Cell Growth Inhibitory Effect of Cinnamic Acid Derivatives from Propolis on Human Tumor Cell Lines

Yukihiro AKAO,*[,a] Hiroe MARUYAMA,[b] Kenji MATSUMOTO,[a] Kenji OHGUCHI,[a] Kumi NISHIZAWA,[a] Takashi SAKAMOTO,[b] Yoko ARAKI,[b] Satoshi MISHIMA,[b] and Yoshinori NOZAWA[a]

[a] *Gifu International Institute of Biotechnology; Kakamigahara, Gifu 505–0838, Japan: and* [b] *API Company Research Institute; Shinhonmachi, Gifu 500–8558, Japan.*

Received February 17, 2003; accepted May 8, 2003

A cell growth inhibitory effect of drupanin and baccharin, ingredients of propolis, was found in human cancer cell lines. These compounds induced apoptosis in the cells characterized by morphological and nucleosomal DNA fragmentation analysis. Their effects were less potent compared with that of artepillin C, which is a known anticancer compound from propolis. Importantly, HL60 cells were more sensitive to drupanin than were Con A-stimulated peripheral blood lymphocytes, whereas the potency of artepillin C was the opposite of that of drupanin.

Key words propolis; drupanin; cell growth inhibition; apoptosis; human tumor cell

Propolis is a sticky mixed substance that is collected from plant materials by honeybees.[1)] It has been considered that propolis is a protective wall against the enemies of bees. Propolis has been uesd as a folk medicine in Europe and Japan, and it is believed that propolis exerts a therapeutic or preventive effect in inflammation, heart disease, and even diabetes mellitus and cancer. Chemical analysis using GC-mass spectrometry demonstrated that approximately 150 polyphenolic compounds including flavonoids and cinnamic acid derivatives are present in propolis.[2)] There have been several reports indicating various biological activities of propolis and its constituents, such as anticancer,[3,4)] antioxidant,[5)] antiinflammatory[6)] and antibiotic[7)] activities.

Evaluation of the biological activities of ingredients in propolis and elucidation of the mechanisms of their functions provide substantial clues for the development of new drug candidates. In the course of phytochemical studies on biologically active compounds from propolis, we examined the anticancer activity of the ethanol extract fraction from propolis and found that two cinnamic acid derivatives showed growth inhibitory activity against human tumor cell lines. It has been reported that artepillin C (3,5-diprenyl-4-hydroxycinnamic acid) from propolis exhibits antitumor activity by induction of apoptosis in human tumor cell lines *in vitro*.[8,9)] In the present study, we demonstrated cell growth inhibition by drupanin and (*E*)-3-prenyl-4-(2,3-dihydrocinnamoyloxy) cinnamic acid, named baccharin by us, in human tumor cell lines and compared them with artepillin C.

Brazilian propolis was extracted with 90% ethanol (EtOH) at room temperature to yield the extract. The EtOH extract was chromatographed over silica gel and the column (70 mm i.d.×330 mm) was eluted stepwise with 2—70% $CHCl_3$–methanol (MeOH). The 11 $CHCl_3$–MeOH-eluted fractions were evaporated and then solubilized with 100% EtOH. The fractions were examined for cell growth inhibition and the fractions showing growth inhibitory effect against human tumor cell lines were further analyzed with HPLC (column: Shiseido AG120; gradient system-A solvent: 20% CH_3CN/2% AcOH and B solvent: 100% CH_3CN/2% AcOH; 20—100%; 0—60 min; flow rate: 1 ml/min; detection: UV 280 nm). After purification of the active compounds, their structures were determined by nuclear magnetic resonance (^{1}H-NMR) analysis. The human leukemia and cancer cell lines were grown in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum under an atmosphere of 95% air and 5% CO_2 at 37 °C. Cell viability following the treatment with the compounds was determined using the trypan blue dye-exclusion test. The cells were seeded at a concentration of 2×10^5/ml. For examination of apoptosis induced by baccharin and drupanin, Hoechst 33342 nuclear staining (5 μg/ml) at 37 °C for 30 min and DNA fragmentation analysis by agarose gel electrophoresis were performed. Differences were statistically evaluated by analysis of variance followed by Sheffe's *F*-test. A p value of less than 0.05 was considered to be statistically significant.

We found that the fractions 5 and 7 of ethanol extract exhibited a cell growth inhibitory effect against colon cancer cell line SW480 for 72 h at a dose of 50 μg/ml. The active compounds were identified to be artepillin C and baccharin in fraction 5, and drupanin in fraction 7 by HPLC (Fig. 1), and NMR analysis which showed the spectra consistent with those previously reported.[10,11)] The structures of these three compounds are shown in Fig. 2. Artepillin C, baccharin, and drupanin were found to be contained in propolis in proportions of approximately 10, 4, and 3%, respectively.

When cells were exposed to these compounds at concentrations between 30 and 200 μM, the growth of all cell lines including human gastric and colon cancer cell lines and leukemia cell lines was markedly inhibited at 150 μM (Table 1). Figure 3 shows the growth curves of SW480 and HL60 cells treated with the compounds at 150 μM, which were relatively sensitive to the compounds. The order of growth inhibitory activity was artepillin C>baccharin>drupanin. The IC_{50} of artepillin C, baccharin, and drupanin in HL60 cells

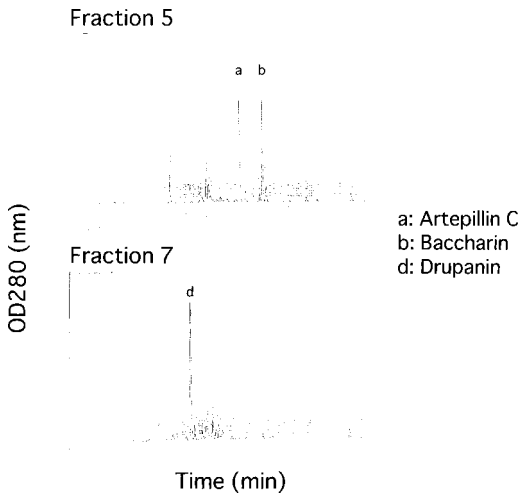

Fig. 1. HPLC Profile of the Ethanol Elute after Extraction of the Components from Propolis and Chromatographic Separation

* To whom correspondence should be addressed. e-mail: yakao@giib.or.jp

(starting cell number; 2×10^5/ml) was 51.1, 78.8, and 85.6 μM, respectively (Table 2). We then examined whether the growth inhibitory activity is due to apoptosis and observed the typical morphologic characteristics of apoptosis, such as nuclear condensation and fragmentation with Hoechst 33342 staining in SW480 and HL60 cells treated with baccharin or drupanin 150 μM for 48 h (Fig. 4A). In the same samples, nucleosomal DNA ladder formation was also observed during the 24 h following treatment (Fig. 4B). The period of appearance and the intensity of DNA ladders which reflect apoptosis-inducing activity, were virtually corrected with the order of potency of cytotoxicity: artepillin C>baccharin>dru-

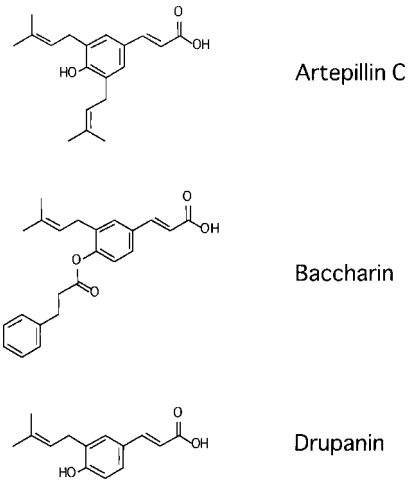

Fig. 2. Chemical Structure of Artepillin C, Baccharin, and Drupanin

Table 1. Comparison of Cell Growth Suppression by Artepillin C, Baccharin, and Drupanin

Cell line		Artepillin C (μM)		Baccharin (μM)		Drupanin (μM)	
		30	150	30	150	30	150
Colon cancer	SW480	+	+++	+	+++	+	++
	DLD-1	−	++	−	+	−	+
	COLO201	+	++	−	+	−	+
Gastric cancer	MKN1	−	+++	−	++	−	++
	MKN28	−	+++	−	+	−	+
	MUGC4	−	+++	−	+	−	+
Leukemia	HL60	+	+++	+	++	+	++
	NB4	+	+++	−	++	+	++
	K562	−	+	−	+	−	−
	U937	−	+	−	+	−	+

The cells were cultured for 72 h and cytotoxicity evaluated. +, 0—50%; ++, 50—75%; +++, 75—100%; −, no effect.

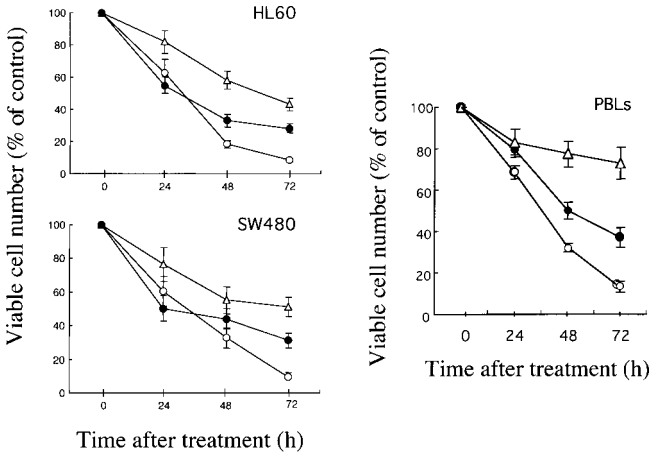

Fig. 3. Effects of Artepillin C (○), Baccharin (●), and Drupanin (△) on Cell Growth of the Human Leukemia Cell Line HL60, Colon Cancer Cell Line SW480, and Mitogen-Stimulated Human Blood Lymphocytes at the Concentration of 150 μM

Human peripheral blood lymphocytes (PBLs) from healthy donors were stimulated with concanavalin A (75 μg/ml) for 48 h and used for control and growth suppression of the compounds. Viable cell numbers of the cells after treatment with artepillin C, baccharin, and drupanin were evaluated by the trypan blue dye-exclusion test and expressed as a percentage of the number in untreated cultures (control). The values represent the mean±S.D. of three independent experiments, each carried out in duplicate. Differences of ones from another are statistically significant at $p<0.01$.

Table 2. IC_{50} Values of Artepillin C, Baccharin, and Drupanin against HL60 and Con-A-Stimulated PBLs

Cell	Artepillin C	Baccharin	Drupanin
HL60	51.1±6.2	78.8±7.2	85.6±7.8
PBLs	19.2±2.1	70.3±6.8	164.8±10.1

PBLs, Con-A-stimulated peripheral blood lymphocytes. The starting number of cells was 2×10^5/ml. The values represent the mean±S.D. of three independent experiments.

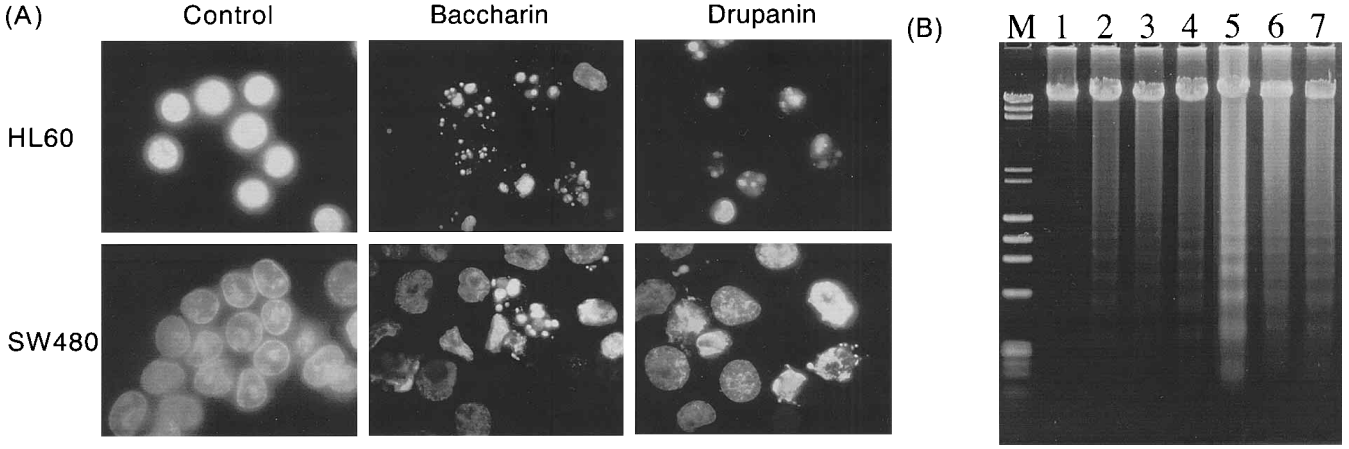

Fig. 4. Apoptosis Induced by Baccharin and Drupanin in SW480 and HL60 Cells

(A) Morphological aspects of the cells. The cells were stained with Hoechst 33342 (5 μg/ml) for 30 min and then observed under fluorescence microscopy. Left panel, cells before treatment; center and right panels, after treatment with each compound at 150 μM for 48 h in each cell line. (B) Nucleosomal DNA fragmentation of HL60 cells after exposure to artepillin C, baccharin, or drupanin 150 μM. Three micrograms of DNA was loaded into each lane. Lane 1, cells before treatment; lanes 2, 3, and 4, cells treated with artepillin C, baccharin, and drupanin for 24 h, respectively; lanes 5, 6, and 7 cells treated with artepillin C, baccharin, and drupanin for 48 h, respectively. Lane M is a DNA size marker.

panin. Thus these findings indicate that drupanin and baccharin inhibited cell growth through the induction of apoptosis in tumor cell lines. Considering the application of both compounds in chemopreventive medicine, we examined their effects on normal human peripheral blood lymphocytes (PBLs) stimulated with concanavalin A (Con-A) in comparison with those of artepillin C. As shown in Fig. 3 and Table 2, although drupanin exhibited no marked growth inhibition at lower doses than 150 μM, artepillin C markedly decreased cell number at the same concentration. Drupanin was observed to be more potent against HL60 cells than against Con-A-stimulated PBLs, whereas the potency of artepillin C was the opposite to that of drupanin.

In the present paper, we first showed that two cinnamic acid derivatives from propolis, baccharin and drupanin, exhibited antitumor activity *in vitro* at doses higher than 30 μM and that the effect is due to apoptosis in human tumor cell lines. It is speculated that the tertiary structures including the prenyl group may contribute to the activity. The anticancer activity of baccharin and drupanin was slightly less than that of artepillin C in HL60 cells. Artepillin C has been reported to exert antitumor activity by induction of apoptosis in human tumor cell lines *in vitro* and *in vivo*[8,9,12,13] and to be safe in animal models.[12,13] However, the cytotoxic effect of drupanin on normal blood lymphocytes stimulated with Con-A was found to be less than that of artepillin C. To evaluate the chemopreventive potency, it should be explored whether these two compounds prevent tumor occurrence or inhibit tumor growth in animal experiments, which are in progress in our laboratory.

REFERENCES

1) Walker P., Crane E., *Apidologie*, **18**, 327—334 (1987).
2) Greenaway W., May J., Scaysbrook T., Whatley F. R., *Z. Naturforsch.*, **46**, 111—121 (1991).
3) Scheller S., Krol W., Swiacik J., Owczarek S., Gabrys J., Shani J., *Z. Naturforsch.*, **44**, 1063—1065 (1989).
4) Grunberger D., Banerjee R., Eisinger K., Oltz E. M., Efros L., Caldwell M., Esterez V., Nakanishi K., *Experientia*, **44**, 230—232 (1988).
5) Pascual C., Gonzalez R., Torricella R. G., *J. Ethnopharmacol.*, **41**, 9—13 (1994).
6) Wang L., Mineshita S., Ga I., Shigematsu T., Matsuno T., *Jpn. J. Pharmacol. Ther.*, **24**, 223—224 (1993).
7) Kujumgiev A., Bankova V., Ignatova A., Popov S., *Pharmazie*, **48**, 785—786 (1993).
8) Matsuno T., Jung S. K., Matsumoto Y., Saito M., Morikawa J., *Anticancer Res.*, **17**, 3565—3568 (1997).
9) Kimoto T., Aga M., Hino K., Koya-Miyata S., Yamamoto Y., Micallef M. J., Hanaya T., Arai S., Ikeda M., Kurimoto M., *Anticancer Res.*, **21**, 221—228 (2001).
10) Shimitt A., Telikepalli H., Mitscher L. A., *Phytochemistry*, **30**, 3569—3570 (1991).
11) Aga H., Shibuya T., Sugimoto T., Kurimoto M., Nakajima S., *Biosci. Biotechnol. Biochem.*, **58**, 945—946 (1994).
12) Kimoto T., Koya S., Hino K., Yamamoto Y., Nomura Y., Micallef M. J., Hanaya T., Arai S., Ikeda M., Kurimoto M., *Pathol. Int.*, **50**, 679—689 (2000).
13) Kimoto T., Koya S., Hino K., Micallef M. J., Hanaya T., Arai S., Ikeda M., Kurimoto M., *Virchows Arch.*, **438**, 259—270 (2000).