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Effects of the special extract ERr 731® from *Rheum rhaponticum* on estrogen-regulated targets in the uterotrophy model of ovariectomized rats

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ABSTRACT

A recent clinical study with a two-year application of the extract ERr 731® from *Rheum rhaponticum* demonstrated its efficacy and potentially suggested it safety regarding unwanted endometrial side effects. The aim of the present study is to provide experimental proof for the latter observation in a preclinical experimental animal model by assessing dose-dependent effects of ERr 731® – either alone or in combination with estradiol (E2) – on growth and proliferation in the uterus of ovariectomized (ovx) rats. ERr 731® was given in a dose corresponding to human therapeutic application and additionally in three pharmacologically relevant doses. In addition to uterine wet weight, this study examines the effects of ERr 731® on the uterine mRNA expression of the proliferation marker Ki67, proliferating cell nuclear antigen (PCNA), insulin-like growth factor-1 (IGF-1), type 1 IGF receptor (IGF-1R), the two estrogen receptor (ER) subtypes α and β (ERα and ERβ) and the estrogen-responsive gene complement C3 (C3). ERr 731® did neither stimulate an uterotrophic response in the uterotrophic assay with ovx rats nor stimulate or modulate the expression of genes associated with proliferation. In combination with E2, ERr 731® reduced the E2-induced uterine growth stimulation. These observations were further substantiated by the expression pattern of genes related to proliferation control, in view of the fact that the E2-induced elevation of Ki67 mRNA and PCNA protein levels in the uterus were counteracted by simultaneous treatment of the animals with ERr 731®. In conclusion, the experimental findings presented here provide further evidence for the safety of ERr 731® towards unwanted uterine and endometrial proliferative events in response to ERr 731® and support observations from recent clinical trials.

1. Introduction

Menopause results from a permanent reduction in the secretion of the ovarian hormones, estrogen and progesterone, and is diagnosed retrospectively after 12 months of amenorrhoea. The decline in the circulating estradiol (E2) levels often results in climacteric complaints, including hot flushes, night sweats, vaginal dryness and the risk of chronic and degenerative diseases such as osteoporosis [1].

Until relatively recently, hormone therapy (HT) was prescribed widely for relieving menopausal symptoms and preventing osteoporosis, but the results of more recent large studies, including the Women's Health Initiative (WHI) study and the Million Women study, have indicated that the risks of combined HT and unopposed estrogen therapy may outweigh the benefits [2,3]. The studies showed that HT is linked to an increased risk of breast cancer and venous thromboembolism. These results have generated growing interest in effective and safe alternatives to the classic HT as treatment regimen of climacteric symptoms. Phytoestrogen-based treatments of menopausal symptoms, for example isoflavones derived from soy or red clover, are among the most commonly used alternatives to HT. However, sufficient information is generally lacking on their efficacy as well as their safety particularly towards potential neoplastic alterations following prolonged use.

The special extract ERr 731® from the roots of *Rheum rhaponticum* (trade name Phytoestrol® N, since 1st September 2007 re-branded as Phyto-Strol® and Phyto-Strol® Loges), was formerly used in Germany for premenopausal women diagnosed with oligomenorrhoea or amenorrhoea. Additionally, it has regularly been taken for the relief of climacteric symptoms without any incidence of safety-related side effects [4]. The clinical efficacy (proof of principle of the biological activity) of ERr 731® was provided in a 12-week double-blind, placebo-controlled clinical trial in 109 perimenopausal women [4]. The study demonstrated that in the ERr 731® group, the Menopause Rating Scale II (MRS II) total score in addition to each of the 11 MRS II criteria representing cardinal symptoms of the climacteric were significantly reduced compared to the placebo group. Additionally, 82 subjects of this clinical trial

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continued intake of ERr 731® in the following 48- and 96-week open-label observational studies which demonstrated the long-term safety of ERr 731®, as no endometrial hyperplasia could be detected and no adverse events related to the study medication occurred [5]. However, in contrast to the clinical effectiveness of ERr 731®, little was known about the molecular mechanism of the extract or its potential metabolites.

R. rhaponticum L., commonly known as rhapontic or Siberian rhubarb, originates from Central Asia and was introduced into Europe in the 17th century. The standardized extract ERr 731® (drug-to-extract ratio 16–26:1, with calciumoxide-to-water 1:38 (mass/mass) as extraction solvent) consists mainly of rhaponticin and desoxyrhaponticin and small amounts of the aglycones *trans*-rhapontigenin and desoxyrhapontigenin (both together about 5%). Natural hydroxystilbenes, including the four hydroxystilbenes: rhaponticin, desoxyrhaponticin, rhapontigenin and desoxyrhapontigenin of ERr 731®, are synthesized in the plant by the same biosynthetic pathway. Resveratrol is the first stilbene compound occurring in this pathway and all other hydroxystilbenes in *R. rhaponticum* L. are derived from it [6].

Only recently, the selectivity of ERr 731® towards the two estrogen receptor (ER) subtypes α and β (ERα and ERβ) was investigated in two human cell culture models representative for the endometrium and bone. ERr 731® activated the ERβ-coupled reporter gene assay in the human endometrial adenocarcinoma cells HEC-1B and the human osteosarcoma cells U2OS. In both cell lines, ERr 731® induced ERβ activation and the effect was completely abolished by the pure anti-estrogen fulvestrant (ICI 182 780), demonstrating that this effect was exclusively mediated through ER activation. In contrast, ERr 731® produced only a weak stimulation of the ERα-coupled reporter gene assay in the U2OS cells [7,8].

As these findings provide a reasonable basis for the *in vitro* mechanism of action, the study presented here focused on investigating potential modulation of growth and proliferation by ERr 731® in the rat uterus *in vivo*. For this purpose, we chose the uterotrophic assay in the ovariectomized (ovx) rat as an experimental model system. The rodent uterotrophic assay is one of the most widely used *in vivo* bio-assays to test estrogenicity by determining the ability of substances to stimulate uterine weight [9]. Following ovariectomy, the uterus undergoes considerable regression to approximately 20% of its original weight. Treatment with estrogens consistently stimulates recovery of ovariectomy-induced uterine weight loss.

In a first experiment, we determined the effects of ERr 731® in ovx rats. One dose of ERr 731® corresponding to the human dosage and three pharmacological doses were tested in comparison with E2 and the uterine wet weight was assessed.

Estrogen-induced uterine proliferation is associated with induction of the expression of a variety of growth factors and cytokines. These factors are thought to mediate and potentiate the estrogen-induced proliferative signal by autocrine and paracrine action [10,11]. One of the best-studied growth factors in the uterine response to E2 is the insulin-like growth factor-1 (IGF-1) [10,12,13]. IGF-1 is regarded as a mediator of E2-induced uterine growth since E2 induces uterine IGF-1 expression in a pattern that correlates with E2-induced cell proliferation. In the process, E2 stimulates the IGF-1 mRNA expression through protein–protein interaction of the ERα–E2 complex with the transcription factor AP-1 on the AP-1 site in the promoter region of the IGF-1 gene [14].

As proteins, which are associated with proliferation, we included for example Ki67 and the proliferating cell nuclear antigen (PCNA). Ki67 is a well-established marker of proliferative activity, because the Ki67 protein is expressed in proliferating cells, but disappears rapidly when cells enter a resting state [15]. PCNA functions as a processivity factor of polymerase δ and ε and is essential for DNA replication [16,17]. Its expression is up-regulated during S phase and it therefore serves as an endogenous marker of cell proliferation.

Consequently, we additionally investigated the effects of ERr 731® on the uterine mRNA expression of IGF-1, type 1 IGF receptor (IGF-1R), the proliferation markers Ki67 and PCNA, the estrogen-responsive gene complement C3 (C3) and ERα and ERβ in ovx rats.

Based on the above-mentioned ERβ specific properties of ERr 731®, which were recently shown *in vitro* [7,8], we hypothesized that the extract might inhibit ERα-induced uterotrophy by activating ERβ, as suggested by Gustafsson et al. in the Yin-Yang hypothesis [18–20]. Therefore, we studied the effects of a co-treatment of ERr 731® with E2 on uterine growth response and the mRNA expression of the marker genes mentioned above.

2. Material and methods

2.1. Substances

17β-Estradiol (E2) was purchased from Sigma–Aldrich (Deisenhofen, Germany).

The extract ERr 731® was provided by Chemisch-Pharmazeutische Fabrik, Carl Müller Apotheker GmbH & Co. KG (Göppingen, Germany).

2.2. Animals

The young adult female Wistar Unilever rats (150 g) were obtained from Harlan-Winkelmann (Harlan-Winkelmann, Borchen, Germany) and were maintained under controlled conditions of temperature (20 ± 1 °C, relative humidity 50–80%) and illumination (12-h light, 12-h dark). All animals had free access to standard rodent diet (Harlan 2019 Rodent Breeding, Harlan-Winkelmann, Borchen, Germany) and water. All animal handling and experimental conditions were in accordance with the Institutional Animal Care and Use Committee guidelines, regulated by the German federal law for animal welfare.

2.3. Treatment of the animals and uterotrophic assay

The animals were ovariectomized and after 14 days of endogenous hormonal decline they were treated subcutaneously (s.c.) once daily for three consecutive days. In a first set of experiments (study 1), animals were treated with E2 (4 μg/kg body weight (bw)/day (d)) and four doses of ERr 731® (0.1, 1, 10 and 100 mg/kg bw/d). In a second set of experiments (study 2), animals were treated with E2 (0.5 μg/kg bw/d) alone or co-incubated with E2 at a suboptimal dose of 0.5 μg/kg bw/d and increasing doses of ERr 731® (0.1, 1, 10 and 100 mg/kg bw/d). Treatment groups were composed of six to eight animals. The animals were randomly selected for treatment and vehicle groups. Substances were dissolved in the carrier castor oil, which was also used as a negative control. After 72 h of treatment, animals were sacrificed by CO_2-inhalation after light anesthesia by O_2/CO_2-inhalation. The uterine wet weight was determined and uteri were snap frozen in liquid nitrogen for later RNA preparation.

2.4. RNA preparation and mRNA quantification

The total RNA was extracted from the rat uteri by peqGOLD TriFast™ according to the manufacturer's instructions (PEQLAB Biotechnologie GmbH, Erlangen, Germany) and the total RNAs of all uteri of the same treatment group were pooled. DNA contamination was enzymatically eliminated by digestion (RQ1 DNase, Promega, Karlsruhe, Germany). The absence of genomic DNA was checked by PCR. For the first-strand cDNA synthesis 3 μg RNA together with

MMLV reverse transcriptase (Promega, Karlsruhe, Germany) and 15mer oligo (dT) primers were used.

Quantitative real-time PCR using an ICycler thermal cycler with iQ real-time detection system (BioRad) was applied for mRNA quantitation. SybrGreen I (Sigma–Aldrich, Taufkirchen, Germany) was used as detection probe. The following primers were used for the real-time PCR experiments:

C3, 5′-ACA GCC TTC CCG GGA GCA TCA ACA-3′ and 5′-AGC GCA CCA CAG GAG GCA CAG AGT C-3′; ERα, 5′-GGAAGCA-CAAGCGTCAGAGAGAT-3′ and 5′-AGACCAGACCAATCATCAGGAT-3′; ERβ, 5′-CTACAGAGAGATGGTCAAAAGTGGA-3′ and 5′-GG-GCAAGGAGACAGAAAGTAAGT-3′; IGF-1, 5′-CTGCTTGCTCACCTT-TACCAG-3′ and 5′-TACATCTCCAGCCTCCTCAGA-3′; IGF-1R, 5′-GTGGAGGAGGTGACAGAAAATC-3′ and 5′-CAAAGATGGAGTTGTG-AAGGAA-3′; Ki67, 5′-AACCAGGACTTTGTGCTCTGTAA-3′ and 5′-CTCTTTTGGCTTCCATTTCTT C-3′; PCNA, 5′-GAGCAA-CTTGGAATCCCAGAACAGG-3′ and 5′-CCAAGCTCCCCACTCGCAG-AAAACT-3′. The Cytochrome-*c*-oxidase subunit I (1A) primers 5′-TGAGCAGGAATAGTAGGGACAGC-3′ and 5′-GAG-TAGAAATGATGGAGGAAGCA-3′ were used for internal control. The expression of all genes was measured at least three times in triplicates using several independently synthesized cDNAs of the same sample. For calculation of the relative rates of gene expression, the $\Delta\Delta C_T$ method [21] was used. Gene expression is shown relative to that in the untreated control animals, which was set to 1.

2.5. Protein extraction and Western blot analysis

The proteins were isolated from the interphase and the phenolic phase of the TriFast™ reagent after chloroform addition and centrifugation according to the manufacturer's instructions (PEQLAB Biotechnologie GmbH, Erlangen, Germany) and the proteins of all uteri of the same treatment group were pooled. Thirty micrograms of total uterine protein were separated by 8.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Immobilon™-P Transfer Membrane, Millipore).

After transfer, membranes were blocked with 5% non-fat dried milk in phosphate buffered saline (PBS) containing 0.1% (v/v) Tween 20 (PBST) as blocking buffer. Membranes were then incubated overnight with the primary antibody anti-PCNA (DakoCytoma) diluted in blocking buffer 1:1000. After three washes with PBST, the membranes were incubated for 1 h with the appropriate secondary antibody conjugated to horseradish peroxidase (HRPO). As a secondary antibody, goat anti-mouse HRPO (Dianova; 1:10,000) was used. Proteins were visualized with an ECL Plus detection kit (Amersham Biosciences) according to the manufacturer's instructions by exposure to Amersham Hyperfilm ECL (Amersham Biosciences). Additionally, membranes were stripped and reprobed with anti-β-actin monoclonal antibody (Sigma) diluted 1:4000 and as secondary antibody, goat anti-rabbit HRPO (Dianova) diluted 1:30,000 was used. Assessment of β-actin was used for normalization. The molecular masses of the detected protein bands were verified using a prestained SDS-PAGE standard (11–170 kDa) from PEQLAB. The films were scanned at GelDoc-It™ Imaging System (UVP BioImaging System) and then transferred to LabWorks image acquisition and analysis software package for quantification by densitometry. The band(s) of interest were identified and the integrated optical density (IOD) representing the amount of PCNA and β-actin respectively was calculated. All Western blot experiments were repeated three times and the mean ± standard deviations (SD) were calculated for the results of each treatment group.

The semi-quantitative results are presented as a ratio of PCNA to β-actin IOD to correct for possible differences of protein loading between samples.

2.6. Statistical analysis

Results of the uterotrophic assay, the quantitative real-time PCR experiments and the densitometric analysis of the protein bands are expressed as mean ± standard deviation (SD). The data were further analyzed by ANOVA followed by the Mann–Whitney-*U*-test and significance was assumed at $p < 0.05$.

3. Results

3.1. Uterotrophic assay

In the first set of experiments (i.e. study 1), the effects of E2 or ERr 731® on the relative uterine wet weight in parallel groups of ovx rats were investigated (Fig. 1A). For this study, an E2 dose that is known to provide the maximal stimulating response was selected, since it is four-fold higher than the dose of ethinylestradiol used in the s.c. treatment arm of the validation program of the OECD guidelines for the uterotrophic assay [22,23]. The vehicle treated ovx control animals had an average relative uterine wet weight of 426 mg/kg bw after three days. With the E2 treatment (4 μg/kg bw/d), the relative uterine wet weight increased significantly to 2455 mg/kg bw after three days. In contrast, the average relative uterine wet weight was not significantly affected by the treatment with any of the four doses of ERr 731®.

In the second set of experiments (i.e. study 2), the effects of E2 alone and the co-treatment with the four doses of ERr 731® on the

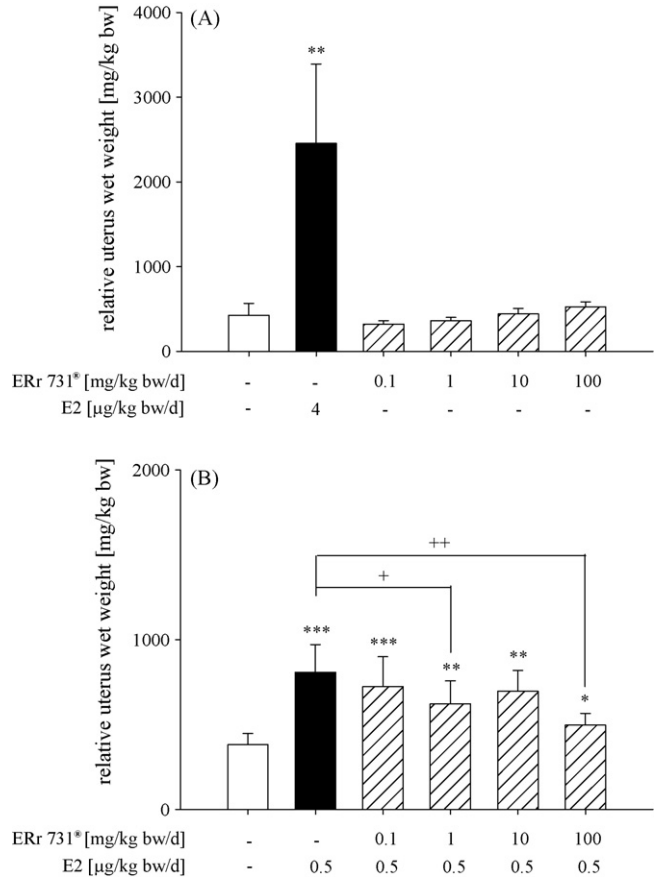

Fig. 1. Uterotrophic assay. Relative uterine wet weights of Wistar rats after three days of treatment with E2 (4 μg/kg bw/d) and ERr 731® (A) and after combinatorial treatment with E2 and ERr 731® (B). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significant differences in comparison to the untreated control group (Co); $^{+}p < 0.05$ and $^{++}p < 0.01$ indicate significant differences in comparison to the E2 treated group.

relative uterine wet weight in ovx rats were investigated (Fig. 1B). For E2, a suboptimal dose of 0.5 μg/kg bw/d was selected.

The vehicle treated ovx control animals had an average relative uterine wet weight of 382 mg/kg bw after three days. The treatment of the animals with E2 resulted in a significant increase in the relative uterine wet weight to 808 mg/kg bw after three days. This increase in uterine weight was partially inhibited by the co-treatment with all tested doses of ERr 731®, whereby the difference

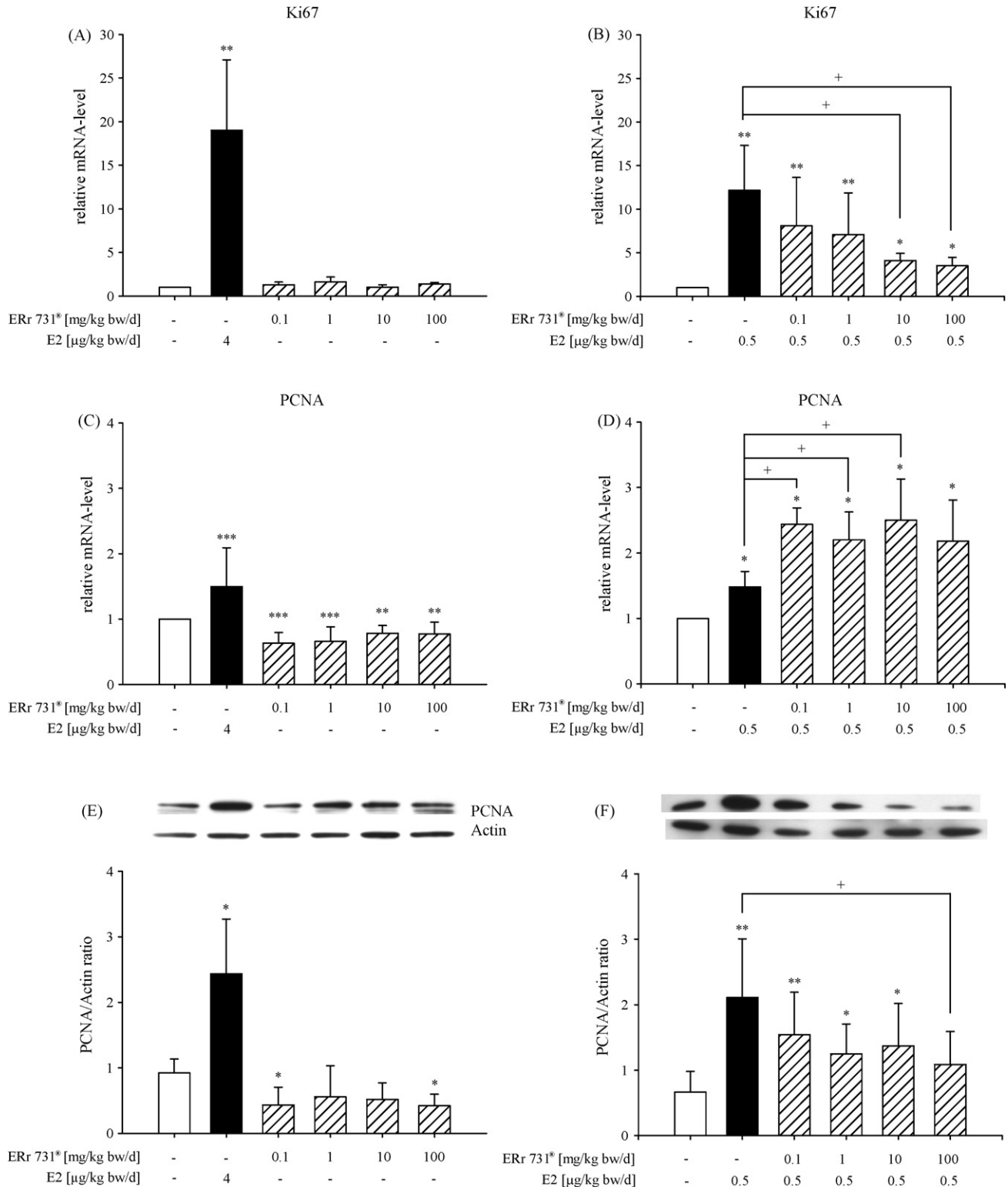

Fig. 2. Expression analysis of the proliferation markers. Effects of E2 (4 μg/kg bw/d) and ERr 731® on the uterine mRNA expression levels of Ki67 and PCNA (A and C) and protein level of PCNA (E). Effects of E2 and the combination of E2 and ERr 731® on uterine mRNA expression levels of Ki67 and PCNA (B and D) and protein level of PCNA (F). For determination of protein levels one representative Western blot analysis of PCNA and β-actin and the densitometric analysis of PCNA bands normalized to corresponding β-actin bands are depicted. Results represent the mean ± standard deviation of integrated optical density (IOD) of at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significant differences in comparison to the untreated control group (Co); $^{+}p < 0.05$ indicates significant differences in comparison to the E2 treated group.

in uterine weight following co-treatment of E2 with ERr 731® in the doses of 1 and 100 mg/kg bw/d as compared to the uterine weight following E2-only treatment reached significance ($p < 0.05$, $p < 0.01$).

3.2. Expression of genes associated with proliferation

As expected, the administration of E2 (4 μg/kg bw/d) resulted in a strong up-regulation of Ki67 and PCNA mRNA expression levels (Fig. 2). Interestingly, the treatment with ERr 731® resulted in a down-regulation of the PCNA mRNA expression levels below the level of the control (Fig. 2C). Ki67 mRNA expression levels were only very weakly up-regulated in response to three pharmacological doses of ERr 731® (Fig. 2A).

Following co-treatment of E2 with different doses of ERr 731® an increase in the Ki67 mRNA expression was observable, but with increasing doses of ERr 731®, the effect of E2 on the Ki67 mRNA expression levels was partially inhibited in a dose-dependent manner (Fig. 2B). The Ki67 mRNA expression levels differed significantly between the E2-only treatment and the co-treatment of E2 with ERr 731® in the doses of 10 and 100 mg/kg bw/d ($p < 0.05$).

The PCNA mRNA expression was found to be up-regulated in response to the co-treatment of E2 with all doses of ERr 731® (Fig. 2D). Regulation of PCNA mRNA expression by estrogens usually follows a fast temporal pattern with a peak of induction 24 h after treatment [24]. Therefore, it is important to additionally assess the PCNA protein levels at the same time point. The E2 treatment induced a significant up-regulation of the PCNA protein level (Fig. 2E). In contrast, the treatment with the different doses of ERr 731® caused a decrease of the PCNA protein level below the control, which corresponds to the mRNA expression pattern (Fig. 2C). The co-treatment of E2 with different doses of ERr 731® tendentially reduced the E2-induced increase in PCNA protein level in contrast to the PCNA mRNA expression. This tendency became significant at a dose of 100 mg/kg bw/d ($p < 0.05$) (Fig. 2F).

In addition, we investigated the expression of IGF-1 and IGF-1R as candidate genes potentially involved in mediating uterine growth responses. The administration of E2 (4 μg/kg bw/d) resulted in a strong up-regulation of IGF-1 mRNA expression (Fig. 3A), whereas the treatment with ERr 731® did not cause any significant changes.

In response to the co-treatment, the IGF-1 mRNA expression levels reached a level similar to that of the E2-only treatment (Fig. 3B).

The IGF-1R mRNA expression was strongly down-regulated by the E2 treatment (Fig. 3C). In the experimental groups treated with the two lower doses of ERr 731® (i.e. 0.1 and 1 mg/kg bw/d), the mRNA expression levels of IGF-1R remained nearly constant, whereas the higher doses (i.e. 10 and 100 mg/kg bw/d) caused a significant decrease of the IGF-1R mRNA expression.

The co-treatment with E2 and at least three of the four doses of ERr 731® resulted in a down-regulation of the IGF-1R mRNA expression even below those levels detectable in the E2-only group (Fig. 3D). In particular, the IGF-1R mRNA expression levels differed significantly between the E2-only treatment and the co-treatment of E2 with ERr 731® in the doses of 0.1 and 10 mg/kg bw/d ($p < 0.05$).

3.3. Expression of both ER subtypes and of complement C3

The treatment with E2 alone markedly down-regulated the uterine mRNA expression of both ERs (Fig. 4). While the two lower doses of ERr 731® did not change the mRNA expression of ERα, it did induce a down-regulation in the two higher doses (Fig. 4A). Even at the lowest dose of ERr 731® (0.1 mg/kg bw/d), an increase in ERα mRNA expression was observed which, however, was not statistically significant.

In contrast to E2, ERr 731® did not consistently alter the mRNA expression of ERβ. Only at the highest dose of ERr 731® (100 mg/kg bw/d) was a significant down-regulation of ERβ mRNA expression observed to a degree comparable to that of E2 (Fig. 4C).

With the co-treatment of E2 and ERr 731®, the mRNA expression levels of ERα remained at a level similar to that found for the E2-only treatment (Fig. 4B).

Regarding ERβ, its mRNA expression was significantly reduced by the co-treatment of E2 with ERr 731® below the level of the

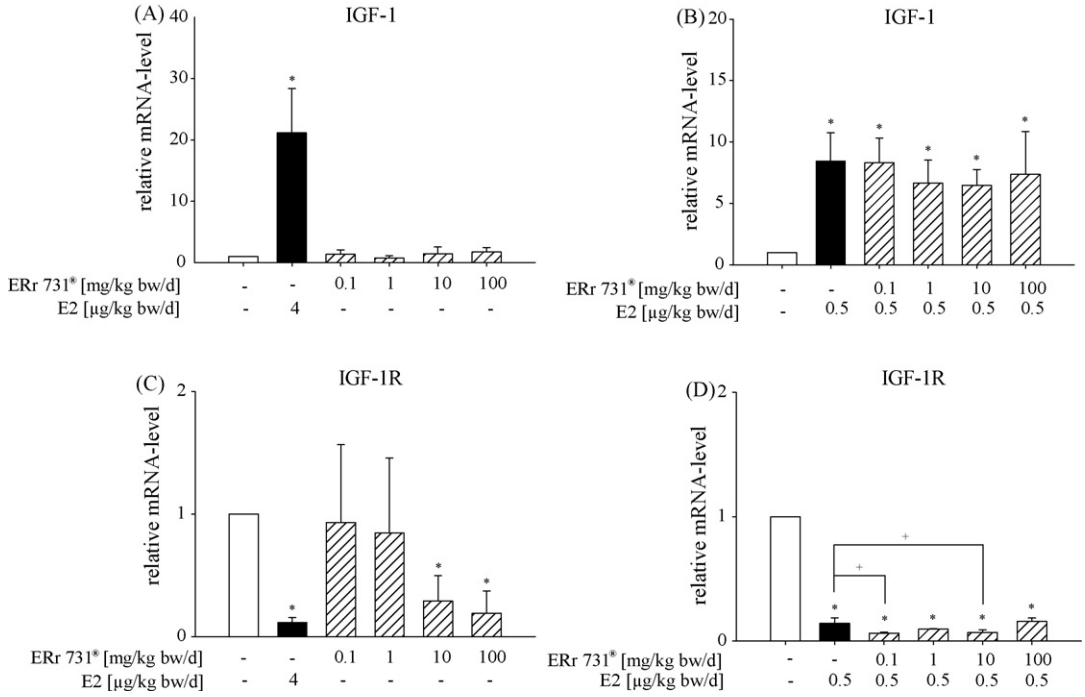

Fig. 3. Expression of proliferation related genes. Effects of E2 (4 μg/kg bw/d) and ERr 731® on uterine mRNA expression levels of IGF-1 and IGF-1R (A and C). Effects of E2 and combination of E2 and ERr 731® on uterine IGF-1 and IGF-1R mRNA expression levels (B and D). *$p < 0.05$ indicates significant differences in comparison to the untreated control group (Co); +$p < 0.05$ indicates significant differences in comparison to the E2 treated group.

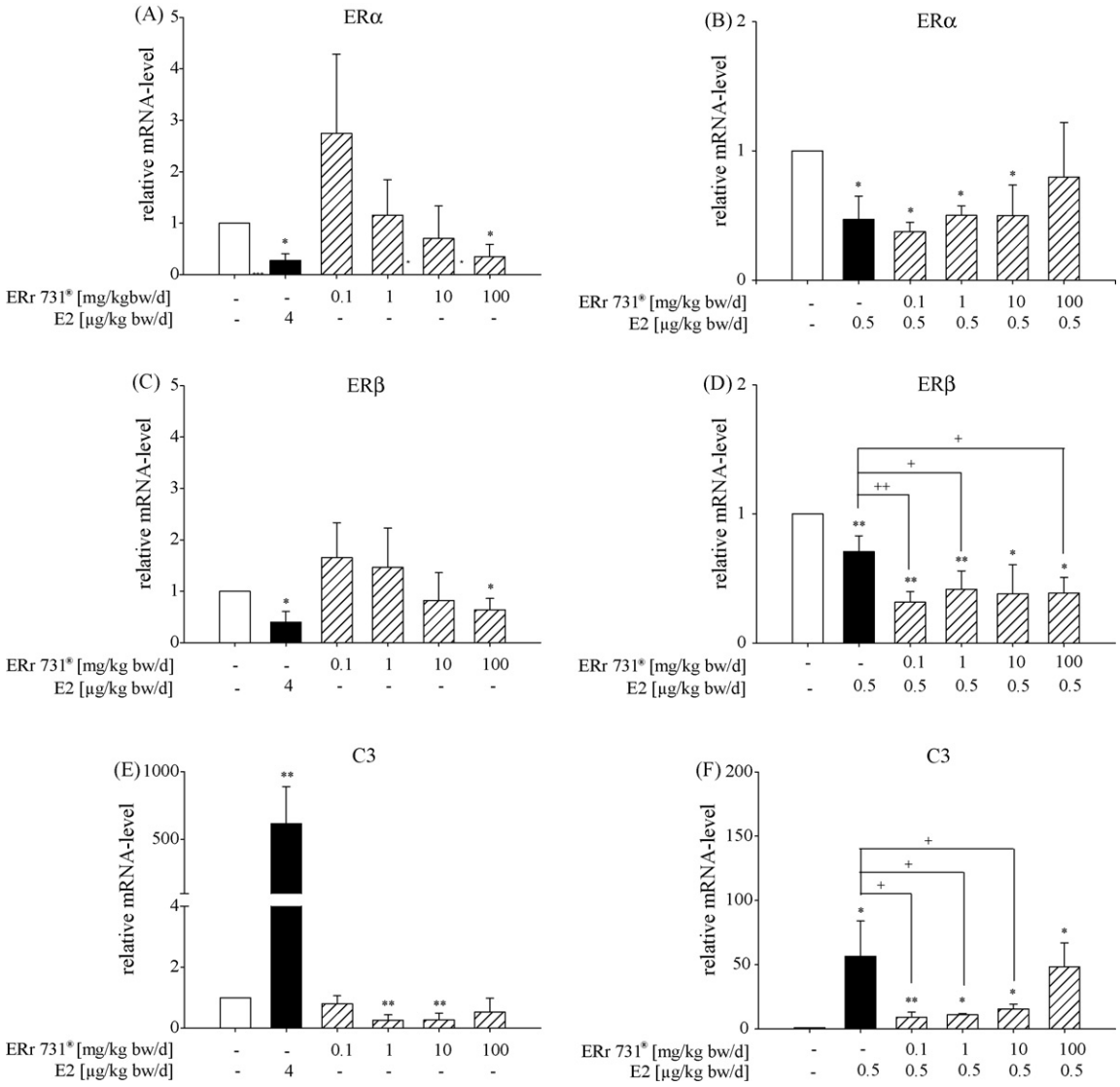

Fig. 4. Expression of the both estrogen receptor subtypes and of complement C3 in the uterus. Effects of E2 (4 µg/kg bw/d) and ERr 731® on uterine mRNA expression levels of ERα, ERβ and C3 (A, C and E). Effects of E2 and combination of E2 and ERr 731® on uterine mRNA expression levels of ERα, ERβ and C3 (B, D and F). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significant differences in comparison to the untreated control group (Co); $+p < 0.05$ indicates significant differences in comparison to the E2 treated group.

E2-only treatment (Fig. 4D). In particular, the ERβ mRNA expression levels differed significantly between the E2-only treatment and the co-treatment of E2 with ERr 731® in the doses of 0.1, 1 and 10 mg/kg bw/d ($p < 0.05$, $p < 0.01$).

The E2 treatment induced a significant up-regulation of the C3 mRNA expression, whereas the treatment with ERr 731® caused a decrease of the C3 mRNA expression below the level of the control (Fig. 4E).

Following co-treatment of E2 with different doses of ERr 731® an increase in the C3 mRNA expression was observable, but the effect of E2 on the C3 mRNA expression levels was almost completely inhibited at the doses of 0.1, 1 and 10 mg/kg bw/d ERr 731® ($p < 0.05$) (Fig. 4F).

4. Discussion

The absence of stimulation of an increased uterine wet weight in the uterotrophic assay is a very important indicator of the safety of estrogenic compounds or herbal extracts regarding the promotion of proliferative alterations within the uterus, which may eventually lead to neoplastic alterations of the endometrial tissue. Furthermore, effects on uterine expression of genes associated with proliferation are considered as more sensitive markers for growth stimulatory effects than the uterine wet weight alone. Hence, the aim of this study was to determine the effects of both therapeutically and pharmacologically relevant doses of ERr 731® on the uterus of estrogen deprived rats to assess any uterotrophic activity of ERr 731®. It was of particular interest to investigate its ability to modulate the uterine expression of ERs and selected genes associated with proliferation. Single treatment of different groups of rats with four different doses of ERr 731® over a period of three days was used to test potential estrogenic effects. In addition, co-administration of the same four doses of ERr 731® with a suboptimal dose of E2 was used to assess possible anti-estrogenic properties.

The effects of E2 observed in this study were consistent with previous studies [24,25] showing that E2 significantly up-regulated the uterine wet weight. In contrast, ERr 731® did not affect the uterine wet weight at any dosage.

The molecular mode of action behind this observation may be explained by the high preference of ERr 731® for ERβ compared to ERα and the lack of any activity on ERα in endometrial cells [7,8]. Similar observations have been reported by Kuiper et al. for various other phytoestrogens, e.g. genistein, daidzein and coumestrol [26]. The lack of stimulation of uterine proliferation by ERr 731® is consistent with the findings that ERβ-selective compounds, e.g. diarylpropylnitrile (DPN) [27] or the synthetic ERβ-selective drug, ERB-041 [28], do not elicit any proliferative effects on the uterus.

It is known that the largest difference between ERα and ERβ protein resides in the N-terminal A/B domain, being identical in only 18% of the amino acid. These differences suggest that the transcriptional activation of different estrogen-responsive genes by ERα and ERβ might show distinct or even opposing mRNA expression patterns [29,30].

Two important parameters that determine the transcriptional potency and agonist/antagonist character of a ligand are: (i) the affinity of the ligand for the receptor and (ii) the conformational change induced by the ligand after its binding to the receptor [31]. Thus it might be possible that the active constituents of ERr 731® interact weakly with ERα, but do not elicit the conformational changes required for dimerization. Furthermore, the opposite transcriptional actions of E2 and ERr 731® probably result from differences in their ability to recruit co-regulators and elicit transcriptional functions of ERα or ERβ. We, therefore, think that the active constituents of ERr 731® selectively recruit co-regulators to ERβ in a way similar to that described for isoflavones [32].

To investigate whether the lack of a uterotrophic response of ERr 731® can be confirmed on a much more sensitive level of gene expression, we studied the mRNA expression of the proliferation markers Ki67 and PCNA and the mitogen IGF-1, which is a potential mediator of the E2-mediated effects on uterine growth [33]. As previously described, E2 up-regulated the mRNA expression of Ki67, PCNA and IGF-1 [12,13,24,34–36]. In contrast, ERr 731® did not affect the mRNA expression of IGF-1, affected the mRNA expression of Ki67 only very slightly and down-regulated the PCNA mRNA expression levels. These data were further substantiated by the decrease of the PCNA protein levels following ERr 731® treatment at all doses (Fig. 2E).

These results clearly demonstrate that ERr 731® does not induce the basic mechanisms involved in the regulation of cell proliferation or growth control, thus confirming and expanding the results of the uterotrophic assay in which ERr 731® did not stimulate a relative uterine wet weight gain.

Diel et al. [25] demonstrated that E2 treatment of ovx rats induces a down-regulation of the IGF-1R mRNA expression. The results of the present study regarding the effects of E2 confirm this finding and additionally show a down-regulation of the IGF-1R mRNA expression. This effect could either be the result of the E2-induced up-regulation of IGF-1 leading to a ligand-dependent down-regulation of its receptor, which has been demonstrated in human fibroblasts and IM-9 lymphocytes [37,38], or it could be a direct inhibitory effect of E2 on the expression of IGF-1R. At therapeutically relevant doses, ERr 731® had no effect on the IGF-1R mRNA expression, but at higher pharmacological doses, ERr 731® treatment correlated with a reduction of the IGF-1R while IGF-1 mRNA levels remained unchanged. This implies a direct effect of ERr 731® on IGF-1R mRNA expression as opposed to the above-mentioned feedback response. The reduced IGF-1R mRNA levels may result in a reduced response to IGF-1. Because IGF-1 is regarded as a potential mediator of the E2 effects on uterine proliferation this may in part explain why ERr 731® has no uterotrophic effect.

Since ERs are key components of mediating estrogenic responses in the uterus, the effects of ERr 731® on the mRNA expression of both ERs were investigated as well. As was observed in previous studies in ovx rats, E2 treatment resulted in a significant down-regulation of the mRNA expression of both ERs representing a negative feedback mechanism [24,36]. ERr 731® treatment did not significantly change the ERβ mRNA level at the doses of 0.1–10 mg/kg bw/d. Only a very mild trend towards a down-regulation of uterine ERβ mRNA levels was observed at the highest dose of 100 mg/kg bw/d. In contrast, it induced a significant down-regulation of the ERα mRNA expression at the highest dose of 100 mg/kg bw/d. In other words, the ERα/ERβ ratio changed dose-dependently in favor of increasing the relative amount of ERβ relative to ERα (Table 1).

To evaluate the estrogen potency of ERr 731®, the mRNA expression of the classical estrogen-responsive gene C3 was determined. Previous studies have demonstrated that E2 treatment resulted in a strong up-regulation of the C3 mRNA expression [39,40]. It is known, that the C3 promoter region contains three estrogen-responsive elements (EREs), whereby the C3 mRNA transcription is induced in the presence of estrogens via the EREs in the promoter region [41].

Therefore, an up-regulation of C3 mRNA expression most probably represents an estrogenic effect and a down-regulation may imply anti-estrogenicity. Although representing a major estrogenic response gene in the rat uterus, estrogen ablation never leads to undetectable C3 mRNA levels. It is well known that nuclear receptors can serve as unliganded transcription factors as well, e.g. following partial activation by phosphorylation initiated by membrane receptor dependent signaling cascades [42]. Interestingly enough, this growth factor initiated activation can be inhibited in the presence of anti-estrogens [11,43].

Whether or not residual estrogen responsiveness regarding C3 expression represent the presence of low levels of estrogens or reflects, e.g. activity of receptor tyrosine pathways remains unclear. However, a down-regulation of C3 mRNA levels below baseline levels in ovx animals as shown here for ERr 731® was previously detected for the pure anti-estrogen fulvestrant [44] and for 7-(O-prenyl)naringenin-4′-acetate in a three-day uterotrophic assay [36].

ERr 731® effectively blocked the E2-induced up-regulation of C3 mRNA expression and in addition it was able to decrease C3 mRNA expression below baseline level when applied by itself. These effects of ERr 731® on the C3 mRNA expression suggest anti-estrogenic properties.

Ultimately, it can be stated that the ERr 731® treatment does not mimic estrogenic growth responses, an observation which in

Table 1
Ratio of the relative mRNA expression rates of ERα and ERβ with the expression in the carrier treated control group set to 1.

Treatment group	Relative mRNA-level ERβ	Relative mRNA-level ERα	Ratio of relative ERβ and ERα mRNA levels
Control	1	1	1
E2	0.4 ± 0.21	0.34 ± 0.033	1.17
ERr 731® 0.1	1.65 ± 0.68	2.75 ± 1.54	0.6
ERr 731® 1	1.47 ± 0.76	1.16 ± 0.69	1.26
ERr 731® 10	0.82 ± 0.54	0.57 ± 0.014	1.43
ERr 731® 100	0.64 ± 0.22	0.35 ± 0.24	1.82

turn serves as a strong argument for the clinical safety regarding endometrial hyperplasia.

The *in vitro* data from ERr 731®, which we have published recently [7,8], suggest an ERβ-selective effect of the extract and its constituents. Several studies have investigated the potential for ERβ-agonists to block the E2-induced increase in uterine wet weight. Malamas et al. [45] described that these compounds were not able to block the E2-mediated uterotrophic response. On the other hand, Frasor et al. [46] reported that the ERβ-selective agonist DPN diminished the uterine weight gain induced by the ERα-selective agonist propyl pyrazole triol (PPT) [47].

It is known that loss of ERβ accompanies proliferative lesions in the diseased mammary gland [48] and prostate [49,50]. Furthermore, knock-out of ERβ leads to hyper-responsiveness of the uterine glands to E2, with exaggerated uterine secretion of proteins, including growth factors [51]. These observations suggest a modulatory role of ERβ in an estrogen-induced proliferation in line with the above-mentioned Yin-Yang hypothesis of ER action [18–20].

With the co-treatment of ovx rats with ERr 731® and E2, the hormonal status of pre- or perimenopausal women with low residual E2-levels was mimicked. The question was whether the ERβ-selective properties of ERr 731® observed *in vitro* could have an impact on the uterotrophic response to E2.

Indeed in combination with E2, ERr 731® treatment partially counteracted E2-induced responses. ERr 731® caused a decrease of the E2-induced stimulation of the relative uterine wet weight and gene-specific effects on the mRNA levels of the investigated genes. It tends to reduce the E2-induced increase in Ki67 mRNA expression levels, C3 mRNA expression levels and PCNA protein level, and it had no impact on the E2-induced up-regulation of IGF-1 and the E2-induced down-regulation of ERα. While these properties are all indications of E2-antagonism, two effects were observed where ERr 731® treatment enhanced E2-responsiveness of proliferation related endpoints. ERr 731® weakly enhanced the E2-induced up-regulation of the PCNA mRNA expression and the E2-induced down-regulation of the IGF-1R mRNA expression.

The ERβ-selective agonist DPN also showed differential effects on the mRNA expression of several genes if used in co-treatment protocols with the ERα-agonist PPT in juvenile mice [46]. In this study, DPN reduced PPT-induced uterine weight gain. In addition, it had no effect on complement C3 or lactoferrin expression, but did have an additive effect with PPT on progesterone receptor and androgen receptor mRNA down-regulation.

These results suggest that the ERβ selectivity of ERr 731® observed *in vitro* may indeed be directly related to the anti-proliferative effects *in vivo*. One explanation might be that ERβ could inhibit estrogen-induced proliferation by inhibiting ERα activation of the cyclin D1 gene and other pro-proliferative targets [30]. Others propose that the inhibitory effects of ERβ on ERα function is related to formation of ERβ/ERα heterodimers [52,53], thereby modulating ERα functions. These observed effects of ERr 731® are consistent with the ERβ-selective properties determined *in vitro,* but especially the inhibitory effect of ERr 731® on the induction of C3 expression by E2 indicates, that contrary to the observed effects *in vitro*, ERr 731® may have some anti-estrogenic properties *in vivo*.

ERr 731® in combination with E2 acted in an additive manner on the ERβ mRNA expression. It enhanced the weak decrease of ERβ mRNA expression following E2 treatment. Ultimately, we show an uncommon mechanism of functional interaction of E2 and natural compounds which needs to be investigated in depth in the near future.

ERr 731® seemed unable to counteract the E2-induced up-regulation of the PCNA mRNA expression and the co-treatment even increased the PCNA mRNA levels after 72 h. Diel et al. [24] demonstrated, that the PCNA mRNA expression follows a fast temporal pattern in which the PCNA mRNA expression is transiently but intensively stimulated by E2 after 7 and 24 h of treatment. However, after 72 h of treatment, like in this study, PCNA expression has been found to bed declined to a lower level again. It may be speculated that the combined treatment with E2 and ERr 731® delays PCNA mRNA turnover by for example a stabilizing effect on the mRNA. Nevertheless this mechanism remains to be investigated in depth.

However, this up-regulation of PCNA mRNA expression resulted neither in elevated protein levels for this proliferation marker in Western blot analysis nor in an increased uterotrophic response as has been shown by assessment of the uterine wet weight.

In conclusion, this report demonstrates for the first time that ERr 731® alone does neither stimulate a uterotrophic response in the classical uterotrophic assay with ovx rats nor stimulate uterine proliferation. Furthermore, the effects of ERr 731® on E2-stimulated endpoints such as uterine wet weight, mRNA expression and protein level were inhibitory to a considerable extent.

With respect to two endpoints, the extract had no influence on the E2-induced effects. Regarding another two endpoints a co-stimulatory effect to E2 treatment could be observed. However, and most importantly, ERr 731® reduced the E2-induced effects on proliferation markers as shown for Ki67 mRNA expression and PCNA protein expression and on estrogen-responsive gene C3.

These experimental findings with ERr 731® in this well-established *in vivo* model system support and expand the *in vitro* results concerning ERβ selectivity in a considerable manner, hint towards some anti-estrogenic properties *in vivo* and provide further support for the safety data on the uterine and endometrial safety of ERr 731® arising from the recent clinical trials.

Conflict of interest

The authors declare that there is no other conflict of interest that would prejudice its impartiality.

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