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Isolation, identification and biological activity of a phyllanthoside metabolite produced in vitro by mouse plasma

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Summary. The antitumor agent phyllanthoside is rapidly metabolized in vitro by mouse plasma [4]. This metabolite has now been isolated from mouse plasma and its structural properties and cytotoxicity characterized. The isolated metabolite was estimated to be >98% pure by HPLC analysis. Mass spectral analysis (fast atom bombardment and tandem mass spectrometry) indicated that the metabolite was the aglycone of phyllanthoside that resulted from the cleavage of the ester bond linking the aglycone and the disaccharide moieties of phyllanthoside. This identification was based on identical collision-induced dissociation spectra of both phyllanthoside and the metabolite. The aglycone was not formed by mouse plasma that had been boiled, filtered to remove proteins, or treated with 1.0 m*M* diisopropyl fluorophosphate. These results suggest that aglycone formation occurs as a result of plasma esterase activity. Michaelis-Menten constants, V_{max} and K_m, for conversion of phyllanthoside to the aglycone at 22° C were estimated to be 1.1 mmol/ml plasma/min and 2.0 m*M*, respectively. Concentrations of phyllanthoside and metabolite required to inhibit cell-colony formation by human A204 rhabdomyosarcoma in vitro were 0.47 n*M* and 24 μ*M*, respectively. The toxicity of phyllanthoside, and perhaps its efficacy as an antitumor agent in mice, may depend on its rate of conversion to the aglycone.

Introduction

Phyllanthoside (Fig. 1), a glycoside obtained from the roots of the Central American tree *Phyllanthus acuminatus* Vahl [5], has recently entered phase I clinical trial in Europe for the treatment of cancer. When given i.p., phyllanthoside exhibits significant antitumor activity in mice against i.p. B16 melanoma and i.p. P388 leukemia but it lacks antitumor activity when given i.v. [Dr. J. A. Mead, personal communication, November 21, 1984: NCI preclinical pharmacology studies on phyllanthoside (NSC 328 426)]. Previous work in our laboratory has shown that phyllanthoside is rapidly metabolized in vitro by plasma from mouse and rat but is more stable in plasma from dog, monkey, and human [6]. Metabolism of phyllanthoside appeared to be related to the drug's toxicity, since nontoxic doses in mice (68 mg/m^2) were much higher than those in dogs (1.5 mg/m^2) and the drug was eliminated in vivo more rapidly from plasma of mice than from that of dogs [4]. In addition, the in vitro cytotoxicity of phyllanthoside to human A204 rhabdomyosarcoma cells was reduced by coincubation of the drug with rat hepatocytes, which also metabolize phyllanthoside [4]. These results suggest that the toxicity of phyllanthoside, and perhaps its efficacy as an antitumor agent, may be decreased by its metabolism to a less toxic metabolite.

We report the isolation by high-performance liquid chromatography (HPLC) and identification by mass spectral analysis of the phyllanthoside metabolite produced by mouse plasma. Metabolism of phyllanthoside by mouse plasma and the cytotoxicity of the metabolite to human A204 rhabdomyosarcoma cells was also characterized.

Materials and methods

Chemicals. Phyllanthoside (NSC 328 426) was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md, USA), and was dissolved in 95% ethanol before use. The purity of the bulk drug was 91.5% by HPLC analysis. The serum esterase inhibitors, bis(*p*-nitrophenyl) phosphate (bNPP), *p*-chloromercuribenzoate (pCMB), and diisopropyl fluorophosphate (DFP) were obtained from Sigma Chemical Co. (St. Louis, Mo, USA). Ethoxypropazine and phospholine iodide were generously donated by Dr. W. Brimijoin (Mayo Clinic, Rochester, Minn, USA).

Animals. Male CDF_1 mice weighing 20–25 g were lightly anesthetized with diethyl ether and were exsanguinated by bleeding from the retro-orbital venous plexus into chilled, heparinized, 2-ml centrifuge tubes. Blood was centrifuged at 12,000 *g* for 3 min to separate the plasma.

Preparation and isolation of metabolite. Phyllanthoside metabolites were isolated from mouse plasma by a modification of the reversed-phase HPLC procedure previously described by Powis and Moore [6]. Briefly, mouse plasma and phyllanthoside (final concentration 0.75 m*M*) were

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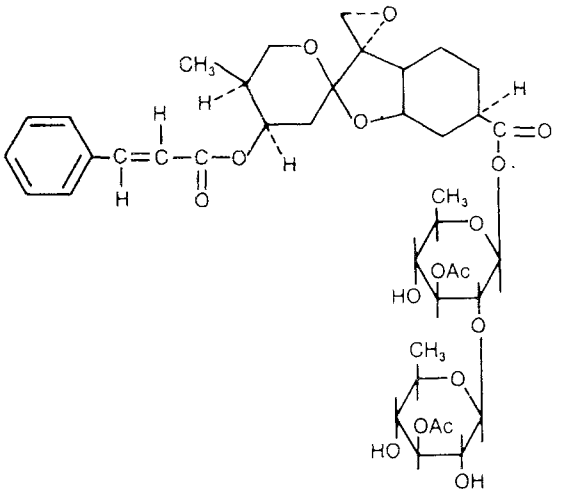

Fig. 1. Structure of phyllanthoside

incubated for 10 min at 22° C, followed by the addition of an equal volume of 0.1 *M* sodium phosphate buffer (pH 7.0) and 10 vol. ethyl acetate. After shaking for 20 min and centrifugation at 1,000 *g* for 10 min, the ethyl acetate was removed and evaporated to dryness with N_2. The residue from the ethyl acetate layer was dissolved in methanol and 10 µl aliquots were injected onto an RP-18 5 mm HPLC column (Hewlett-Packard, Avondale, Pa, USA) with a mobile phase (1 ml/min) of 50% methanol and 50% 1.5 m*M* acetic acid (pH 4.0). Eluting compounds were detected by their absorbance at 270 nm with a Hewlett-Packard diode-array detector. The diode-array detector was also used to record the absorption spectra of phyllanthoside and the metabolite from 200 to 400 nm. The appropriate fractions of column effluent containing the metabolite were collected after the detector and dried with N_2, and the residue was used for mass spectral analysis and in vitro cytotoxicity measurements.

Characterization of mouse plasma metabolite formation. The formation of phyllanthoside metabolite by boiled mouse plasma or mouse plasma deproteinized by filtration was determined by the protocol outlined above. Plasma was boiled for 10 min or deproteinized by filtration through Centrifree micropartition filters with YMT membranes and a molecular weight cutoff of 30 kDa (Amicon, Danvers, Mass, USA) before the addition of phyllanthoside. In addition, mouse plasma was treated with esterase inhibitors for 1 h at 22° C before the addition of phyllanthoside and the determination of metabolite formation. The following esterase inhibitors (final concentrations) were used: $HgCl_2$ (0.25 m*M*), KCN (0.25 m*M*), EDTA (0.1 m*M*), pCMB (0.25 m*M*), phospholine iodide (0.1 m*M*), bNPP (0.5 m*M*), ethoxypropazine (0.1 m*M*), and DFP (1.0 m*M*).

The relationship between the rate of metabolite formation by mouse plasma and phyllanthoside concentration was also determined. Phyllanthoside concentrations were varied from 0.6 to 60 m*M*, and metabolite formation at 22° C was measured for the first 2 min after the addition of phyllanthoside to mouse plasma. Incubations were done in duplicate and 4-hydroxybiphenyl was added as an internal standard [6] prior to HPLC analysis. A nonlinear regression program (Enzfitter, Elsevier-Biosoft, Cambridge, UK) was used to compare rates of metabolite formation to the hyperbolic form of the Michaelis-Menten equation and obtain estimates of the maximal velocity (V_{max}) and Michaelis constant (K_m) for the formation of metabolite in mouse plasma.

Mass spectral analyses of phyllanthoside and metabolite. Phyllanthoside and the metabolite were ionized using fast atom bombardment (FAB) ionization, with glycerol and dithioerythritol:dithiothreitol (1:3, w/w) as the liquid matrices. Mass spectra were obtained on a Nermag R 30-10 triple quadrupole (Nermag, Paris, France) and a VG ZAB (VG Analytical, Manchester, England) using neutral xenon gas accelerated to 8 keV as the bombarding atoms. The mass range of 200–1200 daltons was scanned every 10 s during single-scan analyses. Low-energy collision-induced dissociation experiments (MS/MS) were conducted on the Nermag R 30-10 triple quadrupole using argon (1 ± 10^{-1} torr) as the collision cell gas. The appropriate parent ion was selected and the daughter spectra was obtained by scanning a mass range from 100 daltons up to the parent ion's molecular weight every 10 s. High-energy collision-induced spectra were obtained using a VG ZAB, with helium as the collision gas.

Cytotoxicity studies. The cytotoxicity of phyllanthoside and the isolated metabolite to the human A204 rhabdomyosarcoma cell line growing in soft agarose culture was determined by the procedure previously described by Alley et al. [1]. Briefly, 10^4 A204 cells were grown in 0.5 ml 0.3% soft agarose in 35-mm culture dishes for 24 h. Cultures were overlaid with 1 ml medium containing drug or metabolite (in 95% ethanol) at 0.1, 1, 10, or 100 µg/ml final concentrations. Phyllanthoside or the metabolite was removed 24 h later, and colony formation by A204 cells was quantified by automated image analysis of cells stained with metabolizable 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride after 7 days.

Results

Metabolite formation

Incubation of phyllanthoside with mouse plasma resulted in the formation of a single metabolite that could be separated from the parent drug and isolated by HPLC (Fig. 2). In two separate experiments, recovered metabolite represented 46%±8% by weight of added phyllanthoside and was >98% pure by HPLC analysis. Relative retention times of the metabolite on the RP-18 column were pH-dependent. The retention time of the metabolite decreased from 7.2 min to 2.7 min when distilled water was substituted for 1.5 m*M* acetic acid (pH 4.0) in the mobile phase, whereas retention times for phyllanthoside (6.7 min) did not change.

Metabolite was not formed if mouse plasma was boiled or if plasma proteins were first removed by filtration. In addition, metabolite formation was completely inhibited by preincubation of mouse plasma with 1.0 m*M* DFP. In contrast, the esterase inhibitors $HgCl_2$, KCN, EDTA, pCMB, phospholine iodide, ethoxypropazine, and bNPP did not inhibit metabolite formation by mouse plasma. A hyperbolic curve (Fig. 3) was obtained when the rate of metabolite formation in mouse plasma was plotted against

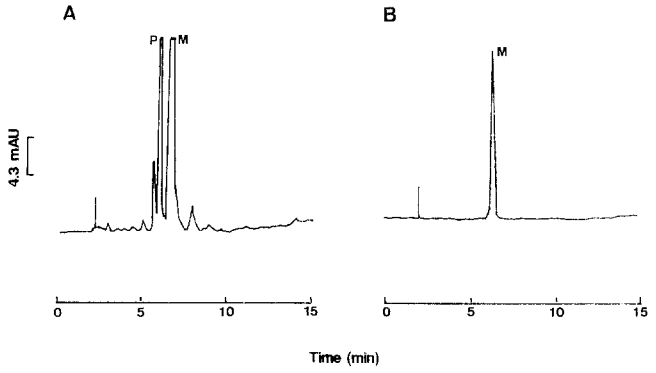

Fig. 2. HPLC chromatograms of phyllanthoside and metabolite. **A** Metabolite (*M*) formed in mouse plasma after incubation with phyllanthoside (0.75 m*M*) for 10 min at 22° C. Phyllanthoside (*P*) was added after extraction of metabolite from plasma. **B** Metabolite (*M*) after isolation and purification by HPLC

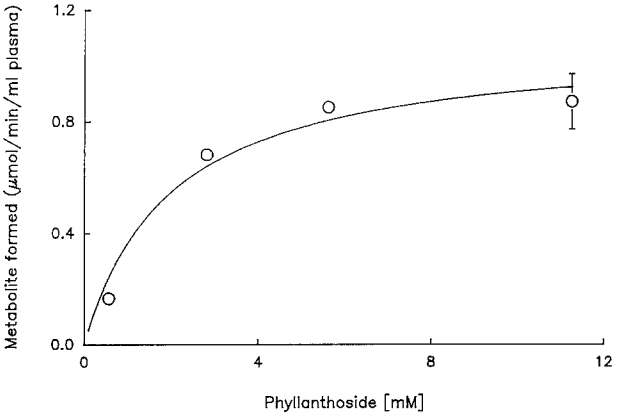

Fig. 3. Metabolism of phyllanthoside in mouse plasma. Phyllanthoside at the appropriate concentration was incubated with mouse plasma for 2 min at 22° C. Values (O) represent the mean ± range of duplicate determinations. The *continuous line* is based on estimates of Michaelis-Menten parameters, V_{max} and K_m, obtained by nonlinear regression analysis of the experimental values

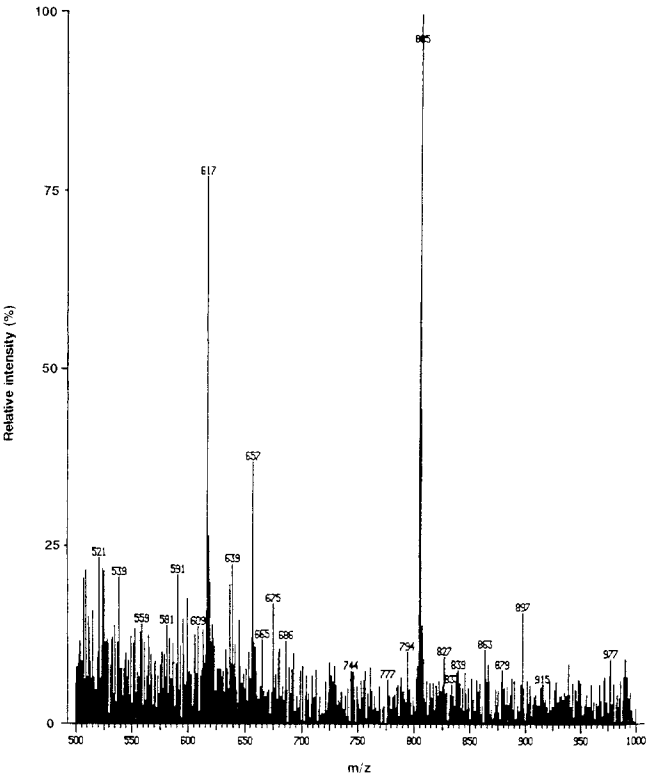

Fig. 4. Positive-ion FAB mass spectrum of phyllanthoside. The molecular ion is at m/z 805

phyllanthoside concentration. The hyperbolic form of the Michaelis-Menten equation was used to model the formation of metabolite in mouse plasma, and V_{max} and K_m values were estimated to be 1.1 mmol/ml per min and 2.0 m*M*, respectively. Phyllanthoside concentrations of >12 m*M* produced a flocculent precipitate in mouse plasma, and the amount of metabolite formed was decreased (data not shown).

Identification of metabolite

Phyllanthoside and the metabolite had very similar UV absorption spectra, characterized by a broad absorption band between 230 and 320 nm and absorption maxima at approximately 278 nm. The mass spectra of the drug showed a $M + H^+$ pseudomolecular ion at 805 daltons. The major fragment at 617 daltons was explained by the loss of one glycosyl group. The presence of sodium in the samples was indicated by the sodium adducts at 827 and 639 daltons (Fig. 4). Low-energy MS/MS analysis of the FAB peaks at m/z 617 and 805 (molecular ion) produced daughter spectra, with an anion at m/z 429 corresponding to the aglycone, thus confirming the identity of the 805- and 617-dalton ions (data not shown). MS/MS spectra of the FAB aglycone fragment of phyllanthoside produced a major daughter ion at m/z 281, indicating a facile loss of a phenyl-CH=CH-C(O)-OH moiety from the aglycone. This ion also predominated the high-energy collision spectra (data not shown) of the aglycone.

The FAB mass spectra of the metabolite had no ions at 805 or 617, but the ions at 451 and 473 daltons corresponded to the mono- ($M + Na^+$) and di-natriated ($M + 2Na^+ - H^+$) adducts of deglycosylated phyllanthoside (Fig. 5). Low-energy MS/MS spectra of the peaks at 917 and 559 daltons did not generate fragments diagnostic of phyllanthoside; it was thus assumed that these ions were not the products of phyllanthoside metabolism. Low-energy MS/MS of the natriated ions did not produce usable fragmentation, but high-energy collision-induced dissociation of the metabolite molecular ion (m/z 429) produced a daughter ion at m/z 281 that was identical to the spectra obtained on the aglycone fragment of phyllanthoside (data not shown).

Cytotoxicity of metabolite

The phyllanthoside metabolite was much less cytotoxic to human A204 rhabdomyosarcoma cells than was the parent drug (Fig. 6). Concentrations of phyllanthoside or metabolite required to produce 50% inhibition of colony formation were 0.47 n*M* and 24 μ*M*, respectively.

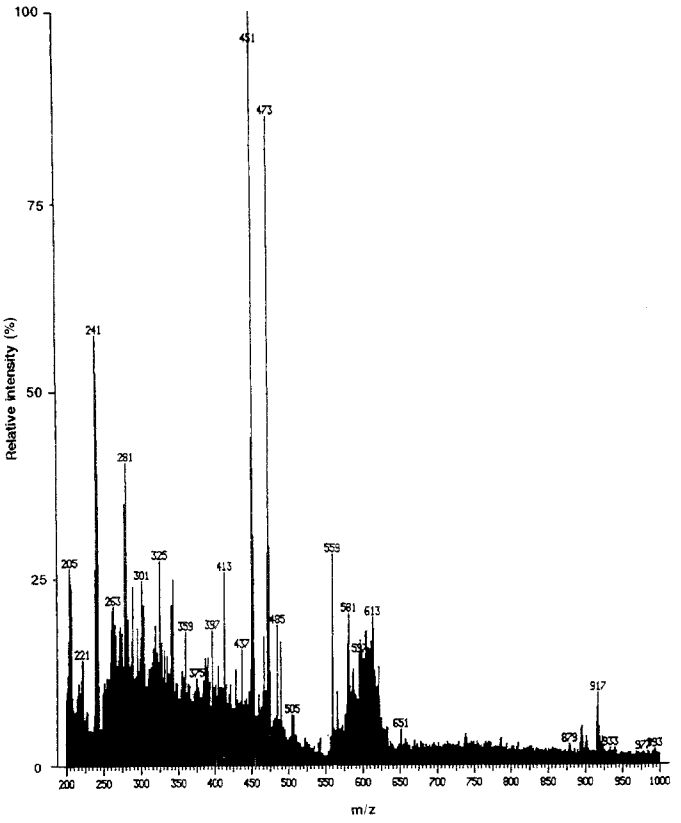

Fig. 5. Positive-ion FAB mass spectrum of the metabolite. The molecular ion of the aglycone (m/z 427) is not present, but peaks at m/z 451 and 473 correspond to the natriated adducts of the aglycone

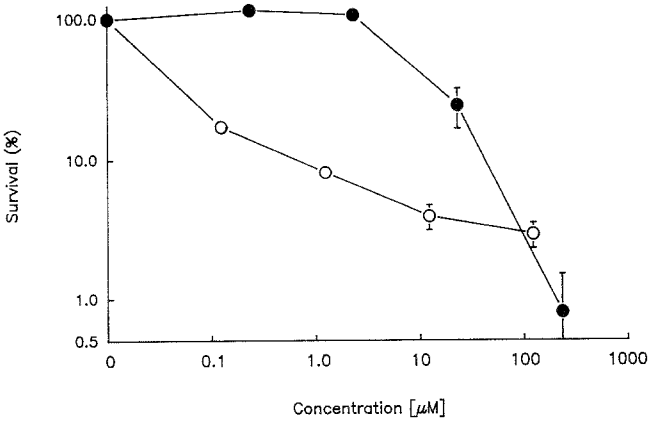

Fig. 6. Cytotoxicity of phyllanthoside and metabolite. Phyllanthoside (○) and the metabolite (●) were incubated with A204 rhabdomyosarcoma cells growing in soft agarose for 24 h. Tumor-cell growth was assessed as colony formation after 7 days. Each point represents the mean ± SD of triplicate assays. The number of colonies at 0 μmol drug/ml was 1,565 ± 123

Discussion

Previous reports have suggested that phyllanthoside is unstable in plasma from several species [6]. In human plasma, phyllanthoside is converted in vitro into several less polar derivatives, and within 2 h the parent drug is no longer detectable. Mouse and rat plasma rapidly convert phyllanthoside into a single metabolite, and the parent drug cannot be detected after incubation of 12 μ*M* phyllanthoside with mouse plasma for 15 s [6].

In the present study, several lines of evidence suggested that the metabolite formed by mouse plasma was the aglycone of phyllanthoside. When distilled water was substituted for 1.5 m*M* acetic acid (pH 4.0), the decreased retention time of the metabolite on an RP-18 column was consistent with the ionizable carboxyl moiety present in the aglycone. In addition, the identical UV absorption spectra obtained for both phyllanthoside and the metabolite suggested that the chromophore responsible for the 278-nm absorption maximum of the parent drug was sill present in the metabolite. This chromophore is assumed to be the phenyl-CH=CH-C(O)-moiety, since the UV absorption maximum for *trans*-cinnamic acid (phenyl-CH=CH-COOH) is 274 nm [2]. The aglycone identity of the metabolite was confirmed by mass spectral analysis of the purified mouse plasma metabolite.

The inability of boiled or filtered mouse plasma to metabolize phyllanthoside suggested that aglycone formation resulted from the enzymatic hydrolysis of the drug. This is in contrast to the nonenzymatic base- or acid-catalyzed acetyl group migration between neighboring hydroxyl groups of the disaccharide of phyllanthoside that has been reported [5]. It is possible that similar acetyl group migrations may occur in plasma or tissues in which phyllanthoside is not rapidly hydrolyzed to the aglycone. The observation that phyllanthoside metabolism is inhibited by the irreversible esterase inhibitor DFP suggested that an esterase is responsible for aglycone formation by mouse plasma. Plasma esterase activities and, in particular, plasma carboxylesterase activities exhibit a marked species variability. Human, dog, and monkey plasma typically exhibit low carboxylesterase activity, whereas rodent and rabbit plasma rapidly metabolize ester-containing compounds such as atropine, cocaine, and etomidate [7]. Rabbit serum atropine esterase activities (0.35 μmol atropine hydrolyzed/min per ml serum) [3] are comparable to the rate of phyllanthoside metabolism observed in the present studies.

The formation of the less cytotoxic aglycone by mouse plasma may have been responsible for the absence of in vivo antitumor activity when phyllanthoside was given i. v. to mice [Dr. J. A. Mead, personal communication, November 21, 1984: NCI preclinical pharmacology studies on phyllanthoside (NSC 328 426)] and may explain the decreased toxicity of phyllanthoside to the mouse relative to the dog [4]. However, in the present studies the aglycone was cytotoxic to human A204 rhabdomyosarcoma cells in vitro at concentrations of >2.3 μ*M*, and the maximal efficacies of the aglycone and phyllanthoside were comparable. This suggests that differences in potency between phyllanthoside and the aglycone may depend on factors other than different biochemical mechanisms of action. For example, cellular uptake of the aglycone may be reduced, relative to that of phyllanthoside, by hydrolysis of the neutral parent drug and formation of the charged aglycone. The mechanism of action responsible for phyllanthoside's inhibition of tumor growth has not been defined, although its inhibition of protein synthesis has been reported [Dr. J. A. Mead, personal communication, November 21, 1984: NCI preclinical pharmacology studies or phyllanthoside (NSC 328426)]. Identification of

the biochemical basis for this drug's action would enable the direct comparison of the pharmacological activities of phyllanthoside with those of the aglycone and perhaps aid in the development of phyllanthoside derivatives with increased antitumor activities.

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