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Antiproliferative activity of lignans against the breast carcinoma cell lines MCF 7 and BT 20

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Abstract
Purpose Phytoestrogens are plant-derived, non-steroidal phytochemicals with anticarcinogenic potential. The major structural classes are the isoflavones and lignans. The aim of this study was to compare the effect of the plant-derived lignans secoisolariciresinol and matairesinol with the human lignans enterodiol and enterolactone as well as with 17β estradiol and tamoxifen on cell proliferation of breast carcinoma cell lines.
Methods The influence of the lignans, 17β estradiol and tamoxifen on cell proliferation was determined using the BrdU test in MCF 7 and BT 20 cell lines.
Results Enterodiol and enterolactone induced a stronger inhibition of cell growth in MCF 7 and BT 20 cells than secoisolariciresinol and matairesinol. The inhibition effects were less expressed in the BT 20 than in the MCF 7 cells.
Conclusions The human lignans enterodiol and enterolactone are more biologically active than their precursors secoisolariciresinol and matairesinol, and may be defined as the real drugs in cancer prevention.

Keywords Lignans · Cell proliferation · MCF 7 · BT 20

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Introduction

In the last decade there has been considerable interest in phytoestrogen intakes in relation to cancer prevention. Phytoestrogens are a diverse group of non-steroidal compounds synthesised by plants. The major structural classes of phytoestrogens are the isoflavones and lignans found at high levels in edible plants such as soybeans, chickpea, flax, fruits and vegetables [1–3]. Genistein and daidzein are the most commonly investigated isoflavones [4, 5]. The most-studied lignans are secoisolariciresinol and matairesinol [6].

Numerous in vitro cell culture studies and in vivo animal experiments demonstrated that phytoestrogens can inhibit tumour growth [7–9]. Some studies described the influence of flax seed on tumour growth of breast cancer cells [10]. A flax seed diet significantly reduced the MCF 7 tumour size in mice [10]. It is known that flax seed contains a high amount of lignans [2, 6]. The previous collaborative work by our group demonstrated that the cell vitality of breast carcinoma and chorion carcinoma cell lines was strongly inhibited after treatment with flax seed and flax root extracts of *Linum usitatissimum* in vitro [11–13]. Pyrolysis field ionisation mass spectrometry documented that the root extracts of *L. usitatissimum* composed of a high amount of polyphenols, including lignin dimers, flavones, isoflavones, lignans and special polyphenols such as suberins, cutins and stilbenes [13]. HPLC–MS analysis demonstrated that the root extracts of *L. usitatissimum* include more representatives of lignans such as secoisolariciresinol, matairesinol, pinoresinol, lariciresinol, isolariciresinol and arctigenin, compared to isoflavones such as genistein, daidzein and biochanin A [12].

In the classical search for anticancer drugs from botanical sources, plant crude extracts or fractions obtained by various means were tested for anticancer properties in a

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variety of in vitro assays [11, 12, 14, 15]. However, the compounds that are present in the plant are not necessarily the compounds that have a pharmacological effect in the human body [16]. This has often been cited as one of the weakness of in vitro tests [17].

The aim of our study was to estimate, if the lignans occurring in the dietary root extract of *L. usitatissimum* [13, 12] are the compounds producing the inhibitory effects of cell proliferation of breast carcinoma cell lines or other chemical components estimated by Szewczyk et al. [13] and Abarzua et al. [12]. Therefore, we compared the effects of the plant-derived lignans secoisolariciresinol and matairesinol with the human lignans enterodiol and enterolactone on the growth of breast carcinoma cell lines individually. Secoisolariciresinol and matairesinol are metabolised into enterodiol and enterolactone by colonic microflora [18]. The effect of the enterolignans enterodiol and enterolactone on breast cancer cell proliferation is already known [19–21], whereas, only a few experiments were performed according to the direct comparison of the effects of the plant-derived lignans with the enterolignans. According to the knowledge of the metabolism of the lignan phytoestrogens enterodiol and enterolactone as human intestinal converted metabolites of lignans were examined first of all. Because the plant-derived lignans are not metabolised completely, the precursors should be compared always with the metabolites according to their biological effect under in vitro conditions. This procedure seems to be convenient for a screening of natural substances, too.

Estrogen receptor (ER) positive as well as ER negative breast cancer cell lines were used to distinguish between ER dependent and independent mechanisms of the lignans tested. While MCF 7 cells were estrogen receptor α and β positive, BT 20 cells did not express these steroid hormone receptors [13]. The effect of the lignans was compared with that of 17β estradiol, a reference for tumour proliferation and with that of tamoxifen, a reference selective estrogen receptor modulator.

Materials and methods

Preparation of lignan-, 17β estradiol- and tamoxifen solutions as test substances

The synthetic lignans secoisolariciresinol, matairesinol, enterodiol and enterolactone as well as the human estrogen 17β estradiol and the anti-estrogen tamoxifen were dissolved in 100% ethanol to provide stock solutions of 1×10^{-1} mol/l for the synthetic lignans, 1×10^{-2} mol/l for 17β estradiol and tamoxifen, respectively. 1 µl of corresponding dilutions of these stock solutions were added to the culture medium to give final concentrations of the lignans (1×10^{-3}, 5×10^{-4}, 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, 1×10^{-7} mol/l), 17β estradiol (1×10^{-11}, 1×10^{-13} mol/l) and tamoxifen (1×10^{-4}, 1×10^{-7} mol/l) (final concentration of ethanol: 1%).

Cell lines and cell culture

The human breast cancer cell lines MCF 7 (HTB 22) and BT 20 (HTB 19) were obtained from the American Type Culture Collection, Cell Biology, LGC Promochem, Wesel, Germany. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, PAA Laboratories GmbH, Coelbe, Germany) with 10% inactivated fetal calf serum (FCS), 1% antibiotics (penicillin/streptomycin) and 0.5% amphotericin at humidified atmosphere (37°C and 5% CO_2).

The assessment of cell proliferation

Cell proliferation of the human breast carcinoma cell lines MCF 7 and BT 20 treated with different concentrations of lignans, 17β estradiol and tamoxifen was analysed using the BrdU Cell Proliferation ELISA kit (colorimetric) as recommended by the manufacturer (Roche, Germany). The test conditions were optimised in preliminary experiments, and the optimal cell number was found to be 5×10^4 cells/well. The experiments were divided into four groups: (1) negative control with 1 µl ethanol, different concentrations (2) of 17β estradiol and (3) tamoxifen as positive controls as well as different concentrations (4) of the test lignans (see above). Each group consisted of 4 replicate wells in 96-well plates. The negative control with 1 µl ethanol is hereafter referred to as 'control'.

BrdU assay

The test is based on the incorporation of 5-bromo-2′-deoxyuridine (BrdU), a pyrimidine analogue, instead of thymidine into the DNA of proliferating cells. MCF 7 and BT 20 cells were grown in 96-well tissue culture plates without phenol red and with hormone-free FCS (Biochrom AG, Berlin, Germany) for 24 h in the absence and presence of different concentrations of the test substances (see above) at 37°C and 5% CO_2. After labelling with BrdU the cells were fixed, and BrdU incorporation into DNA was measured by an ELISA reader (BioRad, Hercules, CA, USA) at 450 nm (reference wavelength: 620 nm). The developed colour and thereby the absorbance values correlate to the amount of DNA synthesis and hereby to the number of proliferating cells in the respective microcultures.

Statistical analysis

The experimental arrangement is based on a three repeats of every test procedure such that each bar in the diagrams

represents the mean ± SD (standard deviation) values of three separate experiments with 12 individual repeats. Statistical analysis was performed using the Student's *t* test for comparison of the means with the control. *P*-values of <0.05 were considered as being statistically significant and denoted by asterisks for the Student's *t* test (*).

Results

Effects of the plant-derived lignans secoisolariciresinol and matairesinol on proliferation of MCF 7 and BT 20 breast carcinoma cells

Secoisolariciresinol and matairesinol significantly inhibited proliferation of MCF 7 cells at concentrations of 1×10^{-5} and 1×10^{-6} mol/l, relative to the control (100%) (Figs. 1a, 2a). Higher concentrations of secoisolariciresinol did not induce inhibition of cell proliferation, whereas, higher concentrations of matairesinol led to a significant blockade of cell growth in a concentration-dependent manner (Figs. 1a, 2a). Matairesinol at a concentration of 1×10^{-3} mol/l reduced the cell growth by about 60% in comparison to untreated control cultures. The negative control 1 (cells in DMEM without ethanol) and negative control 2 (cells in DMEM with ethanol) determinations did not differ in absorbance values, showing that ethanol at 1% did not induce inhibition effects on the growth of MCF 7 and BT 20 cells (data not shown). The influence of secoisolariciresinol on BT 20 cells shows similar effects as on MCF 7 cells (Fig. 1a, b). However, addition of matairesinol to BT 20 cells induced stronger inhibition of cell growth only at the highest concentration of 1×10^{-3} mol/l; the concentration-dependent reduction as demonstrated for MCF 7 cells was not expressed (Fig. 2a, b).

Effects of the human lignans enterodiol and enterolactone on proliferation of MCF 7 and BT 20 breast carcinoma cells

In comparison to the plant-derived lignans secoisolariciresinol and matairesinol, the human lignans enterodiol and enterolactone, which are the metabolised products of the formers, induced a nearly total blockade of cell growth in MCF 7 cells at high concentrations (Figs. 1a, 2a). After addition of enterodiol and enterolactone at a concentration of 1×10^{-3} mol/l, cell proliferation was reduced by about 98 and 97%, respectively. Application of lower concentrations of enterodiol and enterolactone reduced the cell proliferation only weakly or no significant activation of the estrogen-dependent cell line MCF 7 was observed (Figs. 1a, 2a).

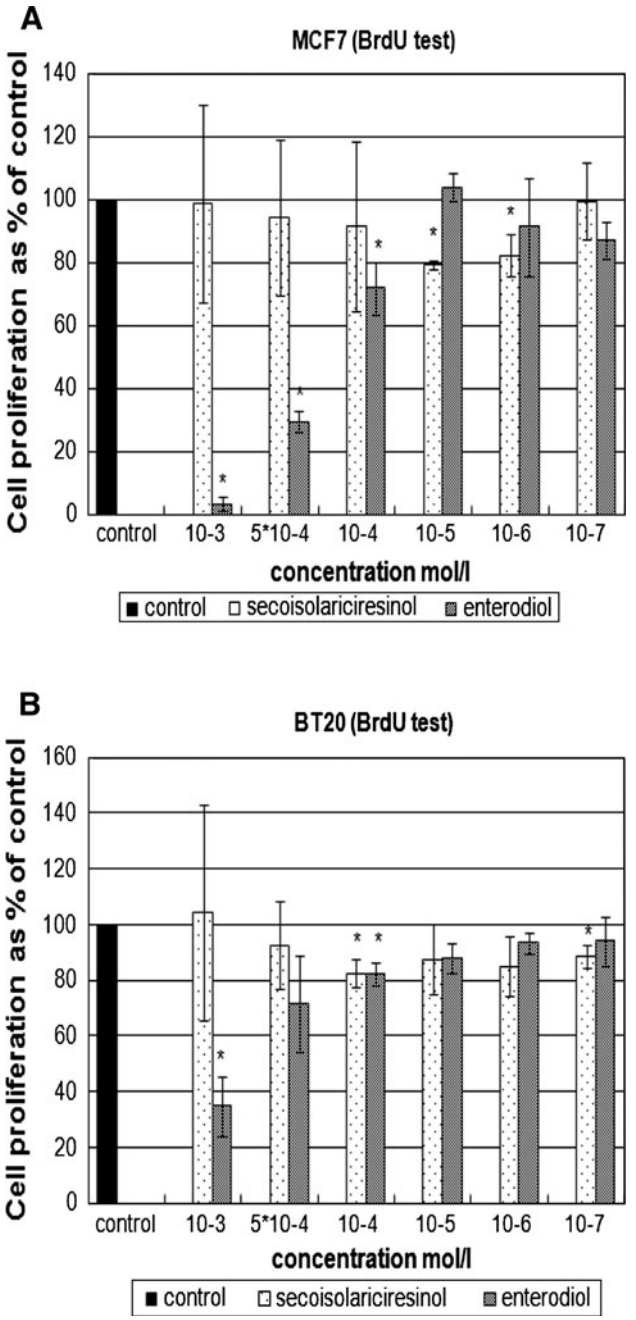

Fig. 1 Effect of different concentrations of secoisolariciresinol and enterodiol on the cell proliferation (BrdU test) of MCF 7 (**a**) and BT 20 (**b**) breast carcinoma cells. Data [mean ± SD (standard deviation)] represent relative number of proliferating cells in % in comparison to control (100%). *Asterisks* (*) indicate significant differences between treated cells and the control ($P < 0.05$)

The impact of enterodiol and enterolactone in BT 20 cells shows stronger inhibition effects in comparison to secoisolariciresinol and matairesinol, too. However, the inhibition effects were less expressed than in the MCF 7 cells (Figs. 1a, b, 2a, b).

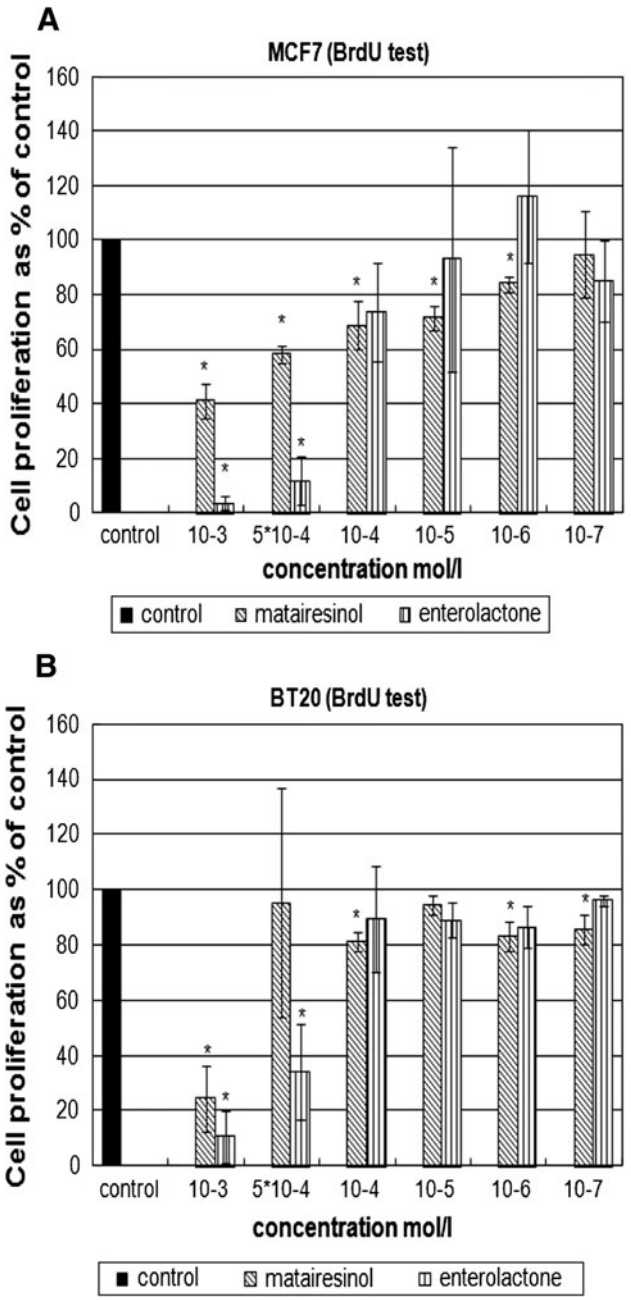

Fig. 2 Effect of different concentrations of matairesinol and enterolactone on the cell proliferation (BrdU test) of MCF 7 (**a**) and BT 20 (**b**) breast carcinoma cells. Data (mean ± SD (standard deviation)) represent relative number of proliferating cells in % in comparison to control (100%). *Asterisks* (*) indicate significant differences between treated cells and the control ($P < 0.05$)

Effects of the human estrogen 17β estradiol and the anti-estrogen tamoxifen on proliferation of MCF 7 and BT 20 breast carcinoma cells

The addition of 17β estradiol at concentrations of 1×10^{-11} and 1×10^{-13} mol/l did not affect the proliferation of MCF 7 and BT 20 cells (Fig. 3a, b). However, application of tamoxifen at a concentration of 1×10^{-4} mol/l significantly reduced the growth by about 85 and 93% in MCF 7 and BT 20 cells, respectively. Lower concentrations of tamoxifen (1×10^{-7} mol/l) inhibited the amount of newly synthesised DNA by about 10 and 15% in MCF 7 and BT 20 cells, respectively (Fig. 3a, b).

Discussion

At present, there is a general consensus that besides plant polyphenols, flavonoids and catechins, lignans are the key constituents in cancer prevention [14, 22]. Our results demonstrated that the mammalian lignans enterodiol and enterolactone are biologically more active than their precursors secoisolariciresinol and matairesinol (Figs. 1a, b, 2a, b). The plant-derived lignan secoisolariresinol did not induce inhibitory effect at the highest concentration applied (1×10^{-3} mol/l) in the MCF 7 cell line, whereas, the mammalian lignan enterodiol produced a 98% inhibition at the same concentration (Fig. 1a). Matairesinol reduced the cell growth up to 60% at this concentration in MCF 7 cell lines, whereas, enterolactone blocked it up to 97% (Fig. 2a). Secoisolariciresinol and matairesinol seem to act as prodrugs with relatively little biological activity. The compounds are substrates for enzymes that are highly expressed in tumour cells (CYP 1A1, CYP 1B1) [17], and the hormone-like conversion products inhibit cell proliferation. Low concentrations of enterodiol and enterolactone (1×10^{-5}; 1×10^{-6}; 1×10^{-7} mol/l) have been found stimulatory or non effecting in the breast cancer cell line MCF 7 as ER transmitted effects (Figs. 1a, 2a), whereas, higher concentrations (1×10^{-4}; 5×10^{-4}; 1×10^{-3} mol/l) lead to growth inhibition as non-receptor effects. These results are in line with the findings of Mousavi and Adlercreutz [21], Wang and Kurzer [23], who demonstrated that the mammalian lignans have stimulatory as well as inhibitory effects on cell growth in breast cancer cells in vitro, depending on the concentrations used. Many investigators have focused on the weak estrogenic and antiestrogenic activity of enterodiol and enterolactone [24–27]. These potencies are confirmed by our results that the stimulatory effects of enterodiol and entrolactone are more expressed in MCF 7 ER positive cells than in BT 20 ER negative cells (Figs. 1a, b, 2a, b). The inhibitory effects at high concentrations of the enterolignans should be similar in MCF 7 and BT 20 cells. These effects are expressed more clearly for enterolactone.

17β estradiol at the concentrations of 1×10^{-11} and 1×10^{-13} mol/l did not induce activation of cell growth in the breast carcinoma cell lines MCF 7 and BT 20 tested (Fig. 3a, b). Cauley et al. [28] demonstrated that high concentrations of estradiol ($<2 \times 10^{-12}$ mol/l) induced a

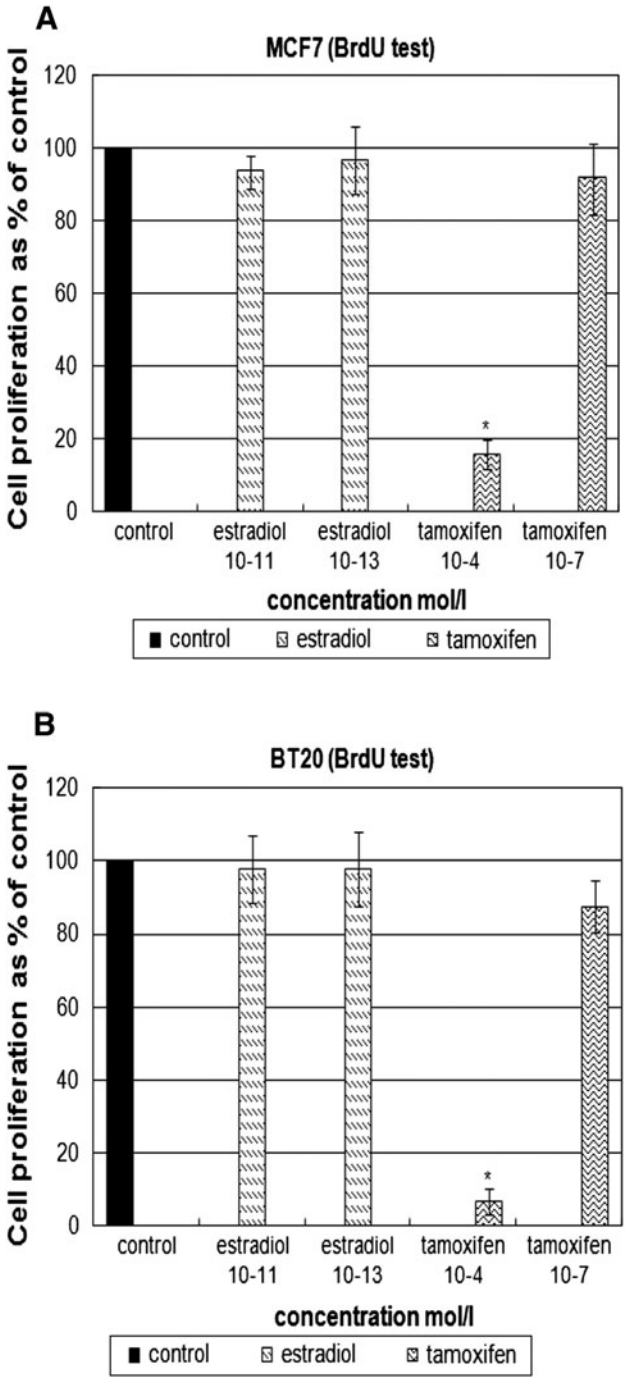

Fig. 3 Effect of different concentrations of 17β estradiol and tamoxifen on the cell proliferation (BrdU test) of MCF 7 (**a**) and BT 20 (**b**) breast carcinoma cells. Data [mean ± SD (standard deviation)] represent relative number of proliferating cells in % in comparison to control (100%). *Asterisks* (*) indicate significant differences between treated cells and the control ($P < 0.05$)

higher risk for breast cancer than low concentrations of estradiol (2–7 $\times$ 10^{-12} mol/l) in clinical studies. It is difficult to compare the observations of endocrinological reactions in clinical studies with those in in vitro assays. But nevertheless, further experiments should be performed with more interval concentration gradations of 17β estradiol in our in vitro studies. CYP1 enzymes are known to play a role in the metabolism of estrogens. Possibly estradiol develops its full activity with the estrogen receptor, only after metabolisation to 4-hydroxyestradiol. The latter compound plays a key role in the development of breast and endometrial cancer [17]. Additional experiments have to be taken into consideration with 4-hydroxyestradiol in future.

Tamoxifen induced a strong inhibition of cell growth in MCF 7 and BT 20 cells (Fig. 3a, b). At concentrations of 1 $\times$ 10^{-4} and 1 $\times$ 10^{-7} mol/l it blocked cell proliferation up to 85% and 93% and 10% and 15% in MCF 7 and BT 20 cells, respectively. The inhibitory activity at these concentrations added is much stronger as for the same concentrations of the lignans enterodiol and enterolactone applicated. The inhibitory effects of tamoxifen in ER positive and negative cells underline numerous results that state that the greatest portion of the growth inhibiting effect of tamoxifen in vitro is not ER dependent [29–32]. Zheng et al. [29] demonstrated that tamoxifen has also non-genomic effects. In concentrations above 5 $\times$ 10^{-6} mol/l it was found that tamoxifen induces apoptosis [29]. At the concentration of 1 $\times$ 10^{-4} mol/l used in our experiments it seems to induce apoptosis. The previous flow cytometric measurements of apoptosis by our working group resulted in a significant increase of apoptosis up to 35% after addition of 1 $\times$ 10^{-4} mol/l tamoxifen to MCF 7 cells [13].

Todorova et al. [30] demonstrated that one of the mechanisms of action of tamoxifen in receptor-negative cells is associated with inhibition of cellular glutamine uptake, oxidative stress and induction of apoptosis. Liang et al. [31] detected that tamoxifen induces apoptosis in ER negative breast cancer cells by DNA fragmentation, loss of mitochondrial cytochrome C and activation of caspase-3. Perry et al. [32] performed long term experiments with tamoxifen in ER negative breast cancer cells, inducing apoptosis by an increased expression of transforming growth factor beta 1.

According to a meta-analysis of randomized trials, a positive effect of tamoxifen was not shown for the ER-negative mammary carcinoma in the clinic. In ER-negative disease, tamoxifen had little or no effect on breast cancer recurrence or mortality [33].

Tamoxifen is a nonsteroidal agent (selective estrogen receptor modulator) with potent antiestrogenic properties, which competes with estrogen for binding sites in breast and other tissues. Tamoxifen itself is a prodrug, having relatively little affinity for its target protein, the estrogen receptor. It is metabolised in the liver by the 450 isoform CYP2D6 and CYP3A4 into active metabolites such as 4-hydroxytamoxifen and N-desmethyl-4-hydroxytamoxifen

(endoxifen), which have 30–100 times more activity with the estrogen receptor than tamoxifen itself [34]. Probably tamoxifen develops it full activity with the estrogen receptor in in vitro experiments only after metabolisation. Additional experiments have to be carried out with the metabolised form of tamoxifen, and the same concentrations as used for the lignans in future.

According to the practical aspects for clinicians in the antitumor therapy, there are no concrete data about the dosages of lignans. Therefore, we can derive only general recommendations such as dietary intake of lignans. It is conceivable to use lignans also in the tumour treatment as adjuvants. There are experimental indications that the dietary flavonol fisetin enhances the apoptosis-inducing potential of TRIAL (tumour necrosis factor-related apoptosis-inducing potential) in cancer cells [35]. In Western populations with low intake of isoflavones, phytoestrogen intake is predominantly derived from intake of plant lignans. Therefore, dietary intake of lignans may be more important for cancer prevention purposes than isoflavone consumption [2].

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Conflict of interest None.

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