer cells the reaction tends to proceed in the reverse direction. Another reason of tumor growth is inactivation of dehydroepiandrosterone **68**, which blocks tumor growth (**Aka et al., 2010**).



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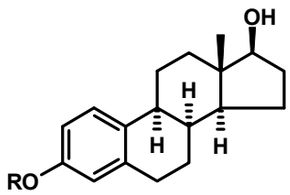
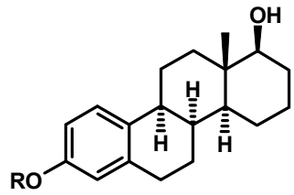
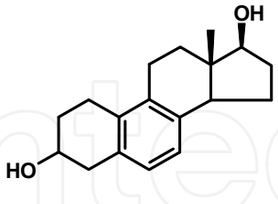


69 a) R=H, b) R= CH_3



70 a) R=H, b) R= CH_3



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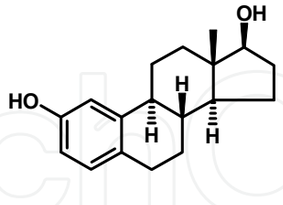

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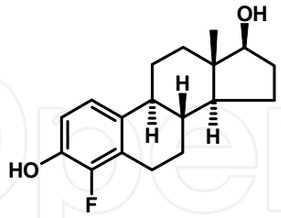

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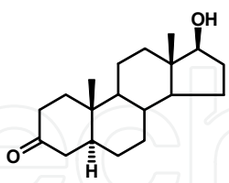
Substrate specificity of available enzyme from human placenta was investigated and it was found that steroids of unnatural series are also substrates for this enzyme (**Egorova et al., 1973**). High substrate specificity was observed only in the case of *trans*-junction of C and D rings. Values of V_{max} of 8α-series steroids may be more than ones for compounds of natural series. For example, V_{max} value of 8α-estradiol **69a** is 219% of V_{max} value of estradiol. D-homo-8α-estradiol **70a** has value of K_m in 5 times less than estradiol value, whereas values of V_{max} are approximately equal. Methylation of phenolic group (analogue **69b**) leads to the decreasing of K_m value in one order, steroid **70b** has value of K_m in 2.5 times less than compound **70a.** It takes attention big values of V_{max} of steroids **71** and **72**, correspondingly

635 and133% in comparison with estradiol (**Langer et al., 1959**). Values of K_m and V_{max} of 2-hydroxyestradiol **72** are comparable with these values for estradiol **1** (**Chernyaeva et al., 1972**), and such modification may be important during the search of inhibitors of 17β-hydroxysteroid dehydrogenase. Hence, the presence of free hydroxyl group at C-3 is not necessary for significant substrate specificity of such steroids. Modified androgens are also substrates for this enzyme, although they are notably less specific (**Chernyaeva et al., 1972**).
The abovementioned fact gives a persuasion that the search of inhibitors of investigated enzyme is most perspective in the series of steroids with unnatural rings junction. As far as we know such investigations have not yet been done.
It is quite interesting, that 2F- and 4F-estradiols (correspondingly **25** and **50**) are not substrates for 17β-hydroxysteroid dehydrogenase (**Langer et al., 1959)**, which may be important during the search of compounds with different spectrum of biological properties.
Primary structure of this enzyme was determinated after the analysis of corresponding cDNA **(Peltoketo et al., 1988**), lately X-rays analysis was obtained **(Ghosh et al., 1995**). The comparison of spatial structures of 17β-hydroxysteroid dehydrogenase and investigated earlier 3α,20β-hydroxysteroid dehydrogenase (**Ghosh et al., 1994**) led to the conclusion about participation of His in the binding of hydroxyl group at C-3 of steroid and structure of transition state with the participation of triad Tyr-Ser-Lys. Authors justly mention the importance of the data obtained for modeling of interactions of 17β-hydroxysteroid dehydrogenase with various ligands for the creation of effective inhibitors of the enzyme. Interesting developing of these investigations was the modeling of spatial structures of the enzymes of humans and rats, which are significantly different in primary structure and substrate specificity. Rat enzyme structure was modeled by the replacement of corresponding amino acids in the structure of human enzyme crystal (**Ghosh et al., 1995**) and following minimization of energy was done (**Putanen et al., 1997**). Results of calculations of rat enzyme are in good correlation with obtained experimental data. Clarification of mechanism of action of both enzymes was done using chimeric enzyme and site-directed substitution.



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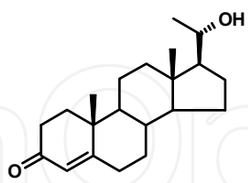

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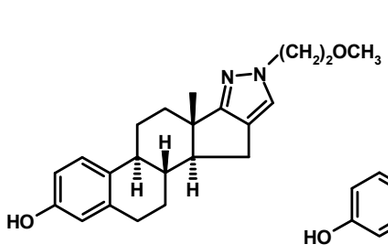
X-rays data of the complexes of 17β-hydroxysteroid dehydrogenase with estradiol **1** (**Azzi et al., 1996**), 5α-dihydrotestosterone **73** and 20α-hydroxy-analogue of progesterone **74** (**Lin et al., 1999**) have been obtained lately. Nevertheless the preliminary evaluation of characteristics for the searching the specific potential inhibitors is quite a difficult task because in human body types 1, 7 and 12 catalyze the transformation of estrone into estradiol (**Blanchard & Luu-The, 2007**). Substrate specificities of 17β-hydroxysteroid dehydrogenases type 1 and 2 are similar, but in the case of second enzyme the transformation reaction estradiol – estrone is mainly directed to estrone formation (**Miettinen et al., 1996**), therefore the inhibition of this enzyme during the treatment of hormone-sensitive breast cancer is quite undesirable. The search of specific inhibitors of

17β-hydroxysteroid dehydrogenases type 1 is complicated task, the attempts to solve this task by replacement oxygen-containing groups at position 17 and introduction of fluorine atoms into the same position of steroid skeleton were unsuccessful (**Deluca et al., 2006**).

Steroid **75** with big value of IC_{50} (530±7 nM) to this enzyme was synthesized, and this analogue has no hormonal activity (**Fisher et al., 2005**). Authors also obtained X-Ray data for the complex of compound **76** with 17β-hydroxysteroid dehydrogenase type 1 that has importance for the solution of this task.

Investigation of inhibitory activity of estrogen analogues with large substituents at C-16 in β-position led to find steroids **77** and **78** with selective action (**Lawrence et al., 2005**). Analogue **77** has value of IC_{50} 0.29 μM to 17β-hydroxysteroid dehydrogenase type 1 (**Purohit et al., 2006**).

Parallel investigations of properties of modified estrogens with substituents in β-position at C-16 led to obtain the compounds of type **79** (**Laplante et al., 2008**), this steroid has value of IC_{50} 44±7 nM for the transformation estrone **2** into estradiol **1** in T-47D intact cells. Unfortunately, analogue **79** possesses estrogenic activity, although this activity is decreased.



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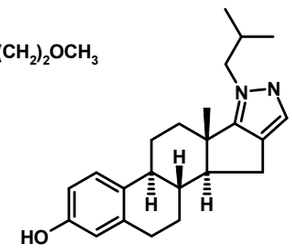

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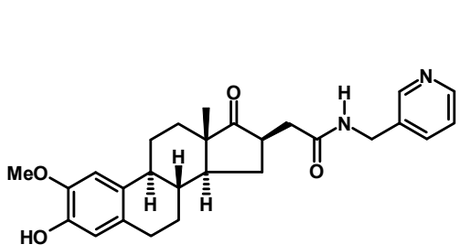

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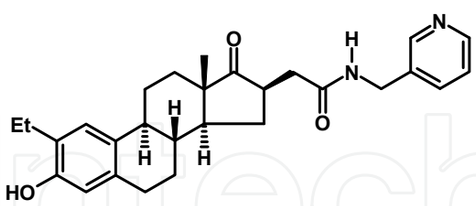

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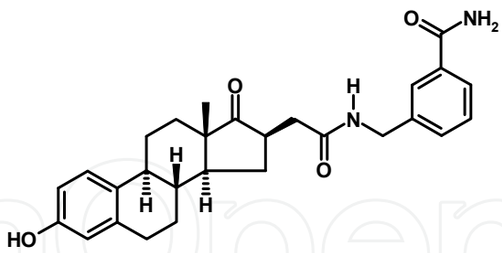

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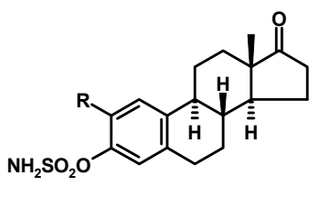
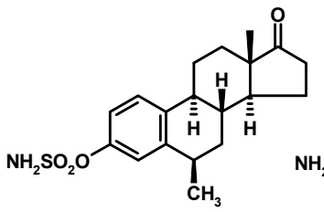
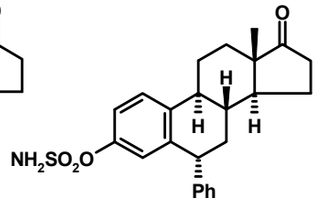
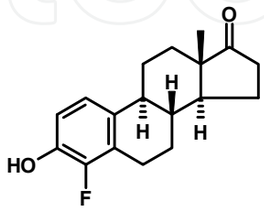
Obviously, clinical application of specific inhibitors of all three discussed enzymes is an intermediate step in the search for medications for the treatment of estrogen-sensitive oncological diseases. The most perspective direction is the search for compounds with simultaneous/synchronous inhibition activity to aromatase, estrone sulfatase and 17β-hydroxysteroid dehydrogenase. The basis for this approach is the possibility for overexpression of aromatase in tumors, which was shown in experiments on MCF-7 cell lines **(Santen et al., 1999**). Aromatase may stimulate the growth of tumors through both autocrine and paracrine pathways (**Chen et al., 1999**). Moreover, long-term estrogen deprivation increases sensitivity to estradiol and enhances aromatase activity in MCF-7 cells (**Yue et al., 1999**).

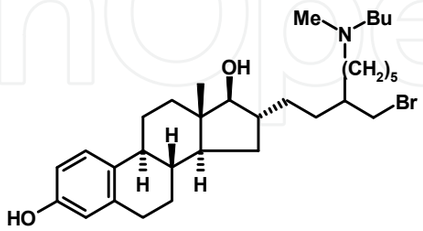




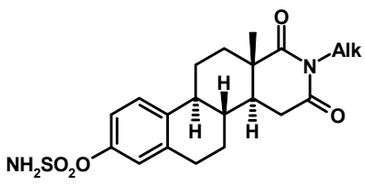


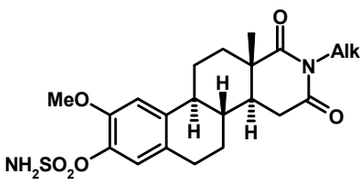
80 a) R=Cl, b) R=Br, c) R=CH_3 **81** **82**





83 **84**





85 **86**

Both substrate specificity of enzymes and potential ways of inhibition metabolism must be taken into account during the search for steroid hormone synthesis inhibitors. The fine example of inhibitors which are able to inhibit the activity of aromatase and estrone sulfatase at the same time was demonstrated by Japanese authors (**Numazava et al., 2005; 2006).** In earlier investigation it was demonstrated that steroids **25**, **47a,b,c** and **48-50** are good inhibitors of aromatase. Sulphamates **80a,b,c** and **81-83** having higher activity in comparison with EMATE have been synthesized. Authors justly think that these results may be useful for the development a new class of drugs having a dual function for the treatment of breast cancer. Additional benefits of the modifications at positions 2-, 4-, and 6 of steroid skeleton are perspective in respect of the fact that they may decrease the potential carcinogenicity of hydrolysis products (if they have) since the decreased possibility of formation of danger o-quinones of type **29** (**Liehr et al., 1995, 1996; Bolton et al., 1998; Bolton & Thatcher, 2008; Zhang, F. et al., 1999**; **Liu, X. et al, 2002; Zhang, Q. et al., 2008)** or 6-hydroxyestrogens (**Itoh et al., 1998**).

Search for non-steroidal inhibitors of aromatase and sulfatase is of interest as well. Such properties belong to heterocyclic sulphamates (**Reed & Potter, 2006**), however the perspectives for its using in clinic is not clear at the moment.

The attempts of synthesis of steroids with multifunctional action, in particular, inhibitors of ERs and 17β-hydroxysteroid dehydrogenase type 1 were done (**Tremblay & Poirier, 1996**).

Steroid **84** was selected as model compound by authors on the basis of previous data. Earlier it was established that in the series of 16-(bromoalkyl)estradiols for the realization of

inhibitory action the presence of side chain (3-4 carbon atoms) at position 16α is optimal. Such substituents contribute to the appearance of antagonist activity to ERα. It is desirable to have the tertiary amide group with substituents on the amide nitrogen. Authors assumed that primary bromide will inactivate 17β-hydroxysteroid dehydrogenase. Thus, steroid was expected to be inhibitor of 17β-hydroxysteroid dehydrogenase and agonist-antagonist to nuclear estrogen receptor. These suppositions have been confirmed: model steroid caused 25% stimulation of cellular growth at concentration 0.1 μM, at the same concentration steroid inhibited by 45% the 0.1 nM estradiol-stimulated growth of ZR-75-1 cells. It means, that compound **84** is partial agonist of ER and inhibitor of 17β-hydroxysteroid dehydrogenase with moderate activity.

Sulphamates **85** and **86** are inhibitors of estrone sulfatase and 17β-hydroxysteroid dehydrogenase (**Potter & Reed, 2002; Messinger et al., 2006).**

The selection of ways for the treatment of oncological diseases mainly depends from individual peculiarities of patients, as result the methods of definition of aromatase, estrone sulphatase, 17β-hydroxysteroid dehydrogenase or mRNA (which realizes their synthesis), content have a crucial importance (**Irahara et al., 2006**).

5. Modified estrogens with antioxidant action as potential neuroprotective agents

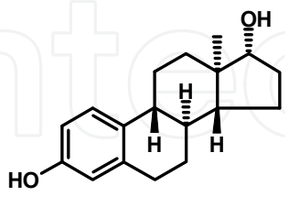
Estradiol and its analogues have been known to have pro- and antioxidant features. These properties of estrogens are still subject of debate and depend on many factors, including animals or tissues, administration routes, concentrations, peroxidative model and so on. Antioxidant action of estrogens has been widely studied in vivo and in vitro. Aside from its effects on LDL-oxidation (**Badeau et al., 2005**), it has reported that estrogens decreased lipid peroxidation in brain homogenates and neuronal cultures (**Thibodeau et al., 2002),** reduced the superoxide anion production in different cells (**Florian et al., 2004),**

In vivo studies allow usage of different experimental models of neurological disease (**Azcoitia et al., 2002**). Among the steroid family, only estrogens have the capability to prevent neuronal cell death caused by oxidative stress. Estradiol has been reported to reduce mortality and cerebral damage in the models of brain ischemia including middle cerebral artery occlusion and common artery occlusion (**Perez et al., 2005**). The neuroprotective effect of estrogens have also been shown in animal model of Parkinson's disease induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and in experimental models with various toxicities including serum deprivation, amyloid β peptide, glutamate-induced excitotoxicity, kainic acid and so on (see **reviews Green & Simpkins, 2000; Amantea et al., 2005**). Estrogens may protect against injury via receptor-dependent and receptor-independent mechanisms and it has been suggested that antioxidant capacity is an important component of the complex neuroprotective effect (**Green & Simpkins, 2000; Prokai & Simpkins, 2007).**

Antioxidant activity of estrogens as well as other known antioxidants in vivo is determined by a lot of factors: concentrations, distribution, localization, fate of antioxidant-derived radical, interaction with other antioxidants, metabolism (**Niki & Noguchi, 2000**). Natural fluctuation of ovarian hormones during estrous cycle may influence the effect of exogenous hormones; therefore ovariectomized animals are often used. Another problem with this approach is that estrogens can be transformed in tissues into metabolites, for example to

catecholestrogens, that have another antioxidant property and can produce prooxidants (**Picazo et al., 2003**). Most animal studied have utilized rodents (especially rats), that have a very high rate of estrogen degradation. Further, in case of brain the synthetic estrogens that are candidates for neuroprotective antioxidants in vivo should be able to cross the blood-brain barrier after their systemic administration. In vivo estrogens probably do not exert their own antioxidant action but interact synergistically with other antioxidants or reductants (**Hwang et al., 2000**). They can also affect the redox state of the cell through alteration of glutathione concentration and enhance the production of high energy compounds, stimulate antioxidant enzymes, such as SOD, catalase or glutathione transferase (**Perez et al., 2005**; **Akcay et al., 2006; Siow et al., 2007; Kumtepe et al., 2009**).
Using in vivo methodology we can not understand molecular mechanisms of certain hormone antioxidant action, but it allows studying the manifestation of complex action of hormone treatment.
More than 12 different types of neuronal cells against the 14 different toxicities were used to investigate neuroprotective effect of estrogens, their analogues and derivatives (**Green & Simpkins, 2000; Wise et al., 2001**). The concentrations of estrogens that have produced protective action in these models vary from physiological (0.1 nM) to pharmacological (50 μM) and it is suggested that different neuronal types may have different sensitivities to estrogen-mediated protection. For example physiological concentrations of 17β-estradiol were neuroprotective in cultures that contain multiple cell types and maintain intact cellular architecture (**Dhandapani & Brann, 2007)**. Several studies provide a positive correlation between in vitro neuroprotective potency and antioxidant activity of estrogens: they inhibited lipid peroxidation induced by glutamate, iodoacetic acid, amyloid β peptide (**Perez et al., 2005; Prokai-Tatrai et al., 2008**), reduced iron-induced lipid peroxidation (**Vedder, et al., 1999**), prevented intracellular peroxides accumulation induced by different toxicities (**Behl et al., 1997**). The neuroprotective antioxidant activity dependents on the presence of OH-group in the C-3 position on the A ring of the molecule. The formation of ether derivatives at C-2 position reduces effect because it abolishes the ability to donate a hydrogen atom (**Prokai et al., 2001**; **Perez et al., 2005**).
However, adjoin electron-donating methoxy groups to the phenolic ring may enhance antioxidant potency by weakening the phenolic O-H bond and provide stability of the formed phenoxyl radical (**Prokai & Simpkins, 2007**). Nevertheless generally higher concentrations of the hormones were required for antioxidant action than were needed for neuroprotection. This observation indicates that antioxidant effect is not a significant mechanism involved in the neuroprotective activity of estrogens in vivo (**Wise et al., 2001; Manthey & Behl, 2006**).
The simplest level of in vitro study of radical scavenging activity is investigation using cell-free models. Oxidation is induced by different systems of free radical generation and different types of prooxidants in order to gain a more precise view of the mechanism of inhibition. This methodology allows to investigate basic chemical properties (antioxidant or prooxidant) of natural estrogen molecules or synthetic analogues and compare compounds in each other.
There are multiple reactive oxygen and nitrogen species (ROS and NOS) and free radicals: superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$), hydrogen peroxide (H_2O_2), nitric oxide ($NO^{\bullet}$), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), peroxyl radical ($ROO^{\bullet}$), lipoperoxy radical ($LOO^{\bullet}$). The reactivity toward various ROS or NOS can be measured by

the inhibition methodology in which free radical species are generated in a special model system and the antioxidant effect is measured by inhibition of the reference reaction (**Sanchez-Moreno C., 2002**). For the superoxide anion radical generation a few model systems can be used: for example, hypoxanthine-xanthine oxidase system, autooxidation of riboflavine or non-enzymatic reaction of phenazine methosulphat in the presence of NADH and O_2. Then $O_2^{\bullet-}$ reduces nitro-blu tetrazolium into formazan at 25^0C and pH=7.4.



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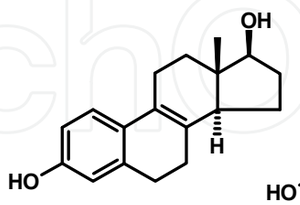

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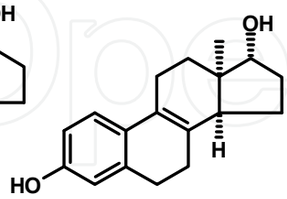

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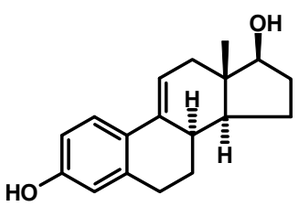

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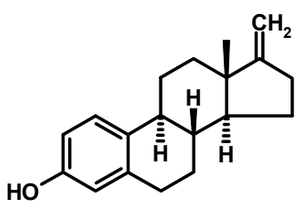

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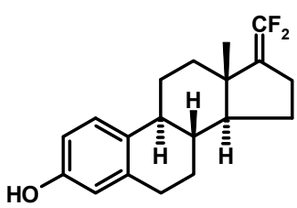

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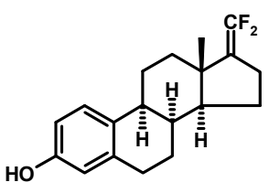

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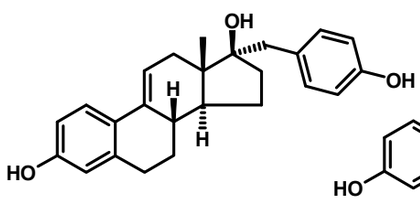

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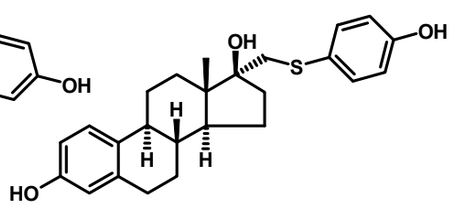

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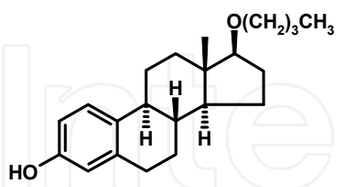

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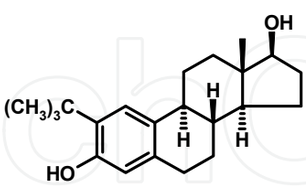

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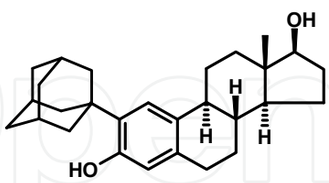

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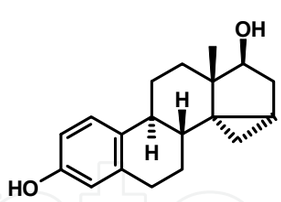
It is important to note that antioxidant action of estrogens is not necessarily depends on their receptors (**Xia et al., 2002**; **Perez et al., 2005**). This allows to search for modified analogues with lowered or depleted hormonal activity. It appeared that, for example, *ent*-estradiol **87** has antioxidant properties **(Simpkins, 2004)**. The introduction of conjugated with aromatic ring double bond into steroid molecule leads to the increased antioxidant activity (steroids **88** and **89)** (**Römer, W.; Oettel, M.; Droescher, P.; Schwarz, S., 1997**) and compound **90** (**Römer et al., 1997**) in comparison with estrogen without double bonds. Steroids of type **88** have significant hypothalamic action (**Berliner, 1996**), that allowed them to be recommended for the treatment of autoimmune diseases.

As we have noted earlier, presence of hydroxyl groups at C-3 and at C17 is important for effective binding of estrogens with estrogen receptors. As the first group is necessary for antioxidant activity of estrogen, synthesis of analogue with substituents at C-17 and/or aromatic ring, which would prevent receptor binding, would be an easy solution of the problem.

Thus, 17-methylensteroid **91** in the experiments on 23-day-old Sprague-Dowley rats exhibits only 1/70 of the uterotropic activity of estradiol. Moreover, this compound blocks the action of 7,12-dimethylbenzanthracene (**Jungblat et al., 1990**). Although in this work the investigation of antioxidant activity of compounds is not mentioned, it must be present in analogy with 17-difluoromethylene derivative **92** (**Bolhman & Rubanii, 1996**). In spite of that steroid has positive action on cardiovascular system; such compounds may have only restricted application because this steroid possesses contraceptive activity. We have to note that biological properties of 8α-analogue **93** are close to properties of steroid **92** (**Bolhman & Rubanii, 1996**). Analogues of type **93** decrease lipoprotein level.

The synthesis of steroid ethers in position 17 also could have lead to compounds having antioxidant activity coupled with lowered uterotropic action, as it was shown with compounds such as **96** (**Prokai et al., 2001; Prokai & Simpkins, 2007)**. Thus, the presence of large substituents in ring D does not result in lowered antioxidant properties, hence synthesis of new compounds which would possess double bond at position 8(9) and large substituents at D-ring can be considered as the next step in the creation of new drugs. It is desirable that these substituents had free phenol hydroxyl group, which would automatically increase its antioxidant properties. In fact, steroids **94** and **95** do have desirable properties (**Römer et al, 1997**).

The introduction of large substituent at position 2 must lead to decrease of estrogenic properties of modifies analogue at the presence of antioxidant activity, that was demonstrated with compounds **97** (**Miller et al., 1996**) and **98** (**Simpkins et al., 2004**) as examples.



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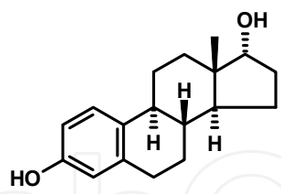

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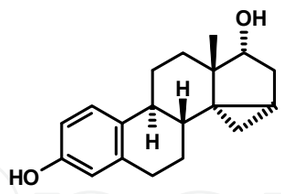


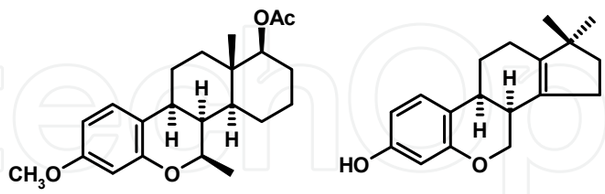
101

The high antioxidant activity of steroid **99** has been shown on different models (**Klinger et al., 2003**). Unfortunately this compound strongly inhibits monooxidaze activity of cytochrome P450 that is undesirable.

Hydroxyl group at C-17 in α-region of molecule does not prevent the appearance of antioxidant properties (compounds **100** and **101)**, which opens additional possibilities for the obtaining compounds with the selective action (**W. Römer, M. Oettel, P. Droescher, S. Schwarz, 1997**).

Antioxidant properties of 6-oxa steroid estrogen analogues have undergone little investigation. Oxygen atom does obviously possess a big influence on electron density distribution in A ring, which can have an impact on antioxidant properties of such compounds. Difference in antioxidant action mechanisms between such compounds and those discussed earlier can be assumed (**Prokai-Tatrai et al., 2008**).

We carried out the search for modified steroid estrogen analogues in the series of steroids with unnatural rings junction having antioxidant properties and lowered/depressed uterotropic activity. Such properties belong to compounds **102** (**Pison et al., 2009**) and **103**. Some results of investigations of antioxidant properties of steroid **102** are presented in the Table 2.



102 **103**

Experimental conditions	Schiff bases, conventional units / mg of phospholipids	Conjugated diene, nmol/mg of phospholipids	Triene conjugates, conventional units / mg of phospholipids	Klein coefficient	Malonic dialdehyde, nmol/mg of phospholipids
Control	129±10	12.2±0.7	0.209±0.022	0.70±0.01	1.65±0.14
Steroid **102**	96±6 P<0.05	9.5±1.0 P<0.05	0.152±0.022 P < 0.05	0.71±0.05 P > 0.05	1.80±0.18 P > 0.05

P – Student' coefficient.

Table 2. Results of investigation of action of steroid **102** on lipid peroxidation in brain of rats

Steroid was given *per os* in olive oil in dose 5 mg per 100 g of weight of rats, for the day before the slaughter. Solutions of the steroids contain 5 mg in 0.3 ml. Control group of animals were treated by olive oil in equal volume.

Solely further investigations of such compounds may show the perspectives of this class of steroids. Depressed hormonal activity of investigated steroids may be a negative factor, inasmuch as during their using the capacity for the induction of the formation of enzymes with antioxidant properties will be decreased.

The presence of antioxidant activity leads to expect neuroprotective action of such modified estrogens, in spite of the fact that exact mechanisms of these properties have not yet been established. A huge amount of patents in this area is a proof of it. We shall cite some of those works (**Simpkins et al, 1994; Covey, 2002; Wuelfert et al, 2002; Peri et al., 2005; Pei, 2005).** Unfortunately clinical trials did not confirm neuroprotective action. Most probably it is connected with multifunctional activity of estrogens, and as a result their positive properties are "compensated" by negative action, but how and which ones are unknown. Further progress in this area must be connected with success in detailed investigation of mechanism of neuroprotective properties and synthesis of analogues with restricted action.

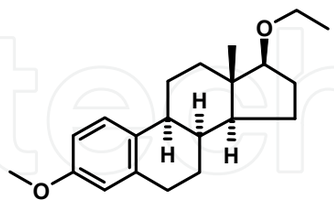
6. Osteoptotective action of estrogen receptors ligands

Several animal modes are used to test new compounds with osteoprotective properties. Ovariectomized mice are the ones most commonly used. Although aging in rats does not trigger osteoporosis development, ovariectomy-driven low estrogen concentration leads to

changes in bone tissue, which mimic those occurring in aged women. At first phase of fast bone tissue loss triggered by accelerated bone remodeling occurs. Later on this process slows down. Trabecular tissue becomes depleted more so then cortical tissue. It should be noted that bone breaking is rarely observed in ovariectomized rats, thus is probably better to call this process osteopeny.

There are known several models for testing steroids on animals. When investigating the action of compounds in mature rats at the age of 3 months intensive growth of bones in longitudinal direction should be taken into account. 75 Days old (**Turner et al., 1994**) rats are more suitable for the investigation; rats model (6-12 months), which has very few skeletal changes, are much harder to work with. To test bone state several parameters are used, such as «bone mineral density» (g/cm^3) and «bone mineral content» (g/sm. length, g/sm^2). Last parameter has linear correlation with bone mass (r=0.999) (**Sato et al., 1995**) and calcium contain in ash after bone burning (r=0.90) **(Gaumet M., 1996)**. By measuring dry weight of the bone (after drying at high temperature until the constant mass is obtained), ash weight after burning **(Bauss et al., 1996)**, and also by investigating the ratio of this two parameters (**Broulik & Schreiber, 1994**) changes in mineral bone content can be measured. Results of dual-energy X-rays absorptiometry BMD correlate with fracture incidence and useful in evaluation progression of osteoporosis (**Sharp et al., 2000**). Yet, the final conclusion about steroid activity can only be given after the investigation of their influence on mechanical durability of the bone (**Turner et al., 1994; Sato et al., 1999**). All the works in this part were investigating naturally occurring steroids.

As we have already noted the significant structural similarity between natural steroids and their 8α-analogues, the osteoprotective and uterotropic action of these compounds were investigated. In experiments with ovariectomized Wistar rats parallel change in those effects was observed. It was suggested that osteoprotective activity is dependent on nuclear α-receptor of estrogens (**Morozkina et al., 2007**). Hence analogues were chosen as model compounds, despite the fact that presence of methoxyl group at C-3 was expected to trigger the lowering of activity. However in this case carcinogenicity was also expected to be lowered. In the experiments with Sprague-Dowley rats compounds **104** and **105** were shown to possess the same osteoprotective activity as ethynylestradiol, although they were administered in higher doses **(Morozkina et al., 2008**). The presence of substituents in positions 2 and 4 cancels the osteoprotective effect.



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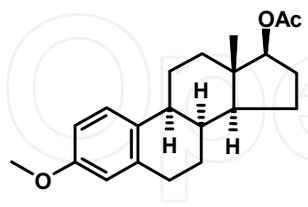

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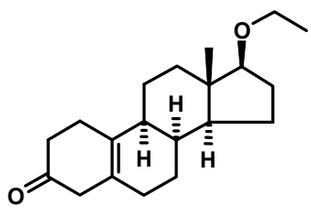
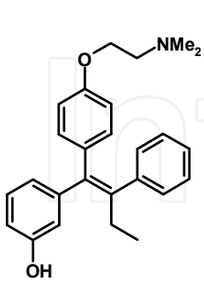


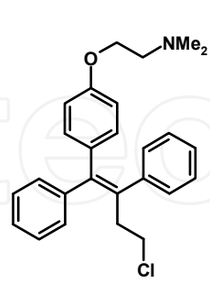
106

We assumed that steroid **106** may have osteoprotective action on the analogy with tibolone. It was confirmed experimentally; however compound **106** has embriotoxic action in experiments of contraceptive properties investigations which possibly decreases significantly the perspectives of such analogues.



Droloxifene

107



Toremifene

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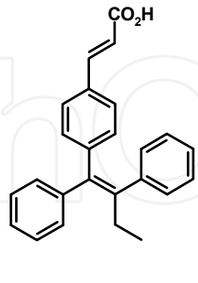
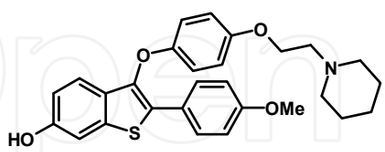


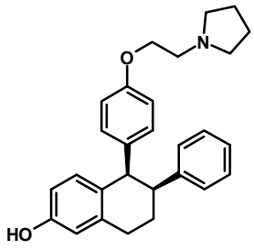
GW5638

109



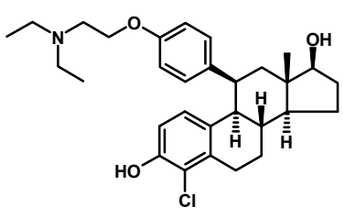
LY353381 (arzoxifene)

110



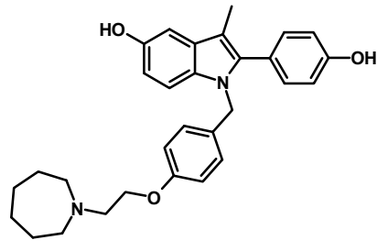
CP335156 (Lasoxifen)

111



HMR3339

112



Bazedoxifene

113

As ER modulators are capable of displaying antagonistic properties in uterus and mammary gland while retaining agonistic features in bone, perspectives of using such compounds as osteoprotectors was investigated. Raloxifene has very effective osteoprotective properties. Clinical studies have shown that, aside from osteoprotective effects, the risk of breast cancer was also lowered (**Fontana & Delmas, 2001**). Interestingly that osteoprotective effect of raloxifene is not always mediated by ERs (**Miki et al., 2009**), and as the result the properties of its analogues may be quite interesting. However it was established that raloxifene in this case has some negative properties, peculiar to typical estrogens (high risk of hot flashes, leg crumps and venous thrombosis (**Deal & Draper, 2006**). In view of the aforesaid it is worth to remember about earlier synthesized compound LY357489 **(Grese et al., 1998),** whose osteoprotective action is significantly higher in comparison with raloxifene. This compound is the conformationally restricted raloxifene' analogue; its selective action was explained by specificity of interaction with ERs. Probably, it is necessary to investigate the mechanism of this compound' action.

Lately a huge number of SERMs with osteoprotective action have been synthesized, for example droloxifene **107 (Ke et al., 1995; Chen, H.K. et al., 1995**), LY 353381 (arzoxifene **110**) (**Sato et al., 1998; Ma et al., 2002**), lasofoxifen (CP-336,156) **111** (**Ke et al., 1998; 2000, 2004)**, GW5638 **109** (**Wilsson et al, 1997**) and many other compounds with interesting biological properties. For example, lasoxifen (CP-336,156) showed osteoprotective and cholesterol-

lowering properties, decreased body weight in the experiments on ovariectomized rats. This compound had osteoprotective action in adult male orchideectomized rats, and has not influence on prostate (**Ke et al., 2000**).

Droloxifene showed interesting biological properties in the experiments with animals, such as osteoprotective action in aged female rats (**Chen, H.K. et al., 1995**). However it was shown to be less effective then tamoxifen in breast cancer treatment clinical studies, so its investigation was stopped (**Vogelvang et al., 2006**).

Lasoxifen showed osteoprotective and cholesterol-lowering action while lowering body weight of ovariectomized rats. This compound also displayed osteoprotective properties in adult male orchideectomized rats while having no effect on prostate (**Ke et al., 2000**). As far as we are aware, effectiveness of lasofoxifen have not undergone clinical studies.

Idoxifene was used in clinical trials on patients, whose tumors were resistant to tamoxifen, however third-generation aromatase inhibitors took its place, clinical data about its osteoprotective properties are conflicting (**Vogelvang et al., 2006).**

Preliminary studies of breast cancer treatment by HMR3339 and its osteoprotective properties provided results that may be considered positive (**Vogelvang et al., 2006**).

Bazedoxifen possesses promising properties. As a result of 5 years long clinical study on 4216 patients in postmenstrual period good osteoprotective properties were found (**Silverman et al., 2010**).

We have already mentioned the possibility of agents' synthesis in 8α-analogues of steroid estrogens series with osteoprotective properties. Preliminary results are presented in Table 3. 17α-ethynylestradiol was used as standard.

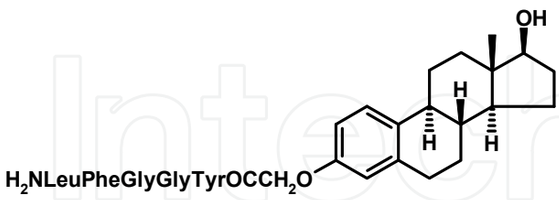
Group of experimental rats (number of animals in the group)	Change of body weight,	Uterine weight, mg/100 g of body weight	Ash femur weight/wet femur weight	Bone mineral density, g/cm2
Sham-operated (10)	29±3*	154 ± 4*	0.432±0.007*	0.258±0.005*
Ovariectomized (15)	60±5	22.7 ± 0.5	0.398 ±0.005	0.231±0.003
Ovariectomized, treated with EE (10)	12±4*	140± 6**	0.433±0.005*	0.255±0.006*
Ovariectomized, treated with steroid **69a** (10)	7 ± 4*	148± 6**	0.425±0.006*	0.258±0.007*
Ovariectomized, treated with raloxifen (10)	33±5*	46.5 ± 2.6*	0.430±0.007	0.245±0.006*
Ovariectomized, reated with raloxifen and steroid **69a** (15)	38±5*	31.2 ± 1.2*	0.425±0.005*	0.248±0.006*

Signs * and ** mean statistically significant difference between studied group and group of ovariectomized animals, $p<0.05$ and $p<0.01$ (Students t-criterion).

Table 3.

According to the data, this compound possesses osteoprotective properties. Also, in contrast to action of 17α-ethynylestradiol and steroid, combination of compound and raloxifene did not trigger hypertriglyceridonemy, yet cholesterol-lowering effect remained. There is no doubt that investigations regarding properties of 8α-series steroids are of great importance.

The new area in synthesis of compounds with osteoprotective properties should be noted – the creation of hybrid molecules having steroid and peptide fragments, for example compounds (**Wang et al., 2003**). Such compounds have stronger action in the experiments on rats in comparison with sum of action of these compounds.



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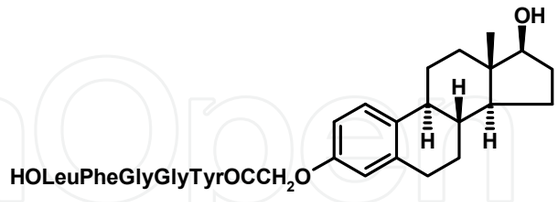

115

Peptidylsteroid **114** has very interesting properties (**Yokogawa et al., 2006**). This hybrid compound during intranasal administration has a potent antiosteoporotic effect without side effects in experiments on female ddY mice.

In the treatment of estrogen-sensitive conditions aromatase inhibitors are to be used sometimes, which triggers estrogen concentration to drop, increasing osteoporosis and cardiovascular disease risk (**Ponzone et al., 2008; Reid, 2009; Gennari et al., 2011**). In each case the decision in using a drug depends on individual features of the patient. Usage combined drugs, which have calcitriol as a part of them, might prove useful to lower osteoporosis (**Krishnan et al., 2010).**

7. Steroid estrogens and cardiovascular system

Coronary heart diseases (CHD) is rare, and the incidence of CHD complications is much lower in premenopausal women then in man or postmenopausal women of similar age. After menopause the gender difference is lost and the incidence of CHD complications in women gradually approach that the men. In addition, menopause adversely affects several risk factors for CHD. It allows to assume the important role of estrogens in the protection of body from coronary heart diseases and expediency of their use as HRT medications.

Protective effect of estrogens has been shown in various animal models.

Rats have been used as a model system to study estrogenic effects on plasma lipid levels (**Lundeen et al., 1997**). On the whole this model has a lot of restrictions, first of all the main one is the predominant plasma cholesterol is HDL, not LDL, as it is in human. There are known: mouse models of atherosclerosis (**Hodgin & Maeda, 2002**) on rabbits (**Haarbo & Christiansen, 1996**) and primates (**Adams et al., 1990**). In all cases the main is the evaluation of influence of estrogens on the development of atherosclerotic plaque or lesion. In most cases it was shown the inhibitory activity of estrogens on atherosclerosis development.

Firstly it was postulated that main antiatherosclerotic action of estrogen is caused by the ability to decrease cholesterol level in serum; however in a number of investigations it was shown that this mechanism is not obligatory (**Shavva et al., 1987; Holm et al., 1997**).

At the present time it is postulated that atheroprotective effects of estrogen may be mediated by their nuclear receptors. ERα was found in endothelial cells (**Venkov et al., 1996**), in smooth-muscle cells (**Karas et al., 1994**) and in myocardial cells. A number of authors maintain the opinion about the importance for protective functions both ERα (**Gerard'es** et al, 2006; **Pare at al., 2002**), and ERβ (**Watanabe et al., 2003).** It is impossible to note the

experimental data, showing the cardioprotective activity of estrogens, which is not mediated by nuclear receptors (**Shavva et al., 1987; Karas et al., 2001**).

Estrogens increase vasodilatation and inhibit the response of blood vessels to injury and the development of atherosclerosis, this action is referred to non genomic, this develops maximum after 20 min after estrogen introduction (**Mendelsohn & Karas, 1999**). Fast vasodilatation is possible due to the influence on both calcium-activated potassium ion-channel function (**Ghanam et al., 2000**), and on the synthesis of nitric oxide (the last relaxes vascular smooth muscle and inhibits platelet activation (**Holm et al., 1997; Node et al., 1997**). More detailed ways of non-genomic effects have been considered by **Mendelsohn & Karas (1999**).

Of crucial importance is the influence of agents on hemodynamic functions (**Borissoff et al., 2011**).

One more factor of risk of arising of atherosclerosis and thrombosis is higher content of oxidized lipoproteins, possible mechanism of these diseases have been considered **(Steinberg et al., 1989; Holvoet & Collen, 1994).**

Antioxidant action of estrogens has been widely studied *in vivo* and *in vitro*. Besides its effects on LDL-oxidation (**Maziere et al., 1991; Markides et al., 1998; Ruiz-Larrea et al., 2000; Badeau et al., 2005**), it has reported that estrogens decreased lipid peroxidation in brain homogenates and neuronal cultures (**Vedder et al., 1999; Thibodeau P. et al., 2002**), reduced the superoxide anion production of different cells (**Bekesi et al., 2000**; **Florian et al., 2004).** All these effects may contribute to the beneficial consequences of estrogen replacement on the cardiac and vascular function, bone and mineral metabolism, brain function.

In the experiments on rabbits with hypercholesterinemic diet it was shown that 17α-dihydroequilin sulphate and 17α-ethynylestradiol significantly reduce atherosclerosis by 35% in the aortic arch and 75-80% in the thoracic and abdominal aorta, in spite of high level of LDL cholesterol (**Sulistiany at al., 1995**). High ration between HDL and LDL level is important but is not absolute parameter in prognosis of cardio-heart diseases risk. Clinical trials of estrone sulphate (at the same time 17α-ethynylestradiol has been investigated) in postmenopausal women have shown the significant improvement of this parameter. It is important that the action of estrone sulphate did not result in triglycerides level increasing in contrast to 17α-ethynylestradiol (**Colvin et al., 1990**).

Thus, numerous investigations on animal models (we described only few of them) give the evidence about the advisability of application of estrogens for HRT. First clinical trials for using of estrogens with this aim gave optimism, however wide-ranging investigations did not confirm the expectations. In older postmenopausal women with established coronary-artery atherosclerosis, 17β-estradiol had no significant effect on the progression of atherosclerosis (**Hodis et al., 2003**). Moreover, the combination: estrogen plus progestin may increase the risk of CHD (**Manson et al., 2003**). This is very important effect, because the content of triglycerides in blood is the independent factor of risk of cardio-vascular diseases (**Koren et al., 1996**).

This difference is very important because the increased level of triglycerides in blood is the independent factor of risk of cardiovascular diseases (**Koren et al., 1996**).

From other side, main and side effects of any medication depend on/from methods of the introduction into the body. Therefore the positive results obtained during transdermal introduction of estradiol takes attention **(Sumino et. al., 2006)**. Oral estrogens raised triglycerides whilst transdermal estradiol lowered those (**Nerbrand et al., 2004**). The

conclusion about propriety of such introduction of estrogens may be done only after wide-ranging long-term investigations.

The investigations of influence of estrogen receptors modulators, being already in clinical application, on coronary heart system have particular interest. First of them is raloxifen. Raloxifene blocks the sedimentation of redundant cholesterol in aorta in the experiments on rabbits (**Bjarnason et al., 1997; 2001**).

One more new steroid has interesting properties (**Pelzer et al., 20**05), this analogue in the experiments in estrogen-deficient spontaneously hypertensive rats has positive influence on hemodynamic function and inhibits cardiac hypertrophy.

8. Conclusion

From the data presented it became obviously the strategy for the searching of new medications on the basis of estrogen receptors ligands with the improved properties in comparison with clinically used. As far as the synthesis of such ligand is very complicated task, on the first stage the group with known structure peculiarities in the solution (including conformational dynamics) and determinate the possibilities for theoretical calculations of their spatial structure. The following docking of new possible agents into structures of macromolecule compounds (receptors, enzymes and other proteins) is used for the selection of most perspective compounds. And it is quite necessary to take into account the metabolic ways of new analogues.

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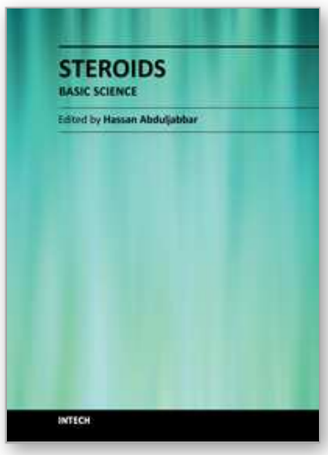

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This book explains the basic science of steroids and is targeted towards professionals engaged in health services. It should be noted that medical science evolves rapidly and some information like the understanding of steroids and their therapeutic use may change with new concepts quickly. Steroids are either naturally occurring or synthetic fat-soluble organic compounds. They are found in plants, animals, and fungi. They mediate a very diverse set of biological responses. The most widespread steroid in the body is cholesterol, an essential component of cell membranes, and the starting point for the synthesis of other steroids. Since the science of steroids has an enormous scope, we decided to put the clinical aspects of steroids in a different book titled "Steroids-Clinical Aspects". The two books complete each other. We hope that the reader will gain valuable information from both books and enrich their knowledge about this fascinating topic.

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