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IN VITRO SCAVENGER EFFECT OF DIHYDROQUINOLINE TYPE DERIVATIVES IN DIFFERENT FREE RADICAL GENERATING SYSTEMS

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The non-toxic and water soluble dihydroquinoline type antioxidants: CH 402 (Na-2,2-dimethyl-1,2-dihydroquinoline-4-yl methane sulphonate) and MTDQ-DA (6,6-methylene bis 2,2-dimethyl-4-methane sulphonic acid: Na-1,2-dihydroquinoline) were studied in various *in vitro* tests in which oxygen free radicals were generated. Both compounds were shown to scavenge superoxide radical anions O_2^- produced in aqueous solution by pulse radiolysis with rate constants k (O_2^- + MTDQ-DA) = 4.10^8 $dm^3 mol^{-1} s^{-1}$ and k (O_2^- + CH 402) = $1.5.10^7$ $dm^3 mol^{-1} s^{-1}$. CH 402 and MTDQ-DA reduced the H_2O_2 produced in the glucose–glucose oxidase reaction, which was detected by the luminol + hemin reaction with a chemiluminometric method. The dihydroquinoline type substrates inhibited the NADPH-induced and Fe^{3+} – stimulated lipid peroxidation and the ascorbic acid-induced non-enzymatic peroxidation pathways in microsomal fractions of rat and mouse liver.

KEY WORDS: Free radicals, lipid peroxidation, dihydroquinoline type antioxidants, pulse radiolysis.

INTRODUCTION

In the pathogenesis of several diseases free radical chain reactions are of importance. Free radical reactions are also involved in the preservation of quality of food of animal and plant origin.[1–5] Toxic reactions induced by free radicals can be inhibited by natural or artificial antioxidants (vitamins, food preservatives, etc.). We have recently described the antioxidant effect of CH 402[6] and MTDQ-DA[7] on brain microsomal and plasma membrane preparations.[8,9] The membrane-protecting effect of these derivatives on membrane fractions was also studied.[10–12] Structures are shown in Figure 1.

In this article we report on the probable routes of scavenging of free radicals by these chemicals in different tests.[13–17] O_2^- is a major factor in oxygen toxicity in aqueous solution. The complex role of superoxide radicals in biological systems has recently been reviewed.[18] O_2^- and H_2O_2 can combine together in the presence of metal ions to generate the highly reactive hydroxyl radical, ·OH.[19] In the present work, we report on the reaction of CH 402 and MTDQ-DA with superoxide radical anion studied by a pulse radiolysis technique.[16,17]

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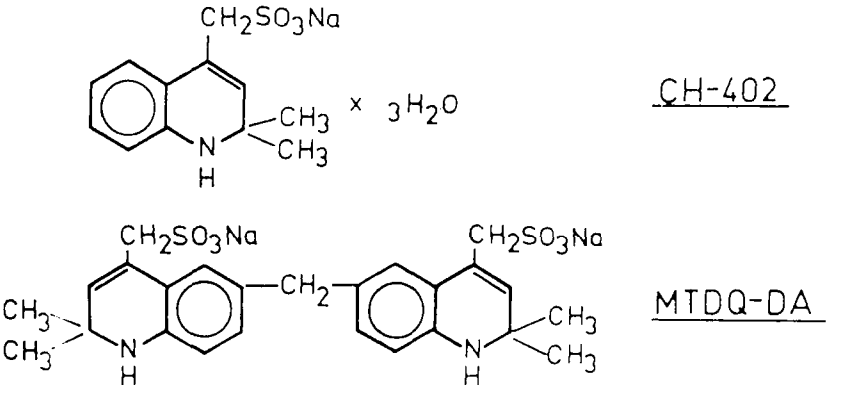

FIGURE 1 Structures of the dihydroquinoline type antioxidants, CH 402 and MTDQ-DA.

A very sensitive assay of H_2O_2 is the luminometric method in which the detection limit is 0.1 pmol. Hydrogen peroxide was generated in a glucose–glucose oxidase system.[20]

Liver microsomes generate free radicals during electron transfer initiated by NADPH and iron. The mechanism of NADPH dependent-and iron-stimulated or ascorbic acid-induced lipid peroxidation is well known.[8,14,21] In this process, the fatty acid side chains of membrane lipids, in particular those containing two or more carbon–carbon double bonds in a divinylmethane structure, are oxidised to hydroperoxides. In the presence of metal catalysts, hydroperoxides decompose to form a complex mixture of hydrocarbons and cytotoxic aldehydes. Several different reduced forms of oxygen participate in the initiation of lipid peroxidation.

MATERIALS AND METHODS

Young male Wistar rats weighing 150–200 g and CBA/Ca mice weighing 25–30 g were used. Animals were killed by decapitation, and their livers were removed. The microsomes were prepared by ultracentrifugation.[15]

Malondialdehyde (MDA) production was detected as an estimate of lipid peroxidation by the thiobarbituric acid test.[22] A molar absorption coefficient E_{532} 1 cm of 156 $mM^{-1} cm^{-1}$ was used. The non-enzymatically induced lipid peroxidation was studied by incubating the protein suspension (1 mg/ml) in a medium of total vol. 0.5 ml and containing 50 mM Tris/maleate buffer, pH 6.8, 1 mM KH_2PO_4 and various concentrations of ascorbic acid and MTDQ-DA.[15]

The enzymatically-induced lipid peroxidation was measured in a medium of total vol. 0.5 ml with a protein content of 1 mg/ml. The medium contained 20 mM Na phosphate buffer, pH 7.5, 0.15 mM KCl, 50 μM $FeCl_3$, 50 μM Na pyrophosphate, glucose-6-phosphate-dehydrogenase 0.6 IU, 0.5 mM NADPH and 10 mM glucose-6-phosphate, plus various concentrations of CH 402 or MTDQ-DA.[14,23]

Protein content of the preparation was determined by Lowry *et al.*[24], using bovine serum albumin as a standard.

Light emission was measured by a chemiluminometric method. The procedure was

carried out in a Medicor Luminometer Model CLD-1 equipped with a MMT microprocessor (MEDILAB, HUNGARY). The measurements were carried out in a double-part cuvette. The contents of the two parts were mixed by centrifugation triggered at the same time as the measurement of chemiluminescence started. The reagent solution, situated in the lower part of the cuvette, contained a mixture of 0.7 mM luminol, 3.8 μM hemin,, which emits luminescence on interaction with free radicals. The pH of the reagent solution was adjusted to 10–11 by adding 11.8 mM Na_2CO_3 prior to deaeration by bubbling with N_2 gas.[13] The solutions which contained the free radical sources, H_2O_2 or the glucose–glucose oxidase system were held in the upper part of the cuvette before starting the experiments. The antioxidants were admixed either to the reagent solution, in the lower part of cuvette (separate part) or to the free radical source solution, in the upper part of cuvette (synchrone part). A comparison of the results of experiments carried out both ways allows conclusions to be drawn on an eventual interaction of the antioxidant with the enzyme by binding to an active site.

When using H_2O_2 as a free radical source, the antioxidants were incubated with the luminol + hemin reagent solution, in order to avoid direct reaction between H_2O_2 and the antioxidants before luminometric detection started. The concentration of H_2O_2 referring to the composed volume of the reagent and radical source solutions, was 4.10^{-7} M. Various concentrations of antioxidants had been prepared in 100 μl volumes and were added to 1000 μl of the reagent solution.

The glucose–glucose oxidase system contained 92 μl of 0.5 M glucose in 0.01 M acetate buffer, pH 5.0, 8 μl glucose-oxidase enzyme from 20 mU/ml suspension.[20] Experiments were carried out with antioxidants held either in the reagent solution or the radical source solution prior to the luminometric measurement. Various concentrations of the antioxidants were prepared in 100 μl volumes; the luminol + hemin reagent mixture comprised 1000 μl volume. The incubating time was 60 sec in either synchrone or in separate phase. The chemiluminometric intensity was expressed in units of mVs. The reaction times were 60 sec.

The computer controlled pulse radiolysis facility and the fast optical detection system of the Institute of Isotopes has recently been described, elsewhere.[16] Briefly, a TESLA made LINAC Model LPR-4 linear accelerator produced 80–2600 ns pulses of electrons with a mean energy of 4 MeV. The dose/pulse was varied between 10 and 60 Gy, as measured by conventional thiocyanate dosimetry.[17] The formation and decay of transient species was followed by a kinetic spectrophotometer (Applied Photophysics, Ltd.) using a Xe arc analysing light source. Analog/digital conversion, handling and storage of data as well as the operation of the accelerator was done by an Iwatsu TS 8123 storage oscilloscope and a Cromemco CS-2H computer.

The L-ascorbic acid was obtained from Merck, Darmstadt, bovine serum albumin, glucose-6-phosphate-dehydrogenase from Calbiochem AG., Lucerne, luminol, hemin and NADPH from Sigma, St. Louis, and all other reagents were from Reanal, Budapest.

Statistical analysis was done by a two-tailed t-test with a present probability level of $P < 0.05$. Mean values represent the data obtained from five experiments.

RESULTS

In general terms, antioxidants are compounds that inhibit lipid peroxidation by interfering with the chain reaction of peroxidation and/or by scavenging reactive

oxygen radicals and/or by repairing damaged sites of biopolymers caused by free radicals. This work was carried out to further demonstrate the antioxidant properties of CH 402 and MTDQ-DA in various systems, in which oxygen free radicals were generated in an enzymatic or non-enzymatic pathway.

In order to demonstrate that CH 402 and MTDQ-DA capture O_2^- and, thus, to verify the former conclusion, pulse radiolysis experiments were performed. Pulse radiolysis offers a convenient way for producing O_2^- anion radicals with high selectivity. On irradiating with pulses of electrons a dilute aqueous solution of sodium formate (0.1–1.0 mol.dm^{-3}) saturated with oxygen (1.3 mmol.dm^{-3}) the major primary transient products of radiolysis (e_{aq}^-, ·OH and H) rapidly convert into O_2^- as follows:

$$e_{aq}^- + O_2 \rightarrow O_2^-$$

$$\cdot OH + HCO_2^- \rightarrow CO_2^- + H_2O$$

$$H\cdot + HCO_2^- \rightarrow CO_2^- + H_2$$

$$CO_2^- + O_2 \rightarrow O_2^- + CO_2$$

In the absence of any other reactive species O_2^- builds up within about a μs using the above experimental conditions and it persists for as long as several milliseconds. In the presence of MTDQ-DA (1 mmol.dm^{-3}) in a buffered aqueous solution of pH 8.2 (30 mmol.dm^{-3} phosphate) a fastly growing transient absorption was observed. The spectrum of the transient, shown in Figure 2, had λ max at 430 nm, and decayed by a second order process in less than a millisecond when pulse doses between 10 and 60 Gy were used. The absorption is attributed to the transient product of the reaction

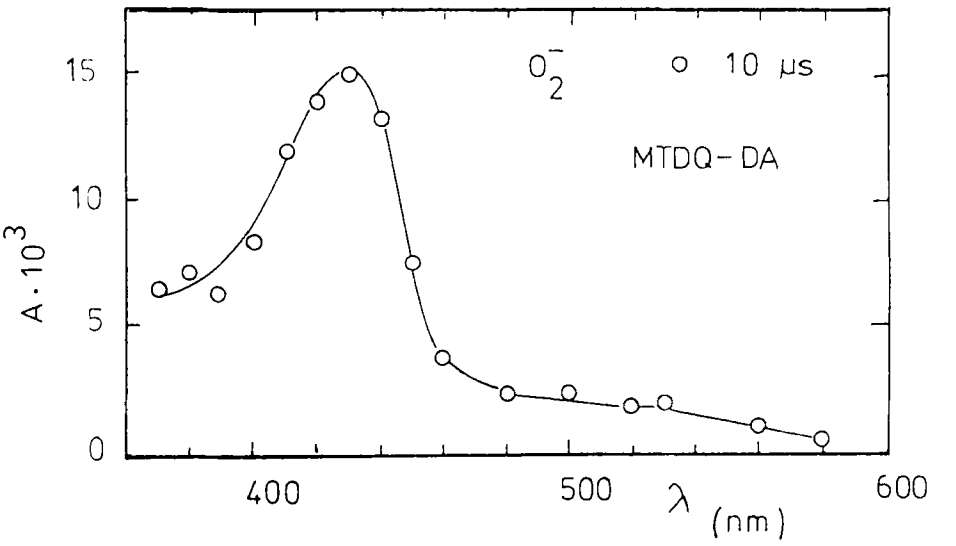

FIGURE 2 Absorption spectrum of the transient product formed in the reaction O_2^- + MTDQ-DA (1 mmol.dm^{-3}) as obtained at 10 μs after the electron pulse of 2.6 μs duration and 54 Gy/pulse. The solution contained 1.3 mmol.dm^{-3} O_2, 0.5 mol.dm^{-3} sodium formate and 30 mmol.dm^{-3} phosphate buffer (pH 8.2).

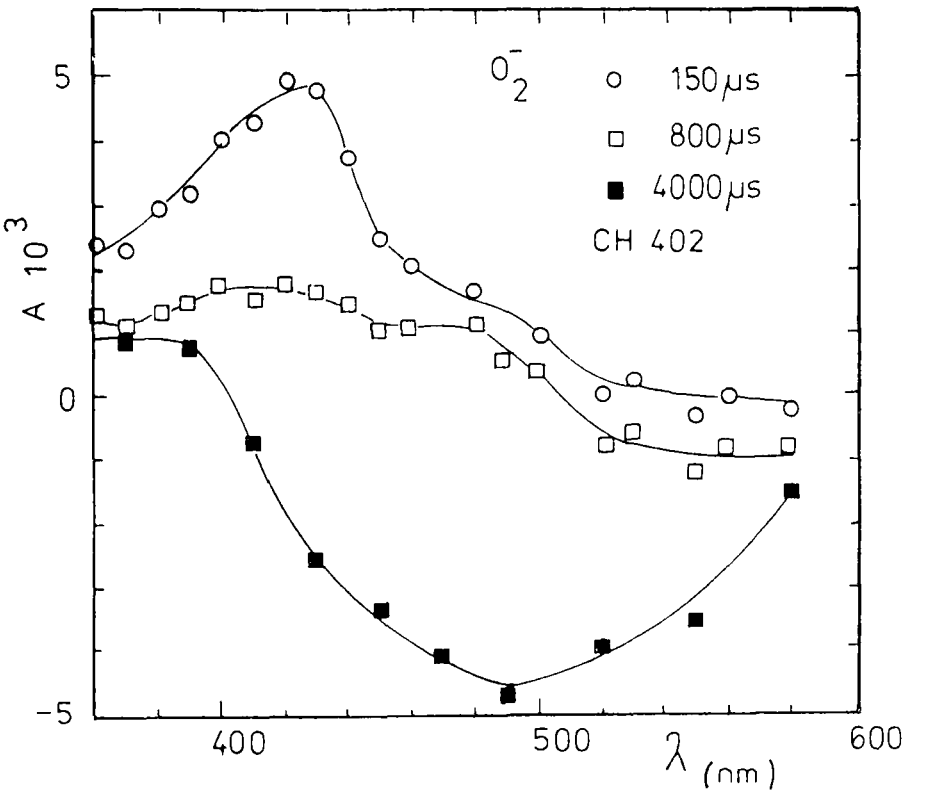

FIGURE 3 Transient absorption spectra obtained at 150 μs (○), 800 μs (□) and 4 ms (■) after the electron pulse of 2.6 μs duration and 29 Gy/pulse incident on an aqueous solution of CH 402 (2 mmol.dm^{-3}). The solution contained 1.3 mmol.dm^{-3} O_2, 0.5 mol.dm^{-3} sodium formate and 30 mmol.dm^{-3} phosphate buffer (pH 8.2).

between O_2^- and MTDQ-DA. From the growth of absorbance at 430 nm a first order rate constant of k (O_2^- + MTDQ-DA) = 4.10^8 dm^3.mol^{-1}.s^{-1} was estimated.

Pulse radiolysis of a dilute aqueous solution of CH 402 (2 mmol.dm^{-3}) using, otherwise, the same experimental conditions as with MTDQ-DA, gives rise to an initial absorption spectrum, which strongly resembles that of MTDQ-DA, except that a minor shoulder grows in between 460 and 510 nm. The evolution of the spectrum is shown in Figure 3. The rate constant of the formation of the transient, as calculated from the exponential growth of absorbance at 430 nm, was k (O_2^- + CH 402) = $1.5.10^7$ dm^{-3}.mol^{-1}.s^{-1}, a value more than an order of magnitude smaller than that obtained with MTDQ-DA. Remarkable differences are observed when the longtime history of the transients formed from the two compounds are compared. In the case of CH 402, a stable product of the reaction sequence absorbing at 370 nm is noticed, while at longer wavelengths significant amount of bleaching, not seen when MTDQ-DA reacted with O_2^-, dominates the spectrum. The bleaching disappeared with a half-time of 30 ms.

The results show that both MTDQ-DA and CH 402 react with superoxide radical

TABLE I
Scavenger effect on H_2O_2 (4×10^{-7} M) by CH 402 and MTDQ-DA: variation of chemiluminescence intensity (mVs) with concentration of antioxidants

Concentrations	Antioxidants	
(M)	CH 402	MTDQ-DA
0	57.511	57.511
8.7×10^{-7}	53.468	55.708
8.7×10^{-6}	30.480	37.609
8.7×10^{-5}	28.587	7.279
8.7×10^{-4}	0.001	0.530

anion at reasonably high rates, the former compound exhibiting the greater reactivity. The initial spectra formed from the two substrates are apparently the same and are, therefore, attributed to the same kind of transient radical ions. However, the mechanism of the process at later stages might be complex, and different for the two compounds, as inferred from the time evolution of the absorption spectra.

Chemiluminescence is a property of excited states of luminol formed in a medium containing free radicals, O_2^-, $^{\cdot}OH$ or H_2O_2. The background of the method as its application to detect free radicals in a wide range of systems has been well documented.[20] We studied the scavenging effect of CH 402 and MTDQ-DA, at increasing concentrations. Hydrogen peroxide is able to induce free radical reactions with organic compounds, presumably via $^{\cdot}OH$ radicals. The data in Table I, show a progressive decrease of luminescence when increasing amounts of CH 402 or MTDQ-DA were added, the effect being attributed to the scavenger activity of the compounds. In these experiments the antioxidants had been incubated with the luminol + hemin reagent solution, separated from H_2O_2, before detection of luminometric intensity, in order to prevent them from direct interaction.

Table II shows the scavenging effect of CH 402 and MTDQ-DA on H_2O_2 generation in the glucose–glucose oxidase system in synchrone and in separate parts. The incubation time of enzyme reaction as well as the time of luminescence detection was 60 sec in these experiments.

It has been known that the redox transformation of iron promotes lipid peroxidation. Reducing enzymes such as NADPH cytochrome P-450 reductase can reduce iron complexes. During NADPH oxidation liver microsomes produce significant amounts

TABLE II
Scavenger effect on H_2O_2 by CH 402 and MTDQ-DA in the glucose–glucose oxidase system: variation of chemiluminescence intensity (mVs) with concentration of antioxidants

(in separate part for 60 sec)			(in synchrone part for 60 sec)		
Concentrations	Antioxidants		Concentrations	Antioxidants	
(M)*	CH 402	MTDQ-DA	(M)**	CH 402	MTDQ-DA
0	47.693	47.693	0	47.693	47.693
8.3×10^{-7}	28.802	31.197	5×10^{-6}	33.165	30.768
8.3×10^{-6}	12.755	25.974	5×10^{-5}	27.222	28.420
8.3×10^{-5}	8.545	3.925	5×10^{-4}	22.403	22.420
8.3×10^{-4}	0.672	0.513	5×10^{-3}	0.615	0.273

* concentrations of antioxidants in the total volume
** concentrations of antioxidants in the upper part of cuvette

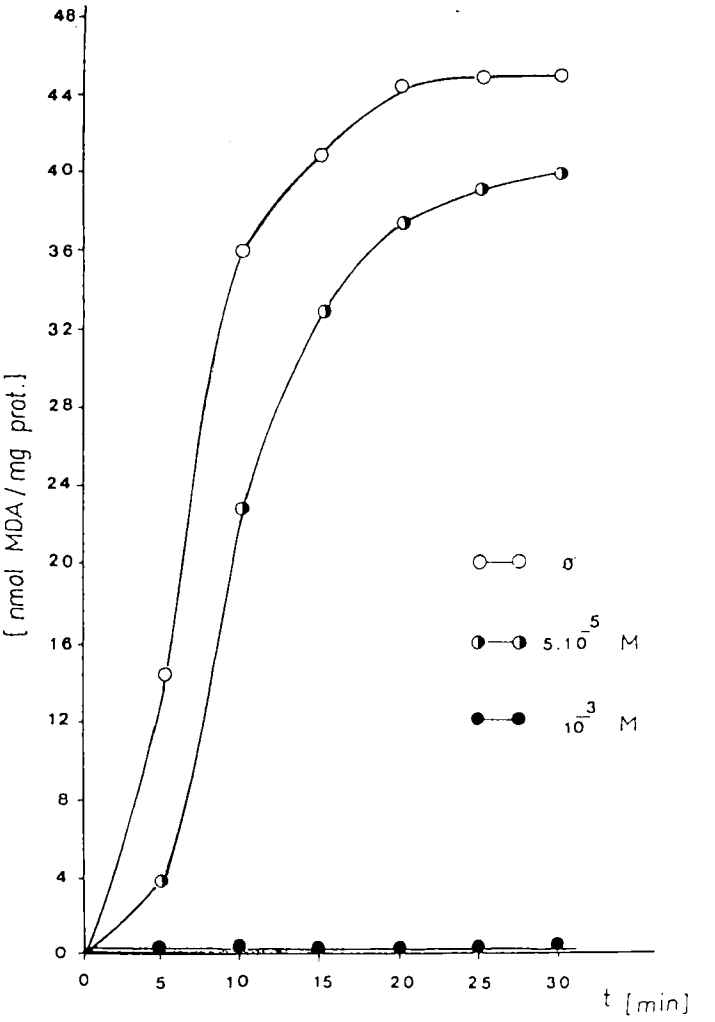

FIGURE 4 Effect of CH 402 on NADPH induced, Fe^{3+} stimulated lipid peroxidation of rat liver microsomal fraction.

of ·OH radicals, which are known to be more active in inducing lipid peroxidation than superoxide radical ions. The NADPH + Fe^{3+}-induced lipid peroxidation was studied *in vitro* using liver microsomal fractions. It was shown previously that none of the antioxidants affected the activity of enzymes, NADPH cytochrome P-450 reductase and polysubstrate monooxygenase system, *in vitro* or *in vivo*, to a significant extent.[23] The CH 402 and MTDQ-DA reduced the induced lipid peroxidation in both cases depending on time and concentration. The peroxidation was expressed in this case as the amount of generated MDA (Figures 4 and 5).

CH 402[9] and MTDQ-DA completely inhibited the ascorbic acid induced lipid peroxidation, as shown in Figure 6.

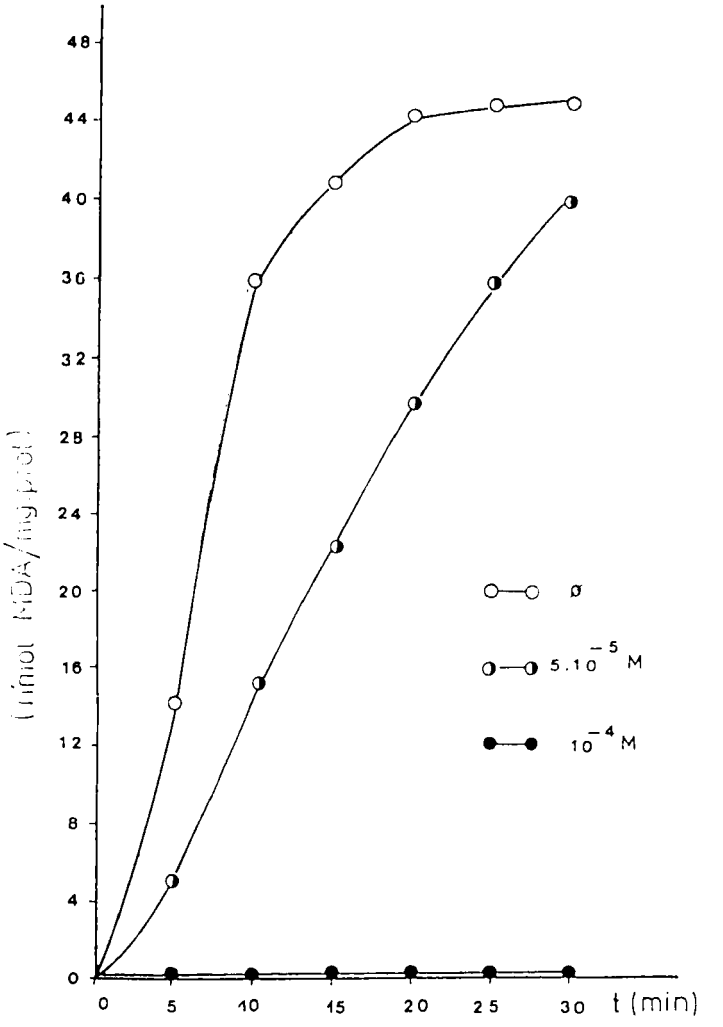

FIGURE 5 Effect of MTDQ-DA on NADPH induced, Fe^{3+} stimulated lipid peroxidation on rat liver microsomal fraction.

DISCUSSION

Drug entrance into the membranes, stability of membranes, the potential for lipid peroxidation, and the activity of phospholipid-dependent enzymes in the membranes vary with the quality and quantity of dietary fat. The membrane associated changes can occur rapidly because of drug-nutrient interactions, especially in nutrient deficiency states.[4,25,26] By decreasing the capacity of natural antioxidants in tissues the yields of free radical chain reactions are rapidly increased and can be observed from the damage they cause.[27–29] The synthetic antioxidants may play an important role in

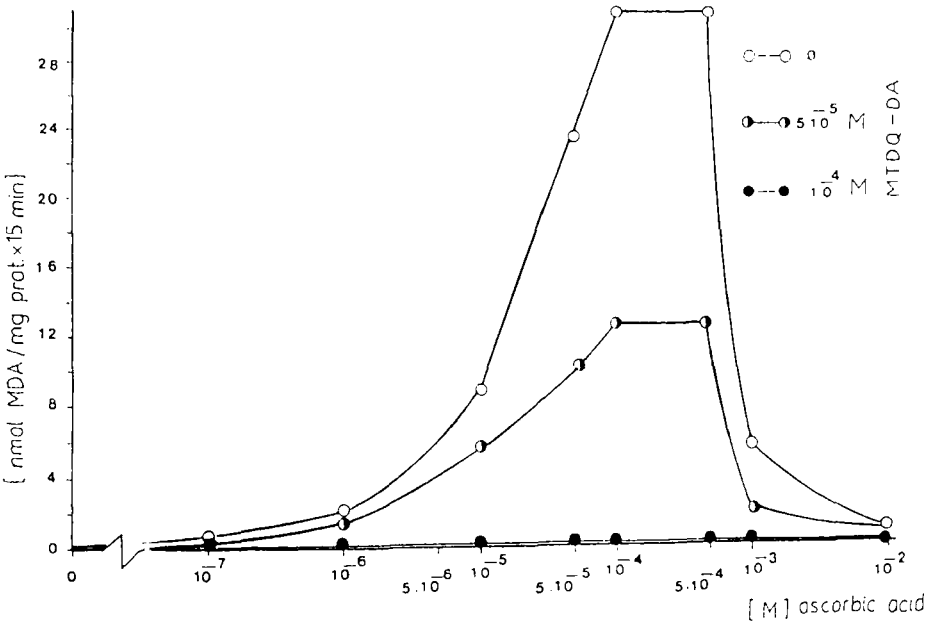

FIGURE 6 Dose dependent inhibitory effect of MTDQ-DA on liver microsomal fraction of mouse.

the protective mechanism both in the therapy and in the conservation of food quality.[1,30,31] The dihydroquinoline type derivatives, CH 402 and MTDQ-DA showed membrane protecting actions in experimental atherosclerosis and in several liver and brain experiments *in vitro*.[1,8-12]

A variety of methods were developed to follow free radicals reactions related to hydrogen peroxide, superoxide anion and hydroxyl free radicals. In this investigation we used enzymatic and non-enzymatic sources of radicals.[13,16,17,20]

The reactivity of MTDQ-DA and CH 402 in capturing superoxide ions and ·OH radicals has been confirmed in this study using a physical/pulse radiolysis/and chemical/H_2O_2/ source of radicals. The results obtained with an enzymatic, glucose–glucose oxidase source can be explained with the same kind of scavenging activity of the compounds. The MTDQ-DA and CH 402 might, however, blockade some sites of the glucose oxidase, among them those responsible for production of H_2O_2. The luminometric data summarized in Tables I and II argue against this as they indicate that the enzymes preserved their activity in producing free radicals, whether the antioxidants had been present during the incubation period or they were admixed to the enzyme together with luminol.

We confirmed that the two substances, CH 402 and MTDQ-DA inhibit both non-enzymatic, ascorbic acid and enzymatic, NADPH induced and iron stimulated lipid peroxidation.

Binding of the antioxidants can greatly influence the mechanism of free radical induced reactions in biological systems, which should be discovered in further experiments. Work is in progress in this field.

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References

1. Fehér, J., Csomós, G. and Vereckei, A., Free Radical Reactions in Medicine, Springer-Verlag, Berlin-Heidelberg, (1987).
2. Pryor, W.A., *Ann. N.Y. Acad. Sci.*, **293**, 1, (1982)
3. Demopoulos, H.B., Flamm, E.S., Pietronigro, D.D. and Seligman, M., *Acta Phys. Scand., Suppl.*, **492**, 91, (1980).
4. Hendrick, S., Eddy, D.E. and Siebold, J., *J. Amer. Geriat. Soc.* **24**, 301, (1976).
5. Dworchák, E. and Prohászka, L., *Z. Ernahrungswissenschaft*, **25**, 96, (1986).
6. USA Patent 4363910.
7. USA Patent 4356306.
8. Blázovics, A., Ambrus, D., Mathiasz, D., Vereckei, A. and Fehér, *J., Exp. Med. (Hung.)*, **38**, 18, (1986).
9. Blázovics, A., Somogyi, A., Lengyel, G., Láng, I. and Fehér, *Free Rad. Res. Comms.*, **4**, 409, (1988).
10. Fehér, J., Bar-Pollák, Zs., Sréter, L., Fehér, E. and Toncsev, H., *Brit. J. Exp. Path.*, **63**, 394, (1984).
11. Fehér, J., Sulyok, S., Pollák, Zs., Toncsev, H., Cornides A., Blázovics, A., Szondy, É. and Geró, S., Atherosclerosis and Cardiovascular Diseases, S. Lenzi and G.C. Descovich eds. Editrice Compositori, Bologna, (1984).
12. Toncsev, H., Pollák, Zs., Kiss, A. and Fehér, J., *Int. J. Tiss. Reac.*, **4**, 325, (1982).
13. Heide, L., Bögl, W., Food. Lab. *Newsletter*, **5**, 21, (1986).
14. Jordan, R.A. and Schenkman, J.B., *Biochem. Pharmacol.*, **31**, 7393, (1982).
15. Player, P.J. and Horton, A.A., *J. Neurochem.*, **37**, 422, (1981).
16. György, I. and Földiák, G., *J. Radioanal. Nucl. Chem.*, **122**, 207, (1988).
17. Baxendale, J.H. and Busi, F., The Study of Fast Processes and Transient Species by Electron Pulse Radiolysis, Reidel, Dordrecht, (1982).
18. Chuaqui, C.A. and Petkau, A., *Radiat. Phys. Chem.*, **30**, 365, (1987).
19. Cadenas, E., Boveris, A. and Chance, B., *Biochem. J.*, **187**, 131, (1980).
20. van Dyke, K., Bioluminescence and Chemiluminescence Instruments and Applications, II., CRC Press, 1985.
21. Svingen, B.A., Buege, J.A., O'Neal, F.O. and Aust, S.D., *J. Biol. Chem.*, **254**, 5892, (1979).
22. Ottolenghi, A., *Arch. Biochem. Biophys.*, **79**, 355, (1959).
23. Blázovics, A., Pénzes, L. and Fehér, J., II. Modulation of Liver Cell Expression, Workshop, Falk Symposium **43**, (1986).
24. Lowry, A.H., Ronsenbrough, N.J., Farr, A.L. and Randall, R.J., *J. Biol. Chem.*, **193**, 265, (1951).
25. Wade, A.E., Norred, W.P. & J.S., Evans, Nutrition and Drug Interrelations, *Acad. Press., N.Y.* (1978).
26. Harmann, D., *Amer. J. Clin. Nutr.*, **25**, 839 (1972).
27. Warts, E.D. and M.N., Gould, *Carcinogenesis*, **7**, 1197, (1986).
28. Miyazawa, T., Ando, T. and Kadena, T., *Agric. Biol. Chem.*, **50**, 71, (1986).
29. Del Maestro, R.F., Thaw, H.H., Björk, J., Planker, M. and Arfors, K.E., *Acta Physiol. Scand., Suppl.*, **492**, 91, (1980).
30. Harmann, D., *J. Amer. Cell. Nutr.*, **1**, 27, (1982).
31. Ueda, S., Hayashi, T. and Namiki, M., *Agric. Biol. Chem.*, **50**, 1, (1986).

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