Studies on the Possible Mechanisms of Protective Activity Against α-Amanitin Poisoning by Aucubin

Dong Hee Lee[1], In Goo Cho[2], Moon Soo Park[2], Ki-Nam Kim[1], Il-Moo Chang[1], and Woongchon Mar[1]

[1]Natural Products Research Institute, Seoul National University, Seoul 110-460, Korea and [2]Chongwae Pharma Corp., P.O. Box Suwon 61, Korea

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Aucubin, an iridoid glucoside, was investigated to determine whether it has a stimulating effect on α-amanitin excretion in α-amanitin intoxicated rats, and whether there is binding activity to calf thymus DNA. High-performance liquid chromatography (HPLC) analysis of α-amanitin in rat urine allowed quantitative measurement of the α-amanitin concentration with a detection limit of 50 ng/ml. In this system, a group treated with both α-amanitin and aucubin showed that α-amanitin was excreted about 1.4 times faster than in the α-amanitin only treated group. Our previous results showed that the toxicity of α-amanitin is due to specific inhibition of RNA polymerase activity and the resultant blockage of the synthesis of certain RNA species in the nucleus. However, no significant activity change on RNA polymerase from Hep G2 cells was observed when aucubin was treated with α-amanitin at any concentration tested. Nevertheless, aucubigenin inhibited both DNA polymerase (IC50, 80.5 μg/ml) and RNA polymerase (IC50, 135.0 μg/ml) from the Hep G2 cells. The potential of both α-amanitin and aucubin to interact with DNA were examined by spectrophotometric analysis. α-Amanitin showed no significant binding capacity to calf thymus DNA, but aucubin was found to interact with DNA, and the apparent binding constant (K_{app}) and apparent number of binding sites per DNA phosphate (B_{app}) were 0.45×10^4 M^{-1} and 1.25, respectively.

Key words: α-Amanitin, Aucubin, DNA polymerase, RNA polymerase, Iridoid glucoside

INTRODUCTION

Aucubin [1,4a,5,7a-tetrahydro-5-hydroxy-7-(hydroxymethyl) cyclopenta (c) pyran-1-yl-β-D-glucopyranoside] is a common iridoid glucoside that is isolated from the leaves of *Aucuba japonica* (Cornaceae) (Bernini *et al.*, 1984), *Eucommia ulmoides* (Eucommiaceae) (Bianco *et al.*, 1974), and *Plantago asiatica* (Plantaginaceae) (Pailer and Haschke-Hofmeister, 1969). It has been well documented that aucubin protects the liver from damage induced by CCl_4 or α-amanitin in mice, rats and beagle dogs (Chang *et al.*, 1983, 1984; Yang *et al.*, 1983; Chang and Yamaura, 1993). It was known as a synthetic intermediate of prostaglandin in the body (Naruto *et al.*, 1979; Berkowitz *et al.*, 1979), and choleretic action was also reported by Takeda *et al.* (1980).

α-Amanitin, a toxic principle from *Amanita* mushrooms (*A. phalloides, A. verna* and *A. virosa*), has been shown to cause hepatic necrosis in both rats mice, with early nuclear damage and fragmentation of the nucleoli (Fiume and Laschi, 1965; Petrov and Sekeris, 1971). Administration of the toxin to mice was followed by a progressive decrease in liver nuclear RNA content, as measured by a marked decrease in RNA polymerase activity of the nuclei isolated. Several compounds, such as cytochrome C, penicillin G, thioctic acid and silymarin, have been recommended on the basis of their alleged clinical effectiveness. However, because of the lack of controlled investigations, their effectiveness could never be substantiated.

Previously, we reported that aucubin produced a markedly increased survival rate in both mice and beagle dogs intoxicated with α-amanitin (Chang *et al.*, 1984; Chang and Yamaura, 1993). Based on this finding, the current study aims to characterize the pharmacological activity of aucubin and evaluate the possible mechanism of protective activity on α-amanitin poisoning.

Correspondence to: Woongchon Mar, Seoul National University, 28 Yungun-dong, Jongro-ku, Seoul 110-460, Korea
E-mail: mars@snu.ac.kr

MATERIALS AND METHODS

Materials

Aucubin was isolated from the fresh leaves of *Aucuba japonica* (Cornaceae) as reported previously (Chang *et al.*, 1983). *N*-succinimidyl-3-(4-hydroxy-3, 5-di[^{125}I]iodophenyl) propionate ([^{125}I]-Bolton-Hunter reagent, 4400 Ci/mmol), [methyl-^{3}H]thymidine 5'-triphosphate (dTTP, 25 Ci/mmol), [5,6-^{3}H]uridine 5'-triphosphate (UTP, 50 Ci/mmol), [20-^{3}H(N)]phorbol-12,13-dibutyrate (PDBu, 20 Ci/mmol), [1 β-^{3}H(N)]androst-4-ene-3,17-dione (30 Ci/mmol) were all purchased from NEN Life Science Products (Boston, USA). L-[1-^{14}C]Ornithine (60 m Ci/mmol), [1-14C]arachidonic acid (56 mCi/mmol), [8-^{14}C]xanthine (60 m Ci/mmol) were purchased from Moravek Biochemicals (California, USA). All other reagents were purchased from Sigma Chem. Co. (St Louis, USA).

The human hepatocellular carcinoma (Hep G2), mouse hepatoma (Hepa-1c1c7), human bladder transitional-cell carcinoma (T_{24}), NIH swiss mouse embryo (NIH/3T3), human erythroleukemia (HEL), mouse macrophage (J774A.1), mouse monocyte-macrophage (RAW 264.7) cell lines were purchased from the American Type Culture Collection (Maryland, USA).

Effect of aucubin treatment on excretion of α-amanitin into urine

Male Sprague Dawley rats weighing 230-280 g were used in all experiments. Food and water were available *ad libitum*. The rats were treated with α-amanitin in a sublethal dose (2 mg/kg) orally and urine samples were collected 5 h, 10 h, 20 h and 40 h after the ingestion of α-amanitin. The excretion of circulation toxins into the urine was detected by high-performance liquid chromatography (HPLC) assay, as described in Table I. Another group was treated orally with a single dose (100 mg/kg) of aucubin at 30 min after α-amanitin ingestion. The effect of aucubin treatment on excretion α-amanitin excretion into the urine was measured by comparing the α-amanitin concentraton in the urine from each group. Urine sample preparation was performed according to the method reported by Jehl *et al.* (1985), which involves a chemical step with deproteinization and organic solvent treatment, and a selective cleanup and concentration step on Sep-Pak Cartridges (Waters SA, Paris, France).

Table I. Analytical condition of HPLC of α-amanitin

HPLC system: Gilson Model 305	
Column: NH_2 DYNAMAX (8 μm, 4.6 × 250 mm)	
Injection volume: 20 μl	
Moble phase:	0-10 min
	Water:acetonitrile (90:10, v/v)
	11-30 min
	Water: acetonitrile (70:30, v/v)
Flow rate: 1.0 ml/min	
Detector: UV detection at 305 nm	
Detector range: 0.01 AUFS	
Chart speed: 0.5 cm/min	
Detection limit : 5 ng/ml	

A Gilson HPLC (Model 305, Villiers-Le Bel, France) equipped with a UV detector set at 305 nm, the wavelength of maximum absorption of α-amanitin, was used. The column system consisted of a NH_2 DYNAMAX column (8 μm, 4.6 × 250 mm; Ranin Instrument, USA) and the mobile phase was a mixture of deionized water and acetonitrile [90:10~70:30 (v/v)].

Interaction of α-amanitin with aucubin

The interaction of α-amanitin with aucubin was studied by thin-layer radiochromatography. The radioactive α-amanitin derivatives were used in thin-layer radiochromatography. [^{125}I]-α-amanitin was synthesized by a previously described method (Bolton and Hunter, 1973). α-Amanitin (10 μg) in 10 μl of 0.1 M sodium borate buffer (pH 8.5) was added to dried *N*-succinimidyl-3-(4-hydroxy-3, 5-di[^{125}I]iodophenyl) propionate ([^{125}I]-Bolton-Hunter reagent) and the reaction mixture was agitated for 15 min at 0°C. The reaction mixture was then reacted for 1 h at 0°C and for an additional 18 h at 4 obviating the need for addition of glycine to destroy any excess reagent. Sephdex G15 gel filtration was used for separating the labeled [^{125}I]-α-amanitin from the hydrolysis products. Aliquots of [^{125}I]-α-amanitin (0.01 μCi) were incubated with a 500 μl binding buffer containing 20 mM sodium phosphate buffer (pH 7.4), 100 mM NaCl, 100 μM aucubin for 1 h at 37°C. The reaction mixtures were then applied to aluminium foil-backed silica gel TLC sheets, and then chromatographed for 90 min at room temperature in a solvent containing a butanol-acetic acid-water mixture (8-2-2, v/v). Autoradiography was performed by placing the TLC plate in contact with imaging plate (Fuji BAS-IIIs, Fuji Photo Film Co., Japan) for 2 h in order to detect any free and bound [^{125}I]-α-amanitin. The imaging plate was scanned by a bioimaging analyzer (BAS-1500, Fuji Photo Film Co., Japan) and then the radioactivity of both the free and bound [^{125}I]-α-amanitin were quantified.

Preparation of partially purified DNA, RNA polymerase

Partially purified DNA polymerase was prepared as specified by Mar *et al.* (1991). Cultured Hep G2 cells were suspended in 1.0 M sucrose buffer (1 × 10^8 cells/ml, 1.0 M sucrose, 2.5 mM KCl, 25 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 50 mM Tris-HCl buffer, pH 7.5), homogenized then centrifuged (1,000 g, 15 min, 4°C. The resulting pellet was resuspended in 10 volumes (v/w) of 0.25 M sucrose buffer (0.25 M sucrose, 2.5 mM KCl, 25 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 50 mM Tris-HCl buffer, pH 7.5) and centrifuged (800 g, 5 min, 4°C). The pellets were

suspended in 9 volumes (v/w) of detergent-containing buffer (0.3% Triton-X100, 1 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 1 mM K_2HPO_4 buffer, pH 6.5) and centrifuged (800 g, 5 min, 4°C). The nuclei were suspended in 9 volumes (v/w) of 0.2 M sucrose buffer (0.2 M sucrose, 1 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 1 mM EDTA, 4 M NaCl, 50 mM Tris-HCl buffer, pH 7.5). EDTA (1 mM final concentration) and NaCl (4 M final concentration) were subsequently added. The mixture was stirred at 4°C for 4 h and dialyzed (12,000-14,000 mol wt cut-off) against the buffer (25 mM K_2HPO_4, 1 mM 2-mercaptoethanol, 1 mM $MgCl_2$, pH 7.5) for 24 h at 4°C. The resulting precipitate was removed by centrifugation and the protein concentration was determined by the method reported by Lowry *et al.* (1951). The preparation was stored as small aliquots at -80°C. Partially purified RNA polymerase was isolated according to the method reported by Mar *et al.* (1991). The nuclei were prepared from cultured Hep G2 cells as described for the preparation of DNA polymerase. The nuclei were suspended in 4 volumes (v/w) of buffer (50 mM Tris-HCl, pH 8.9, 50 mM KCl, 1 mM $MgCl_2$, 0.5 mM phenylmethylsulfonylfluoride, 0.1 mM EDTA, 20% glycerol), and $(NH_4)_2SO_4$ was added to a final concentration of 0.42 μg/ml. The suspension was incubated at 35°C for 10 min then stirred for 3 h at 4°C. The mixture was dialyzed (12,000-14,000 mol wt cut-off) against a buffer solution (50 mM Tris-HCl, pH 7.9, 2 mM dithiothreitol, 0.1 mM EDTA, 25% glycerol, 1 mM $MgCl_2$, 50 mM KCl) for 24 h at 4°C. The resulting precipitate was removed by centrifugation, and glycerol was added to a final concentration of 50%. The extract was stored at -20°C in small aliquots. The protein concentration was determined according to the method reported by Lowry *et al.* (1951).

Effects of α-amanitin and aucubin on the catalytic activity of DNA, RNA polymerase

The assay for DNA polymerase activity was based on that described by Miller and Chinault (1982). Activated calf thymus DNA was prepared as the substrate by the method reported by Schlabach *et al.* (1971). Briefly, calf thymus DNA (100 μM on the basis of phosphate) was dissolved in 10 mM Tris-HCl buffer (pH 7.4) containing 5 mM $MgCl_2$ and pancreatic deoxyribonuclease (40 ng of DNase/mg of DNA). The reaction mixture was incubated at 37°C for 30 min, and DNase was heat-inactivated (10 min, 60°C). The mixture was finally cooled on ice and stored as small aliquots at -80°C. To determine the enzyme activity, the reaction mixtures were prepared in 50 mM Tris-HCl buffer (pH 7.5) containing 8 mM $MgCl_2$, 4 mM KCl, 0.5 mM dithiothreitol, 12.5% glycerol, 20 μM ATP, 5 μM dATP, 5 μM dGTP, 5 μM dCTP, 0.2 μM [methyl-^{3}H] TTP (1.22 μCi, 61 Ci/mmol), 5 μM activated calf thymus DNA, and various concentrations of aucubin (or α-amanitin). The reactions were initiated by the addition of partially purified DNA polymerase (50 μg based on protein), and the mixtures were incubated at 37°C for 30 min. The reactions were then quenched by the addition of 10 μl of 0.25 M EDTA and placed on ice. Aliquots (100 μl) of the reaction mixtures were spotted onto circular discs (2.5 cm) of Whatmann DE18 ion exchange paper and washed with 5% aqueous Na_2HPO_4 and distilled water. The discs were dried, and the radioactivity was determined by a liquid scintillation counter (Microbeta 1450, Wallac Oy, Finland).

The RNA polymerase assay was conducted by a modification of the procedure described by Rose *et al.* (1976). Reaction mixtures were prepared containing 5 μM calf thymus DNA, 1.25 μM [5,6-^{3}H] UTP (5 μCi, 39 Ci/mmol), 3 μM spermidine, 1.67 mM $MnCl_2$, 0.32 mM ATP, 0.32 mM CTP, 0.32 mM GTP, ribonuclease inhibitor (3 Unit), 3.3 mM NaF, 8 mM KCl, 60 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 60 mM Tris-HCl buffer (pH 8.0) and various aucubin (and α-amanitin) concentrations. The reaction was initiated by the addition of partially purified RNA polymerase (100 μg protein) and the remaining procedure was equal to the DNA polymerase assay as described above.

Effects of aucubin on the catalytic activity of quinone reductase

A quinone reductase assay was accomplished by a modification of the procedure described by Prochaska *et al.* (1988). Hepa 1c1c7 cells were plated at a density of 0.5 × 104 cells/well in 200 μl of -minimal essential medium supplemented with 10% fetal bovine serum. The cells were grown for 48 h with various aucubin concentrations in a humidified incubator in 5% CO_2 at 37°C. After the plates were exposed to aucubin for 48 h, the media were decanted, and the cells were lysed by incubation at 37°C for 10 min with a lysis buffer (10 mM Tris-HCl, 140 mM NaCl, 15 mM $MgCl_2$, 0.5% NP-40, pH 8.0). The reaction mixture containing 12.5 mM Tris-HCl (pH 7.4), 0.135 mg BSA, 0.01% Tween 20, 0.05 mM FAD, 1 mM glucose-6-phosphate, 0.03 mM NADP, 0.06 mg MTT, 0.4 U glucose-6-phosphate dehydrogenase, and 0.05 mM menadione was added then incubated for 30 min at 37°C. A blue color developed and the reaction was quenched after 5 min by the addition of 50 μl of a solution containing 0.3 mM dicoumarol. The plates were then scanned at 610 nm with microtiter plate reader (Thermomax, Molecular Device Co., USA).

Effects of α-amanitin and aucubin on the enzyme assay-based model systems for the carcinogenesis and inflammation

ODC assay: T_{24} cells were plated at a density of 0.5×10^4 cells/well in McCoy's 5a medium supplemented with 10% fetal bovine serum. The enzyme assay was based on the method described by Lichti and Gottesman

(1982). After the plates were exposed to aucubin (α-amanitin) for 24 h, the cells were lysed by a method of freezing and thawing. A substrate and cofactor mixture containing 30 mM Tris-HCl (pH 7.5), 1.25 mM ethylene diamine tetraacetic acid (EDTA), 50 mM DTT, 5 mM pyridoxal phosphate, L-[1-14C]ornithine (200 mCi/ml) was added and the resulting mixture was then incubated for 1 h at 37°C. A paper disk (Schleicher and Schuell, # 597) was placed over each well and moistened with 1N NaOH, to allow [1-^{14}C] CO_2 absorption from the L-[1-^{14}C] ornithine. The discs were removed, and the radioactivity was determined by the liquid scintillation counter.

Protein kinase C binding assay: The binding assay was based on the method described by Beutler *et al.* (1989). The assay was performed in 96-well plates. Each reaction mixture contained 50 mM Tris-HCl buffer (pH 7.4), bovine serum albumin (200 μg/well), calf brain particulate (25 protein/well) as a source of protein kinase C, 0.2 mM $CaCl_2$, 20 nCi [^{3}H]PDBu, and aucubin (or α-amanitin). Incubation was performed for 1 h at 37°C and the unbound [^{3}H]PDBu was then removed by filtration with 50 mM Tris-HCl (pH 7.4) through glass fiber filtermats using a cell harvester (Tomtec Inc., USA). The specific binding of [^{3}H] PDBu was calculated using a liquid scintillation counter.

Aromatase assay: The human placental microsomes were obtained as described by Ryan (1959). The aromatase activity was measured based essentially on the assay reported by Tompson and Siiteri (1974). The incubation mixture contained 10 mM potassium phosphate (pH 7.4), 100 mM KCl, 10 mM DTT, 1 mM EDTA, 2.5 mM glucose-6-phosphate, 0.5 mM NADP, 0.1 U glucose-6-phosphate dehydrogenase, 20 nCi [1β-^{3}H]androstenedione, and a placental microsome solution (0.4 protein). Incubation was performed at 37°C for 30 min and terminated by the addition of $CHCl^3$, followed by vortexing for 1 min. Aliquots were removed from the water phase and added to a scintillation cocktail to determine ^3H_2O production.

Xanthine dehydrogenase/oxidase assay: A xanthine dehydrogenase/oxidase assay was performed using a modification of the procedure described by Lin and Shih (1994). NIH/3T3 cells were plated at a density of 0.5 × 10^4 cells/well in Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum. The cells were grown for 24 h with TPA and various aucubin (or α-amanitin) concentrations in a humidified incubator in 5% CO_2 at 37°C. The cells were lysed by incubation at 37°C for 10 min with a lysis buffer (50 mM potassium phosphate, pH 7.8, 1 mM EDTA, 1 mM PMSF, 1 mM DTT, 0.5 % Triton X-100) and further incubated in a reaction mixture containing 50 mM potassium phosphate (pH 7.4), 10 μg/ml superoxide dismutase, 10 μg/ml catalase, 1 mM PMSF, 0.67 mM NAD^+, 50 U/ml lactate dehydrogenase, 2.0 μM pyruvate, 0.4 μCi [8-14C]xanthine at 37°C for 10 min. Small aliquots were removed from the reaction mixture and applied to the aluminium foil-backed cellulose TLC sheets, and then chromatographed for 90 min at room temperature in a solvent containing butanol/acetic acid/water (8/2/2, v/v). Autoradiography was performed to detect any [^{14}C]uric acid by placing the TLC plate in contact with imaging plate for 24 h. The imaging plate was scanned by a bio-imaging analyzer.

Oxygen uptake COX assay: Semi-purified cyclooxygenases (COX) were obtained from bovine seminal vesicles as described by Kulmacz and Wu. (1989). The COX activity was estimated, based on the assay reported by DeWitt *et al.* (1990), by measuring the maximal rate of oxygen uptake in the presence of enzyme and arachidonic acid using a biological oxygen monitor equipped with a Clarke-type electrode (YSI model 5300, Yellow Springs Instument Co., USA). The enzyme reaction was initiated by adding an aliquot of the enzyme solution (ca. 50 μg protein) to a reaction mixture of 1 ml of 0.2 M sodium phosphate buffer (pH 7.4), 3 mM hematin, 0.4 mM hydroquinone, 0.3 mM arachidonic acid and a designated concentration of aucubin (or α-amanitin).

Radiometric COX-I assay: Radiometric COX-I assay was carried out using a modification of the procedure described by Najid *et al.* (1992). Cultured HEL cells were plated at 2×10^6 cells per well in a 24-well plate and incubated for 24 h at 37°C in a humidified atmosphere (5% CO_2) with aucubin (or α-amanitin). The cultured cells were incubated for 10 min at 37°C in the presence of a 1 μM calcium ionophore A23187 and [1-^{14}C]AA solution (0.05 μCi) and extracted with ethyl acetate to isolate the metabolic products of cyclooxygenase. The ethyl acetate layers were applied to aluminium foil-backed silica gel TLC sheets, then chromatographed for 90 min at room temperature in a solvent containing chloroform-methanol-acetic acid-water (v/v, 90:10:1:1). To detect the [^{14}C]-labelled prostanoids, autoradiography was performed by placing the TLC plate in contact with the imaging plate for 24 h. The imaging plate was scanned by a bio-imaging analyzer and the radioactivity of the prostanoid bands was quantified.

Radiometric COX-II assay: Radiometric COX-I assay was conducted by a modification of the procedure described by O'Sullivan *et al.* (1992). Cultured J774A.1 macrophages were plated at 2×10^5 cells per well in a 24-well plate and pre-incubated for 24 h at 37°C in a humidified atmosphere (5% CO_2). The basal release of prostanoids from the untreated J774A.1 macrophages was blocked by pretreatment with an aspirin solution (final concentration, 0.4 μg/ml) so as to exclude the basal COX-I effect. The aspirin pretreated cells were added to aucubin (or α-amanitin) and treated with LPS (2.5 μg/ml) for 18 h to induce COX-II. The inhibitory effects of the test samples against COX-II were determined by the radiometric COX-I assay method as described above.

NOS assay: The nitric oxide synthase activity was estimated, based on the assay described by Hortelano *et al.*

(1993), by observing the magenta-colored azo dye that is formed from NO_2- and Griess reagent. The RAW264.7 cells were plated at a density of 0.5×10^4 cells/well in Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum. The cells were grown for 24 h in both LPS (1.0 μg/ml) and various concentrations of aucubin (or α-amanitin) in a humidified incubator in 5% CO_2 at 37°C. Griess reagent (0.25% naphthylethylenediamine, and 2.5% sulfanilamide in 10% H_3PO_4) was added and the resulting mixture was then incubated for 5 min at 37 °C. The plates were then scanned at 540 nm with a micro-titer plate reader.

Interaction with rat brain tubulin of α-amanitin

The preparation of rat brain tubulin and the binding assay was conducted by a modification of the procedure described by Mandelbaum-Sharit *et al.* (1976). The binding assay mixture contained brain protein (1 mg/ml), 100 mM sodium glutamate, and 10 mM sodium phosphate buffer (pH 6.5). After temperature equilibration at 37°C, α-[^{125}I]-amanitin was added and a binding mixture was adsorbed on a DEAE-cellulose filter (Whatman DE 81). The fiters were washed twice with same buffer and dried. The specific binding of α-[^{125}I]-amanitin was calculated by a liquid scintillation counter

Interaction of aucubin and α-amanitin with DNA

The interaction of aucubin and α-amanitin with calf thymus DNA was investigated by spectrophotometric analysis, as previously described by Komiyama *et al.* (1983). The incubation mixtures were prepared containing 20 mM sodium phosphate buffer (pH 7.4), 100 mM NaCl, calf thymus DNA (0-200 μg/ml), and various concentrations of aucubin (or α-amanitin). The absorbance was measured at 210 nm (α-amanitin; 310 nm) about 30 min after aucubin (or α-amanitin) addition. The absorbance data were used to calculate the Scatchard plot using the following equation

$$r/m = K_{app}(B_{app} - r),$$

where r is the ratio of bound compound to the total DNA nucleotides, m is the concentration of the free compound, K_{app} is the apparent binding constant, and B_{app} is the apparent number of binding sites per DNA phosphate.

RESULTS AND DISCUSSION

The aim of this study was to characterize the biochemical activities of aucubin and to evaluate the possible mechanism of protective activity on α-amanitin poisoning.

As the α-amanitin excretion in urine results show (Fig. 1 and 2), α-amanitin treated rats showed a maximum excretion rate near 5 h and then exhibited minimum constant rate after that time. In order to evaluate the effect of aucubin treatment on excretion of α-amanitin into urine, another group received a single dose (100 mg/kg) of aucubin orally after α-amanitin administration. The group treated with both α-amanitin and aucubin showed that α-amanitin was excreted about 1.4 times faster than in the α-amanitin only treated group (Fig. 2). This result suggests that aucubin or its hydrolyzed products may enhance α-amanitin excretion by displacing it from the binding sites on the plasma albumin molecule. There is a possibility that aucubin is readily hydrolyzed into the its genine form and further polymerized compounds in the gastrointestinal environment. Plasma protein binding of aucubin was confirmed by Suh *et al.* (1991), who conclusively

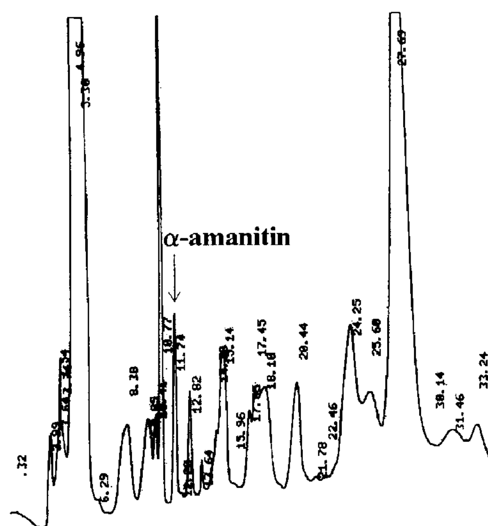


Fig. 1. HPLC chromatogram resulting from a urine containing 9.8 μg/ml of α-amanitin.

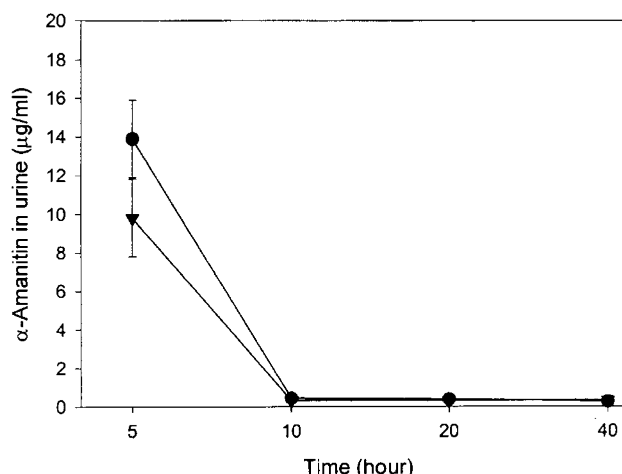


Fig. 2. Effect of aucubin treatment on excretion of α-amanitin into urine when a sublethal dose (2.0 mg/kg) of α-amanitin was administered orally. Each specified time comprised three groups; a control (saline), α-amanitin only treated (▼), α-amanitin plus aucubin treated group (●).

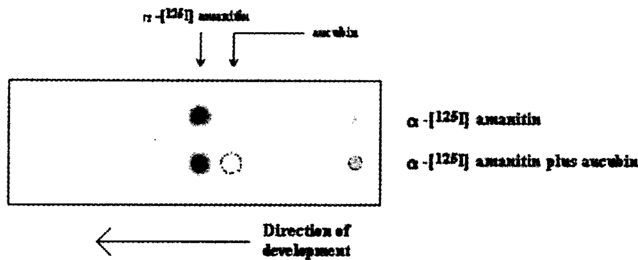
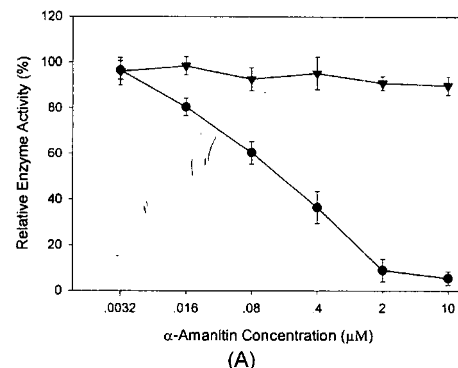


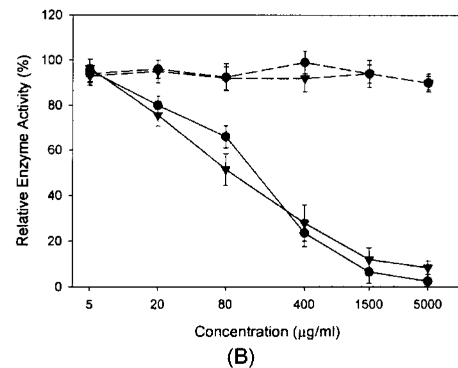
Fig. 3. Thin-layer radiochromatogram of reaction mixture consisted of 20 mM sodium phosphate buffer (pH 7.4), 100 μM NaCl, 0.1 μM α-[^{125}I]amanitin, 10 μM aucubin.

proved that 91% of the aucubin existed in the unbound form. It is reasonable to assume that the metabolized form of aucubin causes a high rate of α-amanitin excretion due to plasma protein binding. After the administration of a sublethal α-amanitin dose, it took only 5 h to decrease the serum concentration to below the limit of detection (50 ng/ml) (data not shown). The results obtained above indicate that a HPLC assay of the urine samples is an appropriate method for diagnosing *Amanita* poisoning.

The α-amanitin and aucubin interaction was investigated using thin-layer radiochromatography but the binding effect between α-amanitin and aucubin was not confirmed (Fig. 3). It was found that the stimulated α-amanitin excretion rate and its protective effect against *Amanita* poisoning were not directly related to the α-amanitin and aucubin interaction.

Partially purified DNA and RNA polymerase were isolated from Hep G2 cells and the specific activities of the purified enzyme were 15 and 5 units/mg, respectively. There is no detectable inhibition of DNA polymerase activity by α-amanitin and aucubin at any concentration tested (up to 10^{-4} M). However, RNA polymerase activity was inhibited by 50% at 10^{-7} M α-amanitin and was completely inhibited at 10^{-5} M under our assay conditions (Fig. 4). Increasing the DNA concentration in the RNA ploymerase assay had no influence on the degree of inhibition. Thus α-amanitin does not react with the DNA itself (Data not shown). This result is consistent with the fact that α-amanitin does not interact with DNA in the spectrophotometric analysis described below. A previous study (Cochet-Meilhac and Chambon, 1974) reported that α-amanitin does not inhibit the free RNA polymerase binding to DNA. Therefore the results demonstrate that α-amanitin interacts with the RNA polymerase-DNA complex to block either initiation, chain elongation or enzyme release. In our experiments, the preventive effect of aucubin on the inhibition of RNA polymerase by α-amanitin was not confirmed (Fig. 4). The protective activities of aucubin against *Amanita* intoxication in mice and beagle dogs (Chang and Yamaura, 1993; Chang *et al.*, 1984) are not directly due to preventive effect of aucubin on the inhibition of RNA polymerase by α-amanitin. However, aucubi-





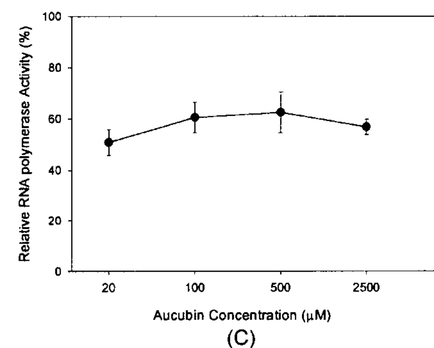


Fig. 4. (A) The effect of α-amanitin on nuclear DNA (▼), RNA polymerase (●) from HepG2 cells. (B) The effects of aucubin (···) and aucubigenin (–) on nuclear DNA (▼), RNA polymerase (●) from HepG2 cells. (C) The effect of aucubin treatment on the inhibition of RNA polymerase by α-amanitin. Relative RNA polymerase activity was made by subtracting the residual activity obtained at an inhibitoy concentration of α-amanitin (0.1 μM) from the control value.

genin exhibited a dose dependent inhibition of the DNA polymerase (IC50, 80.5 μg/ml) and the RNA polymerase (IC50, 135.0 μg/ml) isolated from Hep G2 cells (Fig. 4).

Quinone reductase protects cells against quinone toxicity and their metabolic precursors by promoting the obligatory two-electron reduction of quinones to hydroquinones. In order to investigate mechanical relationship between hepatoprotective effect of aucubin and induction effect of quinone reductase, aucubin was confirmed using a direct assay of quinone reductase from cells grown in microtiter wells. However, there was no detectable induc-

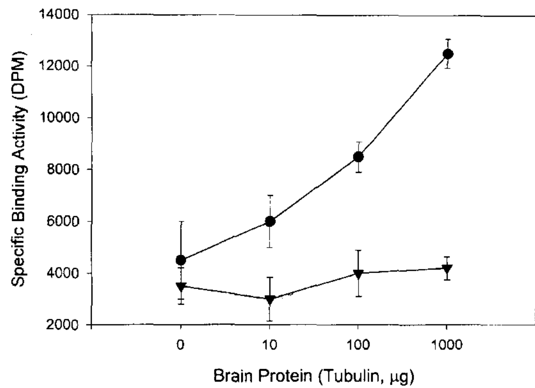


Fig. 5. Binding of α-[^{125}I]amanitin (▼) and [^{3}H]taxol (●) to rat brain tubulin. The specific binding was calculated by subtracting the nonspecific binding from the total binding in the absence of nonradioactive α-amanitin.

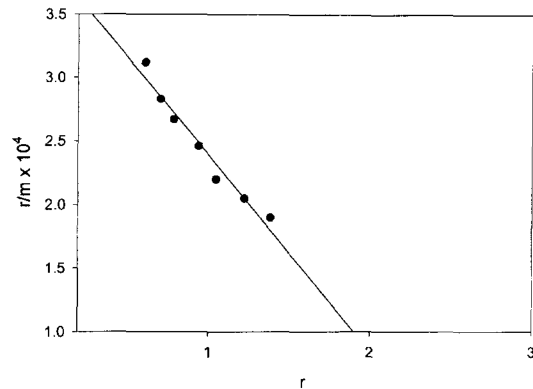


Fig. 6. Isotherms for the binding of aucubin to calf thymus DNA. r, bound aucubin per base pair; m, concentration of free aucubin. Data were obtained from spectrophotometric analysis.

tion effect on the quinone reductase activity of aucubin in Hepa1c1c7 cells.

Phallotoxins and virotoxins are toxic peptides found in *Amanita phalloides, Amanita verna, and Amanita virosa* mushrooms and these toxins bind to the protein microfilaments of the liver cell (Litten, 1975; Wieland and Faulstich, 1978). The relationship between α-amanitin and its inhibitory activity on RNA polymerase has been established. The other possible mechanism of α-amanitin action was examined by the tubulin-binding assay as described above. However its specific binding activity to rat brain tubulin was not observed in our experiments (Fig. 5).

The potentials of α-amanitin and aucubin to interact with DNA were examined by spectrophotometric analysis. α-Amanitin showed no significant binding activity to calf thymus DNA, but aucubin was established as having the potential to interact with DNA. As shown in Fig. 6, a scatchard plot of aucubin binding to calf thymus DNA was obtained from spectrophotometric analysis, and the

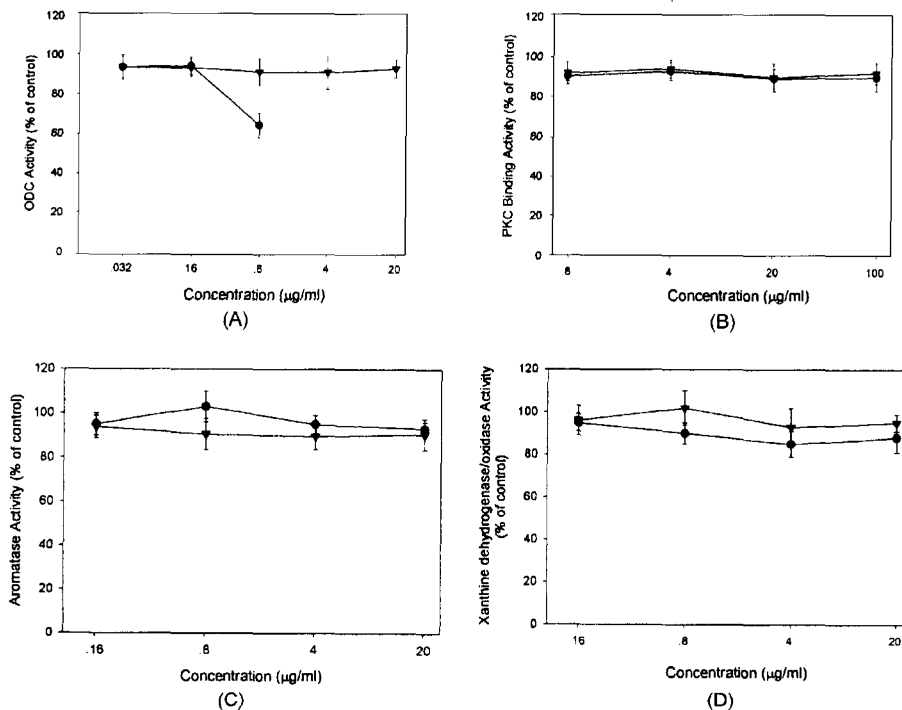


Fig. 7. (A) The effects of α-amanitin (●) and aucubin (▼) on ODC activity in T_{24} cells treated with TPA at 200 nM for 6 h. (B) The effects of α-amanitin (●) and aucubin (▼) on [^{3}H]PDBu binding to protein kinase C. (C) The effects of α-amanitin (●) and aucubin (▼) on human placental aromatase activity. (D) The effects of α-amanitin (●) and aucubin (▼) on TPA-induced elevation of xanthine dehydrogenase/oxidase in NIH/3T3 cells.

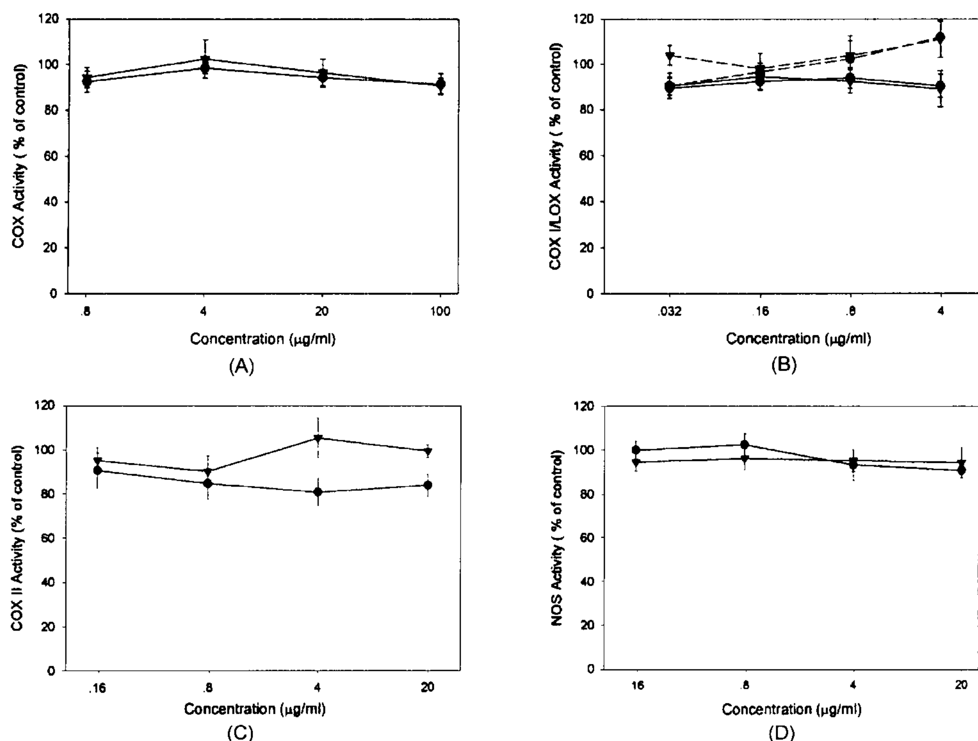


Fig. 8. (A) The effects of α-amanitin (●) and aucubin (▼) on microsomal COX activity semi-purified from sheep seminal vesicles. (B) The effects of α-amanitin (●) and aucubin (▼) on COX I (–)/LOX (···) activity in HEL cells. (C) The effects of α-amanitin (●) and aucubin (▼) on COX II activity in J774A.1 cells treated with LPS at 2.5 μg/ml for 24 h. (D) The effects of α-amanitin (●) and aucubin (▼) on NO synthase activity in RAW264.7 cells treated with LPS at 1.0 μg/ml for 24 h.

apparent binding constant (K_{app}) and the apparent number of binding sites per DNA phosphate (B_{app}) were 0.45×10^4 M^{-1} and 1.25, respectively.

The effects of α-amanitin and aucubin on the enzyme assay-based model system for carcinogenesis and inflammation were studied. However, no detectable activity was observed (Fig. 7 and 8).

In conclusion, aucubin was confirmed to have a stimulating effect on α-amanitin excretion in α-amanitin intoxicated rats and binds to calf thymus DNA. In particular, a HPLC assay of urine samples for α-amanitin has become a useful tool in determining the severity of intoxication, which is a prerequisite for finding the optimum therapy. Our results also showed that aucubigenin exhibited dose dependent inhibition of DNA and RNA polymerase, and the mechanism of action of aucubin is worthy of further investigation.

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