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STUDIES WITH 4′-DEOXYEPIVINCRISTINE (VINEPIDINE), A SEMISYNTHETIC VINCA ALKALOID*

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Abstract—The antitumor activity of 4′-deoxyepivincristine (vinepidine, VNP) was examined against a human rhabdomyosarcoma line, HxRh12, grown as a xenograft in immune-deprived mice. The efficacy of VNP was lower than that of vincristine (VCR) but far superior to that of vinblastine (VLB) in this model. After i.p. administration, accumulation of [G-^{3}H]VNP in tumors was biphasic and progressive for at least 72 hr. In contrast, VCR and VLB achieved maximal tumor levels within 4 hr, after which the level of VCR was maintained but VLB levels decreased 3-fold by 72 hr. Analysis of tumor extracts by high performance liquid chromatography showed that at 72 hr after VNP injection 98% of the radiolabel chromatographed with parent compound. In normal tissues, VNP was cleared less rapidly than VCR or VLB, and analyses of tissue extracts suggested that VNP was less rapidly metabolized than VCR or VLB. This may account for why the potency of VNP is greater than that of VCR in mice.

The dimeric Catharanthus alkaloids vinblastine (VLB) and vincristine (VCR) have an important role in the clinical management of neoplastic diseases, particularly those malignancies afflicting children. Although structurally similar (Fig. 1), these Vinca alkaloids differ in their spectrum of activity against rodent [1] and human [2] tumors, and differ also in the dose-limiting toxicities [3, 4]. In man VLB causes myelosuppression, whereas neurotoxicity is dose limiting for VCR. At present, the structure–activity relationship is unclear with respect to both antitumor actitivy and neurotoxicity, although considerable effort has been directed to these problems [3]. Modification in the velbanamine (upper) or vindoline (lower) moieties may alter antitumor potency and the pattern of toxicity. Thus, substitution of a formyl group on the N_a-atom of VLB yields VCR, which, as mentioned, is neurotoxic in man, and has an altered spectrum of activity against rodent tumors [1]. Modification of the velbanamine moiety has generally been associated with reduced potency. Thus, 4′-deoxy VLB, a natural alkaloid [5], and 4′-deoxyleurosidine (Fig. 1) were less active than VLB as mitotic inhibitors when tested against CHO cells in culture [6], and were less potent against rodent tumors *in vivo*. Leurosidine (epimeric at C-4′; [7]) was also less potent than VLB against P-1534 leukemic mice [1]. However, leurosidine and 4′-deoxyleurosidine demonstrated high therapeutic index against P-1534 leukemia and B16 melanoma [1, 6], with an order of activity equal or superior to VLB.

Vinepidine (VNP), C-4′ deoxyvincristine,

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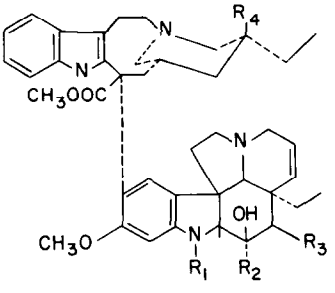

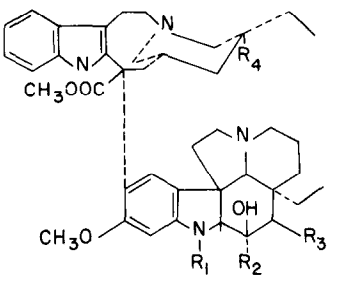

	R_1	R_2	R_3	R_4
Vinblastine	CH_3	$OCOCH_3$	$OCOCH_3$	OH
Vincristine	CHO	$OCOCH_3$	$OCOCH_3$	OH

	R_1	R_2	R_3	R_4
Vinepidine	CHO	$OCOCH_3$	$OCOCH_3$	H
Leurosidine	CH_3	$OCOCH_3$	$OCOCH_3$	OH
4′-deoxy leurosidine	CH_3	$OCOCH_3$	$OCOCH_3$	H

Fig. 1. Structures of Vinca alkaloids.

epimeric at C-4′, differs from deoxyleurosidine in substitution of a formyl group at the N_a-atom of the vindoline moiety (Fig. 1). These modifications to the velbanamine moiety impart some activities new for a Vinca alkaloid [8, 9]. In mice, VNP was more potent than VCR, which is unusual for a Vinca alkaloid modified on the velbanamine structure. In addition, VNP demonstrated 50- to 100-fold less potency in the rat mid-brain cell culture assay [8]. This *in vitro* assay has an excellent correlation with Vinca alkaloid-induced neuropathy in man [9, 10]. As the major dose-limiting toxicity of VCR is neuropathy, this new analogue was of interest to examine.

MATERIALS AND METHODS

Materials. [G-^{3}H]VNP, [G-^{3}H]VCR and [G-^{3}H]VLB (9–12 Ci/mmole) were purchased from Moravek Biochemicals, Brea, CA, and were repurified by high performance liquid chromatography (HPLC) to >92% radiochemical purity. Radioisotopes were stored at −20° and used within 14 days of purification. HPLC grade methanol was purchased from Burdick & Jackson (Muskegon, MI), NCS OCS and ACS solutions from Amersham (Arlington Heights, IL) and other chemicals from the Sigma Chemical Co. (St. Louis, MO). Vincristine, VLB and VNP were gifts from the Eli Lilly Co., Indianapolis, IN.

Immune-deprivation and tumor line. The human rhabdomyosarcoma HxRh12 has been described previously [11]. Briefly, when grown as a xenograft, it has moderately differentiated embryonal histology, demonstrating giant cells, and cross-striations typical of this histiotype. The tumor has demonstrated human lactate dehydrogenase isoenzymes and a human karyotype. Tumor line HxRh12 is very sensitive to VCR, but is intrinsically resistant to VLB [12]. Tumor pieces were implanted s.c. into mice immune-deprived by thymectomy and cytosine arabinoside pretreatment prior to whole body irradiation as described previously [12]. For pharmacokinetic and metabolism studies, tumors weighing between 500 and 1000 mg were used. Mice were administered [G-^{3}H]VNP (2 mg/kg, i.p.; 40 mCi/mmole) and killed at the appropriate interval after injection. Tumor and normal tissues were washed three times in saline (4°), and the intestinal lumen was flushed. Blotted tissues were weighed and digested completely in NCS solution as described [12]. One millilitre of digest was mixed with 10 ml ACS scintillant, and radioactivity was determined.

Determination of [^{3}H]*VNP and metabolites* in vivo. Mice were administered [^{3}H]VNP (0.64 μCi/g body g body weight, 9 Ci/mmole) by i.p. injection. Tumors and normal tissues were excised from groups of mice, 4, 24, and 72 hr after injection, and were immediately immersed in liquid N_2, ground to a fine powder under N_2, and extracted three times with acidified ethanol (95% acidified to pH 4.9 using glacial acetic acid) as previously described [13]. The residual pellet was digested in NCS solution, and total extracted and non-extracted radioactivity was determined in samples taken 4 and 24 hr after [G-^{3}H]VNP injection. Extraction efficiencies for tumor, liver, spleen, intestine and blood were 94.8 ± 0.5, 90.0 ± 1.5, 99.4 ± 0.4, 95.4 ± 0.2 and 99.4 ± 5% respectively. Extracts were lyophilized to dryness, and resuspended in 20% MeOH, pH 4.9, prior to analysis by HPLC [12, 13]. Under these conditions the lower limit of detection was ≥200 fmoles/g tissue (≥5940 dpm/sample).

Metabolism of VNP in vitro. Microsomes were prepared by the method of Connors *et al.* [14]. The final reaction mixture contained 2.8 μmoles glucose-6-phosphate (G-6-P), 17.5 mUnits G-6-P dehydrogenase, 24.8 μmoles $MgCl_2$ 1.4 μmoles NADP 3.3 μmoles [G-^{3}H]VNP, and microsomes equivalent to 1.5 g tissue in a total of 3.81 ml. Reactions were initiated by addition of VNP to the reaction mixture at 37°. Aliquots were removed at various times for up to 4 hr and mixed with 4 vol. of ice-cold acidified ethanol. After centrifuging (12,000 *g*, 4°, 10 min) supernatant fractions were analyzed by HPLC, and radioactivity was determined in 0.5-ml samples. Reactions were carried out as above but boiled microsomes were substituted in order to determine nonspecific degradation of VNP under these reaction conditions.

Measurement of antitumor activity. Mice bearing HxRh12 tumors were given a single administration i.p. of VNP at various dose levels up to the maximum tolerated dose (MTD; 2 mg/kg). Tumor diameters in both control and treated mice were determined at 7-day intervals using vernier calipers. Tumor volumes were calculated assuming that tumor shape was approximately spherical [12].

RESULTS

Tumor HxRh12 was chosen for this study, as it clearly distinguishes between the antitumor activity of VCR and VLB. At their respective MTD, VCR caused complete regression of advanced tumor, with few subsequent regrowths, whereas VLB caused only transient growth inhibition (Table 1). In tumor-bearing and non-tumor-bearing immune-deprived mice (data not shown), the MTD for VNP was lower

Table 1. Comparative antitumor activity of three Vinca alkaloids against human rhabdomyosarcoma xenograft HxRh12*

Agent	Dose (mg/kg)	Sensitivity 0.375	0.75	1.0	1.5	2.0	3.0	4.0
Vinepidine				±		+++++	Toxic	
Vincristine		±	±		+++++		++++++	Toxic
Vinblastine				+		+	+	+

* Tumor response grading system used: (±) transient response with inhibition < T_{D2} (where T_{D2} is mean time for tumor volume to double); (+) growth inhibition ≥ T_{D2}; (+++++) complete regression with subsequent regrowth; and (++++++) complete regression with no regrowth.

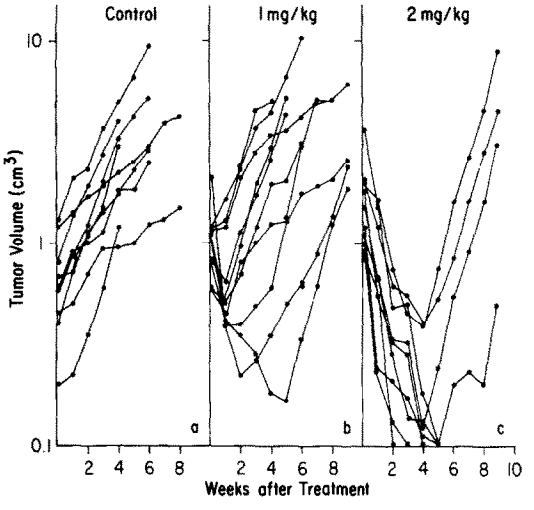

Fig. 2. Antitumor activity of vinepidine against HxRh12 human rhabdomyosarcoma xenografts. Mice bearing bilateral tumors were treated with a single administration of VNP, and tumor growth was subsequently measured. Key: (a) untreated tumors; (b) individual tumors in mice receiving 1 mg/kg VNP, and (c) 2 mg/kg VNP. Results show a representative sample of tumors.

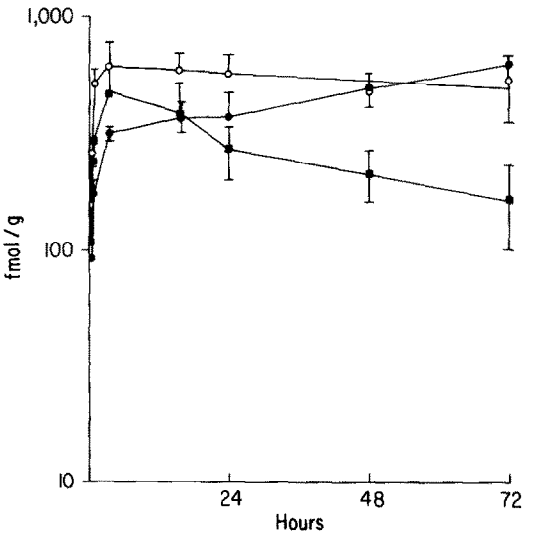

Fig. 3. Accumulation and retention of Vinca alkaloids in HxRh12 xenografts. Tumor-bearing mice were administered radiolabeled VCR (3 mg/kg; ○), VLB (3 mg/kg; ■) or VNP (2 mg/kg; ●) by i.p. injection. At the appropriate time, tumors were excised and digested, and total radioactivity was determined. Results are mean ± 1 S.D. for between eight and sixteen individual tumors at each time point.

than that for VCR (3 mg/kg). Toxicity of VNP was manifest by severe weight loss and ptosis, similar to that observed with VCR. Vinepidine was active against HxRh12 tumors with activity similar to VCR (Table 1). The responses of individual tumors in mice treated with VNP at 1 mg or 2 mg per kg are shown in Fig. 2. At the lower dose, 0/13 tumors regressed completely and 4/13 tumors had a partial response (≥50% volume regression). At the 2 mg/kg dose level (≈ LD_{10}), 9/14 tumors regressed completely and all 14 tumors regressed ≥50%. The responses to VNP, VLB and VCR are summarized in Table 1.

In previous studies we have shown that VCR is tenaciously retained ($T_{\frac{1}{2}}$ = 720 hr) in HxRh12 tumors, whereas VLB is lost from these tumors quite rapidly ($T_{\frac{1}{2}}$ = 48 hr; Ref. 12). It was therefore of interest to examine the accumulation and retention of VNP in these tumors, and to compare this with VCR and VLB. The accumulation and retention of these Vinca alkaloids are presented in Fig. 3. Maximal tumor levels of VCR and VLB were achieved within 4 hr of drug administration, and subsequently VCR was retained whereas VLB was lost in a biphasic manner. In contrast, over the first 4 hr VNP accumulated to a lower level than VCR, but was equivalent to VLB accumulation (on a mg/kg basis). However, in two separate experiments, it was observed that VNP continued to accumulate for up to 72 hr, reaching a concentration similar to VCR in HxRh12 tumors. The concentration of [G-^{3}H]VNP in tumors 72 hr after drug administration was significantly greater than at 24 hr ($P = 0.0009$).

Clearance of total [G-^{3}H]VNP from plasma was rapid, with maximal plasma concentrations approximately 1.5 μM, 10 min after drug injection (Fig. 4). Clearance was compared to that for VCR and VLB in tumor-bearing mice (Fig. 4). The pharmacokinetics of [G-^{3}H]VNP in normal tissues is presented in Fig. 5, and compared to data for VCR where these are available. Even though initial plasma levels of VNP were slightly greater than VCR, tissue levels of VCR were greater at early time points. Vincristine was eliminated from tissues more rapidly than was VNP.

Metabolism of [G-^{3}H]VNP. Pharmacokinetic studies showed that VNP accumulated relatively slowy in HxRh12 xenografts, attaining levels similar to VCR 3 days after administration. It was therefore of considerable importance to determine whether this continued accumulation was due to abstraction of parent compound or due to accumulation of a

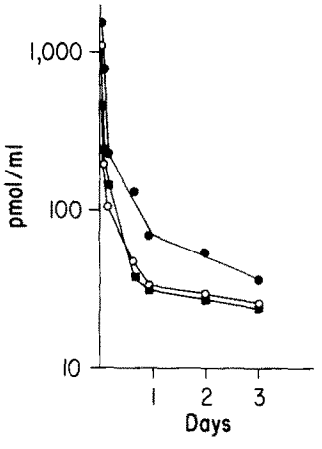

Fig. 4. Clearance of Vinca alkaloids from plasma of tumor-bearing mice. Mice were administered radiolabeled VCR (3 mg/kg; ○), VLB (3 mg/kg; ■) or VNP (2 mg/kg; ●) by i.p. injection. Mice received heparin 15 min before blood was drawn by cardiac puncture under general anesthetic.

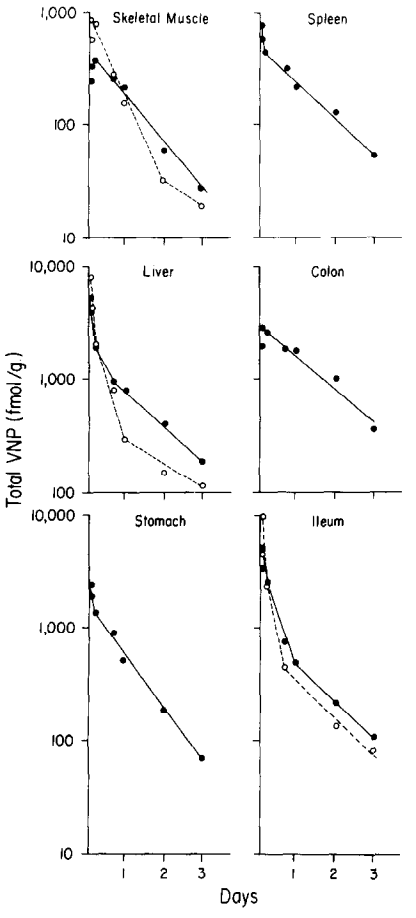

Fig. 5. Accumulation and retention of Vinca alkaloids in normal tissues of tumor-bearing mice. Mice received a single i.p. injection of [G-^{3}H]VNP (2 mg/kg; ●). At various times tissues were excised, washed thoroughly, and digested in NCS solution, and total radioactivity was determined. Pharmacokinetics for VCR (3 mg/kg, ○) are compared where data are available. Each point represents the mean of three to four determinations.

metabolite. Tissues were extracted using mild acidic conditions which did not degrade VNP (data not shown). Extraction efficiencies are presented in Materials and Methods and show that ≥90% of radiolabel could be extracted by acidified ethanol. The proportion of radiolabel that chromatographed

Table 2. Vinepidine as a percentage of total radiolabel in tissue extract*

	% Vinepidine		
	Time after VNP administration (hr)		
Tissue	4	24	72
HxRh12	94.1 ± 1.4	94.7 ± 5.2	98.0 ± 1.1
Intestine	84.9 ± 2.2	68.9	ND†
Liver	82.7	80.3	ND
Spleen	96.0	ND	ND
Blood	80.2	ND	ND

* Values are means ± S.D.; N = 3 or greater.
† ND: tissue levels too low to analyze accurately.

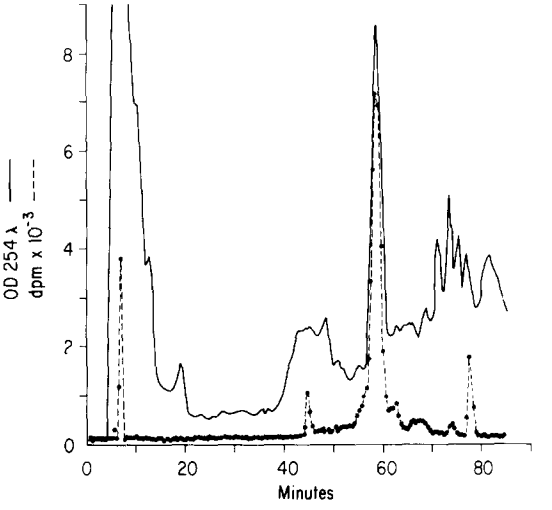

Fig. 6. Elution profile of [G-^{3}H]VNP metabolites extracted from liver. Twenty-four hours after drug administration to tumor-bearing mice, tissues were excised, pulverized under liquid N_2, extracted and analyzed by HPLC as described in Materials and Methods. The major radioactive peak chromatographed with authentic VNP.

with authentic VNP in tumor extracts made at 4, 24 or 72 hr after drug administration is presented in Table 2. At each time point ≥94% of radiolabel chromatographed with VNP in tumor extracts. The proportion of radiolabel that chromatographed with VNP in normal tissue extracts is shown in Table 2, and the elution profile for liver metabolites 24 hr after [G-^{3}H]VNP administration is presented in Fig. 6. At 24 hr, levels of radiolabel were too low to analyze in blood and spleen, and in all mouse tissues 72 hr after VNP injection.

Metabolism in vitro. Incubation of [G-^{3}H]VNP with whole blood (21 hr, 37°) gave a single minor metabolite (retention time = 67 min) constituting approximately 9.8% of the total radiolabel. Incubation of [G-^{3}H]VNP with liver microsomes for up to 4 hr revealed no metabolites, and was thus unlike VLB where desacetyl VLB was formed under identical conditions [13].

DISCUSSION

Vinepidine, 4′-deoxy,4′-epivincristine, is a Vinca alkaloid with modification of the velbanamine moiety [9]. In CBA/CaJ mice, VNP was more potent than either VCR or VLB, and is a somewhat novel modification of the velbanamine moiety as reduction of the C-4′ hydroxyl, or epimerization at the C-4′ have been shown to reduce the potency of VLB [1, 6]. Against human rhabdomyosarcoma HxRh12, growing as a xenograft, VNP exhibited considerable activity, demonstrating a slightly lower therapeutic efficacy than VCR, but being much more efficacious than VLB. Previously, we have shown that, at equitoxic doses, the initial accumulation in HxRh12 xenografts was similar between VLB and VCR, with VCR being selectively retained. Thus, for the drug to be effective it appeared that prolonged retention above some critical concentration was necessary.

The accumulation and retention of VNP showed considerable differences from either VLB or VCR, in that accumulation occurred over the entire 72-hr period examined. Thus, although the initial VNP level was substantially lower than VLB or VCR, by 72 hr VNP concentrations equalled those of VCR. Analysis by HPLC of tumor extracts suggested that it was the unchanged compound that accumulated in these tumors. As VNP is an active agent against HxRh12 xenografts, this would strengthen the suggestion that it is the prolonged exposure to drug, rather than the initial peak concentration (cf. VLB) that determines the magnitude of tumor cell kill *in situ*.

Between 4 and 72 hr after injection of VNP, tumor levels approximately doubled and were significantly greater 72 hr after injection. In contrast, over the same period VLB levels decreased 3-fold and VCR levels remained approximately constant. It was thus pertinent to determine from where VNP was being scavenged. Vinepidine was cleared rapidly from plasma and normal tissues, although clearance was slower than that of VCR. The limited data available indicate that VNP is probably not metabolized rapidly as, at 24 hr, 80% of radiolabel extracted from liver eluted with VNP. Similarly, no metabolism by liver microsomal preparations was measured *in vitro*. In comparison to VCR and VLB [12], a greater proportion of radiolabel chromatographed with parent compound in blood, liver, and spleen samples. This may account for the increased potency of VNP in mice. It is likely, therefore, that VNP released from normal tissues may be taken up by tumor cells. Between 24 and 72 hr plasma levels of total drug (VNP plus metabolites) decreased from 70 to 35 nM, suggesting that these tumors are able to accumulate and trap VNP even when it is presented at very low concentrations.

Vinepidine was selected for further development based upon its activity against a spectrum of rodent tumors, and its low potency in the rat mid-brain assay [8, 9]. With respect to toxicity in the mouse, VNP is similar to VCR in causing ptosis, which was not observed after administration of VLB at an equitoxic dose. This may be indicative of neurotoxicity as preliminary studies in man using a weekly administration schedule have shown that VNP is neurotoxic (G. B. Boder, personal communication). Whether toxicity is due to cumulative effects of VNP caused by its slower elimination or metabolism, using this schedule, is not known. However, data presented in this study suggest that, in HxRh12 human rhadomyosarcomas, drug is accumulated in tumors even at very low plasma concentrations. This raises the possibility that prolonged infusion of VNP at low concentration may achieve cytotoxic concentrations of drug in tumors and possibly reduce the incidence of toxicity to the host.

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