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Structure–Bioactivation Relationship of a Series of Podophyllotoxin Derivatives

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Abstract—*With the aim of elucidating the structural requirements for O-demethylation of the antitumor agent VP-*16-213 *by cytochrome P-*450, *the binding of a series of podophyllotoxin derivatives to rat liver microsomal cytochrome P-*450 *was studied. The examined podophyllotoxin derivatives were: VP-*16-213, *VM-*26, *podophyllotoxin, 4′-demethylepipodophyllotoxin (the aglycone of VP-*16-213 *and VM-*26) *and* 3,5*-dimethoxy-4-hydroxytoluene (a model compound for the E-ring of VP-*16-213). *The binding to phenobarbital (Pb)-induced microsomes was more extensive than that to* 3*-methylcholanthrene (*3*-MC)-induced microsomes. Experiments on the binding to cytochrome P-*450 *in Pb-induced microsomes led to the following findings: (a) the presence of the polycyclic skeleton is necessary for binding; (b) the presence of the sugar moiety gives a further extension of binding, and changes in the sugar moiety affect binding; (c) binding increases on elevation of hydrophobicity; (d) the E-ring itself does not bind. For binding to cytochrome P-*450 *in* 3*-MC-induced microsomes conclusions (a) and (d) appeared to hold true. For the O-demethylation of the podophyllotoxin derivatives containing the dimethoxyphenol ring by Pb- and* 3*-MC-induced microsomes, the following order was observed: VM-*26 > *VP-*16-213 > *aglycone* >> *E-ring. A similar sequence was observed for the cytotoxicity against Chinese hamster ovary cells.*

INTRODUCTION

VP-16-213 [4′-demethylepipodophyllotoxin-1-(4,6-*O*-ethylidene-β-D-glucopyranoside), NSC 141540, etoposide, Fig. 1] is an important antineoplastic agent used against several types of tumors [1]. The cytotoxicity of VP-16-213 is probably caused by DNA damage [2, 3]. Two possible explanations for the mechanism of DNA inactivation caused by VP-16-213 are presented in the literature. One explanation is that VP-16-213 acts by inhibiting the enzyme DNA topoisomerase II, in particular the breakage–reunion reaction of the enzyme, by stabilizing a cleavable complex [4]. The other explanation is that VP-16-213 is metabolized in the dimethoxyphenol ring (the E-ring) to products which cause DNA damage. Loike and Horwitz were the first to suggest that metabolic activation may be a requirement for the cytotoxic and DNA damaging effect of VP-16-213. They observed that the presence of the 4′-OH group in the E-ring of VP-16-213 was necessary for DNA inactivation and that isolated purified DNA was not broken down by the parent drug [5]. O-Demethylation in the E-ring of VP-16-213 by cytochrome P-450 to the orthodihydroxy derivative or catechol of VP-16-213 has recently been reported by us [6, 7] and by Haim *et al.* [8]. Oxidation of the catechol of VP-16-213 led to the formation of the *ortho*-quinone of VP-16-213 [9]. The catechol and *ortho*-quinone of VP-16-213 were found to inactivate ΦX174 DNA, in contrast to the parent drug VP-16-213, supporting the hypothesis that DNA inactivation by VP-16-213 is caused by activation of the dimethoxyphenol ring [9, 10]. In a study on the role of metabolic activation of VP-16-213 by cytochrome P-450 in the process of covalent binding of VP-16-213 to rat liver and HeLa cell microsomal proteins, VP-16-213 was found to bind to cytochrome P-450 giving reversed type I spectral changes [11]. The aim of the present study was to elucidate the structural requirements for binding of VP-16-213 to cytochrome P-450. For this purpose, the binding of a series of podophyllotoxin analogs to rat liver microsomal proteins was

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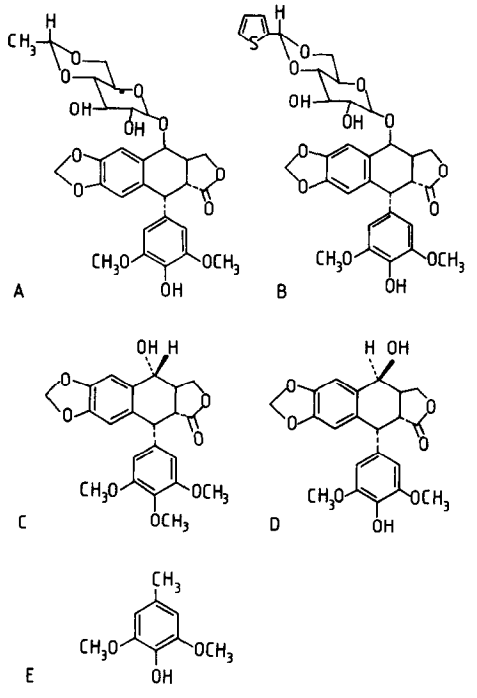

Fig. 1. Structures of VP-16-213 (a), VM-26 (b), podophyllotoxin (c), the aglycone of VP-16-213 and VM-26 (d), and 3,5-dimethoxy-4-hydroxytoluene (e).

studied using phenobarbital- and 3-methylcholanthrene-induced rat liver microsomes. The podophyllotoxin derivatives studied were: VP-16-213, VM-26, podophyllotoxin, 4′-demethylepipodophyllotoxin (the aglycone of VP-16-213 and VM-26), and 3,5-dimethoxy-4-hydroxytoluene (the E-ring of VP-16-213). The structures of these podophyllotoxin analogs are shown in Fig. 1.

MATERIALS AND METHODS

Drugs and chemicals

VP-16-213, VM-26 and 4′-demethylepipodophyllotoxin were gifts from the Bristol Myers Company (Syracuse, NY, U.S.A.). Podophyllotoxin was purchased from Janssen Chimica, Beerse, Belgium. 3,5-Dimethoxy-4-hydroxytoluene, phenacetine and aniline were obtained from the laboratory stock. Sodium phenobarbital was purchased from Brocacef B.V., Maarssen, The Netherlands. 3-Methylcholanthrene was purchased from Eastman Kodak Co., Rochester, NY. All other chemicals used were reagent grade.

Binding of podophyllotoxin derivatives to rat liver cytochrome P-450

Liver microsomes were prepared from male albino Wistar rats (180–200 g) pretreated with phenobarbital (1 g/l drinking water for 10 days) or 3-methylcholanthrene (40 mg/kg/2 ml arachidis oil by i.p. injection 1 × daily for 3 days) by a previously described method [11]. Binding of the podophyllotoxin derivatives to ferric cytochrome P-450 was assayed by difference spectrophotometry using liver microsomal suspensions containing 2 mg/ml protein and 1 mM EDTA in 0.1 M potassium phosphate buffer pH 7.4. The difference spectra were recorded at 25°C on an Aminco DW 2a^{TM} u.v./vis. double beam spectrophotometer. Three milliliters of microsomal suspension was added to the sample and the reference cuvet. After 20 min, a baseline was recorded. The podophyllotoxin derivatives were added as a solution in DMSO to the microsomal suspension in the sample cuvet, in a final concentration range of 34–510 μM. After an equilibration time of 3 min, a spectrum was recorded. Experiments on spectrum identification were performed by the addition of 510 μM VP-16-213 to the sample cuvet and consecutive additions of aniline (3.4 mM) or phenacetin (1 mM) to sample and reference cuvet. The cytochrome P-450 content of the microsomes was determined by the method described by Estabrook *et al.* [12]. O-Demethylation of the podophyllotoxin derivatives by phenobarbital- and 3-methylcholanthrene-induced rat liver microsomes was studied at a concentration of 84 μM, as previously described [7].

Cytotoxicity experiments

A Chinese hamster ovary cell line (Aux B1) was kindly provided by Dr. V. Ling (Toronto, Canada). It was grown in monolayer culture at 37°C in α-minimal essential medium containing 10% fetal calf serum. Cells at concentrations of 1–5 × 10^4/ml were added to 2 ml wells on day 1. On the second day the medium in each well was replaced by medium containing various concentrations of podophyllotoxin derivative. The incubation at 37°C was continued for 3 days. After the incubation, cells were suspended with trypsin-EDTA and their number counted with a cell-counter/hemocytometer. Average numbers of cells upon drug treatment were expressed as a percentage of the average number of cells of untreated controls. The IC_{50} value is the concentration of drug giving a 50% value.

RESULTS

Figure 2 shows the difference spectra obtained for binding of VP-16-213 to Pb-induced rat liver microsomes. The difference spectra showed a minimum at λ = 388 nm and a maximum at λ = 420 nm, indicating that the VP-16-213 difference spectrum is a reversed type I spectrum [13]. However, the effects of the type II substrate aniline and the reversed type I substrate phenacetin on the difference spectrum of VP-16-213 indicate that it could have a mixed reversed type I–type II character, since a residual spectrum was obtained after consecutive additions of an excess aniline or phenacetin to sample and reference cuvets. All other podophyllotoxin derivatives showed difference spec-

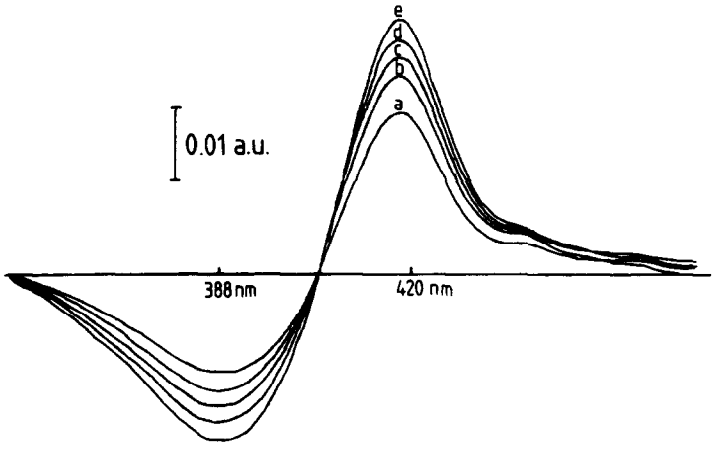

Fig. 2. Binding of VP-16-213 to cytochrome P-450 in phenobarbital-induced rat liver microsomes: difference spectrum. Reference cell: microsomal suspension (MIC), sample cell: MIC + VP-16-213 (a, 34 μM; b, 68 μm; c, 102 μM; d, 170 μM; e, 340 μM).

tra with Pb-induced microsomes similar to that of VP-16-213. Figure 3 shows the relationship between $\Delta A_{420-388}$ and concentration for the binding of the different podophyllotoxin derivatives to Pb-induced rat liver microsomes. At a concentration of about 500 μM, saturation of binding was observed for all podophyllotoxin derivatives, except for podophyllotoxin itself. Figure 4 shows the Eadie–Hofstee plots

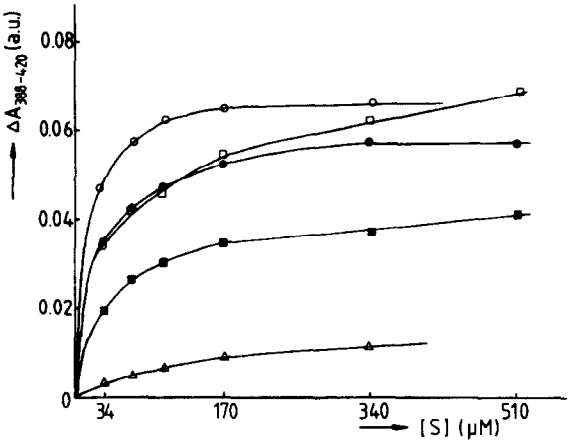

Fig. 3. Relationship between $\Delta A_{420-388nm}$ and concentration for the binding of different podophyllotoxin derivatives to phenobarbital-induced rat liver microsomes; ○, *VM-26;* ●, *VP-16-213;* □, *podophyllotoxin;* ■, *aglycone;* △, *E-ring.*

(ΔA vs. ΔA/[S]) for the binding of the podophyllotoxin derivatives to Pb-induced rat liver microsomes. From these plots, the binding characteristics K_s (the spectral dissociation constant or the concentration at which half of the number of binding sites is occupied) and ΔA_{max} (the maximal spectral change, which is a measure for the total number of binding sites) were calculated (see Table 1). The K_s-values increased in the following order: VM-26 < VP-16-213 < podophyllotoxin, aglycone < E-ring; the ΔA_{max}-values decreased in the following order: VM-26, podophyllotoxin > VP-16-213 > aglycone > E-ring. The difference spectra obtained in the study of the binding of the podophyllotoxin derivatives to 3-MC-induced microsomes showed the same reversed type I characteristics as those obtained with Pb-induced microsomes. The K_s- and ΔA_{max}-values calculated from the Eadie–Hofstee plots for the binding of the podophyllotoxin derivatives to 3-MC-induced rat liver microsomes are also shown in Table 1. The K_s-values increased in the following order: VM-26, aglycone < podophyllotoxin < VP-16-213 < E-ring. The ΔA_{max}-values were found to have the same order of magnitude. The K_s-values for binding to 3-MC-induced microsomes were higher than those for binding to Pb-induced microsomes, indicating a stronger binding to cytochrome P-450

*Table 1. K_s and ΔA_{max}-values for binding of podophyllotoxin derivatives to phenobarbital- and 3-methylcholanthrene-induced rat liver microsomes**

Podophyllotoxin derivative	K_s (μM)		ΔA_{max}/nmol P-450 × 10^{-3}	
	Pb	3-MC	Pb	3-MC
VP-16-213	25 ± 2	215 ± 33	7.0 ± 0.6	3.0 ± 0.5
VM-26	18 ± 1	86 ± 28	8.4 ± 0.5	2.3 ± 0.7
Aglycone	50 ± 4	87 ± 19	5.2 ± 0.4	2.5 ± 0.5
Podophyllotoxin	48 ± 7	138 ± 42	8.4 ± 1.2	2.8 ± 0.9
E-Ring†	166 ± 12	1789 ± 900	2.0 ± 0.1	2.4 ± 1.2

*The results were obtained from two experiments.
†E-Ring = 3,5-dimethoxy-4-hydroxytoluene.

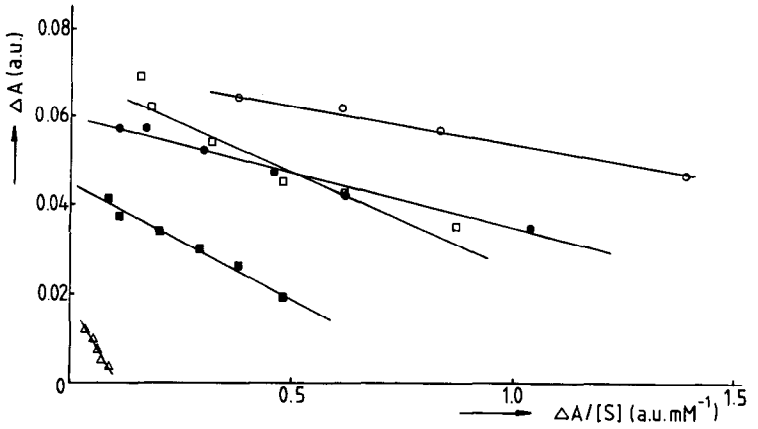

Fig. 4. Eadie–Hofstee plots for binding of podophyllotoxin derivatives to phenobarbital-induced rat liver microsomes. ○, *VM-26*; ●, *VP-16-213*; □, *podophyllotoxin*; ■, *aglycone*; △, *E-ring*. [*P*-450] = 2.84 μM.

in Pb-induced microsomes than to the cytochrome in 3-MC-induced microsomes.

Table 2 shows the results of the experiments on O-demethylation of the podophyllotoxin derivatives by Pb- and 3-MC-induced rat liver microsomes. The extent of O-demethylation decreased in the following order: VM-26 > VP-16-213 > aglycone > podophyllotoxin >> E-ring. The O-demethylation of the podophyllotoxin derivatives by 3-MC-induced microsomes was higher than the O-demethylation by Pb-induced microsomes. The results of cytotoxicity experiments on the podophyllotoxin derivatives in Aux B1 cells are shown in Table 3. The E-ring of VP-16-213 did not show appreciable

Table 2. O-Demethylation by cytochrome P-450 in Pb- and 3-MC induced rat liver microsomes of podophyllotoxin derivatives

Podophyllotoxin derivative	O-Demethylation*† (nmol H_2CO/min. mg protein) Pb	3-MC
VM-26	4.9 ± 0.2	6.5 ± 0.2
VP-16-213	1.6 ± 0.1	2.1 ± 0.2
Aglycone	0.8 ± 0.1	1.1 ± 0.1
Podophyllotoxin	0.6 ± 0.1	0.6 ± 0.1
E-Ring	0.0	0.0

*Mean ± S.D. for three experiments.
†The concentration of the podophyllotoxin derivatives was 84 μM.

Table 3. IC_{50}-values for cytotoxicity of podophyllotoxin derivatives against Aux B1 cells

Podophyllotoxin derivative	IC_{50} (μM)*
VM-26	0.26 ± 0.05
VP-16-213	0.7 ± 0.1
Aglycone	2.5 ± 0.3
Podophyllotoxin	1 ± 0.1
E-Ring	150 ± 10

*Mean ± S.D. for two experiments.

cytotoxicity against Aux B1 cells. The order of cytotoxicity of the podophyllotoxin derivatives against Aux B1 cells was: VM-26 > VP-16-213 > podophyllotoxin > aglycone >> E-ring.

DISCUSSION

From the experiments on the binding of several podophyllotoxin derivatives to Pb- and 3-MC-induced rat liver microsomes it is clear that the podophyllotoxin derivatives bind stronger to cytochrome P-450 in Pb- than in 3-MC-induced rat liver microsomes. This suggests that the podophyllotoxin derivatives bind stronger to cytochrome P-450b than to cytochrome P-450c. The experiments on the binding to cytochrome P-450 in Pb-induced microsomes lead to the following findings:

(a) the presence of the polycyclic skeleton is necessary for binding (aglycone vs. E-ring).
(b) the presence of the sugar moiety gives a further extension of binding (VP-16-213 vs. aglycone), and changes in the sugar moiety also influence the binding (VP-16-213 vs. VM-26).
(c) binding increases on elevation of hydrophobicity (VM-26 vs. VP-16-213, podophyllotoxin vs. aglycone).
(d) the E-ring itself does not bind.

For the binding to 3-MC-induced microsomes the conclusions are less clear, because of the high standard deviations of the K_s-values. The only firm conclusion here is that the presence of the polycyclic skeleton is necessary for binding, the sugar moiety seems to be less important for binding in this case.

From the O-demethylation experiments it can be concluded that the presence of the polycyclic skeleton is necessary for O-demethylation by both Pb- and 3-MC-microsomes, and that the presence of the sugar moiety gives an increase in O-demethylation. Although the podophyllotoxin derivatives seem to bind more strongly to cytochrome P-450 in Pb- than to cytochrome P-450 in 3-MC-induced microsomes, for O-demethylation the reverse holds

true. This indicates that the podophyllotoxin derivatives can bind to several sites of the enzyme including the metabolic site. This is supported by our previous finding that the K_M-value for O-demethylation of VP-16-213 by Pb-induced microsomes is higher than the K_M for O-demethylation by 3-MC-induced microsomes [7]. An important conclusion is that 3,5-dimethoxy-4-hydroxytoluene—the model compound for the E-ring of VP-16-213—did not show appreciable binding to cytochrome P-450 and did not undergo O-demethylation. This indicates that at least the presence of the polycyclic skeleton (the ring system A,B,C,D) is necessary for binding to and O-demethylation by cytochrome P-450 of a podophyllotoxin derivative.

The results of the cytotoxicity experiments with the podophyllotoxin derivatives support the hypothesis of the importance of bioactivation of VP-16-213 for its cytotoxicity. The order of cytotoxicity of the podophyllotoxin derivatives against Aux B1 cells, VM-26 > VP-16-213 > podophyllotoxin > aglycone >> E-ring, is the same as that found for O-demethylation by cytochrome P-450 in Pb- and 3-MC-induced microsomes, but the cytotoxicity of podophyllotoxin is relatively high. This could possibly be explained by the fact that the cytotoxicity of podophyllotoxin is based on another mechanism of action than that of VP-16-213: inhibition of polymerization of microtubuli, resulting in metaphase-arrest. The E-ring of VP-16-213, which does not undergo O-demethylation, shows no cytotoxicity against Aux B1 cells.

In conclusion, in this study evidence was obtained for the necessity of the presence of the polycyclic skeleton in the bioactivation of podophyllotoxin derivatives by phenobarbital- and 3-methylcholanthrene-induced rat liver microsomes.

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