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Modulation of the peritoneal clearance of liposomal cytosine arabinoside by blank liposomes*

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Summary. Liposomes containing cytosine arabinoside (ara-C) release drug slowly and can be used to maintain a locally high concentration of ara-C in the peritoneal cavity for intracavitary chemotherapy. However, a significant amount of active drug does reach the systemic circulation and contributes to systemic toxicity. We have devised a novel method of decreasing toxicity and increasing intraperitoneal half-life by pretreatment with "blank" liposomes containing no active drug. This technique has resulted in prolongation of intraperitoneal half-life of the liposomal ara-C from 21 h to 165 h, enabling maintenance of a therapeutic drug concentration even at 11 days after initial injection. One hundred percent cures (60-day survival) were achieved with a single-dose therapy begun 1 day after i. p. implantation of 10^6 L1210 leukemia cells.

Introduction

Many investigators in the past have attempted to target cytotoxic drugs to the tumor to reduce systemic toxicity and increase tumor kill [6, 7, 12, 21]. One approach is the instillation of drugs directly into a tumor-containing cavity [1]. This is particularly attractive for those cancers that grow and spread within the confines of a cavity, such as ovarian carcinoma [1, 13] or mesothelioma [14].

We chose to study ara-C in the peritoneal cavity because human studies have shown that the peritoneal : plasma concentration ratio is almost 1000 : 1 [9]. To achieve optimal cancer cell kill with a cell cycle phase-specific drug like ara-C, the tumor must be exposed for a prolonged period of time so that all or most of the cancer cells have attempted to synthesize DNA in the presence of the drug [20]. However, prolonged drug exposure is problematic because of the rapid clearance of ara-C from the peritoneal cavity; the half life is only 60–210 min [9]. Therefore, some sort of slow-release form of the drug is needed.

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Previous investigators have shown that encapsulation of ara-C into liposomes results in markedly increased efficacy after a single intraperitoneal injection [2, 3, 5, 10, 17, 19]. However, the systemic toxicity is then also quite prominent. In an attempt to decrease the rate of intraperitoneal clearance of the drug-containing liposomes and thereby perhaps increase the therapeutic index, we have explored the use of "blank" liposomes containing benign substance and no active drug. We employed "multivesicular" liposomes [8] in this study because of the high capture efficiency, ease of scale-up, and stability in storage.

Materials and methods

Materials. Ara-C (Cytosar-U) was a gift from the Upjohn Co., Kalamazoo, Mich; dioleoyl lecithin, dipalmitoyl phosphatidylglycerol, and cholesterol were purchased from Avanti Polar-Lipids, Inc. Birmingham, Ala; triolein and L-lysine, free base, were procured from Sigma, St. Louis, Mo; and nanograde chloroform was from Mallinckrodt, Paris, KY. All were used without further purification. [^{3}H]Ara-C was purchased from Amersham, Arlington Heights, Ill and its purity was checked by high-performance liquid chromatography (HPLC). BDF_1 mice were purchased from Simonsen Laboratories, Gilroy, Calif. The vortex mixer was from American Scientific Products, catalogue # S8223-1, McGaw Pak, Ill.

Synthesis of multivesicular liposomes. The multivesicular liposomes were prepared according to our previously published method [8] with some modifications. For each batch of liposomes prepared, 1 ml of 20 mg/ml ara-C solution in water (with pH adjusted to 1.1 with 1 *N* hydrochloric acid) was added into a 1-dram vial containing 9.3 μmol dioleoyl lecithin, 2.1 μmol dipalmitoyl phosphatidylglycerol, 15 μmol cholesterol, 1.8 μmol triolein, and 1 ml of chloroform. The vial was attached to the head of the vortex mixer and shaken at the maximum speed for 6 min. Each half of the resulting "water-in-oil" emulsion was individually squirted rapidly through a narrow-tip Pasteur pipette into one of two 1-dram vials each containing 2.5 ml water, glucose (3.2 g/100 ml), and free-base lysine (40 m*M*); and then shaken on the vortex mixer for 3 s at "5" setting to form chloroform spherules. The chloroform spherule suspensions in the two vials were transferred into the bottom of a 250-ml Erlenmeyer flask containing 5 ml water, glucose (3.2 g/100 ml), and free-base lysine (40 m*M*). A

stream of nitrogen gas at 7 l/min was flushed through the flask to evaporate chloroform over 10–15 min at 37 °C. The liposomes were then isolated by centrifugation at 600 *g* for 5 min and washed thrice with 0.9% NaCl solution. The resulting liposomes have an average volume-adjusted size (±SD) of 19.4±6.5 μm, percentage of capture of 59%±7% ($n=12$), and capture volume of 36±4 μl/mg of total lipids used. Storage of liposomes at 4 °C resulted in less than 10% leakage at 3 months.

For the blank liposomes, exactly the same procedure was followed except for the use of 4% glucose solution in place of the ara-C solution. The blank liposomes were quantitated after the washings by assaying the glucose content, using the glucose oxidase method [18]. The blank liposomes were diluted to concentration of 11.2 mg/ml glucose content before use.

In vivo studies. Intraperitoneal pharmacokinetic studies were done on male BDF_1 mice weighing 20–25 g. A group of mice was injected intraperitoneally first with 1 ml glucose-containing blank liposomes and then 1 h later with 1 mg encapsulated drug in 1 ml 0.9% NaCl solution. For comparison, a second group of mice was injected with unencapsulated drug alone and a third group with encapsulated drug alone, without the blank liposomes. At appropriate time points, animals were sacrificed by cervical dislocation and peritoneal fluids were first collected into capillary tubes of defined volumes. Then the peritoneal cavities were washed out thoroughly thrice with 1–2 ml of 0.9% NaCl solution. "Free" ara-C concentrations were measured after removal of liposomes and cells from the peritoneal fluids by centrifugation. Next, 40 μ*M* tetrahydrouridine was added to prevent degradation of ara-C, and the specimens were kept frozen at −20 °C until assayed. Fluids containing liposomes were diluted at least 10-fold with distilled water and sonicated for 10 s with a probe sonicator ("50" setting, low power, on Biosonik IV, VWR Scientific, San Francisco, CA) to disrupt liposomal membranes before analysis. Ara-C was quantitated on a Waters and Associates (Milford, MA) HPLC with 280-nm detector as previously described [9], on a C18 reverse-phase Radial-Pak cartridge column, with 5 m*M* potassium phosphate buffer at a pH of 7.0 as mobile phase, running at a rate of 2.0 ml/min. Retention time for ara-C was 5 min and that for the major metabolite, ara-U, was 7 min.

For toxicity and efficacy studies, BDF_1 mice were inoculated with one million L1210 cells i. p. on day 0, given 1 ml blank liposomes i. p. on day 1, and treated i. p. with liposomes containing ara-C suspended in 0.9% saline 1 h after the blank liposome injection. The amount of ara-C in liposomes was measured on a spectrophotometer (UVIKON 810, Kontron International, Zurich) at 280 nm in 100% methanol containing 10 m*M* sodium acetate. The absorbance was linear with respect to concentration in the range measured.

Results

Intraperitoneal pharmacokinetics

Following i. p. injection of unencapsulated ara-C, the amount of the drug in the peritoneal cavity decreased exponentially with a half-life of 16 min (Fig. 1a). Following i. p. injection of liposomal ara-C alone, the clearance of the drug from the peritoneal cavity was markedly reduced and the half-life was about 21 h (Fig. 1a).

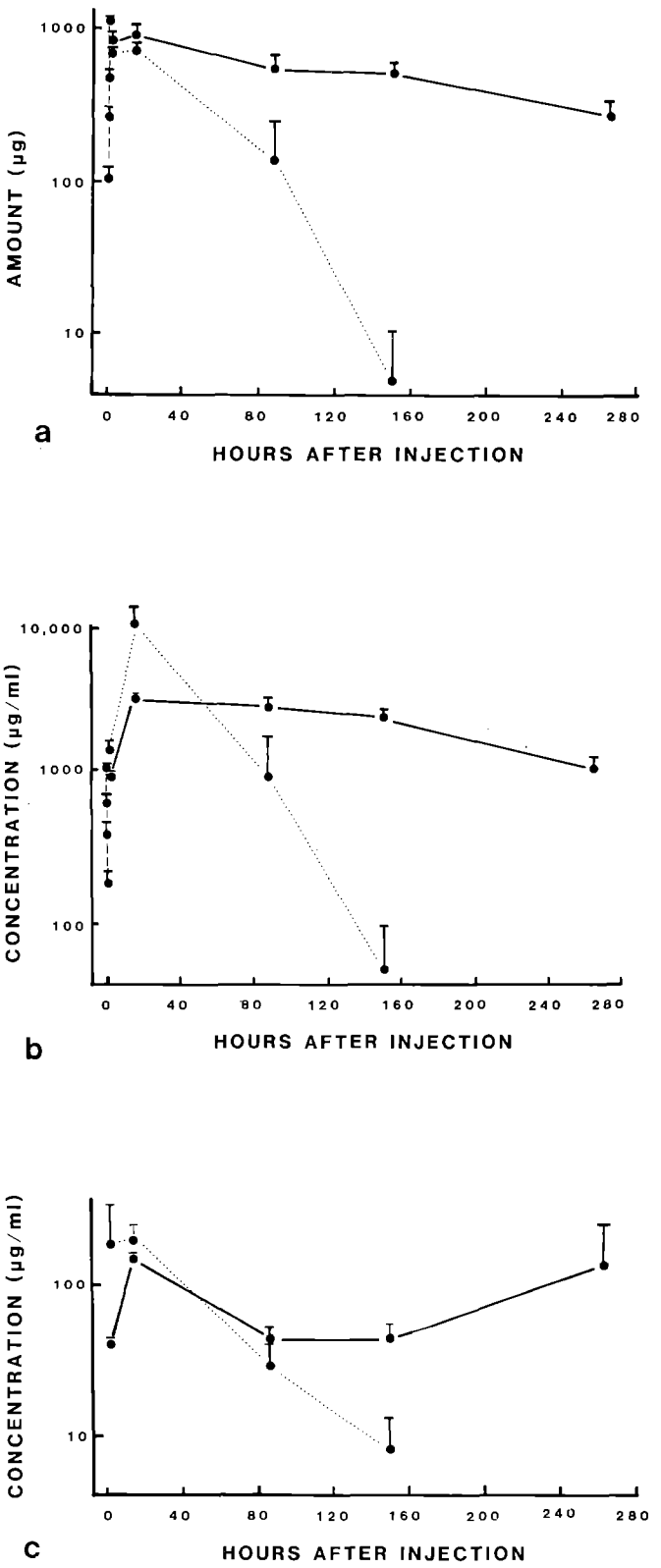

Fig. 1a–c. Intracavitary pharmacokinetics of ara-C after i. p. injection of 1 mg unencapsulated and encapsulated ara-C, with and without blank liposomes. *Dashed line,* unencapsulated ara-C; *dotted line,* encapsulated ara-C without blank liposomes; *solid line,* encapsulated ara-C with blank liposomes. *Points* show mean values and *bars,* the standard deviation, obtained from three mice. **a** Total amount (μg) of drug in peritoneal cavity; **b** total concentration (μg/ml) of drug; **c** free drug concentration (μg/ml) in peritoneal cavity

Table 1. Survival of mice after i.p. innoculation with 10^6 L1210 cells

Liposomal ara-C dosage (mg/kg)	Dummy liposomes[a]	Increased life span (%)		Survival	
				30-day	60-day
0	–	0[b]	0[c]	0/5	0/5
63	–	361	520	4/5	2/5
126	–	314	491	4/5	2/5
178	–	0	0	0/5	0/5
0	+	0	0	0/5	0/5
63	+	457	517	4/5	1/5
126	+	135	509	3/5	3/5
178	+	–	757	5/5	5/5
241	+	–	757	5/5	5/5
305	+	107	497	3/5	3/5
330	+	28	466	3/5	3/5

[a] +, pretreatment with blank liposomes; –, no pretreatment respectively
[b] After exclusion of 60-day survivors
[c] With inclusion of 60-day survivors

The peritoneal clearance of liposomal ara-C was further improved by pretreatment with blank liposomes; the half-life for the total amount (μg) of ara-C in the peritoneal cavity was 165 h (Fig. 1 a). The total concentration of ara-C (free plus entrapped, in μg/ml) in the peritoneal cavity was found to increase threefold over the first 15 h after i. p. injection (Fig. 1 b). During this period of time, the amount of fluid present in the cavity was observed to decrease, with simultaneous increase in turbidity and viscosity of the fluid. At 264 h after the i. p. injection, the total ara-C concentration returned to that found immediately after i. p. instillation. The free ara-C concentration (Fig. 1 c) obtained after removal of liposomes by centrifugation was about a fortieth of the total drug concentration and remained above the minimal cytotoxic level of 0.1 μg/ml [4] up to 264 h.

Efficacy studies

Table 1 shows the survival data for mice treated with varying doses of ara-C encapsulated in multivesicular liposomes. Among those mice treated with a single dose of liposome-encapsulated ara-C alone, 4/5 animals survived 30 days at dosages of 63 and 126 mg/kg, but 60-day survival was 2/5 in each dosage group. At dosage of 178 mg/kg, all mice died from toxicity. Treatment with blank liposomes alone gave no increase in survival.

For those mice treated with blank liposomes and then with active liposomal ara-C, the toxicity was reduced and it was possible to cure 100% of mice at dosages of 178 or 241 mg/kg. At lower dosage levels, there was no significant difference from the group without blank liposomes.

Discussion

Extensive pharmacokinetic studies in various organs have been done in the past with i. p. liposomal ara-C [2, 5], but to our knowledge, no pharmacokinetic study of the drug within the peritoneal cavity itself has been reported. Such information is essential for designing intracavitary chemotherapy. We have shown that liposomal entrapment prolongs the half-life of ara-C in the peritoneal cavity 79-fold (Fig. 1). This was associated with an increase in both efficacy and toxicity compared to unencapsulated drug given on a single-dose schedule. The improved efficacy is probably due to prolonged maintenance of active drug level in both the peritoneal cavity and the systemic circulation, while the increased systemic toxicity results from the latter effect alone. The use of blank liposomes, in conjunction with liposomal ara-C, results in 619-fold prolongation of the drug's intraperitoneal half-life compared to the administration of unencapsulated drug. At the same time, the addition of blank liposomes reduced the systemic toxicity of the liposomal ara-C. The end result is that a high free drug concentration can be maintained in the peritoneal cavity for weeks with reduced systemic toxicity.

How the blank liposomes improve the intraperitoneal pharmacokinetics and reduce the systemic toxicity is not yet fully understood. We speculate that this is due to the blank liposomes' partial blockade of the peritoneal lymphatic clearance of intact drug-containing liposomes, since most liposomes are cleared by that route [16]. This would increase the proportion of ara-C clearance via the portal circulation and hepatic metabolism [1], resulting in decreased systemic ara-C exposure.

It was of interest to find that the total concentration of ara-C in the peritoneal cavity actually rose during the first 15 h, and stayed above the original concentration for a period of 264 h. We attribute this initial rise in concentration to a differential clearance of saline vs the liposomes; the free fluid suspending liposomes was rapidly absorbed, whereas the liposomes themselves were not. Since the amount of ara-C in the liposomes stays about the same, the total amount/volume (i. e., concentration) of the drug increases with time. This may be of potential benefit since it maximizes the concentration of both liposomes and free ara-C in the immediate environment of the intraperitoneal tumor cells.

The decrease in systemic toxicity with blank liposomes enabled us to escalate the dose to the point where 100% of animals could be cured with a single-dose treatment without incurring lethal toxicity. The maximum tolerated dose and the efficacy are significantly better than those found by previous investigators using liposomal ara-C alone [2, 3, 5, 10, 19]. In addition, in previous studies [15, 19], long-term survival was measured at 30 days rather than at 60 days, and some of the "long-term survivors" could have died between 30 and 60 days, as indicated by our data (Table 1) and that of other investigators [11]. We believe this is the first report of 100% cure with a single-dose treatment of mice inoculated with 10^6 L1210 cells.

Since the clearance of liposomes from other extravascular cavities (e. g., subarachnoid and pleural spaces) probably occurs by mechanisms similar to those in the peritoneal cavity, the principle of modulating the liposomal drug with blank liposomes may also apply to those cavities.

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