Cytotoxicity of the Hinokitiol-Related Compounds, γ -Thujaplicin and β -Dolabrin

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γ -Thujaplicin and β -dolabrin, the constituents of the wood of *Thujopsis dolabrata* SIEB. et ZUCC. var. *hondai* showed strong *in vitro* cytotoxic effects against the human stomach cancer cell lines KATO-III and Ehrlich's ascites carcinoma. The cytotoxic effects of the two compounds against both tumor cell lines were clear when cell growth was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. γ -Thujaplicin and β -dolabrin at 0.32 μ g/ml inhibited cell growth of human stomach cancer KATO-III by 85 and 67%, and Ehrlich's ascites carcinoma by 91 and 75%, respectively. There is no large difference in cytotoxicity between these compounds, but the activity of γ -thujaplicin was slightly more potent than that of β -dolabrin. On the other hand, hinokitiol acetate did not show a cytotoxic effect, suggesting that at least a part of the mechanism of the cytotoxic effect of hinokitiol-related compounds is due to metal chelation between the carbonyl group at C-1 and the hydroxyl group at C-2 in the tropolone skeleton of these molecules. The acute toxicities [50% lethal dose (LD₅₀) value: intraperitoneal injection, Van der Waadem] of γ -thujaplicin and β -dolabrin in mice were 277 mg/kg and 232 mg/kg, respectively.

Key words hinokitiol; γ -thujaplicin; β -dolabrin; cytotoxic effect; human stomach cancer KATO-III; Ehrlich's ascites carcinoma

To date, many antitumor agents have been synthesized or isolated as antibiotics, but all have severe adverse effects due to their strong mammalian toxicities. Therefore, the discovery of less toxic antitumor agents is required. As preliminary step to obtain novel antitumor agents, we have been engaged in searching for cytotoxic activity in natural products. As a result, we have already reported that hinokitiol (β -thujaplicin, Chart 1),¹⁾ the major component of *Thujopsis dolabrata* SIEB. et ZUCC. var. *hondai* MAKINO,²⁾ showed *in vitro* cytotoxic effects on five kinds of human and murine tumor cell lines.³⁾ For example, hinokitiol was found to show an inhibitory effect on a colon 26 cell line with a minimal cytopathogenic concentration of 10 μ g/ml.⁴⁾ In addition, there are several reports on the inhibitory effects of tropolone as hinokitiol and its derivatives having the same skeleton on tumor cells *in vitro*.^{5–8)} However, no work has been done on the effects of γ -thujaplicin and β -dolabrin,²⁾ the constituents of the wood of *T. dolabrata* SIEB. et ZUCC. var. *hondai*.

In this work, as a preliminary step to clarify the antitumor activity of hinokitiol-related compounds, the cytotoxic effects of γ -thujaplicin and β -dolabrin (Chart 1), the constituents of *T. dolabrata* SIEB. et ZUCC. var. *hondai* on cell lines of human stomach cancer cell KATO-III and Ehrlich's ascites carcinoma *in vitro* was investigated, in comparison with hinokitiol. As a basic study on the biological activity of hinokitiol-related compounds in mice, acute toxicity following intraperitoneal administration of the three compounds was also examined.

MATERIALS AND METHODS

Chemicals γ -Thujaplicin, β -dolabrin and hinokitiol were isolated from acid oil obtained by distillation of the wood of *T. dolabrata* SIEB. et ZUCC. var. *hondai* MAKINO according to the method of Nozoe *et al.*,⁹⁾ and used for the cy-

totoxic activity tests. However, as the yield of γ -thujaplicin was too small to permit a cytotoxic activity test, a synthetic version was kindly supplied by Takasago Int. Corp.

Medium Complete RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd. Japan) containing 100 μ g/ml streptomycin and 0.0035 μ l/ml 2-mercaptoethanol were used throughout the study. Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical Co., Ltd. Japan) containing 50 μ g/ml kanamycin sulfate and 4mM L-glutamine were used throughout the study.

Cells Human stomach cancer cells KATO-III were maintained in DMEM and RPMI-1640 (1:1) supplemented by 10% heat-inactivated fetal bovine serum (FBS, Gibco, Grand Island, NY, U.S.A.). Ehrlich's ascites carcinoma cells were maintained in DMEM supplemented by 10% FBS. Each cell line was cultured in each medium at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air in a chamber throughout the study.

Animals Male Slc: ddY strain mice weighting 26–29 g were used for the acute toxicity test.

Cytotoxic Assay Two kinds of tumor cells in the exponential growth phase were plated in 96-well flat-bottom microplates at a density of 3×10^3 cells per 100 μ l in each well and grown for 24 h in each medium. After removal of medium, 100 μ l of fresh medium with various concentrations

Chart 1

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of test compounds was added. After 72 h of culture, cell growth was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.¹⁰ Test compounds were dissolved in DMSO and diluted in complete medium at 0.32–20 $\mu\text{g/ml}$. The final concentrations (0.0012–0.08% in complete medium) of DMSO did not influence the cell growth of the three cell lines (data not shown).

Acute Toxicity Test The acute toxicity of γ -thujaplicin, β -dolabrin, hinokitiol, to mice was investigated according to the following method. The mice were divided into groups of 5 mice each. The three compounds were suspended in 5% gum arabic saline solution and intraperitoneally administered. Mortality was followed for 8 d after administration. The LD_{50} was calculated according to the Van der Waerden method.

RESULTS AND DISCUSSION

The cytotoxic activity of γ -thujaplicin, β -dolabrin and hinokitiol on the growth of cell lines of human stomach cancer KATO-III and Ehrlich's ascites carcinoma was examined *in vitro*. These compounds showed strong cytotoxic activities against both kinds of tumor cell lines. As shown in Fig. 1, the growth of both tumor cell lines by test compounds, except for γ -thujaplicine, was suppressed in a concentration-dependent fashion. γ -Thujaplicin, β -dolabrin and hinokitiol at 0.32 $\mu\text{g/ml}$ inhibited cell growth of human stomach cancer KATO-III by 85, 67 and 54%, and Ehrlich's ascites carcinoma by 91, 75 and 58%, respectively. There is no large difference in the cytotoxicity between the three compounds. Among hinokitiol-related compounds, hinokitiol has already been reported to exhibit strong cytotoxic effects on established tumor cell lines such as colon 26, RK and MDCK cells, and minimal cytopathogenic concentration was reported to be around 10 $\mu\text{g/ml}$.⁴ We previously reported that hinokitiol and tropolone showed strong cytotoxic effects *in vitro* on the growth of murine and human tumor cells lines, including RL- δ , MH124, HL60, K562 and KATO-III, and the IC_{50} values for all cell lines were 0.3–0.6 $\mu\text{g/ml}$.³ In our present experiment, in addition to hinokitiol^{3,4} and tropolone derivatives,^{5–8} β -dolabrin and γ -thujaplicin were also found

to show a strong cytotoxic effect *in vitro*.

On the other hand, hinokitiol acetate did not show the same activity (data not shown). These facts suggest that, like other biological activities,^{11,12} at least a part of the mechanism of the cytotoxic effect of hinokitiol-related compounds is due to metal chelation between the carbonyl at C-1 and the hydroxyl group at C-2 in the tropolone skeleton.

As mentioned above, hinokitiol and tropolone have already been reported to show broad and potent cytotoxic activity on various tumor cells.^{3,4} However, the *in vitro* cytotoxic effects of γ -thujaplicin and β -dolabrin are reported for the first time in this paper. Considering that strong cytotoxic activity was found not only with hinokitiol, but also with γ -thujaplicin and β -dolabrin. The activity against various tumor cell lines warrants further investigation.

Control mice gained in weight from 27.4 ± 0.57 g (mean $\pm$ S.E.) to 32.8 ± 0.74 g by administration of 5% gum arabic saline solution at 8 d after the administration. The mortality of mice following intraperitoneal administration of hinokitiol, β -dolabrin and γ -thujaplicin was examined and results are shown in Table 1. When 325 mg/kg of γ -thujaplicin or 310 mg/kg of β -dolabrin were administered, convulsive attack developed at approximately 10 min after administration and all mice died within one hour. In groups given 310 mg/kg of γ -thujaplicin, 295 mg/kg of β -dolabrin or 240 mg/kg of hinokitiol, reduction in voluntary movement began about 15 min after administration and crouching accompanied by eye-closing developed. The animals began to die approximately 30 min later. Although the body weight gain of surviving mice administered each concentration of the three compounds was somewhat lower compared to control mice, there was no significant difference in body weight change between control mice and mice administered compounds (Table 2). The toxicity profiles of the three compounds in mice are summarized in Table 1. The LD_{50} values of γ -thujaplicin, β -dolabrin and hinokitiol were found to be 277 mg/kg, 232 mg/kg and 191 mg/kg, respectively. It should be emphasized that although the cytotoxic effects of γ -thujaplicin and β -dolabrin were stronger than that of hinokitiol, the acute toxicity of the former to mice was weaker than that of the latter. However, the reason for this is not clear at present.

Although the acute toxicity of hinokitiol was already re-

Fig. 1. Effects of γ -Thujaplicin (▨), β -Dolabrin (■), and Hinokitiol (□), on Cell Growth of Human Stomach Cancer KATO-III (A) and Ehrlich Ascites Carcinoma (B)

Values are expressed as % of control and represent the mean $\pm$ S.E. ($n=4$). * $p < 0.05$, ** $p < 0.01$ vs. control.

Table 1. Toxicity Profile of Mice Injected with Hinokitiol, β -Dolabrin and γ -Thujaplicin

Hinokitiol								
Dose (mg/kg)	Mortality (%)							
	1	2	3	day 4	5	6	7	8
240	80	100	100	100	100	100	100	100
225	60	80	80	80	80	80	80	80
210	60	60	60	60	60	60	60	80
195	60	60	60	60	60	60	60	60
180	20	20	20	40	40	40	40	40
165	0	0	20	20	20	20	20	20
150	0	0	0	0	0	0	0	0
LD ₅₀ : 191 mg/kg								
β -Dolabrin								
Dose (mg/kg)	Mortality (%)							
	1	2	3	day 4	5	6	7	8
310	100	100	100	100	100	100	100	100
295	80	80	80	80	80	80	80	80
280	80	80	80	80	80	80	80	80
265	60	60	60	60	60	60	60	60
250	20	60	80	80	80	80	80	80
235	20	40	40	40	40	40	40	40
220	20	40	40	40	40	40	40	40
205	0	20	20	40	40	40	40	40
190	0	20	20	20	20	20	20	20
175	0	0	0	0	0	0	0	0
LD ₅₀ : 232 mg/kg								
γ -Thujaplicin								
Dose (mg/kg)	Mortality (%)							
	1	2	3	day 4	5	6	7	8
325	100	100	100	100	100	100	100	100
310	80	80	80	80	80	80	80	80
295	60	60	60	60	60	60	60	60
280	60	60	60	60	60	60	60	60
265	40	40	40	40	40	40	40	40
250	20	20	20	20	20	20	20	20
235	0	0	0	0	0	0	0	0
LD ₅₀ : 277 mg/kg								

Conditions are as described in the text.

ported by Lee¹³⁾ and Nakano,¹⁴⁾ the activities of γ -thujaplicin and β -dolabrin are reported for the first time. The LD₅₀ value of hinokitiol in the present work [191 mg/kg, animals: ddY strain mice (male), route: intraperitoneal injection] was much lower than in previous papers (395±45 mg/kg¹³⁾ and 760 mg/kg¹⁴⁾). The reasons are thought to be due to differences in the test animals (mongrel mouse)^{13,14)} and route of administration (sub-cutaneous injection¹³⁾ and per oral administration¹⁴⁾). The acute toxicities of hinokitiol, γ -thujaplicin and β -dolabrin in mice were much lower than those of podophyllotoxine (LD₅₀: 5–30 mg/kg, i.p.),¹⁵⁾ vincristine sulfate (LD₅₀: 3.0±0.44 mg/kg, i.p.)¹⁶⁾ and vinblastine sulfate (LD₅₀: 2.7±0.5 mg/kg, i.p.),¹⁷⁾ compounds with cytotoxic activity isolated from higher plants.

Studies on antitumor activity of the three compounds in

Table 2. Body Weight of Mice Injected with Hinokitiol, β -Dolabrin and γ -Thujaplicin

Hinokitiol		
Dose (mg/kg)	<i>n</i>	Body weight
240	0	—
225	1	31
210	1	31
195	2	32.0±1.41
180	3	32.0±0.71
165	4	32.3±0.73
150	5	32.2±0.82
β -Dolabrin		
Dose (mg/kg)	<i>n</i>	Body weight
310	0	—
295	1	30
280	1	31
265	2	31.0±1.41
250	1	31
235	3	31.3±0.86
220	3	31.7±0.41
205	3	31.7±1.08
190	4	32.0±0.82
175	5	32.4±0.84
γ -Thujaplicin		
Dose (mg/kg)	<i>n</i>	Body weight
325	0	—
310	1	30
295	2	31.5±2.12
280	2	32.0±1.41
265	3	31.7±0.81
250	4	32.0±0.75
235	5	32.0±0.61

n: number of surviving mice at 8 d after body weight are the mean±S.E. Other conditions are as described in the text.

mice are in progress, together with the mechanism of their cytotoxic effects.

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