

In Vitro and in Vivo Metabolism of VP 16-213 in the Rat

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Abstract—A high-performance liquid chromatographic procedure utilizing u.v. and radioactivity detection was employed to examine the metabolism of the epipodophyllotoxin derivative VP 16-213 by the rat in vivo and in liver extracts and subcellular fractions. VP 16-213 has been shown to be metabolized extensively by rat liver homogenates and rat liver microsomes, with the formation of one major metabolite. The metabolite formed in vitro was the only metabolite present in plasma samples of rats treated with i.p. injections of VP 16-213. Based on its chromatographic and solubility characteristics, the metabolite is probably a *cis*- or *trans*-hydroxy acid derivative. The liver is involved in the metabolism of VP 16-213, and the localization of the enzyme(s) responsible for the formation of the major metabolite is in the microsomal fraction.

INTRODUCTION

THE ANTITUMOUR epipodophyllotoxin derivative VP 16-213 or 4'-demethylepipodophyllotoxin-1-O-(4,6-O-ethylidene- β -D-glucopyranoside (NSC 141540; Fig. 1) is currently extensively investigated in clinical trials. It is the most active single agent tested against small cell bronchial carcinoma and has also shown activity in non-small cell bronchial carcinoma, acute non-lymphocytic leukemia, Hodgkin's disease, non-Hodgkin's lymphomas and testicular teratomas [1, 2]. Despite the extensive clinical use of VP 16-213, there is little known of the *in vivo* behaviour of the drug, such as bioavailability and metabolism. Advanced studies on the structures, extent of formation and pharmacological and toxicological properties of metabolites have not been performed. There is, however, some evidence from the literature that VP 16-213 can be extensively metabolized. Creaven and Allen [3], using tritium-labelled VP 16-213, found that 15% of the administered dose was excreted in the urine as metabolites. VP 16-213 contains a transfused, highly strained γ -lactone ring and can be chemically converted by base catalysis to a ring-opened *cis*-hydroxy acid derivative [4]. A ring-opened hydroxy acid has been described as being a major metabolite of VP 16-213 by Allen *et al.* [5] and Strife *et al.* [4], but structural evidence was

not conclusive, particularly regarding the metabolite being the *cis*- or *trans*-hydroxy acid derivative. In view of the known possibility of detoxification of podophyllotoxin and analogues by epimerization at the lactone-ring site [6], it is important to know whether a *cis*- or *trans*-hydroxy acid is formed and also whether picro VP 16-213, which is VP 16-213 with the lactone-ring in the *cis*-configuration, is produced in metabolism.

The presence of the sugar moiety in VP 16-213 is of importance regarding its toxicity and biological activity. Earlier derivatives of podophyllotoxin lacking a sugar group and which underwent clinical trials such as podophyllinic acid ethyl hydrazide were found to be too toxic for clinical use [2]. The mechanism of action of podophyllotoxin involves inhibition of microtubule assembly and it arrests cells in metaphase [7]. VP 16-213 arrests cells in the premitotic phase of the cell cycle [2]. It does not inhibit microtubule assembly but, in contrast to podophyllotoxin, it induces intracellular degradation of DNA in Hela cells [8]. 4'-Demethylepipodophyllotoxin, the aglycone of VP 16-213, combines both the inhibitory action on microtubule assembly and the ability to induce breaks in DNA. Regarding these phenomena, it is important to investigate whether the aglycone of VP 16-213 is formed in metabolism.

In view of the above, we have started to investigate in detail the metabolism of VP 16-213. This paper reports on the formation of metabolites of VP 16-213 by subcellular liver fractions of the rat and *in vivo* by the rat.

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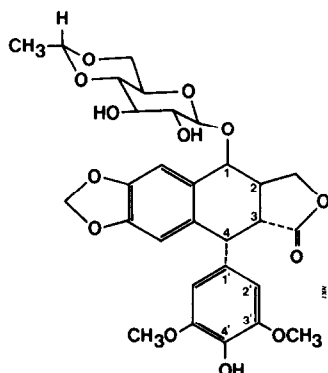


Fig. 1. Structure of VP 16-213.

MATERIALS AND METHODS

In vitro experiments

Adult male rats of the inbred strain R-Amsterdam (R/A) and chinchilla rabbits were used. Livers of rats given 1 g/l sodium phenobarbitone in their drinking water for ten days before use and livers of rabbits were homogenized in 1.15% potassium chloride–0.01 M potassium phosphate, pH 7.4 (6 ml/g liver). Microsomes were prepared by centrifugation of the homogenate at 10,000 *g* for 20 min and recentrifugation of the supernatant at 100,000 *g* for 1 hr. The microsomal pellet was washed by resuspension in fresh extraction buffer and recentrifugation at 100,000 *g* for 1 hr. VP 16-213, specifically labelled with tritium at position C-1 and with a specific activity of 119.8 $\mu\text{Ci}/\text{mg}$, was kindly supplied by Bristol Myers Company (Syracuse, NY) and used for metabolism experiments. Incubations were carried out with liver homogenates and 100,000 *g* supernatant and microsomes which were suspended in 0.01 M phosphate buffer, pH 7.4, for 4 hr at a temperature of 37°C. The total incubation volume was 5 or 10 ml and concentrations of VP 16-213 used were in the range of 10–100 $\mu\text{g}/\text{ml}$. As a cofactor system, 1.43 μmol NADP, 30.6 μmol glucose-6-phosphate, 24.6 μmol $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and 2.5 μl glucose-6-phosphate-dehydrogenase (5 mg/ml, 140 IU/mg) were used per 5 ml of incubation medium. The medium was oxygenated at 0, 1 and 2 hr of incubation or incubations in an atmosphere of nitrogen were carried out. For control incubations VP 16-213 was incubated only in buffer to determine if there was exchange of tritium or decomposition and with homogenates and subcellular fractions inactivated by heating at 85–90°C for 20 min. After 0, 2 and 4 hr of incubation, a 0.5-ml sample of incubate was taken and 1 ml ice-cold ethanol was added. This mixture was centrifuged at 2000 *g* for 30 min. Aliquots of 20 μl of

the supernatants were used for analysis by high-performance liquid chromatography (HPLC; see Assay). Protein was determined by the BioRad protein-assay [9].

In vivo experiments

Male rats were given intraperitoneal injections of [^3H]-labelled VP 16-213 and blood samples were taken for plasma analysis of VP 16-213 and metabolites. In a typical experiment, a rat weighing 175 g was given three consecutive i.p. injections at three-hour intervals of 0.194 mg [^3H]-labelled VP 16-213 in 1,3-propanediol, corresponding to doses of 1.1 mg/kg. At 0.5, 2, 3.5, 5, 6.5, 20, 22 and 24 hr after the first injection, blood samples (0.4–0.6 ml) were taken by orbital puncture under light ether anaesthesia and collected in polypropylene tubes containing 20 μl heparin (5000 IU/ml) to prevent coagulation. The samples were mixed and centrifuged at 1000 *g* for 25 min. The plasma of each sample was divided into two parts (1 and 2, each of approx. 250 μl). Part 1 was diluted with two volumes of ice-cold methanol, the mixture vortexed and centrifuged at 2000 *g* for 25 min. The supernatant was evaporated under nitrogen at 20°C, and the residue dissolved in 100 μl 40% methanol and used for high-performance liquid chromatographic analysis (see Assay). Part 2 was diluted to 1 ml with distilled water, extracted for 1 min with 1 ml chloroform; the mixture was centrifuged at 2000 *g* for 5 min, the chloroform layer evaporated under nitrogen at 20°C and the residue redissolved in 100 μl 40% methanol and used for chromatographic analysis.

Assay

For analysis of metabolism samples, a reverse-phase high-performance liquid chromatographic procedure utilizing u.v.- and radioactivity detection was used. The apparatus consisted of a Kipp LC 771 chromatograph and Rheodyne 7120 injector in combination with a Chrompack Partisil-10 ODS column (25 cm \times 4.6 mm i.d., 10 μm particle size) and a Waters Model 440 u.v. absorbance detector with a fixed wavelength of 254 nm or a Waters 6000 A Solvent Delivery System, a U6K septumless injection system and a Model 440 u.v. absorbance detector with fixed wavelengths of 254 nm and 280 nm in combination with a Chrompack Lichrosorb RP-18 column (30 cm \times 4.6 mm i.d., 10 μm particle size) or a Waters μ Bondapak Alkyl-phenyl column (30 cm \times 3.9 mm i.d., 10 μm particle size). The mobile phase consisted of methanol–water, 40:60 (w/w%), for the *in vivo* experiments and methanol–water

mixtures ranging from 30 to 60 v/v% methanol, buffered at pH 7.1 with 0.01 M potassium phosphate, for the *in vitro* experiments. The flow rates were 1.0 ml/min for the *in vivo* experiments and 1.0 or 1.5 ml/min for the *in vitro* experiments [10]. For radioactivity detection, eluate fractions were collected at intervals of 30 sec and, after addition of 4.5 ml Instagel (Packard), each fraction was subjected to scintillation counting for 5 min in a Beckman LS8000 liquid scintillation counter.

RESULTS

Figures 2 and 3 show the percentage of radioactivity distributions of the chromatograms of metabolism samples obtained after incubation of VP 16-213 with rat liver homogenates and rat liver microsomes using a concentration of VP 16-213 in the incubation

medium of 20 μ g/ml. The data were obtained from three experiments. Under the assay conditions (Partisil-10 ODS column, 40 v/v% methanol as eluent, flow rate 1.5 ml/min) VP 16-213 showed a retention time of 9 min. Figure 2 shows that after 4 hr of incubating VP 16-213 with rat liver homogenate extensive metabolism has taken place. One major radioactive product, with a retention time of 3 min, is formed during the time course of incubation. There was a corresponding decrease in radioactivity and u.v. absorbance of the VP 16-213 peak. Incubations with rat liver homogenates showed the formation of two other radioactive products, with retention times of 5 and 7 min (Fig. 2). However, these products were formed at a lower level than the major product with the retention time of 3 min, and their formation was not reproducible. The

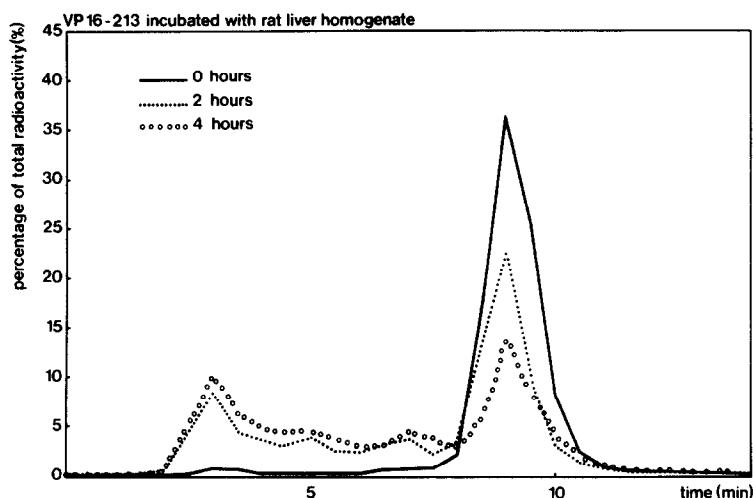


Fig. 2. Radioactivity distributions of chromatograms of incubation samples obtained after incubating VP 16-213 with rat liver homogenate. Eluate fractions of 30 sec were subjected to liquid scintillation counting. Percentage of total radioactivity of the eluate is plotted against retention time. —, 0 hr; ····, 2 hr; ○○○○, 4 hr of incubation.

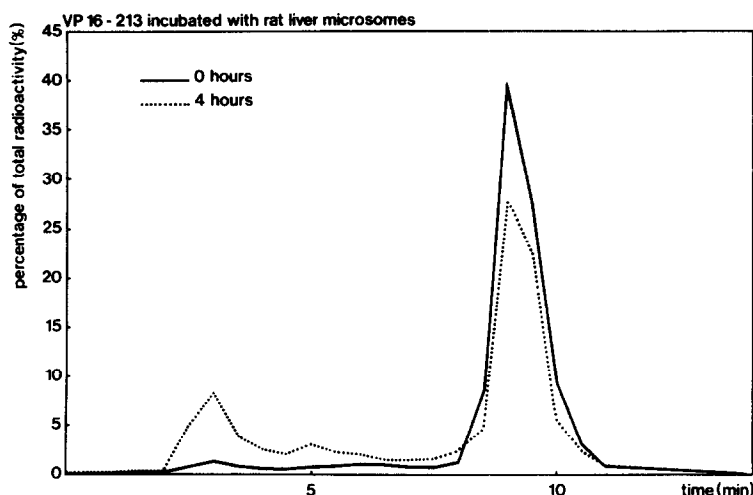


Fig. 3. *Idem* Fig. 2 for incubation with rat liver microsomes. —, 0 hr; ····, 4 hr of incubation.

product with the retention time of 3 min was found to be produced to the same extent by rat liver microsomes (Fig. 3). Incubating VP 16-213 in buffer did show the presence of one radioactive peak for VP 16-213, with a retention time of 9 min, indicating that no exchange of tritium or chemical breakdown of the [^3H]-labelled VP 16-213 occurs during the incubations. Incubation of VP 16-213 with inactivated liver homogenate and microsomes did show a small level of background radioactivity in the chromatogram, comparable to the amount present for the 0 hr incubation samples shown in Figs 2 and 3. These results indicate that the product with the retention time of 3 min is indeed a metabolite of VP 16-213. Incubations with the 100,000 *g* supernatant of rat liver did not show the presence of any radioactive component in the chromatogram besides VP 16-213. The product with the retention time of 3 min was also produced by rabbit liver homogenates to about the same extent as by rat liver homogenates. In the ethanol-aqueous supernatants about 90–95% of radioactivity was recovered from the original incubation samples, 5–10% remaining in the precipitated microsomal or homogenate pellet. About 95% of radioactivity of supernatant samples injected on the HPLC was recovered in the eluates.

Under the conditions of the experiments, the formation of the main metabolite by homogenate and microsomes was linear for no more than two hours and reached a plateau at four hours of incubation. In Fig. 4 the production by rat liver microsomes of the main metabolite given as nmol/hr/mg protein is plotted against concentration of VP 16-213. The amount of product formed after four hours of incubation was used in the calculation of the amount of conversion. At higher concentrations of VP 16-213 the curve reaches a plateau ($V_{\max} = 0.46$ nmol/hr/mg protein). The Lineweaver-Burk plot corresponding to Fig. 4 is shown in Fig. 5. The linear correlation between $1/S$ and $1/V$ indicates the conversion of VP 16-213 to the main product to be enzymatic.

To examine the possible formation of picro VP 16-213 by the rat liver systems, analyses of samples were also carried out on a μ Bondapak Phenyl column. Careful examination of the HPLC-eluates using methanol-water mixtures with a methanol content ranging from 30 to 60% and solvent flow rates of 0.7–1.0 ml/min were carried out, but did not reveal the presence of a component with the same retention time as the picro isomer.

Figure 6 shows the chromatograms of the methanol and chloroform extracts of plasma

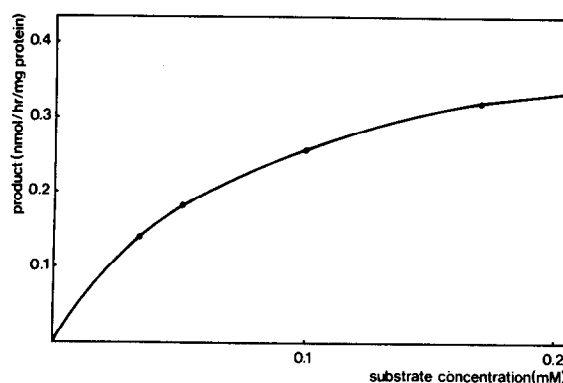


Fig. 4. Formation of the major metabolite by rat liver microsomes given as nmol/hr/mg protein plotted against substrate concentration in mM.

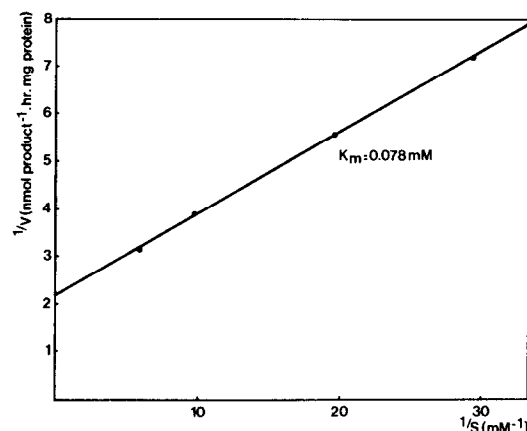


Fig. 5. Lineweaver-Burk Plot ($1/V$ vs $1/S$) for the formation of the major metabolite by rat liver microsomes.

samples obtained from rats treated i.p. with [^3H]-labelled VP 16-213. In the chloroform extracts no extra radioactive peak besides that of VP 16-213 was detected. In the methanol extracts a major radioactive component was present with a short retention time (3 min) compared with that of VP 16-213. This metabolite, which was also found to be produced by rat liver homogenates and microsomes, was present in the methanol extracts of plasma samples after two consecutive injections of VP 16-213 with an interval of three hours. It was present at levels of 36 and 45% relative to VP 16-213 at 3.5 and 5 hr respectively after the first injection.

DISCUSSION

The results of this study show that the epipodophyllotoxin derivative VP 16-213 is extensively metabolized by rat liver homogenates and microsomes and *in vivo* by the rat.

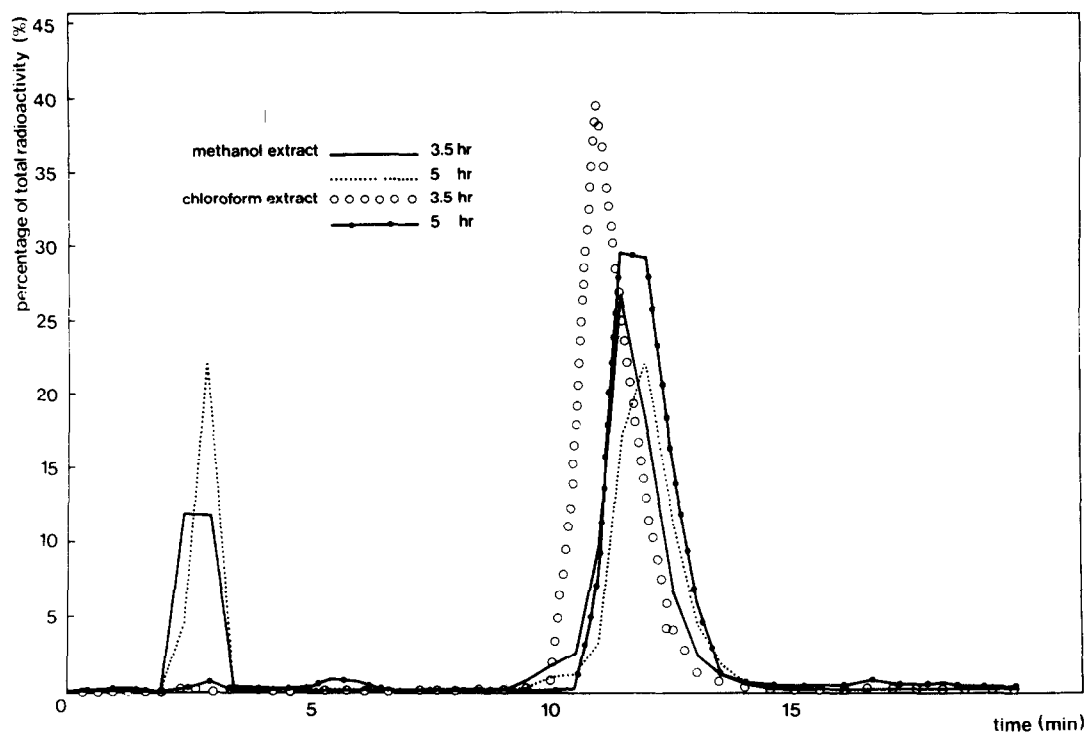


Fig. 6. Radioactivity distributions of chromatograms of methanol extracts (—, 3.5 hr; ····, 5 hr) and chloroform extracts (○○○○, 3.5 hr; —●—●—●, 5 hr) of plasma samples of rats treated i.p. with VP 16-213 at three-hour intervals. The percentage of total radioactivity of the eluate is plotted against retention time.

One major metabolite was formed which could reach high levels: in one *in vivo* experiment it was present in plasma at a level of 45% relative to VP 16-213. Due to its short retention time in reverse-phase chromatography, which matches that of the synthetic *cis*-hydroxy acid derivative of VP 16-213, and solubility properties—insolubility in chloroform, solubility in methanol [4]—it appears to be the *cis*- or *trans*-hydroxy acid derivative of VP 16-213. It was the only metabolite detected in plasma samples of rats to which VP 16-213 was administered. In two incubations with rat liver homogenates the presence of two radioactive components with retention times (5 and 7 min) between that of the major metabolite and of VP 16-213 was seen, but the formation of these products was not reproducible. The product with the retention time of 5 min was also seen in incubations with rat liver microsomes (Fig. 3). The radioactivity remaining in the microsomal pellet after protein precipitation might be due to the presence of a metabolite with higher protein binding than VP 16-213 which could not be detected by the analysis method. In the incubations with liver microsomes, no evidence was obtained for the formation of the aglycone of VP 16-213, 4'-demethylepipodophyllotoxin. The aglycone would be expected to have a

longer retention time than VP 16-213 in reverse-phase chromatography, but no measurable radioactivity was detected in that particular area of the chromatograms. In the *in vivo* experiments no radioactivity was detected in the area with retention times between that of VP 16-213 and 30 min. It has been reported that a microsomal reductive glycosidase is capable of cleaving anthracyclines as adriamycin to a deoxyaglycone [11]. *In vitro* incubation of VP 16-213 with microsomes under similar (anaerobic) conditions also did not reveal the formation of an aglycone.

Picro VP 16-213 cannot be separated from VP 16-213 on reverse phase [^{18}C]-columns, but this separation can be performed on a reverse-phase phenyl column. The presence of picro VP 16-213 in the incubation samples was investigated with this type of column but could not be observed. Because of these results, the presence of picro VP 16-213 in plasma of patients as reported previously [12] might have been due to chemical conversion of VP 16-213 to its *cis*-lactone isomer. In the latter study the detection of a hydroxy acid derivative in plasma of patients was also described. The picro isomer and hydroxy acid were reported to have no measurable cytotoxic effect against CCRF-CEM cells. However, as standard for the

hydroxy acid derivative of VP 16-213, the *cis*-hydroxy acid was used while the *trans*-hydroxy acid might possess cytotoxic activity.

In this paper the formation of one major metabolite *in vitro* and *in vivo* by the rat is described, which appears to be one of the two possible hydroxy acids of VP 16-213. The enzyme responsible for its formation is localized in the microsomal fraction and has a K_m of 0.078 mM for this conversion. This enzyme could be an esterase; its presence in microsomal rat liver fractions would be in accordance with the report on the presence of esterase in such

fractions [13]. Because of the extent of formation of the main metabolite, it would be important to study in detail its structure and cytotoxicity. The formation of this major metabolite of VP 16-213 could have clinical significance and its occurrence in individual patient therapy should be examined.

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