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Brain uptake and anticancer activities of vincristine and vinblastine are restricted by their low cerebrovascular permeability and binding to plasma constituents in rat

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Summary. Unidirectional blood-brain barrier transfer of the lipophilic anticancer agents vincristine and vinblastine was studied in anesthetized rats, using an isolated, in situ brain perfusion technique. Drug binding to plasma constituents was also measured. Despite the high lipophilicity of these agents (the log octanol/physiological saline partition coefficient equalled 2.14 and 1.68, respectively), the cerebrovascular permeability-surface area product, PA, of vincristine in plasma was only 0.49×10^{-4} ml s^{-1} g^{-1} for parietal cerebral cortex, whereas that of vinblastine was too low for determination. These values are similar to those of water-soluble, poorly diffusible nonelectrolytes. The PAs were significantly higher in the absence of plasma protein, being 1.24×10^{-4} and 5.36×10^{-4} ml s^{-1} g^{-1}, respectively. Even these values, determined by brain perfusion of protein-free buffer, were lower than would be expected from the lipophilicity of the agents. The results suggest that additional factors, such as steric hindrance and molecular charge distribution, related to the chemical and geometric structure and the large size of vincristine and vinblastine (molecular weight, 825 and 814 daltons, respectively) restrict their passage across the blood-brain barrier. As a consequence of their paradoxically low permeability at the blood-brain barrier and restrictive binding to plasma and blood constituents, doses of both agents that cause significant inhibition of extracerebral Walker 256 carcinosarcoma tumor implants in rat have no effect on tumor located in the brain.

Introduction

The vinca alkaloids vinblastine and, more particularly, vincristine are important therapeutic agents in the chemotherapy of cancer. They primarily interfere with the polymerization of tubulin to prevent microtubule formation thus arresting cell division [3, 11]. They are large molecules (the molecular weight of vincristine sulfate equals 923 daltons, that of vinblastine sulfate equals 909 daltons) and are lipophilic compounds that are formed of two structurally similar multiringed units, vindoline and catharanthine, linked by a carbon-carbon chain, and differ only in a single substitution on the catharanthine group (Fig. 1). These drugs have a wide spectrum of anticancer activity. Their clinical importance can be gauged by the fact that one or the other is an essential part of standard regimens used for the treatment of Hodgkin's disease, non-Hodgkin's lymphoma, acute leukemia in adults and acute lymphocytic leukemia in children, sarcomas, Wilms' tumor, and cancer of the uterus and lung [2, 7, 14], all of which are potentially curable by chemotherapy.

Whereas the major toxicity of most anticancer agents, including vinblastine, is dose-related bone marrow depression [2, 14], the principal limiting side effect produced by vincristine is neurotoxicity and there is a lack of myelosuppression at conventional doses [14, 31]. Vincristine-induced neurotoxicity is usually manifested by a peripheral,

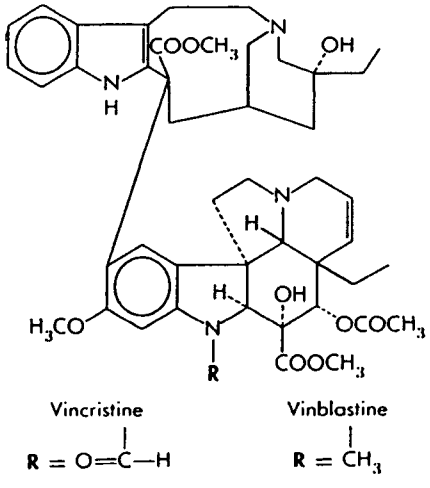

Fig. 1. Chemical structures of vincristine and vinblastine

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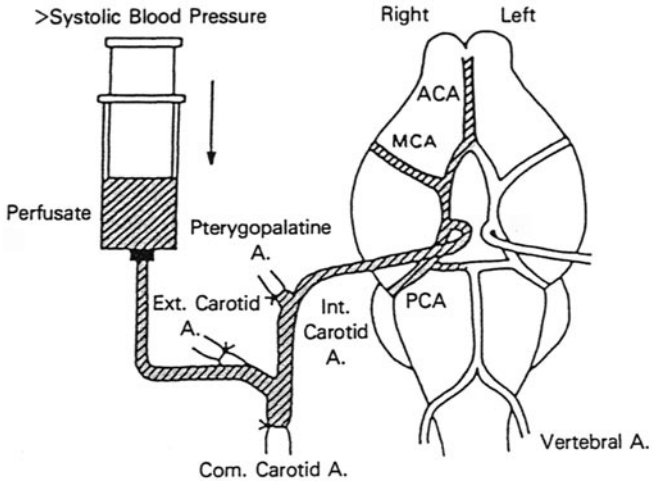

Fig. 2. Diagram of the technique for perfusing the right cerebral hemisphere of a rat. *ACA*, anterior cerebral artery; *MCA*, middle cerebral artery; *PCA*, posterior cerebral artery (from Takasato et al. [48], with permission)

mixed sensoric motor neuropathy, together with symmetrical neurological signs and symptoms [31]. Central neurological manifestations may additionally but less commonly occur, including depression, confusion, hallucinations, insomnia, agitation, psychosis, and hyponatremia. Although neurotoxicity only rarely presents acute clinical problems, as its onset generally occurs after three doses, it can be severe at therapeutic doses of vincristine and life-threatening at higher doses, and it limits the potential usefulness of the drug, as dose reduction or cessation is generally required.

In view of the lipophilicity of these agents and the neurotoxicity associated with vincristine, it is surprising that neither agent has significant anticancer effects on intracerebral deposits of tumors arising from extracerebral neoplasms that are sensitive to these agents [18, 40]. Studies were undertaken to assess the permeability of these compounds at the blood-brain-barrier and to determine the factors that limit their brain uptake. In addition, the anticancer activity of each agent was assessed against peripheral and cerebral implants of tumor in rats such that differences in the activities of each compound could be related to drug access to the brain.

Materials and methods

Anticancer activity studies. The anticancer activity of increasing concentrations of vincristine and vinblastine given in two regimens (as single doses on days 1–5 or on days 1, 3, and 7) was assessed against Walker 256 carcinosarcoma in male Wistar rats (Charles River Laboratories Inc., Wilmington, Mass.) weighing approximately 120 g. For intracerebral implants, tumor (1×10^4 cells) was injected into the parietal cortex through a 30-gauge needle as previously described [22]. For peripheral implants, 0.2 ml minced solid tumor was injected s. c. into the flank [21].

With the exception of control animals, which were given vehicle alone, all animals were injected i. p. with vincristine (0.01–0.4 mg/kg) or vinblastine (0.06–0.35 mg/kg), starting 36 h after tumor implantation. At this time, the blood-brain barrier has reformed following intracerebral tumor implantation [22]. Drug activity against intracerebral tumor was assessed by comparing the mean survival of drug-treated animals with that of controls. Activity against peripheral tumor was assessed by surgically excising tumors 9 days following implantation and comparing the net wet weight to that of controls. Controls were run concurrently with treated animals in all studies, and a minimum of seven animals were used per group. Drug toxicity, TX, was assessed from animal weight reduction and confirmed by postmortem analysis.

Brain perfusion study. Adult male Osborne-Mendel rats (Charles River Laboratories Inc.) weighing 250 g were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The right external carotid artery was canulated with a polyethylene catheter for retrograde infusion; the right pterygopalatine, occipital, and thyroid arteries were ligated; and the right common carotid artery was encircled with silk thread (Fig. 2). Blood flow through the right common carotid artery was never interrupted during the procedure. Following surgery, a heat lamp connected to a feedback device (YSI Indicating Controller, Yellow Springs, Ohio) maintained rectal temperature at 37° C.

The catheter was connected to a syringe containing either [^{3}H]-vincristine or [^{3}H]-vinblastine at 0.5 nmol/ml (1.0 μCi/ml) and [^{14}C]-inulin (0.3 μCi/ml), which were dissolved either in fresh rat plasma or in an HCO_3-buffered physiological saline and prepared immediately prior to use to minimize drug breakdown. Both solutions were filtered, oxygenated with 95% O_2: 5% CO_2, and warmed to 37° C. The pH and CO_2 tensions were 7.40 and 32 mm Hg, respectively. At 1 min before perfusion, the right common carotid artery was ligated and perfusion fluid was infused retrograde into the external carotid artery at a constant rate of 8.3×10^{-2} ml/s using an infusion pump (Model 944, Harvard Apparatus, South Natick, Mass). Measured carotid arterial pressure was between 130 and 140 mm Hg, which does not cause barrier damage, and circulating blood contributed <0.1% of the net flow to the right cerebral hemisphere. After either 25 or 65 s brain perfusion, the animal was decapitated and samples of perfusion fluid and brain were quantified for radioactivity as previously described [23, 48]. Additionally, samples were chromatographed to check the purity of the agents and assess whether metabolism had occurred during the procedure.

Radiochemicals. [^{3}H]-Vincristine (5.9 Ci/mmol), [^{3}H]-vinblastine (10.7 Ci/mmol), and [^{14}C]-inulin (9.0 mCi/mmol) were purchased from Amesham Corp. (Arlington Heights, Ill). The radiochemical purity of [^{114}C]-inulin (>98%) was confirmed by paper chromatography in *n*-propanol, ethyl acetate, and distilled water (7 : 1 : 2, by vol.) as the eluting solvent. The radiochemical purities of [^{3}H]-vincristine (>98%) and [^{3}H]-vinblastine (>98%) were confirmed by thin-layer chromatography on silica gel 60F-254 with chloroform, methanol, and formic acid (70 : 20 : 5 by vol.) as the eluting solvent. No metabolites were detected during the experimental procedures.

Calculations. The brain uptake of a tracer, such as [^{3}H]-vincristine or [^{3}H]-vinblastine, during perfusion is given by a two-compartmental model,

$$dC^*_{br}/dt = k_{in}\, C^*_{pf} - k_{out}\, C^*_{br}, \quad (1)$$

where C^*_{br} represents the concentration of tracer in brain parenchyma (dpm/g); C^*_{pf} is the concentration in the perfusion fluid (dpm/ml), whether plasma or buffered physiological saline; k_{in} and k_{out} are transfer coefficients for influx and efflux, respectively; and t represents the net perfusion time (s). In each study the perfusion time was limited such that only a small quantity of tracer entered the brain. The value for $k_{out}\, C^*_{br}$ remained considerably lower than that for $k_{in}\, C^*_{pf}$, such that back diffusion of tracer from the brain was negligible and could be ignored. Under these circumstances, brain uptake follows unidirectional kinetics and can be described as

$$dC^*_{br}/dt = k_{in}\, C^*_{pf}. \quad (2)$$

In Eqs. 1 and 2, k_{in} is defined as

$$k_{in} = F\,(1 - e^{-PA/F}), \quad (3)$$

where F is the regional cerebral blood flow (ml s^{-1} g^{-1}) and PA is the capillary permeability-surface area product (ml s^{-1} g^{-1}).

Table 1. Cerebrovascular permeability-surface area product, PA, of vincristine and vinblastine in plasma and saline as measured by the brain perfusion technique

Brain area	Vincristine PA		Vinblastine PA	
	Plasma ($\times 10^4$ ml s^{-1} g^{-1})	Saline ($\times 10^4$ ml s^{-1} g^{-1})	Plasma ($\times 10^4$ ml s^{-1} g^{-1})	Saline ($\times 10^4$ ml s^{-1} g^{-1})
Frontal cortex	0.51[a] ±0.09	1.18 ±0.25	–	4.64 ±0.66
Parietal cortex	0.49 ±0.12	1.24 ±0.19	–	5.36 ±1.11
Occipital cortex	0.40 ±0.12	0.98 ±0.15	–	3.49 ±0.04
Hippocampus	0.42 ±0.10	1.27 ±0.37	–	3.29 ±0.90
Caudate nucleus	0.47 ±0.08	0.84 ±0.29	–	3.16 ±0.41
Thalamus/hypothalamus	0.43 ±0.07	0.96 ±0.41	–	3.36 ±0.59

[a] Mean ± SEM. In each case, saline PA values are significantly higher than those measured in plasma, except for vincristine in the caudate nucleus and thalamus/hypothalamus; for vinblastine, saline PA values were significantly greater than zero ($P < 0.05$)
Vincristine and vinblastine saline and plasma concentration = 1 nmol/ml. PA of vincristine in plasma = 43% of PA in saline perfusion study. Concentration of unbound vincristine in plasma, 39.4%; Concentration of unbound vinblastine in plasma, 17.3%

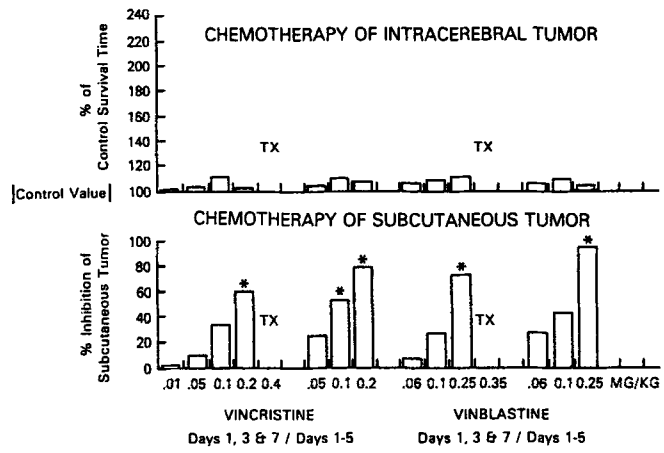

Fig. 3. Anticancer activities of vincristine and vinblastine, injected i.p. on days 1, 3, and 7 or days 1–5 after tumor implantation, against subcutaneous (*bottom*) and intracerebral (*top*) implants of Walker 256 carcinosarcoma tumor in the rat. Each group contained at least seven animals. * $P < 0.05$ for differences between test and control animals. TX, drug-induced toxicity

F was determined in a separate series of experiments from the unidirectional brain uptake of [^{14}C]-diazepam during a 10-s perfusion [48]. To solve for PA, Eq. 2 was integrated from the time at which perfusion fluid entered the cerebral capillaries (t = 0) until the time of decapitation (T):

$$PA = -F \, Ln \, [1 - C^*_{br}(T)/F \, T \, C^*_{pf}]. \quad (4)$$

The cerebrovascular PA of either agent in plasma or buffered physiological saline can be calculated from Eg. 4, by which $C^*_{br}(T)$ is obtained by subtracting the amount of intravascular [^{3}H]-vincristine or [^{3}H]-vinblastine, equal to the product of C^*_{pf} and the regional intravascular volume, from the measured brain concentration of either agnt. [^{14}C]-Inulin, a polysaccharide that does not measurably enter the brain during a 25- or 65-s perfusion, was used to determine the intravascular volume of both agents. In all experiments, 5 s was subtracted from the total perfusion time to obtain T, the actual time during which perfusate was within the cerebral capillaries [48].

Estimation of drug binding to plasma proteins. Binding of [^{3}H]-vincristine and [^{3}H]-vinblastine to plasma proteins in fresh human or rat plasma was measured by centrifugal ultrafiltration at concentrations between 0.1 and 10 nmol/ml as previously described [24].

Estimation of lipophilicity, or log P value. The partition of [^{3}H]-vincristine and [^{3}H]-vinblastine at a concentration of 1.0 nmol/ml was measured between 1-octanol (Sigma Chemical Co.) and buffered physiological saline (pH 7.4). Briefly, 0.5 μCi of each agent was added to 4 ml 1-octanol and 4 ml buffered physiological saline. After vigorous mixing, the two phases were separated by centrifugation and the radioactivity of each was measured. Chromatographic verification of the phases by thin-layer chromatography showed that no degradation occurred during the partition process.

Statistical analysis. A two-tailed Student's *t*-test was performed for the comparison of two means. When more than two means were compared, one-way analysis of variance and the Bonferroni multiple-comparison test were used to compare individual means [33]. In all tests, statistical significance was taken as $P < 0.05$; unless otherwise stated, means ± SE are given throughout.

Results

Brain uptake

The unidirectional movement of vincristine and vinblastine across the blood-brain barrier was measured by an isolated, in situ brain perfusion technique. Table 1 presents the mean PA values (calculated from Eq. 4) for [^{3}H]-vincristine and [^{3}H]-vinblastine in six brain regions perfused with either plasma or buffered physiological saline. The mean PA value for vincristine in plasma was low, varying from 0.51×10^{-4} ml s^{-1} g^{-1} in frontal cerebral cortex to 0.40×10^{-4} ml s^{-1} g^{-1} in occipital cortex. PA values could not be determined for vinblastine in plasma after a 60-s brain perfusion because they were too low. The mean PA values for vincristine and vinblastine in buffered physiological saline were significantly higher than those in plasma. Those of vincristine varied from 1.27×10^{-4} ml s^{-1} g^{-1} in hippocampus to 0.84×10^{-4} ml s^{-1} g^{-1} in caudate nucleus, whereas those of vinblastine were higher still, varying from 5.36×10^{-4} ml s^{-1} g^{-1} in parietal cerebral cortex to 3.16×10^{-4} ml s^{-1} g^{-1} in caudate nucleus.

Vincristine and vinblastine were significantly bound (60.6% and 82.7%, respectively) to plasma constituents at the concentration (1 μ*M*) studied in the brain perfusion

experiments. Furthermore, both agents were lipophilic, preferentially distributing in octanol rather than in physiological saline, with log P (partition) values of 2.14 and 1.68, respectively.

Anticancer activity

Figure 3 shows the effect of vincristine and vinblastine, given at daily doses of 0.01–0.4 mg/kg on either days 1, 3, and 7 or days 1–5, on the growth inhibition of s.c. implanted Walker tumor and on the survival of rats with intracerebral implants of the same tumor. Both agents caused significant, although incomplete, inhibition of s.c. tumor growth. The maximum tolerated dose of vincristine was 0.2 mg/kg i.p. for both regimens, which produced 60% and 80% tumor growth inhibition on the day 1, 3, and 7 and the day 1–5 schedule, respectively; inhibition of 50% was caused by approx. 0.15 and 0.1 mg/kg, respectively.

The highest tolerated dose of vinblastine was 0.25 mg/kg for both schedules. This produced 74% and 94% inhibition of s.c. implanted tumor on days 1, 3, and 7 and days 1–5, respectively; inhibition of 50% was caused by approx. 0.2 and 0.1 mg/kg, respectively. Neither agent significantly lengthened the survival of animals bearing brain tumors (Fig. 3).

Discussion

The apparent inactivity of vincristine and vinblastine against CNS sites of responsive tumors, such as Walker 256 carcinosarcoma, is in accord with that obtained in other animal studies. Both agents have demonstrated poor therapeutic effects in the treatment of murine neuroblastoma [15, 16], and vincristine is inactive against murine glioma [43]. Although a carefully controlled trial has never been conducted using either agent singly in the treatment of CNS tumors in humans, a number of small studies have reported the use of the agents alone and in combination [1, 5, 38, 39]. The results were not dramatic, although in some cases stabilization and remissions of short duration were reported. The development of brain-sequestered disease during the successful treatment of acute lymphoblastic leukemia [6], in addition to pharmacokinetic data, suggests that the poor therapeutic activities of vincristine and vinblastine in brain may be due to poor drug access, as neither agent is found in appreciable quantities in CSF from humans and monkeys or in brain from dogs following systemic administration [8, 28, 29]. This is somewhat surprising, as both agents are lipophilic, with log octanol/physiological saline partition coefficients of 2.14 and 1.68, respectively, and CNS neurotoxicity is sometimes associated with the continued use of vincristine [31].

Greig [19, 20] has reported that a combination of factors determines the time-dependent concentration of an agent that reaches the brain from plasma following its systemic administration. In addition to a required lipophilicity, for an agent that does not share a facilitated transport system at the blood-brain barrier [23], the plasma concentration versus time profile (determined by distribution, metabolism, and elimination processes), binding to plasma constituents and tissues, and cerebral blood flow all combine to determine the final brain concentration of the agent and its alteration over time.

Initial pharmacokinetic studies of vincristine and vinblastine relied on determination of radioactivity following administration of tritiated drug [8, 12, 27]. The later development of radioimmunoassays [30, 42] has led to more detailed reports on the distribution and elimination of these agents. All studies agree that plasma concentrations decline rapidly following their administration to humans and animals. Plasma disappearance is best described by a three-compartmental model, with mean half-life values of 1.9, 19.2, and 1,359 (22.7 h) min for vincristine [42] and 3.9, 53, and 1,173 (19.5 h) min [37] for vinblastine after i.v. administration to humans. A longer terminal half-life of 85 h has also been reported for vincristine [35]. The initial, short half-life values for both agents indicate that plasma drug levels decline rapidly. Not only do both agents have large volumes of distribution, but they are subject to high biliary excretion [27], and extensive metabolism additionally occurs [4, 37]. Metabolism may be species-specific. In humans, vincristine metabolites have been shown to account for up to 30% of plasma radioactivity 5 min following i.v. administration of [^{3}H]-vincristine, and vinblastine metabolites account for up to 13% of radioactivity 4 min after i.v. administration of [^{3}H]-vinblastine [4].

The prolonged terminal half-life values for both drugs indicate avid tissue binding. Disposition studies of the vinca alkaloids in animals have demonstrated that tissue concentrations, with the exception of those in the nervous system, are up to 20-fold those present in blood, following the distributive phase [12]. In addition, analysis of blood has shown that the agents bind significantly to both plasma constituents and blood cells [13]. Our studies determined that 60.6% and 82.7% of vincristine and vinblastine, respectively, were bound to plasma constituents at concentrations between 0.1 and 10 nmol/ml. These results are in accord with those of Donigian and Owellen [13] that report 75% binding of both agents to plasma proteins. Steele and colleagues [46] have demonstrated that the predominant binding constituents are α_1- and α_2-globulins and that up to 99.7% of vinblastine becomes plasma protein-bound at therapeutic concentrations.

Declining plasma concentrations and extensive tissue and plasma protein binding limit the quantity of drug available for uptake into the brain and CSF. Our studies controlled for these confounding parameters by (a) maintaining a constant and high concentration of drug in cerebral capillaries by perfusing drug directly into the internal carotid artery and (b) replacing plasma with a physiological buffer to eliminate binding to plasma constituents. Nevertheless, the measured cerebrovascular permeability of each compound was substantially lower than would be predicted from the log P values. The predicted cerebrovascular permeability of a compound with a log P value of 2 is $>5 \times 10^{-2}$ ml s^{-1} g^{-1} [44], approximately 500 times the measured value for vincristine and vinblastine. Furthermore, extensive studies by Smith and colleagues [34, 45, 48] have demonstrated the accuracy of the isolated brain perfusion technique, used in the present study, in quantitat-

ing the cerebrovascular permeability and transport kinetics of a wide variety of hydrophilic and lipophilic compounds in the rat.

Vincristine and vinblastine are relatively large molecules that are endowed with several highly polarized functional groups, including two basic tertiary amines (p*K*a, 5.0 and 7.4) and several hydroxyl moieties. Repulsive forces between charged and regionally distributed functional groups on the compounds and on lipid molecules of the cerebral capillary membranes may restrict the brain entry of these drugs. In addition, their unusual geometry may limit their ready diffusion into and across the lipid compartment of the blood-brain barier [47]. A similar explanation may account for the inability of the lipophilic immunosuppresive agent cyclosporin to readily enter the brain [9]. This agent is a cyclic endecapeptide that has, together with several amino and carboxylic acid moieties, a large number of alkyl groups that give it a log P value of 2.99 [51]. It is therefore possible that for large molecules, the additive nature of log P values for regionally distributed moieties does not operate for diffusion through biological membranes.

The cytotoxic threshold concentration of vincristine in murine and human cell lines in vitro has been reported to be $>10^{-9}$ *M* [26]. Although this concentration is likely to be achieved and maintained for a significant period of time in peripheral tissues and extracerebral tumors after a clinical dose of <2 mg/m^2, it is unlikely to be achieved throughout a brain tumor except, perhaps, within an area of blood-brain tumor barrier breakdown. As neither vincristine nor vinblastine readily crosses the blood-brain barrier and either can be predicted to have a low permeability at the blood-nerve barrier, how is it possible for vincristine-induced central and peripheral neurotoxicity to occur?

In vitro studies [10, 52] and experiments in rats using (a) osmotic blood-brain barrier opening [36] and (b) cerebral injection [25] have demonstrated that vincristine and vinblastine are directly neurotoxic. However, threshold neurotoxic concentrations are 0.004 μg/ml (5×10^{-9} *M*) [32]. Elaborate studies by Sethi and colleagues [41, 49] have demonstrated that both vincristine and vinblastine undergo extensive and, despite structural similarities, different metabolism/degradation to yield a variety of products of differing lipophilicity. Thompson and colleagues [50] have described an epimer of vincristine, LY 119863, that possesses improved anticancer activity and markedly reduced neurotoxicity as compared with vincristine; others have been reviewed by Gerzon [17]. This indicates that slight modifications in the chemical structure of vincristine, which could be caused by metabolism/degradation, can significantly alter its pharmacology. Furthermore, sensitive radioimmunoassays used to describe the pharmacokinetics of vincristine and vinblastine in plasma have not achieved differentiation between the parent compounds and their degradation products and/or metabolites; the long terminal half-life of vincristine may be due to a combination of these. It is therefore possible that one of these metabolites/degradation products, possessing a greater capacity for penetration of the nervous system than does vincristine, is responsible for the neurotoxicity associated with the latter.

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