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In Vitro and In Vivo Inhibition of Microsomal Lipid Peroxidation by MA-631

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HANNA, A. N., H. M. SHARMA, E. M. KAUFFMAN AND H. A. I. NEWMAN. *In vitro and in vivo inhibition of microsomal lipid peroxidation by MA-631.* PHARMACOL BIOCHEM BEHAV **48**(2) 505–510, 1994.—Excess free radicals are linked to many diseases, including aging, atherosclerosis, and cancer. Previously, we have shown that MA-631 (a complex herbal mixture) inhibits human low-density lipoprotein (LDL) oxidation and may play a role in prevention of atherosclerosis. In this study we further evaluated the in vivo and in vitro antioxidant activity of MA-631. Both the alcoholic and aqueous extracts of MA-631 inhibited enzymatic- and nonenzymatic-induced rat liver microsomal lipid peroxidation in a concentration-dependent manner. The thiobarbituric acid–reactive substances (TBARS) values (nmol malondialdehyde (MDA)/mg microsomal protein) were 1.43 ± 0.18 for microsomes alone (baseline for enzymatic system), 19.63 ± 2.50 for microsomes + reduced nicotinamide adenine dinucleotide phosphate (NADPH) (oxidation without inhibitor), 9.89 ± 1.41 for heated microsomes (baseline for nonenzymatic system), and 27.15 ± 0.08 for microsomes + ascorbate (oxidation without inhibitor). The concentrations (μg/2 ml) of MA-631 which produced 50% inhibition (IC_{50}) of enzymatic- and non-enzymatic-induced lipid peroxidation were 15.2 ± 2.0 and 17.0 ± 2.6, respectively, for the aqueous extract, and 4.3 ± 0.8 and 6.4 ± 1.2, respectively, for the alcoholic extract. A 2% MA-631 (w : w) supplemented diet fed to rats for three weeks inhibited in vivo, toluene-induced microsomal lipid peroxidation in the brain, kidney, liver, and heart. These results imply that MA-631 may be useful in the prevention of free radical–linked diseases.

MA-631 Microsomal lipid peroxidation Thiobarbituric acid–reactive substances Maharishi Ayur-Ved
Toluene Vitamin E Probucol

FREE radical formation during cellular metabolism provides the biochemical basis for oxygen-induced injury to cells and organs. Endogenous antioxidants are the primary means of quenching these free radicals and reactive oxygen species. However, when the number of free radicals exceeds the capacity of antioxidants to remove them, destructive morphological changes occur at the cellular level. Free radicals have been implicated in the pathogenesis of many diseases, including ischemia-reperfusion injury (16,19), CNS injury (5,14,17,33), chronic inflammation (29,34), atherosclerosis (28,32), aging (15,27), rheumatoid arthritis (2), and cancer (4,35). The ingredients of the herbal food supplement MA-631, from the system of natural health care known as Maharishi Ayur-Ved (23), have been shown to be useful in lowering cholesterol levels and treating diabetes mellitus and cancer (1,13,25,31). Also, previously we have shown that MA-631 inhibits Cu^{2+}-catalyzed low-density lipoprotein (LDL) oxidation (24). In this study we evaluated the antioxidant activity of MA-631 on enzymatic (reduced nicotinamide adenine dinucleotide phosphate [NADPH])- and non-enzymatic (ascorbate)-induced rat liver microsomal lipid peroxidation in vitro. We also examined the effect of MA-631 on toluene-induced microsomal lipid peroxidation in various organs in vivo. A widely used organic solvent, toluene is a neurotoxic chemical that acts through increasing the generation of reactive oxygen species (18).

METHOD

Materials

MA-631 was provided by Maharishi Ayur-Ved Products International (Lancaster, MA). Adenosine 5′-diphosphate (ADP), nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis). Thiobarbituric acid and malonaldehyde bis (dimethyl acetal) were obtained from Aldrich Chemical Co., Inc. (Milwaukee). Toluene and sodium ascorbate were purchased from Mallinckrodt Specialty Chemicals Co. (Paris, KY). Fer-

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ric chloride was obtained from Fisher Scientific Co. (Fair Lawn, NJ). Ultracentrifuge tubes were purchased from Seton Scientific (Sunnyvale, CA). Vitamin E was obtained from Gibco BRL (Gaithersburg, MD). Probucol was a gift from Marion Merrell Dow (Cincinnati). Male Sprague-Dawley rats were purchased from Harlan Laboratory (Indianapolis).

MA-631 Constituents

The ingredients of MA-631 are mineral pitch, Indian bedellium, Himalayan silver fir, dry ginger, catkins, *Picrorhiza kurroa,* galls, black pepper, Indian kudju, bamboo, Chinese cinnamon, cinnamon, saffron, and cardamom, processed in the extracts of Indian trumpet flower, cashmere bark, small caltrops, *Aegle marmelos,* trumpet flower, uraria pitch, *Tinospora cordifolia,* country mallow, licorice, Indian asparagus, winter cherry, Indian kudju, raisins, *Mycrostylis wallichii,* globe thistle, cumin seeds, wild cumin, *Pluchea lanceolata, Inula racemosa, Baliospermum montanum,* major catkins, tellycheri bark, nut grass, Indian gooseberry, *Chebulic myrobalans,* and *Beleric myrobalans.* MA-631 contains many biologically active ingredients, such as flavonoids, vitamin C, thiamin, niacin, alkaloids, and rutin. Many of these ingredients are antioxidants (1,8,9,13,21,25,26).

In Vitro Experiment

Agent preparation. Probucol was dissolved in ethanol. Lyophilized vitamin E was dissolved in phosphate buffer. The aqueous and alcoholic extracts of MA-631 were prepared by adding 50 mg MA-631 to 25 ml Ham's F-10 medium or 25 ml 95% ethanol, respectively. The mixtures were vortexed for 5 min, then centrifuged at 2000 rpm. The supernatant was used for the incubation mixtures. To calculate the extracted weight of the MA-631 preparations, 20 ml of the alcoholic extract and ethanol (as a blank) were evaporated to dryness under N_2, and 20 ml of the aqueous extract and Ham's F-10 medium (as a blank) were lyophilized. The value of the extracted weight in the alcoholic and aqueous extractions was used to calculate the weight of the dissolved substances used in the incubation mixtures.

Liver microsome preparation. Male Sprague-Dawley rats were allowed free access to food and water. Rats were sacrificed by exposure to CO_2. Prior to excision, livers were perfused with ice-cold 1.15% KCl buffer. Liver microsomes were prepared by differential ultracentrifugation as described previously (11). The microsomal preparation was suspended in 1.15% KCl so as to contain approximately 10 mg of protein/ml. The protein concentration of the microsomes was determined by the biuret method.

Incubation mixtures for both enzymatic- and non-enzymatic-induced microsomal lipid peroxidation. Liver microsomes (0.5 mg protein) were incubated in the presence or absence of either a reduced NADP (NADPH)-generating system (glucose-6-phosphate, 3 mM; NADP, 0.2 mM; and glucose-6-phosphate dehydrogenase, 1 unit) or ascorbate system (ascorbate, 0.05 mM; $FeCl_3$, 0.001 mM; and ADP, 0.04 mM). In the case of the ascorbate system, the liver microsomes were heat-inactivated before they were added to the incubation mixtures. Each of these systems was employed with or without various concentrations of the agents for 15 min at 37°C. Since some of the agents were dissolved in ethanol, a parallel control was run. The total volume of the incubation mixtures was 2 ml. The degree of microsomal lipid peroxidation was assessed by measuring thiobarbituric acid-reactive substances (TBARS).

Measurement of TBARS. At the end of the incubation, 1 ml of 35% trichloroacetic acid was added to each incubation mixture and vortexed. Then 1 ml of 1.5% TBA was added and the mixtures were incubated at 85-90°C for 15 min. The

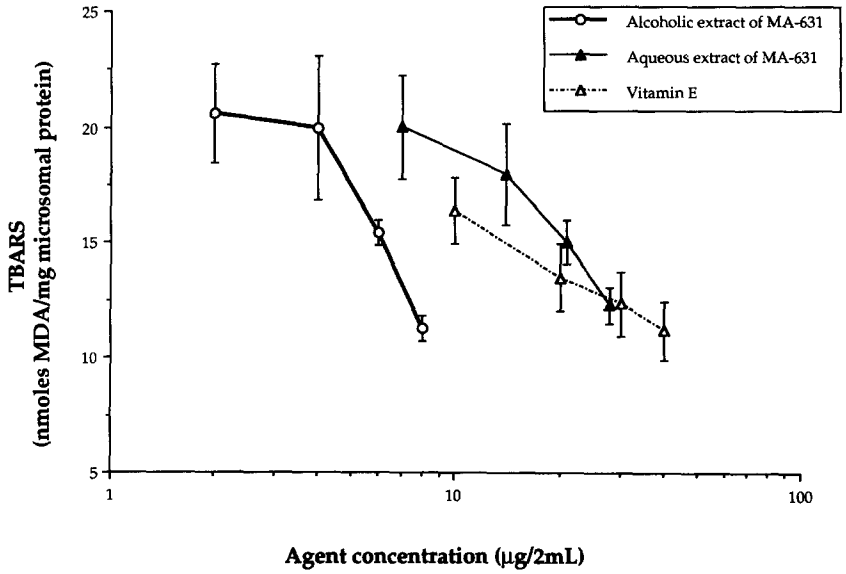

FIG. 1. Concentration-dependent inhibition of ascorbate (non-enzymatic)-induced liver microsomal lipid peroxidation by MA-631. Liver microsomes (0.5 mg protein) were incubated in the presence or absence of an ascorbate system (ascorbate, 0.05 mM; $FeCl_3$, 0.001 mM; and ADP, 0.04 mM), with or without various concentrations of the agents, for 15 min at 37°C. The total volume of the incubation mixtures was 2 ml. The degree of microsomal lipid peroxidation was assessed by measuring TBARS. Values are means ± SDs, $n = 6$.

TBARS content was measured spectrofluorometrically, using excitation and emission wavelengths of 515 and 553 nm, respectively (10).

In Vivo Experiment

Animals and treatment. Male Sprague-Dawley rats were divided into four groups. Each group contained six rats. All rats were allowed free access to food and water. The first and second groups of rats were fed regular chow, and the third and fourth groups were fed regular chow supplemented with 2% MA-631 (w : w). After feeding rats the described diet for three weeks, the first and third groups were injected with a single dose of toluene (1.5 g/kg body weight) IP. Concurrently, the second and fourth groups were injected IP with comparable volumes of normal saline.

Preparation of microsomes. All animals were sacrificed by exposure to CO_2 2 h after the injections. Liver, kidney, heart, and brain microsomes were prepared in the same manner as for the in vitro experiment.

Measurement of TBARS. We used 0.5 ml of the microsomal suspensions, containing 5 mg microsomal protein, to measure TBARS in the same manner as for the in vitro experiment.

Statistical analyses. With this experimental design, the appropriate statistical treatment was an analysis of variance (ANOVA) with repeated measures to determine if there were any differences among the treatment groups. In cases of $p < 0.05$, a post hoc Scheffe's F test was used to compare each treatment to the control group.

RESULTS

In Vitro Experiment

Ascorbate-induced microsomal lipid peroxidation. Both the aqueous and alcoholic extracts of MA-631 and vitamin E, but not probucol, inhibited ascorbate-induced microsomal lipid peroxidation in a concentration-dependent manner. TBARS concentrations decreased as the concentration of the agents increased (Fig. 1). The alcoholic extract of MA-631 was significantly more potent ($p < 0.05$) than vitamin E and the aqueous extract of MA-631. The aqueous extract of MA-631 was significantly less potent ($p < 0.05$) than vitamin E (Table 1). Based on the values of the agent concentrations which produced 50% inhibition (IC_{50}s) of ascorbate-induced microsomal lipid peroxidation (Table 1), the alcoholic extract of MA-631 showed approximately two times more antioxidant potency than vitamin E in inhibiting ascorbate-induced microsomal lipid peroxidation.

NADPH-induced microsomal lipid peroxidation. Probucol, vitamin E, and the aqueous and alcoholic extracts of MA-631 inhibited NADPH-induced microsomal lipid peroxidation in a concentration-dependent manner (Fig. 2). The antioxidant efficiency of the alcoholic extract of MA-631 was significantly more potent ($p < 0.05$) than that of the aqueous extract of MA-631, probucol, and vitamin E. The antioxidant efficiency of probucol was significantly less potent ($p < 0.05$) than the antioxidant efficiencies of the alcoholic extract of MA-631, the aqueous extract of MA-631, and vitamin E. Based on the IC_{50} values in the NADPH-induced lipid peroxidation system, the alcoholic extract of MA-631 exhibited approximately 22, 5, and 4 times more antioxidant potency than probucol, vitamin E, and the aqueous extract of MA-631, respectively (Table 1).

In Vivo Experiment

The average percent weight gain of rats pretreated with MA-631 did not differ significantly ($p > 0.5$) from that of rats fed regular chow (Table 2). The regular chow-fed animals injected with toluene IP produced a four-, two-, and sixfold increase in microsomal lipid peroxidation of the brain, heart, and kidney, respectively, compared to animals injected with saline. Also, the MA-631-supplemented diet significantly low-

TABLE 1

COMPARISON OF PROBUCOL, VITAMIN E, AND AQUEOUS AND ALCOHOLIC EXTRACTS OF MA-631 CONCENTRATIONS, FOR 50% INHIBITION (IC_{50}) OF MICROSOMAL LIPID PEROXIDATION

	IC_{50} (μg/2 ml)	
Agent	Ascorbate-Induced Lipid Peroxidation	NADPH-Induced Lipid Peroxidation
MA-631, aqueous extract	17.0 ± 2.6*	15.2 ± 1.9
MA-631, alcoholic extract	5.4 ± 1.2†	4.3 ± 0.8‡
Probucol	–	90.9 ± 7.4§
Vitamin E	10.6 ± 0.5	19.8 ± 2.7

Values are mean ± SE, $n = 6$. Incubation conditions are the same as described in Figs. 1 and 2. The TBARS values (nmol MDA/mg microsomal protein) were 1.43 ± 0.18 for microsomes alone (baseline for NADPH-induced lipid peroxidation), 19.63 ± 2.50 for microsomes + NADPH (oxidation in absence of inhibitor), 9.89 ± 1.41 for heated microsomes (baseline for ascorbate-induced lipid peroxidation), and 27.15 ± 0.08 for microsomes + ascorbate (oxidation without inhibitor). *The IC_{50} of the aqueous extract of MA-631 was significantly less potent ($p < 0.05$) than those of the alcoholic extract of MA-631 and vitamin E. †The IC_{50} of the alcoholic extract of MA-631 was significantly more potent ($p < 0.05$) than those of either the aqueous extract of MA-631 or vitamin E. ‡The IC_{50} of the alcoholic extract of MA-631 was significantly more potent ($p < 0.05$) than those of either the aqueous extract of MA-631 or probucol. §The IC_{50} of probucol was significantly less potent ($p < 0.05$) than those of the rest of the groups.

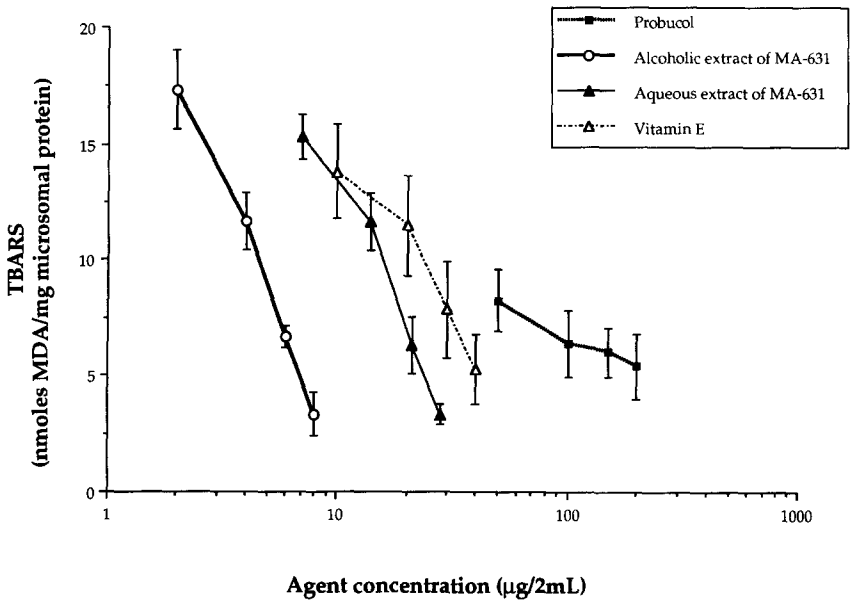

FIG. 2. Concentration-dependent inhibition of NADPH (enzymatic)-induced liver microsomal lipid peroxidation by MA-631. Liver microsomes (0.5 mg protein) were incubated in the presence or absence of an NADPH-generating system (glucose-6-phosphate, 3 mM; NADP, 0.2 mM; and glucose-6-phosphate dehydrogenase, 1 unit), with or without various concentrations of the agents, for 15 min at 37°C. The total volume of the incubation mixtures was 2 ml. The degree of microsomal lipid peroxidation was assessed by measuring TBARS. Values are means ± SDs, $n = 6$.

ered ($p < 0.05$) the basal level of lipid peroxidation in the heart and kidney microsomes, compared to animals fed regular chow. However, MA-631 did not significantly lower the basal level of lipid peroxidation in brain microsomes compared to animals fed a regular chow diet. Feeding the rats regular chow supplemented with 2% MA-631 (w : w) for three weeks completely inhibited toluene-induced microsomal lipid peroxidation in the brain, heart, kidney, and liver (Fig. 3).

DISCUSSION

MA-631 inhibits the initiation and propagation of Cu^{2+}-catalyzed LDL oxidation (24); however, until now no intracellular effects for this herbal food supplement have been measured in vivo or in vitro. In the present work we found that aqueous and alcoholic extracts of MA-631 inhibited both enzymatic- and non-enzymatic-induced liver microsomal lipid peroxidation in a concentration-dependent manner. Both aqueous and alcoholic extracts of MA-631 were more potent than probucol in inhibiting NADPH (enzymatic)-induced liver microsomal lipid peroxidation. Moreover, in the same system the alcoholic extract of MA-631 had more antioxidant potency than vitamin E, but the potency of the aqueous extract of MA-631 did not significantly differ from vitamin E. In nonenzymatic (ascorbate)-induced microsomal lipid peroxidation, the alcoholic extract of MA-631 had significantly higher antioxidant potency compared to that of vitamin E and the aqueous extract of MA-631. However, the aqueous extract of MA-631 had significantly less antioxidant potency than vitamin E in inhibiting ascorbate-induced microsomal lipid peroxidation.

In both the NADPH- and ascorbate-induced microsomal lipid peroxidation, the alcoholic extract of MA-631 had more potency than the aqueous extract, perhaps because it contains more hydrophobic antioxidants which may integrate into membrane structures. This finding is in agreement with the previous work, which showed that the alcoholic extract of MA-631 exhibits more potency than the aqueous extract in inhibiting Cu^{2+}-catalyzed LDL oxidation (24).

The antioxidant activity of MA-631 was further demon-

TABLE 2

EFFECT OF MA-631 PRETREATMENT ON THE AVERAGE PERCENT WEIGHT GAIN OF RATS

Treatment	First Week	Second Week	Third Week
Regular chow alone	15.3 ± 3.6	10.2 ± 4.0	6.5 ± 0.9
Regular chow supplemented with 2% MA-631 (w : w)	13.3 ± 3.1	10.4 ± 2.9	6.5 ± 1.8

Values are means ± SDs, $n = 12$.

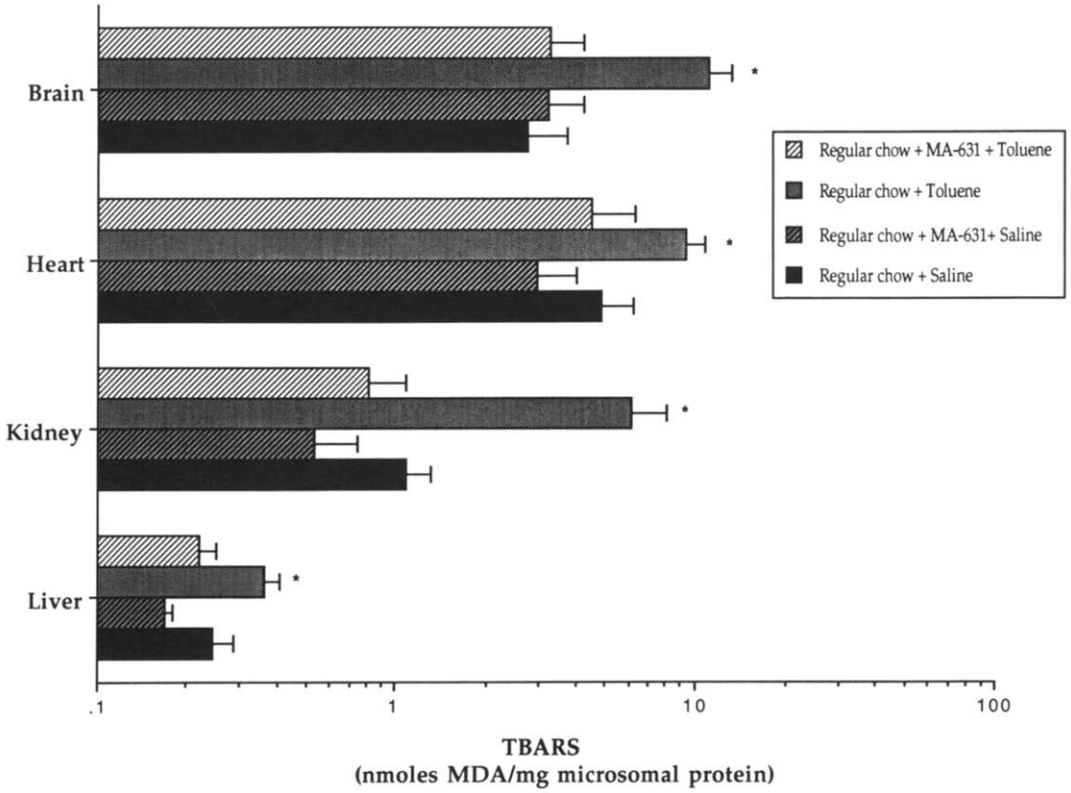

FIG. 3. Effect of in vivo pretreatment with MA-631 on toluene-induced microsomal lipid peroxidation. Animals were fed regular chow or regular chow supplemented with 2% MA-631 (w:w) for three weeks, then injected IP with toluene or a comparable volume of normal saline. Two hours after injection all animals were sacrificed and microsomal lipid peroxidation was assessed by measuring TBARS. Values are means ± SDs, $n = 6$. *Regular chow + toluene is significantly higher ($p < 0.05$) than regular chow supplemented with 2% (w : w) MA-631 + toluene.

strated by the in vivo experiment, which showed that feeding rats regular chow supplemented with 2% MA-631 (w : w) for three weeks inhibited toluene-induced lipid peroxidation in the brain, heart, kidney, and liver microsomes, as assessed by measuring TBARS.

Free radicals and reactive oxygen species attack cellular macromolecules such as proteins, lipids, carbohydrates, and DNA to induce oxidation, cleavage, cross-linking, and modifications, all of which eventually disturb normal cell homeostasis (6,20,30) and are implicated in a variety of pathologic states (2,4,5,14–17,19,27–29,32,34,35). Although there is no specific attributable mechanism by which MA-631 inhibits lipid peroxidation in vivo and in vitro, the antioxidant activity of MA-631 may prevent oxidant stress by destroying peroxides or by chelating metal ions to reduce the generation of free radicals. These substances may also serve as chain-breaking antioxidants which scavenge free radicals and prevent them from attacking lipids. Also, antioxidants may suppress these states through free radical scavenging, and thus prevent these reactive molecules from attacking intracellular macromolecules. Analysis of the components of MA-631 has revealed the presence of various antioxidants, including bioflavonoids (1,8,9,13,21,25,26). These bioflavonoids have demonstrated antioxidant activity, including the inhibition of lipid peroxidation (3,12,22) and LDL oxidation (7). The flavonoids also scavenge hydroxyl radicals by rapidly reacting with them (3). We propose that the mixture of antioxidants present in MA-631 acts synergistically or additively to inhibit lipid peroxidation. Hence, antioxidants such as MA-631 might be beneficial in preventing many free radical–associated diseases.

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