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Novel structural features increase the antioxidant effect of estrogen analogues on low density lipoprotein

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ABSTRACT

Many known estrogens, both natural and synthetic, may act as antioxidants. We designed and synthesized 22 novel estrogen analogues with different ring junctions or substitutions, such as fluorine. We studied the antioxidant capacity *in vitro* of 35 synthetic estrogen analogues in aqueous lipoprotein solution by monitoring the formation of conjugated dienes. In addition to a free C-3 hydroxyl group, the two most active antioxidants had either a methyl group at C-4 and a six-carbon D-ring, or a fluorine atom at C-2 and an unsaturated B-ring. Extension of the D-ring increased the antioxidant capacity of 6-oxa estrogens. Compounds with a fluorine atom at C-2 were similar or more potent antioxidants compared with the principal endogenous estrogen, 17β-estradiol. In compounds with a substituted C-3 hydroxyl group, the antioxidant capacity could be significantly increased by additional double bonds in the C- or D-rings. In conclusion, we show that the antioxidant capacity of estrogen analogues could be increased by structural changes.

1. Introduction

Oxidative damage plays an important role in atherosclerosis [1,2] which is the primary cause of cardiovascular disease. The key events are modification of low density lipoprotein (LDL) and its retention in arterial wall [3]. Oxidative stress and lipid peroxidation have also been implicated in neurodegenerative diseases and aging [4]. Attributed to the presence of endogenous estrogens, the development of atherosclerosis is significantly slower in premenopausal than in postmenopausal women [5]. Upon binding to its receptors (estrogen receptor alpha or beta) 17β-estradiol (17β-E2) may activate transcription of many genes involved in vascular endothelial function and inflammation [6]. Rapid, nongenomic effects may take place through binding of 17β-E2 to cellular membrane receptors (G protein-coupled receptor), and subsequently, lead to for example activation of endothelial nitric oxide synthase and increased production of nitric oxide [7].

At higher concentrations as studied in assays *in vitro*, estrogens inhibit lipoprotein peroxidation [8–10] and act as direct antioxidants by scavenging free radicals [4,11]. Other possible mechanisms include reduction or chelation of redox-active metal ions [12]. Based on previous studies on natural and synthetic estrogens, a phenolic A-ring is an important structural determinant for the *in vitro* antioxidant activity [13,14]. Additional methoxy groups adjacent to the C-3 phenolic hydroxyl have increased antioxidant potency of estrogens [13] or flavonoids [15] in different experimental settings. Moreover, an unsaturated B- or D-ring structure of estrogens and flavonoids has been related to enhanced antioxidant activity [8,13].

In the present study, we were interested in the effects of D-ring modifications on the antioxidant activity of estrogens, such as extension or unsaturation of the D-ring. To investigate antioxidative B- and D-ring structures we included estrogen analogues with a substituted C-3 hydroxyl group. We also studied compounds with different A-ring substitutions, such as fluorine. We synthesized a wide series of steroidal estrogen analogues with unnatural ring junction and having various substituents on the steroidal skeleton. Most of these compounds were new and thus, their antioxidant or other biological effects unknown. We set out to explore the *in vitro* antioxidant capacity of the new estrogen

Abbreviations: 17β-E2, 17β-estradiol; LDL, low density lipoprotein; Log *P*, logarithm of the *n*-octanol/water partition coefficient; SD, standard deviation.

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analogues in aqueous LDL solution, in comparison with the principal endogenous estrogen, 17β-E2.

2. Material and methods

2.1. Synthesis of estrogen analogues

The synthesis and purity of the novel analogues is described in the Experimental section in Supplementary material. The following compounds were synthesized as previously described: 3-hydroxy-7alpha-methyl-6-oxa-estra-1,3,5(10), 8,14-pentaen-17-one (**1**) [16], 3-hydroxy-7α-methyl-6-oxa-estra-1,3,5(10), 8,14-pentaen-17-one acetate (**2**) [17], 7α-methyl-6-oxa-estra-1,3,5(10), 8,14-pentaen-3,17β-diol diacetate (**3**) [17], estra-1,3,5(10), 8,14-pentaen-3,17-diol diacetate (**4**) [18], 7α-methyl-6-oxa-estra-1,3,5(10), 8-tetraen-3,17β-diol diacetate (**5**) [17], 6-oxa-8-alpha-estra-diol diacetate (**8**) [18], 3-methoxy-7alpha,18-dimethyl-estra-1,3,5(10), 8,14-pentaen-17-ol acetate (**11**) [19], 6-oxa-7alpha-methyl-8alpha-estrone methyl ester (**12**) [20], 6-oxa-7α-methyl-8β,14β-estrone (**13**) [21], 6-oxa-8alpha-estrone (**14**) [18], 3-methoxy-17,17-dimethyl-6-oxa-8α-gona-1,3,5(10), 13(14)-tetraene (**19**) [22] 7beta-methyl-D-homo-6-oxa-8alpha-estrone (**21**) [18] and 2-fluoro-3-hydroxy-16-methyl-13α-estra-1,3,5(10), 8(9), 15-pentaen-17-one (**26**) [23] (Fig. 1).

2.2. Isolation and purification of low density lipoprotein

Blood was drawn from healthy, normolipidemic men ($n = 5$) into EDTA-containing vacuum tubes. The study was approved by Ethics committee of Helsinki University Central Hospital, and written informed consent was obtained from the subjects. Plasma was isolated by centrifugation at 2500 × *g*, 15 min, +4 °C and pooled into two different pools. Plasma low density lipoproteins (LDL) were isolated by sequential ultracentrifugation [24] as previously described [13]. The LDL fractions were protected from light at all times and stored at −70 °C. Before the oxidation experiment, a 2 ml aliquot of ultracentrifugally isolated LDL was purified by gel filtration [13] and the concentration of proteins was measured [25]. The lipoprotein-containing fractions were pooled and used for the oxidation study.

2.3. Oxidation of LDL

We used Esterbauer's method [13,26] to study the antioxidant action of estrogen analogues on LDL. The estrogen analogues were dissolved in ethanol and added to LDL (LDL protein concentration of 0.10 mg/ml in PBS). Each experiment included incubations of LDL with two concentrations of 17β-E2 (0.1 μM and 1 μM) as well as control LDL without added estrogen. LDL was subjected to copper-induced oxidation and the formation of conjugated dienes was monitored by UV-spectrophotometer as described in [13]. With each concentration of added estrogen analogues, the percent change in lag time was calculated as follows: (lag time of LDL with added estrogen/lag time of control LDL with solvent only) × 100% [13]. The results are expressed as the mean ± SD of three to five parallel experiments. In 10 consecutive assays, the mean increase in lag time with 1 μM concentration of 17β-E2 was 161 ± 6% (mean ± SD).

Statistical comparisons were made using SPSS Statistics 19.0 software. The level of significance was $P < 0.05$. The logarithm of the *n*-octanol/water partition coefficient (log *P*) values were calculated using MarvinSketch 5.11.4 (ChemAxon Ltd.). The phenolic O—H bond dissociation energies were estimated using Gaussian 09 software.

3. Results and discussion

3.1. Synthesis of the estrogen analogues

The synthetic strategy was based on the method of Torgov and Ananchenko [27], which enabled to synthesize steroidal compounds of diverse structures.

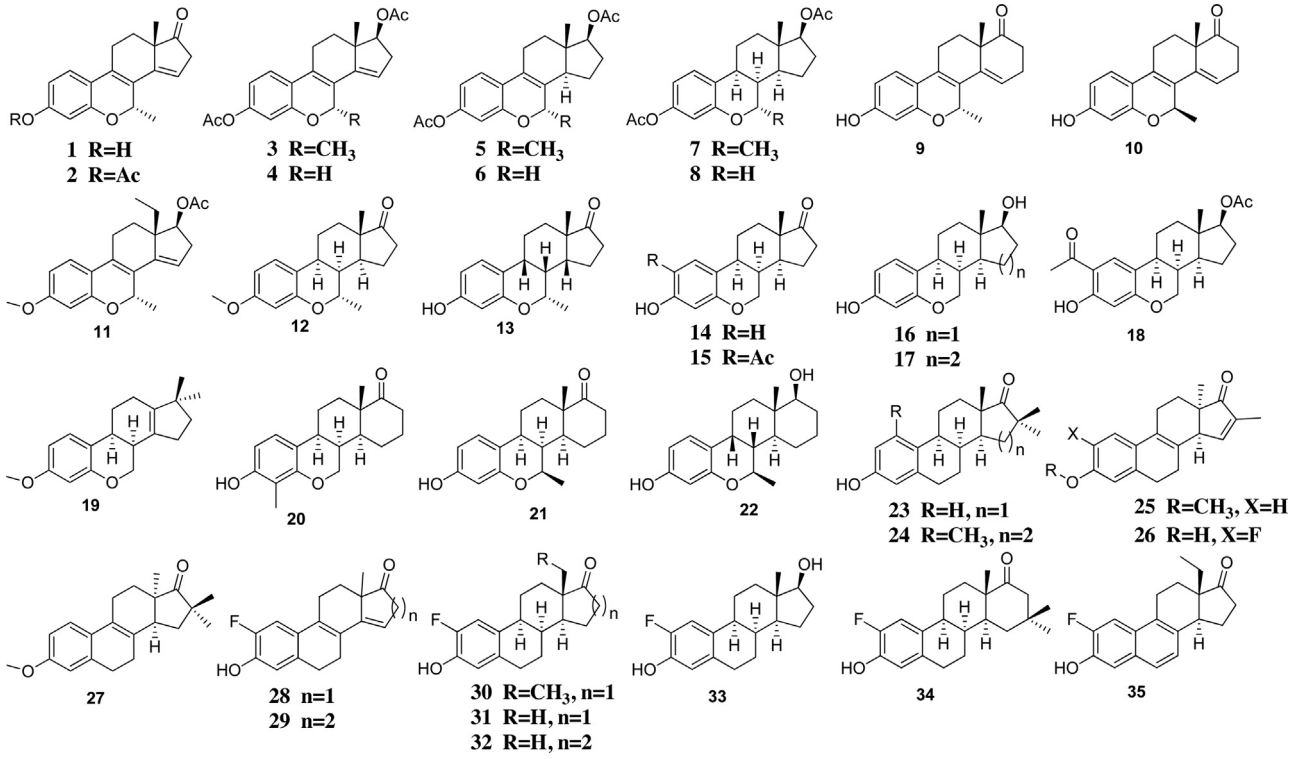

Fig. 1. Structures of the synthetic estrogen analogues used in the LDL oxidation assay. All compounds are racemic.