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Lignans as food constituents with estrogen and antiestrogen activity

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ABSTRACT

Phytoestrogens are plant-derived food ingredients assumed to contribute to the prevention of hormone-dependent cancers, osteoporosis, cardiovascular disease, and menopausal symptoms. Lignans occur in numerous food plants and various structures; they are common constituents of human diet, and estrogen activity has been assessed for lignan metabolites formed in the mammalian intestine. We examined natural lignans and semisynthetic norlignans for estrogen and antiestrogen activity. A transformed yeast strain (*Saccharomyces cerevisiae*) expressing the estrogen receptor alpha and a reporter system was applied as test system. Some plant lignans showed estrogen activity while others and the semisynthetic norlignans were moderately active antiestrogens. Docking of lignans to protein models of estrogen receptor alpha in the active and inactive form sustained the results of the yeast estrogen assay and supported the concept of plant lignans as phytoestrogens.

1. Introduction

Lignans are plant secondary products consisting of mostly two phenylpropanoid moieties connected via their side chain C8 carbons and sometimes by additional ether, lactone, or carbon bonds (Davin and Lewis, 2003). Lignans comprise a large variety of individual structures and are widely distributed in the plant kingdom (Umezawa, 2003) (Fig. 1). Some cytotoxic lignans have attained medicinal importance, e.g. podophyllotoxin, of which derivatives are applied in cancer therapy. Many non-toxic lignans are constituents of human nutrition, present in high concentrations in oilseeds, in grains like wheat, rye, and oat, and many berries (Kuhnle et al., 2008; Smeds et al., 2007). Health supporting effects of dietary lignans have been intensively discussed with a focus on phytoestrogen activity (Adlercreutz, 2007, 2002). Phytoestrogens are non-steroidal phenolic plant compounds that exert the characteristic effects of steroidal estrogens or, as anti-estrogens, prevent steroid estrogen action. Phytoestrogens comprise isoflavonoids, flavonoids, coumestans, stilbenes, and lignans (Dixon, 2004). While the isoflavones genistein and daidzein (Fig. 2) were intensively investigated, fewer studies focused on lignan phytoestrogens. Both, isoflavonoids and lignans are stored in plants predominantly as glycosides and are converted by intestinal bacteria to give metabolites with estrogen activity like equol (Dixon, 2004), enterodiol and enterolactone (Axelson et al., 1982) (Fig. 1). These mammalian lignans in the intestine are produced from, e.g. secoisolariciresinol (Mazur et al., 2000), arctiin (Xie et al., 2003), and sesamin (Penalvo et al., 2005). The estrogen activity of the enterodiol and enterolactone was taken as the mode of action for food lignans (Sonestedt et al., 2009). But non-metabolized plant lignans were also found in human urine indicating that they are absorbed from the intestine as aglycones (Adlercreutz, 2007). This provokes the questions for potential effects of lignans without prior metabolism to mammalian lignans.

Estrogen receptors (ER) belong to the nuclear hormone receptor family of proteins. Upon ligand binding ER dissociate from chaperones and dimerise. In the nucleus, ligand-loaded ER dimers bind to palindromic estrogen response elements and regulate transcription of adjoining genes (Brzozowski et al., 1997; Shiau et al., 1998). Crystal structures of ER ligand binding domains demonstrated that a bound agonist, e.g. 17β-estradiol, allows the C-terminal helix 12 to fold over the ligand binding site closing it like a lid. In this conformation and after recruitment of steroid receptor coactivators 1 and 3, the dimeric ER acts as transcription enhancers. ER-bound antagonists effectuate several alternative positions of helix 12 that all prevent the interaction of the ER dimer with coactivator proteins (Brzozowski et al., 1997; Pike et al., 2001; Shiau et al., 1998). Gene cloning of ER revealed two different sequences named ERα and ERβ, which are similar in the DNA binding region but differ in their ligand binding domain (Pike et al., 1999). Steroid estrogens are bound by both ER types, however, phytoestrogens and synthetic estrogen analogues often show preferential binding to one of the ER (Carreau et al., 2008; Manas et al., 2004; Mueller et al., 2004).

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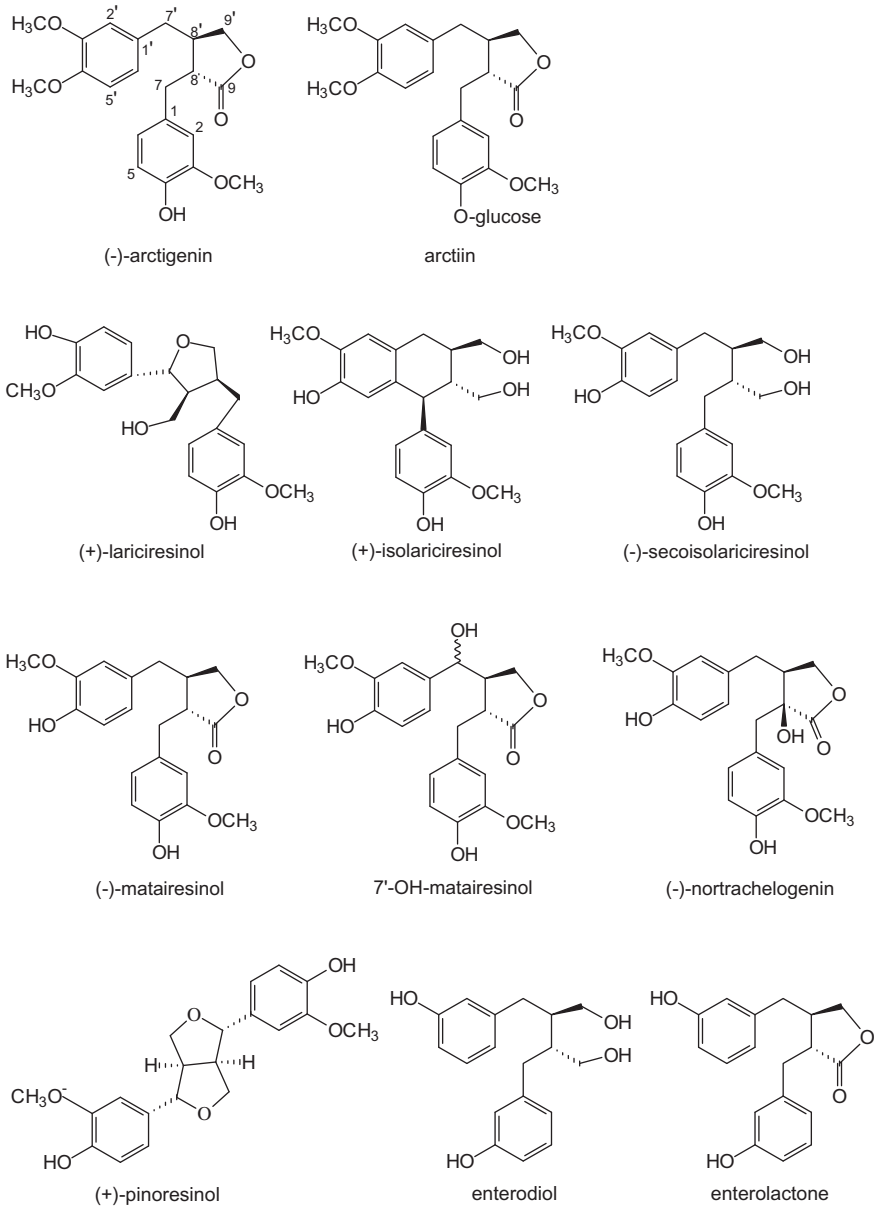

Fig. 1. Natural lignans from plants and from mammalian metabolism.

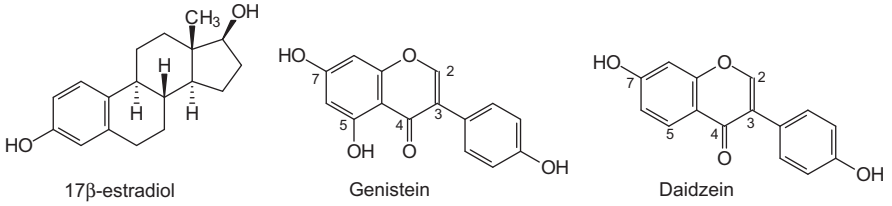

Fig. 2. Ligands for estrogen receptor α.

The yeast estrogen (YES) assay used in this study consists of a transformed yeast strain (*Saccharomyces cerevisiae*) expressing a gene of an estrogen receptor and carrying a plasmid with an estrogen-responsive element located upstream of the reporter gene LacZ that encodes β-galactosidase (Routledge and Sumpter, 1996). Estrogens provoke colour formation by cleavage of a chromogenic galactoside. Combination of test compounds with an effective estrogen enables indication of antiestrogen activity (Sohoni and Sumpter, 1998). Compared to other estrogen assays that use e.g. hormone dependent proliferation or enzyme production of mammalian cells, e.g. Ishikawa cells or MCF-7 cell lines (Mueller et al., 2004; Wang et al., 2006), bioluminescence after receptor dimerisation (Powell and Xu, 2008), or whole animal tests (Owens and Koëter, 2003), YES assays are fast and robust (Bovee et al., 2010). Cell-based estrogen assays have rarely been applied to investigate plant lignans. Lariciresinol, isolariciresinol and

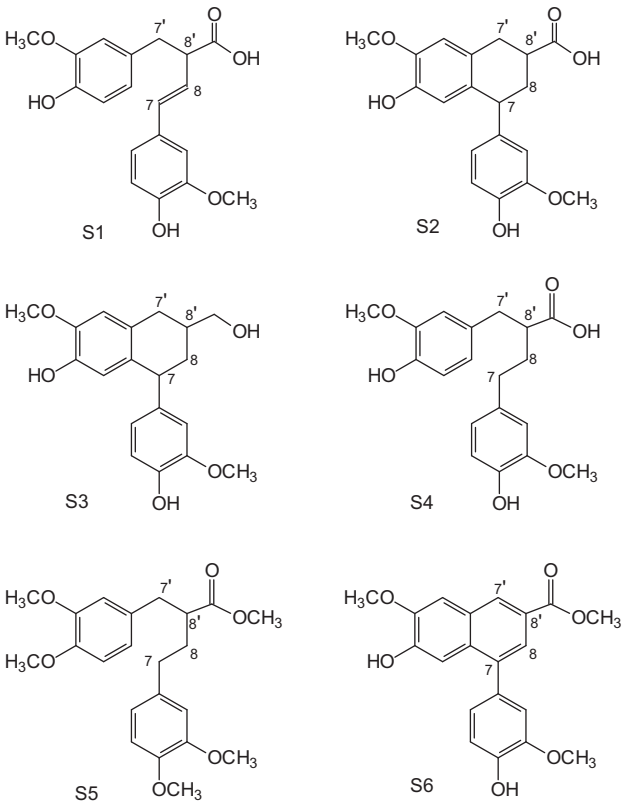

Fig. 3. Synthetic lignan derivatives.

derivatives from *Ephedra viridis* were found to be inactive in a YES assay (Pullela et al., 2005). We applied the yeast cells expressing human ERα to investigate estrogen and antiestrogen activity of various lignans (Fig. 1). Six synthetic norlignans with C6–C5–C6 carbon skeleton (Fig. 3) were included to cover a wide field of lignan structures and to obtain structure activity relationships. 3D models of the estrogen receptor alpha (ERα) in active and inactive conformation were applied for docking analysis of the lignans.

2. Results and discussion

2.1. Estrogen and antiestrogen activity of isolated lignans

17β-Estradiol, genistein, and daidzein were applied to test the YES assay response. Typical sigmoid dose–response curves were obtained (Fig. 4) confirming the correct function of the assay. EC_{50} for 17β-estradiol was found at 35 pM (standard deviation ± 7 pM) and concurred with published data (Routledge and Sumpter, 1996). The EC_{50} values obtained in the YES assay may shift slightly depending on assay parameters like incubation time and temperature (Beresford et al., 2000). For genistein and daidzein EC_{50} were 260 nM (±30 nM) and 5.1 μM (±600 nM) and in the same range as reported from an inter-laboratory comparison of estrogenic ligand efficacies in the YES assay (Dhooge et al., 2006).

All natural lignans (Fig. 1) and six synthetic norlignans (Fig. 3) were individually examined for estrogen activity in concentrations decreasing from *ca.* 1.5 mM to 1.5 μM (500 mg L^{-1} to 500 μg L^{-1}). Estrogen activity was detected for arctigenin, enterodiol, enterolactone, and (−)-7′-hydroxymatairesinol. Among those, arctigenin with EC_{50} of 20 μM (±6.0 μM) was most active and had about twelve times stronger estrogen activity in the YES assay than enterodiol (EC_{50} 240 μM ± 38 μM, Fig. 5). Enterolactone showed only a weak estrogenic effect that increased with the enterolactone concentration in the assay, but an EC_{50} was not determined because saturation was not reached with 1.7 mM. Enterodiol and enterolactone markedly diminished β-galactosidase activity in concentrations above 1 mM (Table 1). Thus the effective affinity to the α-estrogen receptor may be stronger than indicated by the YES assay. Concordantly, transcriptional activation of ERα was found with lower concentrations (1–10 μM) of enterodiol and enterolactone in MCF-7 cells (Carreau et al., 2008). (−)-7′-Hydroxymatairesinol exerted a weak estrogenic effect, which in the highest test concentration of 1.34 mM was *ca.* 10% of the maximal effect (ΔA_{540nm}) achieved by estradiol. The other lignans did not show estrogen activity in the YES assay.

The decrease of the colour production achieved by 220 pM 17β-estradiol was measured for the calculation of antiestrogen activity. The isoflavones genistein and daidzein did not affect the 17β-estradiol response in the YES assay. The synthetic norlignans S3, S5, and S6 exerted the strongest antiestrogen effects (Fig. 6) and showed IC_{50} values between 130 and 590 μM corresponding to 46–220 mg L^{-1}. Plant lignans with antiestrogen activity were matairesinol, isolariciresinol, pinoresinol, nortrachelogenin, and the glycoside arctiin (Table 2). Both, (−)-7′-hydroxymatairesinol and the slightly estrogenic mammalian lignan enterolactone

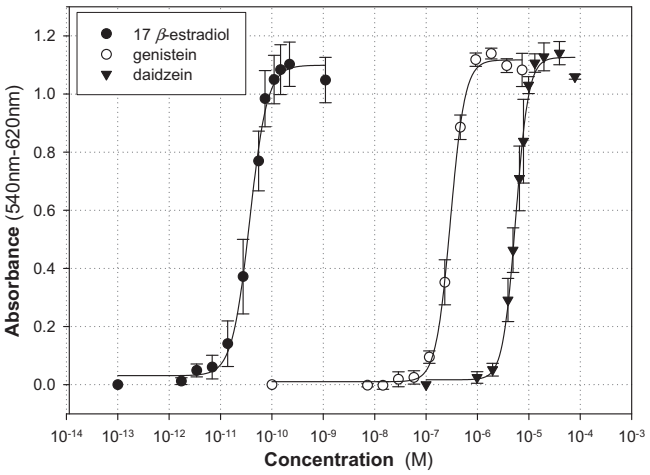

Fig. 4. Dose–response-curves in the YES assay. Vertical bars indicate standard deviation, $n = 4$–26. EC_{50}: 17β-estradiol 3.5×10^{-11} M, genistein 2.6×10^{-7} M, daidzein 5.1×10^{-6} M.

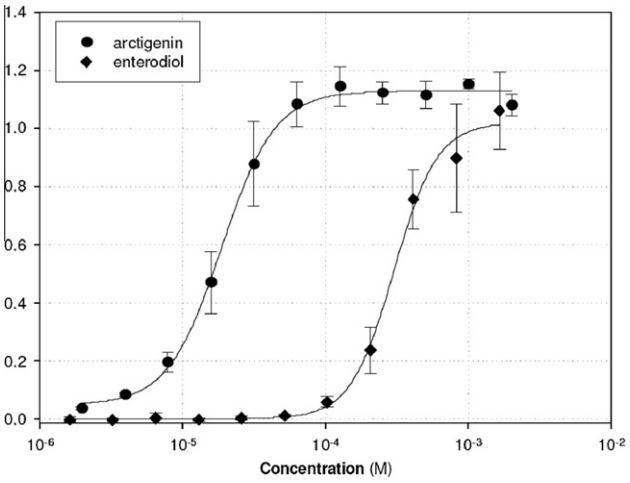

Fig. 5. Estrogen activity of plant lignans in the YES-Assay. Vertical bars indicate standard deviation, $n = 4$–8. EC_{50}: arctigenin 2.0×10^{-5} M, enterodiol 2.4×10^{-4} M.

Table 1
Lignans and control compounds examined with the yeast assay.

Compound	Molecular mass	Max. assay conc.	Growth % of control[a]	β-Galactosidase % of control[b]
17β-Estradiol	272.4	1.1 nM	100	100
Genistein	270.2	7.4 μM	100	100
Daidzein	254.2	79 μM	100	100
(−)-Arctigenin	372.4	1.34 mM	100	100
Arctiin	534.6	0.94 mM	100	100
(+)-Lariciresinol	360.4	1.39 mM	100	100
(+)-Isolariciresinol	360.4	1.39 mM	100	94.8 ± 6.2
(−)-Secoisolariciresinol	362.4	1.38 mM	100	100
(−)-Matairesinol	358.4	1.40 mM	100	100
(−)-7′-Hydroxymatairesinol	374.4	1.34 mM	100	100
(−)-Nortrachelogenin	374.4	1.34 mM	100	94.2 ± 2.4
(+)-Pinoresinol	358.4	1.40 mM	71 ± 3.4	91.2 ± 1.8
(−)-Enterodiol	302.4	1.65 mM	100	10.7 ± 1.1
(−)-Enterolactone	298.3	1.68 mM	100	73.0 ± 5.9
S1	344.4	1.45 mM	100	100
S2	344.4	1.45 mM	100	94.7 ± 2.3
S3	330.4	1.52 mM	100	86.6 ± 3.2
S4	346.4	1.45 mM	100	100
S5	388.5	1.39 mM	100	100
S6	354.4	1.41 mM	87 ± 5.9	94.4 ± 6.0

[a] Growth measured as OD_{620}, control (= 100% reference) with no test compounds added; growth decrease, if registered, is given for the highest (0.94–1.68 mM) lignan concentration ± standard deviation, $n = 4$.
[b] β-Galactosidase activity, control (= 100% reference) with no test compounds added; decrease in β-galactosidase activity, if registered, is given for the highest lignan (0.94–1.68 mM) concentration ± standard deviation, $n = 4$.

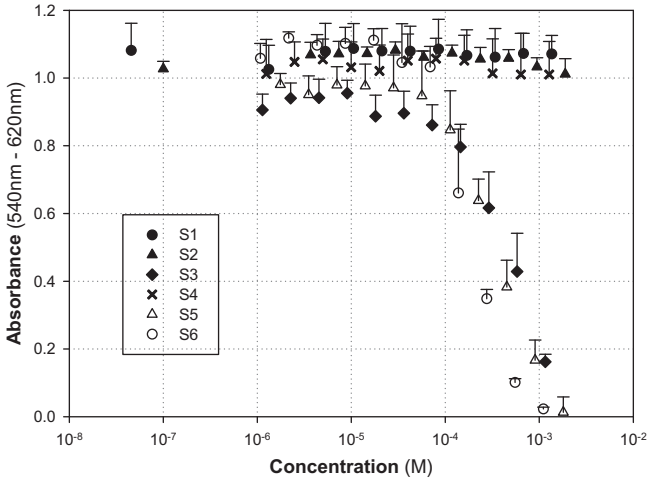

Fig. 6. Dose-dependent inhibition of 17β-estradiol (2.2×10^{-10} M) response by six synthetic norlignans. Vertical bars indicate positive standard deviation, $n = 4$–6, IC_{50}: S3 5.5×10^{-4} M, S5 5.9×10^{-4} M, S6 1.3×10^{-4} M.

Table 2
Inhibition of the 17β-estradiol response (2.2×10^{-10}M) by lignans and comparison of docking score values for the inactive conformation of ERα.

Compound	IC_{50} [M]	Gold score
(−)-Matairesinol	$6.7 \times 10^{-4} \pm 4 \times 10^{-4}$	61.7
(−)-Secoisolariciresinol	–	50.9
(−)-Arctigenin	–	39.1
Arctiin	$6.2 \times 10^{-4} \pm 4 \times 10^{-5}$	69.7
(−)-7′-Hydroxymatairesinol	+	55.9
(+)-Lariciresinol	–	33.5
(+)-Isolariciresinol	$7.0 \times 10^{-4} \pm 4 \times 10^{-4}$	68.8
(+)-Pinoresinol	$1.0 \times 10^{-3} \pm 2 \times 10^{-4}$	48.2
(−)-Nortrachelogenin	$7.3 \times 10^{-4} \pm 3 \times 10^{-4}$	61.8
(−)-Enterodiol	–	30.9
(−)-Enterolactone	+	68.8
S1	–	24.9
S2	–	32.5
S3	$5.5 \times 10^{-4} \pm 2 \times 10^{-4}$	55.8
S4	–	20.8
S5	$5.9 \times 10^{-4} \pm 6 \times 10^{-4}$	60.1
S6	$1.3 \times 10^{-4} \pm 9 \times 10^{-5}$	65.3

– No inhibition at $c_{max} = 1.2$–1.5×10^{-3} M in the YES assay.
+ Weak inhibition, not quantified.

showed weak inhibitory activity, which at 1.7 mM did not reach saturation and left about 60% of maximal estrogen effect. Antiestrogen activity of lignans was weak, and coincidence with fractions from preparative HPLC was tested for matairesinol and pinoresinol. Antiestrogen activity coincided with the major compound peak in the chromatogram and was not found in any other HPLC fraction. Together, these results show for the first time that natural lignan aglycones and a lignan glycoside bind to ERα as activating or blocking ligands albeit with low affinity.

2.2. Lignans in the estrogen receptor model

In an attempt to explain diverging results with lignans that show only small structural differences, protein structure models of the human estrogen receptor α (ERα) in the active, partially active, and inactive form were applied to investigate the positioning of individual lignans in the ligand binding site. Docking of 17β-estradiol, genistein, and 4-hydroxytamoxifen to ERα by the docking programme GOLD confirmed that the program is able to correctly reproduce the experimentally determined respective binding mode. The root-mean-square distance (RMSD) values between the top-ranked docking solutions and the crystal structure were below 1.5 Å for all three ligands. All ligands in ERα formed hydrogen bonds to the carboxylate group of Glu353 and to the guanidinium of Arg394 irrespective of the receptor form (shown for genistein in the active form, Figure 7A). In addition, the ligands showed van der Waals interactions with amino acids in the binding pocket (Leu387, Met343, Leu346, Phe404, Met421, Ile424 and Leu525, shown as far as possible in Fig. 7). Genistein docked into the active form of ERα formed a hydrogen bond like estradiol to the imidazole ring of His524 located nearby helix 12, which is the activation helix of nuclear receptors. Daidzein docked to ERα

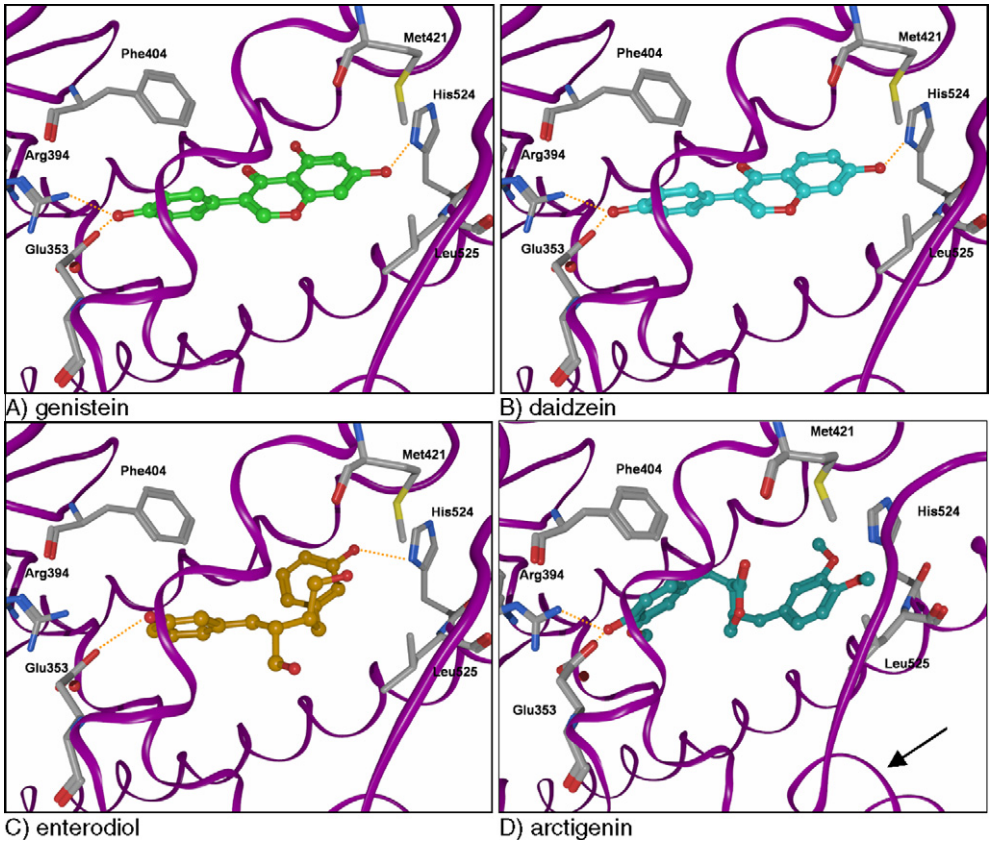

Fig. 7. Docking results for (A) genistein, (B) daidzin, (C) enterodiol, and (D) arctigenin into the active form of the estrogen receptor α. Only relevant amino acids shown. Black arrow in (D) points to a part of helix 12.

displayed hydrogen bonds like genistein (Fig. 7B). The hydroxyl group at C5 of genistein, which is the only structural difference to daidzein, may contribute to receptor binding (Manas et al., 2004), as the EC_{50} of the genistein is about one order of magnitude lower (Fig. 4). Score values of the docking program GOLD (Table 3) summarise protein–ligand hydrogen bond energy, protein–ligand van der Waals energy, ligand internal van der Waals energy, and ligand torsional strain energy (Nissink et al., 2002; Verdonk et al., 2003). For the compound tested here, docking score values decrease with rising EC_{50} values. The mammalian lignan enterodiol formed a hydrogen bond only to Glu353, whereas the distance between the hydroxyl group and Arg394 was above 4 Å. A weak hydrogen bond (3.5 Å) was observed to His524 (Fig. 7C). Agonist activity of enterodiol in the YES assay was low (EC_{50} 240 μM). Arctigenin, in contrast, showed the same hydrogen bonds to Glu353 and Arg394 like the natural agonist 17β-estradiol and other agonists. In addition, the dimethoxyphenyl ring of arctigenin made favourable hydrophobic interactions with Phe404, Met421, and Leu525 (Fig. 7D), visible by hydrophobic interaction fields (Fig. 8A) in addition to polar interactions of arctigenin with ERα (Fig. 8B). Extensive interactions confirmed the assignment of arctigenin as agonist of ERα. The other lignans were tested on the active form of ERα, but no energetically favourable docking solutions were obtained. The basic side chain of the antagonist 4-hydroxytamoxifen, which projects perpendicularly from the plane of the aromatic systems, fitted into an additional cavity formed solely in the inactive form of the estrogen receptor (3ERT), in which helix 12 is displaced (Fig. 9A). Matairesinol, isolariciresinol, and nortrachelogenin were docked into similar positions in the inactive form of ERα. Matairesinol differs from arctigenin only by a hydroxyl group on C4′ instead a methoxy group. Comparison of arctigenin (Figs. 7D and 8) and matairesinol (Fig. 9B) positioning revealed that fewer hydrophobic interactions prevented matairesinol to bind tightly. Instead, the molecule protruded from the ligand binding site more than arctigenin and impeded helix 12 to close the ligand binding site and adopt the conformation of active ERα. Similar to matairesinol, steric clashed were observed with isolariciresinol and nortrachelogenin in the active form of ERα due to the size and the number and positions of non-methylated hydroxyl groups. These lignans were then docked into the inactive form of the ERα. All lignans with antagonistic activity (arctiin, matairesinol, (+)-isolariciresinol, (+)-pinoresinol, (−)-nortrachelogenin) were able to form the essential hydrogen bonds with Glu353 and Arg394, whereas no hydrogen bond was observed with His524 due to the displacement of helix 12 (Fig. 9). Interestingly, the docking of arctiin showed that the hydroxyl groups of the glucose moiety were able to form hydrogen bonds with Glu353 and Arg394 as well with the backbone CO of Leu346 and Leu387. The aglycone part of the ligand was placed in the open channel observed in the inactive form of ERα (Fig. 10A). Docking of the synthetic norlignans S1–S6 gave favourable solutions for S3, S5, and S6 with the inactive ERα. The hydroxyl groups on C4 of S3 and S6 formed hydrogen

Table 3
Docking scores for agonists docked to the active conformation of ERα.

Compound	EC_{50} [M]	Gold scores
17β-Estradiol	$3.5 \times 10^{-11} \pm 7 \times 10^{-12}$	63.1
Genistein	$2.6 \times 10^{-7} \pm 3.0 \times 10^{-8}$M	59.1
Daidzein	$5.1 \times 10^{-6} \pm 6.0 \times 10^{-7}$	52.1
(−)-Arctigenin	$2 \times 10^{-5} \pm 6.0 \times 10^{-6}$	58.6
(−)-7′-Hydroxymatairesinol	Weak	49.1
(−)-Enterodiol	$2.4 \times 10^{-4} \pm 3.8 \times 10^{-5}$	50.1
(−)-Enterolactone	Weak	51.1

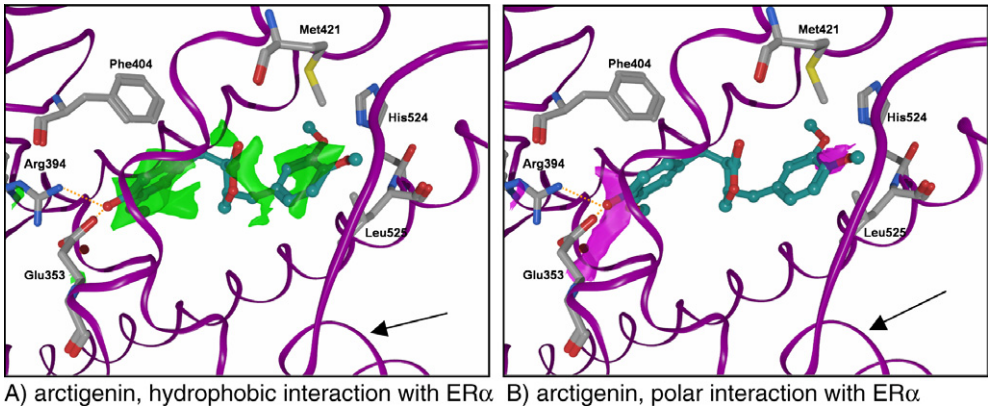

Fig. 8. Molecular interaction fields of arctigenin in ERα. (A) Hydrophobic interaction field (methyl probe contour level −2.5 kcal/mol, green) calculated for arctigenin (cyan) in the active form of ERα. (B) Polar interaction field (OH probe, contour level −7.5 kcal/mol, purple) calculated for arctigenin (cyan) in the active form of ERα. Only relevant amino acid residues are displayed. Black arrows indicate a part of helix 12 in the active form of ERα. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

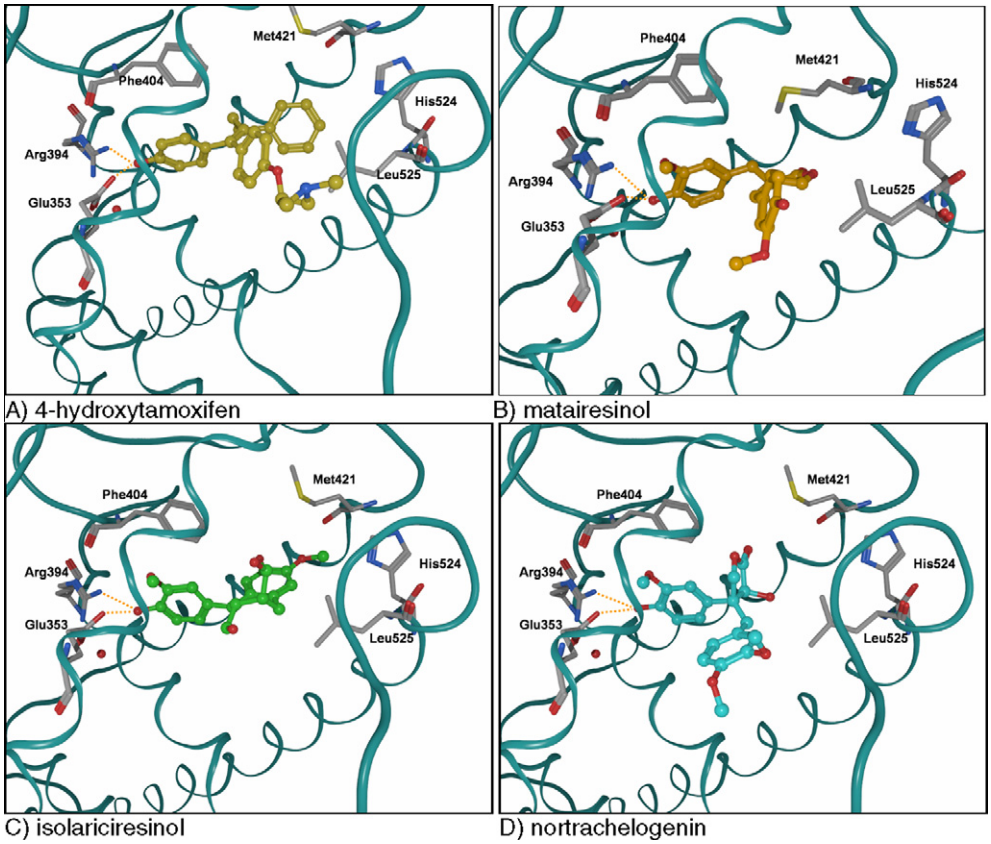

Fig. 9. Docking results for inactive form of ERα IC_{50}: (-)-matairesinol 6.7×10^{-4}M, (+)-isolariciresinol 7.0×10^{-4}M, (-)-nortrachelogenin 7.3×10^{-4}M. Only relevant amino acids are shown. Hydrogen bonds are shown as dashed lines. Helix 12 is displaced and not visible in this section.

bonds to the carboxylate of Glu353 (shown for S6 in Fig. 10C). The bulky substituents, e.g. ester and methoxy groups, were located in the hydrophobic channel formed in the inactive form of ERα after displacement of helix 12. Interestingly, docking solutions were also obtained for S5, which does not possess a phenolic hydroxyl group. S5 was bound mainly by hydrophobic interactions and it could be fitted into the ligand binding site of inactive ERα irrespective of the configuration at C8′ (Fig. 10D). Docking of S1 and S4 was possible, but as the deprotonated carboxylate groups of S1 and S4 were located in the hydrophobic part of the binding pocket (Fig. 10E and F), the positions were highly unfavourable. For S2 no docking solution was obtained. For all docking solutions in the inactive conformation of ERα, docking scores were obtained (Table 2). They correlate roughly with the biochemical IC_{50} data by showing higher scores for lignans that were antiestrogenic *in vitro*. The model-guided structure activity comparisons allow to conclude that aromatic rings should carry one phenol hydroxyl group imitating the estradiol A-ring to enable tight binding to the active conformation of ERα. Additional hydroxyl groups and carboxyl groups in the lignan or norlignan skeleton (e.g. in matairesinol, nortrachelogenin, S1, S2, and S3) rather impede binding to the hydrophobic ligand binding pocket of active ERα. Less polar methoxy groups on

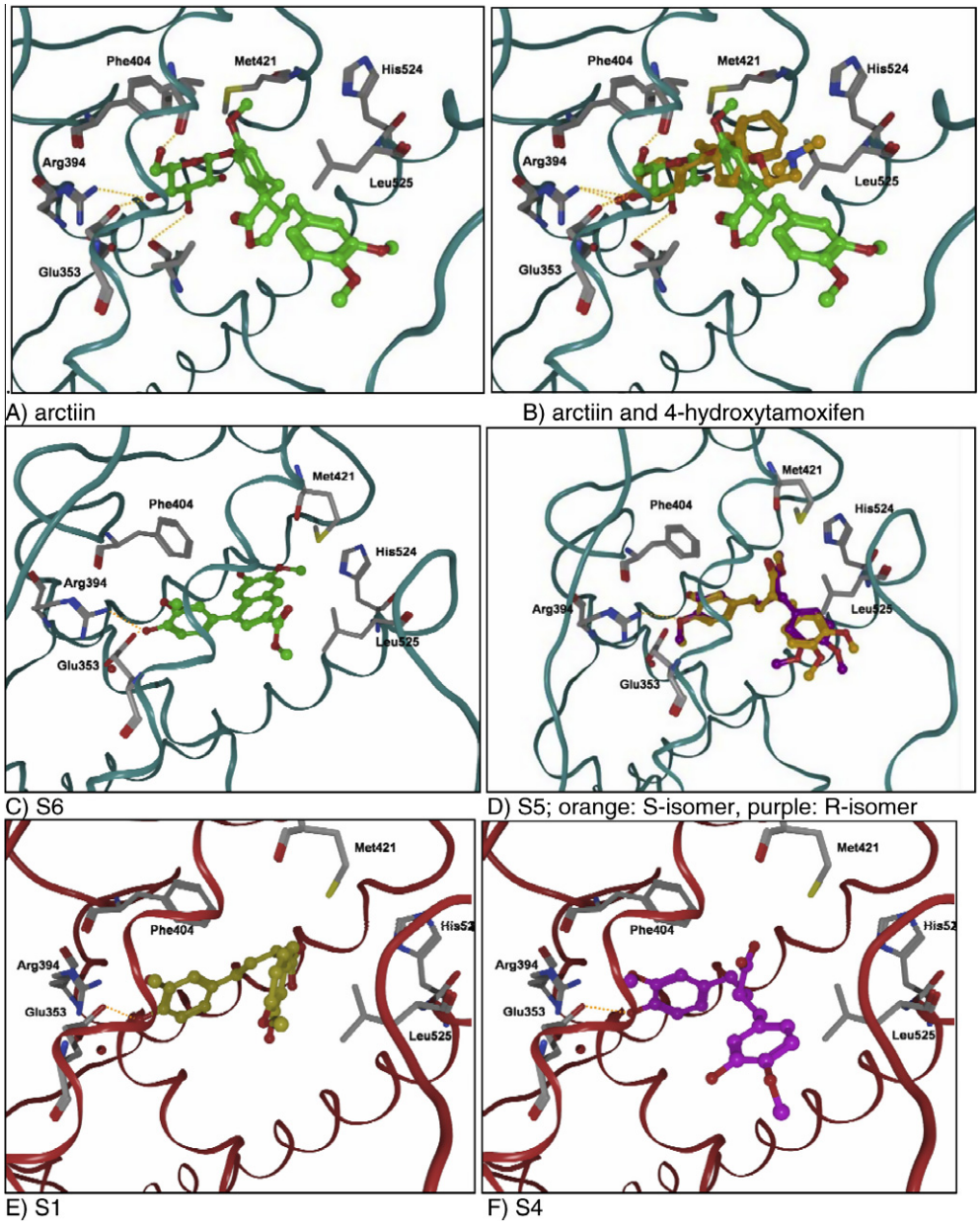

Fig. 10. Docking to the inactive form of ER. (A) arctiin (green) alone, (B) arctiin (green) in comparison to the co-crystallized ligand 4-hydroxytamoxifen (orange). (C) S5 and (D) S6 gave favourable solutions, while free carboxyl groups of (E) S1 and (F) S4 protruded into the hydrophobic cavities, which is unfavourable. Only relevant amino acids are shown. Hydrogen bonds are shown as dashed lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the aromatic rings may allow binding, but, depending on their positions and on the rigidity of the lignans skeleton, appear too bulky for the active ERα conformation. Ring-substituted lignans with a rigid aryltetraline (isolariciresinol, S3) or arylnaphtalene skeleton (S6) protrude from the binding site allowing only binding to the inactive ERα form with helix 12 displaced.

3. Conclusions

Ligand docking sustained YES assay results indicating that some plant lignans may exert estrogen or antiestrogen activity. The agonist activity of arctigenin in the YES assay is weak; the EC_{50} value is one to two orders of magnitude higher than those of genistein and daidzein, however in the same range as the mammalian lignans enterodiol and enterolactone. Quantitative relations among estrogen agonist and antagonist activities may vary depending on the cell type of the activity assay. Alterations in strength may be due to e.g. non-receptor proteins binding to ER and modifying the responses or multidrug resistance efflux pumps (Zachrewski, 1997). Agonist and antagonist activity derived from the YES assay, therefore, are a strong indicator, but not a definite rating of strength of effects.

Isoflavones are limited to a few selected foods, predominantly soy bean products and other pulses. Lignans, in contrast, are common constituents in the human diet and could exert an influence by the amount of intake. Arctigenin, showing estrogen activity in this study, is consumed with foods like safflower seeds (Sakakibara et al., 2007), sesame seeds (Smeds et al., 2007), and wheat flower (Dinelli et al., 2007). Also vegetables with regional importance like *Arctium lappa* root may add to the arctigenin intake. Arctigenin content in wheat flower is usually low (*ca.* 1 mg/kg), but some her-

bal medicines prepared from *A. lappa* fruits and *Saussurea* herb contain high arctigenin levels, up to 3%. A serving containing 20 g safflower seeds will provide *ca*. 60 mg arctiin or 40 mg arctigenin. Quantitative relations in epidemiological studies comparing lignan intake and frequency of hormone dependent cancer, however, have been difficult to establish. Large variations in were attributed to variability of intestinal flora and thus the differential metabolism of food lignans to enterolactone and enterodiol as effector molecules. We now suggest that the lignans themselves, prior to metabolism to mammalian lignans, contribute to the phytoestrogen effect of a diet rich in vegetables, fruits, and whole grain products. Lignans as non-metabolized molecules, besides the intestinal metabolites, are absorbed and may be metabolized in the liver (Nurmi et al., 2003; Papadakis et al., 2008). Quantities of absorption, however, need to be examined in more details and will certainly be subject to variation. With the weak biological activity shown in the YES assay physiological relevance of lignan serum levels may appear questionable. But as lignans are frequent dietary constituents, additive effects must be considered, like it was proven for environmental estrogens (Silva et al., 2002).

Additional food constituents of plant origin, e.g. ellag tannins (Larrosa et al., 2006) are currently identified that after intestinal metabolism exert estrogen-like effects, thus the impact of diet on the hormonal balance is underpinned. Certainly, further assay systems for estrogen activity, e.g. estrogen responsive cells like MCF-7, should be applied in the future to endorse estrogen and antiestrogen effects of lignans and other plant constituents in mammalian cells, and the studies should be extended to estrogen receptor β.

4. Experimental

4.1. Materials

Arctiin, arctigenin (purity > 99%), and matairesinol (purity > 97%) were obtained from BioSolutions Halle, Germany. All other natural lignans, including enterodiol and enterolactone, were purchased from ArboNova, Turku, Finland. Test compounds were isolated from natural sources and purified by chromatography. GC, GC–MS, and HPLC showed the respective lignan as the major compound (>95%). ^{1}H and ^{13}C NMR spectra confirmed that impurities were of very low quantity. Contaminations of isolated lignans with traces of steroid estrogen were considered. If such contaminations appeared in some arctigenin preparations, it should, however, not be constant in quantity. Several lots of arctigenin were compared and the estrogen effect was stable in strength, allowing the conclusion that estrogen activity must be due to the major compound arctigenin. Similar conformity tests were performed with batches of enterodiol and enterolactone. Semisynthetic norlignans S1–S6 were prepared starting from 7′-hydroxymatairesinol and purified by column chromatography. Identities and purity of the products were analysed by GC–MS and by NMR. Impurities (<5%) were starting materials, isomers, or minor quantities of side products from the syntheses. Synthesis was done essentially as described (Eklund et al., 2002). Shortly, compound S2 was obtained from S1 by an acid catalyzed ring-closure reaction with TFA in dichloromethane. Compound S3 was obtained by lithium aluminium hydride reduction of S2. Compound S4 and S5 were obtained by hydrogenation with Pd/H_2 in ethanol and by methylation with MeI/K_2CO_3 in dry acetone, respectively. Compound S6 was obtained by aromatization of methyl ester of S2. The complete experimental procedure and characterization of these norlignan derivatives will be published elsewhere. 17β-Estrogen, genistein, and daidzein (purity > 98%) were obtained from Sigma. Yeast medium constituents were obtained from Merck, Germany. *Escherichia coli* β-galactosidase for control assays, *o*-nitrophenyl β-D-galactoside, and the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG) were from Sigma. All other chemicals, solvents, and buffer constituents were from Merck or Sigma and of analytical grade. Plasticizer phthalates may influence the YES assay (Sanfilippo et al., 2010). All solvents and water used for the YES assay were tested individually to confirm that they did not interfere with the assay. Each micro-well plate contained blank assays with yeast cells and solvents only. Analysis and isolation procedures, including chromatography, were performed in glass or steel material to avoid plasticizers.

4.2. HPLC

Lignans were separated by HPLC (Agilent series 1100) on a C18 column (Merck LiChrospher RP18, 5 μm, 4 × 250 mm) in a solvent system consisting of A methanol and B water with a gradient of 20% A to 70% A in 30 min. Column temperature was 22 °C; flow was 1 mL/min; injection volume was 20 μL. Detection was performed in a diode array detector, quantitation was done at 278 nm.

4.3. Yeast estrogen assay

The transformed yeast cells (*S. cerevisiae*) (Routledge and Sumpter, 1996) were kindly provided by Dr. Christoph Schäfers, Fraunhofer Institute for Molecular Biology and Applied Ecology, Schmallenberg, Germany. Cells were cultivated in yeast minimal medium as described (Routledge and Sumpter, 1996). Estrogen activity assay was performed as described (Routledge and Sumpter, 1996) in 96-well plates. Yeast cells were pre-cultured in 50 mL growth medium at 28 °C under shaking at 150 rpm until an optical density (620 nm) of 1.0. Before the assays yeast cells were diluted 1:25 (v/v) into fresh medium containing 170 μM (100 μg mL^{-1}) chlorophenol red-β-D-galactopyranoside (CPRG). Wells of the 96-well plates were supplied with (nor)lignans or isoflavonoids and with 17β-estradiol, dissolved in 20 μL methanol. After evaporation of the solvent 200 μl freshly prepared yeast cell suspension containing CPRG was filled into every well. The plates were incubated for three days at 32 °C before absorbance was measured at 540 nm. Estrogenic or anti-estrogenic activity was calculated by subtraction of absorbance at 620 nm (to account for turbidity) from absorbance at 540 nm. Dose curves were generated by plotting ($\Delta A_{540-620nm}$) against test compound concentrations in the assay. The concentration that produced half of the maximal effect ($\Delta A_{540-620nm}$) of that individual compound was taken as EC_{50}. IC_{50} was the concentration that reduced the effect ($\Delta A_{540-620nm}$) of 220 pM 17β-estradiol to half. Data were calculated using SigmaPlot (Systat Software GmbH, Erkrath, Germany). Each experiment was at least repeated four times.

4.4. Control assays

As some lignans are cytotoxic and cause DNA strand breaks (Slevin, 1991), growth of yeast cells in presence of lignans was monitored in preliminary assays recording turbidity at 620 nm after 3 days. Lignans and norlignans up to 0.94–1.7 mM (500 mg L^{-1}) alone or in combination with 220 pM 17β-estradiol did not compromise growth of yeast cells (Table 1). Exceptions were 1.4 mM pinoresinol and 1.4 mM S6, which diminished yeast growth after 5 days to 71% and to 88%, respectively, of control dishes. Yeast cells also tolerated without any growth reduction daidzein up to 79 μM, genistein up to 7.4 μM, and 17β-estrogen up to 1.1 nM. Estrogenic activity of compounds is reported in the yeast assay *via* synthesis and excretion of the enzyme β-galactosidase. Inhibition of β-galactosidase activity by any of the test compounds was examined with all lignans up

to 0.9–1.7 mM (500 mg L^{-1}) alone or in combination with 220 pM (60 ng L^{-1}) 17β-estradiol. The assay consisted of β-galactosidase from *E. coli* (Sigma) 450 pkat/well, o-nitrophenyl β-D-galactoside (Sigma) 2.3 mM, lignans, isoflavonoids, and 17β-estradiol 35 pM–1.7 mM, $MgCl_2$ 1 mM, 2-mercaptoethanol 113 mM, dissolved in sodium phosphate buffer 100 mM, pH 7.3, total volume 100 µl in 96-well plates. After pre-incubation at 37 °C for 1 h without substrate, absorbance was read 4 min after substrate addition for 20 min at 414 nm. Enzyme activity was calculated after calibration with *o*-nitrophenol in 96-well plates. Most lignans did not inhibit the β-galactosidase assay, or their inhibitory activity was weak and only visible in the highest concentration in the assays (Table 1). Enterodiol, enterolactone, and S3 (1.5–1.7 mM) reduced β-galactosidase activity to 10%, 73%, and 87%, respectively, of control. Daidzein, genistein, and 17β-estrogen did not interfere with the β-galactosidase assay.

4.5. Computational modelling

To model estrogen receptor-ligand complexes, the coordinates of the human ERα-ligand binding domain liganded with 17β-estradiol (1ERE), genistein (1X7R), and 4-hydroxytamoxifen (3ERT) were taken from the Protein Databank. The three crystal structures represent the active (17β-estradiol), the partially active (genistein), and the inactive form (4-hydroxytamoxifen) of the estrogen receptor (Brzozowski et al., 1997; Manas et al., 2004; Shiau et al., 1998). Hydrogen atoms were added and charges from AMBER (Case et al., 2005) were loaded. The default charge states of titratable sites on the protein were chosen and all histidine residues were treated uncharged. AM1 electrostatic potential (ESP) charges were calculated for the ligands. All water molecules observed in the receptor crystal structure were deleted with one exception: the conserved water molecule bound to Glu53 and Arg394 was included. The estrogen receptor structures were energy-minimized using a tethering constant of 100 kcal/mol on the protein backbone atoms. Docking of potential ligands was examined by program GOLD 3.2 using the minimized structures of the estrogen receptor in the active as well in the inactive conformation. All torsion angles in each ligand were allowed to rotate freely. The binding site was defined on Phe404 with a radius of 15 Å. Gold scores were calculated using default parameters in the programme and chosen as fitness function. The fitness score is taken as the negative of the sum of the component energy terms (protein–ligand hydrogen bond energy, protein–ligand van der Waals energy, ligand internal van der Waals energy, ligand torsional strain energy), so that larger numerical fitness indicate a better fitness in the model (Nissink et al., 2002; Verdonk et al., 2003). For each molecule 10 docking runs were performed. The resulting solutions were clustered on the basis of the heavy atom rmsd values (1 Å). The top-ranked poses for each ligand were retained and analyzed graphically within MOE 2008.10 (Chemical Computing Group). To validate the results obtained by the automated docking procedure, a series of GRID calculations were performed. Program GRID is an approach to predict non-covalent interactions between a molecule of known three-dimensional structure (i.e. estrogen receptor) and a small group as a probe (representing chemical features of a ligand). The calculations were performed using version 23 of the GRID program and the crystal structures mentioned above. The calculations were performed on a cube (13 × 15 × 12 Å) including the binding pocket in order to search for binding sites complementary to the functional groups of the ligands. Several probes were used to study the active site of the estrogen receptor (hydroxyl, methyl and hydrophobic DRY probe). The calculated GRID contour maps were then viewed superimposed on the crystal structure of the estrogen receptor using the MOE 2008.10 software.

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