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Antioxidant and antitumor effects of hydroxymatairesinol (HM-3000, HMR), a lignan isolated from the knots of spruce

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The antioxidant properties of hydroxymatairesinol (HM-3000) were studied *in vitro* in lipid peroxidation, superoxide and peroxyl radical scavenging, and LDL-oxidation models in comparison with the known synthetic antioxidants Trolox (a water-soluble vitamin E derivative), butylated hydroxyanisol (BHA) and butylated hydroxytoluene (BHT). On a molar basis HM-3000 was a more effective antioxidant than Trolox in all assays and more effective than BHT or BHA in lipid peroxidation and superoxide scavenging test. The *in vivo* antioxidative effect (evaluated as the weight gain of C57BL/6J mice fed an α-tocopherol-deficient diet) of HM-3000 (500 mg/kg per day) was comparable to that of DL-α-tocopherol (766 mg/kg per day). The antitumor activity of HM-3000 was studied in dimethylbenz[*a*]anthracene (DMBA)-induced rat mammary cancer. HM-3000 had a statistically significant inhibitory effect on tumor growth. Prevention of tumor formation was also evaluated in the Apc^{Min} mice model, which develops intestinal polyps spontaneously. HM-3000 was given in diet at 30 mg/kg per day and decreased the formation of polyps and prevented β-catenin accumulation into the nucleus, the pathophysiological hallmark of polyp formation in this mouse model. In short-term toxicity studies (up to 28 days) HM-3000 was essentially non-toxic when given p.o. to rats and dogs (daily doses up to 2000 and 665 mg/kg, respectively); HM-3000 was shown to be well absorbed (>50% of the dose) and rapidly eliminated. In human studies HM-3000 has been given in single doses up to 1350 mg to healthy male volunteers without treatment-related adverse events. Rapid absorption from the gastrointestinal tract and partial metabolism to enterolactone in humans was demonstrated. In summary, HM-3000 is a safe, novel enterolactone precursor lignan with antioxidant and antitumor properties.

Key words: Hydroxymatairesinol, enterolactone, lignans, antioxidant, cancer, functional food.

Introduction

Lignans are a class of phenolic compounds with a 2,3-dibenzylbutane skeleton (see structure of HM-3000, Figure 1). They exist in wide variety of plants and are distributed among different parts (roots, leaves, stem, seeds, fruits) but mainly in small amounts. In many sources (seeds, fruits) lignans are found as glycosidic conjugates associated with the fiber component of plants. Among the most common dietary sources of lignans are unrefined grain products. Very high concentrations in edible plants have been found in flaxseed (Thompson *et al.*, 1996), and lower concentrations in tea and unrefined grain products (e.g. in rye) (Mazur and Adlercreutz, 1998).

Plant lignans are converted by gut microflora to mammalian lignans, such as enterolactone and enterodiol (Axelson *et al.*, 1982). These undergo an enterohepatic circulation and are excreted in the urine mainly as glucuronides (Axelson and Setchell, 1981).

Lignan-rich diets inhibit development of experimental melanoma, mammary, prostate and colon cancers (Serraino and Thompson, 1992; Jenab and Thompson, 1996; Thompson *et al.*, 1996; Landström *et al.*, 1998; Li *et al.*, 1999). Furthermore, rye inhibits the inulin-promoted adenoma formation in Apc^{Min} mice (Mutanen *et al.*, 2000). There are also epidemiological data to support the cancer-preventive actions of such diets in humans, as the urinary excretion and serum concentrations of enterolactone are low in women diagnosed with breast cancer (Ingram *et al.*, 1997; Pietinen *et al.*, 2001). However, the mechanisms of the antitumor action of lignan-rich diets are not well understood.

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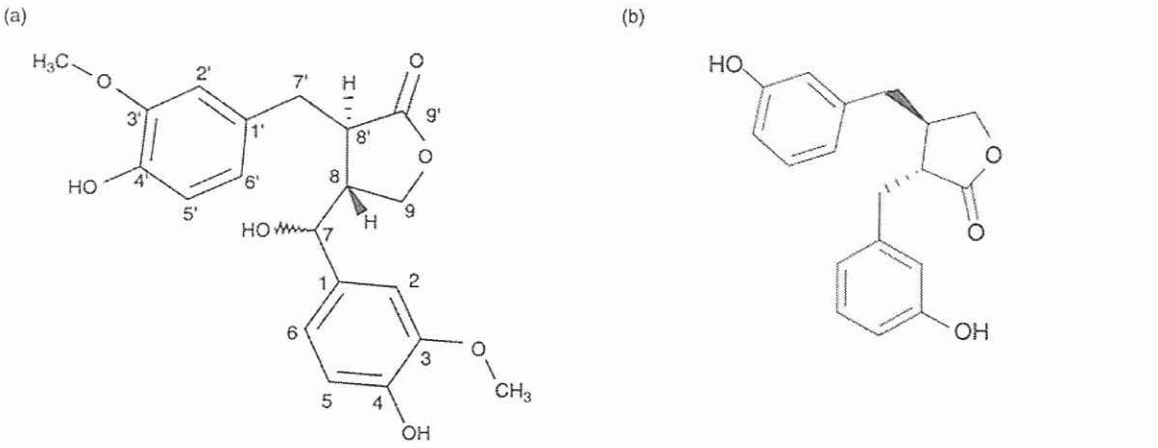

Figure 1. Structure of HM-3000 and enterolactone. (a) Structure of HM-3000, numbering of the carbon atoms, and natural isomers. The suggested configurations of two natural isomers are 8′R*, 8R*, 7R* and 8′R*, 8R*, 7S* (lactone ring is *trans*). (b) Structure of enterolactone. Enterolactone is formed by gut microbial flora.

Many lignans are strong antioxidants. This could represent one mechanism responsible for their anticancer action. On the other hand, antioxidant deficiency is associated with an increased risk of atherosclerosis, cancer and (neuro)degenerative diseases (Halliwell, 1994), although there are also other opinions (De Gaetano *et al.*, 2001). The role of oxidized LDL has been emphasized as a significant independent risk factor for cardiovascular diseases (Schroeter *et al.*, 2000).

Many plants are known to contain compounds with estrogen-like properties, although the role of lignans as hormonally active plant components is far from clear. Flaxseed in the human diet has been shown to cause changes in menstrual cycle (Phipps *et al.*, 1993). In healthy young men, the short-term (6 weeks) flaxseed supplementation of the diet (10 g/day in muffins) had no significant effect on plasma testosterone concentrations (Shultz *et al.*, 1991) indicating a lack of hormonal activity in men.

There are no data on the effects of pure lignans in humans, simply because they have not been available in sufficient amounts for adequate biochemical, pharmacological, toxicological and human studies.

So far, the highest concentrations of HM-3000 have been found from the Norweigian spruce tree (*Picea abies*) branch knots, which remain inside the stem during the growth of the tree. The concentrations of lignans within the knot may reach 4–20% (dry weight %) and HM-3000 may represent about 60% of all lignans (Ekman, 1976). Such knots are therefore an excellent source of HM-3000. In the thermomechanical pulp industry spruce knots are an undesired quality-decreasing component of the fiber-making process due to their hardness and colour. Thus, the pulp industry may be an almost unlimited source of HM-3000. In the spruce, HM-3000 is 1:1 mixture of two stereoisomers.

The aim of the present study was to evaluate the mode of action and the antitumor properties of HM-3000 *in vitro* and *in vivo*. A further interest for us was to estimate whether HM-3000 itself or its metabolite enterolactone is the biologically active component. We propose HM-3000 as a putative functional food ingredient to elevate enterolactone levels and thereby lower the risk of certain cancers.

Materials and methods

Isolation and purification of HM-3000

The knots were milled and extracted with different methods to obtain HM-3000. In principle, two products were used: free HM-3000 and K-acetate adduct of HM-3000. Free HM-3000 was a mixture of two isomers. So far, no difference in the activity of isomers has been found. HM-3000 and its K-acetate adduct also have similar biological activity on a molar basis. Initially, HM-3000 was extracted with dichlormethane and later with ethanol or water. The purity of the free HM-3000 varied between 85% and 95% except for the Apc^{Min} mouse study, where it was about 50%. K-acetate adduct has been prepared from ethanol or water extracts by addition of K-acetate. The adduct precipitated so that HM-3000 and K-acetate were in equimolar ratio. The purity of K-acetate adduct used in safety pharmacology, toxicology and clinical studies was about 95%.

Antioxidative properties of HM-3000 in vitro

The antioxidative capacity of HM-3000 and reference compounds was estimated by several methods: (1) inhibition of lipid peroxidation (Ahotupa *et al.*, 1997), (2) inhibition of low-density lipoprotein (LDL) oxidation using both biochemical (Ahotupa *et al.*, 1996) and NMR techniques, (3) superoxide and peroxyl radical scavenging assays (Ahotupa *et al.*, 1997) and (4) inhibition of oxidative burst in human monocytes. LDL oxidation was studied by NMR techniques according to Laatikainen *et al.* (1996). LDL was diluted to a final concentration of 0.46 mg/ml by 0.15 mol/l NaCl in D_2O:H_2O (1:9). $CuCl_2$ was added so that the final concentration of metal ion was 40 µmol/l. Also 5 µl of 30% H_2O_2 was added to the samples. Samples were incubated at room temperature for 3 hours. α-Tocopherol or HM-3000 was added so that their final concentration was 60 µmol/l. After the incubation the samples were extracted using deuterochloroform. The spectra of $CDCl_3$ extracts were recorded by a Bruker AVANCE DRX 500 spectrometer in a routine way. The quantification of the signals was performed using the total line shape procedure of the PERCH software (Laatikainen and Niemitz, 2002). The confidence limits for the signal intensities are about 5% or, for the arachidonic acid signal, about ±5 area units.

The effects of HM-3000 and enterolactone on the oxidative burst were studied in buffy coat preparations (Finnish Red Cross, Helsinki, Finland), which contain circulating monocytes and granulocytes. These cells are responsible for burst of free radicals (oxidative burst) (i.e. chemically reactive compounds responsible for microbicidal activity). *In vitro* the oxidative burst can be induced by stimulating the cells by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). The assay was completed essentially as described by Ristola and Repo (1989).

Antioxidative properties of HM-3000 in vivo

The *in vivo* antioxidant properties were investigated by specific 'experimental oxidative stress model' in laboratory mice. Oxidative stress was induced in 12-week-old male C57BL/6J mice by combined effects of vitamin E-deficient diet and a chemical pro-oxidant (CCl_4 13 mmol/kg p.o. diluted in rapeseed oil 1:2 v/v). Mice were divided into four different semipurified diet groups: (1) Vitamin E-deficient group (control); (2) vitamin E-deficient diet supplemented with 766 mg DL-α-tocopherol/kg diet; (3) vitamin E-deficient diet supplemented with 300 mg HM-3000/kg diet; (4) vitamin E-deficient diet supplemented with 766 mg DL-α-tocopherol and 300 mg HM-3000/kg diet. Half of the mice in each treatment group were sacrificed after 2 weeks treatment and another half after 4 weeks treatment.

To estimate the level of oxidative stress, two different methods for lipid peroxidation were used: diene conjugation (in serum) and thiobarbituric acid reactive substances (in liver). Animal weight gain was also recorded.

The antioxidant capacity of serum was analyzed to determine the concentrations of α- and γ-tocopherols, β-carotene and ubiquinol by HPLC (Ahotupa *et al.*, 1997).

Hormonal effects of HM-3000

The estrogen receptor-binding capacity of HM-3000 was measured with fluoropolarometry by determining its ability to displace the labeled ligand specifically bound to recombinant estrogen receptor alpha protein (Estrogen Receptor-alpha Competitor Assay, Panvera, USA).

The estrogen receptor-mediated reporter activity was estimated in a mammary cancer cell line MCF-7 harboring an estrogen response element–luciferase gene construct. The cells expressing the reporter construct were treated with HM-3000 (up to 10 µmol/l) in the presence or absence of 17-β-estradiol (1 nmol/l).

The estrogenicity and aromatase inhibiting activity of HM-3000 in female animals was evaluated by the uterotropic assay in immature rats as described earlier (Saarinen *et al.*, 2000). The treatment time, however, was 7 days because of the expected low hormonal activity of the test compound.

Estrogenic (antiandrogenic) and antiestrogenic effects of HM-3000 were studied in intact and hypoandrogenic Noble strain male rats (age 6–9 months) (for exact methods, see Saarinen *et al.*, 2000). The chronic hypoandrogenic state with both structural and functional changes in the male reproductive tract was induced by neonatal estrogenization (diethylstilbestrol, 10.0 µg/kg body weight in rape oil s.c. on postnatal days 1–5). Twelve animals, both intact and hypoandrogenic, were gavaged daily with 50 mg/kg HM-3000 in rape oil. Another 12 animals from both animal models were gavaged with rape oil only as a placebo treatment. After 4 weeks of treatment the testes and accessory sex glands (ventral prostate, seminal vesicles and coagulating gland) were weighed and the concentrations of serum and testis testosterone and pituitary and serum luteinizing hormone (LH) levels were measured.

Antitumor effects of HM-3000 in vitro

Antiproliferative and apoptotic effects of HM-3000 and enterolactone were studied in cancer cell lines. HeLa (cervical adenocarcinoma), MCF-7 (breast adenocarcinoma) and Caco-2 (colorectal adenocarcinoma) cells were

cultured in complete growth media consisting of Dulbecco's Modified Eagle Medium (DMEM, Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Bioclear), penicillin and streptomycin (Sigma, 100 IU/ml and 0.1 mg/ml final concentrations, respectively) and L-glutamine (Sigma, final concentration 4 mmol/l).

Cells were plated on a 96-well plate (Costar) at 5000 cells per well. Lignan compounds were dissolved in ethanol and the desired amount was applied to the well prior to cell plating, and ethanol was evaporated. After evaporation, cells were plated at 100 µl volume of complete growth medium per well. Four days after treatment/plating, the number of cells per well was estimated by adenosine triphosphate (ATP)–luciferase assay. Three to four replicates were used per exposure group. In short, 100 µl of 1% trichloroacetic acid was added to a well, incubated for 10 min, and a 50-µl aliquot was removed to another plate. The reconstituted ATP monitoring reagent/Tris buffer was added to a well and luminescence was measured.

The level of apoptotic cells was estimated by staining the cellular specimen with acridine orange and counting the cells with apoptotic nuclear morphology under an epifluorescence microscope essentially as described in McGahon *et al.* (1995). The specimens were counted blind with no information as to the treatment group. Three hundred cells per sample were counted to obtain a distribution on normal (smooth, homogeneous) and apoptotic (condensed, fragmented bright) nuclear DNA staining.

Antitumor effects in vivo

The antitumor activity of HM-3000 in rat DMBA-induced mammary cancer was studied in female Sprague–Dawley rats as described earlier (Saarinen *et al.*, 2000, 2002). The rats were allocated to different treatment groups so that the total number of tumors at the beginning of the experiment was similar in each group. Early treatment (prevention of tumors) as well as late treatment of existing tumors was studied separately. HM-3000 was given p.o. at dose levels varying from 5 to 15 mg/kg or mixed in the diet. During the feeding period, the tumors were palpated weekly and their size was measured.

The chemopreventive activity of HM-3000 in intestinal neoplasia was evaluated in the Apc^{Min} mouse model as described by Oikarinen *et al.* (2000). HM-3000 was given in the diet. The number of polyps and intracellular distribution of β-catenin were assessed.

Pharmacokinetics of HM-3000

Absorption and distribution of HM-3000 has been studied using 5,5′-tritium-labeled HM-3000 (for positions see Figure 1). For the whole-body autoradiography HM-3000 was administered i.v. or p.o. to male Sprague–Dawley rats. Quantitative whole-body autoradiography was performed at 0.5, 2, 8, 24 and 72 hours after dosing (total dose 250 mg/kg). The test was performed according to Inveresk Research Laboratory guidelines (Inveresk Research, Tranet, Scotland).

For elimination studies urine was collected from rats after the administration of various doses of HM-3000. For urine collection, animals were transferred to a metabolic cage. The centrifuged urine volumes were measured and stored at –20°C. For pretreatment 750 µl 0.2 mol/l acetate buffer (pH 4.0 ± 0.1) was added to 3.0-ml thawed urine aliquots. Sep-Pak C18 columns (100 mg silica-based resin/column) were used for urine extractions. Columns were preconditioned with 3.0 ml H_2O, 3.0 ml methanol and 3.0 ml acetate buffer. After urine was filtered through the column and washed with 3.0 ml of acetate buffer, polyphenolics were eluted with 3.0 ml methanol. The eluate was evaporated to dryness under nitrogen in a +45°C water bath and dried residues were redissolved in 3.0 ml of 0.2 mol/l acetate buffer. *Helix pomatia* enzyme (30 µl) mix was added and the solutions were incubated in +37°C overnight to hydrolyze glucuronides and sulfates. Three hundred microliters of flavone stock solution (100 µg/ml in EtOH) was added to the hydrolyzed samples. The samples were extracted in C-18 columns and evaporated to dryness as described above and stored at –20°C until analyzed with GC-MS as described in Saarinen *et al.* (2000).

Results and discussion

Antioxidative properties of HM-3000 in vitro

HM-3000 was compared with the well-known antioxidants Trolox, which is a water-soluble vitamin E derivative, and BHA and BHT in their abilities to inhibit lipid peroxidation, to inhibit of LDL oxidation, and to scavenge superoxide and peroxyl radicals (Table 1). Considering all the tested compounds and tests together, HM-3000 was the strongest antioxidant among the tested molecules. HM-3000 was more effective than BHA or BHT in all assays, and stronger than Trolox in all assays except for the lipid peroxidation inhibition assay, where the compounds were almost equally active.

The LDL oxidation study with a NMR method using Cu^{2+} as oxidant proved that HM-3000 was an effective antioxidant of LDL (Figure 2). HM-3000 had a slightly different antioxidant profile from that of α-tocopherol. While similar in preventing arachidonic acid oxidation, α-tocopherol was not able to prevent CH=CH oxidation

Table 1. Antioxidant effect of HM-3000 and enterolactone when compared with reference antioxidants

	HM-3000	Trolox	BHA	BHT	ENL
Inhibition of lipid peroxidation (μmol/l)[a]	0.06	0.02	1.1	15.3	133
Inhibition of LDL oxidation (nmol/mg LDL)[b]	6.0	22.0	n.d.	n.d.	66.7
Superoxide anion scavenging (μmol/l)[c]	5.6	25	15	>1000	>200
Peroxyl radical scavenging (stoichiometric ratio)[d]	1:4	1:2	n.d.	n.d.	n.d.

HM-3000; Trolox, water-soluble vitamin-E derivative; BHA, butylated hydroxyanisol (synthetic antioxidant); BHT, butylated hydroxytoluene (synthetic antioxidant); ENL, enterolactone; n.d., not determined.

[a]Peroxidation of microsomal lipids was initiated by *tert*-butylhydroperoxide, peroxidation were detected by chemiluminescence; results are given as IC_{50} values (that is concentration of test material that inhibits lipid peroxidation by 50%).

[b]Isolated human low-density lipoprotein (LDL) was oxidized *in vitro* by copper, oxidation was detected by spectrophotometric diene conjugation analysis; results are given as IC_{50} values.

[c]Superoxide anion was produced by xanthine–xanthine oxidase system and detected by chemiluminescence; results are given as IC_{50} values.

[d]Peroxyl radicals was generated by thermal decomposition of 2,2′-azobis(2-amidinopropane)HCl and detected by chemiluminescence; results are given as the stoichiometric factor (that is moles of peroxyl radicals scavenged by one mole of test compound).

at all and only inhibited linoleic acid oxidation slightly. HM-3000 at a concentration of 60 μmol/l inhibited roughly 50% of all oxidative reactions.

In summary, HM-3000 appears to be an effective and multifaceted antioxidant in comparison to previously well-known antioxidants. Inhibition of LDL oxidation is of special importance in humans as the concentration of oxidized LDL in serum is considered to be one of the independent predictors of atherosclerosis and cardiovascular diseases.

HM-3000 and enterolactone were both effective inhibitors of oxidative burst in human monocytes/granulocytes (Figure 3). A 10 μmol/l concentration of HM-3000 was required to diminish the oxidative burst, while enterolactone had an effect at 1 μmol/l concentration. Thus, enterolactone appears to be more potent than HM-3000 in inhibiting the activity of monocytes/granulocytes induced by TPA.

Antioxidative properties of HM-3000 in vivo

At the start of the study the average body weight in all groups of animals was not significantly different: group 1 (vitamin E-deficient diet only) 28.8 ± 1.6 g; group 2 (diet with vitamin E) 29.2 ± 2.1 g; group 3 (diet with HM-3000) 28.9 ± 1.5 g; and group 4 (diet with vitamin E and HM-3000) 28.5 ± 1.6 g. Body weight gain after 2 weeks in mice in group 1 was 4% slower than in mice in the groups on a vitamin E-supplemented diet, and after 4 weeks treatment it was 6% lower than the other groups. Weight gain of the mice on HM-3000-supplemented diet was similar to that of mice in the vitamin E-supplemented diet.

After 2 weeks feeding, diene conjugation (DC) was 10% elevated among mice in the vitamin E-deficient group. After 4 weeks, group 1 had 12% higher DC than mice on either vitamin E- or HM-3000-supplemented diet. Serum DC was clearly lowest in those mice who received diet supplemented by both vitamin E and HM-3000 (26% lower compared with mice in group 1). The effect was not due to vitamin E itself, because HM-3000 alone did not influence α-tocopherol concentrations (in deficient control mice and mice on HM-3000 alone it was 2.4 and 2.7 μmol/l, respectively). The figures were 12.2 and 14.3 μmol/l in animals with α-tocopherol supplementation and with α-tocopherol + HM-3000, respectively. Analysis of thiobarbituric acid-related substances in the liver as an indication of oxidative stress clearly showed that HM-3000 alone and in combination with vitamin E effectively prevented oxidative stress. However, HM-3000 and vitamin E did not affect the level of oxidative stress in normal mice.

The study showed that HM-3000 acts as an antioxidant *in vivo*. Moreover, it demonstrated that HM-3000 can prevent the influence of vitamin E deficiency, and also that HM-3000 fortifies the antioxidant effects of vitamin E.

Hormonal effects of HM-3000

HM-3000 did not bind to the ERα even at 10 μmol/l concentration. Nor did HM-3000 (up to 10 μmol/l) induce any estrogen receptor-mediated gene expression as measured with estrogen response element luciferase-expressing MCF-7 cell line. Likewise, HM-3000 did not inhibit 1 nmol/l 17-β-estradiol-induced gene expression. This indicates a lack of estrogenic and antiestrogenic activity of HM-3000 and confirms the earlier findings of Saarinen *et al.* (2000).

HM-3000 had no significant estrogenic effect on the uterine weight of the immature rat (for details, see Saarinen *et al.*, 2000). Neither did HM-3000 reduce the weight gains, indicating no antiestrogenic effect. The results in male animals have been reported elsewhere (Saarinen *et al.*, 2000). To summarize, HM-3000 had no detectable

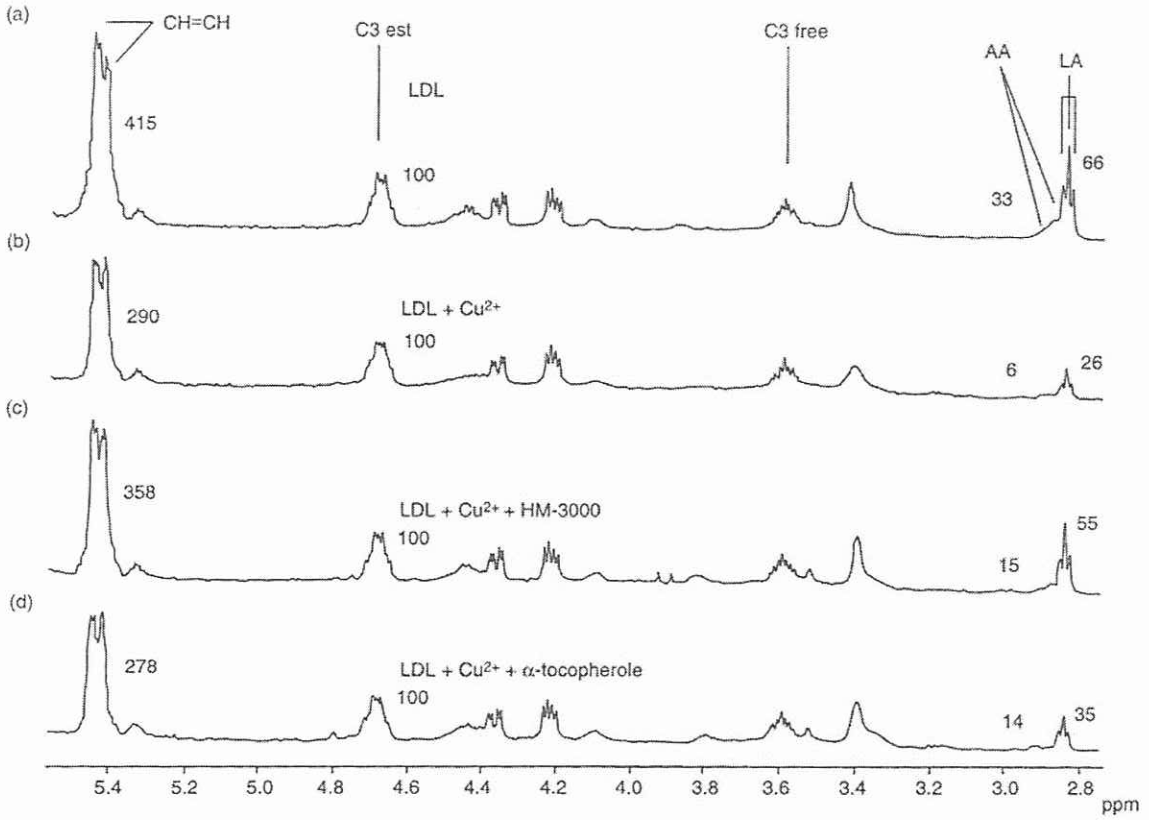

Figure 2. A part of 500 MHz ^{1}H-NMR spectrum of LDL after 3 h incubation and extraction with $CDCl_3$. (a) Control: $CDCl_3$-extracted non-oxidized LDL; (b) $CDCl_3$-extracted LDL oxidized with Cu^{2+}; (c) $CDCl_3$-extracted LDL oxidized with Cu^{2+} in the presence of 60 µmol/l HM-3000; (d) $CDCl_3$-extracted LDL oxidized with Cu^{2+} in the presence of 60 µmol/l α-tocopherole (vitamin E). The signal assignments are: CH=CH refers to double bond protons; C3 est to esterified cholesterol C3 proton; C3 free, to free cholesterol C3 proton; AA to arachidonic acid and LA to linoleic acid protons. The numbers on the side of the signals give their areas when the signal of esterified C3 proton of cholesterol is considered as internal standard and given the value of 100. Oxidation decreased the CH=CH signals from 415 to 290 and α-tocopherol was not able to inhibit this oxidation (value 278) while HM-3000 (value 358) had about 50% inhibition. Similarly, only weak inhibition of linoleic acid oxidation was observed with α-tocopherol, while HM-3000 was effective antioxidant. α-Tocopherol and HM-3000 had comparable effect on arachidonic acid oxidation.

estrogenic, antiestrogenic or antiandrogenic action in rats as indicated in the weights of uterus, prostate, seminal vesicles, or concentrations of testosterone and LH.

Antitumor effects of HM-3000 in vitro

HM-3000 affected the proliferation in different cancer cell lines only marginally at concentrations up to 100 µmol/l (Figure 4). HM-3000 did not affect the number of apoptotic cells after 3 days in culture medium (data not shown). Enterolactone dose-dependently inhibited cellular growth at concentrations above 10 µmol/l. At 100 µmol/l enterolactone produced the largest effect. Enterolactone added to the culture medium at 100 µmol/l did not activate any extra apoptosis.

Antitumor effects of HM-3000 in vivo

The antitumor effects of HM-3000 have been studied in DBMA-induced mammary carcinoma model in the rat (Saarinen *et al.*, 2000, 2002). HM-3000 had a statistically significant antitumor effect in reducing total tumor volume at a dose level of 15 mg/kg (Figure 5). However, in subsequent studies at a lower dose (5 mg/kg) the effect did not reach statistical significance (data not shown).

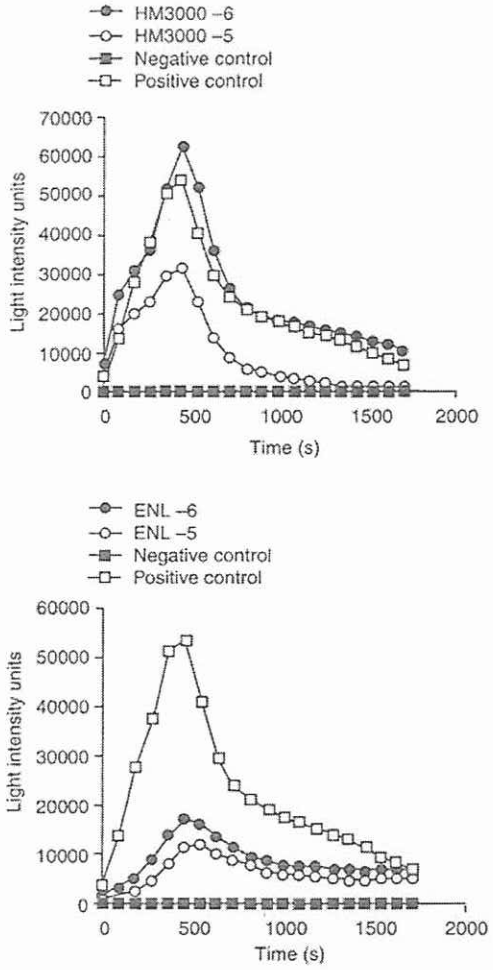

Figure 3. The effect of HM-3000 or enterolactone (ENL) on the oxidative burst in human venous blood monocytes/granulocytes. The oxidative burst was initiated with a protein kinase C-activator TPA. Concentrations of HM-3000 and enterolactone: –6 indicates 1 µmol/l, –5 indicates 10 µmol/l.

HM-3000 showed a significant chemopreventive effect by especially preventing the formation of new intestinal adenomas in Apc^{Min} mice. The mean number of adenomas in the small intestine was 26.6 ± 11.0 (mean ± SD) in mice fed with HM-3000 while it was 39.6 ± 8.9 ($P = 0.031$ compared with HM-3000 group) in inulin-fed mice and 36.0 ± 7.4 ($P = 0.049$ compared with HMR group) in inulin/rye-fed mice. HM-3000 did not affect the growth of existing adenomas since there were no differences in mean diameter, tumor size (% of total), and distribution of adenomas along the length of the intestine between the diet groups. In the colon and cecum, the incidence of tumors (60–75%) and the

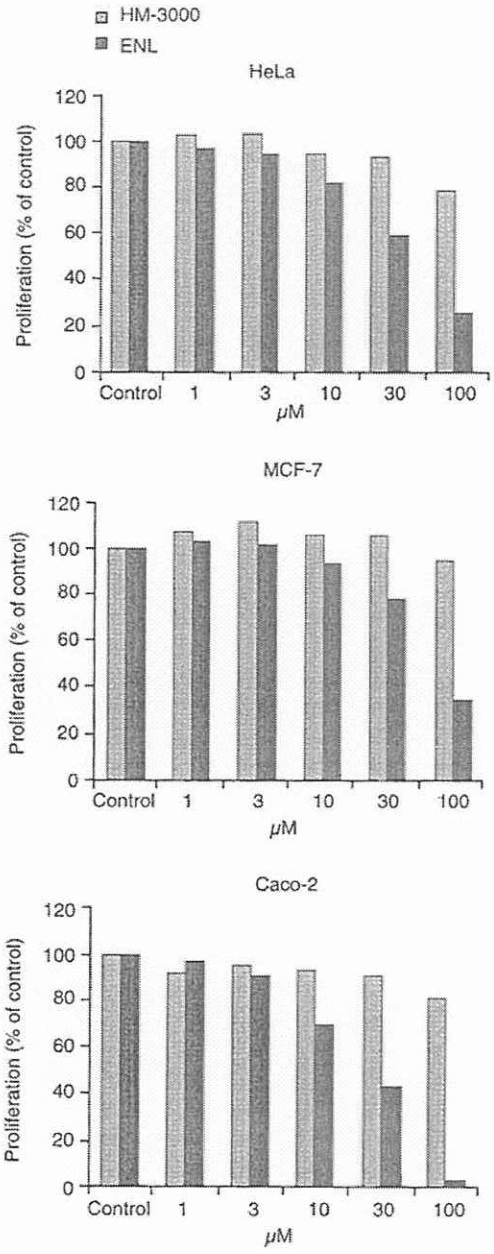

Figure 4. The effect of HM-3000 or enterolactone (ENL) on cell proliferation in HeLa, MCF-7 and Caco-2 cells. Cells were plated in the presence of varying concentrations of test compound and 4 days later the number of cells on culture plate was estimated by measuring the concentration of ATP in each well. A mean of at least a quadruplicate measurement from single experiment has been presented. The amount of ATP in solvent control wells was set at 100%.

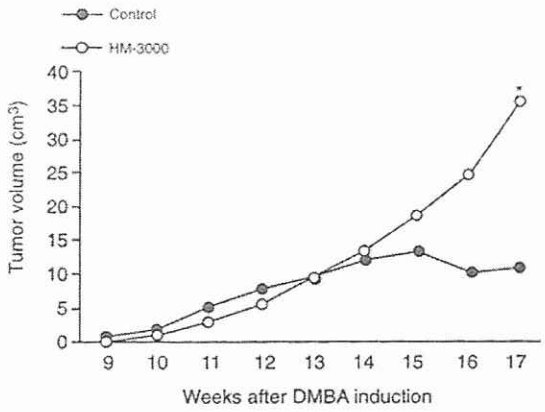

Figure 5. Total tumor volume during placebo or HM-3000 treatment in dimethylbenz[*a*]anthracene (DMBA)-treated rats. Each time point represents one palpation. Change in tumor volume is statistically significant ($P < 0.05$) from control group at 17 weeks (Saarinen *et al.*, 2000 with permission).

number of adenomas (0.9–1.3) did not differ between the groups.

HM-3000 resulted in normalization of β-catenin levels in adenoma tissue of Apc^{Min} mice, indicating that HM-3000 may mediate its chemopreventive effect through the Apc–β-catenin pathway. Wild-type Apc protein regulates intracellular β-catenin level and prevents the entrance of β-catenin into the nucleus, where excess β-catenin could trigger abnormal gene transcription and thus promote tumor development (Korinek *et al.*, 1997; Morin *et al.*, 1997; Beherens *et al.*, 1996). In the cytosolic fraction, β-catenin level in adenoma tissue was significantly elevated ($P = 0.008$–0.013) in all the diet groups as compared to that of the surrounding mucosa. In the nuclear fraction, β-catenin in the inulin and inulin/rye groups was also significantly higher ($P = 0.003$–0.009) in the adenoma tissue when compared to the surrounding mucosa. However, HM-3000 was able to restore the nuclear β-catenin level of the adenoma tissue to the level found in the surrounding mucosa. Since there was also a tendency for cytosolic β-catenin to be low in HM-3000-fed mice, it is possible that HM-3000 might both enhance degradation of cytosolic β-catenin and prevent β-catenin transport to the nucleus.

Pharmacokinetics of HM-3000

The tissue distribution of radioactivity after tritium-labeled HM-3000 administration was studied both after i.v. and p.o. dosing. The radioactivities in general were low in all tissues except for gastrointestinal tract and bladder. Very low levels of radioactivity were observed in the brain and spinal cord, as shown in Figure 6. The serum kinetics of radioactivity was comparable after i.v. and p.o. dosing. The initial distribution phase was rapid, but the terminal elimination was relatively slow with a half-life of about 24 h. Following oral administration the peak plasma concentrations were observed as early as 1 h post dose. About 56% of the radiolabeled dose was absorbed following oral administration.

HM-3000 was found at detectable levels in the urine and serum in rats after dietary exposure (Table 2). In addition, enterolactone was markedly elevated in rats fed with HM-3000. Thus, HM-3000 appears to be a novel enterolactone precursor (Saarinen *et al.*, 2000).

Safety and toxicology studies

In general, HM-3000 was well tolerated in all animal studies performed so far. No differences in body weight gain were found between the groups during the course of various animal experiments. However, 50% of the Apc^{Min} animals in the HM-3000-fed group had some shedding of hair, while only one in the inulin-fed and none in the inulin/rye-fed groups had such disorders.

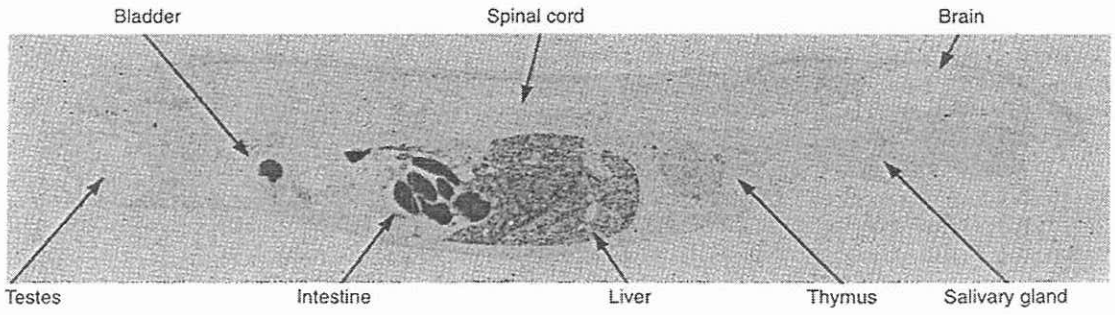

Figure 6. Whole-body autoradiogram of a section through a male rat 30 min following oral administration of [^{3}H]-HM-3000. Total dose of HM-3000 was 250 mg/kg.

Table 2. Excretion of HM-3000 and enterolactone in the rat urine and their levels in serum after 8 weeks dietary administration of HM-3000 (ca. 5 mg/kg/day). The animals received a single dose of DMBA one week after the start of HM-3000 diet

	Urine excretion (μg/24 h)		Serum concentration (ng/ml)	
Group	HM-3000	Enterolactone	HM-3000	Enterolactone
Control	not detectable	4 ± 1	not detectable	8.2 ± 10.3
HM-3000 8 weeks	126 ± 43	234 ± 106	93 ± 34	105 ± 44

From Saarinen *et al.* (2002).

A GLP level regulatory toxicology program with HM-3000 is underway. Short-term toxicology refers to very low toxicity. The LD_{50} has not been reached since all animals tolerated HM-3000 well at doses up to 2 g/kg per day (for 14 days), which is in practice the highest dose that can be administered. In 28-day repeated dose toxicity studies in rats and dogs (1 g/kg per day and 665 mg/kg per day, respectively) no treatment-related toxicity was observed. In addition, the genotoxicity studies (AMES, chromosome aberration *in vitro* and rat micronucleus tests) indicate lack of genotoxicity.

Human studies

A single-dose study up to a dose of 1200 mg (orally) indicated an excellent tolerability as no HM-3000-related adverse reactions were recorded. Absorption of HM-3000 was rapid and T_{max} was achieved at 0.5 h. C_{max} and AUC were in correlation with the dose. Enterolactone was detected, mainly as conjugates, in serum. Enterolactone concentrations increased as the function of HM-3000 dose.

Future goals and prospects

The completed and ongoing toxicological studies indicate that HM-3000 poses an extremely low toxic potential. The safety evaluation program continues with chronic and reproduction toxicity studies.

Human studies have also been initiated, and preliminary results from a single-dose study showed that HM-3000 is well tolerated among healthy subjects.

Thus, we propose that HM-3000 could be a promising functional food ingredient to be added to suitable foodstuffs to lower the risk of certain cancers and cardiovascular diseases.

Conclusion

HM-3000 is a strong antioxidant and experimental anticancer lignan. It is converted by the gut flora partly to enterolactone, which may be responsible for the anticarcinogenic actions of HM-3000. HM-3000 is absorbed rapidly from the gastrointestinal tract, but its concentrations in serum and tissues is relatively low due to its rapid metabolism.

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