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Time dependency of uterine effects of naringenin type phytoestrogens *in vivo*

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Abstract

Phytoestrogens exhibit significant estrogen agonistic/ antagonistic properties in animals and humans. Naturally occurring flavonoids with a naringenin backbone like 8-prenylnaringenin (8-PN) and 6-(1,1-dimethylallyl)naringenin (6-DMAN) are considered to be some of the most potent phytochemicals activating nuclear

receptors. 8-PN is a more potent estrogenic substance while 6-DMAN appears to have a higher antiandrogenic potency, however these are less well characterized compared to other phytoestrogens such as genistein. The aim of this study was to assess the estrogenic properties of 8-PN and 6-DMAN in an ovariectomized *in vivo* rat model. 8-PN and 6-DMAN were applied at concentrations of 15 mg/kg bw. We assessed the uterotrophic response after 7h, 24h and 72h of treatment. In contrast to 8-PN, 6-DMAN did not alter uterine wet weight or the level of expression of proliferation markers at any time point. In contrast to the uterotrophic response, 6-DMAN stimulated uterine mRNA expression of estrogen responsive genes carrying an estrogen response element (ERE) in the ovariectomized rats, but to a lesser extent than E2 and 8-PN. In all treatment regimens the mRNA expression of estrogen receptors α and β mRNA was measured. In summary, we assessed the time dependent uterine responses and estrogenic activities of 6-DMAN and 8-PN. In contrast to 8-PN which mimicked the E2 induced responses on uterine wet weight and gene expression, 6-DMAN has no uterotrophic effect and only regulated the mRNA expression of genes carrying an ERE. Therefore, 6-DMAN is an exciting candidate molecule for future investigations and potentially a natural occurring selective estrogen receptor modulator.

Keywords: Phytoestrogens, 6-(1,1-dimethylallyl)naringenin, 8-prenylnaringenin, Uterotrophy, Estrogen receptor, Estradiol

1. Introduction

The environmental estrogens from plants called phytoestrogens (PEs), have received extensive attention, for more than 60 years now, due to their widespread occurrence in a variety of products to which humans and animals are exposed to on a regular and sometimes continuous basis (Bennetts et al., 1946; Brown and Setchell, 2001; Singleton and Khan, 2003). Over the last few years, public as well as scientific interest in phytoestrogens, like genistein, has intensified due to their potential health benefits as soy supplements and health foods containing concentrated amounts of phytoestrogens are being marketed extensively. However, PEs are a diverse group of plant-derived compounds that structurally or functionally mimic mammalian estrogens partial or in total and therefore may have a significant impact on human health (Cos et al., 2003; Ososki and Kennelly, 2003). However,

regarding their use as pharmacological compounds, there is still an ongoing risk/benefit debate. Estrogenic effects are mostly mediated through the estrogen receptors (ER). Since the discovery of a second ER β (to differentiate from the original, subsequently named ER α (Kuiper et al., 1996; Byers et al., 1997), led to a re-evaluation of the physiology and toxicology of the estrogen signaling system. The biological significance of the two ER subtypes still remains unresolved, but may explain the selective and diverse effects of estrogens occurring in different target tissues. Whereas ER α seems to be the prevalent ER subtype in the female reproductive tract, levels of both ERs are easily detectable in the gonads (Byers et al., 1997; Saunders et al., 1997). The preference of PEs for ER subtypes (Kuiper et al., 1998), leading to a potential organ selectivity of their actions and therefore being a potential naturally occurring selective estrogen receptor modulator (SERM), is of high pharmacological relevance. SERMs are distinguished from pure receptor agonists and antagonists. Their function is estrogen-like, but in contrast to the natural ligand estradiol they act in an organ selective and gene specific way (Vollmer and Zierau, 2004). The estrogenic effects of specific phytoestrogens have been characterized to different degrees (Whitten and Patisaul, 2001).

Phytoestrogens have been proposed as an alternative to classical hormone replacement therapy (HRT) treatment regimes. However, despite a vast number of studies published so far, evidence of the clinical effectiveness of PEs is very limited. A current metaanalysis proved that the beneficial effect of PEs on menopausal complaints are comparably small (Krebs et al., 2004). On the other hand, high dietary intake from soy PEs has been demonstrated to be connected to positive effects on (peri-)menopausal complaints (Nagata et al., 2001).

A group of recently described relatively potent phytoestrogens are of the naringenin type (Milligan et al., 1999; Milligan et al., 2002; Zierau et al., 2002). Especially 8-prenylnaringenin (8-PN) from hops, *Humulus lupulus* (L.) and beer, initially described as 8-isopentenyl naringenin (Kitaoka et al., 1998) has been characterized as an extremely potent phytoestrogen (Kitaoka et al., 1998; Milligan et al., 1999; Schaefer et al., 2003). The estrogenic activity of 8-PN *in vitro* is the highest of all plant derived estrogens known so far (Matsumura et al., 2005). Another of these “novel phytoestrogens” is the flavonoid 6-(1,1-dimethylallyl)naringenin (6-DMAN) (Fig. 1) found so far only in the leaves of the African tree *Monotes engleri* (Gilg.) (Seo et al., 1997). We recently have described the estrogenic (Zierau et al., 2002) and

antiandrogenic properties of 6-DMAN (Zierau et al., 2003). For both naringenins low toxicity and weak cytostatic properties were reported, making them good candidate molecules for further studies (Tokalov et al., 2004). 8-PN has been demonstrated to be a potent ER α selective phytoestrogen (Schaefer et al., 2003). For 6-DMAN the selectivity towards the two ER subtypes has not been investigated so far. The activity spectrum of a number of 8-alkylnaringenins spans from full E2-agonism to partial agonism to antagonism, including diverse ER-subtype activity, only depending on the length and the bulkiness of the substituent (Roelens et al., 2006). In this study, to further elucidate the molecular basis of the difference of the estrogenic properties of 6-DMAN and 8-PN. To evaluate the specificity of these compounds for the ER α and - β we tested them using a reporter gene assay in bone-derived *U2OS cells* as has been described before (Moller et al., 2007). In a second setting the estrogenic properties of 6-DMAN and 8-PN have been analyzed *in vivo* and compared to the estrogenic activity of E2. We performed a time resolved uterotrophic assay in ovariectomized female Wistar rats. The rats were treated with 15 mg/kg BW of 8-PN or 6-DMAN per day, the positive control was E2 at 10 μ g/kg BW per day and the negative control was the carrier castor oil. Animals were treated time dependently for 7 h, 24 h and 72 h to assess the uterotrophic response profile of the substances. The genes for the real-time-PCR quantification were chosen to reflect a range of target processes and regulatory mechanisms. Furthermore they allow for comparability with previous studies (Diel et al., 2004; Zierau et al., 2004; Kretzschmar et al., 2007).

2. Materials and Methods

2.1. Substances

17 β -estradiol (E2) was obtained from Sigma (Deisenhofen, Germany), 6-(1,1-dimethylallyl)naringenin (6-DMAN) and 8-prenylnaringenin (8-PN) were synthesized from naringenin as described previously (Gester et al., 2001). The purity of the used compounds was assessed to be >99 % (gas chromatography and HPLC). 6-(1,1-dimethylallyl)naringenin and 8-prenylnaringenin were used as racemic mixtures.

2.2. Cell culture systems and plasmids

U2OS cells were from ATCC/Promochem (Wesel, Germany). U2OS cells stably transfected with the ER α (U2OS-ER α cells), the ER β expression plasmid and the (ERE)2-tk-Luc reporter plasmid were kindly provided by Dr. Luisella Toschi (Schering AG, Berlin).

2.3. Reporter gene assays in U2OS cells

U2OS cells were stably transfected with ER α (U2OS-ER α cells) and cultured routinely in DMEMF12 medium containing 10% fetal calf serum (FCS) and 0.5 mg/ml G418 (Calbiochem/VWR, Darmstadt, Germany). Experiments were performed in DMEM-F12 Medium containing 5% dextran coated charcoal (DCC) stripped FCS and 0.5 mg/ml G418. For transfection, U2OS-ER α cells were transferred to a 24 well plate (30,000 cells/well) and transfected with 100 ng of the (ERE)2-tk-Luc reporter plasmid using a liposome protocol (DOTAP; Roth, Karlsruhe, Germany) and a DOTAP:DNA ratio of 3:1. Luciferase activity was measured with a commercial kit (Promega, Mannheim, Germany) according to the manufacturer's instructions. Reporter gene assays in U2OS cells transiently transfected with ER β (U2OS-ER β cells), which do not express any ERs, were cultured routinely in DMEM-F12 Medium containing 10% FCS. Experiments were performed in DMEM-F12 Medium containing 5% DCC. For transfection, U2OS cells were transferred to a 24 well plate (45,000 cells/well) and co-transfected with 100 ng of the ER β expression plasmid and 600 ng (ERE)2-tk-Luc reporter plasmid using a liposome protocol (DOTAP; Roth, Karlsruhe, Germany) and a DOTAP:DNA ratio of 3:1. Luciferase activity was measured with a commercial kit (Promega, Mannheim, Germany) according to the manufacturer's instructions.

2.4. Treatment of cell cultures with the test substances

All test substances were dissolved initially in DMSO and were used at a final DMSO concentration of 0.1%. All assays were performed with different concentrations of the test substances specifically adapted to the respective assay in order to obtain dose–response relationships (see legends to Figures).

2.5. Animals

Juvenile female Wistar rats (150 g) were obtained from Charles River (Charles River Laboratories, Sulzfeld, Germany) and were maintained under controlled conditions of temperature ($20^{\circ}\text{C} \pm 1$), relative humidity (50-80%) and illumination (12 h light, 12 h dark). All rats had free access to standard rat diet (SSniff R10-Diet, SSniff GmbH, Soest, 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.

2.6. Uterotrophic assay

Animals were ovariectomized (ovx). After 14 days of endogenous hormonal decline the animals were treated for 7 h, 24 h and 72 h s.c. with the respective compounds. Negative control was the carrier castor oil. The animals were randomly selected for treatment and vehicle groups ($n = 6$). E2, 8-PN and 6-DMAN were administered s.c. with doses of 10 $\mu\text{g/kg/d}$ E2 and 15 mg/kg/d for the PEs dissolved in carrier castor oil. These values correspond to the most potent doses of 6-DMAN and 8-PN tested in previous *in vivo* experiments (Diel et al., 2004; Zierau et al., 2004). The treatment of the animals was carried out two hours after the start of the light period of the day and subsequently the animals were sacrificed by CO_2 inhalation after light anaesthesia with O_2/CO_2 inhalation after 7 h, 24 h and 72 h of treatment respectively. The uterus was excised, trimmed free of fat and cut just above its junction with the cervix and wet weight was determined. Thereafter the uteri were snap frozen in liquid nitrogen for RNA preparation.

2.7. RNA-preparation, reverse transcription and Real-time PCR

The total cytoplasmic RNA was extracted from the rat uteri by the standard TRIzol® method (Life Technologies). DNA residues were enzymatically eliminated by digestion (Deoxyribonuclease I, Ambion) and removal was checked by PCR. SUPERSRIPT™ II Reverse Transcriptase (Life Technologies) and Oligo (dT) 12-18 were used for the first-strand cDNA synthesis

2.8. mRNA quantification using quantitative real time PCR

Quantitative real-time PCR was carried out by Platinum® Taq DNA polymerase (Life Technologies) using the iCycler Thermal Cycler with iQ real-time Detection System. The reactions were run three times in triplicates. After vortexing 50 µl aliquots of the mix was pipetted in each well of the 96 well thin-wall PCR plate (Bio-Rad). PCR reactions consisted of a first denaturing cycle at 95°C 3 min, followed by 50 cycles of 10 sec at 95°C, 15 sec at 60°C and 30 sec at 72°C. Fluorescence was quantified at the end of the 60°C annealing step and product identity was confirmed by a melting curve analysis (60°C–95°C). Primer sequences and amplicon sizes are summarized in table 1.

The relative mRNA amounts of target genes ER α and β, complement C3, progesterone receptor (PR), proliferation-related Ki-67 antigen, proliferating cell nuclear antigen (PCNA), cyclooxygenase 2 and vascular endothelial growth factor receptor 2 were calculated after normalization to an endogenous reference gene (Cytochrome C Oxidase subunit 1). Results were expressed as relative amounts of mRNA compared to the vehicle control animals using the $2^{-\Delta\Delta CT}$ formula (Winer et al., 1999).

2.9. Western blot analysis of uterine protein expression

Uteri were dissected from the animals and immediately stored in liquid nitrogen. Pooled frozen tissues was pulverized in liquid nitrogen and homogenized in a mixture (1 : 1) of preparation buffer (240mM saccharose; 20mM PIPES; 10mM EDTA; 50mM NaH₂PO₄; 1mM PMSF in 2-propanol; pH 7,4) and freezing buffer (400mM saccharose; 5mM PIPES; 5mM Tris; 10mM EDTA; 50mM NaH₂PO₄; pH 7,2).

Protein concentrations were determined using bicinchoninic acid (VWR, Darmstadt, Germany). Equal amounts of protein samples (30µg) were loaded onto a 8,5 % SDS-polyacrylamide gel, electrophoresed and transferred to 0,45 µm PVDF membranes (Millipore, Massachusetts; USA). The PVDF membranes were blocked with 5 % skim milk powder and incubated with specific antibodies for PCNA (1 : 1.000, Dako Cytomation, Glostrup, Denmark) and Actin (1 : 4.000; Sigma, Saint Louis; Missouri, USA) at room temperature for 1 h, followed by incubations with Goat-Anti-Mouse horseradish-peroxidase (HRPO)-conjugated secondary antibody (1:10.000, Dianova, Hamburg, Germany) and Goat-Anti-Rabbit HRPO conjugated secondary antibody (1 : 30.000, Dianova, Hamburg, Germany), respectively, at room temperature for 1 h. The Western blots were developed with a chemiluminescence ECL Plus western blotting detection system (GE Healthcare, Buckinghamshire, UK) and exposed to Amersham Hyperfilm ECL (GE Healthcare, Buckinghamshire, UK) at room temperature. The protein bands were quantified by densitometry using the LabWorks 4.6 software (UVP Ltd, Cambridge, UK). To account for inhomogeneous protein loading of the slots the ratio of the reference protein (Actin) to the target protein (PCNA) was calculated.

2.10. Statistical analysis

Statistical analysis of the data in this work was performed using two way analysis of variance followed by pair wise comparison of selected means using the Student's t-test. The criterion for significance was set * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to the negative control.

3. Results

3.1. Effect on ERα and ERβ activation

The effect on ERα dependent activity was tested in stably transfected U2OS-ERα cells. For the ERβ dependent activity U2OS cells transiently transfected with ERβ (U2OS-ERβ cells) were used. While for 8-PN even at the lowest tested concentration a statistically significant induction luciferase reporter gene activity was detectable in

U2OSER α , 6-DMAN showed this activity only starting at a concentration of 100 nM (Fig. **2A** and **2C**)

After stimulation of the U2OS-ER β cells with 6-DMAN and 8-PN a statistically significant ER β -mediated induction of reporter gene activity was detected (Fig. **2B** and **2D**). The maximal activation for both substances was already present at concentrations of 10 nM and was not much enhanced by further increasing the concentration.

3.2. Uterus wet weight

The uterotrophic response in ovariectomized Wistar rats was tested time dependent manner after 7h, 24h and 72h of treatment. 8-PN and 6-DMAN were applied at concentrations of 15 mg/kg BW per day (Fig. **3**). Positive control was E2 at 10 μ g/kg BW per day and as negative and the carrier control castor oil was used. Control animals had an average uterus wet weight of 0.185 g $\pm$ 0,012 g after vehicle treatment at any time point. E2 treatment over 7h and 24h caused a significant increase in uterus wet weight between 1.7 to 1.9 fold, while uterus wet weight after 72h increased even further to 3.7 fold. 8-PN like E2 caused a significant increase in uterus wet weight between 1.6 to 1.8 fold after 7h up to 2.7 fold after 72h treatment compared to the carrier control. In contrast to 8-PN, 6-DMAN did not show any significant effect on uterus wet weight at any time point.

3.3. Gene and protein expression in the uterus

The mRNA expression of ER α and β have been detected in the rat uterus under all treatment conditions. Following 72 h of treatment with E2, in addition to the two naringenins, the uterine ER α mRNA showed a four fold down-regulation compared to in the carrier control animals (Fig. **4A**). The same result was consistent for levels of ER β . The strongest response in the uterus was observed after 7h to 24 h treatment for all test substances, while after three days of treatment the expression of ER β mRNA recovered to higher levels which were still below baseline expression (Fig. **4B**).

The regulation of mRNA expression of other estrogen sensitive genes in the uterus by E2 followed the expected response pattern. The complement C3 and the PR

mRNA levels followed a time dependent pattern of expression (Fig. **5A** and **5B**). While complement C3 reached the highest expression levels already after 24 h of E2, 8-PN and 6-DMAN treatment, only for E2 treatment a significant increase persisted for 72 h. Complement C3 expression follows a similar pattern of regulation in all treatment groups, while the PR mRNA expression pattern is substance dependent. PR expression is already up regulated following 7h of either E2 or 8-PN treatment. 6-DMAN treatment leads to a delayed upregulation of gene expression following 72h of treatment, while PR expression was already back to baseline levels after equally long E2 treatment.

mRNA expression of cyclooxygenase 2 (COX2) was transiently up-regulated after 7h, dropped to control levels at 24h and appeared down regulated after 72h of either E2 or 8-PN treatment, while 6-DMAN showed no effect on the expression level of COX2 at any of these time points (Fig. **6A**). Clusterin gene expression was also significantly down regulated following administration of E2 at either the 24h or 72h time point, while 8-PN treatment only led to a significant down regulation of clusterin mRNA expression at the 24 h time point. 6-DMAN had no effect at any time point (Fig. **6B**).

The mRNA expression of the vascular endothelial growth factor (VEGF) and its receptor 2 (VEGFR2) was transiently upregulated in response to E2 and 8-PN following 7h of treatment. 6-DMAN treatment had no significant impact on the expression of VEGFR2 and VEGF mRNA (Fig. **7A** and **7B**). The mRNA expression of proliferation markers PCNA and KI67 was transiently up-regulated by E2 as well as by 8-PN in a clear time dependent manner while 6-DMAN treatment at none of the time points showed an impact on the regulation of proliferation associated gene expression (Fig. **8A** and **8B**). To validate the findings on mRNA expression of proliferation markers and uterotrophy data we additionally investigated the PCNA protein levels in all treatment groups. We see significantly higher PCNA protein levels following E2 treatment at the 24h and 72h time point. While 8-PN in tendency mimics E2 with regard to it's effect on the PCNA protein expression pattern, 6-DMAN treatment had no effect on PCNA levels at any of the time points, according to the densitometric analysis(Fig. **8C**).

4. Discussion

It is well known that proliferation of endometrial cells is under the control of estrogens and that the risk of endometrial carcinoma increases with estrogen replacement therapy, (Ito, 2007). To study the influence of phytoestrogens in the absence of endogenous estrogen stimuli, we used ovariectomized female rats. Although widely used, the efficacy and safety of PEs as pharmacological compounds is still controversial. The preference of phytoestrogens for ER subtypes may result in a potential organ selectivity of their actions and is therefore of high pharmacological relevance. We therefore evaluated the receptor selectivity of 8-PN and 6-DMAN on the level of transactivation in bone-derived U2OS cells.

While 8-PN had a similar transactivation efficiency through both receptors 6-DMAN showed activity via the ER β at 10fold lower concentrations than via the ER α . This demonstrates a modest ER β selectivity of 6-DMAN which has been observed for some other phytoestrogens as well (Kuiper et al., 1998).

A further aim of this study was to better characterize the estrogen-like substances naringenin-type phytoestrogens 8-PN and 6-DMAN *in vivo*.

We applied the three day uterotrophic assay in ovariectomized rats which can answer two fundamental questions related to the properties of candidate phytoestrogens: 1) Proof of principle of the *in vivo* estrogenicity, 2) assessment of potential uterotrophic effects and impact on uterine proliferation. The latter is related to safety issues because exclosure of uterine stimulation would suggest safety towards unwanted intermediate uterine bleeding or hyper proliferation of the uterus in a menopausal setting.

We analyzed the time course of the uterotrophic response in ovariectomized Wistar rats at 7h, 24h and 72h time points respectively. E2 and 8-PN but not 6-DMAN significantly stimulated the uterine wet weight and this uterotrophic response for E2 and 8-PN followed as expected a clear time course. The lack of uterine stimulation of 6-DMAN as seen in the absence of uterotrophy was reflected in the deficiency of stimulating the mRNA expression of proliferation markers at any investigated time point. Conversely, E2 and 8-PN as expected from the observed uterotrophic response showed an upregulation of mRNA expression of the proliferation markers PCNA and KI67. These findings were confirmed by the PCNA protein data demonstrating that the observed changes in PCNA mRNA expression subsequently lead to altered protein levels in the E2 and 8-PN treated rats. In contrast to the

uterotrophic response, 6-DMAN stimulated increased uterine mRNA expression of some estrogen responsive genes in the ovariectomized rats, but not as pronounced as seen with E2 and 8-PN.

In all treatment regimens, the expression of ER α and β mRNA, essential for the mediation of estrogenic effects, could be demonstrated. While application of E2 and 8-PN caused a similar response pattern on the expression of all E2-regulated genes, confirming the mostly E2-agonistic nature of 8-PN, 6-DMAN treatment lead to a more complex pattern of gene regulation. With genes containing EREs or ERE half sites a 6-DMAN dependent regulation of expression could be shown. However the PR mRNA expression pattern apparently shows a delayed onset of upregulation following 6-DMAN treatment compared to E2 and 8PN treatment. On the other hand 6-DMAN had no observable effect on E2 regulated genes that do not possess EREs as regulatory elements.

The standard path of estrogen action is via ER dimerisation and binding to EREs in the promoter regions of target genes and leading to transcription. But additional pathways for estrogen action have been suggested (Coleman and Smith, 2001) including ER modulating gene expression via protein-protein binding with c-Fos/c-Jun B (AP-1), Sp1 or through NF- κ B (Ray et al., 1994; Kushner et al., 2000; Safe, 2001). These alternative response mechanisms have been referred as non classical pathways. To investigate these non classical pathways a non classical ER knock-in (NERKI) mouse was established on the background of an ER α knockout mouse supplemented with a zinc finger deficient ER α knock-in. In these mice, the ERE dependent pathway of estrogen action is compromised while tethering mechanisms by protein/protein interactions are retained (Jakacka et al., 2002). These NERKI mice have enlarged uteri with cystic hyperplasia (Jakacka et al., 2002) suggesting that the uterine weight is not regulated via ERE pathway. In addition, a more recent study has shown that uterine proliferation appears to be mediated by tethering mechanisms (O'Brien et al. 2006). Apparently estrogenic effects of 6-DMAN are mediated by binding to EREs in the promoter regions of target genes and not by the described non classical pathways, which could explain the lack of an uterotrophic effect.

The 8-alkylnaringenins investigated by Roelens *et al.* (Roelens et al., 2006) show an activity spectrum spanning from full E2-agonism to partial agonism to antagonism, including differential subtype activity, only depending on the length and the bulkiness of the substituent. 6-DMAN, although possessing a different substituent, fits nicely

within this range. Some authors suggest that 8-PN may have beneficial effects in postmenopausal women and may become an alternative to classical HRT treatment regimes (Rad et al., 2006). Our data indicate that 6-DMAN with its lack of uterine stimulation might match the criteria for HRT much better than the pure E2 agonist 8-PN, because it lacks the proliferational effect on the endometrial cells which may increase the risk of formation of endometrial carcinoma.

In conclusion, we have identified several important differences between the two examined naringenin compounds in a time dependent protocol *in vivo*. 8-PN acts as a pure E2 agonist in the uterus, based on its uterotrophic effect but also from the very similar pattern of gene expression following time dependent treatment. However, 6-DMAN seems to primarily effect the expression of ERE dependent genes. Interestingly, in contrast to E2 treatment, no uterine wet weight gain was detected following 6-DMAN further highlighting the potential safety of 6-DMAN with respect to uterine stimulation and cancer development. The results from the bone-derived U2OS cells demonstrate that small structural differences of PEs lead to explicit differences in the receptor preference. The preference of 6-DMAN towards transactivation via the ER β might add to the explanation of the observed differences in the regulation of gene expression *in vivo* and are one of the desired characteristics for a medication of menopausal complaints.

These findings highlight the significance of a systematic approach to investigate the differences in estrogenic effects of structurally related PEs and provide valuable information for future drug or tool compound discovery. They also indicate that 6-DMAN acts as a naturally occurring SERM since it differentially regulates the expression of only a subset of estrogen responsive target genes in the rat uterus. 6DMAN might constitute an attractive candidate molecule for future investigations into its usefulness in HRT.

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Table 1: Primers used for real-time-PCR quantification

Fig. 1: Chemical structures of (a.) 8-prenylnaringenin and (b.) 6-(1,1-dimethylallyl)naringenin.

Fig. 2: Effects of 6-(1,1-dimethylallyl)naringenin and 8-prenylnaringenin on the activation of ER α and ER β in U2OS cells. (A) The effect of 6-(1,1-dimethylallyl)naringenin (6-DMAN) and (C) 8-prenylnaringenin (8-PN) on ER α activity in the stably transfected U2OS-ER α cells was investigated by measuring reporter gene-coupled luciferase activity. The relative luciferase units (RLU) were measured in the presence of DMSO (¼ 100%), E2 (10^{-8} M) and 6-DMAN. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (B) and (D) ER β dependent activation of reporter gene activity was investigated by transient cotransfection of U2OS osteosarcoma cells with an ER β expression plasmid and (ERE)₂-tk-luc reporter construct (for details see Material and methods). ERr 731s activated the ER β -coupled reporter gene activity in a statistically significant manner, reaching an activity level similar to that exerted by 10^{-8} M E2. Significance against DMSO control (¼ 100%): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 3: Uterine wet weight following treatment of ovariectomized animals:

Uteri of untreated ovariectomized control animals (Co), ovariectomized animals treated with 10 µg/kg BW/ d estradiol (E2) and ovariectomized animals treated with 6-(1,1-dimethylallyl)naringenin (6-DMAN) and 8-prenylnaringenin (8-PN) at a concentration of 15 mg/kg/d BW. Asterisks indicate values significantly different from the respective controls. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 4: Real-time RT-PCR analysis of uterine gene expression:

Expression of estrogen receptors (ER) α and β mRNA was analyzed by quantitative real-time RT-PCR analysis. Data of uterine gene expression of untreated ovariectomized control animals (Co), ovariectomized animals treated with 10 µg/kg BW/ d estradiol (E2) and ovariectomized animals treated with 6-(1,1-

dimethylallyl)naringenin (6-DMAN) and 8-prenylnaringenin (8-PN) at a concentration of 15 mg/kg/d BW. Significancies are indicated as described in Fig.3.

Fig.5: Real-time RT-PCR analysis of uterine gene expression:

Expression of Complement C3 and the progesterone receptor (PR) mRNA was analyzed by quantitative real-time RT-PCR analysis. Data of uterine gene expression of untreated ovariectomized control (Co), ovariectomized animals treated with 10 µg/kg BW/d estradiol (E2) and ovariectomized animals treated with 6-(1,1-dimethylallyl)naringenin (6-DMAN) and 8-prenylnaringenin (8-PN) at a concentration of 15 mg/kg/d BW. Significancies are indicated as described in Fig.3.

Fig.6: Realtime RT-PCR analysis of uterine gene expression:

Expression of the Cyclooxygenase 2 (COX2) and clusterin mRNA was analyzed by quantitative real-time RT-PCR analysis. Data of uterine gene expression of untreated ovariectomized control (Co), ovariectomized animals treated with 10 µg/kg BW/d estradiol (E2) and ovariectomized animals treated with 6-(1,1-dimethylallyl)naringenin (6-DMAN) and 8-prenylnaringenin (8-PN) at a concentration of 15 mg/kg/d BW. Significancies are indicated as described in Fig.3.

Fig.7: Realtime RT-PCR analysis of uterine gene expression:

Expression of the vascular endothelial growth factor (VEGF) and it's receptor 2 (VEGFR2) mRNA was analyzed by quantitative real-time RT-PCR analysis. Data of uterine gene expression of untreated ovariectomized control (Co), ovariectomized animals treated with 10 µg/kg BW/d estradiol (E2) and ovariectomized animals treated with 6-(1,1-dimethylallyl)naringenin (6-DMAN) and 8-prenylnaringenin (8-PN) at a concentration of 15 mg/kg/d BW. Significancies are indicated as described in Fig.3.

Fig.8: Realtime RT-PCR analysis of uterine gene expression and uterine PCNA protein level:

Expression of the proliferation markers KI67 and PCNA mRNA was analyzed by quantitative real-time RT-PCR analysis (A +B) and densitometric analysis of PCNA protein levels after Western Blot analysis (C). Data of uterine gene expression respectively PCNA protein level of untreated ovariectomized control (Co),

ovariectomized animals treated with 10 µg/kg BW/d estradiol (E2) and ovariectomized animals treated with 6-(1,1-dimethylallyl)naringenin (6-DMAN) and 8-prenylnaringenin (8-PN) at a concentration of 15 mg/kg/d BW. Significancies are indicated as described in Fig.3.

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Fig. 1 **a.** (±) 8-prenylnaringenin **b.** (±) 6-(1,1-dimethylallyl)naringenin

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8

Table 1

genes	primer	amplicon size
Cytochrome C Oxidase I (1A):	forward 5' TGA GCA GGA ATA GTA GGG ACA GC 3' reverse 5' GAG TAG AAA TGA TGG AGG AAG CA 3'	261 bp
Estrogen receptor (ER) α	forward 5' GGA AGC ACA AGC GTC AGA GAG AT 3' reverse 5' AGA CCA GAC CAA TCA TCA GGA T 3'	383 bp
Estrogen receptor (ER) β	forward 5' CTA CAG AGA TGG TCA AAA GTG GAA 3' reverse 5' GGG CAA GGA GAC AGA AAG TAA GT 3'	216 bp
Complement C3 (C3):	forward 5' ACA GCC TTC CCG GGA GCA TCA ACA3' reverse 5' AGC GCA CCA CAG GAG GCA CAG AGT C3'	276 bp
Progesterone receptor (PR):	forward 5' CCC AGA CGA AAA GAC ACA AAA T 3' reverse 5' CCA AAG AGA CAC CAA GAA GTG AT 3'	222 bp
Clusterin (CLU)	forward 5'-CCC TCC AGT CCA AGA TGC TCA ACA C-3' reverse 5'-CCA TGC GGC TTT TCC TGC GGT ATT C-3'	303 bp
Proliferation-related Ki-67 antigen (KI67):	forward 5'-AAC CAG GAC TTT GTG CTC TGT AA-3' reverse 5'-CTC TTT TGG CTT CCA TTT CTT C-3'	209 bp
Proliferating cell nuclear antigen (PCNA):	forward 5'-GAG CAA CTT GGA ATC CCA GAA CAG G-3' reverse 5'-CCA AGC TCC CCA CTC GCA GAA AAC T-3'	158 bp
Cyclooxygenase 2 (COX2):	forward 5'-AAC TGT ATC CCG CCC TGC TGG TG-3' reverse 5'-AGA CAG CCA CCA TCA ACG CAA GT-3'	284 bp
Vascular endothelial growth factor receptor 2 (VEGFR2):	forward 5'-GAA AAA GCC GGC CAG TGA GTG TAA-3' reverse 5' AGC GCA GTG TGG TCC CAG CAG AGT CAA-3'	345 bp

Table 2

	E2				6-DMAN				8-PN		
	7h	24h	72h		7h	24h	72h		7h	24h	72h
ERα			↓↓				↓				↓↓
ERβ	↓↓	↓↓	(↓)		↓	↓			↓↓	↓↓	
C3	↑	↑↑	↑↑		(↑)	↑↑			↑	↑↑	
PR	↑↑	↑					↑		↑↑	↑	↑
Cox 2	↑↑		(↓)						↑↑		(↓)
Clusterin		↓↓	↓↓							↓↓	
VEGF	↑↑	↑							↑↑	↑	(↑)
VEGF-R2	↑	↑↑	(↓)						↑	(↑)	
Ki67		↑↑	↑							↑↑	↑
PCNA	↑	↑↑	(↑)						↑	↑↑	