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Disposition and metabolism of the antitumor glycoside phyllanthoside in mouse and beagle dog*

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Summary. Phyllanthoside is a naturally occurring glycoside with activity against IP transplantable murine tumors. Phyllanthoside administered IV, to mice at a nontoxic dose of 16 mg/kg could not be detected in blood or plasma even 30 s after administration. There was rapid formation of a less polar metabolite, which disappeared with a half-life of about 10 min. When phyllanthoside was administered as an IV bolus to beagle dogs at doses of 0.1, 0.5, and 3.0 mg/kg the mean half-life of phyllanthoside elimination from plasma was 1.3 min and total body clearance 85.8 ml min^{-1} kg^{-1}. A second phase of elimination was seen but could not be accurately defined. Only trace amounts of the less polar metabolite were detected in dog plasma. Infusion of phyllanthoside to beagle dogs at doses of 0.5 and 3.0 mg/kg over 70 min gave values for an initial half-life of 0.3 and 0.6 min, a terminal half-life of 99.4 and 16.5 min, and a total body clearance of 11.2 and 49.2 ml min^{-1} kg^{-1}, respectively. The highest nontoxic dose of phyllanthoside in dog was 0.1 mg/kg, while doses of 0.5 mg/kg and 3.0 mg/kg resulted in ataxia and death of the dog. There was no difference in toxicity to dog according to whether phyllanthoside was given by IV bolus or continuous infusion. Isolated hepatocytes from rat metabolized phyllanthoside at a rate of 4.4 μg/min per 10^6 cells to form the less polar metabolite. Coculture with isolated hepatocytes decreased the cytotoxicity of phyllanthoside to A204 human rhabdomyosarcoma cell line growing in soft agarose. It is suggested that rapid metabolism of phyllanthoside in mouse as against dog might account for the lower toxicity of phyllanthoside in mouse, and might also account for the reported poor antitumor activity of IV-administered phyllanthoside in the mouse.

Introduction

Phyllanthoside is a glycoside derived from the roots of *Phyllanthus acuminatus* Vahl, a tree found in abundance in Central and South America [5]. The structure of phyllanthoside has been determined by Pettit et al. [6] (Fig. 1). Phyllanthoside is a potent inhibitor of leucine incorporation in leukemia P-388 cells in vitro, producing delayed dose-dependent inhibition of DNA synthesis, but has no effect on RNA synthesis. This pattern of inhibition is typical of cytotoxic protein synthesis inhibitors.[1] In mice phyllanthoside administered IP exhibits antitumor activity against IP B-16 melanoma and IP P-388 leukemia.[1] Phyllanthoside is currently being considered for eventual clinical trials by the National Cancer Institute, USA. Previous work has shown phyllanthoside to be rapidly degraded by plasma of mouse and rat, but to be more stable in plasma of dog, monkey, and human [7]. We now report the disposition of phyllanthoside in mouse and dog and its conversion to a less cytotoxic metabolite.

Materials and methods

Drugs. Phyllanthoside (NSC 328426) was supplied as bulk drug by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md, USA. The purity of the bulk phyllanthoisde was determined by high-performance liquid chromatography (HPLC) to be 93.7%. Phyllanthoside was formulated in flint glass vials containing 10 mg drug and 100 mg mannitol and stored under N_2 at −70 °C until use, when it was reconstituted with 4 ml 5% ethanol: 2.5% Cremophore EL. Cremophore EL was obtained from Sigma Chemical Co., St. Louis, Mo, USA. For bolus injection phyllanthoside was used as the reconstituted solution at 2.5 mg/ml. For continuous infusion the solution was further diluted to 0.1 mg phyllanthoside/ml with 0.9% NaCl containing 2.5% ethanol and 1.2% Cremophore EL. All solutions were kept on ice until use.

Pharmacokinetic studies. Phyllanthoside, at a dose of 16 mg/kg, was administered by rapid IV injection (< 30 s) to male CDF_1 mice weighing 23–25 g. Mice were held in a Broome-type restraint and given a 2.5 mg/ml solution of formulated phyllanthoside by injection into a tail vein with a 25-gauge needle. Blood was collected from groups of three mice at 0, 0.5, 1, 2, 5, 10, 30, 60, 120, 180, and 360 min. Mice were lightly anesthetized with diethyl ether and as soon as rapid limb movements had ceased animals were exsanguinated by bleeding from the retro-orbital venous plexus [4] into chilled, heparinized 2-ml centrifuge tubes. For the earlier time points mice were already anesthetized when they received the drug. Times are the

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[1] Preclinical Pharmacology Studies on Phyllanthoside (NSC 328426), National Cancer Institute, Bethesda, Md, USA (Dr J. A. R Mead, personal communication November 21, 1984

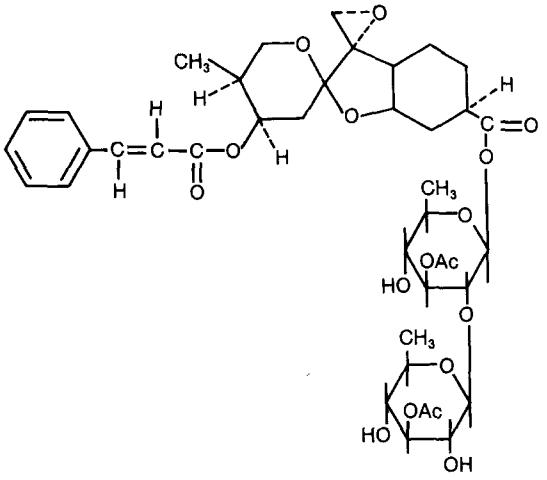

Fig. 1. Structure of phyllanthoside

midpoint of the blood collection period, which took about 30 s. The blood was immediately centrifuged at 15000 rpm on a Beckman B microcentrifuge (Beckman Instruments, Irvine, Calif) for 2 min, and 0.2 ml plasma was taken for immediate extraction of phyllanthoside and its metabolites and HPLC assay the same day. A group of three mice received formulated phyllanthoside, 16 mg/kg, by rapid IV injection and were placed in an all-glass metabolism chamber. Urine was collected for 24 h on dry ice and assayed for phyllanthoside and metabolites.

Phyllanthoside was administered to male beagle dogs weighing 11–14 kg by IV bolus injection over 1 min, or as a continuous infusion over 70 min at doses of 0.1 mg/kg, 0.5 mg/kg, and 3 mg/kg. Dogs were placed in a Pavlov type dog sling, and phyllanthoside was injected through a Teflon catheter (Angiocath 18 gauge, Deseret Co., Sandy, UT, USA) into a cephalic vein. Samples of blood, 5 ml, were drawn into a syringe from a Teflon catheter in the jugular vein at 0, 1, 2, 3, 5, 8, 10, and 12 min following bolus administration and at 0, 3, 5 min and 5 min intervals thereafter during the 70 min continuous infusion. Blood was immediately transferred to chilled heparinized tubes, which were centrifuged for 5 min at 4600 rpm at room temperature to separate plasma. Duplicate samples of plasma were immediately taken for extraction of phyllanthoside and its metabolites and HPLC assay the same day.

Microsomes were prepared from the liver of male Sprague-Dawley rats by the method of Ernster et al. [2] and suspended at a protein concentration of 10 mg/ml in 0.15 *M* KCl. Microsomal incubations were conducted at 37 °C with gentle shaking in 100-ml Erlenmeyer flasks in a final volume of 8.0 ml, with a mixture containing 30 m*M* Tris-HCl buffer, pH 7.4, 2.5 m*M* $MgCl_2$, 0.5 m*M* $NADP^+$, 1 m*M* glucose-6-phosphate, and 3.5 units/ml glucose-6-phosphate dehydrogenase. After a 5-min preincubation at 37 °C the reaction was started by the addition of phyllanthoside at a concentration of 20 μg/ml. Samples of incubation medium, 0.5 ml, were taken for assay of phyllanthoside and metabolites at 0, 2.5, 5, 10, 20, and 30 min.

Rat hepatocytes were prepared from the livers of male Sprague-Dawley rats by the methods of Stewart and Inabe [9]. Hepatocyte viability determined by trypan blue exclusion was always greater than 90%. Hepatocytes, 8×10^6 cells, were incubated in 8 ml 0.2 *M* Tris-HCl buffer, pH 7.4, 5 m*M* $MgCl_2$ containing phyllanthoside at a concentration of 20 μg/ml in open 100-ml Erlenmeyer flasks with gentle shaking at 37 °C for 30 min. Samples, 0.5 ml, of incubation medium and hepatocytes were removed at different times for assay of phyllanthoside.

The effect of rat hepatocyte coculture on the antiproliferative activity of phyllanthoside against the A204 human rhabdomyosarcoma tumor cell line growing in soft agarose culture was determined by a procedure previously described [1], Briefly, 10^4 A204 cells were grown in 0.5 ml 0.3% soft agarose in 35-mm culture dishes for 24 h then overlaid with 1 ml medium containing phyllanthoside at various concentrations, with or without 10^5 rat hepatocytes. Drug and hepatocytes were removed 24 h later, and colony formation by A204 cells was quantified by automated image analysis of cells stained with metabolizable 2-(*p*-idophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride after 10 days.

Assay. Phyllanthoside and metabolites were assayed by a reversed-phase HPLC procedure as previously described [7]. Brielfy, 0.5-ml aliquots of plasma or incubation medium were mixed with 1 μg 4-hydroxybiphenyl internal standard and 0.5 ml 0.1 *M* sodium phosphate buffer, pH 7.0, and shaken in closed tubes with 5 ml ethyl acetate for 20 min. The tubes were centrifuged at 1000 *g* for 10 min at room temperature, after which the ethyl acetate layer was removed and evaporated to dryness under N_2 at room temperature. The residue was dissolved in 100 μl methanol and 60 μl was injected onto a RP-18, 5-μm HPLC column (Merck, Darmstadt, FRG) with a mobile phase of a 50%–100% linear gradient of methanol in 0.3 *M* sodium acetate buffer, Ph 4.0. Eluting compounds were detected by their absorbance at 270 nm. The lower limit of sensitivity of the assay for phyllanthoside was 0.1 μg/ml, and the coefficient of variation was ±3.3% at 10 μg/ml and ±7.4% at 1 μg/ml.

Pharmacokinetic analysis. Plasma drug concentration data were subjected to nonlinear least-squares regression analysis using the NONLIN pharmacokinetic computer program [3] with a weighting factor of $1/y^2$. Pharmacokinetic parameters were calculated according to Wagner [10].

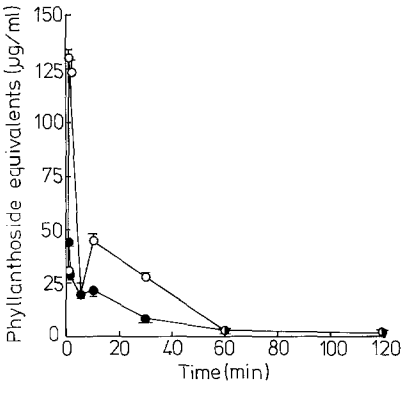

Fig. 2. Phyllanthoside metabolite in plasma (○) and blood (●) of mouse. Phyllanthoside was administered IV to groups of three mice at a dose of 16 mg/kg. Blood and plasma were obtained from different groups of mice. No parent phyllanthoside could be detected in blood or plasma at any time point. Phyllanthoside metabolite is expressed as phyllanthoside equivalents, assuming the same extinction coefficient as phyllanthoside at 270 nm

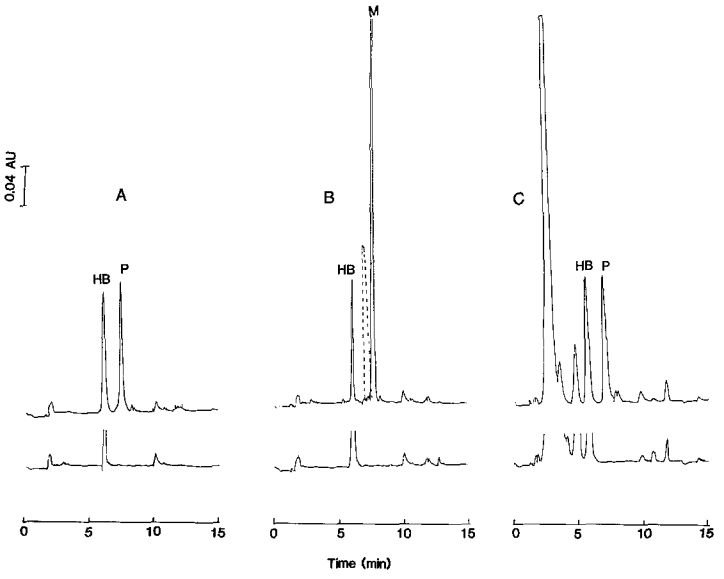

Fig. 3 (A–C). Chromatograms of phyllanthoside in plasma. *Upper traces* phyllanthoside *(P)* or metabolite *(M)* in plasma with internal standard 4-hydroxybiphenyl *(HB)*. Lower traces, control plasma with internal standard only. **A** Phyllanthoside added to heat-treated mouse plasma (100 °C for 3 min) to give 10 μg/ml; **B** mouse plasma 30 s after IV administration of phyllanthoside, 16 mg/kg. The position of the phyllanthoside peak is indicated by the *dotted line;* **C** dog plasma 2 min after IV administration of phyllanthoside, 3 mg/kg

Results

Pharmacokinetic studies in mouse

Unchanged phyllanthoside could not be detected in plasma of mouse even 30 s after IV administration of phyllanthoside, 16 mg/kg, although a less polar metabolite was present which disappeared with a half-life of approximately 10 min. The metabolite was undetectable 120 min after the administration of phyllanthoside (Fig. 2). A typical chromatogram of mosue plasma containing the less polar metabolite is shown in Fig. 3. Phyllanthoside was assayed in whole blood of a separate group of mice to rule out the possibility that phyllanthoside was taken up by red blood cells, thus accounting for its rapid disappearance from plasma. The results were similar to those obtained with plasma, and no unchanged phyllanthoside could be detected in whole blood (Fig. 2).

No parent phyllanthoside could be detected in the urine of mice 24 h after administration of phyllanthoside, but there were at least four more polar metabolite peaks (results not shown).

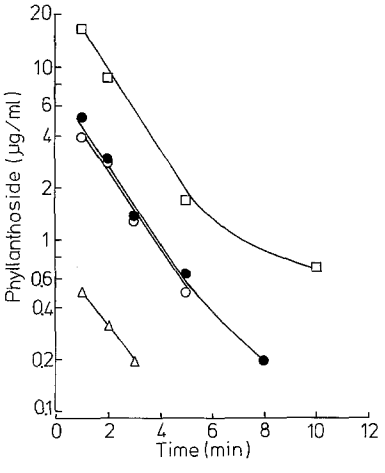

Fig. 4. Phyllanthoside plasma elimination in dog after bolus administration. Phyllanthoside was administered as a 1-min IV bolus to beagle dogs at doses 3 mg/kg (□) one dog; 0.5 mg/kg ■, two dogs; and 0.1 mg/kg △ one dog. *Continuous lines* are computer generated fits to the data

Pharmacokinetic studies in dog

Bolus administration. Plasma concentrations of phyllanthoside in beagle dogs given IV bolus doses of phyllanthoside of 0.1, 0.5, and 3 mg/kg are shown in Fig. 4. A typical chromatogram of plasma from a dog given phyllanthoside is shown in Fig. 3. Elimination of phyllanthoside appeared to be biphasic at the doses of 0.5 and 3 mg/kg, but insufficient data points were obtained for accurate definition of the second phase of elimination. The mean half-life for the initial phase of phyllanthoside disappearance was 1.3 min, the mean apparent volume of distribution, 163 ml/kg, and assuming a singel phase of elimination, the mean total body clearance was 85.8 ml min^{-1} kg^{-1}. The value for total body clearance may be an overestimate because of our inability to define the second phase of phyllanthoside elimination. There was no detectable metabolite formation from phyllanthoside except in one dog receiving 0.5 mg/kg, where a very small peak of less polar metabolite was seen 12 min after phyllanthoside was given (results not shown).

Continuous infusion. Plasma concentrations of phyllanthoside in dogs given, phyllanthoside by continuous IV infusion over 70 min are shown in Fig. 5. Phyllanthoside was barely detectable in plasma after a dose of 0.1 mg/kg. At phyllanthoside doses of 0.5 and 3.0 mg/kg the rise in plasma phyllanthoside was biphasic, with initial half-lives of 0.3 and 0.6 min and terminal half-lives of 99.4 and 16.5 min, respectively. Total body phyllanthoside clearance at a phyllanthoside dose of 0.5 mg/kg was 11.2 ml min^{-1} kg^{-1} and at a dose of 3.0 mg/kg was 49.2 ml min^{-1} kg^{-1}. No phyllanthoside metabolites were detected in the plasma of these dogs.

Toxicity. Phyllanthoside administered by IV bolus injection was toxic to dogs at doses of 0.5 mg/kg and 3 mg/kg. At both doses lethargy was apparent immediately on phyllanthoside administration and emesis occurred within 1 h. The dog given a bolus dose of 3 mg/kg developed diarrhea, was ataxic by 6 h and died overnight. One of the two dogs given bolus phyllanthoside 0.5 mg/kg died after 72 h,

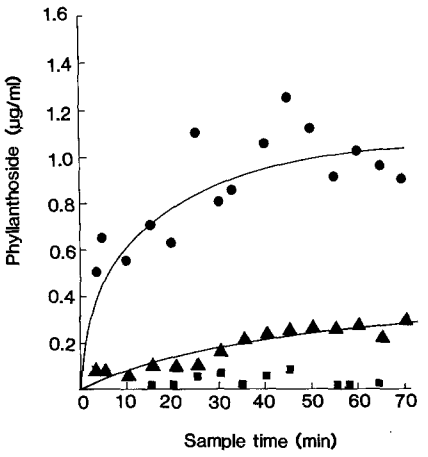

Fig. 5. Plasma phyllanthoside concentrations during IV infusion. Phyllanthoside was administered by 70-min continuous infusion to beagle dogs at doses of 3 mg/kg (●), 0.5 mg/kg (▲), and 0.1 mg/kg (■). *Continuous lines* are computer-generated fits to the data

the other dog was moribund and was killed at 48 h. The dog receiving bolus phyllanthoside 0.1 mg/kg experienced no lethargy or emesis and at 48 h appeared only slightly ataxic. The toxicity of phyllanthoside given by 70-min infusion was similar to that of phyllanthoside administered by bolus injection. At the phyllanthoside dose of 3 mg/kg the dog showed no toxic effects during infusion, but died overnight. The dog receiving phyllanthoside at 0.5 mg/kg was lethargic by the end of the infusion. It became progressively weaker over the next few days and was moribund on day 4, when it was killed. The dog receiving phyllanthoside at 0.1 mg/kg experienced mild emesis during the infusion, but exhibited no lethargy or other adverse side effects and was alive and healthy 3 weeks later.

Liver metabolism studies

Phyllanthoside was metabolized by rat liver microsomes to the same less polar metabolite as was seen in plasma (Table 1). The breakdown was not dependent upon added NADPH and was not inhibited by N_2 or 3 m*M* cyclohexane, 1,2-oxide, an inhibitor of microsomal epoxide hydrolase [8]. Preheating microsomes to 100 °C for 3 min completely inhibited the breakdown of phyllanthoside. Isolated rat hepatocytes also converted phyllanthoside to the less polar metabolite (Table 1). The reaction was not inhibited by anaerobic conditions or 3 m*M* cyclohexane 1,2-oxide, but was inhibited by preheating the hepatocytes to 100 °C for 3 min.

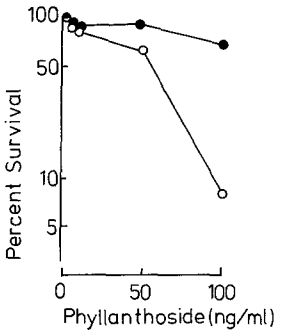

Fig. 6. Inactivation of phyllanthoside by hepatocyte coculture. Phyllanthoside was incubated with A204 rhabdomyosarcoma cells growing in softagarose for 24 h in the absence (○) and in the presence (●) of isolated rat hepatocytes (10 hepatocytes: 1 tumor cell). Tumor cell growth was assessed as colony formation after 10 days. Each *point* is the mean of quadruplicate determinantions

Table 1. Metabolism of phyllanthoside by rat liver

Incubation	Phyllanthoside utilization (μg/min per mg or 10^6 cells[a])	Metabolite appearance[a] (μg/min per mg or 10^6 cells[b]
Microsomal Fraction		
Air, −NADPH	0.43	0.78
Air, +NADPH	0.71	0.64
N_2, +NADPH	0.71	0.71
Air, +NADPH, +3 mM CHO	0.71	1.00
Hepatocytes		
Air	4.4	2.0
N_2	6.0	2.2
Air, +3 mM CHO[c]	5.4	2.3
Air, boiled	–	–

Results are means of three determinations in each case, and are initial rates of metabolism measured over 5 min

[a] Expressed as μg/min per mg protein for the microsomal fraction or μg/min per 10^6 viable cells for hepatocytes

[b] Expressed in phyllanthoside equivalents, assuming a similiar extinction coefficient at 270 nm

[c] CHO, cyclohexane, 1,2-oxide

Phyllanthoside was markedly cytotoxic to A204 human rhabdomyosarcoma cells in soft agarose culture. The concentration for 50% inhibition of colony formation (IC_{50}) was 50 ng/ml (Fig. 6). Coincubation of the A204 cells with isolated hepatocytes for 24 h led to a marked decrease in the cytotoxicity of phyllanthoside, and the IC_{50} was greater than 100 ng/ml, the highest concentration tested.

Discussion

Phyllanthoside is very rapidly eliminated from plasma when administered to mice. No parent phyllanthoside could be detected in plasma, even within 30 s of IV administration, and all the phyllanthoside was converted to a less polar metabolite. It has been reported previously that when it is added to fresh mouse plasma in vitro, disappearance of phyllanthoside is very rapid, so that no parent drug can be detected even after 15 s of incubation and there is formation of a less polar derivative [7]. Metabolism by plasma could account in part for the failure to detect parent drug in vivo. Isolated hepatocytes were also found to metabolize phyllanthoside, and in vivo the liver and other tissues might contribute to phyllanthoside metabolism. Elimination of phyllanthoside from plasma of beagle dog was slower than in mouse and was biphasic. The initial half-life calculated from bolus administration data was 1.3 min, and the terminal half-life calculated from infusion data was 16.5–99.4 min. The total body clearance of phyllanthoside calculated from bolus administration data was 85.8 ml min^{-1} kg^{-1}, and that calculated from infusion data was 11.2 to 49.2 ml min^{-1} kg^{-1}. The value for total body clearance calculated from bolus administration

data may be an overestimate because of our inability to accurately define a second phase of phyllanthoside elimination following bolus administration. Only traces of phyllanthoside metabolite were detected in the plasma of dogs receiving phyllanthoside.

In vitro studies have shown that metabolism of phyllanthoside with the formation of a less polar metabolite by isolated hepatocytes is associated with loss of cytotoxicity to A204 rhabdomyosarcoma cells. It is probable, therefore, that the rapid disappearance of phyllanthoside and the formation of a less polar metabolite in mouse accounts for the absence of antitumor activity of IV phyllanthoside against IP and SC, tumors, compared with the activity of IP phyllanthoside against IP tumors.[1] Whether IV phyllanthoside might exhibit more antitumor activity in dog, where phyllanthoside is eliminated from plasma more slowly, is not known. Phyllanthoside does, however, appear to be much more toxic to dog than to mouse. The highest nontoxic dose of phyllanthoside in dog is approximately 0.1 mg/kg (1.5 mg/m^2), whereas a dose of 16 mg/kg (68 mg/m^2) is nontoxic to mouse. Human plasma is similar to dog plasma, in that it breaks down phyllanthoside more slowly than plasma from mouse or rat [7]. Phyllanthoside might, therefore, also exhibit a greater relative toxicity in human than in mouse.

The identity of the less polar metabolite is not known, and work is in progress to identify its structure. It was not possible to inhibit the formation of the phyllanthoside metabolite by rat hepatocytes by an inhibitor of epoxide hydrolase, or its formation in mouse plasma by a variety of inhibitors of plasma esterases (unpublished observations).

In summary, phyllanthoside is very rapidly broken down to a less polar metabolite when injected IV in mice, but is more slowly eliminated from plasma of dog. The less polar metabolite has not yet been identified, but in vitro studies have suggested that it is less cytotoxic than parent phyllanthoside. This may explain the greater toxicity of phyllanthoside to dog than mouse.

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References

1. Alley MC, Powis G, Appel PL, Kooistra KL, Lieber MM (1984) Activation and inactivation of cancer chemotherapeutic agents by rat hepatocytes cocultured with human tumor cell lines. Cancer Res 44: 549
2. Ernster L, Siekvitz P, Palade G (1962) Enzyme-structure relationships in the endoplasmic reticulum of rat liver. J Cell Biol 15: 541
3. Metzler CM, Elfring G, McEwen AJ (1974) A package of computer programs for pharmacokinetic modeling. Biometrics 30: 562
4. Migdalof BH (1976) Methods for obtaining drug time course data from individual small animals: Serial microblood sampling and assay. Drug Metab Rev 5: 295
5. Pettit GR, Cragg GM, Gust D, Brown P, Schmidt JM (1982) The structures of phyllanthostatin 1 and phyllanthoside from the Central American tree *Phyllanthus acuminatus* Vahl. Can J Chem 60: 939
6. Pettit GR, Cragg GM, Niven ML, Nassimbeni LR (1983) Structure of the principla antineoplastic glycosides of *Phyllanthus acuminatus* Vahl. Can J Chem 61: 2630
7. Powis G, Moore DJ (1985) A high performance liquid chromatography assay for the antitumor glycoside phyllanthoside and its stability in plasma of several species. J Chromatogr 342: 129
8. Seidegard J, DePierre JW (1983) Microsomal epoxide hydrolase properties, regulation and function. Biochim Biophys Acta 695: 251
9. Stewart AJ, Inabe T (1976) *N*-Demethylation of aminopyrine in vivo and in the isolated hepatocyte of the rat. Biochem Pharmacol 28: 461
10. Wagner JG (1976) pharmacokinetic equations allowing direct calculation of many needed pharmacokinetic parameters from the coefficients and exponents of polyexponential equations which have been fitted to the data. J Pharma Biopharm 4: 443

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