Cholesterol Biosynthesis Inhibitory Component from *Zingiber officinale* ROSCOE

Masahiro TANABE,[a] Yuh-Dan CHEN,[b] Ken-ichi SAITO[c] and Yoshihiro KANO*,[c]

Nagakura Pharmaceutical Company Ltd.,[a] 3–1–16, Shotenshita, Nishinari-ku, Osaka 557, Japan, Brion Research Institute of Taiwan,[b] 116, Chung-Ching South RD Sec. 3, Taipei, Taiwan and Hokkaido Institute of Pharmaceutical Sciences,[c] Katsuraoka 7–1, Otaru-shi, Hokkaido 047–02, Japan. Received August 10, 1992

We previously reported on the isolation and identification of (*E*)-8β,17-epoxylabd-12-ene-15,16-dial (ZT) from ginger (rhizome of *Zingiber officinale* ROSCOE, Zingiberaceae).

In this paper, the pharmacological effects of ZT are reported. The experimental mouse hypercholesterolemia induced by Triton WR-1339 was treated after oral administration of ZT. In homogenated rat liver with ZT, cholesterol biosynthesis was decreased. In addition, the same activity was observed in the homogenated rat liver which was resected after the oral administration of ZT.

According to the results of general pharmacological screening, no remarkable activity of ZT was observed except for an inhibitory effect on the cholesterol biosynthesis.

Keywords cholesterol biosynthesis inhibitor; *Zingiber officinale*; Zingiberaceae; (*E*)-8β,17-epoxylabd-12-ene-15,16-dial; diterpene; hypercholesterolemia

Arteriosclerosis is considered to be related to serum lipids.[1,2] It is said that coronary arterial disease can be controlled by the treatment of hypercholesterolemia and/or hyperlipemia. Regarding serum cholesterol, the general opinion is "The lower, the better." Therefore, the development of a therapeutic agent that will reduce high serum cholesterol levels is desired.[3–5] Recently, hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitory agents have been developed for the therapeutic treatment of hypercholesterolemia.

We had reported on the isolation and identification of (*E*)-8β,17-epoxylabd-12-ene-15,16-dial (ZT) from ginger (rhizome of *Zingiber officinale* ROSCOE, Zingiberaceae)[6] (Fig. 1).

From the results of pharmacological screening of ZT, the inhibitory effect of ZT against cholesterol biosynthesis was concluded and ZT was assumed to be an HMG-CoA reductase inhibitor.

Materials and Methods

Animals Wister rats (male, 150—200 g) obtained from Sankyo Laboratory Service and ICR mice (male, 18—22 g) obtained from the Experimental Animal Center of the Medical Institute of Taiwan University were used.

The animals were kept in a room at 23±1 °C with a 12-h dark–light cycle and fed normal food and tap water for one week before the experiment.

Chemicals ZT isolated from ginger by us was used. [1-^{14}C]sodium acetate, Triton WR-1339, digitonin and Atromid were purchased respectively from New England Nuclear Ltd., Ruger Chemical Co., Ltd., Wako Pure Chemicals Co., Ltd. and Sigma Chemical Co., Ltd.

General Pharmacological Screening Experiments of ZT on the 45 trial items of the general pharmacological screening test were carried out. These methods were based on the literature.[7] In the *in vivo* experiment, one group was composed of four mice and the *in vitro* experiment was repeatedly carried out.

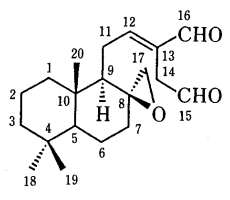

Fig. 1. Structure of (*E*)-8β,17-Epoxylabd-12-ene-15,16-dial (ZT)

On the Treatment of the Experimental Hypercholesterolemia Induced by Triton Eight mice were used per each group. Mice that were fasted overnight were injected with Triton WR-1339 (600 mg/kg dissolved in 0.9% NaCl solution) *via* the caudal vein. Just after injection, ZT (suspended in 0.25% methylcellulose solution) was administered orally to the mice in varying dosages: 25, 50, 100 and 200 mg/kg. Furthermore, 20 h later, the same dose of ZT was re-administrated orally. At 43 h after Triton injection, blood samples were collected from the carotid arteries and put into test tubes.

In order to prevent blood coagulation, the samples were kept cool for 40 min before centrifugation (800 rpm, 15 min). Then the supernatant serum was collected. The amount of serum cholesterol was measured twice by Technicon Autoanalyzer (Technicon Instruments Co., New York). Atromid (100 mg/kg, dissolved in 0.25% methylcellulose solution) was used as a positive control. The total cholesterol value in the serum sample was calculated against the control case of only 0.25% methylcellulose administration.

Inhibitory Effect on Cholesterol Biosynthesis in Rat Liver *in Vitro* Twelve rats were divided among three groups. After fasting the rats for 18 h, rat liver was taken out and washed with normal saline. After washing, the liver was homogenated and then filtrated through a 300 μm filter.

Next, approximately 50 mg of the homogenated liver was put into a test tube and centrifugated (2300 rpm, 15 min). After removal of the supernatant liquid, the precipitate was washed with Kreb's–Ringer bicarbonate buffer (components in mmol: NaCl 126.5, KCl 2.4, $MgCl_2$ 0.83, $CaCl_2$ 1.1, Na_2SO_4 0.5, $NaHCO_3$ 27.5, KH_2PO_4 0.5, glucose 5.9, ventilation 95% O_2, 5% CO_2, pH 7.4).[8,9]

Solutions of varying ZT concentration were added to each homogenated liver suspended by 25% Tween 80 in the Kreb's–Ringer bicarbonate buffer (37 °C).[10] This mixture was incubated for 30 min.

Then, 100 μl of 180 μCi/ml [1-^{14}C]sodium acetate was added to the mixture. The incubation was continued. Approximately two more hours later, 3 ml of 20% KOH ethanol solution was added. The mixture was divided into two test tubes. Using one of them, the protein content in the mixture was measured by Lowry's method.[11] Another fraction was extracted three times by 3 ml of petroleum ether and all extracts were combined. The extract was centrifugated (3000 rpm, 10 min) and a supernatant petroleum ether fraction was taken. After adding 1% digitonin solution into this fraction, centrifugal separation (3000 rpm, 10 min) of this mixture was done. The precipitate was collected and dissolved in ethanol. The radioactivity of ethanol solution was measured by the scintillation counter (Aloka Co.) and the amount of cholesterol biosynthesized per protein content was calculated.

The Influence on Cholesterol Biosynthesis in Rat Liver after Oral Administration of ZT Rats were fasted for 18 h and orally administered ZT (100, 200 mg/kg) dissolved in 0.5% methylcellulose normal saline.

Two hours later, rat liver was taken out and washed with normal saline.

After washing, the liver was homogenated and then filtrated through a 300 μm filter.

The homogenated liver was divided into two test groups. One group was incubated with Tween 80 and another was without.

Approximately 50 mg of the homogenated liver was put into a test tube and the previous procedure was followed, but without the addition of ZT in the test tube.

Calculation The formula used to calculate the inhibitory rate on cholesterol biosynthesis in mice serum was as follows. The amount of cholesterol was shown as mg%. In both the *in vivo* and *in vitro* experiments in rats, the sterol in the precipitate generated by digitonin was considered a biosynthetic cholesterol and calculated as dpm/mg based on the protein amount.

The results were statistically analysed by the Student's *t*-test.

$$\text{the inhibitory rate } (\%) = \{(A-B) \div A\} \times 100$$

A: mean value in control (mg%)
B: mean value in experimental subjects (mg%)

Results

The Effects of ZT Related to General Pharmacological Screening The results of the general pharmacological screening test are shown in Table I.

In the acute toxicity test on mice, no death cases were observed at either the 600 mg/kg dose by the oral route or the 25 mg/kg dose by intra-abdominal administration. Therefore, ZT was considered to be a low toxic substance. According to our pharmacological test, the same result was found. The suppressive rate on rat stress ulcer was 85%

TABLE I. The General Pharmacological Screening

	Test item	Administration method	Dose[a)]	Judgement standard	Response	Relative agent
1	Toxicity	*p.o.*	600	NT		
2	Reflex depressant	*p.o.*	600	<16	24/24	
3	Behavior depressant	*p.o.*	600	<40	58/60	
4	Muscle relaxant	*p.o.*	600	<20	29/29	
5	Motor stimulant	*p.o.*	600	>12	0/0	
6	Antidepressant	*p.o.*	100	> 3	0	
7	Antimetarazol	*p.o.*	600	> 3	0	
8	Autonomics	*p.o.*	600		−	
9	Anticonvulsant	*p.o.*	200	< 2	3	
10	Analgesic	s.c.	10	>50	0	
11	Anesthetic	s.c.	10	< 3	6	
12	Antiparkinson	s.c.	10	< 2	3	
13	Narcotic antagonist	s.c.	10	< 2	3	
14	Neuroleptic	*p.o.*	200	>12	0	
15	Catalepsy	*p.o.*	200	+	−	
16	Ataxia	*p.o.*	200	+	±	
17	Antiulcer	*p.o.*	50	>60	85	
		p.o.	25	>60	0	
		p.o.	4	>60	70	Atropine
18	Antidiarrheal	*p.o.*	200	< 2	3	
19	Vasodilation	i.p.	10	+	−	
20	Anorexic	*p.o.*	100	>80	15	
21	Intestine rx	*in vit.*	20	+	−	
22	Spasmogdic	*in vit.*	20	+	−	
23	Antihistamaminic	*in vit.*	20	+	−	
24	Anticholinergic	*in vit.*	20	+	+	
		in vit.	10	+	−	
		in vit.	0.1	+	+	Atropine
25	Anticerotonine	*in vit.*	20	+	+	
		in vit.	10	+	±	
		in vit.	5	+	−	
		in vit.	2	+	+	Prometazine
26	Uterine rx	*in vit.*	20	+	−	
27	β-Adrenergic stimulant	*in vit.*	20	+	−	
28	β-Adrenergic inhibitor	*in vit.*	20	+	−	
29	Coronary dilator	*in vit.*	20	+	−	
30	Cardiotropic	*in vit.*	20	I/C	−/−	
31	Antiedema	*p.o.*	200	>30	5	
32	Hypoglycemic	*p.o.*	200	>20	0	
33	Hypotensive	*p.o.*	100	>10	0, 1, 5	
34	Pressor	*p.o.*	100	>10	−	
35	Platelet agg. adp.	*in vit.*	200	>50	19	
36	Platelet agg. coll.	*in vit.*	200	>50	19	
37	Fat clearing	*p.o.*	200	> 1	0	
38	Antiarrhythmic	i.p.	10	< 2	3	
39	Diuretic	*p.o.*	40	> 2	0.3	
40	Res stimulant	*p.o.*	400	> 0.0	0.014	
41	Anticoagulant	*p.o.*	400	+	−	
42	Antiasthmaic	*p.o.*	200	>50	43	
43	Syst anaphylaxia	*p.o.*	200	< 1	1	
44	Ca-Antagonism	*in vit.*	20	+	−	

a) *In vivo*: doses are in mg/kg, *in vitro*: concentrations are in μg/ml.

TABLE II. The Effects on Triton Secondary Hypercholesterolemia; ZT Decreased the Cholesterol in Mice

Sample	(mg/kg)	Cholesterol (mg %)	Average (%)	Decrease (%)
Control		26, 28, 24, 26		
		28, 26, 29, 31	29.88	
		36, 31, 33, 34		0
		30, 32, 31, 33		
(*E*)-8β,17-Epoxylabd	25	34, 31, 30, 37	33.00	—
12-ene-15,16-dial	50	25, 26, 27, 26	24.75	17[a]
		24, 20, 25, 25		
	100	18, 20, 20, 20	22.50	25[b]
		25, 29, 24, 24		
	200	15, 16, 12, 19	16.00	46[c]
		16, 19, 12, 19		
Atromid	100	18, 19, 20, 23		
		18, 17, 25, 20	22.50	25[b]
		26, 28, 25, 27		
		24, 22, 24, 24		

a) $p<0.05$. *b*) $p<0.01$. *c*) $p<0.001$.

at a 50 mg/kg dose of ZT in oral administration. In this test, the inhibitory rate of atropine (4 mg/kg dose) as a positive control was 70%.

The anti-acetylcholine action of ZT on guinea pig ileum by the Magnus method revealed 1/200 of atropine potential and the anti-serotonin action showed 1/10 of promethazine potential.

Each trial of reflex repression, behavior depression, muscle relaxation and motor stimulation by means of the Irwin method disclosed no score at 600 mg/kg oral dose of ZT.

Furthermore, a neurological trial of antidepressants, antimetrazols, autonomics, anticonvulsants, analgesics, anesthetics, anti-parkinsons, narcotic antagonisms, neuroleptics, anti-cataleptics and anti-ataxics disclosed no significant response.

The effects on the circulatory system such as coronary dilatation, cardiotonic, anti-edema, hypotension, and platelet aggregation (ADP and collagen) were not revealed.

The Inhibitory Effects of ZT on Experimental Hypercholesterolemia Induced by Triton in Mice As shown in Table II, there was no significant difference in the comparison between the control group and the 25 mg/kg oral dose group of ZT.

However, the decrease of serum cholesterol value in mice was found to be dose-dependent at 17% ($p<0.05$), 25% ($p<0.01$) and 46% ($p<0.001$) for doses of 50, 100 and 200 mg/kg, respectively.

Additionally, the same doses of ZT and Atromid as a positive control showed the same efficacy.

The Inhibitory Effects of ZT on Cholesterol Biosynthesis (*in Vitro*) in Rat Liver Using rat liver sections, the effect of ZT on cholesterol biosynthesis was examined.

As shown in Table III, the results indicated a dose-dependent effect as ZT decreased the cholesterol biosynthesis in rat liver.

The Influence on Cholesterol Biosynthesis in Rat Liver after Oral Administration of ZT After oral administration of ZT, the influence on cholesterol biosynthesis in rat liver was examined. As shown in Table IV, ZT dose-dependently decreased cholesterol biosynthesis in rat liver. Examinations both with Tween 80 and without Tween 80 showed suppressive effects, but the former was stronger than the latter.

TABLE III. The Biosynthetic Suppressive Effects of Cholesterol by ZT in the Rat Liver *in Vitro*

Concentration (M)	Biosynthesis of cholesterol (dpm/mg protein)	Inhibitory rate (%)
0	969.1±19.3	—
10^{-6}	835.5±53.7	13.6±6.6
10^{-5}	741.5±17.9[a]	23.4±2.4
10^{-4}	614.2±43.9[a]	36.6±4.5
10^{-3}	606.4±59.0[a]	37.5±5.7
10^{-2}	563.4±45.5[a]	41.6±5.2

a) $p<0.01$.

TABLE IV. Cholesterol Suppressive Effects in the Rat Liver after Oral Administration of ZT

	Dose (mg/kg)	Biosynthesis of cholesterol (dpm/mg protein)	Inhibitory rate (%)
Absence of Tween 80	0	159.0± 7.4	
	100	132.6± 5.7[a]	16.60
	200	113.6± 8.2[b]	28.55
Presence of Tween 80	0	915.2±71.0	
	100	697.5±49.8[a]	23.79
	200	588.8±27.8[b]	35.66

a) $p<0.05$. *b*) $p<0.01$.

Discussion

Following the identification of ZT in ginger, a traditional Chinese medicine, general pharmacological screening tests of ZT were examined.

The serum cholesterol decreasing effect of ZT was revealed by a test on experimental hypercholesterolemia induced by Triton, and its effect had the same potency as that of Atromid, which is the common anti-hypercholesterol agent and positive control in this experiment.

In the normal human body, the amount of serum cholesterol is basically regulated by three factors: cholesterol intake from food, cholesterol biosynthesis in the liver and excretion of cholesterol. Cholesterol biosynthesis is the main factor for the regulation of the body's serum cholesterol level.[12]

Thus, the decreasing effect of ZT on cholesterol biosynthesis in rat liver was studied. The results of *in vivo* and *in vitro* experiments showed an inhibitory effect of ZT on cholesterol biosynthesis. Furthermore, the decreasing effect on the cholesterol biosynthesis after oral administration of ZT proved that ZT was absorbed intact from the intestinal tract and distributed into rat liver. Following incubation with the cholesterol biosynthesis stimulator Tween 80, the cholesterol value in rat liver was higher than that following the incubation without Tween 80. Consequently, the more active the cholesterol biosynthesis, the more noticeable was ZT's effects.

Therefore, ZT is assumed to be an effective compound as an HMG-CoA reductase inhibitor because its structure and radical groups are analogues of compactin and its allied compounds.

On the other hand, compactin type agents have no effect

on the serum cholesterol level in rodents such as rats and mice. But ZT was able to decrease the amount of serum cholesterol in rodents.

According to the general pharmacological screening, it was considered that ZT could be regarded as an anti-hypercholesterolemic agent, since it did not reveal any remarkable effects except for the anti-hypercholesterolemic effect, revealing ZT as a low toxitic compound.

We intend to study the mechanism of ZT in detail as well as HMG-CoA reductase inhibitory activity.

Acknowledgement We are very grateful to Dr. Xue-Hui Zhao for helping us on this experiment.

Reference

1) Lipid Research Clinics Program, *JAMA*, **251**, 351 (1984).
2) Lipid Research Clinics Program, *JAMA*, **251**, 365 (1984).
3) H. Mabuchi, J. Koizumi and M. Takeda, *Coronary*, **4**, 281 (1987).
4) J. Stamler, D. Wentworth and J. D. Neaton, *JAMA*, **256**, 2823 (1986).
5) M. J. Martin, S. B. Hulley and W. S. Browner, *Lancet*, ii, 933 (1986).
6) Y. Kano, M. Tanabe and M. Yasuda, *Shoyakugaku Zasshi*, **44**, 55 (1990).
7) R. A. Turner (ed.), "Screening Methods in Pharmacognology," Academic Press, New York, 1969.
8) X.-H. Zhao and Y. Nomura, *Int. J. Devl. Neuroscience*, **8**, 267 (1990).
9) S. J. Plotch, M. Bouloy and R. M. Krug, *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 1618 (1979).
10) Y. Nomura, I. Yotsumoto and Y. Nishimoto, *Dev. Neurosci.*, **5**, 198 (1982).
11) O. H. Lowry, N. J. Rosebrough, A. L. Parr and R. J. Randall, *J. Biol. Chem.*, **1193**, 265 (1951).
12) D. K. Spady and J. M. Dietschy, *J. Lipid Res.*, **24**, 303 (1983).