Enterolactone Inhibits the Growth of 7,12-Dimethylbenz(*a*) anthracene-induced Mammary Carcinomas in the Rat[1]

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Abstract

The inverse association between a high enterolactone (ENL) concentration in both urine and serum, and the risk of breast cancer found in epidemiological studies suggests a chemopreventive action for ENL. However, no causal relationship has been established in clinical studies or in experimental models for breast cancer. In the present study, the potential chemopreventive action of p.o. administered ENL (1 or 10 mg/kg of body weight) was tested in 7,12-dimethylbenz(*a*)anthracene-induced mammary cancers of the rat. Rats were maintained on a standard open-formula chow diet. Daily p.o. administration of ENL at a dose of 10 mg/kg of body weight for 7 weeks significantly inhibited tumor growth. The growth-inhibitory effect of ENL was more pronounced on the new tumors, which developed during the treatment period, but ENL also inhibited the growth of those tumors established before the start of the lignan administration. The rat serum concentration of ENL, which illustrated a permanent positive effect on breast cancer growth, was 0.4 μM, which is >10-fold as compared with the serum concentrations found in the general human population. The effect of ENL was not restricted to any specific histological tumor type. ENL was demonstrated to act as a weak aromatase inhibitor *in vitro* and to reduce the relative uterine weight of the 7,12-dimethylbenz(*a*)anthracene-treated nonovariectomized rats. However, in a short-term assay ENL had no effect on the uterine growth of the intact or androstenedione-treated immature rats. Thus, the mechanism of the ENL action and its minimum or optimal daily dose remains to be clarified.

Introduction

Plant lignans, such as SECO and matairesinol found in many edible plants, are transformed by intestinal microbiota into mammalian lignans END[3] and ENL, respectively (1–4; Fig. 1). ENL is the quantitatively most important lignan in human serum. An inverse association between the urinary or serum ENL contents and the risk of breast cancer has been described in several epidemiological studies (5–7). The finding has stimulated considerable interest in the possible chemopreventive action of ENL and its precursors.

Flaxseed is the richest known dietary source of mammalian lignans (8). Thompson *et al.* (9) demonstrated that SDG, isolated from flaxseed, was metabolized into both END and ENL, and showed chemopreventive properties in the DMBA-induced mammary tumor model in rats. Administration of SDG (1.5 mg/day, *i.e.,* 4–9 mg/kg of body weight) starting 1 week after the DMBA induction reduced the tumor multiplicity (number of tumors per rat) and tumor incidence (number of animals with tumors). SDG also inhibited the growth of the established tumors (mean total tumor volume per animal) and number of tumors appearing during the late stage of carcinogenesis (10). A plant lignan HMR was metabolized into ENL in rats in a previous study (11). HMR inhibited the growth of DMBA-induced mammary tumors and increased the proportion of the stabilized and regressing tumors. HMR administration via diet in an average daily dose of 4.7 mg/kg of body weight starting 1 week before DMBA induction reduced both tumor volume and tumor growth, but no significant reduction in the tumor multiplicity was observed (12). At the end of the 8- and 17-week dietary supplementation with HMR, the serum ENL concentrations (0.35 μM) were >12-fold as compared with the controls.

In pilot human studies, dietary flaxseed given preoperatively to newly diagnosed breast cancer patients markedly reduced the Ki67 labeling index and c-erb2 of the breast cancer tissue, suggesting direct antiproliferative effects (13). As expected, there was a marked increase in the urinary excretion of mammalian lignans after the ingestion of flax. A recent study of short-term flaxseed supplementation and fat restriction in diet additionally showed significantly lower proliferation rates and higher rates of apoptosis in prostate cancer of men before operation (14). However, the observed effects of flax do not allow for any conclusions on the possible role of lignans in the inhibition of cancer growth, be-

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[3] The abbreviations used are: END, enterodiol; ENL, enterolactone; SDG, secoisolariciresinol diglucoside; DMBA, 7,12-dimethylbenz(*a*)anthracene; HMR, 7-hydroxymatairesinol; SECO, secoisolariciresinol; MR, (8R,8′R)-(−)-matairesinol; EQ, equol; *O*-DMA, *O*-desmethylangolensin; DA, daidzein; GEN, genistein; arom+HEK 293, human embryonic kidney cells transfected with human aromatase gene; HPLC, high-performance liquid chromatography; AN, androstenedione; Pd-C, palladium on carbon; BHT, butylated hydroxytoluene.

cause other potentially bioactive multiple compounds are present in flaxseed.

ENL is assumed to mediate the anticarcinogenic action of plant lignans shown in experimental breast cancer and account for the antiproliferative effects seen in the clinical studies of flaxseed supplementation. However, there is no direct evidence for the anticarcinogenic action of ENL in humans or experimental cancer models in animals. The possible anticarcinogenic effects of ENL on breast cancer were tested for the first time by using a DMBA-induced mammary carcinoma of the rat in this study.

Materials and Methods

Synthesis and Isolation of Lignans. A racemic mixture of ENL was essentially synthesized as described by Kirk *et al.* (15). The structure was confirmed by ^{1}H and ^{13}C nuclear magnetic resonance spectrometry (in $CDCl_3$) with a JEOL JNM-A500 spectrometer at 500 and 125 MHz, respectively. All of the spectra were concordant with published data (16). The exact mass determined by mass spectroscopy (Fisons Zab Spec-oaTOF) was 298.120100 (calculated for $C_{18}H_{18}O_4$; 298.120509). SECO was extracted from *Araucaria angustifolia* and HMR from *Picea abies* with a method described previously (17). The crude acetone extracts were evaporated totally in a rotary film evaporator, and the dry extract was mixed with three parts of silica gel (Fluka silica gel 60). The mixture was placed in a sample injection module of a Flash 40i chromatography apparatus (Biotage Inc., Charlottesville, VA). Purification was performed on a Flash 40M cartridge (Silica, 32–63 μm, 60A; Biotage Inc.) with an eluent composition of 3:97 (ethanol:dichlorometane) for HMR and 5:95 for SECO. Fractions of 50 ml were collected, and each fraction was examined using TLC. The purest fractions as judged by TLC were collected and evaporated to dryness. An analysis by gas chromatography after sialylation with bis(trimethylsilyl)trifluoroacetamide (Acros, Geel, Belgium) and trimethylchlorosilane (Fluka Chemicals, Buchs, Switzerland) showed a purity of 95% for HMR. SECO was additionally recrystallized from dilute ethanol, resulting in a purity of 97% as analyzed by gas chromatography. MR was synthesized by additional development of a method used by Freudenberg and Knof (18). The hydrogenolysis reaction starting from HMR was performed in a 50-ml double-necked roundbottomed flask with vigorous magnetic stirring. Exactly, 1 g of Pd-C 10% (Acros) was mixed with 1,2-dichloroethane (J. T. Baker, Deventer, Netherlands) and kept in an ultrasonic bath for 5 min. The Pd-C was activated with H_2 at atmospheric pressure (balloon) for 3 h. A 3:1 mixture of (−)-HMR and (−)-*allo*-HMR (2 g) was added to the catalyst suspension. The reaction was continued at atmospheric pressure for 99 h. The temperature of the mixture was kept at 60°C. According to TLC on silica (1:10 methanol:dichloromethane), unreacted HMR remained in the reaction mixture. Then 1.2 g of Pd-C 10% was added to the mixture, and the reaction was continued at 50°C for 24 h. The reaction mixture was filtered through a filter paper and purified by flash chromatography with a toluene:ethylacetate (100:22) solvent system. The yield was 57%, *i.e.,* 1.078 g of a white solid was obtained. $(a)^{24}_D = -36.9°$ [c = 3.33 in tetrahydrofuran (mg/ml)], in literature $(a)^{25}_D = -42.2°$ [c = 2 in acetone (mg/ml); Ref. 19]. Nuclear magnetic resonance data were in accordance with that reported previously (20). High resolution mass spectroscopy, *m/z* calculated for $C_{20}H_{22}O_6$ (M^+), is 358.141639, and was found to be 358.141900. A radioimmunoassay kit for rat serum prolactin was procured from Immunotech Ltd., Marceille, France.

Effects of ENL in the DMBA-induced Mammary Tumor Model. Anticarcinogenic activity of ENL was studied using the mammary tumor model and female Sprague Dawley rats. These rats were obtained from Harlan Netherlands (Horst, the Netherlands) at the age of 40 days. They were housed (2–3 animals/cage) under a 12:12-h light-dark cycle at 21°C with 50% humidity, and with free access to water and a standard open-formula RM 1 diet (SDS, Special Diet Services, Whitham Essex, England). At the age of 50 days, these rats were induced p.o. with 12 mg of DMBA (Sigma Chemical Co., St. Louis, MO) in 1 ml of sesame oil (Acros). One of the DMBA-induced animals was sacrificed before the start of the lignan feedings. Nine weeks (63 days) after induction, these animals were divided into three treatment groups (13 rats in each). The open-labeled groups (with the total number of the tumors similar in each group at the beginning of the experiment) were: (*a*) control group; (*b*) ENL 1 mg/kg of body weight; and (*c*) ENL 10 mg/kg of body weight. ENL dissolved into 50% polyethylene glycol (MW 3400; ICN Biomedicals Inc., Aurora, OH) in water containing 10% ethanol or placebo (50% polyethylene glycol in water and containing 10% ethanol) was administered daily p.o. starting 9 weeks after the DMBA-induction. The rats were gavaged daily for 50 days. These rats were weighed and tumors palpated once a week. The width (w) and the length (l) of the tumors were measured weekly to determine the tumor volumes according to a formula $V = [\pi \times (\text{width})^2 \times \text{length})]/12$. The minimum width and length of the palpable tumors both were 0.1 cm. The total tumor volume (per number of animals in each group) and tumor multiplicity (the number of tumors per rat in the treatment group) were followed weekly during the treatment period. In the control group and in the ENL 1 mg/kg group, 2 animals had to be sacrificed because of a tumor bursting (bleeding tumor) before the end of the experiment. In the follow-up of tumor growth, the tumor volumes of rats sacrificed before the end of the experiment were calculated with stabilized volume during the last weeks of the experiment. Animal weight gain and diet consumption per animals in one cage was followed weekly as well. At the end of the experiment (113 days after induction), animals were weighed and anesthetized with carbon dioxide, and blood was collected by heart puncture before decapitation. Serum was separated by centrifugation and the samples stored at −20°C until analyzed. Tumor samples were dissected and fixed in neutral buffered formalin for histopathological examination. Liver and uterus were weighed when wet.

The established tumors were classified into two groups according to their growth pattern: (*a*) growing tumors: tumors with an increasing volume, tumors appearing in the last week of the experiment, and tumors with a fluctuating growth pattern (only temporal regression in growth during experiment); and (*b*) nongrowing tumors: stabilized tumors and

Table 1 The numbers of the tumors in treatment and control groups

Group	The number of rats at the start of the experiment	Total number of established tumors	Number of rats at the end of the experiment	Tumors present at the end of the experiment	CR[a]
Control	13	30	11	22	3
ENL 1 mg/kg	13	24	12	16	5
ENL 10 mg/kg	13	25	13	17	6

[a] Completely regressed tumors.

regressing tumors. Those tumors that disappeared during the experiment were included in the group of nongrowing tumors.

Urine Collections. Individual 24-h urine samples were collected in two sets on 2 consecutive days during the last week of the experiment (*i.e.,* 16 weeks after DMBA induction). At the time of the urine collection, the rats were treated with ENL (1 mg/kg or 10 mg/kg) or with a placebo daily for 46 or 47 days. The collection jars of the metabolic cages contained 120 μl of 0.56 M ascorbic acid (Sigma Chemical Co.) and 120 μl of 0.15 M sodium azide (Merck, Darmstadt, Germany) as preservatives. The centrifuged urine volumes were measured and the urine samples stored at −20°C until analyzed.

Analysis of Lignan Metabolites from Rat Urine and Serum. Lignans and isoflavones in urine and serum were measured using solid-phase extraction followed by HPLC and tandem mass spectrometry as described previously by Valentín-Blasini *et al.* (21). All of the urine and serum samples were spiked with a mixture of stable isotope-labeled internal standards ([$^2H_6$]-ENL, [$^2H_6$]-END, [$^2H_6$]-MR, [$^2H_4$]-EQ, [$^2H_5$]-*O*-DMA, [$^2H_3$]-DA, [$^2H_4$]-GEN) and deconjugated internal standards (4-methylumbelliferone glucuronide and 4-methylumbelliferone sulfate). Serum was diluted and buffered with ammonium acetate [1 ml of 250 mM (pH 5.0)]. Conjugated analytes were hydrolyzed by the addition of *Helix pomatia*, H-1, and incubated overnight at 37°C. Deconjugated samples were extracted with Oasis hydrophilic-lipophilic balanced solid-phase extraction (60 mg hydrophilic-lipophilic balanced copolymer, 3 ml; Waters Scientific, Beverly, MA) as described by Valentín-Blasini *et al.* (21) and resuspended in mobile phase. Extracted samples were separated by a reversed-phase HPLC (50 mm Prism column; Keystone Scientific, Bellafonte, PA) followed by tandem mass spectrometry on a triple quadrupole instrument (Sciex API III and/or API 3000; Perkin-Elmer Sciex Instruments, Wellesley, MA) using heated nebulizer atmospheric pressure chemical ionization in the negative ion mode.

Histological Examination. For the purposes of light microscopy, the formalin-fixed tumor samples were embedded in paraffin, cut at 5 μm, and stained with H&E. The numbers of tumors to be evaluated in each treatment group are presented in Table 1. Fully necrotic tumors and tumors from rats sacrificed before the end of the experiment were not available for histological examination. Four histological subcategories or types of tumors were identified as described previously by Archer and Orlando (22): A = poorly differentiated; B = well differentiated; C = atrophic; and D = secretory. The mitotic count (number of mitoses per 10 high power fields with ×40 objective) was examined.

***In Vitro* Aromatase Inhibition Assays.** The potential aromatase inhibition of ENL, SECO, MR, and HMR was tested in HEK-293 cells transfected permanently with the human aromatase gene (Arom+HEK 293; Ref. 23). The inhibition assays were performed as described previously (11). Briefly stated, Arom+HEK 293 cells were cultured in DMEM (Life Technologies, Inc., Paisley, Scotland) containing 10% of two times charcoal-stripped heat-inactivated FCS (Life Technologies, Inc.) for 2–4 days before the experiments. The cells were detached with trypsin-EDTA mixture (Life Technologies, Inc.). Centrifuged cells were resuspended in serum-free DMEM and counted. The incubation mixture contained 50 μl of [^{3}H]androst-4-ene,3,17-dione (NEN Life Science Products, Zaventem, Belgium; in DMEM, final concentration 0.5 nM), 50 μl of unlabeled AN (Sigma Chemicals Co.; in DMEM, final concentration 0.5 nM), 100 μl of DMEM solution containing lignan in ethanol, and 800 μl of cell suspension (*i.e.,* 0.5 × 10^6 cells). Aromatase inhibition was tested at 1, 2, 5, and 10 μM concentrations for the determination of the IC_{50} values. After incubation for 3 h in Arom+HEK 293 cells, unlabeled carriers AN, testosterone, 17β-estradiol, and estrone (all obtained from Sigma Chemicals Co.) were added. Steroids were twice extracted with 3 ml of dichloromethane (Rathburn, Peebleshire, United Kingdom). The tubes containing combined dichloromethane extracts were totally evaporated in a nitrogen flow at +45°C. The extracted steroids were dissolved in 35% acetonitrile (Rathburn) in water before HPLC run. HPLC was used for a separation of steroid metabolites and a quantification of [^{3}H]AN and [^{3}H]estrone. An analysis was completed with the HPLC system (Waters 2690) equipped with Waters 996 photodiodearray detector and flow scintillation analyzer (model 150TR; Packard Bioscience Company). Nova-Pak C18 column (3.9 × 150 mm; Waters Co.) with Nova-Pak C18 guard column was used for the separation of conversion products. The mobile phase was 35% acetonitrile in water, and the flow rate was 1 ml/min. Aromatase activity (formation of [^{3}H]estrone) was calculated as the percentage of [^{3}H]AN converted to estrone. The IC_{50} values for each compound were calculated by using the GraphPad Prism 3.0 program (GraphPad Software Inc., San Diego, CA).

Uterotropic Estrogenicity and Aromatase Inhibition Assays in Immature Rats. Potential estrogenicity, and an ability to inhibit aromatase of mammalian lignan ENL and plant lignans MR, SECO, and HMR were *in vivo* evaluated

using uterotropic tests in immature rats as described previously (11). Briefly stated, 18-day-old female Sprague Dawley pups (dams originally obtained from Harlan) were weaned and evenly randomized to treatment groups. Immature rats were housed (3–5 per cage) under a 12:12-h light-dark cycle at 21°C with 50% humidity, and they had free access to water and a RM 3 soy-free diet (SDS Special Diet Services).

In estrogenicity assays, control rats were administered p.o. with rapeseed oil (Kultasula; Raisio Ltd., Turku, Finland) containing 10% of ethanol (100 μl/10 gram of body weight), which was used as a vehicle for feedings. DES (2 μg/kg of body weight, positive control) or lignans (50 mg/kg of body weight in each treatment group) were administered to rats daily. On day 8 after weaning (*i.e.,* after 7 days of treatment), 28-day-old animals were anesthetized with carbon dioxide, weighed, and their uteri were immediately removed and weighed after a gentle removal of possible fluid from uterine cavity.

On the basis of the screening of different doses of AN (11) a dose of 30 mg/kg of body weight was selected for *in vivo* aromatase inhibition assays with lignans. A daily p.o. dose of 50 mg/kg of body weight of each lignan (ENL, MR, SECO, and HMR) was tested. In the negative control group, 18-day-old rats were injected s.c. with rapeseed oil. Positive control rats were treated s.c. with AN. In both control groups, the animals were administered daily p.o. with rapeseed oil. Aromatase inhibitor finrozole (Hormos Medical Corp., Turku, Finland; 10 mg/kg of body weight) was administered p.o. to immature rats treated with AN. In lignan groups, AN-treated rats were gavaged with ENL, MR, SECO, or HMR in oil vehicle as described above. The animal body weight gain was followed daily. Twenty-five-day-old animals were anesthetized with carbon dioxide, weighed, decapitated, and their uteri weighted wet.

Statistical Analysis. Statistica version 5.1 software for Windows was used for the statistical analysis (Stat Soft, Inc., Tulsa, OK). Normally distributed data of urine and serum phytoestrogen concentrations, and animal weights in DMBA-induced animals were analyzed by using one-way ANOVA followed by Tukey's least significance test. In the case of relative uterine weights, least significant difference test was used after the ANOVA. Non-normally distributed data of uterine weights of immature animals, serum prolactin, and relative tumor growth were analyzed by Kruskal-Wallis median test followed by Mann-Whitney *t* test. The independent growth of each tumor was confirmed by using Mann-Whitney *t* test. The acceptable level of significance was $P \leq 0.05$.

Results

Chemopreventive Properties of ENL in DMBA-induced Mammary Tumors. ENL at 10 mg/kg significantly reduced the total tumor volume during the 7-week period (Fig. 2). A similar trend was seen in the ENL 1 mg/kg treatment group during the first 4 weeks, but the total tumor volume reached the control values during the last 2 weeks of the treatment, and the difference did not reach statistical significance at any time point ($P \geq 0.11$; Fig. 2). In the animals

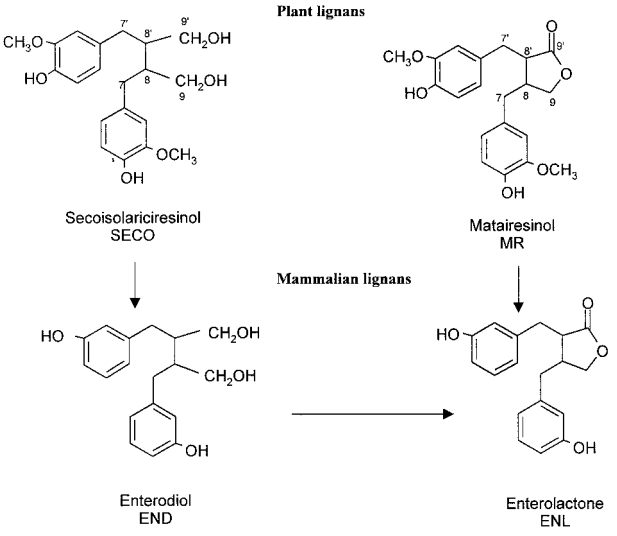

Fig. 1. Metabolism of plant lignans to mammalian lignans.

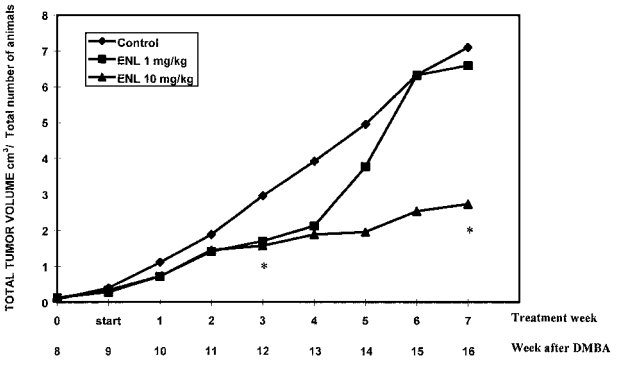

Fig. 2. Growth of all tumors. *, growth significantly reduced as compared with the control.

treated with the higher dose of ENL (10 mg/kg), the total tumor volume remained below the control value toward the end of the treatment. The inhibition of tumor growth was more pronounced in tumors that developed during the 7-week ENL treatment period (Fig. 3), but ENL 10 mg/kg also inhibited the growth of those tumors established before the start of the treatment (Fig. 2). At the end of the experiment, the number of the tumors (per number of rats) tended to be smaller in ENL 1 mg/kg and 10 mg/kg groups when compared with the control group (1.45 and 1.31 *versus* 2.00, respectively), but the difference did not reach significance ($P > 0.05$).

An increased proportion of the nongrowing (regressing, stabilized, or totally disappeared) tumors and a decreased proportion of the growing tumors was observed in the ENL 10 mg/kg group (Fig. 4). At the end of the experiment, no differences in tumor proportions were observed between ENL 1 mg/kg and control groups. In both control and ENL 1 mg/kg groups, a part of the growing tumors (4.0 and 4.5%, respectively) showed a fluctuating growth pattern, *i.e.,* the tumors first regressed but started to grow again during the

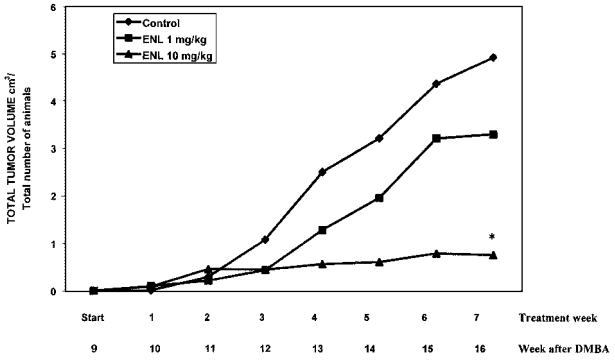

Fig. 3. Growth of tumors established during the ENL treatment period.

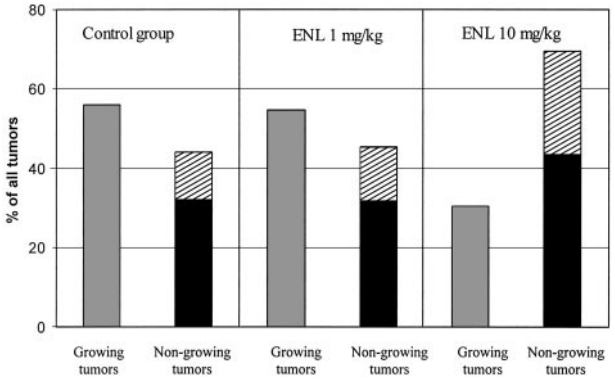

Fig. 4. Anticarcinogenic activity of ENL in DMBA-induced mammary carcinomas in female rats. *Gray bars,* growing tumors; *black bars,* regressing and stabilized tumors; *striped bars,* completely regressed tumors.

experiment. No fluctuating growth was observed in ENL 10 mg/kg group.

The DMBA-induced tumors were histologically verified to be adenocarcinomas. The tumors to be evaluated (Table 1) were additionally histologically characterized according to Archer and Orlando (22) as types A, B, C, or D (Table 2). The growth inhibition effect of ENL was not restricted to any specified histological type of the tumor. The average mitotic count in the control samples (6.1 ± 6.4) was not significantly different from the mitotic count in ENL 1 or 10 mg/kg treatment groups (5.6 ± 3.8 and 4.3 ± 2.7, respectively). Furthermore, slightly elevated prolactin serum concentrations were measured both in ENL 1 mg/kg and 10 mg/kg treatment groups (53 ± 39 ng/ml and 62 ± 71 ng/ml, respectively) as compared with the controls (23 ± 14 ng/ml), but the differences did not reach statistical significance ($P = 0.11$).

The body weight gain and average food consumption were similar in the three treatment groups throughout the experimental period (data not shown). At the end of the experiment, the relative uterine weight in the ENL 10 mg/kg group was significantly reduced as compared with the control group (Table 3). No differences in liver weights were observed (Table 3).

Table 2 Histological types of DMBA-induced rat mammary tumors, which could be evaluated in treatment and control groups

Group	Number of evaluable tumors	Histological type[a] (% of tumors)			
		A	B	C	D
Control	21	19	57	19	5
ENL 1 mg/kg	15	40	53	7	0
ENL 10 mg/kg	17	18	65	18	0

[a] Classified according to Archer and Orlando (22).

Table 3 Relative uterine and liver weights of DMBA-treated rats in treatment and control group at the end of the experiment

	Relative uterine weight (mg/100 g bw)	Relative liver weight (mg/100 g bw)
Control group	210 ± 59	3167 ± 223
ENL 1 mg/kg	182 ± 44	3182 ± 348
ENL 10 mg/kg	163 ± 21[a]	2935 ± 171

[a] Significantly different from control group ($P = 0.02$).

Lignans and Phytoestrogens in Urine and Serum. The p.o. administration of ENL in a dose of 1 mg/kg of body weight did not significantly increase the basal ENL concentrations in urine or serum (Table 4, *A and B,* respectively). However, after the daily administration of the higher oral dose of ENL (10 mg/kg of body weight), the ENL concentrations in serum and in urine were significantly increased. The administration of ENL (10 mg/kg) also increased END concentration in serum but not in urine. Increased serum concentrations of EQ and *O*-DMA were similarly measured after exposure to a higher dose of ENL (Table 4, *A* and *B*). No significant differences were observed in GEN or DA concentrations of either urine or serum (Table 4, *A* and *B*).

Aromatase Inhibition of Lignans *in Vitro* and *in Vivo*. The IC_{50} concentrations for aromatase inhibition were tested for several lignans including ENL in HEK 293 cells transfected with human aromatase gene. ENL was the most potent inhibitor of aromatase with IC_{50} value of 8.9 μM. The IC_{50} values for SECO, END, MR, and HMR were >10 μM, the highest tested concentration.

The aromatase inhibition *in vivo* was tested by measuring the reduction of the uterine growth as judged by its wet weight in control and AN-treated immature rats (Tables 5 and 6). Oral administration of ENL and plant lignans (SECO, MR, and HMR) in a dose of 50 mg/kg of body weight for 7 days showed neither growth-promoting nor growth-inhibiting actions on uterine growth.

Discussion

ENL was shown to inhibit tumor growth in the DMBA model for breast cancer. Inhibition was more pronounced in tumors that developed during the 7-week treatment period, but ENL also inhibited the growth of the tumors established before the start of lignan administration. An increased proportion of nongrowing (regressing, stabilized, or totally disappeared) tumors and a decreased proportion of growing tumors were observed in the rats treated with the dose of 10 mg/kg of

Table 4 Lignans and phytoestrogens in urine and serum by treatment and control group

A. Urine Lignans and phytoestrogens

Group[a]	Lignans and phytoestrogens in urine μg/24 h					
	ENL	END	GEN	DA	EQ	O-DMA
Control	223.93 ± 78.38[b]	8.34 ± 3.57[b]	43.65 ± 19.93[b]	70.35 ± 29.85[b]	44.78 ± 11.27[b]	2.54 ± 2.67[b]
ENL 1 mg/kg	298.15 ± 70.99[b]	8.61 ± 5.91[b]	43.43 ± 15.26[b]	60.27 ± 21.21[b]	52.01 ± 13.40[b]	1.64 ± 1.56[b]
ENL 10 mg/kg	610.71 ± 234.9[c]	10.13 ± 5.66[b]	35.14 ± 14.66[b]	77.30 ± 35.34[b]	30.38 ± 8.02[c]	5.40 ± 3.00[c]

B. Serum lignans and phytoestrogens in treatment and control groups

Group[a]	Lignans and phytoestrogens in serum ng/ml (nM)					
	ENL	END	GEN	DA	EQ	O-DMA
Control	62.5 ± 26.2[b]	5.5 ± 2.7[b]	29.2 ± 14.7[b]	22.2 ± 9.6[b]	138.0 ± 59.9[b]	13.3 ± 9.3[b]
	(209.5 ± 87.9)	(18.2 ± 8.94)	(108.1 ± 54.4)	(87.3 ± 37.8)	(569.5 ± 247.2)	(51.6 ± 36.0)
ENL 1 mg/kg	68.6 ± 17.3[b]	4.6 ± 1.7[b]	30.3 ± 7.8[b]	25.3 ± 8.0[b]	161.0 ± 49.3[b]	9.4 ± 6.2[c]
	(230.2 ± 58.1)	(15.4 ± 5.6)	(112.1 ± 28.9)	(99.5 ± 31.5)	(664.4 ± 203.5)	(36.4 ± 24.0)
ENL 10 mg/kg	120.8 ± 37.0[c]	8.4 ± 2.4[c]	25.8 ± 11.0[b]	25.0 ± 10.9[b]	189.0 ± 82.3[c]	22.4 ± 13.2[d]
	(405.4 ± 124.2)	(27.8 ± 7.9)	(95.5 ± 40.7)	(98.3 ± 42.9)	(780.0 ± 339.7)	(86.8 ± 51.2)

[a] Rats were gavaged with placebo or with ENL for 7 weeks.
[b–d] The letters [b, c, d], indicate statistically significant differences in concentrations of measured compounds between the groups ($P \leq 0.05$).

Table 5 Uterine weights of immature rats treated with AN in treatment groups

Treatment group	s.c. injections	Administered p.o.	*n*	Relative uterine weight (mg/100 g bw)
Control[a]	Oil	Oil	7	41 ± 3[b]
Positive control[c]	AN 30 mg/kg	Oil	13	119 ± 51[a]
Aromatase inhibitor	AN 30 mg/kg	Finrozole 10 mg/kg	9	67 ± 19[c]
ENL	AN 30 mg/kg	ENL 50 mg/kg	9	119 ± 35[a]
MR	AN 30 mg/kg	MAT 50 mg/kg	4	146 ± 29[a]
SECO	AN 30 mg/kg	SECO 50 mg/kg	5	135 ± 22[a]
HMR	AN 30 mg/kg	HMR 50 mg/kg	10	102 ± 14[a]

[a–c] Relative weights with the different letters [a, b, and c] indicate statistically significant differences ($P < 0.05$) between the treatment groups tested by Kruskal-Wallis median test followed by Mann-Whitney *U* test.

Table 6 Estrogenicity of lignans in immature rats as measured by uterine weight

Treatment group	*n*	Relative uterine weight (mg/100 g bw)
Control	9	46 ± 5[a]
Control + 10% ethanol	5	48 ± 8[a]
Diethylstilbestrol 2 μg/kg bw	8	164 ± 20[b]
Finrozole 10 mg/kg bw	6	31 ± 3[c]
ENL 50 mg/kg bw	6	47 ± 5[a]
MR 50 mg/kg bw	4	55 ± 9[a]
SECO 50 mg/kg bw	6	49 ± 6[a]
HMR 50 mg/kg bw	6	49 ± 7[a]

[a–c] Relative weights with the different letters [a, b, and c] indicate statistically significant differences. ($P < 0.05$) between the treatment groups tested by Kruskal-Wallis (median) test followed by Mann-Whitney *U* test.

body weight. The dose of 10 mg/kg of body weight doubled both the serum and urine concentrations of ENL. The serum and urine ENL concentrations did not significantly differ from the controls when the lower dose of ENL (1 mg/kg of body weight) was used, and no inhibition of tumor growth was observed in rats treated with this dose. This is most likely because of the use of open-formula chow as the basal diet, which resulted in relatively high basal ENL concentrations. We conclude that ENL may, at least in part, mediate the chemopreventive actions of plant lignans, SDG, and HMR seen in the experimental breast cancer models, and play an essential role in the antiproliferative actions of a flax-containing diet observed in the clinical studies.

The mechanism(s) of the ENL action as a chemopreventive agent is not known. Only two *in vivo* studies of the ENL action have been published. Waters and Knowler (24) have shown that ENL given in a dose of 1 mg/kg (s.c.) reduced the estradiol-stimulated RNA synthesis in uterus (ENL administered 22 h before estradiol). When estradiol was administered at the same time or up to 12 h before ENL, no significant effect on the stimulation by estradiol was seen. The authors pointed to the structural resemblance between ENL and antiestrogens such as tamoxifen, and suggested the competition with estradiol for the receptor binding as a possible mechanism of action. On the other hand, Setchell *et al.* (2) showed that ENL administered s.c. (6.7 and 20 mg/kg of body weight) did not have an estrogenic effect (*i.e.,* increase in uterine weight) in immature female mice. Similarly, in a study by Saarinen *et al.* (25), ENL showed no significant estrogenic or antiestrogenic activity via ERα or ERβ *in vitro* at concentrations <1 μM. In agreement with these findings, no significant effect was seen on the uterine growth of intact immature rats in the present study. These findings are in contradiction to the hypothesis that the ER-mediated-pathways would be the major targets of the ENL action. Similarly

to some other plant phenols such as chrysin or apigenin (11), ENL is a weak inhibitor of aromatase *in vivo*. Inhibition of aromatase by pharmaceuticals has been demonstrated to inhibit mammary cancer growth in humans and experimental models, and would, therefore, be an attractive explanation here as well. It is possible that the statistically significant decrease in uterine weight seen among the DMBA-treated female rats after the chronic administration of ENL may partly be because of aromatase inhibition. However, we could not demonstrate aromatase inhibition by ENL in a short-term test *in vivo, i.e.,* an AN-induced uterine growth assay.

DMBA-induced rat mammary carcinomas are shown to be prolactin dependent (26). The reoccurrence of mammary tumors in ovariectomized rats treated with estradiol can be prohibited by hypophysectomy (26), and the antiestrogen-induced regression of tumors can be reverted by increasing the release of prolactin (27). However, the increase in serum prolactin alone is not sufficient for regrowth of the DMBA-induced mammary tumors (27). In previous studies, nonsteroidal aromatase inhibitors have been shown to inhibit the incidence and growth of DMBA-induced mammary carcinomas and to decrease serum prolactin concentrations (28–30). In this study, no decrease was observed in serum prolactin concentrations. Instead, slightly elevated concentrations were measured. This suggests that ENL is not decreasing prolactin concentration in serum. Interestingly, Hutchins *et al.* (31) reported increased serum prolactin concentrations, and decreased serum 17β-estradiol and estrone concentrations in postmenopausal women after a 7-week administration of flaxseed in a daily dose of 10 g.

The chemopreventive actions of plant lignans SDG (9, 10) and HMR (12) were evident when the administration was started weeks before the tumor induction with DMBA or 1 week after the induction, suggesting that mechanisms other than DMBA detoxification may be involved. Antioxidativity of dietary components is one suggested mechanism of action in mammary carcinogenesis. McCormick *et al.* (32) documented that synthetic antioxidants BHT and butylated hydroxyanisole showed antitumor activity when administered to animals via undefined laboratory rodent diet in a dose of 2500 mg/kg of diet or 5000 mg/kg of diet. Furthermore, Cohen *et al.* (33) reported a significant inhibitory effect of BHT in DMBA-induced tumor incidences in rats exposed to dietary BHT in a dose range from 300 to 6000 mg/kg of diet. The antioxidative activity of ENL is comparable with that of BHT. However, high doses of BHT (300–6000 mg/kg of diet) were required for tumor inhibition as compared with the ENL doses used in this study. Thus, it is unlikely that ENL action would be based solely on its antioxidative activity.

It is possible that a long-term exposure to elevated concentrations of lignans may alter the composition and metabolic activities of intestinal microbiota, possibly resulting in altered metabolism of other dietary polyphenols. In this study, elevated serum concentrations of EQ and *O*-DMA, metabolites of DA, were observed. In premenopausal women, high EQ excretion has been associated with lower serum concentration of estrogens and androgens, and higher concentration of sex hormone-binding globulin, a hormonal pattern consistent with lower risk for breast cancer (34). Thus, the increased serum concentrations of EQ and *O*-DMA could account for chemopreventive effects observed in this study.

In a population study (7), the significant difference in serum ENL was observed between breast cancer patients and their healthy controls (20 nM *versus* 26 nM, respectively). A wide variation in the ENL concentration was found between individuals. The mean serum ENL concentration was 3 nM in the lowest quintile and 54 nM in the highest. In this study, the mean ENL concentration in rat serum was 405 nM after 7 weeks of daily gavage with ENL at a dose of 10 mg/kg of body weight. After an 8- and 16-week dietary HMR administration, 351 nM concentration of ENL similarly showed a positive effect in the DMBA-induced rat mammary carcinoma model (12). These measured ENL concentrations are >10-fold when compared with those found in the general population. However, the lowest observable effects level is not known for ENL or any other lignan, either in humans or in the experimental cancer models. The minimum or optimal daily dose of lignans that could be associated with a reduced risk of cancer in humans thus remains to be established.

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