Free Rad. Res., Vol. 32, pp. 255–264
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Antioxidative Property of T-0970, A New Ureidophenol Derivative

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Accepted by Prof. A. Bast

(Received 20 April 1999; In revised form 8 July 1999)

We investigated the antioxidative property of T-0970, a newly synthesized ureidophenol derivative. The inhibitory effect of T-0970 on spontaneous lipid peroxidation in rat brain was 10 times greater than those of well-known antioxidants such as butylhydroxytoluene (BHT), probucol and α-tocopherol. T-0970 also showed dose-dependent free radical scavenging activities *in vitro* for both superoxide anions and hydroxyl radicals. The radical-scavenging potencies of T-0970 were about 10–30 times stronger than those of BHT. We evaluated the *in vivo* antioxidative ability of T-0970 in the animal model of acute oxidative tissue injury in rats. Intraperitoneal injection of ferric nitrilotriacetate (Fe/NTA) caused an acute and remarkable increase in the level of thiobarbituric acid-reactive substances (TBARS) in both plasma and the liver, and also resulted in a considerable elevation of the plasma levels of GOT and GPT indicative of hepatic injury. Both oral and intravenous administration of T-0970 dose-dependently depressed these diagnostic parameters. These results indicate that T-0970 may have a therapeutic potential in various diseases associated with oxidative tissue injury.

Keywords: Radical scavenger, superoxide anion, hydroxyl radical, lipid peroxidation, ferric nitrilotriacetate, antioxidants

INTRODUCTION

Reactive oxygen species such as superoxide anions and hydroxyl radicals have been known to play an important role in the development of various diseases such as ischemia–reperfusion injury, inflammation and atherosclerosis.[1,2]

Since the discovery of superoxide dismutase (SOD),[3] several attempts of the therapeutic use of SOD have been performed against some diseases involving oxidative tissue injury. However, the therapeutic potential of SOD was limited because of its antigenecity,[4] rapid clearance from the circulation[4,5] and difficulty in passing through the cell membranes.[6] Furthermore, SOD cannot be orally administered because it is a high-molecular weight enzyme. Although many kinds of chemically modified SOD have been designed to resolve the inconvenience of native SOD,[7] there are few reports of successful clinical therapies using them.

On the other hand, a number of studies have indicated that synthetic and natural antioxidants

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may protect cells and tissues from oxidative injury.[8–10] A well-known natural antioxidant, α-tocopherol, showed protective effects against oxidative tissue injury by long-term oral administration.[8,10] A synthetic antioxidant, probucol, has been reported to protect low-density lipoprotein (LDL) from oxidative modification and reduce the development of atherosclerosis by mechanisms other than its hypolipidemic action in animal models.[10–12]

Therefore, chemical compounds with a strong antioxidative property may have a good therapeutic potential against diseases associated with oxidative tissue injury.

We planned to synthesize water-soluble and potent antioxidants which could be applied both orally and intravenously. Both α-tocopherol and uric acid are well-known natural antioxidants, but their antioxidative mechanisms have been reported to be different from each other.[13,14] We designed several compounds by combining para-alkoxyphenol structure of α-tocopherol and urea structure of uric acid[15] (Figure 1). The antioxidative property of the compound varies significantly with substituent/structural modifications. Many kinds of ureidophenol derivatives were synthesized and their antioxidative profiles were evaluated. Part of it was presented in the reports by Nakao *et al.*[15] T-0970 was finally selected as a water-soluble strongest antioxidant (Figure 1).

In order to evaluate the antioxidative potency of T-0970 *in vivo*, we employed the ferric iron/nitrilotriacetate (Fe/NTA)-induced tissue injury model in rats.[16] Fe/NTA is known to generate reactive oxygen species in the body following administration, and causes lipid peroxidation and DNA damage in the liver and kidney.[17,18] Furthermore, it is well documented that Fe/NTA causes severe hepatic and renal carcinomas after long-term administration, and that the reactive oxygen radicals contribute to the generation of carcinomas. We estimated the *in vivo* antioxidative activity of T-0970 by evaluating its protective effect on the acute hepatic injury induced by Fe/NTA after either oral or intravenous administration in rats.

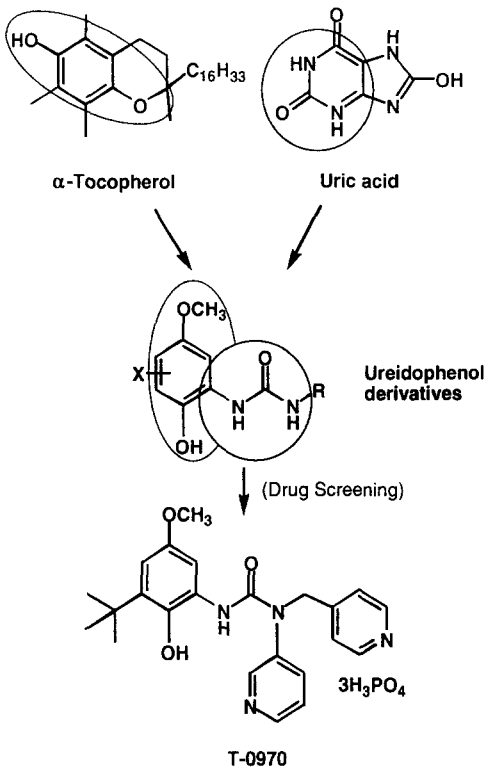


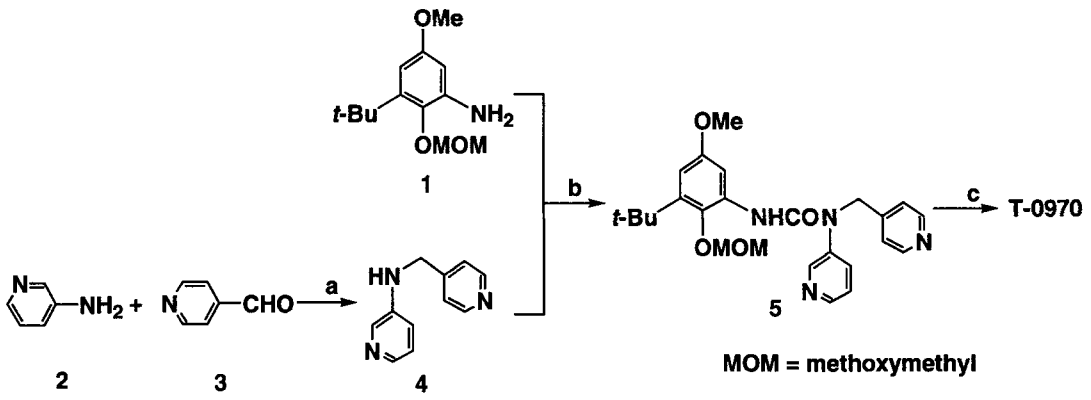
FIGURE 1 Design of T-0970, 1-(3-tert-butyl-2-hydroxy-5-methoxyphenyl)-3-(3-pyridyl)-3-(4-pyridylmethyl) urea triphosphate.

In this paper, we report and discuss the *in vitro* and *in vivo* antioxidative properties of this novel ureidophenol compound T-0970.

MATERIALS AND METHODS

Chemicals

Probucol was purchased from Daiichi Seiyaku Co. Ltd. (Tokyo, Japan). SOD prepared from bovine kidney was purchased from Calzyme Laboratories (California, USA). α-Tocopherol, butylhydroxytoluene (BHT), dimethylthiourea (DMTU), ferric nitrate, disodium nitrilotriacetate were purchased from Nacalai Tesque (Kyoto, Japan). All other reagents used were of the highest grade commercially available.



SCHEME 1 (a) (1) *p*-TsOH, toluene, (2) H_2, Pd-C, MeOH; (b) triphosgen, Et_3N, CH_2Cl_2; (c) (1) conc. HCl, MeOH, (2) K_2CO_3, (3) H_3PO_4.

Synthesis of T-0970

T-0970 was synthesized by the synthetic route shown in Scheme 1. 3-tert-Butyl-5-methoxy-2-methoxymethoxyaniline (**1**) was synthesized according to our previous report.[15]

N-(4-Pyridylmethyl)-3-aminopyridine (4)

A mixture of 3-aminopyridine **2** (117.7 g, 1.25 mol), isonicotinic acid **3** (140.6 g, 1.31 mol), *p*-toluenesulfonic acid (1.5 g) and toluene (600 mL) was heated under reflux for 3 h with a Dean-Stark apparatus. After removal of the solvent, the residue was dissolved in MeOH (1200 mL), and the mixture was hydrogenolyzed in the presence of 10% palladium on carbon (6.0 g) using Parr apparatus (H_2, 3.5 atm) for 3 h at room temperature (r.t.). After the catalyst was filtered off, the filtrate was concentrated *in vacuo*. The residue was purified on silica gel chromatography using $CHCl_3$/MeOH (15 : 1) as an eluent to afford **4** (116.0 g, 50%) as a brown oil.

1-(3-tert-Butyl-5-methoxy-2-methoxymethoxy)-3-(3-pyridyl)-3-(4-pyridylmethyl)urea (5)

To a solution of triphosgen (18.99 g, 64 mmol) in CH_2Cl_2 (1000 mL) was added dropwise a solution of **1** (35.34 g, 148 mmol) and triethylamine (62 mL, 443 mmol) in CH_2Cl_2 (300 mL) at −78°C, and the reaction mixture was warmed up to r.t. for 30 min. After removal of the solvent, the residue was dissolved in CH_2Cl_2 (600 mL) and the solution of **4** (27.35 g, 148 mmol) and triethylamine (31 mL, 222 mmol) in CH_2Cl_2 (250 mL) was added dropwise at r.t. After stirring for 1 h at r.t. and for 5 h under reflux, the reaction mixture was washed with H_2O, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified on silica gel chromatography using $CHCl_3$/MeOH (20 : 1) as an eluent to afford **5** (40.10 g, 60%) as a brown viscous oil.

1-(3-tert-Butyl-2-hydroxy-5-methoxyphenyl)-3-(3-pyridyl)-3-(4-pyridylmethyl)urea triphosphate (T-0970)

To a solution of **5** (76.39 g, 170 mmol) in MeOH (750 mL) was added conc. HCl (80 mL) and the mixture was stirred for 2 h at r.t. After removal of the solvent, the residue was dissolved in H_2O (1000 mL), and the solution was neutralized with K_2CO_3. The mixture was extracted with AcOEt, the organic layer was washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. The crude crystals were recrystallized from AcOEt to afford T-0970 free base (46.41 g, 67%) as colorless needles: m.p. 152–153°C. The free base (97.80 g, 241 mmol) was dissolved in EtOH (1000 mL) with heating, and the solution of phosphoric acid (71.92 g, 734 mmol) in EtOH (450 mL) was added.

After cooling, the resulting solid was collected by filtration and recrystallized from acetone/H_2O (19 : 10) to afford T-0970 (101.60 g, 60%) as colorless needles: m.p. 196–198°C; ^{1}H-NMR (DMSO-d_6) δ 1.31 (9H, s), 3.65 (3H, s), 5.02 (2H, s), 6.53 (1H, d, J = 3 Hz), 6.89 (1H, d, J = 3 Hz), 7.37 (2H, d, J = 6 Hz), 7.46 (1H, dd, J = 5 and 8 Hz), 7.80–7.95 (2H, m), 8.45–8.90 (5H, m); IR (KBr) 3400, 1658, 1643, 1600 cm^{-1}; SIMS m/z 407 (base). Anal. Calcd. for $C_{23}H_{26}N_4O_3 \cdot 3H_3PO_4$: C, 39.44; H, 5.04; N, 8.00. Found: C, 39.55; H, 5.16; N, 7.88.

Animals

Adult male Sprague Dawley rats (250–300 g) were purchased from Charles River Japan, Ltd. (Kanagawa, Japan). All animals were allowed free access to standard laboratory chow and water, and housed in a room controlled at 24°C with a 12 h light/dark cycle. Rats were sacrificed under ethylether-anesthesia.

Inhibitory Effect on Lipid Peroxidation

The whole brain was homogenized in 4 volumes of cold phosphate-buffered saline (PBS) and centrifuged at 4°C for 20 min (10 000 × g). The supernatant was stored at −80°C until use. Prior to the assay, the stored supernatants of the homogenates were further diluted with 4 volumes of PBS, and incubated at 37°C for 15 h in the presence or absence of test compound (0, 0.1–10 μM) under air. The extent of lipid peroxidation was assessed by measuring the thiobarbituric acid-reactive substances (TBARS).[19,20] The amount of TBARS was calculated as nanomole equivalents of malondialdehyde (MDA) as a standard compound. The inhibition percentage was calculated by comparison with the fully-oxidized and non-oxidized controls. Concentration–inhibition relationships were fitted to a sigmoidal model of the form log-concentrations versus inhibitory activity, and IC50 values were determined. The direct interference between MDA and the test compounds was checked by adding test compounds to the MDA solution in the TBA reaction and comparing the values of TBARS with that of corresponding MDA standard. These compounds did not show any interference in the TBA reaction.

Superoxide Anion-Scavenging Activity

The superoxide anion-scavenging activities of test compounds were determined by the NADH/phenazine methosulfate (PMS)/nitroblue tetrazolium (NBT) system.[21,22] Briefly, generation of superoxide anions was measured in a reaction mixture containing 160 μM NADH, 40 μM NBT and 8 μM PMS in PBS at pH 7.4. The reduction of NBT was followed by measuring the change in absorbance at the wavelength of 560 nm for 2 min, a period in which the absorbance increased linearly. Test compounds were prepared in dimethylsulfoxide (DMSO) and added to the reaction mixture to give a final concentration of 0.7% DMSO which showed almost no effect in this system. These compounds showed no direct effects on NBT. In our preliminary experiment, SOD (100–10 000 U/mL) dose-dependently depressed the increasing rate of absorbance in this system, suggesting that the increase in absorbance mainly reflects the amounts of superoxide anion generation. However, there is a possibility that part of its reaction proceeded independent from superoxide anion generation because high amount of SOD (10 000 U/mL) did not fully suppress the reaction in this system.

Hydroxyl Radical-Scavenging Activity

The hydroxyl radical-scavenging activities of test compounds were determined by the method reported by Russell *et al.*[23]

Briefly, a solution of methyl orange containing suspended zinc oxide was prepared in 5 mM sodium borate buffer (pH 9.2) to give a final concentration of 40 μM and equivalent to 6 mM respectively. Each test compound was prepared in dimethylformamide (DMF) and added to the above solution to give a final concentration of

0.1% which showed almost no effect in this system. The mixture was placed 20 cm away from a 100-W light source for 2 h. Blank mixtures were also prepared by the same procedure and stored in the dark for 2 h. All the vessels were centrifuged at 1500× *g* for 5 min to remove suspended zinc oxide. The decrease in absorbance of each sample at the wavelength of 465 nm was recorded in comparison with the blank which was stored in the dark. The maximal amount of hydroxyl radicals generated in this system was determined by the decrease in absorbance of the control sample which contained only 0.1% DMF. As the decrease in absorbance of methyl orange reflects the amount of hydroxyl radicals not scavenged by test compound, the hydroxyl radical-scavenging activity of a test compound was determined in comparison with the control sample.

Inhibitory Effect on the Fe/NTA-Induced Oxidative Injury Model

We slightly modified the acute Fe/NTA-induced toxicity model reported by Shiomi *et al.*[16] Briefly, rats were fasted overnight before the experiment and were intraperitoneally administered an Fe/NTA solution in a dose equivalent to 10 mg/kg of ferric iron. In the case of oral administration, test compound was administered 30 min prior to Fe/NTA-treatment. For intravenous administration, the infusion of test compound was started 30 min prior to Fe/NTA-treatment and continued at the dose of 50–200 μg/kg/min for 60 min under urethane-anesthesia. Three hours after Fe/NTA-treatment, the animals were deeply anesthetized with ethylether and heparinized blood and the liver were collected. The blood was immediately centrifuged at 4°C for 15 min (2000× *g*) to obtain plasma. The levels of TBARS in plasma were determined and the values were expressed as nanomole equivalents of MDA.[19,20] The liver was perfused with cold saline immediately after excision, homogenized with 4 volumes of cold PBS and centrifuged at 4°C for 20 min (10 000× *g*). The level of TBARS of the supernatant was determined. The levels of GOT and GPT in plasma were determined by using an automatic biochemical analyzer, Model 705 (Hitachi Co. Ltd., Tokyo, Japan).

Statistics

All data were expressed as the means ± SE of 4–6 experiments. Statistical comparison among the groups was made by analysis of variance (ANOVA) followed by the Tukey–Krammer's test. The probability below 5% was considered statistically significant.

RESULTS

Inhibitory Effect of T-0970 on Lipid Peroxidation

In order to evaluate the effect of T-0970 on the oxidation of tissue lipids, we investigated the inhibitory effect on spontaneous lipid peroxidation in brain homogenates. The extent of lipid peroxidation was assessed by measuring TBARS after incubation at 37°C for 15 h under air. Table I shows the IC50 values for T-0970, BHT, α-tocopherol and probucol to inhibit lipid peroxidation. T-0970 showed a potent inhibitory effect with its IC50 10 times lower than those of the well-known standard antioxidants BHT, α-tocopherol and probucol.

TABLE I Inhibitory effects of T-0970 and reference antioxidants on lipid peroxidation in brain homogenates

Compounds	IC50: μM
T-0970	0.30 ± 0.05
BHT	3.80 ± 0.25*
α-Tocopherol	3.81 ± 0.17*
Probucol	4.06 ± 0.27*

Rat brain homogenates were incubated at 37°C for 15 h in the presence or absence of various concentrations of test compound (0, 0.1–10 μM). The extent of lipid peroxidation was assessed by measuring the TBARS as described in the text. The IC50 values were determined as described in the text ($n = 4$). *Significantly different from T-0970.

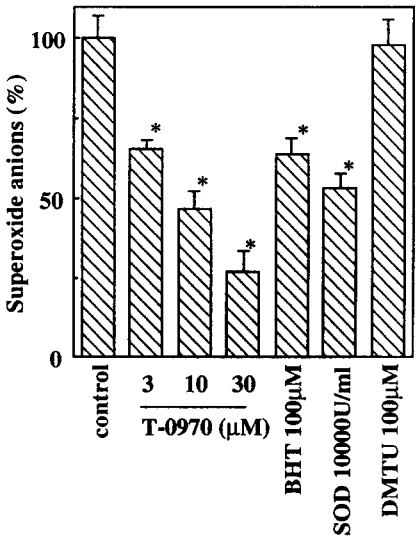


FIGURE 2 Scavenging effect of T-0970 on superoxide anions *in vitro*. The activities of T-0970, BHT, SOD and DMTU to scavenge superoxide anions were determined as described in the text. Data are expressed as the means ± SE ($n = 4$). *Significantly different from control. $p < 0.05$.

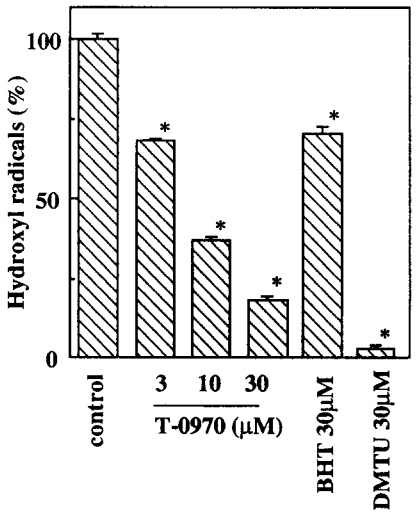


FIGURE 3 Scavenging effect of T-0970 on hydroxyl radicals *in vitro*. The activities of T-0970, BHT and DMTU to scavenge hydroxyl radicals were determined as described in the text. Data are expressed as the means ± SE ($n = 4$). *Significantly different from control. $p < 0.05$.

Superoxide Anion-Scavenging Effect of T-0970 *In Vitro*

The superoxide anion-scavenging activities of the test compounds were determined by the NADH/PMS/NBT system. Figure 2 shows the scavenging activities of T-0970, BHT, SOD and DMTU. T-0970 (3–30 μM) showed a dose-dependent superoxide anion-scavenging activity. This scavenging potency of T-0970 was about 30 times stronger than that of BHT. SOD at 10 000 U/mL also showed a significant potency in this system. DMTU is known as a hydroxyl radical scavenger, and it did not show any superoxide anion-scavenging effect even at the concentration of 100 μM in this system.

Hydroxyl Radical-Scavenging Effect of T-0970 *In Vitro*

Figure 3 shows the hydroxyl radical-scavenging activities of T-0970, BHT and DMTU as determined by the methyl orange–zinc oxide system. T-0970 (3–30 μM) showed a dose-dependent hydroxyl radical-scavenging activity. This scavenging potency of T-0970 was about 10 times stronger than that of BHT. DMTU showed an almost complete scavenging effect at the concentration of 30 μM.

Protective Effect of T-0970 on Fe-NTA-Induced Injury *In Vivo*

As shown in Figure 4, intraperitoneal injection of Fe/NTA markedly elevated the levels of GOT, GPT and TBARS in plasma. Oral administration of T-0970 (3–10 mg/kg) dose-dependently depressed the elevation of these parameters. In this experiment T-0970 also depressed the elevated hepatic level of TBARS in a dose dependent manner as shown in Figure 5.

Figure 6 shows the effect of intravenous administration of T-0970 in this model. T-0970 (50–200 μg/kg/min, 60 min) also showed dose-dependent lowerings of the levels of these parameters.

DISCUSSION

We evaluated the antioxidative property of T-0970 both *in vitro* and *in vivo*. Our results

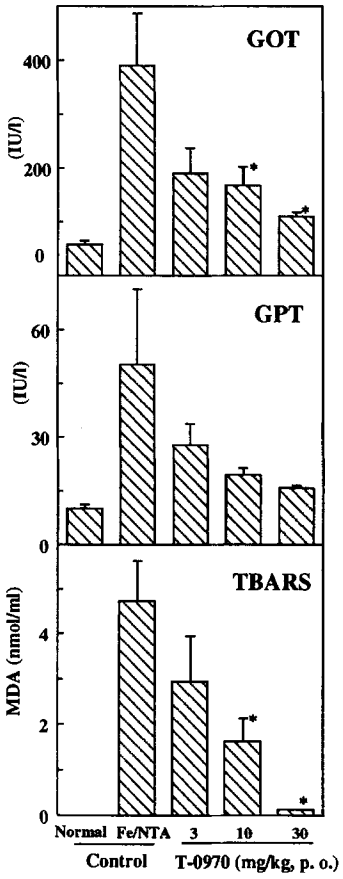


FIGURE 4 *In vivo* effect of orally administered T-0970 on the plasma biochemical parameters of Fe/NTA-treated rats. Effects of orally administered T-0970 on the elevated levels of plasma GOT, GPT and TBARS of Fe/NTA-treated rats were determined as described in the text. Data are expressed as the means ± SE ($n = 6$). *Significantly different from control. $p < 0.05$.

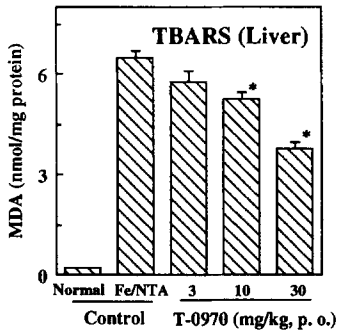


FIGURE 5 *In vivo* effect of orally administered T-0970 on the hepatic level of TBARS of Fe/NTA-treated rats. Effects of orally administered T-0970 on the elevated hepatic level of TBARS in Fe/NTA-treated rats were determined as described in the text. Data are expressed as the means ± SE ($n = 6$). *Significantly different from control. $p < 0.05$.

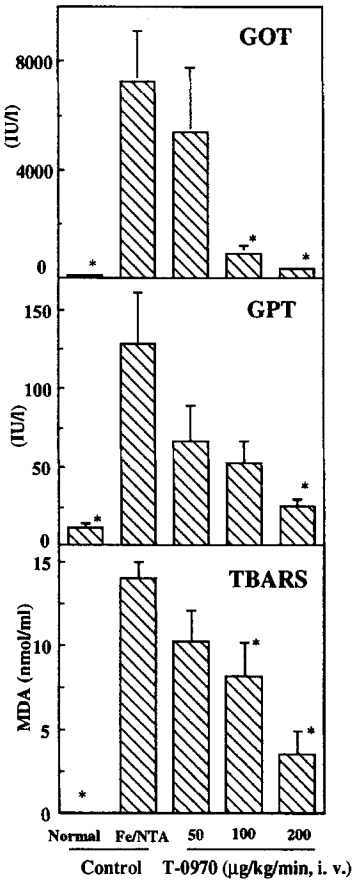


FIGURE 6 *In vivo* effect of intravenously infused T-0970 on plasma parameters of Fe/NTA-treated rats. Effects of intravenously infused T-0970 on Fe/NTA-treated rats were evaluated. T-0970 (50–200 μg/kg/min) was infused via the tail vein from 30 min prior to Fe/NTA-treatment for 60 min under urethane-anesthesia. The levels of plasma GOT, GPT and TBARS of Fe/NTA-treated rats were determined as described in the text. Data are expressed as the means ± SE ($n = 6$). *Significantly different from control. $p < 0.05$.

clearly indicate that T-0970 has a potent radical-scavenging ability both *in vitro* and *in vivo*.

The synthesis of T-0970 was designed in our laboratory by the idea of combining para-alkoxyphenol structure of α-tocopherol and urea structure of uric acid[15] (Figure 1). Phenolic antioxidants such as α-tocopherol terminate a radical chain reaction by donating a hydrogen atom from their phenolic hydroxyl group to the peroxidized lipid radical and they turn themselves into phenoxy radicals.[13] Uric acid reacts with a free radical to form a urate radical which is stabilized by delocalization of the unpaired

electron in the π electron system of the urea moiety.[14] The potency of these antioxidants is determined by the ease of releasing a hydrogen atom and the stability of the generated intermediate radical. The stability of intermediate radical of T-0970 may be enhanced by the electron-donating substituents (CH_3O–) on the benzene ring, and bulky substituents ($(CH_3)_3C$–) at the ortho-positions of the phenolic hydroxyl group. Urea moiety may also contribute to the enhancement of the activity. Quantitative structure–activity analysis of the antioxidative property of ureidophenol derivatives has been reported by Nakao *et al.*[15] Although the report did not mention the antioxidative activity of T-0970, they discussed that these type of modification in the substituents of ureidophenol structure enhances the antioxidative potency. T-0970 was selected as a strongest water-soluble antioxidant in our series of ureidophenol derivatives. As shown in Table I, T-0970 showed strongest activity and actually has 10 times stronger antioxidative activity than that of α-tocopherol.

There are many long-term treatment studies in disease models involving oxidative tissue injury.[9,10] However, there are few reports of simple and rapid *in vivo* screening systems to evaluate the potency of antioxidants. We used the Fe/NTA-induced acute toxicity model reported by Shiomi *et al.*[16] with a slight modification. In this model, both GOT and GPT levels in plasma and the hepatic level of TBARS are acutely elevated as a result of the oxidative hepatic injury. Fe/NTA is known to cause oxidative tissue injury by generating reactive oxygen species such as hydroxyl radicals *in vivo*.[16] As T-0970 effectively scavenged hydroxyl radicals *in vitro*, it was expected to show a protective effect on the tissue injury *in vivo*. The finding that both intravenous and oral administration of T-0970 effectively depressed the elevation of GOT and GPT in plasma, suggested that T-0970 could cross the cell membranes, and exert protective effect on the hepatic injury by its antioxidative ability.

The lowering effects on the levels of GOT and TBARS in plasma were statistically significant at doses higher than 10 mg/kg (p.o.) and 100 μg/kg/min (i.v.), respectively. From our unpublished data, the plasma level of T-0970 30 min after oral administration of 30 mg/kg was found to be above 5 μM and its total bioavailability was 40% as compared with intravenous injection. The plasma levels of T-0970 during intravenous infusion of 100 and 200 μg/kg/min were also found to be 5 and 10 μM, respectively. Therefore, the plasma concentrations of T-0970 in our experiments seem to be of μmolar levels. These levels are sufficient to demonstrate the antioxidative effect *in vitro*. T-0970 did not show any other typical pharmacological effects on the general behavior, hemodynamics and plasma biochemical parameters such as GOT, GPT after oral administration of 30 mg/kg in this experiment. Further, 2-week oral administration of T-0970 (100 mg/kg/day) caused almost no typical histopathological changes in organs (such as liver, kidney, thymus, testis, epididymis, prostate, lungs, salivary glands, esophagus, pancreas and brain.) (unpublished data). From these considerations, we concluded that T-0970 protected tissues from oxidative damage owing to its potent antioxidative property with no toxicological effects in this experiment.

There are many reports on diseases and tissue injuries which are closely related to free radical generation.[1,2,8] For example, ischemia–reperfusion injury, inflammation, atherosclerosis, photosensitization, radiation damage, some types of cancer and toxic organ injuries are linked with free-radical chain reactions. Furthermore, free radicals are believed to be causally related to aging.[24,25]

We have previously reported that the lipid-soluble antioxidant T-2591, one of the present series of ureidophenol derivatives,[26] effectively suppressed the foam cell formation in cultured macrophages. In an experiment with the high cholesterol-fed hamster model, long-term oral administration of T-2591 effectively reduced the oxidizability of LDL and inhibited the lipid accumulation in the aorta. T-2591 is currently under development as a drug for atherosclerosis.

The lipophilic antioxidants such as probucol or α-tocopherol can be used by intravenous injection. However these compounds do not easily solve in water. T-0970 was designed as a water-soluble potent antioxidant to overcome the above inconveniency.

The present data showed that T-0970 chemically scavenged the reactive oxygen species and inhibited the lipid peroxidation of tissues *in vitro*. Furthermore, we presented evidence that T-0970 showed *in vivo* antioxidative effects by both oral and intravenous administration in the rat model. Therefore, we consider that T-0970 may serve as a useful drug for both chronic and acute phases of various diseases which are associated with oxidative tissue injury. Further studies of T-0970 with various animal models such as the model of ischemia–reperfusion injury and inflammation are now in progress and will be reported elsewhere.

Acknowledgments

We are grateful to Drs. K. Matsumoto, Y. Honma and T. Iwasaki for their encouragement during the study. We would also like to thank Dr. S. Murata for valuable discussions.

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