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Effect of 6-(10-Hydroxydecyl)-2,3-dimethoxy-5-methyl-1,4-benzoquinone (CV-2619) on Microsomal Lipid Peroxidation

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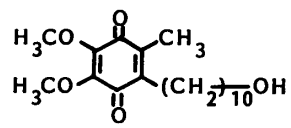
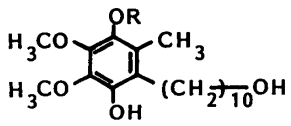
6-(10-Hydroxydecyl)-2,3-dimethoxy-5-methyl-1,4-benzoquinone (CV-2619) and related compounds (the corresponding hydroquinone and hydroquinone monosulfate forms) inhibited lipid peroxidation, especially that initiated with ferrous ion in microsomes of rat liver and canine brain. The fact that CV-2619 inhibited the Fe^{2+}-dependent peroxidation of the membrane lipid in microsomes and linolenic acid indicated that CV-2619 itself possesses antioxidant activity, like its hydroquinone form. However, CV-2619 barely inhibited lipid peroxidation in aged membranes and did not quench hydroxy radicals. It showed some stimulation of peroxide quenching systems, such as horseradish peroxidase and rat liver cytosomal peroxidation-inhibiting protein. These results suggest that CV-2619 mainly inhibits the initial steps of lipid peroxidation.

Keywords——lipid peroxidation; CV-2619; 2H-CV-2619; 2H-CV-2619-S; microsome; rat liver; canine brain; linolenic acid; antioxidant activity

Lipid peroxidation of subcellular fractions, mitochondria and microsomes, has been well studied,[1] and the formation of active oxygen and free radicals in these fractions has been examined in connection with brain vascular damage.[2] Recently, 6-(10-hydroxydecyl)-2,3-dimethoxy-5-methyl-1,4-benzoquinone (CV-2619) has been reported to offer protection against cerebral vascular disorders.[3] Since CV-2619 is mostly changed to its hydroquinone form, 2H-CV-2619, during incubation with brain homogenate,[4] 2H-CV-2619 was considered to inhibit lipid peroxide production, and consequently to prevent tissue damage.

In this report, we describe the effect of CV-2619, 2H-CV-2619 and its monosulfate (2H-CV-2619-S) (Table I) on lipid peroxidation in microsomes of rat liver and canine brain.

TABLE I. Structure of CV-2619 and Related Compounds Tested in This Study

Chemical structure	R	Abbreviation
	—	CV-2619
	H	2H-CV-2619
	SO_3K	2H-CV-2619-S

Experimental

Visible spectra were recorded with Hitachi 200-20 and Titertek® Multiskan spectrophotometers, and electron spin resonance (ESR) spectra with a JEOL JES-PE spectrometer. CV-2619,[5] 6-(10-hydroxydecyl)-2,3-dimethoxy-5-methylhydroquinone (2H-CV-2619) and 6-(10-hydroxydecyl)-2,3-dimethoxy-5-methylhydroquinone 4-sulfate (2H-

CV-2619-S)[6] were synthesized in our laboratories. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) and 5,5′-dimethyl-1-pyrroline-1-oxide (DMPO) were purchased from Sigma, horseradish peroxidase (HRP) from Boehringer Mannheim, linolenic acid from P-L. Biochemicals, and other chemical reagents from Wako Pure Chemical Industries. Protein concentrations were determined by the Lowry method.[7]

Preparation of Microsomes[8]——Livers of female Sprague-Dawley rats, 6—8 weeks old, or brains (cerebrum) of mongrel dogs were isolated and minced thoroughly with scissors. The minced tissue was washed several times with ice-cold buffered potassium chloride (0.15 M KCl, 5 mM Tris-maleate, pH 7.4) and homogenized in the same buffered solution, using a Teflon-glass homogenizer. The homogenate was centrifuged at $15000 \times g$ for 15 min. Microsomes were obtained by centrifugation at $100000 \times g$ for 1 h from the $15000 \times g$ supernatant. The pellet was washed once by suspension in the buffered potassium chloride. The resulting pellet was resuspended in the same solution at about 5 mg protein/ml and stored at −80 °C. It was thawed just before use.

Preparation of Dialyzed Cell Sap (Cytosolic Peroxidation-Inhibiting Protein)[9]——The $100000 \times g$ supernatant fraction in the preparation of the microsomes of rat liver was dialyzed overnight at 4 °C against 20 volumes of 0.1 M Tris–HCl buffer, pH 7.5. This cell sap was added to the microsomal suspension accompanied by glutathione to determine the inhibitory activity of the microsomal lipid peroxidation.

Assay of Lipid Peroxidation[8]——The reaction mixture contained 40 mM Tris-maleate, pH 7.4, 0.5 mg microsomal protein, 60 μM NADPH, 5 μM $FeSO_4$, and a solution of test compound in dimethyl sulfoxide (DMSO) (for CV-2619, 2H-CV-2619 and 2H-CV-2619-S) or ethanol (for α-tocopherol) in a total volume of 2.5 ml. The oxidation reaction was initiated by adding NADPH and $FeSO_4$. After incubation for 30 min at 37 °C, 1 ml of the reaction mixture was rapidly transferred to a mixed solution consisting of 50 μl of 0.2% butyrated hydroxytoluene (BHT) in ethanol, 300 μl of 20% trichloroacetic acid (TCA), and 600 μl of 0.05 M thiobarbituric acid (TBA). The mixture was centrifuged at $1400 \times g$ for 10 min, and the resulting supernatant was removed and placed in a glass tube, which was then capped and placed in boiling water for 20 min. The amount of colored product (thiobarbituric acid-reactive substance, TBARS) was measured by determining the absorbance at 532 nm and converting it to nanomoles of malondialdehyde (MDA) using tetraethoxypropane as a standard. Percent inhibition was calculated as [1 − TBARS in test group/TBARS in control group] × 100. In this calculation, TBARS is the difference between the values before and after incubation. The control group contained vehicle (DMSO or ethanol) alone instead of the test compound solution. For the peroxidation of linolenic acid, the reaction mixture contained 1 mM linolenic acid, 9.8×10^{-5} M $FeSO_4$, 4.9×10^{-5} M ethylenediaminetetraacetic acid (EDTA), 1.8×10^{-4} M H_2O_2 (or 9.8×10^{-5} M $FeSO_4$ only), and 0.15 M KCl in 1 mM phosphate buffer, pH 7.4, in a total volume of 2 ml. The reaction was stopped by adding 0.1 ml of BHT, 0.6 ml of 20% TCA, and 1.2 ml of 0.05 M TBA. The reaction mixture was boiled as described above, and $A_{532-600}$ was measured.

Measurement of ESR Spectra[10]——Samples were prepared for ESR measurement by mixing the reaction mixture containing 1 mM linolenic acid, 2.45×10^{-4} M $FeSO_4$, 4.9×10^{-5} M DETA, 1.8×10^{-4} M H_2O_2, 10 mM DMPO and 0.15 M KCl in 1 mM phosphate buffer, pH 7.4. The samples were placed in the ESR spectrometer immediately after mixing. Linolenic acid was dissolved in acetonitrile at 50 mM as a stock solution.

Assay of HRP[11]——HRP was dissolved in 0.02 M phosphate buffer, pH 6.4, at 4 μg/ml and stored. Before being used, the HRP solution was diluted with citrate buffer, pH 5.5, (containing 0.01% sodium merthiolate) at 800 pg/ml. A 100 μl portion of this solution was placed in a microplate well and the test compound in ethanol (5—10 μl) was added to it. Next, 100 μl of solution containing *o*-phenylenediamine (44 mg) in citrate buffer–30% H_2O_2 (1500 : 1 (v/v) 10 ml) was added to the reaction mixture. After 15—20 min, 4 N H_2SO_4 (100 μl) was added and the absorbance at 492 nm was measured.

Determination of NADPH–Cytochrome c Reductase Activity[12]——The reaction mixture contained 0.05 M phosphate buffer, pH 7.7, 21 μg (rat liver) or 400 μg (canine brain) protein of microsomes, 34 nmol of cytochrome c, 3 μmol of KCN and 100 nmol of NADPH, in a total volume of 3.0 ml. The reaction was initiated by adding NADPH. The absorbance increase at 550 nm was measured.

Results

Effect of CV-2619 on Peroxidation of Microsomal Lipids and Linolenic Acid

Lipid peroxidation in liver microsomes requires NADPH and is markedly stimulated by iron salts.[13] The role of NADPH appears to be to maintain iron in the reduced state (Fe^{2+}).[8] To study the effect of CV-2619 on the peroxidative decomposition of membrane lipid, we investigated the effect of CV-2619 and related compounds on NADPH/iron-dependent peroxidation of lipid in microsomes of rat liver and canine brain. The lipid peroxidative reaction was determined in terms of the amount of TBARS[1a] formed by the reaction of TBA with MDA derived from peroxide. Since ethanol and DMSO did not affect the TBARS value under the experimental conditions described above, test compounds were dissolved in these

Table II. Effect of CV-2619 and Related Compounds on the Formation of TBARS[a] in Lipid Peroxidation of Rat Liver and Canine Brain Microsomes (RLMs and CBMs)[b]

Compound	Concentration (M)	% inhibition	
		RLMs	CBMs
CV-2619	1×10^{-5}	97.3±1.2	26.5± 9.4
	1×10^{-4}	98.5±0.5	91.0±10.0
2H-CV-2619	1×10^{-5}	—	70.4
	1×10^{-4}	96.0	96.3
2H-CV-2619-S	1×10^{-5}	36.0±5.2	15.4± 3.6
	1×10^{-4}	96.2±2.6	55.7± 1.8
α-Tocopherol	1×10^{-5}	10.3±2.5	N.E.[c]
	1×10^{-4}	12.2±2.4	

a) TBARS: Thiobarbituric acid-reactive substance. *b*) The reaction mixture contained 0.5 mg of microsomal protein, 5 μM $FeSO_4$, 60 μM NADPH, a solution of test compound in DMSO (for CV-2619, 2H-CV-2619 and 2H-CV-2619-S) or ethanol (for α-tocopherol) and 40 mM Tris-maleate buffer, pH 7.4 in a total volume of 2.5 ml. Incubations and assays were performed as described under Experimental. % inhibition with respect to the control is shown (control 0%). *c*) No effect.

Table III. Recovery of 2H-CV-2619-S after Lipid Peroxidation in Canine Brain Homogenate

Incubation time at 37 °C (min)	Amount added (μg)	Recovery (%)	
		Expt. I[a]	Expt. II[b]
0	229	100	85.5
30	229	100	95.4

a) After incubation, the reaction mixture (2.5 ml) as described in the legend to Table II containing 1.01 mg protein of homogenate was lyophilized, and extracted with MeOH. The extract was separated by thin layer chromatography, developing with $CHCl_3$–MeOH (3 : 1 (v/v)). A band corresponding to 2H-CV-2619-S was extracted with MeOH and evaporated. The residue was dissolved in H_2O to determine A_{280}. Recovery was calculated based on $E^{1\%}_{1\,cm}=24$ at 280 nm ($\lambda^{H_2O}_{max}$) of 2H-CV-2619-S. *b*) The aqueous solution obtained in Expt. I was acid-hydrolyzed, and extracted with isobutanol. The extract was oxidized with Ag_2O to obtain CV-2619. The UV absorption, A_{280}, of the resulting isobutanol solution was determined. Recovery was calculated based on $E^{1\%}_{1\,cm}=451$ at 280 nm ($\lambda^{iso\text{-}BuOH}_{max}$) of CV-2619.

solvents and added to the incubation mixture.

Under these experimental conditions, CV-2619, 2H-CV-2619 and 2H-CV-2619-S showed strong antioxidant activities, while α-tocopherol had much weaker activity than CV-2619 (Table II). The hydroquinone compound was not the only active form of CV-2619, because 2H-CV-2619 could not be detected in the incubation mixture of CV-2619 (data not shown). Since 2H-CV-2619-S would be hydrolyzed to 2H-CV-2619 in the tissue homogenate, it was incubated with the canine brain homogenate. Though the mixture was analyzed to detect 2H-CV-2619, the original sulfate was recovered quantitatively (Table III).

As α-tocopherol is a well-known lipid antioxidant for microsomes[14] and mitochondria,[15] the weak antioxidant activity found in the above experiment may have resulted from its being more lipophilic than CV-2619 and the related compounds.[16] We next tried fractionating the microsomes after the test compounds had been added to the tissue homogenates and inducing lipid peroxidation with NADPH/Fe^{2+}. In this case, the TBARS value in the α-tocopherol-treated microsomes was the lowest (Table IV), thus indicating that the incorporation of α-tocopherol in the lipid phase depends on the experimental conditions. It has been reported that inhibition of lipid peroxidation by vitamin E in phospholipid complex system may

Table IV. Effects of CV-2619 and α-Tocopherol on TBARS Formation in Canine Brain Microsomes in the Presence of NADPH and Fe^{2+} a)

Compound	Amount added/g tissue	TBARS nmol/mg protein (% inhibition)
None	—	15.6 (0)
CV-2619	35 μmol	4.2 (73)
α-Tocopherol	35 μmol	1.6 (90)

a) Assay details were as described in Experimental and the legend to Table II, except that test compounds were added to the homogenate prior to the fractionation. The test compound was added to the homogenate (1 g of wet tissue in 5 ml of the buffer) as an ethanolic solution (0.5 ml).

Table V. Effect of CV-2619 on Peroxidation of Linolenic Acid in the Presence of Fe^{2+} a)

Compound	Concentration (M)	TBARS nmol/tube (% inhibition)	
		Linolenic acid solution	
		Freshly prepared	Stored for one month
Control		11.2 (0)	17.4 (0)
CV-2619	1×10^{-5}	4.9 (56)	19.1 (−10)
	1×10^{-4}	3.8 (66)	15.6 (10)

a) The reaction mixture contained 1 mM linolenic acid, 9.8×10^{-5} M $FeSO_4$ and 0.15 M KCl in 1 mM phosphate buffer, pH 7.4, in a total volume of 2 ml. Reactions were initiated by addition of $FeSO_4$. The reaction was stopped by adding 0.1 ml of BHT, 0.6 ml of 20% TCA, and 1.2 ml of 0.05 M TBA. Visible absorption, $A_{532\text{-}600}$, of the reaction mixture (3.9 ml) was measured after boiling of the mixture for 20 min.

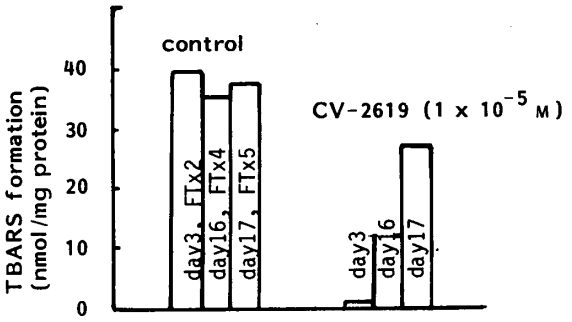


Fig. 1. Effect of Aging and Freezing-Thawing (FT) on the Formation of TBARS in Rat Liver Microsomes

The number of days after the preparation of microsomes and the number of FT cycles are shown. Assay details are given in Experimental and in the legend to Table II.

require its incorporation and susequent solubilization within the membrane matrix.[14] Note that CV-2619 showed considerable antioxidant activity in both systems.

Under aerobic conditions, polyunsaturated fatty acids form various radicals, then peroxidized fatty acids appear. To find the step of oxidation upon which CV-2619 acts as an antioxidant, we investigated the relation of the aging time of the target lipids to the CV-2619 activity. The study of the effect of CV-2619 on lipid peroxidation in aged microsomes showed that the antioxidant activity decreased with aging time under aerobic conditions (Fig. 1). To simplify the assay system, one of the membrane fatty acids, linolenic acid, was used as the target of lipid peroxidation. A solution of linolenic acid in acetonitrile was stored in a refrigerator (5 °C) to obtain an aged sample. CV-2619 showed strong antioxidant activity in freshly prepared linolenic acid solution, but only weak activity in a solution which had been aged for a month (Table V). This result agrees with that for microsomes. These findings suggest that CV-2619 acts on the initial steps of lipid peroxidation.

Table VI. Effects of CV-2619 and 2H-CV-2619 on Peroxidation of Linolenic Acid in the Presence of Fe^{2+}, EDTA and H_2O_2[a]

Compound	Concentration (M)	TBARS nmol/tube (% inhibition)
Control		3.1 (0)
CV-2619	1×10^{-5}	3.1 (0)
	1×10^{-4}	3.2 (−3)
2H-CV-2619	1×10^{-5}	2.9 (6)
	1×10^{-4}	3.6 (−16)

a) The reaction mixture contained 1 mM linolenic acid, 9.8×10^{-5} M $FeSO_4$, 4.9×10^{-5} M EDTA, 1.8×10^{-4} M H_2O_2 and 0.15 M KCl in 1 mM phosphate buffer, pH 7.4, in a total volume of 2 ml. Other assay details were as described in Experimental and in the legend to Table V.

Table VII. Effect of CV-2619 on the Activity of Horseradish Peroxidase[a]

Compound	Amount added	Absorbance at 492 nm			Relative activity (%)
		−HRP	+HRP	Difference	
Control (ethanol)	10 μl	0.043	0.757	0.714	100
CV-2619	1.5×10^{-5} M	0.058	0.801	0.743	104
	1.5×10^{-4} M	0.110	0.946	0.836	117

a) Assay details as described in Experimental.

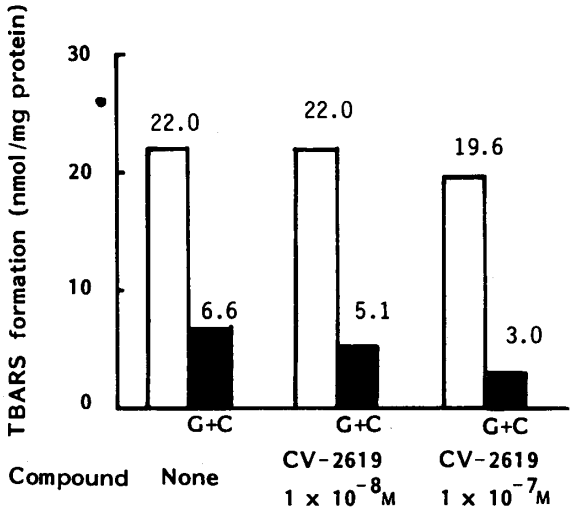


Fig. 2. Effects of Cell Sap, Glutathione and CV-2619 on TBARS Formation in NADPH/Fe^{2+}-Dependent Rat Liver Microsomal Lipid Peroxidation

The reaction mixture (2.5 ml) was consisted of 5 μM $FeSO_4$, 60 μM NADPH, 2.5 mM glutathione, 1.36 mg protein of rat liver cell sap obtained as described in Experimental, 0.83 mg protein of microsomes, a solution of CV-2619 in DMSO and 40 mM Tris-maleate buffer (pH 7.4). Reactions were initiated by addition of $FeSO_4$ and NADPH. Incubations and assays were performed as described under Experimental and in the legend to Table II.

G, glutathione; C, cell sap; ■, in the presence of G and C.

Effect on Active Oxygens

In the 1970's, ESR spectroscopy indicated that NADPH-dependent microsomal lipid peroxidation involves the hydroxy radical (·OH).[12] To study the effect of CV-2619 on ·OH, we measured the ESR spectra of a reaction mixture in which ·OH was generated by means of the Fenton reaction using Fe^{2+} and H_2O_2. CV-2619 could not scavenge ·OH trapped by DMPO (data not shown), and this result was supported by the formation of TBARS in the incubation mixture containing linolenic acid and the Fenton reagent (Table VI). These results suggest that CV-2619 hardly scavenges ·OH. Under these conditions, the amount of peroxide induced by ·OH was less than that by Fe^{2+} alone.

Peroxidase, such as glutathione peroxidase (GSH-Px), can catalyze the reduction of hydroperoxides as well as hydrogen peroxide. In addition, peroxidation-inhibiting proteins

TABLE VIII. Effect of CV-2619 on NADPH–Cytochrome c Reductase Activity[a]

Microsomes	Compound	Amount added (μmol/mg protein)	NADPH-cyt. c reductase activity (nmol/min per mg protein)
Rat liver	Control (DMSO)	—	47.5
	CV-2619	2.4	63.3
Canine brain	Control (DMSO)	—	5.7
	CV-2619	1.5	7.1

a) Assay details as described in Experimental.

which exhibit GSH-Px activity have been obtained from pig and rat liver cytosol.[17] Here we examined the effect of CV-2619 on HRP and rat liver cytosolic peroxidation-inhibiting protein with regard to active oxygen-quenching systems. As shown in Table VII and Fig. 2, CV-2619 tended to stimulate HRP and the rat liver cytosolic peroxidation-inhibiting protein, though the enhancements were not significant.

Effect on Microsomal Electron Transport System

The microsomal electron transport system is related to the biological oxidation of various materials. Since CV-2619 has inhibitory activity on the NADH oxidase system in rat and canine brain mitochondria,[18] we investigated the effect of CV-2619 on NADPH–cytochrome c reductase in rat liver and canine brain microsomes. However, CV-2619 did not inhibit the NADPH–cytochrome c reductase activity in either microsomes (Table VIII), suggesting that the antioxidant activity of CV-2619 did not arise from enzym inhibition.

Discussion

CV-2619 has been reported to inhibit lipid peroxidation in rat brain homogenates and mitochondria as its hydroquinone, 2H-CV-2619.[4,18] On the other hand, the ubiquinone homolog having a short prenyl chain, ubiquinone-3, has been reported to show antioxidant activity in the quinone form as well as the hydroquinone form.[19] The active form of CV-2619 needs to be identified in order to elucidate its antioxidant activity. For these reasons, we investigated the effect of CV-2619 and related compounds on NADPH/Fe^{2+}-induced lipid peroxidation in rat liver and canine brain microsomes, which do not contain any system that reduces quinone to hydroquinone, such as mitochondria. We also studied the effect of CV-2619 on the Fe^{2+}-induced oxidation of linolenic acid. Our results showed that CV-2619 has strong antioxidant activity in these assay systems (Tables II and III), leading to the conclusion that CV-2619 functions as its quinone form in microsomes. This study also showed that CV-2619 displays little antioxidant activity on aged, partially peroxidized microsomal lipid or linolenic acid (Fig. 2 and Table V), suggesting that it may affect the initial steps of lipid peroxidation. A related compound, 2H-CV-2619-S, was found to act as an antioxidant without being hydrolyzed to 2H-CV-2619 (Table III). It is not surprising that 2H-CV-2619 has this action, because phenols (including α-tocopherol) are known to have antioxidant activity. As reduction of the quinone to the semiquinone has been reported in microsomes,[20] the semiquinone form of CV-2619 may be the active form; further study is needed to check this.

There is still controversy about the initiators of lipid peroxidation, and the hydroxy radical (·OH) has been reported to be one of them. However, CV-2619 did not inhibit the lipid peroxidation induced by ·OH which was produced by the Fenton reaction (Table VI). It

seems likely that CV-2619 could stimulate the active oxygen-quenching enzyme, HRP (Table VII), and the activity of rat liver cytosolic peroxidation-inhibiting protein (Fig. 2). There has been a report that the initiation of oxidative decomposition of phospholipid may involve the perferryl ion, FeO_2^{2+}, but not ·OH in microsomal NADPH-dependent lipid peroxidation.[21] The production of a smaller amount of peroxide from linolenic acid by ·OH than by Fe^{2+} alone (Tables V and VI) might suggest that initiation of peroxidation of fatty acids involves FeO_2^{2+} regardless of the presence of microsomal enzyme.

In view of these results, CV-2619 might scavenge fatty acid radicals which were produced by the abstraction of hydrogen from polyunsaturated fatty acids in NADPH/Fe^{2+}-dependent microsomal lipid peroxidation, in addition to enhancing the biological hydroperoxide quenching system to some extent. We have reported that CV-2619 has a membrane-stabilizing activity based on experiments using rat liver lysosomes as a membrane model.[5] This membrane-stabilizing activity is assumed to depend upon scavenging of radicals produced in the membrane on aging. Under our experimental conditions, CV-2619 could not scavenge ·OH. However, 2H-CV-2619 scavenges the stable radical of 1,1-diphenyl-2-picrylhydrazyl (DPPH)[4] and ubiquinone-10 scavenges an adriamycin radical.[22] Radical scavenging with CV-2619 requires further examination using other radicals.

As the brain is the tissue most easily damaged by free radicals,[23] the antioxidant activity of CV-2619 is of great interest. This activity in microsomes, in addition to that of 2H-CV-2619 formed in mitochondria, seems to play an important role in delaying the appearance of cerebral apoplexy and in alleviating the condition after a stroke. Further studies are needed along these lines.

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