Two Related Cinnamic Acid Derivatives from Brazilian Honey Bee Propolis, Baccharin and Drupanin, Induce Growth Inhibition in Allografted Sarcoma S-180 in Mice

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Honey bee propolis is rich in cinnamic acid derivatives. Baccharin and drupanin from Brazilian honey bee propolis are cinnamic acid derivatives that contain prenyl moieties. We previously isolated these two compounds and demonstrated that they induce an apoptotic event in several tumor cell lines. In this study, we examined the tumoricidal activity of baccharin and drupanin in mice allografted with sarcoma S-180 and also studied the genotoxic effects on normal splenocytes using the alkaline single cell gel (comet) assay. We found that both baccharin and drupanin effectively suppressed growth of the tumor. Furthermore, these compounds induced a significant genotoxic effect on the tumor cells in comparison with normal splenocytes. Thus, baccharin and drupanin are potent tumor suppressive components of honeybee propolis.

Key words cinnamic acid derivative; baccharin; drupanin; cytotoxicity *in vivo*; genotoxity

Propolis, a resinous aromatic substance collected by honeybees from the bud or bark of certain coniferous trees, has long been used in folk medicine. Several studies on the isolated chemical components of propolis have reported a number of biological responses including antibacterial, anti-fungal, anti-inflammatory and anti-tumor activities. In particular, 3,4-dihydroxycinnamic acid phenetyl ester (CAPE) displays various potent anti-tumor effects.[2,3] CAPE suppresses TPA-induced tumor promotion,[4] adenovirus E1A-mediated transformation[5] and COX-2 expression,[6] which contributes to neo-angiogenetic effects in the *in situ* tumor mass[3] and inhibits immune responses.[7,8] Cinnamic acid also exhibits anti-tumor activity, although somewhat weaker than CAPE.[9] It is possible that other as yet unidentified chemical components of propolis, including cinnamic acid derivatives also exert anti-tumor activity.

Baccharin and drupanin among the cinnamic acid derivatives which have prenyl moieties have been reported to possess tumoricidal activity and induce apoptosis against the myelocytic leukemia cell line HL60.[10] Apoptosis plays an important role in the tumorcidal activity of chemotherapeutic agents.[11] Baccharin and drupanin effectively kill leukemic cells through the apoptotic process, but the tumoricidal effects on other tumor cell lines have not been tested.

Here we studied the *in vitro* tumoricidal activity of these two derivatives against various tumor cell lines. For *in vivo* experiments baccharin and drupanin were separately administered orally to DDY mice allografted with mouse sarcoma S-180. The results indicate that both compounds have *in vitro* tumoricidal activity against various cell lines and also *in vivo* activity against allografted sarcoma S180. Moreover, although baccharin and drupanin effectively induce a genotoxic effect on sarcoma S-180, normal splenocytes are less affected.

MATERIALS AND METHODS

Materials Baccharin and drupanin were extracted as described previously.[2] In short, Brazilian propolis (Minas Gerais green propolis) was extracted with 90% ethanol and subjected to chromatography over silica gel, evaporated and then solubilized in 100% ethanol. The ethanol extracts were evaporated to dryness and kept at −20 °C.

Cell Culture All cell cultures were performed at 37 °C in an atmosphere of 5% CO_2. We used 6 human tumor cell lines in this study: NCI-H460 (non-small cell lung cancer), MCF7 (breast cancer), PC3 (prostate cancer) and LNCaP (prostate cancer) as adherent (solid) tumor cells, and HL60 (myelocytic leukemia) and U937 (lymphoma) as non-adherent (non-solid) tumor cells. We also used a mouse transplantable tumor cell line, sarcoma S-180 to compare *in vitro* and *in vivo* testing. The tumor cell lines (NCI-H460, MCF7, U937, HL60, S-180) were purchased from the American Type Culture Collection (Manassas, VA). LNCaP and PC3 were supplied by Dr. T. Deguchi (Graduate School of Medicine, Gifu University, Japan). All cell lines were maintained and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin.

Normal mouse splenocytes were prepared as follows. Six to 8 week-old male mice were used in all experiments. Total cell suspension was prepared by mincing spleens with frosted slide glasses and red blood cells were eliminated from the cell suspension by treatment with hemolytic buffer (150 mM ammonium chloride, 149 mM PBS) for 2 min. After centrifugation, cells were washed twice with PBS and resuspended in RPMI 1640 supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. After stimulation with 2 μg/ml concanavalin A for 48 h, splenocytes were used for the experiments.

Analysis of Cell Proliferation Cell proliferation assay was performed as described by Monks *et al.*[12] Briefly, cells were harvested and plated at 75—80% confluence, detached by mild trypsinization for adherent cell lines NCI-H460, MCF7, PC3 and LNCaP. Cells were seeded in 100 μl of RPMI 1640 in each well of 96-well flat-bottomed microplates and incubated. Baccharin and drupanin (500 mM in DMSO) were serially diluted with RPMI 1640 (1 : 2 dilution) and added to each well in triplicate. Solvent control (DMSO) was confirmed to produce no cytotoxic effects. After 48 h in-

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cubation, a TCA-treated sample (50 μl of 50% TCA for adherent cell line or 50 μl of 80% TCA for non-adherent cell lines) was subjected to *in situ* fixation and cells were further incubated for 1 h at 4 °C. The supernatant was discarded and cells were washed with distilled water 3 times. The microplates were dried at room temperature and Sulfo Rhodamine B (SRB) solution (0.4% SRB in 1% acetic acid) was added and incubated for 10 min. Unbound SRB supernatant was then discarded and each well was washed with 1% acetic acid 5 times and air-dried. Tris base (10 mM) was added to each well and the sample was dissolved thoroughly. The absorbance at 515 nm of the samples was measured using a microplate (ELISA) reader. All assays were performed in triplicate and mean values were expressed. GI_{50}, TGI and LC_{50} parameters were calculated according to Monks *et al*.[12]

Animals Male 5 week-old DDY mice were purchased from Nihon SLC (Hamamatsu, Japan). All animals were fed a standard diet (Charles River) and given distilled water *ad libitum*. The test groups (n=6 to 7) were given baccharin or drupanin orally at 100 mg/kg/d (0.4 ml). The control group (n=6) was given 0.4 ml of the vehicle solution (5% arabic gum solution) per day. After 28 d of treatment, animals were sacrificed by exsanguination under ether anesthesia. The entire tumor mass was dissected and immediately placed in PBS (pH 7.2) which had been pre-cooled to 4 °C to avoid dryness. The tumor mass and body were weighed. All experiments were performed according to the National Research Council Guide for the Care and Use of Laboratory Animals.[13] Data were expressed as means±S.E. One-way ANOVA test was used to assess the difference in the tumor mass and body weight between the treatment and control groups.

Comet (Single Cell Gel Electrophoresis (SCGE)) Assay The comet assay was performed according to Olive *et al*.[14] with slight modification. Frosted slides were precoated with a thin layer of normal electrophoresis-grade agarose and allowed to dry. Single cell suspensions (3.5×10^4 cells) were added to 100 μl of 0.5% low melting point agarose gel solution. An aliquot of 75 μl was pipetted onto a precoated slide. After solidification, the slides were placed in an alkaline lysis solution (30 mM NaOH, 1.2 M NaCl, 1% *N*-laurylsarcosine) and the cells were lysed in the dark at room temperature for 1 h. Slides were then immersed for 1 h in an alkaline solution (30 mM NaOH, 2 mM EDTA). After rinsing with TBE (8.9 mM Tris Base, 8.9 mM boric acid and 2.5 mM EDTA), slides were placed in a horizontal electrophoresis chamber filled with fresh TBE and then subjected to electrophoresis at 1 V/cm for 10 min. After electrophoresis, the slides were rinsed with distilled water and stained for 20 min in a 2.5 μg/ml propidium iodide in 0.1 M NaCl. Slides were rinsed again, dehydrated with 70% ethanol and dried in a lightproof container at 4 °C until analysis. Samples were viewed by fluorescence microscopy (excitation at 488 nm) and photographed. For evaluation of the comet moment, 100 randomly selected cells were analyzed with the Scion image Beta 4.0.2 loaded macro-program provided on the internet by Dr. Herbert M Geller (at http://dir.nhlbi.nih.gov/labs/ldn/macroanalysis.asp).

Immunoblotting Whole cell lysates were prepared by lysing cells in RIPA (PBS, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 40 μg/ml PMSF, 0.5 μg/ml aprotinin, 0.5 μg/ml pepstatin, 1 mM EDTA, 0.5 μg/ml pepstatin and 10 mM sodium fluoride). The protein concentration of cell lysate was determined by protein assay CBB kit (Nacalai, Japan). About 50 μg of protein was separated by electrophoresis on 10% SDS-polyacrylamide gel. Gels were electroblotted to nitrocellulose membranes for 1 h at 2 mA/cm^2 using a semi-dry transfer apparatus. The primary antibody was anti pp53 (ser-15) rabbit antibody (sc-11764-R, Santa Cruz Biotechnology) and the secondary antibody was HRP-conjugated anti-rabit IgG antibody (W401B, Promega). Immunoblots were developed by using the ECL detection system (Amersham Bio-

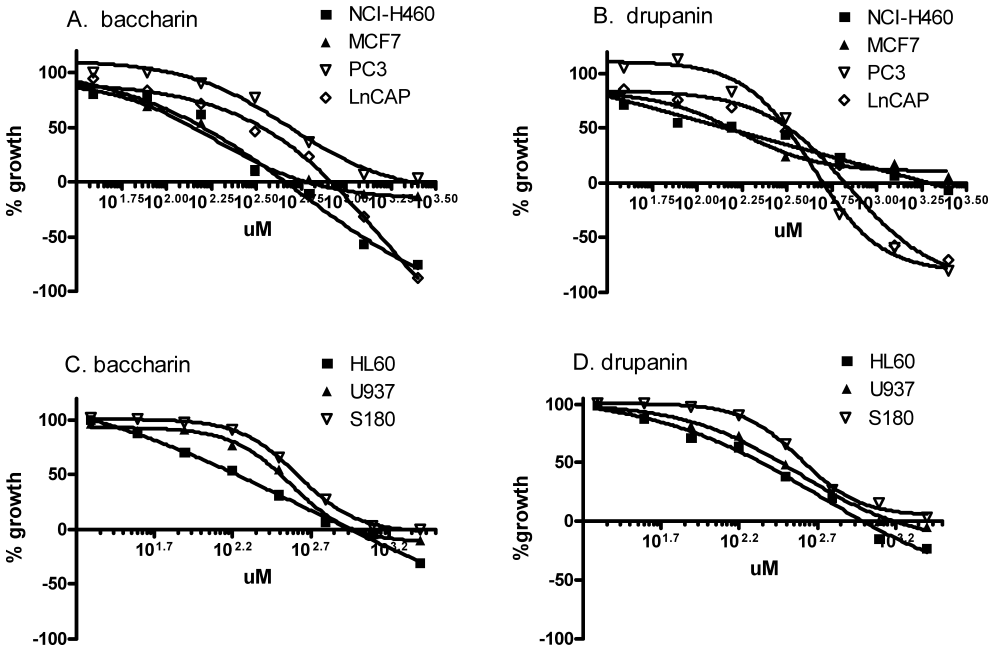

Fig. 1. Dose-Dependent Anti-proliferative Effects of Baccharin and Drupanin

Cells were cultured for 24 h in RPMI1640 supplemented with 10% FCS and exposed to various concentrations of baccharin and drupanin for 48 h. Each point represents the mean±S.E. of 3 independent experiments. (A) Effect of baccharin on adherent type tumor cell lines. (B) Effect of drupanin on adherent type tumor cell lines.

sciences) according to the manufacturer's instructions.

RESULTS

Growth Inhibition of Various Cancer Cells by Baccharin and Drupanin Cinnamic acid shows cytotoxicity toward cancer cell lines.[9] To further assess the growth inhibitory activity of baccharin and drupanin, we exposed various cancer cell lines to different concentrations of each compound for 48 h and examined the dose–response effecs of cell growth (Fig. 1). Baccharin was cytotoxic to NCI-H460 LNCaP and HL60. While drupanin showed cytotoxic effects on PC3 and LnCaP. Overall, both baccharin and drupanin showed growth inhibitory effects on all the cell lines examined, however, the apparent sensitivity of these effects seemed somewhat cell type-dependent as determined by GI_{50}, TGI and LC_{50} values (Table 1), suggesting that each compound has a distinct cellular target for this effect. These results indicate that baccharin and drupanin are potent and effective cytotoxic or growth inhibitory compounds against tumor cells.

Baccharin and Drupanin Reveal Anti-tumor Activity *in Vivo* To investigate *in vivo* antitumor activity of baccha-

Table 1. Growth Inhibitory Effect of Baccharin and Drupanin

A. Cellular Responses to Baccharin

	NCI-H460	MCF7	PC3	LnCaP (μM)	HL60	U937	S-180
GI_{50}	158.5	141.3	501.2	338.8	166.0	323.6	416.9
TGI	457.1	588.8	2290.9	812.8	891.3	933.3	1995.3
LC_{50}	1230.3	N.D.	N.D.	1548.8	N.D.	N.D.	N.D.

B. Cellular Responses to Drupanin

	NCI-H460	MCF7	PC3	LnCaP (μM)	HL60	U937	S-180
GI_{50}	143.9	154.9	319.2	319.2	218.8	288.4	407.4
TGI	1927.5	N.D.	503.5	418.8	977.2	1548.8	N.D.
LC_{50}	N.D.	N.D.	885.1	1331.4	N.D.	N.D.	N.D.

The tumor cell lines were exposed to the various concentrations of baccharin and drupanin for 48 h. The activity of each compound is expressed as the GI_{50}, TGI and LC_{50} values, which indicate the molar concentrations required to cause half growth inhibition, total growth inhibition or net 50% loss of initial cells at the end of incubation period, respectively. N.D.; not determined.

Table 2. The Effect of Oral Administration of Baccharin and Drupanin on Body Weight

	Administration (mg/kg)	*n*	Body weight (g)
Baccharin (100 mg/kg)	100	7	36.4±1.9
Baccharin (30 mg/kg)	30	6	42.1±0.3
Drupanin (100 mg/kg)	100	6	37.3±0
Drupanin (30 mg/kg)	30	6	38.1±1.5
Vehicle	—	6	41.7±3.0

Data shown are mean±S.E.M.

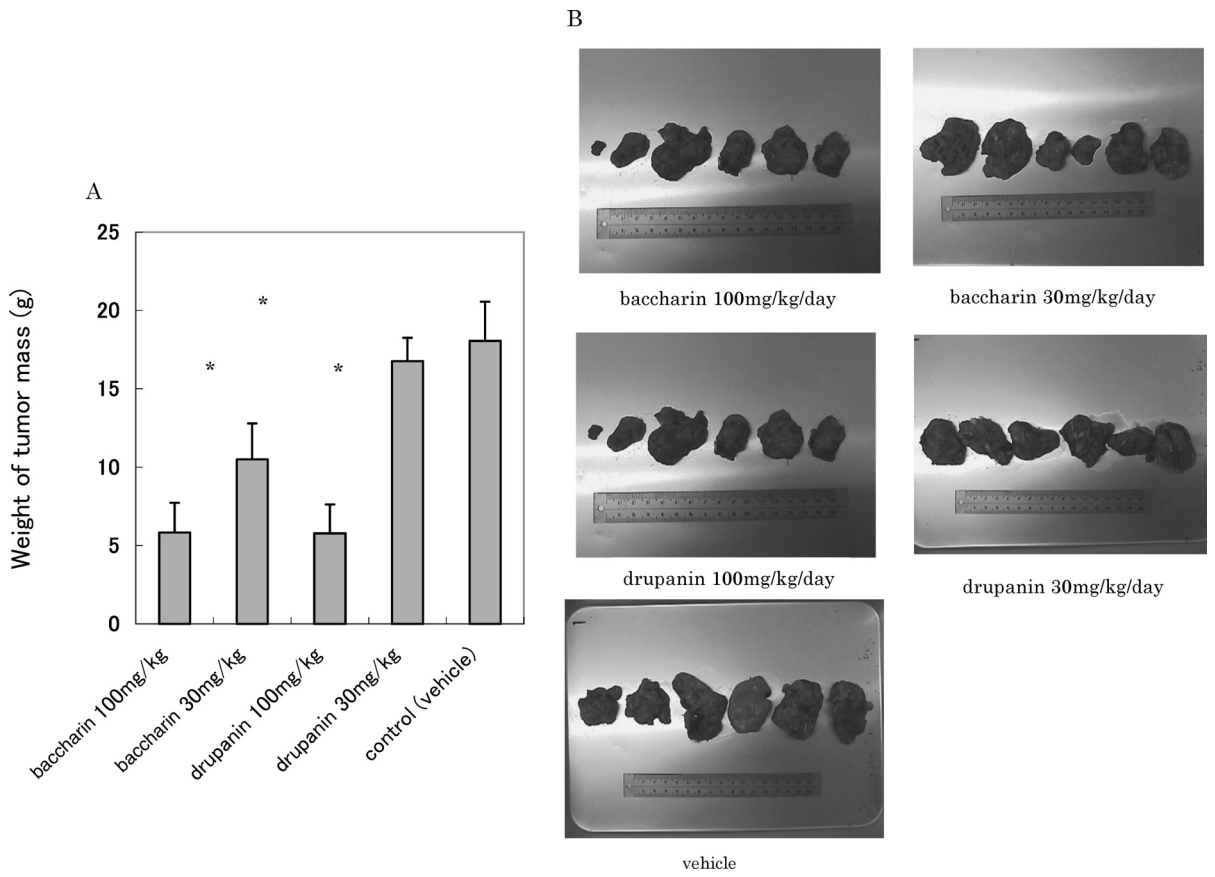

Fig. 2. *In Vivo* Growth Inhibition of Sarcoma S-180 Cells

Sarcoma S-180 cells (1×10^7 cells) were transplanted subcutaneously to male DDY mice. Baccharin or drupanin was orally administered daily at 30 or 100 mg/kg/d in 5% gum arabic solution for 28 d. Each tumor mass was excised and weighed. (A) Comparison of solid tumor mass weights. Data represents the mean±S.E. $n=6$—7. Asterisks denote significant differences at $p<0.05$; data were compared using one-way ANOVA test. (B) Macroscopic profiles of solid tumor mass.

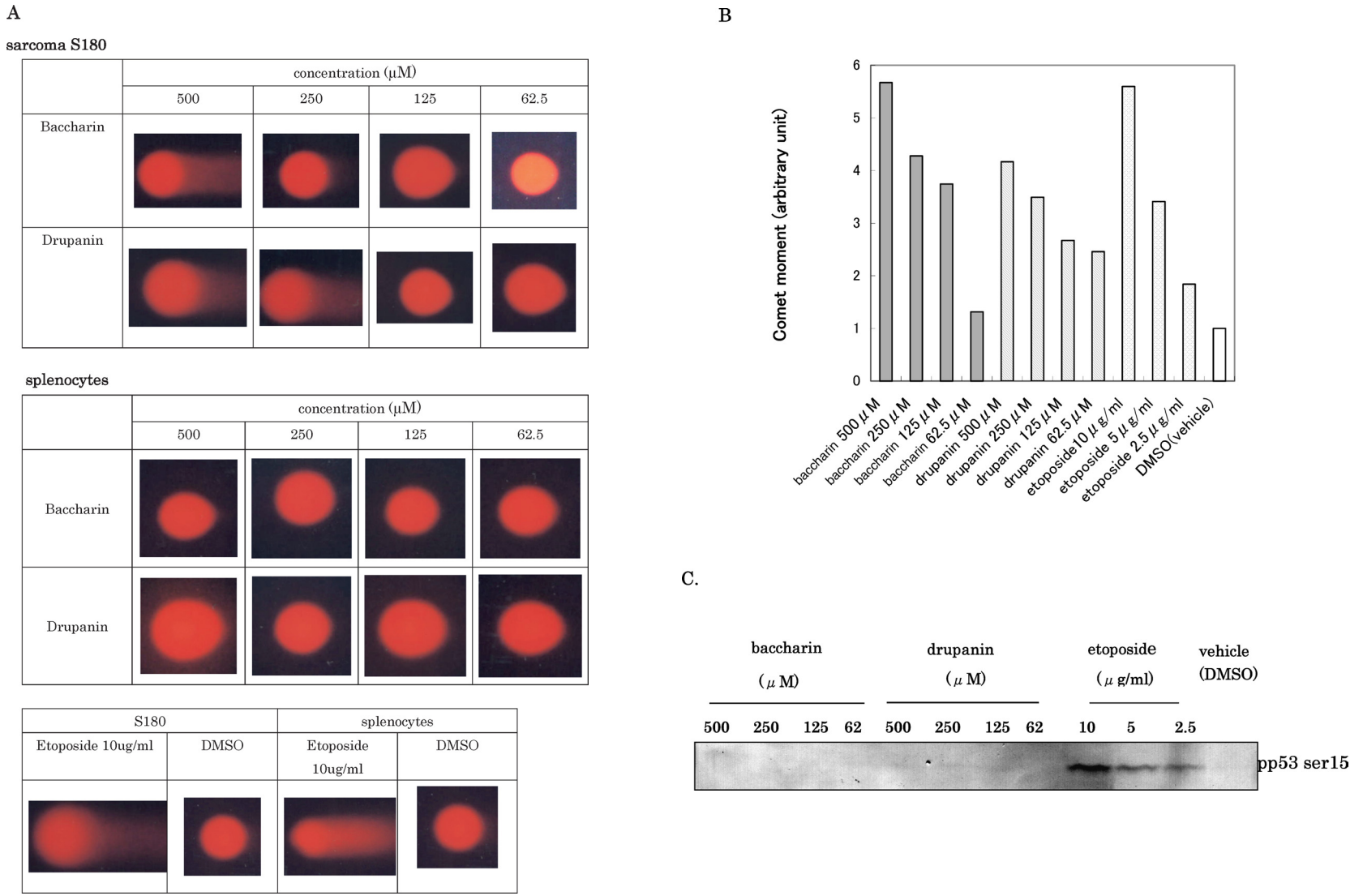

Fig. 3. Detection of Baccharin and Drupanin-Induced DNA Damage by Alkaline Single Cell Gel Electrophoresis (Comet Assay) and Immunoblot Analysis with pp53 (ser-15) Antibody

Sarcoma S-180 cells and splenocytes were exposed to various concentrations of baccharin or drupanin for 30 h. As a positive control, etoposide, a topoisomerase II inhibitor, was used to induce DNA damage, and the DNA damage was determined using the alkaline comet assay as described in Materials and Methods. (A) Typical DNA migration patterns of sarcoma S-180 cells and mouse normal splenocytes. (B) The comet tail moment of sarcoma S-180 cells, which was calculated as the integrated DNA density in the comet tail multiplied by the distance from the center of the nucleus to the center of mass of the tail. The comet tail moment is normalized to the negative control (DMSO-treated cells) and expressed as arbitrary units. (C) Immunoblot analysis of normal splenocytes treated with baccharin and drupanin. The splenocytes were treated as described above. Equal amounts of cell lysate (50 μg of protein per lane) were analyzed by SDS-polyacrylamide gel electrophoresis and subjected to immunoblotting analysis using polyclonal anti-pp53 (ser-15) antibody.

rin and drupanin, we orally administered these compounds to DDY mice allografted with sarcoma S-180. After 28 d of treatment, baccharin significantly suppressed the tumor mass growth at a dosage of either 30 mg/kg/d or 100 mg/kg/d ($p<0.05$) (Figs. 2A, B). The same treatment with drupanin resulted in a significant decrease of tumor mass at a dosage of 100 mg/kg/d ($p<0.05$) but not at 30 mg/kg/d. After excision of the tumor, we examined the body weight of each mouse (Table 2). A slight but not a marked decrease in body weight was observed among the baccharin, drupanin and vehicle administered groups. These results indicate that orally administered baccharin or drupanin inhibits the tumor growth.

Baccharin and Drupanin Induce Genotoxic Damage in S-180 Various anticancer drugs induce apoptosis by irreversible DNA damage leading to cell death. Baccharin and drupanin have been reported to induce apoptosis[2] and we tested whether either of them induces genotoxic damage in sarcoma S-180. Sarcoma S-180 was exposed to baccharin or drupanin and then analyzed by a single cell gel electrophoresis (SCGE) assay under the alkaline condition (Fig. 3A). Etoposide-treated cells were used as a positive control since this agent specifically inhibits nuclear type II topoisomerase. DNA damage caused by etoposide treatment was detected in both sarcoma S-180 and normal splenocytes as shown by comet tailing in the assay procedure (*i.e.* broken DNA strands). Treatment with baccharin or drupanin also caused DNA damage in sarcoma S-180. The degree of cellular DNA damage for all compounds tested was dose-dependent, as measured by the comet tail moment (Fig. 3B). It is of worthy to note that neither baccharin nor drupanin induced DNA damage in normal splenocytes (Fig. 3A). Immunoblot analysis also confirmed that neither compound induced p53 expression whereas etoposide did in normal splenocytes (Fig. 3C). The results suggest that baccharin and drupanin are preferential inducers of cytotoxicity and genotoxicity in tumor cells.

DISCUSSION

In this study we showed the *in vitro* cytotoxicity of cinnamic acid derivatives, baccharin and drupanin, in several human cancer cell lines and *in vivo* tumoricidal activity in mice bearing sarcoma S-180 cells. Oral administration of baccharin (30 mg/kg/d) or drupanin (100 mg/kg/d) for 4 weeks caused a significant reduction in tumor mass growth (Fig. 2). This is the first demonstration that cinnamic acid derivatives exert growth inhibitory effects in an allografted tumor in mice.

It was also shown that both baccharin and drupanin induce a genotoxic effect in sarcoma S-180 cells while normal splenocytes are only marginally affected. Single cell gel electrophoresis (SCGE) assays under alkaline condition confirmed that exposure to *ca.* 62.5 μM baccharin or drupanin significantly induced DNA damage in sarcoma S-180 cells (Fig. 3A). It should be noted that DNA damage was not observed in normal splenocytes after exposure to these compounds under the same condition (Fig. 3A), but the mechanism for this resistance to genotoxicity in normal splenocytes upon exposure to baccharin or drupanin remains to be determined. Many anti-cancer compounds are known to affect normal cells, leading to adverse effects such as suppression of hematopoiesis.[15] In this context, baccharin and drupanin are thought to be safe and advantageous as chemopreventive agents for cancer.

Of interest is that the cytotoxic effect and DNA damage in sarcoma S-180 exposed to baccharin or drupanin were not accompanied by expression of p53 (unpublished data). Several anti-cancer drugs, such as alkylating agents, induce the genotoxic effect and p53 expression in tumor cells leading to apoptotic cell death.[16] Therefore, baccharin and drupanin cause cytotoxic or growth inhibitory effects in tumor cells *via* a mechanism independent of p53. Artepillin C, another cinnamic acid derivative from honeybee propolis, has been reported to induce apoptosis in HL60,[17] however, it remains to be clarified how this is achieved in this cell line. Likewise, baccharin and drupanin also induce apoptosis in leukemic cell lines HL60 cells[10] and U-937 (unpublished data). Further studies are required to define the mechanism by which the cinnamic compounds induce a cytotoxic effect in tumor cells.

Baccharin and drupanin are purified from Brazilian propolis (Minas Gerais green propolis), which is originally gathered from young buds of plants (*Baccharis dracumculifolia*) by honey bees. Brazilian propolis is rich in baccharin and drupanin, the content of which is 2.5% and 1%, respectively (our unpublished data), and contains various cinnamic acid derivatives.[18,19] Other sources of bee propolis (European and Chinese) contain only a small amount of cinnamic acid derivatives, which in turn is rich in flavonoids.[18,20] Thus, Brazilian bee propolis is a natural compounds library of cinnamic acid derivatives and a screening of this for useful compounds can be a good tool to develop medicines or nutritional supplements.

In conclusion, two related cinnamic acid derivatives, baccharin and drupanin, possess *in vivo* tumoricidal activity in mice bearing sarcoma S-180 cells. Furthermore, these compounds may induce tumor cell death, with less genotoxic to normal hematopoietic cells than anti-cancer drugs.

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