Action of penta-acetyl geniposide on aflatoxin B_1-induced DNA repair synthesis in rat primary hepatocytes

Tsui-Hwa Tseng, Jin-Ming Hwang, Chia-Yih Chu and Chau-Jong Wang

Penta-acetyl geniposide [$(Ac)_5$-GP] was isolated from *Gardenia fructus* (San-jee-chee in Chinese) through a chemical modification of crude extract. The present study demonstrated that $(Ac)_5$-GP was a potent compound on inhibiting AFB_1-induced unscheduled DNA synthesis in rat primary hepatocytes. Time course and dose response of $(Ac)_5$-GP on glutathione S-transferase (GST) and dose response on glutathione peroxidase (GSH-Px) in hepatocytes were studied. The GST activity was increased significantly at 3 hr and in the dose of 0.1mM. The GSH-Px activity was increased in all of the test doses (0.02-0.1mM). It showed that enzyme activities of GST and GSH-Px in AFB_1-treated hepatocyte cultures were enhanced significantly in the pretreatment of $(Ac)_5$- GP (0.1mM, $p < 0.01$). From these results, it was supposed that $(Ac)_5$-GP reduced AFB_1-induced DNA repair synthesis via increasing AFB_1 detoxication.

Key words: penta-acetyl geniposide, AFB_1, hepatocyte, DNA repair synthesis, glutathione S-transferase, glutathione-peroxidase.

Introduction

Penta-acetyl geniposide [$(Ac)_5$-GP] was obtained from modified extract of *Gardenia Fructus* [1]. *Gardenia jasminoides* Ellis, a plant in the family of *Rubiaceae*, grows widely in the central and southern parts of Taiwan. Its fruit, *Gardenia fructus*, has been used as a herbal medicine to cure liver and gall bladder disorders, hepatitis, and acute jaundice [2- 5]. The components of *Gardenia Fructus* were categorized into two classes, carotenoids (eg. crocetin) and iridoid glycosides (eg. geniposide). Crocetin was shown to decrease the number of tumors as well as delay the onset of rat and chicken tumors [6,7]. Kimura et al. reported that geniposide reduces serum triglyceride, lipid peroxide and phospholipid in high sugar diet [8]. Lau et al. [9] proposed that geniposide may facilitate the billiary extraction of α -naphthylisothiocyanate and/or its toxic

Institute of Biochemistry, Chung Shan Medical and Dental College, Taichung, Taiwan.

metabolites. Our previous study has showed that $(Ac)_5$-GP inhibits the growth of C6 glioma cell *in vitro* and *in vivo* [10-11]. $(Ac)_5$-GP also inhibits DNA damage to AFB_1-treated C3H10T1/2 cells(1). However, the mechanism remains unclear. In this study, the action of $(Ac)_5$-GP on DNA damage induced by AFB_1 in rat hepatocytes was investigated.

Cultured hepatocytes derived from various species have been increasingly important because of their differentiated procarcinogen-metabolizing capacity [12]. This capacity makes liver cells a probable target for early interaction with carcinogen in rats and humans. The toxic and carcinogenic effects of AFB_1 take place only after it is metabolized by cytochrome P-450 enzymes. The reactive metabolites will interact with macromolecules, i.e. DNA [13-16]. One or more of the following mechanisms may be involved in the protection against AFB_1-DNA binding: (i)inhibition of AFB_1 activation pathways, (ii)induction of detoxication pathways, and (iii) electrophile/radical scavenging. In this experiment, the action of $(Ac)_5$-GP on AFB_1-induced unscheduled DNA synthesis were studied. The related enzymes of AFB_1 biotransformation were investigated, and possible mechanism of the chemopreventive action of $(Ac)_5$-GP on AFB_1-induced hepatic damage is discussed.

Materials and Methods

Chemicals

AFB_1, calf thymus DNA, Tris, proteinase K, glutathione (GSH), 3,-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 1-chloro-2,4-dinitrobenzene (CDNB), hydrogen peroxide (H_2O_2), GSH reductase, NADPH, and [methyl-^{3}H] thymidine were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Aquasol-2 (Scintillation liquid) was obtained from New England Nuclear, Boston, MA, U.S.A. and a protein assay kit from Bio-Rad Lab., Ltd., Watford, Herts., U.K..

Primary culture of rat hepatocyte

Hepatocytes were isolated from adult male Sprague-Dawley rats (220-280g, fed *ad libitum*) by the collagenase perfusion method [17]. They were plated at a cell density of 1.5 x 10^5 cells/ml in 4 ml of Williams E medium (Gibco) supplemented with 10% calf bovine serum, 1% PSN (Gibco) antibiotic mixture, and 1% glutamine. The medium was changed 3 hr after plating to remove nonadeherent cells. The culture was maintained for the following assay.

Isolation of penta-acetyl geniposide

Dried and peeled fruits of *Gardenia jasminoides* Ellis (*Rubiaceae*) was refluxed with 500 ml of ether to get rid of lipids and then extracted twice with 500 ml of hot methanol for 3 hr. Methanol layer was collected and concentrated to half of its volume. The resultant solution was stored at 0°C for two days and then filtered. The filtrate was evaporated to dryness and partitioned in 300ml solvent of n-butanol/water (1:1). Water layer was reextracted with butanol. All butanol soluable phases were collected and again evaporated to dryness. A viscous brick red concentrate was obtained. A mixed solvent of pyridine and acetic anhydride (2:1) was added into the concentrate to affect acetylation for 24 hr. The reacting product was extracted with n-butanol/water (1:1). Butanol layer was collected and washed with water twice. An adequate amount of anhydrous sodium sulfate was added to dehydrate the butanol extract. Removal of the butanol gave a yellow solid, from which a pale yellow compound was purified by recrystalization or liquid chromatography. The compound is confirmed by its characterization as described by our previous study (10) and the structure was shown in Fig.1.

Effect of $(Ac)_5$-GP on GST activity

The cytotoxicity assay of $(Ac)_5$-GP was performed according to the MTT colorimetric assay [18]. Nontoxicity dose (0.02-0.1mM) was used in the following assay. To determine the time course effect of $(Ac)_5$- GP on GST activity, 0.1mM concentration of $(Ac)_5$-GP was added into culture and incubated for 0, 1, 2, 3, 6 hr respectively. The cells were harvested and homogenized in Tris-sucrose buffer (pH =7.5) and centrifuged at 105,000 g. The resulting supernatants were used for the estimation of GST (CDNB as substrate) [19]. Protein concentration was determinated by a commercial kit (Bio-Rad Lab. Ltd., Watford, Herts., U.K.) with BSA as standard. The data was expressed as % of individual control.

Measurement of DNA repair synthesis

The effect of $(Ac)_5$-GP on AFB_1-induced DNA repair synthesis was measured by determining the amount of [methyl-^{3}H] thymidine incorporated into nuclear DNA in the presence of hydroxyurea(15mM) [20]. Hepatocyte cultures were first pretreated with $(Ac)_5$-GP at the desired concentration and then hydroxyurea was added and the cultures were preincubated for 1 hr. Finally, AFB_1 and [methyl-^{3}H] thymidine (1 μ Ci/ml) were added and incubated for 18 hr. The cell monolayer was washed with PBS and harvested at the end of culture. The cells were loaded onto a 25mm, 2 μm pore size PC filter through a swinnex polyethylene filter holder to lyse the cells with 10ml lysing buffer containing 2% SDS, 0.025 M EDTA, 0.5 mg/ml thymidine, 0.1M glycine (pH=10), plus 0.15 mg/ml proteinase K and washed with 3ml of the same buffer without proteinase K. The filter was carefully transferred to a scintillation vial. 1.5ml of 0.5N $HClO_4$ was added to the vial and heated at 60 ℃ in a water bath for 90 min. An aliquot was taken for the determination of radioactivity by liquid scintillation counter LSC-900 and DNA content was measured using a modification of the diphenylamine reaction method [21]. Data was expressed as dpm / μg DNA.

GST and GSH-Px assay

To examine the dose effects of $(Ac)_5$-GP on the activities of GST and GSH-Px, the cultures were maintained for 6 hr. The cells were homogenized to determine GST (CDNB as substrate) and GSH-Px (H_2O_2 as substrate) [22] (Fig.3). Protein was quantified with a commercial kit (Bio-Rad). The enzyme activities were expressed as nmol/min/mg protein. To evaluate the effects of $(Ac)_5$-GP on the activities of GST and GSH-Px in hepatocyte treated with AFB_1, the cells were pretreated with $(Ac)_5$GP in various concentrations for 1 hr, then 0.01mM AFB_1 was added. After 5 hr, the cells were harvested and homogenized. The activities of GST (CDNB as substrate) and GSH-Px (H_2O_2 as substrate) were determined and expressed as nmol/min/mg protein (Table II).

Statistics

Results were reported as mean ± standard deviation from three individual determinations. Statistical differences were analyzed by Student t test and significant differences were established at the $p< 0.01$ level.

Results

Preparation of $(Ac)_5$-GP

The products of isolation were rechecked in TLC with authentic sample (Fig.1). It was dissolved in DMSO and distilled water for the follwing studies. The final concentration of DMSO was no more than 0.2%.

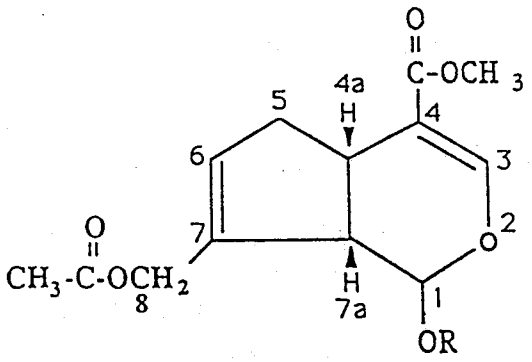

Fig.1 The structure of penta-acetyl geniposide, $(Ac)_5$-GP.
R= β -D-2, 3, 4, 6-tetra-acetyl-glucose

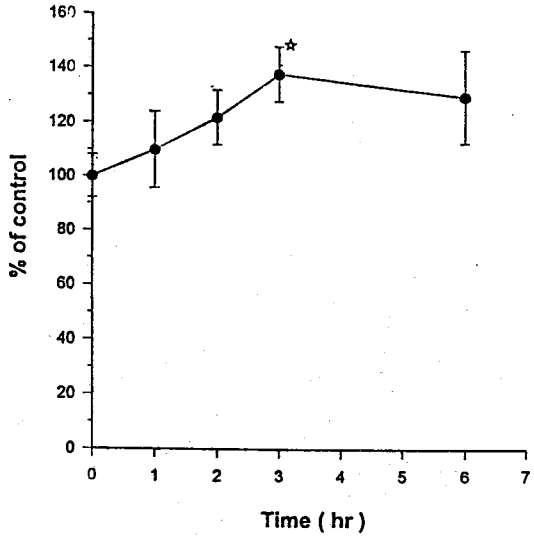

Fig.2 Time course effect of $(Ac)_5$-GP induced GST activity at the concentration of 0.1mM. At indicated time, cells were harvested and homogenized for the determination of GST activity with CDNB as substrate and expressed as % of control. Values are mean ± SD,n=3. ☆p< 0.01

Time-dependent effect of $(Ac)_5$-GP on GST activity

In this study, 0.1mM of $(Ac)_5$-GP was added to hepatocyte cultures. The cells were harvested at 0, 1, 2, 3, 6 hr. The results showed that the GST activity of $(Ac)_5$-GP treated hepatocytes was elevated at 1 hr and significantly ($p< 0.01$) at 3 hr (Fig.2).

Effect of $(Ac)_5$-GP on AFB_1 induced DNA repair synthesis

The effect of $(Ac)_5$-GP on AFB_1 induced DNA repair synthesis was evaluated with unscheduled DNA synthesis (Table.I). $(Ac)_5$-GP significantly inhibited AFB_1-induced DNA repair synthesis at concentration of 0.02mM ($p< 0.01$), 0.05mM, and 0.1mM ($p< 0.001$). $(Ac)_5$-GP showed the protective effect against AFB_1-induced DNA damage.

Table I. Effect of $(Ac)_5$-GP on AFB_1-induced unscheduled DNA synthesis in rat primary hepatocytes

Treatment[a]	dpm/ μg DNA	% of AFB_1 control
DMSO (0.2%)	37±3[b]	
AFB_1 (0.01 mM)	101±18	100
AFB_1 (0.01 mM) plus		
$(Ac)_5$-GP (0.02 mM)	44±4*	44
$(Ac)_5$-GP (0.05 mM)	38±2**	38
$(Ac)_5$-GP (0.1 mM)	34±5**	34

a. Primary hepatocyte cultures were pretreated with hydroxyurea and various concentration of $(Ac)_5$-GP for 1 hr, then added AFB_1 and [methyl-^{3}H] thymidine (1 μ Ci/ml) for 18 hrs. The cells were harvested and lysed for radioactivity counting and DNA quantitation. DNA damage was determined by UDS and expressed as dpm/ μg DNA.

b. Mean±SD, values are the average of triplicate determinations.* $p<0.01$, ** $p<0.001$ compared with AFB_1-treated group.

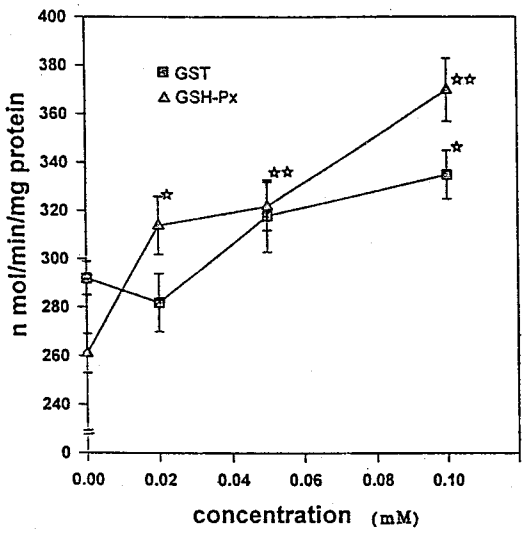

Fig.3 Effect of $(Ac)_5$-GP on the activities of GST and GSH-Px in primary hepatocytes. Hepatocyte cultures were treated with $(Ac)_5$-GP in designated concentration for 6 hr. Cells were harvested, homogenized and centrifuged for the enzyme activity assay. Values are mean ±SD, n=3. ☆p<0.01, ☆☆p<0.001

Effect of $(Ac)_5$-GP on the activities of GST and GSH-Px induced by AFB_1

The dose response of $(Ac)_5$-GP on the activities of GST and GSH-Px was shown in Fig.3. The activity of GSH-Px was significantly increased in the presence of $(Ac)_5$-GP in all test concentration (0.02mM, $p<0.01$; 0.05 and 0.1mM, $p<0.001$) and GST in 0.1 mM ($p<0.01$) (Fig.3). When compared with AFB_1 alone the activities of GST and GSH-Px were increased by pretreatment of $(Ac)_5$-GP and significantly ($p< 0.01$) in the concentration of 0.1mM (TableII).

Discussion

Many chemical carcinogens, including AFB_1, undergo mixed function oxidase-mediated oxidative reactions generating active and inactive metabolites by the cytochrome P-450 dependent oxidative reaction. The 8,9-epoxide was a carcinogenic species of AFB_1 metabolites [23, 24]. Apart from binding to macromolecules, alternative fates for AFB_1-8,9-epoxide include hydrolysis to 8,9-dihydrodiol [25] and conjugation with GSH to form 8-(S-glutathionyl)-9-hydroxy-8,9-dihydro-AFB_1 (AFB_1-SG) [26, 27]. The formation of AFB_1-GSH conjugate through the GST catalysis is a detoxication reaction. GSH and its related en-

Table II. The effect of $(Ac)_5$-GP on the activities of GST and GSH-Px in rat primary hepatocyte culture treated with AFB_1

Treatment[a]	GST nmol/min/mg protein	GSH-Px nmol/min/mg protein
DMSO (0.2%)	262±9[b]	212±15
AFB_1 (0.01 mM)	298±13	291±19
AFB_1 (0.01 mM) plus		
$(Ac)_5$-GP (0.02 mM)	295±10	295±18
$(Ac)_5$-GP (0.05 mM)	310±8	324±20
$(Ac)_5$-GP (0.10 mM)	331±6**	368±16*

a. Primary hepatocyte cultures were pretreated with $(Ac)_5$-GP under various concentration for 1 hr, then AFB_1 was added for 5 hr prior to cell harvest. The cell suspension was homogenized and centrifuged for enzyme activity assay.

b. Mean±SD, values are the average of triplicate determinations. * $P<0.01$ compared with AFB_1-treated group.

zymes, including GST and GSH-Px, would therefore provide an important detoxication pathway.

Our previous studies suggested that geniposide (GP) inhibits AFB_1-induced DNA repair synthesis through increasing AFB_1 detoxication metabolism [4-5]. $(Ac)_5$-GP, the derivative of GP, showed more potent effect on the inhibition of AFB_1-induced DNA repair synthesis in our investigation (data not show). Thereby, action of $(Ac)_5$ GP on AFB_1-induced genotoxicity was studied in this work. It was found that the activity of GST was increased significantly at 3 hr and in 0.1mM of $(Ac)_5$-GP. The GSH-Px activity was increased in all of the test doses (0.02-0.1mM). It also showed that activities of GST and GSH-Px in AFB_1-treated hepatocytes were enhanced significantly in the presence of $(Ac)_5$-GP (0.1mM,$p< 0.01$). However, it might not selectively inhibit a class of P-450 isoenzymes involved in the metabolic activation of AFB_1(unpublished data). From these data, the effectiveness of $(Ac)_5$-GP in inhibiting DNA damage may be related at least in part to its ability to induce GST or GSH-Px and to its lipophilic character.

In conclusion, $(Ac)_5$-GP showed chemopreventive effect on AFB_1-induced DNA damage in rat primary hepatocytes. One of the mechanisms was probably the induction of a detoxication pathway via conjugation with GSH, then decreasing DNA binded by AFB_1 metabolites.

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戊乙醯去羥梔子甘對黃麴毒素誘發肝細胞不正常DNA修補合成作用之影響

曾翠華　黃俊銘　朱嘉一　王朝鐘

梔子（*Gardenia jasminoides* Ellis）為茜草科（*Rubiaceae*）植物，在中國神農本草經中屬於中品藥，漢方中梔子柏皮湯（梔子，柏皮，甘草），茵陳蒿湯（梔子，茵陳，大黃），黃連解毒湯（梔子，黃連，黃芩，黃柏）…等皆含之，臨床上認為具消炎，解熱，利尿等療效，民間廣用於治肝炎，因此在生藥上占有相當重要之角色。其主要成分一類為類胡蘿蔔素如藏紅花酸，藏紅花素，一類為配糖體如梔子甘，去羥梔子甘等，前一類廣泛應用於食品色素中，具有延遲或降低老鼠腫瘤之發生，另有抑制肝毒性及保肝之作用，後一類報告指出有利膽及保肝之作用。黃麴毒素是廣布於花生，玉米，稻米之一種黴菌所分泌之毒素，其中以AFB_1毒性最強，對肝臟影響很大，根據流行病學調查顯示黃麴毒素可能是亞洲及非洲地區居民肝癌發生率偏高之主因之一。本研究將梔子乾燥果實之甲醇粗萃取液經過乙醯化，再以再結晶或管柱層析法分離，即可得到戊乙醯去羥梔子甘（$(Ac)_5$-GP），方法簡易且產率高，擬對其生物活性做深入的探討，本研究將其對黃麴毒素AFB_1誘發肝細胞DNA修補合成之作用，進行一連串之探討，發現戊乙醯去羥梔子甘對黃麴毒素誘發肝細胞DNA 修補合成有抑制作用，此一作用可能是透過增強肝臟中GST及GSH-Px等酵素活性，將毒性較強之黃麴毒素代謝物除去。

關鍵詞：戊乙醯去羥梔子甘，黃麴毒素，肝細胞，DNA修補合成作用。

中山醫學院生化所