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Combination chemotherapy of human ovarian xenografts with intraperitoneal liposome-incorporated valinomycin and *cis*-diamminedichloroplatinum(II)

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Abstract. Intraperitoneal administration of liposomal valinomycin (MLV-VM) with *cis*-diamminedichloroplatinum(II) (cDDP) had significant antitumor activity against murine P388 leukemia and inhibited the growth of OVCAR-3 tumors in a nude mouse model of human ovarian cancer. This tumor is a teratoma originating in the ovary with pathogenesis and metastatic properties similar to those of human ovarian cancer. Drug was given to the mice once every 5 days for 4 doses beginning 1 day after i.p. implantation of 10^7 or 5×10^7 OVCAR-3 tumor cells. For P388 leukemia, drug was given i.p. once or on days 1 and 5 after tumor inoculation. Despite the use of low doses of MLV-VM, the antitumor activity of the combination [increase in life span (%T/C), 289%–294%] represents a 4-log cell kill over the additive effect of the two drugs, indicating a synergistic interaction between MLV-VM and cDDP. Likewise, low doses of the drug combination produced a synergistic interaction on human ovarian OVCAR-3 tumors, and tumor-free, long-term survivors were obtained. Combined therapy of liposome-incorporated valinomycin and cisplatin was well tolerated and produced no overlapping nephrotoxicity, although a decrease in liver enzyme markers (alkaline phosphatase and/or alkaline aminotransferase) with MLV-VM was observed. These results appear to suggest that MLV-VM with cDDP may have considerable potential for the treatment of ovarian cancer disseminated within the peritoneal cavity, although the frequency and sequence of drug administration may need to be improved.

Abbreviations: cDDP, cisplatin, *cis*-diamminedichloroplatinum(II); VM, valinomycin; MLV-VM, liposomal valinomycin; PKC, protein kinase C; AP, alkaline phosphatase; ALT, alkaline liver transaminase; BUN, blood urea nitrogen

Introduction

Ovarian cancer is one of the most frequent fatal gynecological malignancies occurring in women in highly industrialized countries [38]. It is often a regional disease, with growth being confined to the peritoneal cavity even in advanced stages [41]. In most patients who display only a partial response to chemotherapy, the disease will recur within the abdomen; this can lead to bowel obstruction and death by inanition, infection, and hemorrhage [29]. Since the metastatic spread of ovarian carcinoma is often limited to the peritoneal cavity, i.p. administration of certain chemotherapeutic agents can result in a significantly greater exposure of the tumor to the active drug [12]. Recent studies have demonstrated that delivery of various anticancer agents via the peritoneal route is a well-tolerated and feasible approach [22–25; 30–33]. For example, cDDP is one of the most effective drugs available for i.p. treatment of ovarian carcinoma [28]. Trials of single-agent cDDP or cDDP-based combination i.p. therapy in patients with residual small-volume ovarian cancer who have failed to respond to i.v. cDDP treatment have demonstrated beneficial clinical results, with 30% of patients achieving complete remissions after single-agent cDDP treatment [39] and 65% being clinically free of disease after cDDP-based combination therapy [21]. However, the failure to achieve a complete remission rate of 100% could be due to the rapid absorption of these low-molecular weight compounds from the peritoneal cavity and their systemic distribution into the blood. Consequently, some of cDDP's systemic toxicities such as nephrotoxicity remain clinically intact [18]. Thus, strategies designed to retain these drugs in the peritoneal cavity could be of value for the treatment of ovarian cancer.

One of these strategies is the i.p. delivery of anticancer drugs in liposomal form, either alone or in combination with platinum-based drugs. As microparticulates, liposomes are likely to be retained in the peritoneum for longer periods than would most free drugs [6, 34]. The retention of drug incorporated into liposomes in the peritoneum would further permit the sustained exposure of tumor cells

to high concentrations of anticancer drugs, with lower systemic toxicity. Thus, cytotoxic modulation of i.p. delivered cDDP with liposomal drugs may be beneficial in local or regional chemotherapy of ovarian cancer.

We have previously shown that the toxicity of valinomycin (VM), a membrane-active agent with antineoplastic activity, can be dramatically reduced and its antitumor activity can be maintained, if not enhanced, by its incorporation into liposomes [7]. Further, we have recently shown that VM as well as liposomal valinomycin (MLV-VM) exhibit synergistic action with platinum drugs (cisplatin, carboplatin, and ormaplatin) on human ovarian cancer in vitro [5, 10]. Thus, the ability of MLV-VM to potentiate the cytotoxicity of cisplatin can conceivably be an asset in regional drug delivery and chemotherapy of tumors confined to the peritoneal cavity as in the case of ovarian cancer.

In the present report, we describe the results of treating i.p. human OVCAR-3 ovarian cancer xenografts in nude mice with i.p. administrations of liposome-incorporated valinomycin and cisplatin. In addition, the effect of simultaneous i.p. administrations of both drugs on P388 leukemia-bearing mice was studied. To assess the possible overlapping toxicity between MLV-VM and cisplatin, the hematological and renal plasma biochemical indices were also determined after i.p. drug administration. The results suggest that the combination chemotherapy of MLV-VM and cisplatin may have merit as a form of localized drug delivery for the treatment of ovarian cancer disseminated within the peritoneal cavity with minimal overlapping host toxicity.

Materials and methods

Materials. VM was obtained from the Natural Products Branch of the National Cancer Institute (Bethesda, Md.); cisplatin was obtained from Bristol Myers Co. (Evansville, Ind.); dimyristoylphosphatidylcholine and phosphatidylserine were purchased from Avanti Polar Lipids, Inc. (Birmingham, Ala.); and cholesterol was obtained from Sigma Chemical Co. (St. Louis, Mo.).

Animals and tumors. Human ovarian OVCAR-3 xenografts were obtained from Dr. Joseph G. Mayo, National Cancer Institute (Frederick, Md.). All procedures involving the use of animals were approved in advance by the Committee for Animal Research at Washington State University, and the committee's guidelines for humane treatment of animals were strictly followed. Animals were maintained in microisolator cages under sterile conditions and fed ad libitum. Animal handling was carried out under sterile conditions by a limited number of persons either in laminar flow hoods or within isolators. OVCAR-3 was passaged in female athymic nude (nu/nu) mice (Simonsen Laboratories, Gilroy, Calif.) as previously described [19]. At about 30- to 35-day intervals, ascites was harvested from donor animals and cells were washed once by centrifugation at 400 *g* for 5 min and then resuspended in phosphate-buffered saline at a concentration of 1×10^8 cells/ml; 0.5 ml was transferred to recipient mice by i.p. injection. Animals developed voluminous ascites tumors, and death was observed at about 40–45 days after tumor inoculation. The P388 mouse leukemia was maintained in ascites form by weekly implantation of 10^6 cells into DBA/2 mice, and the experimental inocula of leukemia cells were injected i.p. at 10^6 cells into female $B_6D_2F_1$ mice that weighed between 18–22 g at the beginning of the experiments. These mice were supplied by The Jackson Laboratory (Bar Harbor, Me.).

Liposome preparation. MLV-VM was prepared as previously described [8, 9]. The preparations used in the present study comprised dimyristoylphosphatidylcholine, cholesterol, and phosphatidylserine (molar ratio, 10:4:1) and included 10% (w/w) VM. The phospholipid concentration was determined by phosphorus assay [15], and the multilamellar vesicles (MLVs) produced were sterilized by passage through a 0.22 μM filter.

Treatment protocols and evaluation of antitumor effect. Ascitic OVCAR-3 xenografts were obtained by peritoneal aspiration from nude mice previously injected with tumor cells. The tumors were diluted with an equal volume of RPMI-1640, and 0.5 ml of the resulting suspension ($1-5 \times 10^7$ cells) was injected i.p. per mouse. The number of vital OVCAR-3 cells in the cell suspension was determined by trypan blue dye exclusion. After 24 h, i.p. injections of saline, "empty" liposome, MLV-VM, cDDP, and/or the drug combination were given to each group of mice (8–10 mice/group) every 5th day for 2 weeks as shown in Fig. 1. Antitumor effects were expressed as the percentage of survival based upon the median survival of treated versus control (untreated) groups. Tumor-bearing mice were killed when they had developed a tumor burden that would lead to death in 24 h as established from previous experiments. The same approach used for the P388 leukemia model was taken. On day 0, female $B_6D_2F_1$ mice were inoculated i.p. with 10^6 P388 leukemia cells. There were 6–8 mice in each drug treatment group and 10–12 mice in each vehicle-treated control group. On day 1, the various treatments began as indicated in Table 1. The mortality was monitored daily and the increase in life span (%T/C) was used as an index to assess the antitumor effectiveness as we have previously reported [7]. Synergy of activity was analyzed by determining the treatment log cell kill as described by Corbett and Valeriote [3] using the following equation:

$$\log_{10} \text{cell kill} = [T-C]-[\text{duration of treatment}]/3.32\ t_d,$$

where T and C represent the survival of treated and control groups, respectively, and $t_d = 12$ h, which is the doubling time of P388 leukemia in our system.

Biochemical plasma indices. The acute lethal toxicity as well as the possible overlapping toxicity of the drug combination was examined by determining serum chemistry profiles in nude mice injected with cumulative doses of cDDP, MLV-VM, and/or the drug combination as indicated in Table 2 and Fig. 2. Athymic nude mice with no tumor, ascitic tumor, or solid tumor were given i.p. injections of 12 mg/kg cDDP, 4 mg/kg MLV-VM, and/or the drug combination. Control mice were injected i.p. with saline or "free" liposomes. On days 1 and 5 after drug injection, pooled blood samples were obtained by cardiac puncture from four mice per group on each day. Blood urea nitrogen (BUN) and plasma creatinine values as indicators of renal toxicity and the activity of alanine aminotransferase (ALT) and alkaline phosphatase (AP) as indicators of hepatotoxicity were determined by the Veterinary Clinical Diagnostic Laboratory at Washington State University. Similarly levels of blood glucose as well as plasma electrolytes were also determined.

Results

In our initial experiments, we studied the effectiveness of i.p. injected low doses of cDDP and MLV-VM against P388 leukemia in mice; a representative result of these experiments is shown in Table 1. Mice ($n = 10-12$) injected i.p. with 10^6 P388 leukemic cells on day 0 were treated i.p. on day 1 with cDDP (6 mg/kg) and MLV-VM (0.3 mg/kg) once or on days 1 and 5. No antagonism was found between i.p. cDDP and i.p. MLV-VM when the two drugs were tested together against i.p. P388 leukemia in mice, as indicated by the high increase in the life span (T/C, 288% vs 294%) of treated mice versus controls. For example, the antitumor activity of the individual agents given in single doses was 175% and 129% for cDDP and MLV-VM, respectively. The dose of cDDP used in this study was half of the maximum tolerated dose (MTD), whereas that of MLV-VM was 0.1 MTD [7].

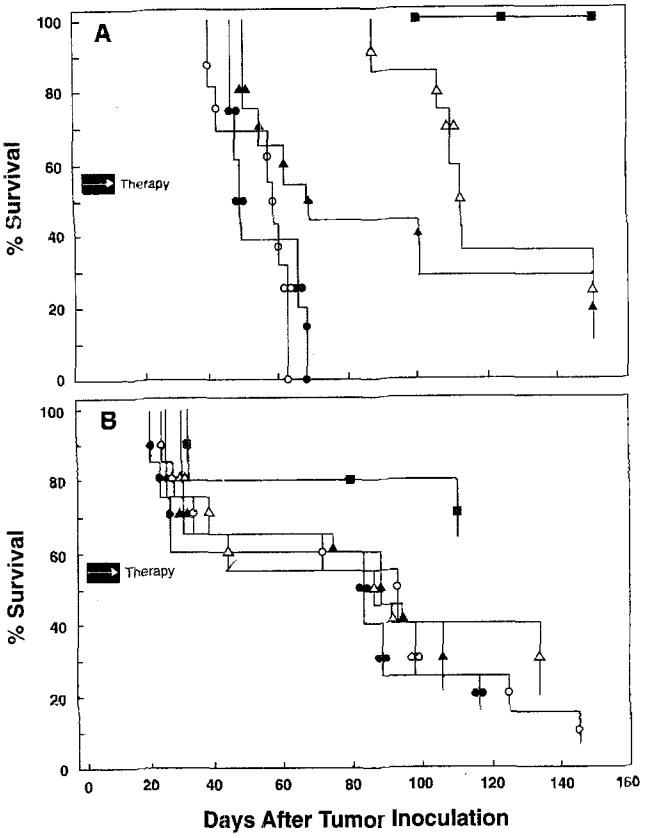

Fig. 1 A, B. Survival of mice bearing i.p. ascitic xenografts of human OVCAR-3 ovarian cancer following i.p. treatment with MLV-VM and cDDP. Nude mice ($n = 8-10$) were given injections of 5×10^7 cells (**A**) or 1×10^7 cells (**B**) i.p. On days 1, 5, and 9, 13 mice were treated with (△) 3 (**A**) or 1 mg/kg cDDP (**B**), (▲) 1 mg/kg MLV-VM, or (■) the drug combination. Control mice received saline (○) or "empty" liposomes (●). The animals were monitored, and the duration of survival was noted

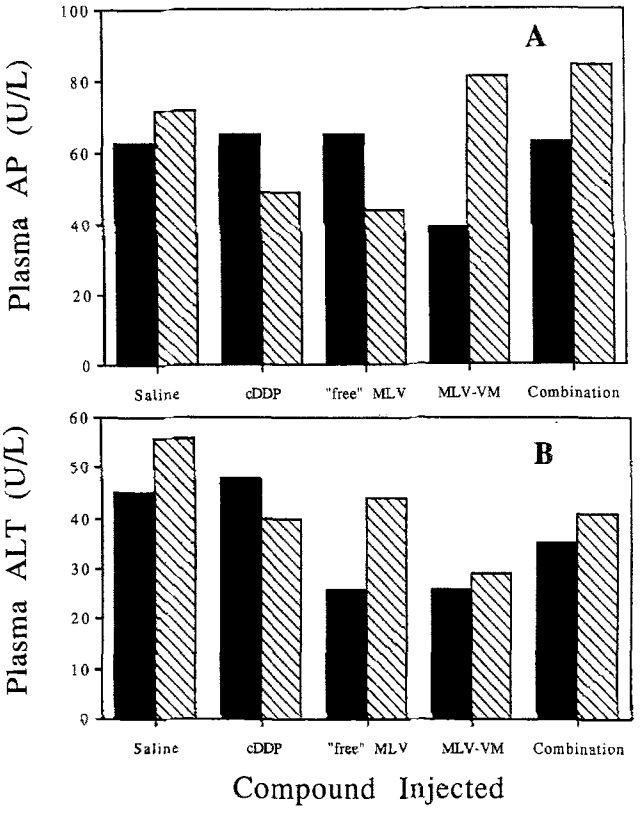

Fig. 2 A, B. Effect of cisplatin and liposomal valinomycin on liver enzyme markers. Animals were treated with i.p. injections of cDDP, MLV-VM, and/or a combination of the two. Control mice received either saline or "free" liposomes. The activity of pooled-plasma AP (**A**) and ALT (**B**) of 4 mice was then determined on day 1 (■) and day 5 (▨)

Table 1. Antitumor activity of i.p. cisplatin and liposomal valinomycin against ascitic P388 leukemias[a]

Compound	Dose (mg/kg)	Schedule (days)	MST (days)	%T/C
cDDP	6	Single	18	175
	6	1, 5	19.7	191
MLV-VM	0.3	Single	13.3	129
	0.1	1, 5	18	175
Drug combination	6+0.3	Single	29.5	289
	6+0.3	1, 5	30	294
Control	0	0	10.2	

[a] Tumor cells were inoculated i.p. at 10^6 cells/mouse on day 0. Treatment was given i.p. on day 1 or days 1 and 5

To evaluate the extent of drug synergy in the P388 model, the tumor $\log_{10}$ cell kill of the drug combination was determined relative to that of the individual drugs [3]. In this case, the effect of the drug combination (T/C, 288%) represents a 4-log cell kill over the additive effects of the individual drugs given in single doses. The same was true for the drug combination given in multiple injections. This clearly indicates that the in vivo interaction between low doses of MLV-VM and cisplatin in P388 leukemia-bearing mice was truly synergistic. Thus, the study was extended further to ascitic human ovarian OVCAR-3 cells xenografted into the peritoneal cavity of female nude mice. Mice ($n = 8-10$) were xenotransplanted i.p. with 5×10^7 cells (Fig. 1 A) or 1×10^7 cells (Fig. 1 B). On day 1, multiple i.p. treatment was started with MLV-VM (1 mg/kg), cDDP (3 mg/kg, Fig. 1 A; 1 mg/kg, Fig. 1 B), and the drug combination. Control mice were injected with saline or "empty" liposomes. Mice treated with control solutions died at a median of 50–90 days after the inoculation of either 5×10^7 or 1×10^7 OVCAR-3 cells (Fig. 1). Mice treated with MLV-VM alone survived marginally better with median durations of survival of 70–95 days, and two mice from each treated group were alive at 150 days after treatment (Fig. 1 A, B). The administration of cDDP resulted in a dose-dependent prolongation of the life of test animals.

In this experiment, treating mice with low doses of cDDP (1 mg/kg) did not significantly extend animal survival beyond that of control mice given saline injections (Fig. 1 B). However, 3 mg/kg cDDP extended the life span of mice by 60 days (Fig. 1 A). Treating mice with a combination of low doses of MLV-VM and either dose of cDDP did in fact extend the median duration of survival beyond

Table 2. Effect of cisplatin and liposomal valinomycin on plasma parameters in athymic nude mice[a]

Treatment	BUN (mg/dl)	Creatinine (mg/dl)	Glucose (mg/dl)	Na^+ (mEq/l)	K^+ (mEq/l)	Cl^- (mEq/l)
Day 1:						
Saline	30	0.3	163	145	7.4	104
"Empty" liposomes	21	0.3	172	147	7.9	107
12 mg/kg cDDP	24	0.3	155	146	7.9	106
4 mg/kg MLV-VM	26	0.3	169	145	7.7	105
Drug combination	25	0.3	155	148	7.5	109
Day 5:						
Saline	25	0.3	170	146	8.4	98
"Empty" liposomes	23	0.3	157	144	8.0	109
12 mg/kg cDDP	29	0.3	154	148	8.0	105
4 mg/kg MLV-VM	23	0.3	186	143	8.6	107
Drug combination	21	0.3	191	144	7.5	103

[a] Animals were treated with i.p. injections of cDDP, MLV-VM, and/or a combination of the two. Control mice received either saline or "free" liposomes. Parameters of pooled plasma samples from 4 mice were determined on days 1 and 5

that reached by treatment with either drug alone. Mice treated with 3 mg/kg cDDP and 1 mg/kg MLV-VM (Fig. 1A) showed 100% survival and appeared healthy at 150 days after treatment. There was no gross evidence of neoplasia in the peritoneum, and only small tumor implants at the site of tumor inoculation were observed. Mice treated with the same doses of MLV-VM and inactive doses of cDDP (Fig. 1B) showed 70% survival over that of animals treated with each drug alone. This indicates that the interaction of cDDP and MLV-VM in human ovarian OVCAR-3 xenografts is truly synergistic as in the case of the P388 leukemia model.

To study the possible overlapping toxicity between MLV-VM and cDDP, cumulative doses of each drug alone and in combination were given i.p. to athymic nude mice and changes in pooled plasma markers for kidney and liver function as well as serum electrolytes were determined. Animals treated with either cDDP, MLV-VM, or the drug combination showed no change in BUN or creatinine plasma values (Table 2), indicating a lack of significant overlapping renal toxicity for the drug combination. However, animals treated with either "empty" liposomes or MLV-VM showed significant changes in plasma ALT and/or AP activity. For example, Fig. 2 shows that plasma ALT values significantly decreased on days 1 and 5 following i.p. administration of "empty" liposomes or MLV-VM. Similarly, cDDP-treated animals also showed a marked decrease in plasma ALT and/or AP values at 5 days following drug administration. Likewise, the plasma ALT values were low after treatment with the drug combination, indicating possible overlapping hepatotoxicity with coadministration of both drugs. There was no significant change in serum electrolyte levels as indicated in Table 2, although the blood glucose level was relatively high at 5 days following treatment with MLV-VM and the drug combination as compared with that observed in the untreated mice.

Discussion

The chemotherapy of cancer may be the most difficult of all therapeutic enterprises. Antineoplastic drugs should selectively kill or impair the growth of malignant cells, which differ only in subtle ways from normal host cells [4]. The creation of truly selective chemotherapeutic agents has proven to be an elusive goal. Numerous agents are highly toxic to tumor cells; however, these agents also display very predictable host toxicities, most commonly affecting rapidly dividing normal cell populations. The low toxic-to-therapeutic ratio of most anticancer drugs has prompted a search not only for new chemotherapeutic entities but also for innovative ways to improve the utilization of existing drugs.

One such approach has been the use of drug-delivery technologies such as liposomes [6]. Liposomes as drug carriers can sometimes modify the therapeutic profile of selected antitumor drugs in a very favorable manner. This often comes about through a reduction in drug toxicity to critical host tissues. Good examples of this would be the many interesting studies of liposomal doxorubicin and the antifungal polyene antibiotic amphotericin B [13, 16, 25, 27, 35]. The use of drug-delivery systems can not only extend therapeutic possibilities for well-established drugs but also create opportunities for therapeutic application of chemical moieties not previously considered for drug use. Such a case would be our utilization of lipid-soluble, membrane-active ionophoric agents such as VM as anticancer drugs, an application made posible by the characteristics of liposomes as a delivery system [7]. In contrast to other studies of liposome-incorporated antitumor drugs, we have chosen to utilize, in conjunction with liposomes, drugs that act primarily on cell membranes and have strong affinities for lipid bilayers; by this means we can modulate their undesirable side effects and potentiate the cytotoxic effects of other antineoplastics such as DNA-interacting drugs used in such a combination. To a considerable degree, this goal has been realized in potentiating the cytotoxicity of platinum-based antitumor drugs. We have recently reported that MLV-VM enhances the cytotoxic effects of cisplatin, ormaplatin, and carboplatin against human ovarian carcinoma OVCAR-3 and CaOV-3 cells in vitro [5, 10]. This synergistic interaction between a membrane-active drug incorporated into liposomes and DNA-intercalating drugs in human ovarian carcinoma cells has prompted us to extend this observation to P388 leukemia-bearing mice and to human OVCAR-3 tumor xenografts that are restricted to the peritoneal cavity. The OVCAR-3 cell line was established from malignant ascites of a patient with progressive adenocarcinoma of the ovary [19]. These cells are resistant to clinically relevant concentrations of Adriamycin, melphalan, and cisplatin and appear to serve as a suitable model for i.p. therapy of ovarian cancer.

The results presented herein show that simultaneous i.p. treatment with low doses of MLV-VM greatly enhanced the antitumor activity of cisplatin against the P388 murine leukemia model and against human OVCAR-3 xenotransplants confined to the peritoneal cavity of mice. No antagonistic effect of this treatment was noted, and the extent of synergy in the P388 model was a 4-log cell kill over the

additive effect of the drug combination. Likewise, this positive response was observed in athymic nude mice bearing human ovarian cancer. Multiple i.p. administrations of low doses of MLV-VM (1 mg/kg) greatly enhanced the antitumor activity of inactive doses (1 mg/kg) of cisplatin. In this experiment, 70% of the animals receiving this drug combination appeared to be tumor-free and survived beyond 150 days. This observation confirms the finding in our recent in vitro studies that low doses of MLV-VM can potentiate the cytotoxicity of cDDP toward human ovarian cells in a synergistic interaction [5]. However, membrane-active drugs like VM are likely to be toxic to vital tissues and organs whose physiological functions depend on the maintenance of an ion gradient, such as the kidneys [7]. Likewise, the clinical use of cDDP is limited by its severe side effects, mainly renal toxicity [11, 26]. Several trials have been carried out in attempts to improve the efficacy of cDDP by reducing its renal toxicity [14, 42].

In the present study, we found no overlapping renal toxicity between MLV-VM and cDDP when the drugs were given i.p. in cumulative doses. Our data also indicate a lack of significant changes in Na^+, K^+, and Cl^- serum electrolytes following treatment with the drug combination. Therefore, it is conceivably possible to use low doses of cisplatin in combination with MLV-VM to achieve the desirable therapeutic effects with no overlapping nephrotoxicity. However, i.p. administration of cDDP, MLV-VM, and/or the drug combination resulted in a decrease in ALT and/or AP enzyme activity, indicating possible hepatotoxicity. Large liposomes such as MLVs are primarily taken up by the mononuclear phagocytic cells of the reticuloendothelial system; thus, these liposomes tend to accumulate in organs rich in reticuloendothelial (RE) cells, especially the liver and spleen [34]. Therefore, accumulation of liposomes in RE-rich cells such as liver Kuppfer cells can result in the observed changes in liver enzyme markers. However, Wang and Huang [43] showed low liver uptake (1.5%–4.5%) of i.p. injected liposomes in athymic nude mice. They also observed a relatively high uptake of liposomes in the spleen and stomach. It was suggested that this observation may be unique to athymic mice, since i.p. injected liposomes would normally accumulate in the mouse liver. For this reason, several attempts have been made to "steer" liposomes to non-RE-cell targets by making selective changes in their lipid composition so as to prolong the liposomes' circulation half-life and, thus, avoid possible toxicity to the reticuloendothelial system [1, 17]. Repeated administration of cisplatin can also result in liver toxicity in mice; this effect could be modulated by coadministration of cisplatin with selenite [37]. Currently, we are trying to confirm this possible overlapping hepatotoxicity by determining the effect of the drug combination on liver histopathological changes as well as measuring the activity of other liver enzymes such as glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT). Furthermore, modulating the lipid composition to avoid possible liver uptake in this case would also be very useful.

The molecular event responsible for the observed synergism between MLV-VM and cDDP has not been fully established. Previously we have demonstrated that low doses of VM can increase the cellular platinum uptake by a factor of 2 upon simultaneous treatment for 3 h. This modest increase in intracellular platinum accumulation does not seem to be sufficient to explain fully the cytotoxic synergism we previously observed [5]. However, we recently reported that membrane-associated protein kinase C (PKC) can also be activated (2-fold) upon cotreatment with both drugs for 3 h [10]. Membrane-active antitumor drugs such as Adriamycin have been shown to activate PKC [20, 40]. Likewise, activation of PKC has recently been documented to be involved in cisplatin-induced antitumor activity [2, 36]. Thus, it is conceivably possible to assume that a good correlation between the activation of PKC signal-transduction pathways and an increase in cellular platinum uptake may exist that is related to the observed synergistic interaction between MLV-VM and cDDP. We are currently investigating this possible correlation.

In conclusion, our study has shown that i.p. therapy with MLV-VM and cisplatin can be an asset in the locoregional therapy of ovarian cancer with no overlapping nephrotoxicity. Neither the frequency nor the sequence of drug administration was optimized in the present work; thus, the results obtained might be improved further.

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