Anticancer Effects of a Plant Lignan 7-Hydroxymatairesinol on a Prostate Cancer Model *In Vivo*

ANNIKA BYLUND,*[,2] NIINA SAARINEN,†[,2] JIE-XIAN ZHANG,‡ ANDERS BERGH,§ ANDERS WIDMARK,‖ ANDERS JOHANSSON,‡ EVA LUNDIN,§ HERMAN ADLERCREUTZ,¶ GÖRAN HALLMANS,‡ PÄR STATTIN,§ AND SARI MÄKELÄ†[,1]

Department of Community Medicine and Rehabilitation Geriatric Medicine, Umeå University Hospital, S-901 87 Umeå, Sweden; †University of Turku, Functional Foods Forum and Institute of Biomedicine, University of Turku, FI-20014 Turku, Finland; ‡Department of Public Health and Clinical Medicine, Nutritional Research, Umeå University, S-901 87 Umeå, Sweden; §Department of Medical Biosciences, Pathology, Umeå University Hospital, S-901 87 Umeå, Sweden; ‖Department of Oncology, Umeå University Hospital, S-901 87 Umeå, Sweden; ¶Department of Clinical Chemistry, Institute for Preventive Medicine, Nutrition and Cancer, Folkhälsan Research Center, and University of Helsinki, FI-00014 Helsinki, Finland

Clinical intervention studies and experimental studies with lignan-rich diets suggest that lignans may have inhibitory effects on prostate cancer, but no clinical or experimental studies with purified lignans have been published. The purpose of this study was to investigate the effect of a plant lignan 7-hydroxymatairesinol (HMR) on LNCaP human prostate cancer xenografts in athymic mice. Athymic nude male mice were injected subcutaneously with LNCaP cells. Starting 3 days after tumor cell injections, a control diet or a control diet supplemented with 0.15% or 0.30% of HMR was administered to mice and the tumor take rate and growth was observed for 9 weeks. HMR diet inhibited the growth of LNCaP tumors. Mice treated with HMR had smaller tumor volume, lower tumor take rate, increased proportion of nongrowing tumors, and higher tumor cell apoptotic index compared with controls. Furthermore, the cell proliferation index was reduced in mice receiving the 0.30% HMR diet compared with mice receiving the control diet. Our results suggest that dietary HMR started at the early phase of the tumor development inhibits the growth of the LNCaP human prostate cancer xenografts in athymic male mice. Exp Biol Med 230:217–223, 2005

Key words: phytoestrogens; 7-hydroxymatairesinol; diet; LNCaP human prostate adenocarcinoma; athymic nude mice

Introduction

The hypothesis that a lignan-rich diet inhibits the development and growth of hormone-sensitive prostate cancer is supported by the data from several experimental studies. A diet high in rye bran (30%) increased apoptosis in Dunning R3327 PAP prostate tumor implants in rats (1, 2), and in the human androgen-sensitive LNCaP xenografts in BALB/c athymic mice (3) when compared with animals receiving a semipurified control diet. Furthermore, in a transgenic adenocarcinoma mouse prostate (TRAMP) model, dietary flaxseed (5%) inhibited the growth and progression of the cancers (4).

To determine if lignans *per se* are biologically active, purified compounds have to be investigated. We chose to use a plant lignan 7-hydroxymatairesinol (HMR) because it is structurally closely related to matairesinol (MR; Fig. 1). MR is one of the plant lignans found in many foods but only in small quantities (5, 6), and rich dietary sources suitable for MR isolation are not available. HMR, in contrast, is found in very high quantities in knots of Norway spruce (*Picea abies*) (7), and can be extracted in large amounts sufficient for a long-term *in vivo* experiment. Similar to abundant dietary plant lignans secoisolariciresinol diglycoside (SDG) and MR, HMR is also metabolized into mammalian lignan enterolactone (ENL) *in vivo* (8–13).

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[1] To whom correspondence should be addressed at University of Turku, Functional Foods Forum, FI-20014 Turku, Finland. E-mail: sarmak@utu.fi

[2] These two authors have made an equal contribution to this article.

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Table 1. Composition of the Diets (g/100 g Diet)[a]

Ingredient	Control	HMR 0.30 Diet	HMR 0.15 Diet
Low-fat milk protein	27.4	27.4	27.4
Corn starch	21.0	20.7	20.75
Corn oil	1.6	1.6	1.6
Lard	1.3	1.3	1.3
Flaxseed oil	9.5	9.5	9.5
Sucrose	21.0	21.0	21.0
Vitamin mixture	1.4	1.4	1.4
Mineral mixture	3.6	3.6	3.6
Cellulose	13.2	13.2	13.2
HMR	0	0.30	0.15

[a] HMR, 7-hydroxymatairesinol.

Because of the limited availability of most lignans in large amounts, only a small number of studies have been conducted using purified compounds. Plant lignans, such as SDG, sesamin, and HMR have been tested in experimental models for hormone-dependent cancers. SDG isolated from flaxseed and HMR have both been shown to inhibit the growth of mammary carcinoma and intestinal tumors in rodent models (11, 12, 14–16), whereas sesame seed lignan sesamin added to semipurified diet has been demonstrated to decrease the multiplicity of the mammary tumors (17).

In this study, we examined the effect of dietary HMR on growth of LNCaP xenografts in athymic nude mice. LNCaP is an androgen-sensitive human prostate adenocarcinoma cell line (18, 19). To our knowledge, this is the first study done with a purified lignan in an experimental prostate cancer model *in vivo*.

Materials and Methods

Extraction of 7-Hydroxymatairesinol. HMR extracts were isolated from Norway spruce (*P. abies*) as previously described (12, 20). Lignans were the major component of the isolate (63.9% of the total weight analyzed with a gas chromatography–mass spectroscopy [GC-MS] method), and HMR was the major lignan (72.9% of the lignans). Other minor lignan components (each less than 3%) were liovil, secoisolariciresinol, conidendric acid, matairesinol, lignan A, conidendrin, and lariciresinol. The rest of the isolate consisted of polar, nonvolatile, high molecular weight organic components.

Diets. The semipurified experimental diets used in these experiments were prepared in our laboratory. The major ingredients of the diets were cornstarch (Sigma Chemical Co., St. Louis, MO), sucrose (Danisco Sugar AB, Malmö, Sweden), low-fat milk protein (Semper AB, Stockholm, Sweden), cellulose (a mixture of 50% Dicalcel 2 and 50% Dicalcel 4; Hope Farms, Woerden, Netherlands), corn oil (CPC Foods AB, Kristianstad, Sweden), lard (EllcoFood AB, Kävlinge, Sweden), and flaxseed oil (Alternativ förädling AB, Örebro, Sweden). The cellulose was used to adjust the fiber content and energy density in the diet. The diets containing 0.15% of HMR (HMR 0.15 group) and 0.30% of HMR (HMR 0.30 group) were made by supplementing the semipurified control diet with 0.15 and 0.30 g of HMR per 100 g of diet, respectively. All diets had a similar energy density and were isocaloric in percentage energy content from the nutrients: 20% of the energy from protein, 30% from fat, and 50% from carbohydrates (excluding the energy values contained in dietary fiber and derived from fermentation). The composition and the nutritional content of the diet are listed in Tables 1 and 2. All diets were pelleted, dried at 40°C, and stored at −20°C.

Cell Culture. The LNCaP cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 50 μg/ml of gentamicin and 11 ng/ml methyltrienolone R1881 (DuPont NEN, Boston, MA), and 10% fetal calf serum in an atmosphere of 5% CO_2 and air at 37°C. All cell medium components except R1881 were obtained from Lab Kemi (Stockholm, Sweden). Cells were harvested from subconfluent cultures after a 5-min exposure to a solution of 0.02% EDTA and 0.2% trypsin. After suspension in nonsupplemented RPMI-1640 medium, the cells were counted with Coulter Counter (Coulter Electronics Ltd., Luton, UK). Cultures used for experiments were in exponential growth phase.

Animals. Male athymic mice of the BALB/cABom strain, age 6–8 weeks, were obtained from the Bomholtgård

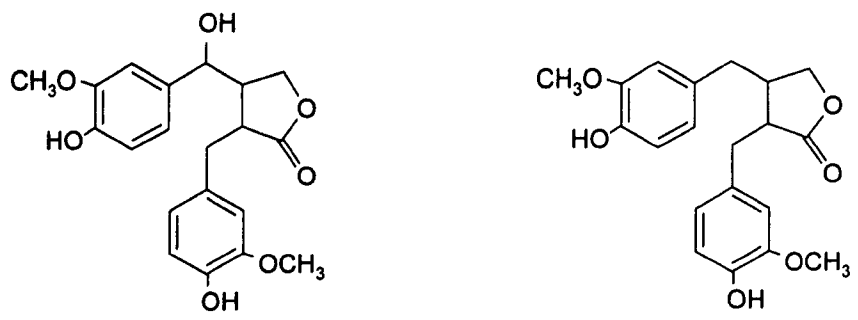

Figure 1. Chemical structures of plant lignans 7-hydroxymatairesinol (HMR) and matairesinol (MR).

Breeding and Research Center Ltd. (Ry, Denmark). The animals were housed, six animals per cage, in a pathogen-free environment with air filter tops in filtered laminar airflow hoods. Standard vinyl cages with a stainless steel layer were used to prevent animals from accessing wooden chips, which were used to absorb excretions. Cages and bedding were autoclaved before use. During the course of the experiments, all animals were cared for in accordance with institutional guidelines. The research ethical committee for animal studies at Umeå University approved the design of this study.

Experimental Design. After a 1-week acclimatization period, 36 male athymic mice were randomized into three groups with 12 mice in each group and six animals in each cage. Each mouse was inoculated subcutaneously with 0.05 ml of Matrigel (BD Bioscience, Franklin Lakes, NJ), supplemented with 11 μg/ml of basic fibroblast growth factor (Sigma-Aldrich, St. Louis, MO), followed by inoculation of 4×10^6 tumor cells, on both sides of the back. Starting 3 days after tumor cell injections, the mice were fed the semipurified test diets. The control group received control diet, the HMR 0.15 group received the 0.15% HMR diet, and the HMR 0.30 group received the 0.30% HMR diet. The dietary treatment period was 9 weeks. The mice were weighed and the tumor take rate (percentage of sites injected with tumor cells that developed into palpable tumors) and the tumor sizes were measured with a caliper once per week starting 4 weeks after tumor cell implantations (i.e., for 5 weeks). The palpations were carried out in blinded manner (i.e., by a person who had no information on the treatments of the mice). The volumes of established tumors were calculated by using the formula: volume (mm^3) = (length × width × height) × π/6.

At sacrifice, the tumors, liver, ventral and dorsolateral lobes of prostate, and right testis were removed, weighed, and processed for histological examination as described in "Analysis of General Tumor Morphology, Cell Proliferation, and Apoptosis." As the growth rate of LNCaP tumors varied, the tumors were classified in two groups according to their growth characteristics at the end of the experiment: (i) growing tumors (tumors that increased in volume during the treatment and tumors that became palpable during the last week of the experiment), and (ii) nongrowing tumors (tumors with no change in volume for at least the 2 last weeks of the experiment and tumors that regressed in size compared with their maximum volume).

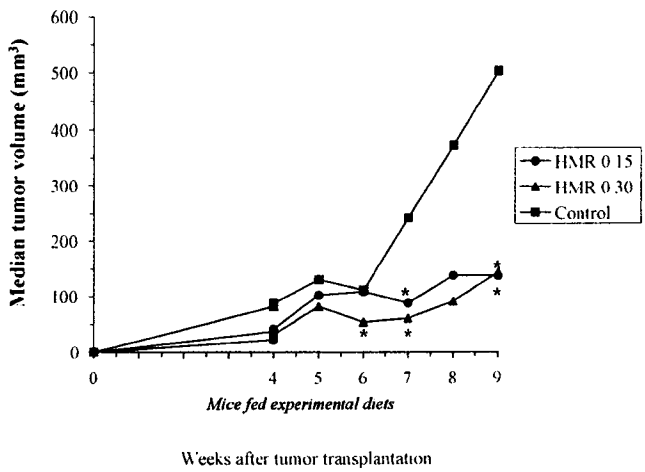

Figure 2. Median tumor volumes in tumor-bearing mice. 7-Hydroxymatairesinol (HMR) diets were started 3 days after tumor cell implantations. The time points when median tumor volumes in the HMR groups significantly differed from controls are indicated with stars: (A) At 6 weeks, control (13 tumors) vs. HMR 0.30 (16 tumors; $P < 0.017$). (B) At 7 weeks, control (13 tumors) vs. HMR 0.15 (nine tumors; $P < 0.017$) and control vs. HMR 0.30 (16 tumors; $P < 0.003$). (C) At 8 weeks, HMR groups were not significantly different from controls ($P > 0.05$). (D) At 9 weeks, control (16 tumors) vs. HMR 0.15 (13 tumors; $P < 0.022$) and control vs. HMR 0.30 (18 tumors; $P < 0.018$).

Metabolic Studies. The animals underwent a metabolic study 2 weeks after the injection of the tumor cells. Twelve animals from each dietary group were maintained in metabolic cages (six mice per cage) for 72 hr, and their body weight, food, and water intake were recorded. Urine was collected in 24-hr intervals from jars containing 10 mg of ascorbic acid as preservative, pooled, and stored at −30°C.

Analysis of Phytoestrogen in Food and Urine. Isotope dilution gas-liquid chromatography–mass spectrometry was used to analyze the isoflavones and lignans in experimental diets as previously described (5). Diet samples from each experimental group were collected in the middle and at the end of the experiment, and duplicated lignan analyses for HMR, secoisolariciresinol, matairesinol, genis-

Table 2. Nutritional Content of the Raw Material in the Diets (%)

Ingredient	Protein	Fat	ω-6 Fatty acids	ω-3 Fatty acids	Carbohydrates	Moisture	Ash	Dietary fiber
Low-fat milk protein[a]	67.1	0.9	—	—	20.4	3.00	8.58	1.2
Corn starch[a]	0.2	0.2	—	—	90.3	9.21	0.09	0.8
Sugar[b]	0.0	0.0	—	—	100.0	-	-	0.0
Cellulose[a]	0.0	0.1	—	—	93.0	6.90	0.05	85.1
Corn oil[b]	0.0	100.0	59.1	1.2	0.0	-	-	0.0
Lard[b]	0.0	100.0	8.5	0.7	0.0	-	-	0.0
Flaxseed oil[c]	0.0	100.0	15.2	58.8	0.0	-	-	0.0

[a] Analyzed values.
[b] Values obtained from Swedish food tables.
[c] Values provided by the supplier.

Table 3. Tumor Data at the Time of Sacrifice

Parameter	Control	HMR 0.15 group	HMR 0.30 group
No. of tumors	16	13	18
Tumor take rate[a] (%)	67	54*	74
Tumor weight (mg)	237 ± 317	95 ± 129*	83 ± 184*
Apoptotic index[b]	0.15 ± 0.08	0.68 ± 0.54***	0.69 ± 0.28***
Proliferation index[b]	24 ± 6	25 ± 7	19 ± 2**

[a] Proportion of sites injected with tumor cells that developed into palpable tumors after 9 weeks (i.e., until the end of the experiment).
[b] Expressed as percentage of terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick-end labeling–stained (apoptosis) or Ki-67–stained (proliferation) tumor epithelial cells out of 1500–2000 cells evaluated in nonnecrotic areas.
*$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$.

tein, and daidzein were performed. The dietary intake of lignans was calculated based on the amount of diet intake during the metabolic registration period and the mean concentration of lignans in the diet.

The urinary ENL concentrations were measured by using a time-resolved fluoroimmunoassay (TR-FIA) method as previously described (21). Because the TR-FIA values were approximately 30% higher than the values obtained with our GC-MS reference method, the TR-FIA values were corrected according to the following formula: corrected value = $-0.14804 + 0.82102 \times$ TR-FIA value. In this way the values corresponded to those measured by GC-MS (21). In the HMR 0.15 group, one of the urine samples was not analyzed because of contamination of the collected sample with food.

Analysis of General Tumor Morphology, Cell Proliferation, and Apoptosis. For histological analysis, the tumor samples were fixed in formalin for 24 hr and processed for paraffin embedding. Four-millimeter–thick sections were cut and used for hematoxylin-eosin and immunostaining. General tumor morphology of each tumor was assessed from sections stained with hematoxylin-eosin.

For immunostaining of the cell proliferation marker Ki-67, the sections were heated on a microwave oven and incubated overnight with a polyclonal antibody to human Ki-67, diluted to 1/100 (MIBI, Immunotech SA, Marseille, France) as described previously (22). The terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick-end labeling method was used to stain cells with fragmented DNA (i.e., apoptotic or necrotic cells). The analysis was performed using the *In Situ* Cell Death Detection Kit (Boehringer Mannheim, Mannheim, Germany) as described previously (22).

The cell proliferation and apoptotic indexes of each tumor were investigated in nonnecrotic areas. The apoptosis and proliferation indexes (percentage of stained tumor epithelial cells) were determined by evaluating 1500–2000 cells per tumor at ×400 magnification. All samples were analyzed in a blinded procedure.

Statistics. The comparisons between groups were made using the Kruskal-Wallis one-way analysis of variance followed by Mann-Whitney *U* test. The chi-square test was used to test the tumor take rate between dietary groups. A P value < 0.05 was considered statistically significant. The data are expressed as mean ± standard deviation (SD) except that non–normally distributed tumor volume and weight data are expressed as median ± SD. The statistical analysis was performed using Statistical Package for the Social Sciences (SPSS 10.0; SPSS Inc., Chicago, IL).

Results

Effect of HMR on Tumor Take Rate and Growth. When the dietary treatment was started early (i.e., before the appearance of the palpable tumors) the tumor take rate was significantly reduced in the animals fed the HMR diet. At the start of the recording period (i.e., 4 weeks after tumor cell injections), a lower tumor take rate was recorded in the HMR 0.15 group (4%; one tumor) compared with control and HMR 0.30 groups (33%; eight tumors in both groups; $P < 0.05$). Similarly, at the end of the experiment (i.e., after 9 weeks of dietary treatment) tumor take rate was lower in HMR 0.15 group compared with the control or HMR 0.30 group (Table 3).

The suppressing effect of dietary HMR on tumor growth started 6 weeks after tumor cell injections and it lasted from that point forward throughout the experiment. The tumor volumes were significantly smaller in both HMR 0.15 and HMR 0.30 groups than in the control group (Fig.

Table 4. Dietary Intake of Lignans and Isoflavones During the Metabolic Observation Period (mg/kg)[a]

Diet group	SECO	MR	HMR	GEN	DA
Control	nd	nd	nd	0.03	nd
HMR 0.15 group	1.00	0.57	13.44	0.04	nd
HMR 0.30 group	1.75	1.04	25.75	0.04	nd

[a] Calculated value from the lignan and isoflavonoid concentrations analyzed in the diets and the mean value of two pooled dietary intake data from six animals in one metabolic cage for 3 days. SECO, secoisolariciresinol; MR, matairesinol; HMR, 7-hydroxymatairesinol; GEN, genistein; DA, daidzein, nd, not detectable.

Table 5. Daily Urinary Excretion of ENL During the Metabolic Observation Period[a]

Diet group	Urinary ENL (nmol/animal/day)	Urinary ENL (pmol/day/g of body weight)
Control	0.026	0.94
HMR 0.15	2.878	111
HMR 0.30	1.592	60.9

[a] Urine samples collected from six mice in one metabolic cage for 3 days. In control and HMR 0.30 groups, $n = 2$; in HMR 0.15 group, $n = 1$. ENL, enterolactone; HMR, 7-hydroxymatairesinol.

2). The tumor growth-inhibiting effect of HMR was also seen as an increased proportion of nongrowing (regressing and stabilized) tumors in HMR 0.15 and HMR 0.30 groups compared with the control group (46%, 26%, and 13%, respectively). Accordingly, the tumor weight at sacrifice was higher ($P < 0.05$) in control mice compared with the HMR 0.15 and 0.30 groups (Table 3).

Organ Weights. There were no significant differences in body weights between the treatment groups, except for the last week when the animals in the HMR 0.30 group were smaller (22.68 ± 0.98 g) compared with controls (25.98 ± 1.05 g; $P < 0.05$). No significant differences in prostate or liver weights between control and HMR treatment groups were observed at the time of sacrifice (data not shown). However, the average testis weight was significantly higher in the HMR 0.15 group (96 ± 3 mg), but not in the HMR 0.30 group (91 ± 3 mg) compared with the control group (89 ± 3 mg).

General Tumor Morphology, Cell Proliferation, and Apoptosis. No apparent differences in general morphology of the tumors between the three groups were observed. However, the tumor epithelial cell proliferation index was significantly lower in the HMR 0.30 group compared with the control group ($P = 0.001$; Table 3). Moreover, the tumor epithelial cell apoptotic index was significantly ($P < 0.0001$) higher in the HMR 0.15 and HMR 0.30 groups compared with the control group (Table 3).

Dietary Intake and Urinary Excretion of Lignans and Isoflavones. No significant difference in food or energy intake was observed between the groups during the metabolic registration period (data not shown). The consumption of lignans was calculated on the basis of food intake and the concentration of HMR in the diet. As expected, the animals receiving the HMR diets consumed more plant lignans compared with the animals receiving control diet, and no differences in isoflavone intake between control and HMR groups were measured (Table 4). Accordingly, a higher daily urinary excretion of the mammalian lignan ENL was found in both HMR dietary groups compared with the control dietary group (Table 5). Surprisingly, there was no difference between the two HMR dose groups with regard to daily urinary ENL excretion.

Discussion

In the present study we demonstrate that a long-term administration of dietary HMR inhibits the growth of LNCaP human prostate cancer xenografts in athymic nude male mice. Animals given HMR starting 3 days after tumor cell injection until the end of the experiment (i.e., 9 weeks) had reduced tumor take rate, smaller total tumor volume, increased proportion of nongrowing tumors, and a higher tumor cell apoptotic index compared with animals receiving the control diet. Furthermore, in the HMR 0.30 group the tumor cell proliferation index was also lower than in controls.

The growth-inhibiting effects of HMR diets on LNCaP prostate cancer xenografts are in accordance with our earlier findings with rye (3). A diet containing 30% of rye inhibited the LNCaP tumor growth and increased tumor apoptosis when tested in an experimental design similar to that of the present study (3). Our results also are in agreement with the small clinical intervention studies done with lignan-rich diets. Flaxseed supplementation combined with fat restriction increased the apoptosis labeling index in prostate tumor tissue (23), and in a controlled short-term intervention study the apoptotic rate was higher in prostate tumor biopsies from men with a high intake of rye bread than in biopsies from men consuming a control diet (22).

In diet, lignans occur as plant lignans, which are at least partly converted by gut microbiota to the mammalian lignans enterodiol and ENL (8, 10, 11, 24). Previous studies in rats have shown that orally administered HMR is metabolized to ENL, which is also the major mammalian metabolite of matairesinol (11, 12). The mammalian metabolites, rather than the plant lignans, are thought to be lignans with anticancer activity, although very limited *in vivo* data are available. So far, the tumor growth-inhibiting effect of mammalian lignan ENL has been demonstrated only in 7,12-dimethylbenz(a)-anthracene-induced rat mammary carcinoma (25). Administration of ENL to rats inhibited the growth of the established mammary tumors, supporting the idea of ENL as a biologically active compound that can be partly responsible for the lignan effects.

Lignan-containing diets have shown promising results in experimental prostate cancer. In the TRAMP model, flaxseed reduced the progression of prostate cancer (4). Accordingly, rye diets have been shown to inhibit the growth of androgen-sensitive Dunning prostate tumor implants in rats as well as human LNCaP xenografts in mice (1–3). The inhibitory effects of both flaxseed and rye on tumor development and growth were measured mainly as increased tumor cell apoptosis and decreased proliferation.

In humans, in the two nested case-control studies on circulating ENL and prostate cancer risk, no decrease in risk was observed for increasing levels in serum or plasma (26, 27). In these studies, however, as well as in many population-based studies, the serum ENL concentrations

were rather low and may have been too low for a direct protective effect. In these studies, ENL concentrations may merely have been a marker for a fiber-rich and healthy diet in general. However, diet supplementation with flaxseed or rye has been reported to increase the serum ENL concentration up to more than 100 n*M* (22, 28) demonstrating that high serum lignan levels in humans are achievable. Furthermore, both flaxseed and rye have demonstrated prostate tumor growth-inhibiting effects in men (22, 23). It is thus possible that ENL is a biologically active compound also able to inhibit prostate cancer.

Immune-deficient athymic mice are generally used to study human cancer xenografts *in vivo*. However, the impact of immune deficiency of these mice on gastrointestinal microbiota and the ability to form mammalian lignans from plant lignan precursors is poorly understood. In this experiment ENL was detected in the urine of HMR-treated athymic mice, demonstrating that these mice were able to convert plant lignans to mammalian lignans even although the mice were kept in a pathogen-free environment. However, the proportion of the daily HMR dose recovered as urinary ENL was much lower in these male nude mice (6% and 3% in the HMR 1.5 and 3.0 groups, respectively) than in previous studies with female rats (26%) (11). This may be due to species, gender, or differences in the duration of lignan administration. Furthermore, it is possible that the differences in ENL excretion are associated with differences in gut microbiota of different species, and consequently, the capacity to form ENL from the plant lignan precursors.

The urinary ENL excretion in this study was somewhat puzzling. Despite the higher HMR content in diet (0.15% HMR diet vs. 0.30% HMR diet groups), there was no increase in the daily urinary excretion of ENL (2.88 and 1.59 nmol/day, respectively). At present, there is no obvious explanation for the lack of increase in ENL excretion. There were no major differences between these two groups with regard to tumor growth inhibition either, which would be consistent with the hypothesis of ENL being the active compound. In HMR-exposed rats, however, HMR is present in the urine in high concentrations, also indicating that this lignan is absorbed (11). It is thus possible that plant lignans could be biologically active. Whether HMR is absorbed also in the athymic mice to the same extent as in rats, and if any of the tumor-inhibiting effects are caused by HMR itself, remains to be investigated.

The possible cellular targets and mechanisms of lignan actions in prostate cancer are poorly understood. *In vitro* experiments with prostate cancer cells suggest that lignans may act as endocrine modulators, and inhibit cell proliferation in hormone-sensitive prostate cancer cell lines such as LNCaP, whereas in hormone-insensitive cell lines such as DU145 and PC3 (29), the antiproliferative effect is weaker. Lignans have also been shown to inhibit the androgen-metabolizing enzymes 5α-reductase, hydroxysteroid oxidoreductase, and aromatase (30–32). It has also been suggested that lignans influence the bioavailability of androgens by decreasing the binding of sex hormone–binding globulin to dihydrotestosterone, and thus accelerate the metabolic clearance of endogenous androgens (33, 34). Furthermore, in relatively high concentrations (>1 μ*M*) *in vitro*, mammalian lignan ENL has been demonstrated to compete with 17-β-estradiol for binding to estrogen receptors (ERs) α and β (concentration that inhibits 50%, 6.7 and 39 μ*M*, respectively) (35) and subsequently to activate protein expression (12, 35). However, the role of estrogens in prostate cancer is still unclear and therefore it is impossible to judge if ER-mediated mechanisms play any role in prostate cancer prevention.

In conclusion, our results support the hypothesis that lignans have anticarcinogenic properties, and suggest that the favorable effects of fiber-rich diets, at least partly, may be related to lignans. However, several critical questions with regard to the biologically active lignan(s), optimal doses, mechanisms, and molecular targets of lignan action still remain to be explored.

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