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In vivo characterization of estrogen receptor modulators with reduced genomic versus nongenomic activity *in vitro*

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ABSTRACT

Estrogen receptor (ER) ligands that are able to prevent postmenopausal bone loss, but have reduced activity in the uterus and the mammary gland might be of great value for hormone therapy. It is well established that the classical ER can activate genomic as well as nongenomic signal transduction pathways. In this study, we analyse the *in vivo* behaviour of ER ligands that stimulate nongenomic ER effects to the same extent as estradiol, but show clearly reduced activation of genomic ER effects *in vitro*. Using different readout parameters such as morphological changes, cellular proliferation, and target gene induction, we are able to demonstrate that ER ligands with reduced genomic activity *in vitro* show a better dissociation of bone versus uterine and mammary gland effects than estradiol that stimulates genomic and nongenomic effects to the same extent. We conclude that pathway-selective ER ligands may represent an interesting option for hormone therapy.

1. Introduction

Estrogens influence a variety of biological functions such as reproduction, mammary gland development [1], bone turnover [2], and modulation of the cardiovascular system [3]. The estrogen receptor (ER) can activate different signal transduction pathways. In the classical pathway, ER acts as a ligand-dependent transcription factor that binds to estrogen response elements (EREs) in promoters of target genes. In contrast, rapid nongenomic effects are initiated outside the nucleus and lead to the activation of cytoplasmic signal transduction cascades [4]. The establishment and maintenance of reproductive function critically depends on genomic effects. Mice harbouring point mutations within the DNA binding domain of the ER do not show uterine growth in response to estradiol and this therefore underlines the importance of genomic ER signalling for uterine growth [5]. Nongenomic effects are suggested to be partly involved in mediating the effects of estrogen in bone [2] and the cardiovascular system [6]. In bone, it has been demonstrated that estrogen can exert antiapoptotic effects on osteoblasts and osteocytes. These effects have been shown to be partly mediated via nongenomic activation of the SRC/Shc/ERK pathway and repression of JNK signalling cascades leading to the modulation of the activity of transcription factors such as Elk-1, CREB, and c-Fos/c-Jun [7]. In endothelial cells, the ERα/PI3K interaction promotes the rapid activation of eNOS leading to enhanced NO production and vasorelaxation [8].

Estradiol and conjugated equine estrogens (CEE) have successfully been used in hormone therapy to treat postmenopausal symptoms such as hot flushes, sleep disturbance, vaginal dryness, and osteoporosis. As outlined before, the beneficial effects on bone and the vasculature seem to be partly mediated via nongenomic effects. In contrast, the stimulation of uterine epithelial cell proliferation – an unwanted side effect of estrogen-only therapy – depends on binding of the ER to EREs and thus genomic effects [5]. These findings may lead to the conclusion that pathway-selective ER ligands with reduced genomic versus nongenomic activity may be valuable tools for hormone replacement therapy. In line with this hypothesis, estren (4-estren-3α,17β-diol) has been claimed to be a pathway-selective ER ligand that prevents ovariectomy-induced bone loss via its nongenomic effects but does not have any stimulatory action on the uterus due to its reduced genomic activity [2]. However, one major drawback of estren is its potent androgenic

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activity *in vivo*. Estren increases musculus levator ani and seminal vesicle weights in male orchidectomized mice [9], and promotes androgen phenotypes in primary lymphoid organs that are clearly independent of the presence of ERs [10]. Such strong androgenic compounds are not of interest for hormone replacement therapy.

We previously identified several estrogen receptor ligands that stimulate nongenomic ER-mediated effects to the same extent as estradiol but exhibit strongly reduced genomic activity *in vitro* [11]. In contrast to estren, these compounds lack any androgenic activity both *in vitro* and *in vivo*.

Here we describe the *in vivo* characterization of two tool compounds in comparison to estradiol and estren. We were interested in analysing how the pathway-selectivity of these compounds *in vitro* translates into their *in vivo* activity, and whether such compounds with reduced genomic activity might be of interest for hormone therapy.

2. Materials and methods

2.1. Animals and drugs

17β-Estradiol and 5′-bromo-2′-deoxyuridine (BrdU) were purchased from Sigma. Estren (estr-4-ene-3α,17β-diol), compound A (2-(4-hydroxyphenyl)-3-methylbenzo[*b*]thiophen-5-ol) (structure disclosed in Ref. [11]), and compound C (non-steroidal compound, structure not yet disclosed) were synthesized in the laboratories of Bayer Schering Pharma AG. Swiss Webster and C57BL/6 mice (Charles River) were maintained on a 14-h light/10-h dark cycle and provided with food and water ad libitum. All animal procedures were run according to German animal welfare law and with permission of the District Government of Berlin.

2.2. Protection from ovariectomy-induced bone loss

Female Swiss Webster mice ($n = 120$, 6 months of age, 10 mice per group) were randomized into 12 groups. Group 1 was sham-operated and groups 2–12 were ovariectomized. The day after ovariectomy daily subcutaneous treatment with either vehicle (PEG) (groups 1 and 2) or test compounds was started for 6 weeks. Group 3 received estradiol (6.4 μg/kg), groups 4–6 were treated with estren (0.1, 1, 5 mg/kg), groups 7–9 were treated with compound C (0.1, 1, 5 mg/kg) and groups 10–12 were treated with compound A (0.1, 1, 5 mg/kg). Animals were sacrificed after 6 weeks of treatment. *Ex vivo* bone mineral density (BMD) of the tibia was measured with pQCT. The bone-protective dose, i.e. the minimal dose of each test compound leading to bone mineral densities that were not significantly different from the bone mineral density of sham-operated animals, was determined. In the same animals, uterine effects of the test compounds were assessed by analysis of relative uterine weight and uterine epithelial cell height.

2.3. Combined mammary gland and uterine growth assay

To assess the dissociation of bone versus uterine and bone versus mammary gland effects for the test compounds in more detail, we performed combined uterine growth and mammary gland assays within the same cohort of mice. In preliminary experiments, using different mouse strains as well as mice of different ages we did not observe any gross differences between Swiss Webster and C57BL/6 mice regarding the uterine responses after stimulation with estradiol. These results were in line with published data [12]. Since younger mice, i.e. mice that did not reach full puberty, are required for the analysis of estrogenic effects on the mammary gland and most of the published reference data are generated in C57BL/6 mice, we decided to use this strain of mice for the subsequent analysis. Mammary gland readout parameters were endbud formation, epithelial cell proliferation, and target gene induction. Readout parameters for uterine estrogenic action were epithelial cell height, epithelial cell proliferation, and target gene induction.

Female C57BL/6 mice were ovariectomized at the beginning of the sixth week of age. Two weeks after ovariectomy, the animals were treated with daily subcutaneous Injections of either vehicle (ethanol/arachisoil (1 + 9, v/v)), 6.4 μg/kg estradiol, compound A (0.1, 0.3, 1, 3 mg/kg), compound C (0.1, 0.3, 1, 3 mg/kg), or estren (0.1, 0.3, 1, 3 mg/kg). Treatment was performed for 3 weeks. Group size was eight animals per treatment group. Two hours before sacrifice animals received an injection of BrdU dissolved in PBS (70 mg/kg body weight i.p.). Animals were killed by cervical dislocation. After sacrifice, the left inguinal mammary gland was removed and spread on a glass slide and fixed for 48 h at room temperature in Carnoy's fixative (six parts 100% ethanol, three parts chloroform, one part glacial acetic acid). After staining in carmine alum (0.2% carmine alum, 0.5% aluminium potassium sulphate, one crystal of thymol), the mammary glands were dehydrated, cleared in xylene, and stored in Protaqstura. Photographs were taken at 12× magnification. Endbuds per mm^2 were counted by an investigator blinded to the experimental treatment the animals had received.

The dorsal 2/3 of the right mammary gland and one uterine horn were fixed in 4% formalin at 4 °C overnight and embedded in paraffin for BrdU immunostaining and determination of uterine epithelial cell height. The ventral third of the right mammary gland and the other uterine horn were rapidly frozen in liquid nitrogen and used for the analysis of target gene induction.

2.4. Determination of mammary and uterine epithelial cell proliferation

Paraffin-embedded uteri and mammary glands were cut in 5-μm thick slices. BrdU immunostaining using the mouse monoclonal anti-BrdU antibody from DAKO (M0744) was performed as described previously [13]. The percentage of BrdU-positive cells in the uterine epithelium was determined by evaluation of 300–500 uterine epithelial cells per animal. In the mammary gland, the amount of BrdU-positive cells was determined by evaluation of all ducts on four different slices per animal.

2.5. Quantitative RT-PCR

RNA was isolated after homogenization of uteri and mammary glands in guanidinium thiocyanate [14]. 5 μg of RNA were digested with DNase I and reversely transcribed with random hexamers using the SuperScript III First-Strand Synthesis System (Invitrogen). Real-time Taqman PCR analysis was performed using the ABI Prism 7700 Sequence Detector System according to the manufacturer's instructions (PE Applied Biosystems). Prevalidated probes and primers for murine INDO (catalog no. Mm00492586_m1), Cytokeratin 18 (catalog no. Mm01601702_g1), LTF (lactotransferrin) (catalog no. Mm00434787_m1), Cyclin E1 (catalog no. Mm 00432367_m1) and TBP (TATA-box-binding protein) (catalog no. Mm00446973_m1) were purchased from PE Applied Biosystems. All experiments were carried out in duplicate. Relative mRNA levels were calculated by the comparative ΔCT-method. In the mammary gland, the expression level of INDO was normalized to Cytokeratin 18, whereas in the uterus the expression level of LTF and Cyclin E1 was normalized to TBP.

2.6. Statistical analysis

Data are represented as mean ± S.D. To analyse whether compounds show a dissociation of bone versus uterine and