IN VITRO STUDIES ON THE ACTIVITY OF JAPANESE KAMPO HERBAL MEDICINES OREN-GEDOKU-TO (TJ-15) AND TOKI-SHAKUYAKU-SAN (TJ-23) AS SCAVENGERS OF FREE RADICALS

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SUMMARY

Oren-Gedoku-To (TJ-15) inhibited enzymatically (NADPH or CumOOH) and non-enzymatically (Fe-ascorbate) induced lipid peroxidation in rat liver microsomes as assessed by TBA-reactive product accumulation. Toki-Shakuyaku-San (TJ-23) had little effect on either system. The protective effect of TJ-15 against lipid peroxidation was not dependent upon the presence of microsomal drug-metabolizing activity and could not be fully accounted for by its action on microsomal electron transfer, as evaluated by studying the kinetics of cytochrome *c* reduction.

Both TJ-15 and TJ-23 reduced the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), indicating their free radical scavenging ability. The reactivity of TJ-15 was comparable with that of standard ascorbate, while the activity of TJ-23 was approximately 10 times lower.

In a free solution TJ-15 and TJ-23 effectively scavenged $OH^{\bullet}$ radicals, as indicated by the inhibition of ethylene production from 2-keto-4-methiolbutyric acid (KMBA), and $O_2^{\bullet-}$ anion radicals, as assessed by the inhibitory effect on the rate of nitro blue tetrazolium (NBT) reduction by the $O_2^{\bullet-}$ generating system xanthine/xanthine oxidase.

KEY WORDS

Oren-Gedoku-To (TJ-15), Toki-Shakuyaku-San (TJ-23), antioxidant activity, free radical scavenging activity

INTRODUCTION

Free radicals are highly reactive molecular species causing cell metabolic disturbances, cell injury and even cell death. In particular, active oxygen species are possibly involved in cancer, atherosclerosis, Alzheimer's disease, ischemic tissue damage and inflammation, and they are considered the principal causative agents of aging /1-4/. Free radical mediated lipid peroxidation is presumed to be one of the primary molecular events leading to these abnormal responses. The mechanism of lipid peroxidation and the effect of antioxidants on this process has therefore been extensively studied.

The medicines used in traditional Japanese Kampo practice are of plant origin. Clinical experience to date suggests that most Kampo preparations have a mild but progressive action with few associated side-effects, making them particularly suitable for the treatment of chronic diseases. The Kampo drug TJ-15 (Oren-Gedoku-To) is widely used for the treatment of post-stroke syndromes and hypertension - diseases in which the deleterious role of free radicals may be involved.

Hydroxyl radical scavenging activity and lipid peroxidation inhibitory effect of a series of Kampo preparations, including TJ-15 and TJ-23, in phosphatidylcholine liposomes has been demonstrated recently /5-7/. In the light of the suggested role of free radicals as mediators of tissue injury and disease, this may have important therapeutic implications.

The present study was conducted to compare the ability of TJ-15 and TJ-23, two Kampo preparations with different antioxidant efficiencies /5/, to scavenge free radical species responsible for lipid peroxidation. First, the inhibitory effects of TJ-15 and TJ-23 on *in vitro* rat liver microsomal lipid peroxidation was examined and their action on the microsomal electron transfer system was compared. Second, the free radical scavenging activity of the Kampo preparations was examined using a stable free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH). Third, the reactivity of TJ-15 and TJ-23 with hydroxyl and superoxide radicals was investigated. A preliminary account of this work was presented elsewhere /8,9/.

MATERIALS AND METHODS

Chemicals

Oren-Gedoku-To (TJ-15) and Toki-Shakuyaku-San (TJ-23) were obtained from Tsumura & Co. Ltd. (Tokyo, Japan). $NADP^+$, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, cytochrome *c*, xanthine, xanthine oxidase, 2-keto-4-methiolbutyric acid (KMBA), nitro blue tetrazolium (NBT) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were from Sigma Chemical Co. (St. Louis, USA). Cumene hydroperoxide (CumOOH) and thiobarbituric acid (TBA) were from Fluka AG (Buchs, Switzerland).

Liver preparations

Male Wistar rats (220-270 g) fasted overnight were used as liver donors. Livers were homogenized in 3 volumes of 1.15% KCl and the microsomal fraction was isolated /10/.

Incubations and assays

For the assay of NADPH-dependent lipid peroxidation, the incubation mixture consisted of liver microsomes (1.2-1.7 mg protein/ml), the NADPH-generating system (0.6 mM NADP, 6.0 mM glucose-6-phosphate, 6 mM $MgCl_2$ and 1 U/ml glucose-6-phosphate dehydrogenase) and 12.5 mM potassium phosphate buffer, pH 7.5. Incubation was started by the addition of the microsomal suspension. For the CumOOH-induced lipid peroxidation, the above incubation mixture contained 50 μM CumOOH instead of the NADPH-generating system. For the iron/ascorbate-induced lipid peroxidation, the incubation mixture consisted of microsomes pre-heated to 100°C for 5 min, 50 μM $Fe(NH_4)_2(SO_4)_2$, 0.5 mM ascorbate and 12.5 mM potassium phosphate buffer, pH 7.5. The reaction was started by adding ascorbate. Kampo preparations were added dissolved in water. All incubations were conducted aerobically at 37°C in a final volume of 1.0 ml. The formation of malondialdehyde in the incubation mixture was assayed by the method of Buege and Aust /11/.

The activity of microsomal NADPH-cytochrome P-450 reductase was evaluated by following the kinetics of reduction of cytochrome *c* at 550 nm, according to Williams and Kamin /12/. The assay was conducted at 37°C in 0.5 cm cells containing 33 μM potassium

phosphate buffer (pH 7.5), 1 mM KCN, 50 μM cytochrome *c*, approximately 10 μg/ml of microsomal protein, and 100 μM NADPH in a final volume of 1.5 ml. The reaction was initiated by the addition of the NADPH. The rate of cytochrome *c* reduction was calculated using an extinction coefficient of 21 cm^{-1} mM^{-1} /12/.

Free radical scavenging activity was determined by the method of Mellors and Tappel /13/. The ethanolic solution (0.1 ml) of the test Kampo preparation was added to 3 ml of 50 μM solution of DPPH in ethanol. The decrease of absorbance during the reduction of DPPH was continuously monitored at 518 nm.

The iron/ascorbate model system [0.1 mM $Fe(NH_4)_2(SO_4)_2$, 1.7 mM ascorbate and 0.2 mM EDTA in 0.05 M potassium phosphate buffer] was used to generate hydroxyl radicals in the absence of microsomes. The production of hydroxyl radicals was assayed by measuring the generation of ethylene from 0.25 mM 2-keto-4-methiolbutyric acid (KMBA) as described by Cohen and Cederbaum /14/.

The xanthine/xanthine oxidase model system (0.1 mM xanthine, approx. 10^{-2} U/ml of xanthine oxidase and 1 mM DETAPAC in 0.05 M potassium phosphate buffer) was used to generate a reproducible flux of superoxide anion radicals in the absence of microsomes, and 56 μM nitro blue tetrazolium (NBT) was used as an indicator of superoxide production /15/.

RESULTS AND DISCUSSION

To evaluate the properties of TJ-15 and TJ-23 relevant to free radical-mediated processes, we analyzed quantitatively their effect on *in vitro* microsomal lipid peroxidation. Liver microsomes have been successfully used as a model to study enzyme-catalyzed lipid peroxidation since microsomes, disrupted endoplasmic reticulum vesicles, contain unsaturated fatty acids in high proportions, and at the same time they contain enzymes of the electron transfer system which make them capable of producing free radical species.

In our experiments we used three different modes of initiation of oxidative damage. The first required NADPH, NADPH-cytochrome P-450 reductase and chelated iron. In the second system the oxidative stress was induced by cumene hydroperoxide (CumOOH), bypassing requirements for reducing equivalents and iron. These two enzymatic systems contrast with the non-enzymatic iron/ascorbate induced lipid

peroxidation in heat-inactivated microsomes used as the third mode of the initiation of oxidative damage.

From the results shown in Figure 1 it is obvious that TJ-15 inhibited lipid peroxidation initiated both enzymatically and non-enzymatically. This indicates that the inhibition of lipid peroxidation by TJ-15 was not dependent upon the presence of microsomal drug metabolizing activity. On the other hand, TJ-23 did not significantly inhibit the non-enzymatic iron/ascorbate induced lipid peroxidation and only slightly depressed the enzymatically initiated peroxidation processes.

The protective effect of TJ-15 against NADPH-induced lipid peroxidation cannot be fully accounted for by the observed inhibition of microsomal electron transfer, shown in Figure 2, since TJ-15 affected the activity of NADPH-cytochrome *c* reductase only marginally in the concentration region where more than 90% inhibition of lipid peroxidation was observed (compare Figs. 1 and 2).

Considering the possible metal chelating properties of TJ-15, they do not appear to be important in its inhibitory action since TJ-15 effectively inhibited lipid peroxidation initiated by cumene hydroperoxide alone, which neither requires iron nor is affected by its presence /16/.

As reported recently /5,6/, Kampo medicines considerably inhibited iron/hydrogen peroxide-initiated lipid peroxidation in phosphatidylcholine liposomes. In this completely non-enzymatic process, TJ-15 was found to be more effective than TJ-23, which is in agreement with our above presented results recorded in the microsomal preparations.

On the basis of the results obtained so far, we suggest that the antioxidant effect of Kampo medicines may operate predominantly by a direct interaction of their active component(s) with free radical species involved in the initiation and/or propagation of the chain peroxidation reaction. To investigate the molecular mechanism of the antioxidant action of Kampo medicines, we chose the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) to assay the free radical scavenging activity of TJ-15 and TJ-23. As shown in Figure 3, the stable free radical DPPH was reduced by the test Kampo preparations. The reactivity of TJ-15 was comparable with that of the standard ascorbate, while the activity of TJ-23 was approximately 10 times lower. The high reactivity of TJ-15 with the stable radical DPPH indicates that it is the ability to eliminate free radicals which

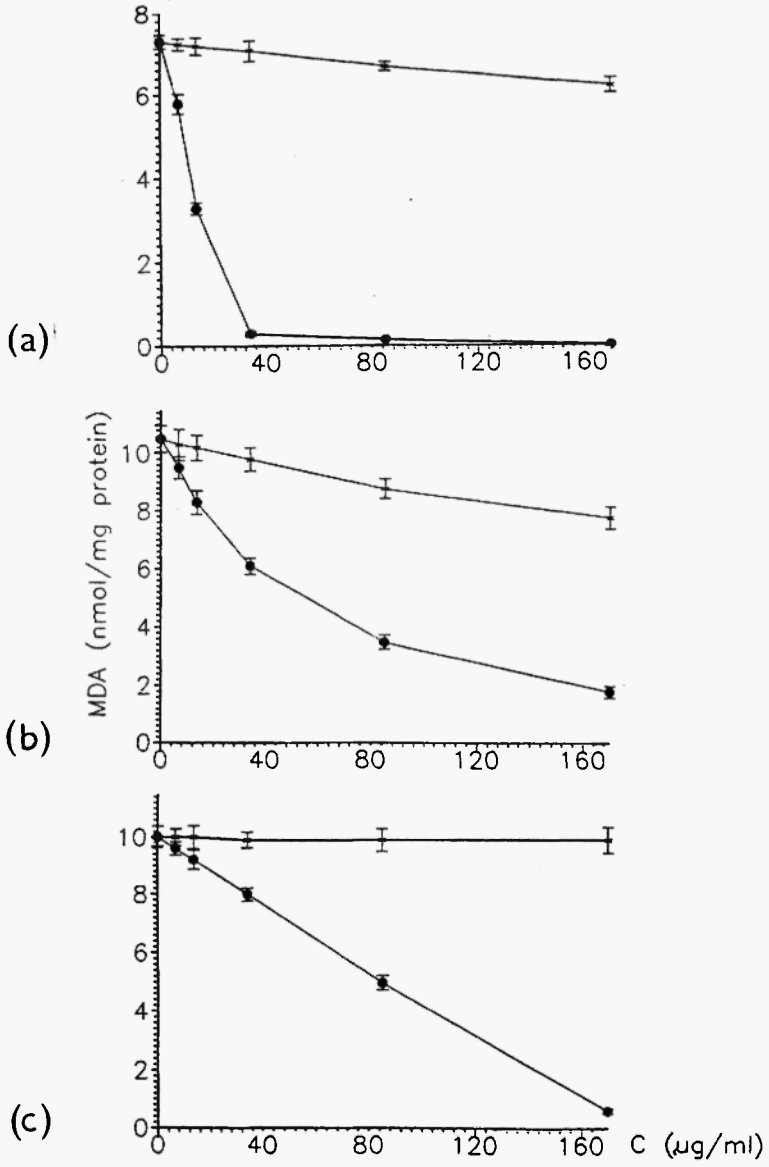

Fig. 1: Effect of TJ-15 (●) and TJ-23 (*) on lipid peroxidation in rat liver microsomes. (a) NADPH-induced lipid peroxidation, time of incubation 30 min. (b) Cumene hydroperoxide-induced lipid peroxidation, time of incubation 10 min. (c) Iron/ascorbate-induced lipid peroxidation in boiled microsomes, time of incubation 60 min. Results are mean values ± SEM from 5 experiments.

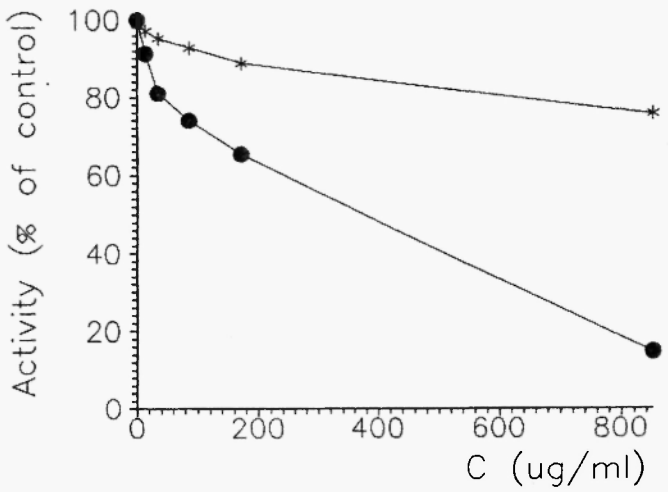

Fig. 2: Effect of TJ-15 (●) and TJ-23 (*) on the activity of microsomal NADPH-cytochrome *c* reductase. The control activity was 31.7 nmol of cytochrome *c* reduced in 1 ml of the incubation mixture per 1 min. For experimental details see Materials and Methods. Results are mean values from two experiments.

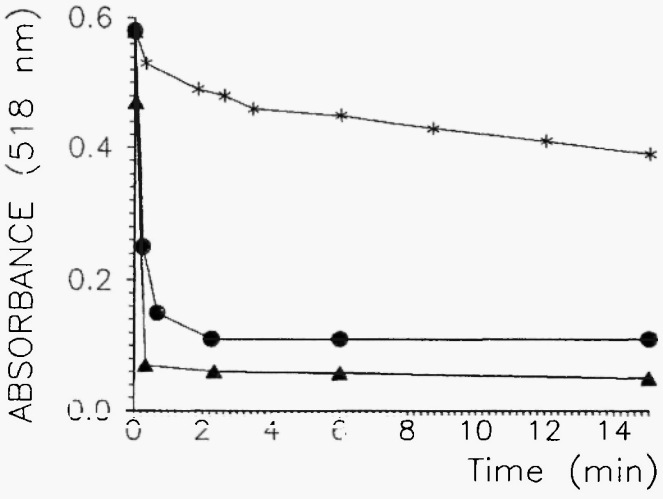

Fig. 3: Reduction of 1,1-diphenylpicrylhydrazyl (DPPH) by TJ-15 (●), TJ-23 (*) and ascorbate (▲). Results are mean values from 3 experiments. SEM bars are within the symbols.

predominates in the molecular mechanism of the antioxidant action of Kampo preparations.

From the results shown in Figure 1 we can speculate that TJ-15 may scavenge alkoxyl ($LO^{\bullet}$) and peroxyl ($LOO^{\bullet}$) radicals, since these active oxygen species are thought to be the main causative agents in CumOOH- /16/ or iron/ascorbate-induced lipid peroxidation /17/. In a recent report /7/ the ability of Kampo preparations to scavenge hydroxyl radicals was demonstrated, using the ESR technique.

In the present work we compared the effect of TJ-15 and TJ-23 on hydroxyl radical-dependent degradation of the selective hydroxyl radical scavenger, 2-keto-4-methylthio-butyric acid (KMBA). As a source of hydroxyl radicals in this study, we used a pure chemical model system based on the oxidation of ascorbate by ferric ions. As shown in Figure 4, both TJ-15 and TJ-23 significantly inhibited the production of ethylene from KMBA, which is a measure of the activity of hydroxyl radicals generated in the system. TJ-23 was found to be a slightly more effective scavenger of hydroxyl radicals than TJ-15.

We compared the effect of TJ-15 and TJ-23 on superoxide anion radical-dependent reduction of nitro blue tetrazolium (NBT). The rate of reduction of the originally yellow dye NBT to a blue-black product is a measure of superoxide generated in the system. In our experiments we used the xanthine/xanthine oxidase model system to generate superoxide anion radicals. As shown in Figure 5, both TJ-15 and TJ-23 inhibited the rate of reduction of NBT, with TJ-15 being a more effective scavenger of superoxide than TJ-23. As was shown in control sets of experiments the Kampo preparations under test neither directly reduced the NBT dye in the concentration range employed nor did they significantly effect the activity of xanthine oxidase as determined by colorimetric assay of uric acid formation /18/ (data not shown).

Other Kampo preparations have recently been reported to have antioxidant and free radical scavenging effects. TJ-960 (Sho-Saiko-To-Go-Keishi-Ka-Shakuyaku-To) was found to scavenge superoxide anions, hydroxyl and peroxyl radicals in aqueous solutions with an activity similar to that of vitamins C and E /19/. While TJ-8007 (Zokumei-To) inhibited lipid peroxide production with lower potency than alpha-tocopherol, its free radical scavenging activity was similar to that of alpha-tocopherol /20/. TJ-114 (Sairei-To) was reported to inhibit active oxygen generation *in vitro* in isolated hepatocytes /21/.

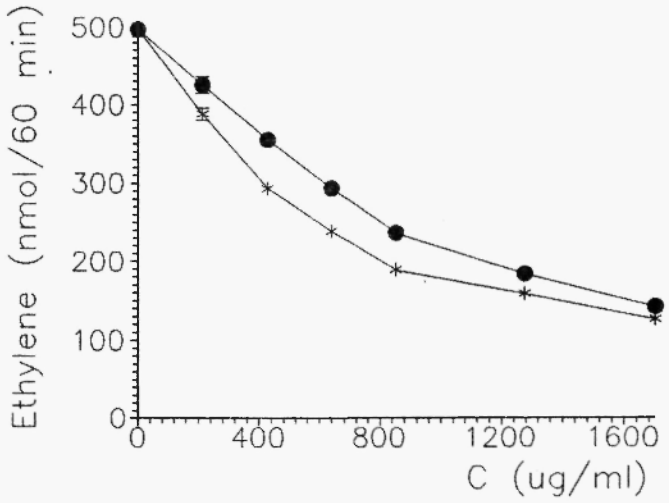

Fig. 4: Effect of TJ-15 (●) and TJ-23 (*) on hydroxyl radical-dependent formation of ethylene from 2-keto-4-methylthiobutyric acid (KMBA) in the iron/ascorbate model system free of microsomes. Results are mean values ± SEM from 5 experiments.

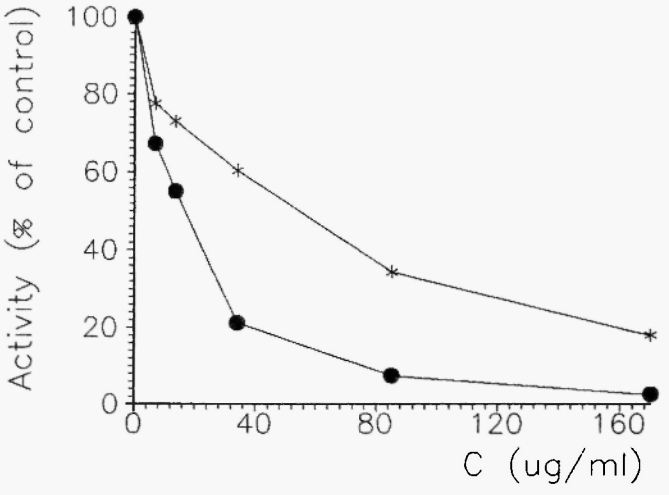

Fig. 5: Effect of TJ-15 (●) and TJ-23 (*) on superoxide-dependent reduction of nitro blue tetrazolium (NBT) in the xanthine/xanthine oxidase model system free of microsomes. Results are mean values from 3 experiments. SEM bars are within the symbols.

TJ-9 (Sho-Saiko-To) markedly inhibited iron-induced lipid peroxidation in microsomes and mitochondria, yet it failed to quench superoxide anion radicals in neutrophils /22/.

The pharmacognosy of Kampo medicines is currently being extensively investigated. Tannins, flavonoids and saikosaponins were suggested to be the main ingredients responsible for the free radical scavenging activities of Kampo preparations /20-23/. However, interactions between the various components of a Kampo medicine are believed to play a role in the therapeutic effects of the drugs which require further study.

In conclusion, both of the two Kampo preparations studied, TJ-15 and TJ-23, were found to be effective scavengers of hydroxyl radicals and superoxide anoin radicals generated in a free solution. However, the results indicate that only TJ-15 effectively scavenges the stable free radical DPPH and is capable of reaching hydrophobic intra-membrane sites at concentrations which make TJ-15 effective as an antioxidant.

On balance, the present results suggest that TJ-15 may be a potentially useful protective agent against free radical-mediated toxic damage. Studies on the antioxidant activity of Kampo preparations in *in vivo* conditions are in progress.

ACKNOWLEDGEMENTS

This work was supported by a grant from Tsumura & Co., Tokyo, Japan.

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