IN VITRO TESTS OF 1,3-DITHIA-2-THIOXO-CYCLOPENT-4-ENE TO EVALUATE THE MECHANISMS OF ITS HEPATOPROTECTIVE ACTION

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ABSTRACT — Some *in vitro* tests were performed using 1,3-dithia-2-thioxo-cyclopent-4-ene (DT827A) to evaluate its mode of action on hepatoprotection. Experiments with leucine, uridine and thymidine uptakes using L-132 cells showed no increase in leucine uptake, but did show a tendency toward increases in both uridine and thymidine uptakes, which suggested a stimulating effect on RNA and DNA synthesis *in vitro*. DT827A did not influence GSH levels in the HepG2 cultured cell system, and did not show inhibition of cytochrome P450 2E1 in microsomes obtained from mouse liver. There may be no possibility for DT827A to protect a liver injury through its influence on liver GSH levels and inhibiting metabolic activation of CCl₄ in this *in vitro* system. However, the inhibitory effect of DT827A on lipid peroxidation was observed *in vitro*, the same as the observation *in vivo*. Furthermore, when DT827A was incubated with Alamar Blue in the presence of a mixture of mouse liver S9 and mitochondrial fractions for 20 hr, DT827A showed more reducibility than it did in a case of non-incubation, and it was suggested that DT827A would be metabolized in the S9-mitochondrial fraction *in vitro* to show an increase of Alamar Blue reducibility.

The protective effect of DT827A on a liver injury is due neither to its influence on liver GSH levels nor inhibition of the metabolic activation of CCl₄, but a possible mechanism of action for the DT827 series of compounds to indicate an antioxidative effect would be brought about by the role of the compounds as a radical scavenger as well as its reductive effect.

KEY WORDS: Hepatoprotective effect, *In vitro* test, Lipid peroxidation, Radical scavenging effect, SH-group

INTRODUCTION

Over the past years we have studied the pharmacological profile of the three compounds, namely 1,3-dithia-2-thioxo-cyclopent-4-ene (DT827A; chemical structure is shown in Fig. 1), and its two derivatives of 4-phenyl-1,3-dithia-2-thioxo-cyclopent-4-ene (DT827B) and 4-(4-fluorophenyl)-1,3-dithia-2-thioxo-cyclopent-4-ene (DT827C). DT827A was synthesized by F. Challenger *et al.* (Challenger *et al.*, 1952), and has been used as an intermediate for the synthesis of other compounds. We paid attention to these compounds' structure of dithiol function on the assumption that they have a hepatoprotective potential, since, as far as we know, those phar-

macological effects had not been studied. We previously reported their *in vivo* pharmaceutical profiles using animal models with liver injury caused by single or repeated exposure to carbon tetrachloride (CCl₄), repeat-

Fig. 1. Chemical Structure of DT827A.
DT827A: 1,3-dithia-2-thioxo-cyclopent-4-ene.

ed exposure to orotic acid, repeated exposure to a combination of D-galactosamine (D-GalN) plus CCl_4 (D-GalN+ CCl_4), and, furthermore, repeated exposure to thioacetamide by measurements of some biochemical parameters such as GOT, GPT, alkaline phosphatase and total cholesterol values as well as liver weights and histopathology (Sadanobu *et al.*, 1997 and 1998). In consideration of these results, it is clearly suggested that the three DT827 compounds have a hepatoprotective potential for relieving liver damage caused by various substances. In particular, they showed relief in liver necrosis dose-dependently, which could suggest an inhibitory effect on lipid peroxidation. We further speculated that inhibition of lipid peroxidation is a possible mechanism of action for the protective potential. However, we pointed out the necessity of *in vitro* tests to confirm the mechanism.

The purpose of these experiments reported herein is to discuss the mechanism of the DT827 series of compounds. The compound used in these tests is DT827A only by reason of its solubility. DT827A is more soluble in various solvents than either DT827B or DT827C, and accordingly DT827A is most appropriate for *in vitro* tests among the three compounds.

MATERIALS AND METHODS

Measurements of protein, RNA and DNA syntheses in L132 cells

DT827A was supplied from the laboratories of SSP Co., Ltd. (Tokyo, Japan), which synthesized the compound. Measurements of leucine, uridine and thymidine uptakes were performed to evaluate an effect on stimulation to protein synthesis, RNA synthesis and DNA synthesis, respectively. Human alveolar type II cells (L-132 cells; Tezuka *et al.*, 1993) obtained from Dr. Yamanaka at our university were seeded onto Dulbecco's modified Eagle medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) at a concentration of 1.25×10^5 cells/dish, and DT827A was added. For measurement of leucine uptake, L-132 cells were added to the medium with 37 kBq/dish of [4,5- ^3H] leucine (9.25 GBq/m mol, Moravek Biochemicals, Inc., USA) and the uptake was measured 6 hr, 24 hr and 48 hr after addition of DT827A at 0, 1, and 10 μM . For measurement of uridine uptake, L-132 cells were added to the medium with 3.7 kBq/dish of [2- ^{14}C] uridine (2.072 GBq/m mol, Moravek Biochemicals, Inc., USA), and the uptake was measured at the same three periods. For measurement of thymidine uptake, L-132 cells were added to the medium with 37 kBq/dish of [methyl- ^3H]

thymidine (259 GBq/m mol, Moravek Biochemicals, Inc., USA), and the uptake was measured 6 hr and 24 hr after addition of DT827A at 0 and 10 μM . In all cases, a liquid scintillation counter was used for measurement of radioactivity after 1-hr pulse labeling of each isotope, and protein contents were calculated by the Bio-Rad Protein Assay method (Bradford, 1976) with measurement of absorbance at 595 nm.

Determination of reduced-type glutathione levels in cultured hepatoma cells and mouse liver

Human hepatoma (HepG2) cells were obtained from the Cell Resource Center for Biomedical Research Institute of Department, Aging and Cancer, Tohoku University. Male mice of the ddY strain were purchased from Japan S. L. C., Shizuoka, Japan, at 5 weeks old, and acclimated for 1 week to use for this experiment. All through the acclimation period and experimental period, water and animal diet were given freely. A reduced form of glutathione (GSH) was obtained from Wako Pure Chemicals, Tokyo, Japan.

HepG2 cells were seeded to plates at 5×10^5 cells/well, and incubated at 37°C for 72 hr. After removal of a medium, 1.98 mL of DMEM containing 10% fetal bovine serum (JRH Bioscience, USA) was added, and then 20 μL of DT827A (solution with DMSO at 10^{-2}M) was added to obtain a final drug concentration of 0.1 mM. After incubation for 6 hr, 12 hr or 24 hr, the test solutions were washed with Ca^{2+} - and Mg^{2+} -free phosphate buffered saline (PBS(-)) to collect cells. The cells were homogenized and centrifuged at 15,000 rpm for 30 min, and 4.5 mL of 0.1 M of PB-EDTA (0.2 M sodium phosphate — 10 mM EDTA buffer, pH 8.0) was added to 0.5 mL of the supernatant. Next, 1.8 mL of 0.1 M PB-EDTA and 100 μL of o-phthalaldehyde (OPT) were added to 1 mL of the test solution, and the spectrum was measured to calculate GSH levels with a fluorescence spectrometer (Hissin and Hilf, 1976).

DT827A at a dose of 0.1 mg/0.1 mL/10 g body weight was orally administered to the mice, and after either 24 hr or 48 hr, the liver was isolated and homogenized to obtain its supernatant. GSH levels were measured using the same method.

Measurement of cytochrome P450 2E1 activity in mouse liver

Male mice of the ddY strain at 5 weeks old were purchased from Japan S. L. C. and acclimated for 3 days to use in this experiment. β -NADPH was obtained from Wako Pure Chemicals.

DT827A was orally administered to mice at 0.1 mg/0.1 mL/10 g body weight, and after 12 hr the liver was isolated. Through exsanguination and perfusion, the liver was isolated to be homogenized, and then a microsomal fraction of the liver was obtained by centrifugation at $105,000\times g$.

Protein assay was made using Protein-Assay CBB ($5\times$) solution (Nacalai Tesque, Inc., Kyoto, Japan) and calculated by absorbance rate at 595 nm. To measure drug metabolic enzyme activity, potassium phosphate buffer (pH 7.4, PPB), *p*-nitrophenol (PNP) and NADPH were added to the liver microsomal suspension for incubation, and then levels of 4-nitrocatechol generated through the PNP hydroxylation process were measured by the absorbance method (Koop, 1990).

Determination of inhibition of lipid peroxidation

Double lipid layers of endoplasmic reticulum in microsomal fraction are peroxidized by Fe-ADP solution (a mixture of iron(III) chloride solution and ADP solution) in the presence of NADPH. Malonaldehyde produced by the peroxidation reacts with thiobarbituric acid (TBA) to form a red color. This experiment was performed to measure lipid peroxidation using the above-mentioned method (Ohkawa *et al.*, 1978). DT827A was dissolved in DMSO to yield solutions of $10^{-1}M$, $10^{-2}M$, and $10^{-3}M$, and they were then mixed with PBS to obtain solutions of 1 mM, 0.1 mM, and 0.01 mM. In both the Fe-ADP added group (Fe-ADP(+)) and Fe-ADP non-added group (Fe-ADP(-)), the above 3 concentrations of DT827A and 0 mM of DT827A were used for this experiment. In each test tube, 300 mM of Hepes (pH 7.4) and 10 mM of NADPH were added. After Vortex mixing, 25 μL of microsomal solution was added for incubation at 25°C for 15 min. Next, 300 μL of 70% trichloroacetic acid (TCA) solution and 300 μL of $CHCl_3$ were added to centrifuge at 2,350 rpm for 15 min. Absorbance of the supernatant was measured at 532 nm in comparison with Fe-ADP(-) as the reference standard.

Alamar Blue reductive activity in the presence of rat liver S9 fraction and mitochondrial fractions

Male rats of the Wistar strain at the age of 5 weeks were obtained from Japan S. L. C. Alamar Blue was purchased from Bio Source, USA. The isolated rat liver obtained by perfusion was centrifuged at $9,000\times g$ for 20 min to obtain the S9 fraction, and otherwise the mitochondrial fraction was prepared in accordance with the method of Hogeboom modified by Utsumi (Utsumi, 1963). Protein assay was performed with the

same procedure as the above-mentioned cytochrome P450 2E1 activities' experiment using a preparation of 1 mg/mL by PBS(-).

For measurement of reducibility of the Alamar Blue, either DT827A or GSH was added to PBS(-) at a final concentration of 0.1 mM, and immediately 250 μL of Alamar Blue was also added in order to measure absorbance 30 min after the addition. To measure the reducibility of Alamar Blue in the presence of a mixture of S9 fraction and mitochondrial fraction, DT827A or GSH was added at the same concentration as above for non-incubation groups, where absorbance was measured 30 min after the addition, and DT827A or GSH was added at 0.1 mM and 0.01 mM for 20-hr incubation groups.

Statistical analysis

Student *t*-test (Snedecor and Cochran, 1980) was used for statistical analysis.

RESULTS

Influence of DT827A on protein, RNA and DNA syntheses in L132 cells

The values of leucine, uridine, and thymidine uptakes are shown in Fig. 2. There was no significant difference in leucine uptake values among the three DT827 groups in the three exposure periods of 6 hr, 24 hr and 48 hr. As to uridine uptake, a dose-dependent increase of the uptake was observed after 48-hr exposure, and a tendency toward a dose-dependent increase was observed on thymidine uptake.

Influence on reduced-type glutathione levels in cultured hepatoma cells and mouse liver

There was no significant difference in GSH levels between the DT827A group and control group 6 hr, 12 hr, and 24 hr after addition of the test compound to HepG2 cells, and there was no significant difference in GSH levels of the liver between the DT827A group and control group 24 hr and 48 hr after administration of the test compounds to mice.

Influence on mouse cytochrome P450 2E1 activity in the liver

There was no significant difference in *p*-nitrophenol hydroxylation activities between the DT827A group and control group.

Inhibition of lipid peroxidation

As shown in Fig. 3, absorbance by a thiobarbituric

rate-reactive substance (TBARS) in the DT827A group was decreased dose-dependently, and 1 mM of DT827A was decreased in almost one-tenth of the control group.

Alamar Blue reductive activity in the presence of rat liver S9 fraction and mitochondrial fraction

Reduction is shown in Fig. 4. There was no significant difference in absorbance between the DT827A group and GSH control group 30 min after addition of Alamar Blue to DT827A and GSH at the final concentration of 0.1 mM.

Without incubation in the presence of a mixture of S9 fraction and mitochondrial fraction, 0.1 mM of GSH showed a statistically significant reducibility in comparison with 0.1 mM of DT827A. When incubated for 20 hr in the presence of a mixture of S9 fraction and mitochondrial fraction, DT827A showed more reducibility at 0.01 mM than 0.1 mM, but the degree of the reducibility was less than that of the GSH group at 0.1 mM.

Fig. 3. Inhibitory effect of DT827A on lipid peroxidation. 1 mM, 0.1 mM, and 0.01mM of DT827A were added to microsome suspension in the presences of NADPH and Fe-ADP, and malone dialdehyde levels produced were regarded as an index of degree of lipid peroxidation by measurement of thiobarbituric acid-reactive substances (TBARS). Each value represents mean ± S.D. (n=3). **: Statistically significant from values in the control (0 μ M of DT827A) group, $p < 0.01$.

Fig. 2. Leucine, uridine, and thymidine uptakes in L132 cells after addition of DT827A. Leucine uptake (left), uridine uptake (middle) and thymidine uptake (right) were measured 6 hr (upper), 24 hr (middle) and 48 hr (bottom) after addition of 1 μ M and 10 μ M of DT827A to human alveolar type II cells (L-132 cells) seeded at 1.25×10^5 cells/dish (10 μ M of DT827A only for measurement of thymidine uptake). Each value represents mean ± S.E. (n=2). *, **: Statistically significant from values in the control (0 μ M of DT827A) group, $p < 0.05$ and $p < 0.01$, respectively.

DISCUSSION

Some *in vitro* tests were performed on DT827A to examine its mechanism of action for liver protection. First, using L-132 cells, leucine, uridine and thymidine uptakes were examined. The results in uridine and thymidine uptakes, and a dose-dependent increase or tendency toward increase in the uptakes was observed, particularly in the final observation period of the uridine uptake test. It was accordingly suggested that DT827A would have a stimulating effect on RNA synthesis and DNA synthesis. However, there was no dose-dependent increase in leucine uptake in all exposure periods of the DT827A group, and accordingly it was suggested that in such an *in vitro* experimental condition as that of pulse labeling for the last 1 hr of exposure, no influence of DT827A on an index for protein synthesis could be observed.

Using hepatoma-origin HepG2 cells and mouse liver, the influence of DT827A on GSH level was tested. Between the DT827A groups and control groups, there were no significant differences in GSH levels measured 6 hr, 12 hr, and 24 hr after addition of DT827A to HepG2 cells and, additionally, in the experiment with mouse liver, GSH levels of the DT827A group were almost similar to the control group. There may be no possibility that DT827A can protect a liver injury through its influence on liver GSH levels.

The possible influence of DT827A on the enzyme activity of cytochrome P450 2E1, which would effect the metabolic activation of CCl₄, was examined using

mouse liver microsomes and *p*-nitrophenol, which is a typical substrate for the 2E1 enzyme. There was no significant difference between the DT827A group and control group, and it was considered that DT827A had no effect on inhibiting metabolic activation of CCl₄ mainly catalyzed by CYP2E1 (Johanssen and Ingelman-Sundberg, 1985). The characteristics of DT827A in not influencing the drug metabolism enzymes would be a certain advantage of the compound from the viewpoint of *in vivo* safety.

Consequently, a possible mechanism of action by DT827A to inhibit lipid peroxidation was examined using an *in vitro* model. In the presence of NADPH, DT827A was added to the medium in which lipid peroxidation had been induced by Fe-ADP. Both 1 mM and 0.1 mM of DT827A significantly inhibited lipid peroxidation dose-dependently, and it was considered that the inhibitory effect on lipid peroxidation observed in animal models had been reproduced in this *in vitro* model.

Lastly, the reductive effect of DT827A was examined using Alamar Blue in comparison with GSH as a reference compound under the two conditions of a simple addition of DT827A or GSH to Alamar Blue in the absence of S9 and mitochondrial fractions, and exposure to DT827A or GSH in the presence of a mixture of S9 fraction and mitochondrial fraction (with and without incubation). Alamar Blue is usually used for evaluation of the proliferative ability of cell lines *in vitro*, incorporating a colorimetric growth indicator based on detection of metabolic activity (Ahmed *et al.*, 1994).

Fig. 4. Alamar Blue reduction activity in the presence of rat liver S9 fraction and mitochondrial fraction. DT827A or GSH was added at 0.1 mM to Alamar Blue (A). DT827A or GSH was added to a mixture of S9 fraction and mitochondrial fraction obtained from rat liver of male Wistar strain, and 250 μ L of Alamar Blue, with either no incubation (B) or 20-hr incubation (C). Either DT827A or GSH was added at the concentration of 0.1 mM for groups (A) and (B), and both 0.1 mM and 0.01 mM for group (C). Each value represents mean $\pm$ S.D. (n=3); No inter-group statistical analysis was performed.

However, in this experiment, we focused on such merits of the reagent as a simple assay to perform, since the indicator is water-soluble, and we used it in order to discuss the reductive activity of DT827A *in vitro*. Because a reasonable reducibility was observed in the GSH group, it was considered that the system did work under this condition. By the results of the simple addition of DT827A or GSH to Alamar Blue, both agents showed little reducibility, and their degrees were almost the same. When incubation was not done to the system of either DT827A or GSH added to a mixture of S9 fraction and mitochondrial fraction, the reducibility of DT827A was almost the same as that in the above-mentioned simple addition, but GSH showed a higher reducibility at a dosage of 0.1 mM. Apart from the case of non-incubation, when the test specimens were incubated for 20 hr, 0.1 mM of DT827A showed a little reducibility quite a bit less than the GSH group at the same concentration. However, 0.01 mM of DT827A showed more reducibility than that of 0.1 mM of the compound. As one of the possible reasons for the reducibility in a lower-concentration group, the presence of an optimal concentration to show an antioxidative effect may be considered for this *in vitro* system. We may recall a similar experience reported by Ek *et al.* (Ek *et al.*, 1995). In their report on uptake of ascorbic acid into human umbilical vein endothelial cells, they suggested that there was an optimal concentration range for the uptake process, and they further discussed a higher concentration lacking a protective effect. In our *in vitro* system, an optimal concentration would be less than 0.1 mM.

The results of the experiment using Alamar Blue suggested that DT827A would show a reductive effect to Alamar Blue with any components contained in the S9-mitochondrial fraction *in vitro*.

In our previous pharmacological studies using the three DT827 series compounds *in vivo* (Sadanobu *et al.*, 1997 and 1998), we discussed a possible mechanism of the series of compounds as similar to diisopropyl 1,3-dithiol-2-ylidenemalonate (malotilate), showing a hepatoprotective effect on liver injury induced by various substances (Imaizumi *et al.*, 1981). Namely, according to Katoh and Sugimoto the effect of malotilate may be due to any or all of 1) inhibition of CCl₄ activation, 2) inhibition of lipid peroxidation, and 3) acceleration of liver function (Katoh and Sugimoto, 1982), and our previous study results suggested a possible role of the DT827 series of compounds to inhibit lipid peroxidation.

As we discussed in our previous reports, a possi-

ble mechanism of action for the DT827 series of compounds to indicate an antioxidative effect would be brought about by one role of the compounds as a radical scavenger. We can refer to a series of studies on 5-(*p*-methoxyphenyl)-3*H*-1,2-dithiol-3-thione (anethole dithiolethione; ADT) (Christen *et al.*, 1995; Rat *et al.*, 1995), which has a chemical structure similar to the DT827 series of compounds. Christen *et al.* reported that ADT scavenged a radical of a chemical substance which would cause a liver injury, and its action resulted in prevention of lipid peroxidation (Christen, 1995). In conclusion on our *in vivo* and *in vitro* experiments on DT827 series compounds, they do not work on relieving liver damage by enhancement of GSH activity, but may work by a reductive effect via any components contained in S9 and mitochondrial fractions and by an inhibition of lipid peroxidation through radical-scavenging, presumably via microsomes.

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