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Liposome encapsulated vincristine: preclinical toxicologic and pharmacologic comparison with free vincristine and empty liposomes in mice, rats and dogs

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A preclinical toxicology study of liposome encapsulated vincristine, free vincristine and empty liposomes was carried out in mice and dogs by single and multiple (daily for 5 days) intravenous injection. Single and multiple dose intravenous injection studies in mice showed the encapsulated form of vincristine to be less toxic than free vincristine. Empty liposomes injected intravenously into dogs were without significant toxicity. In dogs, the toxicities seen with liposomal vincristine were qualitatively similar to those of free vincristine with only minor quantitative differences. The principal toxicities of free and liposomal vincristine in dogs were anorexia, weight loss, pyrexia, myelosuppression and gastrointestinal toxicity. After single high doses of either formulation gastrointestinal toxicity was the dose-limiting toxicity, while either hematologic or gastrointestinal toxicity was dose limiting after multiple dose administration of either drug. Histopathologic lesions of importance were bone marrow atrophy, necrosis and atrophy of the lymphoproliferative tissues, necrosis of gastrointestinal tract mucosa, liver and pancreas, and hemorrhage. Distribution studies in rats showed significantly higher vincristine levels in serum, spleen, liver, trachea, jejunum, cerebrum, lung, ischiatic nerve and heart, and significantly lower levels in colon, stomach, salivary gland, thymus esophagus and pancreas after injection of the liposome-associated agent. No toxicities were seen that should preclude safe clinical trial of liposomal vincristine in man.

Key words: Dogs, liposomes, mice, pharmacokinetics, rats, toxicity, vincristine.

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Introduction

Formulation of antineoplastic agents in liposomes has generated interest because of the potential for decreased systemic and local toxicity and increased efficacy in preclinical test systems. It is not yet clear if increased efficacy is due to slow release of the agent from the carriers or passive targeting of the liposomes to tumor target sites. Reduction of organ specific toxicity, however, is likely due to decreased drug exposure to susceptible tissues. Liposomal encapsulation of doxorubicin has been shown to decrease the cardiotoxic potential of this agent,[1,2] due apparently to decreased exposure of the heart muscle to this toxic agent.[3] Liposomal encapsulation of doxorubicin has also been shown clinically to lessen the severity of a number of other side effects such as gastrointestinal toxicity and dermal toxicity after extravasation of the compound.[4] Increased efficacy in a wide variety of murine test systems has also been demonstrated for liposomal doxorubicin.[5–7] This may be a result of the potential for increased dose intensity due to decreased toxicity.

The Vinca alkaloids are an important class of antineoplastic agents with clinical activity against a variety of neoplasms such as leukemia, lymphomas and embryonal tumors. The major and dose-limiting toxicity of vincristine is neurotoxicity, with the most frequent toxicity a peripheral neuropathy. The drug also causes autonomic (constipation and abdominal cramps) and CNS toxicity (altered consciousness and mental changes). Previous studies have demonstrated increased efficacy of vincristine encapsulated in liposomes versus the free drug in tumor model systems such as P388 and L1210 lymphocytic leukemia[8,9] and mouse mammary carcino-

ma MC2[7], as well as decreased systemic toxicity in mice.[9] Clinical studies have demonstrated increased efficacy of vincristine when infused,[10,11] which has also generated interest for a long circulating formulation of vincristine. Vincristine has poor solubility characteristics in aqueous systems and a relatively high permeability to membranes which has limited the development of a stable liposomal formulation. Recently, a remote-loading technique for liposomal encapsulation of vincristine has been developed.[9] This pharmaceutically acceptable formulation allows for the encapsulation of vincristine in liposomes at the clinical site using an active trapping system. This system is based upon the movement of lipophilic cations (i.e. vincristine) across a lipid membrane due to a pH gradient.[8,9] This process results in the production of stable liposomes with trapping efficiencies approaching 100% and very high levels of entrapped drug.

Because of the promise of greater specificity of antitumor activity and the expectation from previous studies that liposomal vincristine may be less toxic, a preclinical safety and distribution study of liposomal vincristine, in comparison with free drug and empty liposomes, was carried out prior to human clinical trial of the vincristine formulated in 120 nm distearylphosphatidylcholine (DSPC)/cholesterol liposomes.

Materials and methods

Liposomal vincristine formulation characteristics

The liposomes utilized for entrapment of vincristine were composed of DSPC and cholesterol combined at a molar ratio of 55 : 45. Liposomes (100 mg lipid/ml) were hydrated in 300 mM citrate buffer adjusted to pH 4.0, extruded through 100 nm pore size polycarbonate filters at 65 °C[9] and filter sterilized through 0.22 micron pore size filters (Millipore, Bedford, MA). This process yielded homogeneously sized liposomes with a mean diameter of approximately 120 nm as determined by quasielastic light scattering employing a Nicomp 370 particle sizing apparatus. The liposomes were stored at 4 °C until use.

Vincristine was encapsulated in the preformed DSPC/cholesterol liposomes just prior to use using the pH gradient-dependent remote loading technique.[9] This entrapment procedure utilizes the response of lipophilic amines to transmembrane pH gradients to actively load the drug into the liposome interior.[9] Major advantages of this system are that trapping efficiencies approaching 100% are readily achieved and problems associated with long-term storage of drug loaded liposomes is avoided. In this procedure liposomes (0.2 ml) were mixed with 1.0 ml commercially available vincristine (1.0 ml) (Oncovin; Eli Lilly, Indianapolis, IN) at room temperature in a sterile empty vial. Then, 5 ml of 100 mM Na_2HPO_4 was added and the sample was then incubated at 65 °C for 10 min with intermittent mixing. Column (Sephadex G-50) chromatography analysis of the final solution (0.16 mg vincristine/ml) indicated that the trapping efficiency was more than 95% for this procedure. In addition, HPLC analysis of encapsulated vincristine has demonstrated that the vincristine was more than 95% pure after entrapment.[9] The material was used for experimental purposes within 8 h of entrapment.

Empty liposomes were prepared essentially as described above, except saline (non-preserved sodium chloride injection 0.9%, USP) was substituted for the vincristine sulfate. Free vincristine sulfate (Oncovin) was diluted with saline prior to injection.

Test animals and husbandry

Male and female Ha/ICR mice (4–8 weeks of age, 20–30 g) were obtained from the breeding colony of Roswell Park Cancer Institute. The animals were housed in stainless steel cages on hardwood sawdust bedding in rooms with 12 h light/dark cycles, and fed Teklad Laboratory Animal Chow (6% fat content) and acidified tap water. Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were housed in suspended stainless steel cages (wire mesh floors), and fed Teklad Laboratory Animal Chow (Madison, WI) (6% fat content) and acidified tap water. Young (4–13 months) male and female pure-bred beagle dogs from the dog colony of Roswell Park Cancer Institute were housed individually in stainless steel cages in rooms with 12 h light/dark cycles, and fed Wayne Dog Chow (Chicago, IL) (300 g/day) and tap water (1500 ml/day).

Toxicity studies in mice

Male and female Ha/ICR mice (4–8 weeks of age, weighing 20–30 g) were intravenously administered single or multiple (daily for five consecutive days) doses of saline, empty liposomes, free vincristine or liposome encapsulated vincristine at varying con-

centrations. Group size was 5 mice/sex/dosage group. Mice were observed and weighed daily. The experimental observation period was 28 days.

Toxicity studies in beagle dogs

Beagle dogs (one to two males and one to two females per group) were injected intravenously with single (0.05, 0.1 and 0.2 mg/kg) doses of free or liposomal vincristine, or multiple doses of free (0.01 and 0.02 mg/kg for five consecutive days) or liposomal vincristine (0.01, 0.02 and 0.04 mg/kg for five consecutive days); or multiple (daily for 5 days) doses of the amount of lipid needed to encapsulate a dose of 0.4 mg vincristine/kg.

Evaluation of toxicity was carried out by monitoring rectal temperature (10 and 3 days prior to injection, 1 and 3 h after injection, daily for 14 days after injection, and weekly thereafter); evaluation of clinical signs (daily); weekly EKG analyses; weekly neurologic exams; daily measurement of food and water consumption and body weight; weekly urinalysis (male dogs only); and serial serum chemistry and hematology evaluations (10 and 3 days prior to dosage, 1, 4, 7 and 11 days after injection, and weekly thereafter).

Hematology measurements were made with Baker 7000 and 810 analyzers (Baker Instruments, Allentown, PA), and serum chemistry evaluations with a Gemeni centrifugal analyzer (Electro-Nucleonics, Fairfield, NJ) and an Orion Na/K 1020 analyzer (Orion Research, Cambridge, MA).

The hematology measurements carried out included: erythrocyte, total white blood cell (WBC), platelet and reticulocyte counts, WBC differential, hemoglobin, hematocrit, prothrombin time, partial thromboplastin time, and erythrocyte indices (MCV, MCH, MCHC). The serum chemistry analyses include: glucose, BUN, creatinine, electrolytes (Na, K, Cl, Ca, P, Mg), cholesterol, total bilirubin, serum enzymes (alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, creatinine phosphokinase, amylase, lactate dehydrogenase), total CO_2, total protein and albumin.

Neurological evaluations consisted of assessment of spinal reflex function (knee jerk, tonic neck reflexes, supporting reaction, tactile placing reaction, hopping reaction and the test of Romberg) and cranial nerve exam (cranial nerves II, V, VIII, IX, X, XI and XII).

All dogs were sacrificed by exsanguination following anesthesia with sodium pentobarbital. One pair of dogs from each group were sacrificed 1 week after dosage and another pair 2 months after dosage. Tissue samples from the eye, lip, tongue, palatine tonsil, parotid salivary gland, thyroid, trachea, esophagus, mammary gland, thymus, heart, lung, spleen, lymph nodes (mandibular, bronchial, mesenteric), colon, jejunum, duodenum, stomach, liver, kidneys, suprarenals, urinary bladder, pancreas, aorta, testicle and prostate (male), ovary and uterus (female), diaphragm, femoral bone marrow, femur, rib, vagus nerve, brain, and spinal cord were removed, fixed in buffered 10% formalin, and stained with hematoxylin and eosin. If animals died before the scheduled necropsy, they were necropsied immediately.

Distribution studies in rats

[^{3}H]Vincristine (6.67 Ci/mmol, 7.22 mCi/mg; Amersham, Arlington Heights, IL) was mixed with unlabeled vincristine sulfate to yield a formulation containing 0.5 mg vincristine/ml plus 50 μCi [^{3}H] vincristine/ml. Male rats were injected intravenously (tail vein) with free or liposomal labeled agent (2 mg vincristine/kg). At 4 and 24 h after treatment three rats from each group were sacrificed, organs and tissues removed, and solubilized by addition of 1 ml SOLUSOL (National Diagnostics, Manville, NJ) to 200 mg of tissue followed by heating (60 °C, 1 h) in a tightly stoppered container. Ecosint A (9.0 ml) (National Diagnostics, Manville, NJ) and 100 μl glacial acetic acid were added to the samples, placed in the dark for 48 h and counted (#1 c.p.m.) in a Beckman LS1801 scintillation counter (Beckman Instruments, Irvine CA). An internal standard of [^{3}H]-vincristine was added, the samples recounted (#2 c.p.m.) and a quench coefficient calculated as follows:

$$\text{quench coefficient} = \frac{\text{internal standard c.p.m.}}{(\#2\ \text{c.p.m.}) - (\#1\ \text{c.p.m.})}$$

The quench coefficient was used to calculate the [^{3}H]vincristine concentration.

Results

Studies in Mice

HA/ICR mice were injected intravenously with varying doses of liposomal vincristine to evaluate the potential for lethality of this preparation in comparison with free drug. Other groups were treated with empty liposomes, saline or received no treatment

Table 1. Lethality of free and liposomal vincristine in HA/ICR mice[a]

Dose (mg/kg/day)	Cumulative dose (mg/kg)	No. of doses	Dead/treated	Day of death
Liposomal vincristine				
2.0	2.0	1	0/10	
4.0	4.0	1	6/10	4,5,7,8,8,9
6.0	6.0	1	10/10	0,4,4,4,5,5,6,6,7,7
0.8	4.0	5	0/10	
1.2	6.0	5	10/10	5,5,5,5,5,6,6,7,7,11
Free vincristine				
2.0	2.0	1	0/10	
4.0	4.0	1	10/10	4,4,5,5,5,6,6,7,7,8
0.4	2.0	5	0/10	
0.8	4.0	5	0/10	

[a] Mae and female HA/ICR mice were given single or multiple (daily for five consecutive days) intravenous doses of free or liposome encapsulated vincristine. Deaths were recorded from days 0 to 28.

whatsoever for comparative purposes (data not shown).

No mice treated with free or liposomal vincristine (single dose study, Table 1) died at the 2.0 mg/kg dose level. When the dose of liposomal vincristine was doubled to 4.0 mg/kg, six of 10 mice died (four or five male mice died and two of five female mice died). In comparison, all mice that received free drug at this dose died. If the dose of liposomal agent was increased to 6.0 mg/kg, all mice died. Mice were given five consecutive daily doses of each agent to evaluate the potential for cumulative toxicity. All mice that received 0.8 mg liposomal vincristine/kg (cumulative dose of 4 mg/kg) survived, whereas this dose was lethal to six of 10 animals if given as a single dose. All mice died that received a cumulative dose of 6.0 mg/kg (1.2 mg/kg daily for 5 days), as did the mice that received it as a single dose. In contrast, mice that received 0.8 mg free vincristine/kg per day for five consecutive days all survived, whereas this dose when given as a single dose (4.0 mg/kg) was lethal to all animals.

Mice that received toxic doses of either liposomal vincristine or free vincristine had roughened fur coats, and became weak and thin. One female mouse that received 4.0 mg liposomal vincristine/kg and another mouse that received 6.0 mg liposomal vincristine/kg developed signs of diarrhea. At the highest dose of free vincristine tested (4.0 mg/kg), the last surviving male mouse on day 7 developed possible neurologic signs (i.e. poor coordination). On day 8 the last surviving female mouse displayed posterior paralysis.

One group of mice was treated with the same amount of lipid needed to encapsulate the highest dose of vincristine given as encapsulated drug (6.0 mg/kg) and another group was treated with saline at the same volume needed to deliver the 6.0 mg liposomal vincristine/kg dose. None of the control groups (empty liposomes; saline; non-injected controls) developed any patterns of weight loss or physical abnormalities. There were no deaths (injection related deaths or delayed deaths) in the saline or non-injected groups. However, there were three deaths shortly (minutes) after injection in the empty liposome groups. These deaths (two of five males and one of five females) may have been due to the large volumes infused (0.9–1.0 ml in these mice that weighed 23–27 g) rapidly (3–5 s).

Studies in beagle dogs

Single dose studies. A total of 10 dogs were treated with liposomal vincristine and for comparative purposes 10 were treated with free vincristine (Table 2). One dog of four died that received liposomal vincristine at a dose of 0.1 mg/kg, while none of the dogs that received free drug died at this dose. All of the animals that received 0.2 mg/kg of either drug formulation died rapidly (within 2–3 days) after injection of overwhelming gastrointestinal toxicity. A summary table (Table 3) details the significant toxicities seen in these dogs. Gastrointestinal toxicity was seen in both groups that received the lowest dose of agent, 0.05 mg free or liposomal vincristine/kg. Two of the four dogs treated with free drug developed significant gastrointestinal toxicity as manifested by diarrhea (one dog with short lived gastrointestinal hemorrhage) lasting 4–5 days.

Table 2. Dose and schedule of free and liposomal vincristine and empty liposomes administered to beagle dogs.[a]

Treatment (mg/kg×days)	Cumulative dose (mg/kg)	Day of necropsy[b]
Free vincristine		
0.05 × 1	0.05	8,8,63,64
0.1 × 1	0.1	8,8,63,64
0.2 × 1	0.2	3*,3*
0.01 × 5	0.05	63,64
0.02 × 5	0.1	7,7,8*,64
Liposomal vincristine		
0.05 × 1	0.05	8,8,63,63
0.1 × 1	0.1	3*,7,65,65
0.2 × 1	0.2	2*,2*
0.01 × 5	0.05	65,65
0.02 × 5	0.1	7,7,65,65
0.04 × 5	0.2	6*,6*
Empty liposomes[c]		
0.4 × 5	2.0	7,7,64,64

[a] Equal numbers of male and female beagle dogs per group were injected intravenously with free vincristine, liposomal vincristine or empty liposome on day 0.
[b] Drug-related death post-treatment(*).
[c] The amount of empty liposome that would have been needed to encapsulate a dose of 0.4 mg vincristine/kg.

Table 3. Summary of toxicities of free (F) and liposomal (L) vincristine in beagle dogs: single intravenous dosage[a]

Dose (mg/kg)	0.05F	0.05L	0.1F	0.1L	0.2F	0.2L
N	4	4	4	4	2	2
Anorexia	1/4	1/4	4/4	4/4	2/2	2/2
Weight loss (>10%)	1/4	1/4	4/4	4/4	2/2	2/2
Pyrexia (>103.5 °F)	0/4	0/4	0/4	1/4	0/2	1/2
Vomiting	0/4	3/4	3/4	3/4	2/2	1/2
Diarrhea	4/4	3/4	4/4	4/4	2/2	2/2
GI bleeding	1/4	1/4	2/4	4/4	2/2	2/2
>Grade III myelo[b]	0/4	0/4	2/4	3/4*	**	**
Muscle enzyme elevation	4/4	4/4	4/4	4/4	2/2	2/2
Amylase elevation	0/4	2/4	2/4	2/4	2/2	1/2
Hepatic enzyme elevation	1/4	2/4	2/4	1/4	0/2	1/2
Dead/treated	0/4	0/4	0/4	1/4	2/2	2/2
Day of death				3	3,3	2,2
Histopathology lesions						
GI	0/4	0/4	1/4	3/4	2/2	2/2
liver	0/4	3/4	0/4	3/4	2/2	1/2
bone marrow	0/4	0/4	0/4	1/4	2/2	2/2
muscle	0/4	0/4	0/4	0/4	0/2	0/2
pancreas	0/4	0/4	1/4	1/4	1/2	2/2
lymphoproliferative	1/4	1/4	2/4	3/4	2/2	0/2

[a] Male and female (two to four per group) beagle dogs were injected intravenously with free or liposomal vincristine at doses of 0.05, 0.1 or 0.2 mg/kg.
[b] WHO toxicity grading system. Grade III toxicity defined as leukocytes 1000–1900/mm^3, granulocytes 500–900/mm^3 or platelets 25 000–490 000/mm^3.
* Drug-related death post-treatment prior to expected onset of myelosupression.

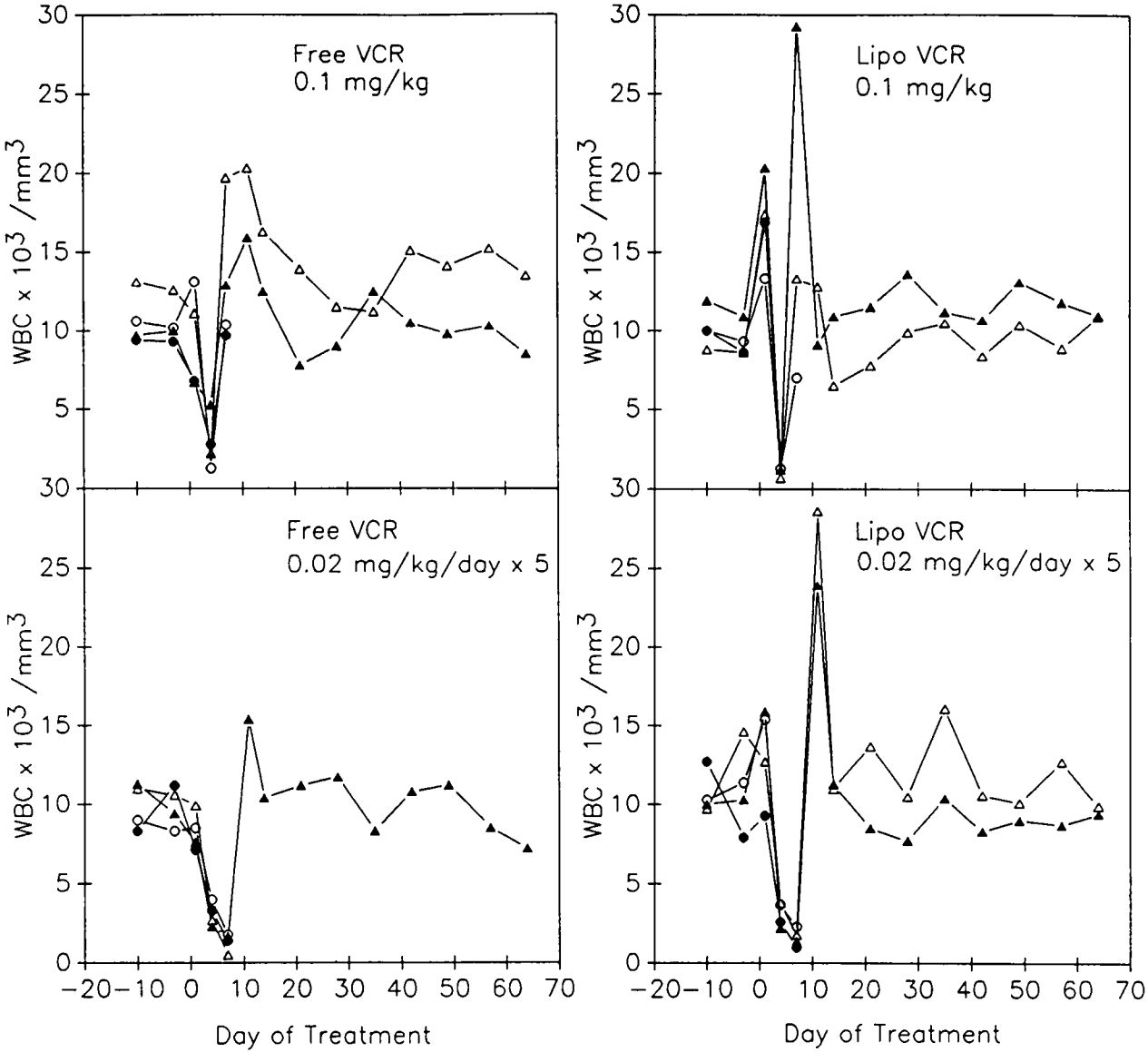

Figure 1. Fluctuations in peripheral total white blood cell counts of beagle dogs injected intravenously day 0 with free or liposomal vincristine by single (0.1 mg/kg) or multiple (0.05 mg/kg daily for five consecutive days; cumulative dose 0.1 mg/kg) dose schedules.

Severe gastrointestinal toxicity was seen in one of the four dogs that received the liposomal agent (diarrhea and hemorrhage) and it lasted for nearly 1 week. Much milder toxicity was seen in the other three dogs in this group (i.e. loose stools/diarrhea for 1–3 days). All dogs that received 0.1 mg free or liposomal vincristine developed severe gastrointestinal toxicity, as manifested by severe diarrhea (all eight dogs) and gastrointestinal hemorrhage (four of four that received liposomal agent and two of four that received free drug). Gastrointestinal toxicity (vomiting, diarrhea and gastrointestinal hemorrhage) was overwhelming, and led to the death of dogs treated at 0.2 mg/kg.

Hematologic toxicities were similar after treatment with free or liposomal vincristine, and can best be appreciated by evaluation of the 0.1 mg/kg treatment groups (Figures 1 and 2). All treated dogs developed lymphopenia by day 1, with recovery to pretreatment levels by day 4 in the majority of dogs from each group. Dogs that received liposomal vincristine developed a marked neutrophilia at 1 day after treatment (Figure 1). The fall in total WBC count (due primarily to neutropenia) occurred rapidly (by day 4 in all dogs) and recovery to normal WBC and platelet values was complete by days 8–11 (Figure 1). Neutrophil counts fell below 500/mm^3 in three of four dogs that received 0.1 mg free or

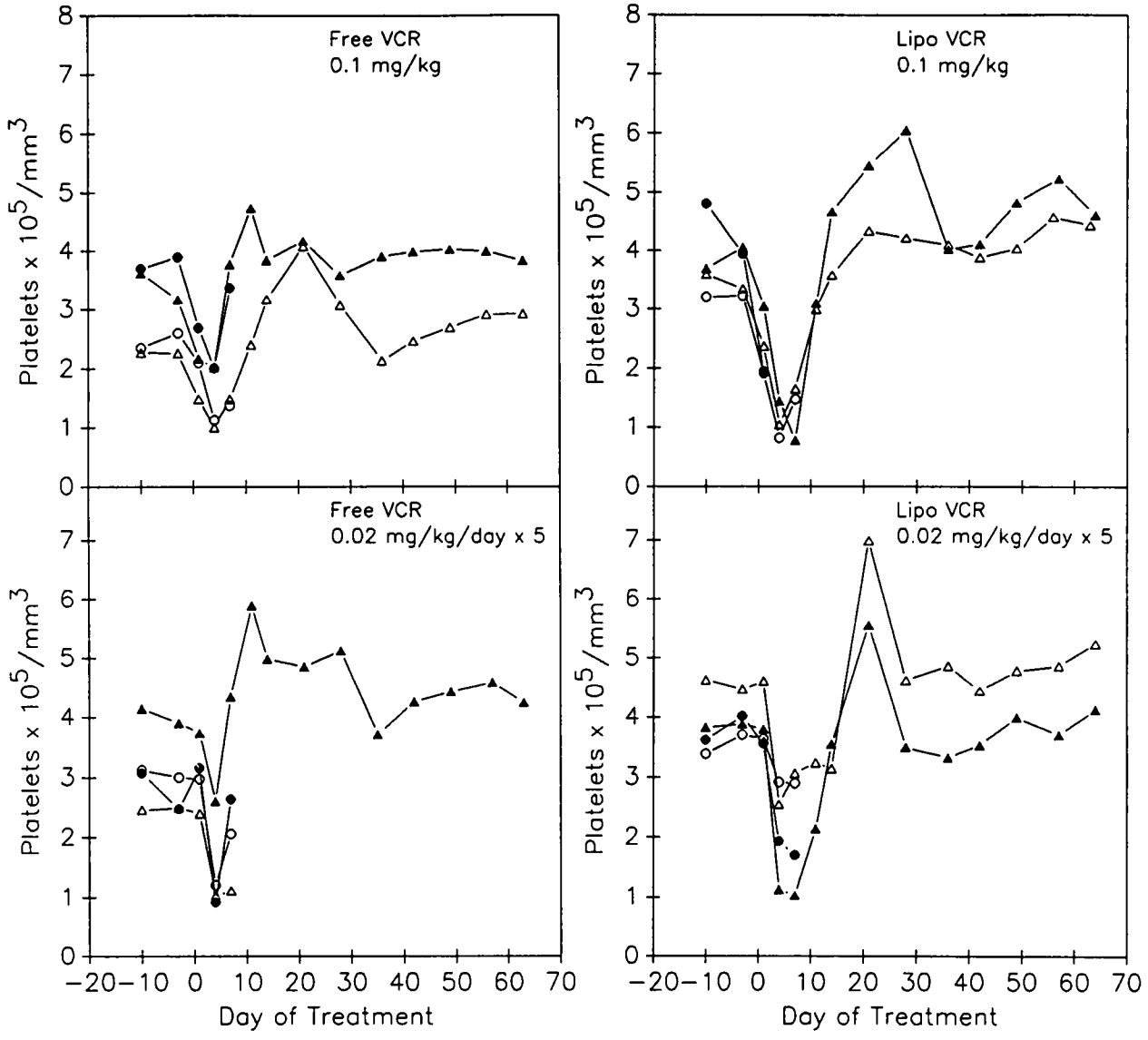

Figure 2. Fluctuations in peripheral platelet counts of beagle dogs injected intravenously day 0 with free or liposomal vincristine by single (0.1 mg/kg) or multiple (0.05 mg/kg daily for five consecutive days; cumulative dose 0.1 mg/kg) dose schedules.

liposomal vincristine/kg on day 4 (one dog from the liposomal group died prior to day 4). Platelet numbers were decreased in all dogs; nadir values of 77 000–195 000 in dogs treated with liposomal drug (0.1 mg/kg) and 101 000–202 000/mm^3 in dogs treated with the same dose of free vincristine (Figure 2). Recovery to normal levels in these two groups was by day 7 or 11 in the dogs that survived or were permitted to recover. The documented myelosuppression in dogs that receive 0.2 mg free or liposomal vincristine/kg consisted only of lymphopenia on day 1 in all dogs. The expected drop in platelets and neutrophils was not seen due to the rapid demise of the dogs from gastrointestinal toxicity.

Serum chemistry perturbations included elevations in muscle enzymes (all dogs of every treated group at all doses) shortly (day 1) after treatment with rapid return to normal. There were no physical manifestations of this toxicity (i.e. pain, reluctance to move).

Elevations in liver enzymes indicative of transient hepatotoxicity and elevations of amylase indicative of pancreatic toxicity were seen with approximately equal frequency after administration of either drug formulation (Table 3). Elevations in serum alkaline phosphatase and electrolyte disturbances were seen in both treatment groups, and probably were a reflection of the gastrointestinal toxicities.

Histopathologic lesions in these dogs were gastrointestinal (dilated crypts, crypt necrosis, hemorrhage, epithelial degeneration and regeneration), hepatic (bile duct proliferation, epithelial degeneration and necrosis), bone marrow (aplasia), pancreatic (mononuclear infiltrates, multifocal areas of degeneration and coagulation necrosis) and lymphoproliferative (involution, hyperplasia, lymphoid necrosis). There were no clear differences between groups in either the incidence or severity of lesions (Table 3). Neurologic and EKG exams were negative for drug induced toxicity in all dogs of either drug group.

Multiple-dose studies. The initial dose of active agent (free and liposomal) tested was 0.01 mg/kg/day, equivalent (as a cumulative dose) to the lowest dose used in the single-dose trial (0.05 mg/kg) (Table 2). Two dogs were treated with liposomal agent and two dogs with free drug. Four dogs were treated with 0.02 mg free vincristine/kg for 5 days and four with the same dose of liposomal agent. One of the free drug dogs died on day 8, while all of the dogs that received liposomal agent survived to the scheduled sacrifice times. The dose of liposomal drug was doubled to 0.04 mg/kg/day (cumulative dose of 0.2 mg/kg). Dogs were not treated with free drug at this dose as lethality and serious toxicity had already been seen at the 0.02 mg/kg/day level.

Moderate to severe gastrointestinal toxicity (with evidence for gastrointestinal hemorrhage) was seen in one dog treated with a dose of 0.01 mg free drug/kg/day. The other three dogs in this group and the dogs that received the same dose of liposomal drug showed few gastrointestinal toxicities of significance. All dogs that received the next escalation (0.02 mg/kg) showed moderate to severe gastrointestinal toxicity. Only one of the eight dogs (the dog that received free drug and that died) had treatment-related vomiting. All eight dogs suffered from some degree of diarrhea with two of four dogs that received free drug and one of four dogs that received the liposomal agent had evidence of gastrointestinal hemorrhage. Gastrointestinal toxicity after a dose of 0.04 mg liposomal vincristine/kg was very severe, consisting of vomiting in one of two dogs; with diarrhea and gastrointestinal hemorrhage in both dogs.

Myelosuppression was rather modest at a dosage of 0.01 mg/kg of either drug. The total fall in WBC number in the four dogs ranged from 26 to 50% with similar decreases in platelets. All parameters returned to normal by days 11 or 14. Significant myelosuppression was seen in all dogs that received 0.02 mg free or liposomal vincristine/kg (Figure 2). All of the free drug dogs showed grade III (WHO grading system) toxicity to leukocytes and three of four dogs that received liposomal drug had grade III toxicity (one dog with grade II). Severe drops in neutrophils and lymphocytes were recorded, with less toxicity to platelets (Figure 2). Recovery to normal in the dogs that had sufficient time to recover was rapid (days 11–14). Myelosuppression was severe (grade IV leukocyte toxicity) in dogs that received 0.04 mg liposomal vincristine/kg/day. Serum chemistry perturbations included elevations in muscle, pancreatic and hepatic enzymes (Table 4). The types of lesions seen in this multiple dose series was similar to that seen after single dose administration. No new toxicities were induced by the 5 day treatment regimen.

Empty liposomes. A comparative study of empty liposomes was also conducted to gain an appreciation of the contribution of liposomes to the overall toxicity of the encapsulated agent. Four beagle dogs were administered a dose of empty liposomes equivalent to the amount needed to encapsulate a 0.4 mg/kg dose of vincristine, corresponding to 10 times the lethal level (0.04 mg liposomal vincristine/kg) determined in this study, for five consecutive days. Few toxicities were noted with this preparation that seemed to be drug related. Two of the four dogs developed slight inappetence during treatment, but this did not result in significant weight loss in either dog. Drug-related gastrointestinal toxicity may have occurred in two of the four dogs but in very mild form. One dog had a slight amount of diarrhea (10% of total stool volume) on day 2. Another dog passed small amounts of bloody mucous on days 3 and 7, and small amounts of diarrhea on days 8, 10 and 56. These toxicities may well be considered background problems and not drug-related. The total leukocyte count dropped slightly in two dogs (nadir values of 6.3 and 6.7/mm^3). In one dog this finding had a good temporal relationship to treatment (day 7), while in the other there was a poor temporal relationship to treatment (day 42). There were no serum chemistry findings to suggest drug-related toxicity. The only potential treatment-related histopathologic lesions were seen in one dog (mottling of the liver, prominent bile ducts). However, these lesions are more likely to be incidental and not treatment-related.

Table 4. Summary of toxicities of free (F) and liposomal (L) vincristine and empty (E) liposomes in beagle dogs: multiple-dose intravenous study (daily for five consecutive days)

Dose (mg/kg)	0.01F	0.01L	0.02F	0.02L	0.04L	0.4E
Cumulative dose	(0.05)		(0.01)		(0.2)	(2.0)
N	2	2	4	4	2	4
Anorexia	0/2	0/2	4/4	3/4	2/2	2/4
Weight loss (>10%)	0/2	0.2	4/4	3/4	2/2	2/4
Pyrexia (>103.5 °F)	0/2	0/2	1/2	3/4	2/2	0/4
Vomiting	0/2	0/2	1/4	0/4	0.2	0/4
Diarrhea	1/2	0/2	4/4	4/4	2/2	0/4
GI bleeding	1/2	0/2	2/4	1/4	2/2	0/4
Grade III myelo[b]	0/2*	0/2	4/4	3/4	2/2	0/4
Muscle enzyme elevation	0/2	0/2	4/4	3/4	2/2	0/4
Amylase elevation	0/2	0/2	0/4	3/4	0/2	0/4
Hepatic enzyme elevation	0/2	0/2	0/4	0/4	0/2	1/4
Dead/treated	0/2	0/2	1/4	0/4	2/2	0/4
Day of death			8		6,6	
Histopathology lesions						
GI	0/2	0/2	2/4	2/4	2/2	0/4
liver	0/2	2/2	1/4	1/4	0/2	1/4
bone marrow	0/2	0/2	3/4	2/4	1/2	0/4
muscle	2/2	0/2	0/4	0/4	0/2	0/4
pancreas	0/2	0/2	0/4	0/4	0/2	0/4
lymphoproliferative	2/2	1/2	3/4	1/4	1/2	3/4

[a] Male and female (two to four per group) were injected with free or liposomal vincristine at doses of 0.01, 0.02 or 0.04 (liposomal vincristine only) by a multiple dose schedule (daily for five consecutive days). Control dogs were administered intravenously daily for 5 days the amount of empty liposomes that would have been needed to encapsulate a dose of 4.0 mg vincristine/kg.
[b] WHO toxicity grading system. Grade III toxicity defined as leukocytes 1000–1900/mm^3, granulocytes 500–900/mm^3 or platelets 25000–490 000/mm^3.
* Drug-related death post-treatment prior to expected onset of myelosuppression.

Pharmacology studies

Radioactively labeled preparations of free and liposomal vincristine were administered to male rats, and tissue levels determined 4 and 24 h after injection. The tissue levels (corrected for plasma drug level) are shown in Table 5. For ease of comparison, the differences in vincristine levels found in animals treated with the free or liposomal vincristine are summarized in Table 6. At the 4 h time point, significantly less drug was found in the skeletal muscle, colon, stomach, salivary gland, thymus, esophagus and pancreas, while significantly more drug was found in the serum, spleen, liver, trachea and jejunum. At the 24 h time point levels in the skeletal muscle, salivary gland and thymus were still significantly lower, while higher in the serum, spleen, liver, trachea, cerebrum, lung, ischiatic nerve and heart. The largest differences in vincristine content between free and liposomal drug was observed in serum where L/F ratios were 147.95 and 35.45 at 4 and 24 h post-injection, respectively.

Discussion

Vincristine is a widely used antineoplastic antibiotic owing to its efficacy against a variety of neoplasms and its utility in combination with other drugs because of its relative lack of myelosuppressive toxicity. Although this agent is phase specific, conventional administration of this agent is typically by bolus injection. This is a consequence of the difficulties involved with prolonged intravenous infusions of drugs with strong vesicant characteristics. This may be a significant problem since it has

Table 5. The 4 and 24 h distribution of free and liposomal vincristine in rats[a]

	Tissue levels (μg vincristine/g)							
	4 h sacrifice				24 h sacrifice			
	free VCR		lipo-VCR		free VCR		lipo-VCR	
Tissue	average	SD	average	SD	average	SD	average	SD
Ischiatic nerve	0.148	0.016	0.177	0.057	0.048	0.010	0.092	0.016
Salivary gland	2.788	0.175	1.180	0.101	0.892	0.158	0.477	0.077
Thymus	0.971	0.070	0.422	0.159	1.149	0.142	0.680	0.183
Lymph node	0.632	0.211	0.681	0.452	0.396	0.061	0.421	0.034
Lung	1.761	0.162	1.706	0.432	0.352	0.023	0.796	0.158
Diaphragm	0.749	0.084	0.535	0.125	0.211	0.024	0.273	0.084
Skeletal muscle	0.642	0.076	0.202	0.062	0.327	0.047	0.193	0.020
Esophagus	1.457	0.264	0.679	0.066	0.324	0.092	0.351	0.088
Trachea	0.917	0.331	3.786	1.358	0.222	0.131	2.107	0.418
Heart	1.020	0.202	0.806	0.031	0.124	0.011	0.191	0.012
Kidney	1.282	0.221	0.962	0.143	0.257	0.030	0.301	0.077
Testes	0.071	0.015	0.077	0.040	0.064	0.016	0.068	0.006
Pancreas	1.424	0.300	0.843	0.025	0.222	0.033	0.196	0.036
Liver	0.803	0.167	4.060	0.467	0.306	0.039	1.813	0.263
Spleen	3.092	0.054	49.995	19.990	1.353	0.070	21.281	6.067
Stomach	0.756	0.191	0.308	0.115	0.781	0.626	0.664	0.630
Jejunum	1.193	0.203	2.412	0.619	0.432	0.045	1.558	0.871
Duodenum	1.431	0.214	1.873	0.248	0.494	0.163	1.057	0.492
Colon	0.838	0.182	0.298	0.084	0.361	0.127	0.640	0.701
Cerebrum	0.015	0.006	0.005	0.005	0.007	0.001	0.026	0.004
Cerebellum	0.015	0.007	0.018	0.021	0.011	0.004	0.037	0.021

[a] Rats were injected intravenously with 2 mg free of liposomal [^{3}H]vincristine/kg, and sacrificed 4 and 24 h later. Tissues were removed and vincristine amounts calculated as detailed in Methods.

been shown that prolonged infusion of vincristine enhances its therapeutic activity.[10,11] Of special interest is a clinical study that has shown efficacy of vincristine given by prolonged infusion after the same patients failed to respond to bolus infusions.[10]

Laboratory studies of vincristine have demonstrated that its cytotoxic effects are both concentration and time dependent.[14] Pharmacokinetic studies in man have demonstrated rapid disappearance of vincristine from blood.[15] Such studies strongly suggest that efficacy of vincristine may be enhanced by prolonging exposure of cells to potentially cytotoxic drug levels. Encapsulation of the agent in liposomes leads to significantly higher blood levels of the agent for prolonged periods of time (Table 6) and therefore may enhance the therapeutic effectiveness of the agent.

The purpose of the study reported here was to evaluate the toxicity of liposomal vincristine in comparison with the well studied free drug and to determine if tissue distribution correlated with the organ-specific toxicities of the drugs. Both free and liposomal vincristine caused lethality in mice. The liposomal vincristine was somewhat less toxic; higher doses were needed for complete lethality. In light of the lethal, weight loss and physical effects of liposomal vincristine in comparison with the clinically well accepted vincristine, there are no findings in the murine study that should preclude its clinical use. The data indicate that similar doses of free and liposomal vincristine may be given.

The toxicities caused by free and liposomal vincristine after single intravenous injection to dogs were similar, and included anorexia with concomitant weight loss, gastrointestinal toxicity (the dose-limiting toxicity), myelosuppression (decreases in lymphocytes, neutrophils and platelets), loss of red cells (probably secondary to gastrointestinal hemorrhage), and serum chemistry perturbations secondary to muscle, hepatic, gastrointestinal and pancreatic toxicity. There were no patterns of toxicity of liposomal drug that clearly differentiated it from free drug and there were no new toxicities demonstrated in the dog study that should exclude this agent as a candidate for clinical trial. No neurologic toxicities, either of peripheral or central

Table 6. Biodistribution of free and liposomal vincristine in rats

Tissue type	L/F Ratio[a]	
	4 h	24 h
Significantly less ($p < 0.05$)[b]		
skeletal muscle	0.31	0.59
colon	0.36	NS
stomach	0.41	NS
salivary gland	0.42	0.53
thymus	0.43	0.59
esophagus	0.47	NS
pancreas	0.59	NS
Significantly greater ($p < 0.05$)		
serum	147.95	35.45
spleen	16.17	15.73
liver	5.06	5.98
trachea	4.13	9.49
jejunum	2.02	NS
cerebrum	NS	3.71
lung	NS	2.26
ischiatic nerve	NS	1.92
heart	NS	1.54

[a] Vincristine levels found in tissues of animals treated with liposomal vincristine divided by the level of drug found in tissues of animals treated with free vincristine.
NS is not significant ($p > 0.05$).
[b] p-values were determined employing a standard two-tailed Student's t-analysis.

origin, were detected by functional and morphologic evaluations. This is not an unexpected finding, as the dog is not considered to be a good model for vincristine-induced neuropathy. Due to the steep dose–response relationship to toxicity, a conservative escalation scheme would be prudent.

The toxicities after multiple-dose treatment were similar to those after single-dose treatment, although myelosuppression became more apparent, as the dogs were surviving long enough to develop myelosuppression, and not dying quickly from gastrointestinal toxicity. While at single high doses of drug it was clear that gastrointestinal toxicity was the dose-limiting toxicity, by the multiple-dose schedule (daily for 5 days) both gastrointestinal toxicity and myelosuppression appeared serious, and it is difficult to determine which toxicity may have led to the death of the dogs. In addition, by stretching the total single dose out for 5 days, there appeared to be less vomiting after treatment with either free or liposomal agent, and fewer elevations in amylase and alkaline phosphatase (indicators of pancreatic and gastrointestinal toxicity respectively).

A toxicity not seen in this trial was pyrexia in dogs within 24 h of dosage. This toxicity was noted in a previous toxicity trial of liposomal doxorubicin[13] and is probably due to interleukin-1 release.[16] Like liposomal doxorubicin, liposomal vincristine did induce a neutrophilia within 1 day of dosage in dogs (Figure 1), an effect which is also probably mediated by interleukin-1. Because of the limited number of large animals used in this study, it is difficult to state that either formulation (free or liposomal) causes more or less toxicity than the other. One dog in the free drug group died at the 0.02 mg/kg/day level, while all of the other dogs that received liposomal drug survived. Doubling the dose of liposomal drug led to rapid death of all of the treated dogs.

While no new or enhanced toxicities were demonstrated here with the encapsulated form of vincristine, the distribution data indicate higher drug levels of the encapsulated agent in critical target tissues, i.e. liver, cerebrum and ischiatic nerve. The potential for enhanced hepatic and neurotoxicity therefore exists, given the higher drug levels in these organs. It should be noted, however, that these properties are accompanied by dramatic increases in plasma (Table 6) and tumor (L. Mayer, unpublished observations) vincristine levels observed with the liposome formulation.

Conclusion

The routine basis for advancement to clinical trial of a new analog or formulation of an established agent is either increased therapeutic activity in preclinical test systems, decreased or changed toxicity, or a change in pharmacology that may enhance the antitumor properties of the agent. While this study does not reveal any significant change in toxicity characteristics (other than decreased lethality in mice), no new toxicities were revealed which would preclude human clinical trial. Given the advantages inherent in increasing drug exposure time for a phase specific agent and the demonstrated enhanced activity in murine test systems, liposomal vincristine appears to be an excellent candidate for phase I clinical trial in man. A phase I trial of liposomal vincristine is currently underway at the British Columbia Cancer Agency (Vancouver, BC, Canada).

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