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ORIGINAL ARTICLE

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The effect of vincristine–polyanion complexes in STEALTH liposomes on pharmacokinetics, toxicity and anti tumor activity

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Abstract Poly(ethylene glycol) (PEG)-derivatized liposome vehicles improve antitumor effectiveness of entrapped anthracyclines and vinca alkaloids. However, the plasma clearance of entrapped vincristine is substantially faster than the lipid phase or other entrapped aqueous markers, suggesting leakage out of the liposome during transit in the blood compartment. We tested the effect of altering the drug's in vivo leakage rate on pharmacokinetics, toxicity, and antitumor activity of entrapped drug in rodent models. Suramin, heparin, and dextran sulfate were tested for their ability to produce a precipitable complex in vitro. PEG-derivatized liposomes were prepared with the complexing agent inside, and vincristine was driven inside using an ammonium gradient. The resulting preparations were found to have plasma distribution half-lives significantly longer than the formulation without a complex-forming agent. There was no increase in acute lethality, and in the case of the suramin-vincristine complex, the acute lethality was significantly reduced at the highest does level. Anti-tumor activity against the mouse mammary carcinoma MC2 was tested in a multiple-dose study. Free vincristine did not affect the tumor growth rate significantly, but at the same dose level all PEG-coated liposome formulations inhibited tumor growth markedly. The suramin containing formulation was as effective as the formulation lacking polyanion, but the heparin and dextran sulfate containing formulations were less effective. Thus, compounds which form insoluble complexes with vincristine alter in vivo plasma distribution phase pharmacokinetics without increasing acute lethality, but without a corresponding increase in anti-tumor activity.

Key words Dextran sulfate · Heparin · Liposomes · Suramin · Vincristine

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Introduction

Numerous preclinical studies [8, 10, 12] and clinical trials [6, 16] have established that PEG-derivatized STEALTH* liposomes containing entrapped doxorubicin improve therapeutic efficacy markedly. The improvement in antitumor activity is thought to be due to increased tumor tissue drug levels achieved after treatment with long-circulating liposomes [4, 12, 17]. In rodents, PEG-derivatized liposomes have demonstrated distribution phase half-lives in excess of 15 h using lipid phase markers like ^{3}H-cholesteryl hexadecyl ether [13] and entrapped aqueous phase markers like ^{67}ga desferroxamine [12], ^{125}I-tyraminylinulin [1], epirubicin [10] and doxorubicin [10, 20]. In the doxorubicin studies cited above, the loading method caused doxorubicin to accumulate above its solubility limit inside the liposomes and so form an entrapped colloidal gel [9]. Although a very similar method was used to load vincristine into the same STEALTH lipid matrix, the distribution half-life of this drug was considerably shorter (10 h) [2]. Nevertheless, vincristine entrapped in PEG-derivatized liposomes improves antitumor therapeutic efficacy significantly [2, 17, 19]. Because vincristine is substantially more water soluble than anthracyclines at physiological pH values, we wondered if entrapped vincristine that leaked out of the liposomes during the distribution phase had reduced its therapeutic potential. In this study we report on the use of polyanionic compounds to form an entrapped

complex with vincristine inside STEALTH liposomes, and the resulting changes in the pharmacokinetics, acute toxicity, and antitumor activity.

Materials and methods

Materials

Unless otherwise specified, reagents were ACS grade purity or better and obtained from J.T. Baker (Philipsburg, N.J.). Sodium suramin, USP, was bought from CBChemicals, (Woodbury, Ct.). Heparin sulfate and dextran sulfate were purchased from Sigma Chemical Co (St. Louis, Mo.). The methoxypoly(ethylene glycol) carbamate of distearoyl phosphatidylethanolamine (PEG-PE) was from Genzyme/Sygena (Cambridge, Mass.), hydrogenated soy phosphatidylcholine from Natterman (Koln, Germany) and cholesterol, NF from Croda (Fullerton Calif.).

Liposome preparation

Liposomes were prepared as described previously [2]. The liposome matrix, hydrogenated soy phosphatidylcholine, cholesterol, and PEG-PE at a lipid weight ratio of 3:1:1 respectively, was dissolved in ethanol and injected into a 60 °C solution of 125 m*M* ammonium citrate with suramin, heparin, dextran sulfate, or no precipitating agent as described below. The hydrated multilamellar vesicles were extruded ten times through double-stacked 0.1 μm pore size polycarbonate membranes (Costar/Nuclepore, Cambridge, Mass.), and then ten times through a single 0.05 μm pore size polycarbonate membrane (same source) in a high-pressure extrusion apparatus (Lipex, Vancouver, B.C., Canada). Mean particle size was determined by dynamic light scattering (Coulter N4, Hialeah, Fl.). External ammonium citrate was removed by dialysis, and the liposomes were loaded by adding drug to the liposome preparation, incubating in a 60 °C water bath for 10 min, and transferring to an ice waterbath to stop the loading. [G-^{3}H]vincristine sulfate (Amersham, Arlington Heights, Ill.) was entrapped in liposomes, as described above, at a specific activity of 20 mCi/mg for pharmacokinetic studies. Unentrapped drug was removed by dialysis into 10% w/v sucrose, 3.1 mg/ml sodium citrate and dihydrate, 0.9 mg/ml citric acid monohydrate at pH 5.5

Analytical methods

Vincristine concentration in test-tube precipitants was determined by UV spectrophotometry at 297 nm. The stability-indicating HPLC method in the US Pharmacopeia [15] was used with minor modification to determine vincristine concentration in suramin test-tube precipitant experiments and all liposome formulations.

Pharmacokinetics

Pharmacokinetic studies in rats were performed with male and female adult Sprague Dawley rats (250–400 g) as previously described [2]. A 300–400-μl sample (0.25 mg/kg vincristine) was administered by tail vein injection. Whole blood samples of 400 μl were collected from the retroorbital sinus of anesthetized rats at selected time-points. The blood samples were distributed into 100-μl aliquots and bleached by adding 0.2 ml of 30% hydrogen peroxide and incubated at 60 °C for 1 h before addition of scintillation cocktail (Beckman ReadyGel) Disintegrations per minutes (DPM) in each sampler were determined using a Packard TriCarb CA-2000 liquid scintillation counter.

Singe dose toxicity in mice

Acute toxicity was determined in non-tumor-bearing male ICR mice (10–15 g) by intravenous tail vein Injection. The experiment was terminated at 14 days, as previous experiments had established no delayed deaths after this time [2].

Mouse tumor model

Efficacy studies of STEALTH liposomal vincristine (S-VCR) in the C3H/He mouse mammary carcinoma MC2 model are described in detail elsewhere [17]. Tumor tissue was removed from anesthetized (Penthrane, Abbott, N. Chicago, Ill.) female C3H/He strain mice and cut into 1-mm^3 pieces. Single tumor pieces were implanted subcutaneously in the left and right flank of anesthetized mice on day 0. Ten tumors were implanted in five mice per treatment group. Freshly diluted drug formulations were administered by tail vein injection on days 3, 10 and 17. Mice were killed by carbon dioxide asphyxiation when at least one tumor had grown beyond possible regression but prior to signs of discomfort.

Statistical methods

Pharmacokinetic data were analyzed using a non-linear least-squares fitting program (Rstrip, Micromath, Salt Lake City, Italy). Survival data was evaluated by Chi-squared analysis. Statistical significance between treatment groups was determined by one-way ANOVA followed by the Student-Newman-Keuls multiple comparison procedure [5].

Results

The polyanionic, sulfated polysaccharide heparin has been reported to be compatible (no precipitation) [3] or incompatible [7] with vinca alkaloid depending on the ratio of drug to polyanion. Vincristine is an alkaloid with two positively charged amine groups of pK_A 5.0 and 7.4, and these reports suggest that other sulfated polyanions might cause precipitation at an optimal ratio. We also obtained dextran sulfate, another anionic sulfated polysaccharide, and suramin, a hexyl-sulfonated napthylurea reported to have antiproliferative activity by itself [14].

We confirmed that sulfonated oligosaccharides could form an insoluble complex with vincristine by titrating dextran sulfate with various drug concentrations (Fig. 1). Up to 90% of the vincristine could be rendered precipitable with low speed centrifugation. Additional experiments were done by titrating a fixed drug concentration with suramin or heparin (Fig. 2). Excess drug or excess polyanion resulted in a less than complete formation of insoluble complex Suramin and heparin precipitated peak amounts of vincristine at a drug/polyanion weight ratio of about 2.5:1 (w/w), which is a molar ratio of 3.8:1 for vincristine/suramin.

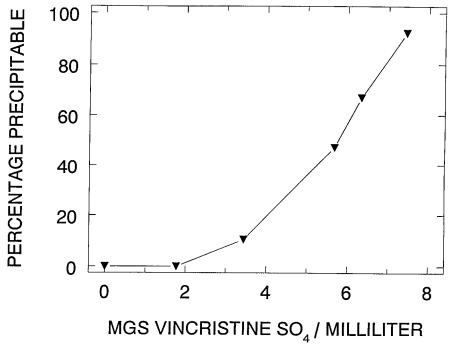

Fig. 1 Dextran sulfate precipitation of Vincristine. Dextran sulfate (5 mg/ml) was aliquoted into test tubes and concentrated stock solution of vincristine was added to give the final concentrations shown. Turbid solutions formed immediately, and after incubation at room temperature the flocculates were spun down at 500 g. Vincristine concentration in pellet and supernatant was assayed to determine the percent precipitated

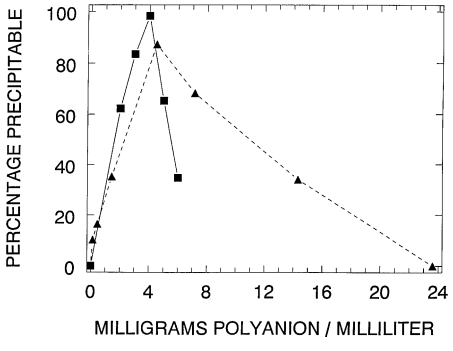

Fig. 2 Vincristine precipitation by suramin or heparin. Vincristine sulfate (10 mg/ml) was aliquoted into test tubes and concentrated stock solutions of suramin (■) or heparin (▲) were added to give the final concentrations shown. Work-up of the samples was as in Fig. 1 (140 IU/mg heparin)

Table 1 Characteristics of liposomal vincristine formulations used in animal studies. The weight ratio of drug to polyanion was estimated by dividing the encapsulated drug concentration by the polyanion concentration in the hydration buffer, and by the capture volume of STEALTH liposomes in this size range [8, 11]

	S-VCR	S-VCR-DS	S-VCR-H	S-VCR-S
Polyanion	None	Dextran sulfate	Heparin	Suramin
Vincristine (mg/ml)	0.9	1.0	1.0	1.0
Estimated drug/polyanion (w/w)	–	8.7/1	1.8/1	2.8/1
Phospholipid (m*M*)	14.8	13.2	12.3	17.1
Drug entrapped (%)	98	97	96	90
Mean vesicle diameter, (nm)	84	92	100	79

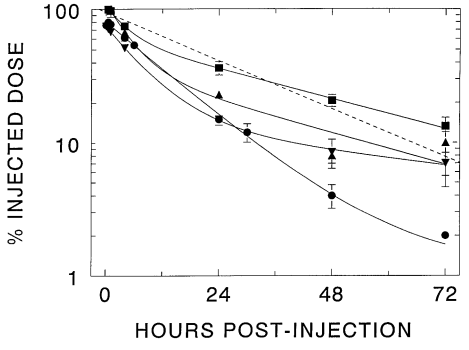

Fig. 3 Plasma pharmacokinetics of STEALTH [G-^{3}H] vincristine formulations. Rats were given a single dose injection of formulations containing suramin (■), heparin (▲) or dextran sulfate (▼), or without insoluble complex-forming agent (●) at 0.25 mg vincristine/kg body weight. Solid lines are the non-linear least-squares fit to the data. The dashed line is the theoretical time course with a 20-h half-life. Data points are the group mean and standard deviation using three animals per treatment group

It was not possible to calculate the stoichiometric ratio for dextran sulfate and heparin because the oligosaccharides have a distribution of different molecular weights and degrees of sulfonation.

Liposomes were prepared as described above, resulting in formulations with the characteristics presented in Table 1. The concentration of dextran sulfate encapsulated within the liposomes was limited to 5 mg/ml. Doubling the concentration in the hydration buffer caused extreme foaming and an inability to extrude the liposomes down to less than 150 nm mean diameter. Thus, the estimated ratio of drug to polyanion was roughly fourfold higher than for the heparin and suramin preparations. If the complexation phenomena in Fig. 2 held within the liposome-entrapped compartment, approximately 80% and 90% of the drug was complexed to heparin and suramin, respectively.

We hypothesized that the relatively fast distribution phase of S-VCR was not due to clearance of the intact liposome particle, but due to drug leakage out of the liposome into the plasma. Leaked drug would be cleared from plasma within minutes [2]. If drug leakage was the rate-limiting step, a more stable, insoluble complex might be expected to increase the plasma distribution phase half-life. Figure 3 indicates that the presence of the polyanions increased the plasma residence time of vincristine. Significantly increased vincristine plasma concentrations were seen 24 h postinjection for the heparin and suramin formulations. By 48 h, the drug plasma concentration of the dextran sulfate formulation was significantly greater. The vincristine-suramin formulation, had a distribution phase comparable to the half-lives observed for PEG liposome-entrapped gallium [12], tyraminylinulin [1], and anthracyclines [10, 20].

The lethality of the different formulations in mice was compared after a single dose (Table 2). The 3 mg/kg dose level of the suramin-vincristine formulation was the only formulation that was statistically and substantially less toxic than the free drug or other liposomal formulations.

Table 2 Single dose lethality study of liposomal vincristine formulations. The fraction is the proportion of animals per group dying within 14 days after a single injection of a specified formulation at a given dose level. Percentage group mortality is in parentheses (*ND* not determined)

	1.5 mg/kg	2.0 mg/kg	2.5 mg/kg	3.0 mg/kg
S-VCR-S	0/10	1/10 (10%)	0/10	0/10*
S-VCR	ND	0/10	1/10 (10%)	6/10 (60%)
Free VCR	0/7	0/7	ND	5/7 (71%)
S-VCR-H	0/10	0/10	2/10 (20%)	8/10 (80%)
S-VCR-DS	ND	0/10	1/10 (10%)	9/10 (90%)

*$P < 0.005$ vs free VCR , Chi squared analysis

The effectiveness of the various formulations was compared to saline- and free drug-treated animals. A previous study had established that S-VCR at 2.0 mg/kg almost completely prevents tumor growth [17]. Therefore, we dosed the animals at suboptimal levels to determine if any therapeutic gain would be provided by inclusion of suramin (S-VCR-S), heparin (S-VCR-H) or dextran sulfate (S-VCR-DS) in the STEALTH formulation. Figure 4A shows that at 1.0 mg/kg unencapsulated drug had no effect on the tumor growth rate. All of the PEG-coated liposome formulations significantly inhibited the tumor growth rate. Analysis using the Student-Newman-Keuls multiple comparison *t*-test procedure indicated that the mean tumor volumes at day 51 were all significantly different from saline-treated controls ($P < 0.05$). The most effective formulations were S-VCR and S-VCR-S which were not significantly different from each other, and inhibited tumor growth almost completely for 1 month posttreatment. S-VCR-DS and S-VCR-H were less effective. At the 1.3 mg/kg dose level (Fig. 4B) all the formulations were significantly different from saline-treated control. The responses of groups treated with S-VCR-DS and S-VCR-H 1.3 mg/kg were significantly improved over the 1.0 mg/kg dose level of the same formulation. Multiple comparison analysis indicated there was no significant difference in tumor volume between the S-VCR-, S-VCR-S-, and S-VCR-H-treated groups.

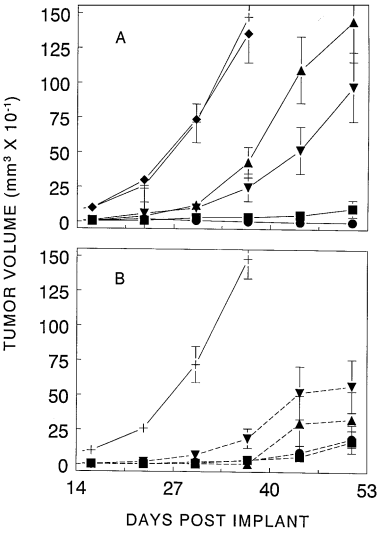

Fig. 4A, B Antitumor activity against mouse mammary carcinoma. Mice were implanted with a 1-mm^3 piece of MC2 carcinoma in each flank on day 0. On days 3, 10, and 17 the mice were given tail vein injections of saline (+) or vincristine at (**A**) 1.0 mg/kg, or (**B**) 1.3 mg/kg formulated as free drug (◆), in STEALTH liposomes without complex-forming agent (●), or with suramin (■), heparin (▲) or dextran sulfate (▼). Data points are the mean and standard deviation of tumor volume

Discussion

In this study we tested the hypothesis that including a complex-forming agent with vincristine in PEG-derivatized liposomes would increase the plasma distribution phase and increase antitumor activity by reducing the amount of drug lost during transit in the blood.

Suramin, heparin, and dextran sulfate were all able to form insoluble complexes with vincristine in the test-tube. By encapsulation of these polyanions in STEALTH liposomes, it was possible to increase the plasma distribution phase half-life considerably. This is consistent with a reduced amount of drug leaking out of the liposomes and being rapidly cleared from the plasma compartment. S-VCR-S alone had a changed toxicity, being quite significantly less toxic than S-VCR and free drug. This may be related to the prolonged plasma half-life because of reduced drug leakage and distribution to systemic target tissues.

Antitumor activity was tested at suboptimal dose levels in a murine carcinoma model to see if there was enhancement of the therapeutic effect. Although all PEG-derivatized liposome formulations were substantially better at retarding tumor growth than free drug, the formulations containing dextran sulfate or heparin were less effective than liposomes without a complex-forming agent. We speculate that the reduced effect may be due to a portion of the dose not being available at the tumor site, perhaps by incomplete disruption of the drug–polyanion complex. Alternatively, the vincristine–polyanion complex itself may have an inherently reduced cytotoxicity. Chemical inactivation is also possible, although analysis of the formulations by our stability-indicating HPLC method showed no significant degradation of vincristine. In the case of S-VCR-S being similar to S-VCR in antitumor activity, we cannot exclude the possibility that tumor growth was also inhibited by suramin's antiproliferative activity [14]. In conclusion, it seems that the plasma half-life of cationic drugs in long-circulating liposomes can be increased by including a polyanion capable of forming an insoluble complex. Our studies show that although plasma residence time is significantly increased, there is no corresponding increase in antitumor activity. While

complexation may yet prove to be useful for cytotoxic agents with in vivo liposome leakage rates much faster than 10 h, a minimum 10-h plasma distribution phase is sufficient to produce the full therapeutic benefit of vincristine.

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