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In vitro interaction of liposomal valinomycin and platinum analogs: cytotoxic and cytokinetic effects

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Cisplatin is the most active agent in the chemotherapy of ovarian cancer and this activity can be enhanced by liposomal valinomycin (MLV-VM) *in vitro*. To test whether MLV-VM is capable of augmenting the cytotoxic and cytokinetic effects of other platinum analogs, drug combinations of MLV-VM and platinum drugs were tested against two human ovarian cancer cell lines (OVCAR-3 and CaOV-3) and on Chinese hamster ovary (CHO) cells *in vitro*. MLV-VM enhanced the sensitivity to cisplatin, ormaplatin and carboplatin on human ovarian carcinoma cells that show various degrees of drug sensitivity. This interaction was shown to be truly synergistic by median-effect analysis up to 90% cell kill. The combination index at 50% cell kill (CI_{50}) was also used to quantitate the extent of drug synergy. In the OVCAR-3 cell line, for example, the CI_{50}s were 0.62, 0.85 and 0.8 for cisplatin, ormaplatin and carboplatin, respectively. DNA histograms obtained by flow cytometry showed that CHO cells treated with cisplatin alone accumulated in the S–G_2 segment, with a partial G_2 block. The addition of 2 μM VM with cisplatin, significantly enhanced the accumulation of cells at the G_2/M phase. Our results further demonstrate that *in vitro* treatment with VM, cisplatin and/or combination is associated with an increase in protein kinase C (PKC) activity. These findings suggest that accumulation of cells at G_2/M phases and modulation of PKC activity could be among the basis for the cytotoxic synergism observed between cisplatin and VM.

Key words: Cytotoxic synergism, flow cytometry, liposomal valinomycin, ovarian carcinoma, platinum analogs, protein kincase C.

Introduction

Cisplatin is one of the most active compounds in the antineoplastic pharmacopeia and the single most active agent for treating ovarian carcinoma.[1,2] However, the therapeutic effectiveness of cisplatin is accompanied by strong toxic effects, the prime one being nephrotoxicity, as well as severe nausea, vomiting, myelotoxicity and ototoxicity.[3] Further, there is frequent progression of the tumor cells to a cisplatin-resistant state.[4] These undesirable effects have stimulated the search for platinum analogs with a better therapeutic index and a similar or improved antitumor activity.[5,6] Of primary interest are carboplatin (*cis*-diamine-1,1-cyclobutanedicarboxylate platinum(II) [NSC 241240]) and ormaplatin (tetrachloro (*d,l-trans*)1,2-diaminocyclohexane platinum(IV) [NSC 363812]). Both agents appear to have excellent antitumor activities and, unlike cisplatin, myelosuppression is the dose-limiting side-effect.[7,8] Therefore, it is possible to use these analogs in combination chemotherapy with cisplatin to improve efficacy while avoiding overlapping host toxicity.[9] However, the possibility of cross-resistance between some of these compounds such as carboplatin and cisplatin might limit the generalization of this approach.[10] Thus, an alternative approach is to develop rational drug combinations in which the individual agents interact to potentiate the cytotoxic effects in a synergistic manner. We have recently adopted this approach and reported that valinomycin (VM), a membrane active antitumor agent, when incorporated into liposomes, significantly enhances cytotoxic effects of cisplatin against human ovarian carcinoma cells.[11,12] Our findings led us to test, *in vitro*, the interaction between liposomal VM (MLV-VM) and 'second generation' platinum compounds (carboplatin and ormaplatin) to determine whether the observed synergism between MLV-VM and cisplatin is specific for cisplatin or whether it is a more general phenomenon exhibited by other platinum drugs. Further, we wished to gain a better understanding of the mechanism(s) involved in such an interaction. Thus, we report here that liposomal valinomycin can markedly enhance the sensitivity of human ovarian carcinoma cells to platinum analogs, that the interaction is truly synergistic and that it is associated with the blocking of cell cycle progression in G_2 M phase.

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Both agents are also involved in the activation of membrane-associated protein kinase C (PKC). Parts of this report were presented previously as an abstract.[13]

Materials and methods

Materials

VM was obtained from Natural Products Branch, National Cancer Institute (Bethesda, MD), cisplatin was obtained from Bristol Myers (Evansville, IN), carboplatin and ormaplatin were provided by the Drug Synthesis & Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD), cholesterol, phosphatidylserine, thiazolyl blue, propidium iodide and RNase A were from Sigma (St Louis, MO).

Dimethylsulfoxide was from Fisher Scientific (Fair Lawn, NJ). Dimyristoylphosphatidylcholine was purchased from Avanti Polar Lipids (Birmingham, AL). [γ-^{32}P]ATP was purchased from New England Nuclear (Boston, MA). All other chemicals were reagent grade.

Drugs

VM stock solutions were prepared at 10^{-4} M in ethanol and 20 μl aliquots from appropriately diluted samples were added to the tissue culture plates. The final concentration of ethanol in culture was always $<0.1\%$, which is essentially non-toxic to cells. Platinum drugs were reconstituted immediately before the experiments with 0.9% saline to yield a solution containing 1 mg/ml; all subsequent dilutions were made in either α-minimal essential medium (MEM) or RPMI 1640. MLV-VM was prepared as described previously.[14,15] The preparations used were composed of dimyristoylphosphatidylcholine, cholesterol and phosphatidylserine (molar ratio, 10:4:1) and included 10% of VM (w/w). The liposomes produced were multilamelar vesicles (MLV) and were sterilized by passage through a 0.2 μm filter.

Cells and culture conditions

The human ovarian tumor cell lines used in this study, CaOV-3 and OVCAR-3, were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in monolayer cultures in α-MEM or RPMI 1640 (Gibco, Grand Island, NY), supplemented with 10% (v/v) fetal calf serum (HyClone, Logan, UT), at 37°C in humidified air containing 5% CO_2. Under these conditions, the doubling times of CaOV-3 and OVCAR-3 were 72 and 54 h, respectively. Chinese hamster ovary (CHO) cells were grown as monolayers in α-MEM, supplemented with 10% fetal bovine serum, penicillin and streptomycin.

Cell treatment and data analysis

In this study, cells were treated with MLV-VM or platinum drugs individually or with drug combination, at fixed dose ratios for 72 h. The dose ratios (interactive ratio) were chosen so that the IC_{50}s of the individual drugs would fall in the middle of the range of doses tested. The IC_{50} was defined as the drug concentration necessary to produce 50% inhibition of cell growth compared to untreated controls. The cell surviving fraction was determined by the MTT assay as described previously.[14,15] Synergy of activity was analyzed as recently reported by our laboratory[12] using the median-effect principle of Chou and Talalay[16] and plotted as combination index (CI) versus fraction affected (Fa). The combination index at 50% cell kill (CI_{50}) was used to quantitate the extent of synergy.[17]

Flow cytometric analysis and cell cycle distribution

The flow cytometric assay was performed after staining of DNA utilizing the propidium iodide method.[18] Briefly, exponentially growing CHO cells were treated with VM or platinum drugs for 1 h. The cells were washed, trypsinized, suspended in medium and centrifuged at 500 *g* for 5 min. The pellet was then resuspended in 1 ml phosphate buffered saline (PBS) and fixed in 3 ml 95% ethanol. The fixed cells were centrifuged, rinsed in PBS and resuspended in 1 ml propidium iodide staining solution (50 μg/ml propidium iodide, 100 μg/ml RNase A and 0.1% Triton X-100 in PBS). This mixture was incubated for 4 h on ice and the cells were analyzed on a coulter Epics 751 System (Epics Division, Coulter, Hialeleah, FL). Computer-generated 'gated' analysis was used to determine cell cycle position (DNA content) of drug-treated cells. The percentage of cell cycle distribution phases was calculated in a channel by channel *t*-test of the pair

of mean histograms (PARA 1 and NON-PARA programs). The experiments were repeated at least twice and 10 000 cells were counted per sample.

Preparation of membrane and cytosolic fractions of PKC assay

Cytosolic and membrane-associated PKC activity was prepared as described.[19] CHO cells at 5×10^7 were initially treated with 100 μM cisplatin or VM 100 and 200 nM, and/or combination for 3 h. Cells were then harvested, pelleted and resuspended in ice-cold phosphate buffer saline without any divalent cations and homogenized with 40 strokes (Wheaton Dounce homogenizer, type A pestle).

Centrifugation of the homogenate was at 100 000 *g* for 1 h at 4°C. The supernatant was stored on ice while the pellet was washed with buffer B (20 mM Tris, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 2 mM phenylmethansulfonyl fluoride) by centrifugation (15 min, 12 000 *g*). The pellet was again resuspended with 4 ml of buffer B and homogenized with 10 strokes, as described above. Partial purification of the enzyme was then performed at 4°C by ion exchange chromatography on columns containing 1 ml DEAE–cellulose (Whatman DE52) equilibrated with 10 ml of buffer B. The solubilized membrane and cytosolic PKC fractions were applied to separate columns, followed by washing of the columns twice with 3 ml of buffer B. PKC was then eluted with 2 ml of buffer B containing 100 mM sodium chloride. Leupeptin was added to the elute at a concentration of 25 mg/ml. The enzyme was stored at −80°C until assay was performed.

PKC assay

PKC activity was assayed by the transfer of γ-^{32}P-labeled phosphate from ATP to histone, as described in the protein kinase C assay system (Gibco). All assays were performed in both the presence and the absence of a PKC pseudosubstrate inhibitor peptide (Gibco). The samples were dried on phosphocellulose and washed with 1% H_3PO_4 and the amount of phosphorylated peptide was quantified by scintillation counting using a Tri-Carb 1500 liquid scintillation counter. Protein was determined by the method of Bradford[20] using bovine serum albumin as a standard.

Results

Cytotoxicity studies

We previously reported[11,12] the existence of a marked cytotoxic synergism between MLV-VM and cisplatin on human ovarian carcinoma CaOV-3 cells in culture. To examine this interaction more closely, we first conducted a series of experiments to study the response of human ovarian tumors (OVCAR-3 and CaOV-3) to other platinum complexes upon continuous exposure for 72 h. Growth inhibition was determined by the MTT assay. Each of the four experiments used platinum drugs at concentrations ranging from 1.25 to 40 μM and MLV-VM at concentrations from 0.06 to 1.0 nM VM. The values for 50% growth inhibition of the three platinum complexes evaluated in this study are shown in Figure 1(A). Ormaplatin has IC_{50} values of 7.3 and 2.5 μM using OVCAR-3 and CaOV-3 cells, respectively. In OVCAR-3 cells, cisplatin has an IC_{50} value of 8.2 μM. In contrast, carboplatin was consistently the least potent drug with IC_{50} values of 21 and 4.3 μM for OVCAR-3 and CaOV-3 cells, respectively. Nonetheless, this indicates that all platinum analogs studied produced dose-dependent cytotoxicity in human ovarian cell lines but the molar potency was variable between each cell line. Figure 1(B) clearly shows that MLV-VM inhibited the growth of human OVCAR-3 and CaOV-3 cells at very low concentrations with IC_{50} values of 0.3 and 0.7 nM, respectively. Thus, our result indicates that both platinum drugs and liposome-incorporated VM are cytotoxic to both cell lines, permitting the evaluation of their interaction by median effect analysis. Median-effect analysis yields an index of the extent of interaction (CI) for the two drugs being examined. A CI of 1 at the 50% growth inhibition (IC_{50} value) indicates an additive interaction, a CI > 1 indicates antagonism and a CI < 1 indicates synergism. In this study, drug interaction was examined by treating the ovarian cells in culture with MLV-VM and platinum drugs individually or in combination, over a wide range of doses but at a fixed dose ratio for 72 h. In each case the molar ratio of platinum drug : MLV-VM was 1 : 0.0001. Each agent alone and in combination with MLV-VM produced a linear median effect plot with regression coefficients >0.90 indicating that the dose–response relationships followed the basic mass action principle.

Figure 2 summarizes the interactions of cisplatin : MLV-VM (A), ormaplatin : MLV-VM (B) and carboplatin : MLV-VM (C) on CaOV-3 and OV-

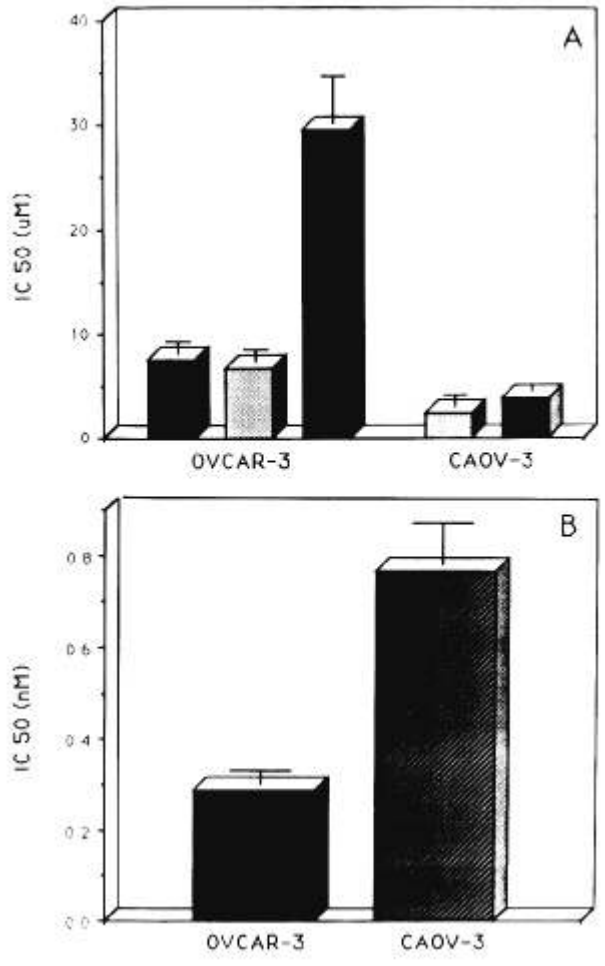

Figure 1. Sensitivity patterns of human ovarian carcinoma cell lines to (A) platinum analogs. Exponentially growing ovarian cells were seeded in 10% serum supplemented α-MEM medium (CaOV-3) or RPMI 1640 (OVCAR-3) at 8×10^3/well and 1×10^3/well, respectively. Seventy-two hours later, the cells were treated with different doses of platinum analogs (■, cisplatin; □, carboplatin; ▨, ormaplatin) or MLV-VM for 72 h. The cells were allowed to grow in drug-free medium for an additional 3 days and the cell survival was determined by the MTT assay. The IC_{50} values were determined from survival curves generated for each experiment. Data are the means (±SE) of at least three independent experiments.

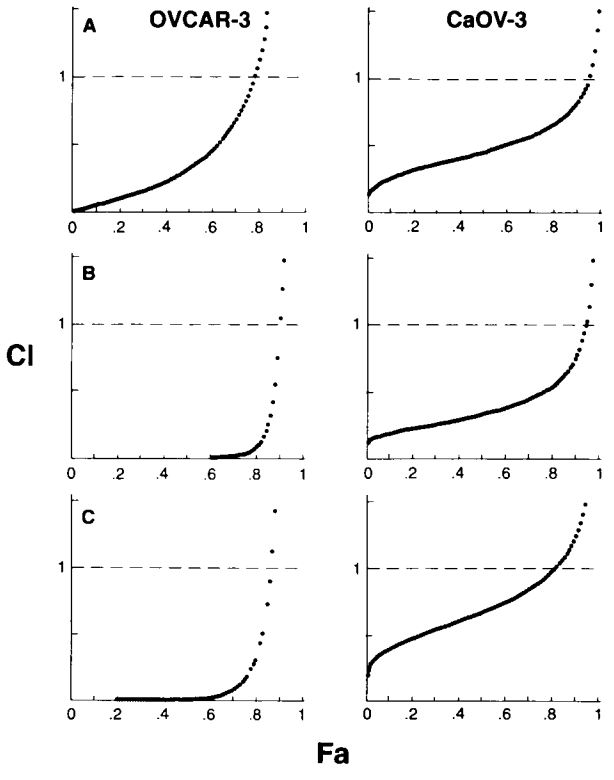

Figure 2. Computer-generated curves describing the combined effects of platinum drugs and MLV-VM. Median effect plot of interaction of MLV-VM and cisplatin (A), ormaplatin (B) or carboplatin (C) in CaOV-3 or OVCAR-3 cell cultures. All points above a CI of 1 are antagonistic, those below are synergistic and those equal to 1 are additive.

CAR-3 cells, using computer-generated curves (Fa versus CI). These curves are graphic representations of the CI as described by Chou and Talalay.[16] As indicated in Figure 2, MLV-VM markedly enhanced the sensitivity of both OVCAR-3 and CaOV-3 cells to all three platinum drugs and this interaction was truly synergistic up to 90% cell kill. In CaOV-3 cells, MLV-VM was most effective in enhancing sensitivity to carboplatin (median CI = 0.2) and least effective for ormaplatin (median CI = 0.75). In OVCAR-3 cells, MLV-VM was most effective in enhancing sensitivity to cisplatin and least effective for ormaplatin and carboplatin.

Table 1 presents quantitative results of the interaction of platinum analogs : MLV-VM (1 : 0.002 molar ratios) using CHO cells. The results demonstrate clear synergistic interaction between cisplatin : MLV-VM and ormaplatin : MLV-VM; a marked reduction in the effective drug concentration could be achieved at the ED_{50}, ED_{70} or ED_{90}. In contrast, the carboplatin : MLV-VM combination produced no synergy at the same molar ratios.

Cytokinetic effects

We previously reported[15] that VM enhances the cytotoxic effect of cisplatin against CHO cells. To ascertain this effect on CHO cell cycle, cells were incubated with the drug for 1 h and samples were then taken at various time periods of post-treatment incubation, stained with propidium iodide and analyzed by flow cytometry. DNA histograms of

Table 1. Cytotoxic effects of platinum analogs or MLV-VM as single agents or when used in combination on CHO cells[a]

	Concentration of single agent				Concentration of drug combination[b]		
	cisplatin	ormaplatin	carboplatin	MLV-VM	cisplatin: MLV-VM	ormaplatin: MLV-VM	carboplatin: MLV-VM
ED_{50}	9.81	7.12	24.45	0.0013	0.61	0.41	4.31
ED_{70}	16.14	11.34	39.23	0.0017	0.94	0.69	5.81
ED_{90}	35.68	24.11	83.29	0.0027	1.85	1.58	9.31

[a]Drug doses are in μM.
[b]The drug molar ratios were 1 : 0.002 (platinum analogs : MLV-VM).
The concentrations of MLV-VM and platinum drugs needed to achieve an ED_{50}, ED_{70} or ED_{90} are shown for each drug used alone or in combination.

samples taken at 1, 3, 6, 24 and 48 h are shown in Figure 3. Incubation with 2 μM VM for 1 h, resulted in a gradual decrease in the fraction of cells in the initial two phases (G_1 and S) during the first 6 h period with a prominent decrease after 24 h. VM caused a decrease in the fraction of cells in S phase from 46 to 20% during the 24 h period, while the fraction calculated to be in G_2/M increased from 25 to 53%. In contrast, incubation of cells with cisplatin (20 μM) for 1 h resulted in a different cytokinetic effects than VM. As shown in Figure 3, cells treated with cisplatin resulted in a transient 20 and 8% increase of cells in S and G_1 phases, respectively, with modest changes in G_2/M phases during the 24 h period. The incubation with the combination of VM and cisplatin resulted in essentially the same cytokinetic changes noted with VM alone after a 24 h period. However, when cell cycle analysis was followed up to 48 h, additional cytokinetic changes were observed. Figure 4 shows representative histograms of the population distribution of DNA contents at 48 h post-treatment in drug-free medium. Control CHO cells had approximately 15% of their cells in the G_2/M phases of the cell cycle. In cells treated with VM or cisplatin, there was a higher proportion of cells in G_2/M phases and a pronounced decrease in the proportion of cells in G_1 phase. Thus, the predominant effect of incubating CHO cells with the drug combination was a 3-fold increase of cell accumulation in G_2/M phases.

PKC activation

Previous studies have demonstrated that *in vitro* treatment with cisplatin is associated with an increase in PKC activity.[21] To establish whether the modulation of this enzyme is linked to the observed synergism between VM and cisplatin in CHO cells, we measured PKC activity in cells treated with VM, cisplatin and/or combination by phosphorylation of a synthetic peptide derived from myelin basic protein that serves as a specific substrate for this enzyme.[22] As illustrated in Table 2, only membrane-associated PKC was optimally activated (>2-fold) by treatment with VM (100 and 200 nM), cisplatin (100 μM) as well as drug combination. Our data also indicates that the activation of PKC with the drug combination is mainly contributed by VM treatment, although both drugs stimulate only membrane-associated PKC.

Discussion

The plasma membrane as well as the internal cellular membranes of the mitochondria, endoplasmic reticulum and golgi apparatus, play important roles in cell architecture and function. Thus, they would seem to be appropriate targets for cytotoxic drugs to be used in cancer therapeutics.[23] However, as is obvious from the dearth of antitumor agents which act at this level, membranes have been sighted as a target for drug development. For the past 3 years, our laboratory has been involved in the development of membrane active drugs of ionophoric types such as VM to be utilized in the control of neoplasia, both as direct cytotoxic agents and as modulators of the cytotoxic action of other antitumor drugs. It is on this premise that our laboratory has begun to explore the cytotoxic interaction between liposome-incorporated VM and DNA-acting drugs on tumor cells.

MLV-VM synergistically enhances the cytotoxicity of cisplatin to CaOV-3 human ovarian carcinoma cells and is associated with only 2-fold increase

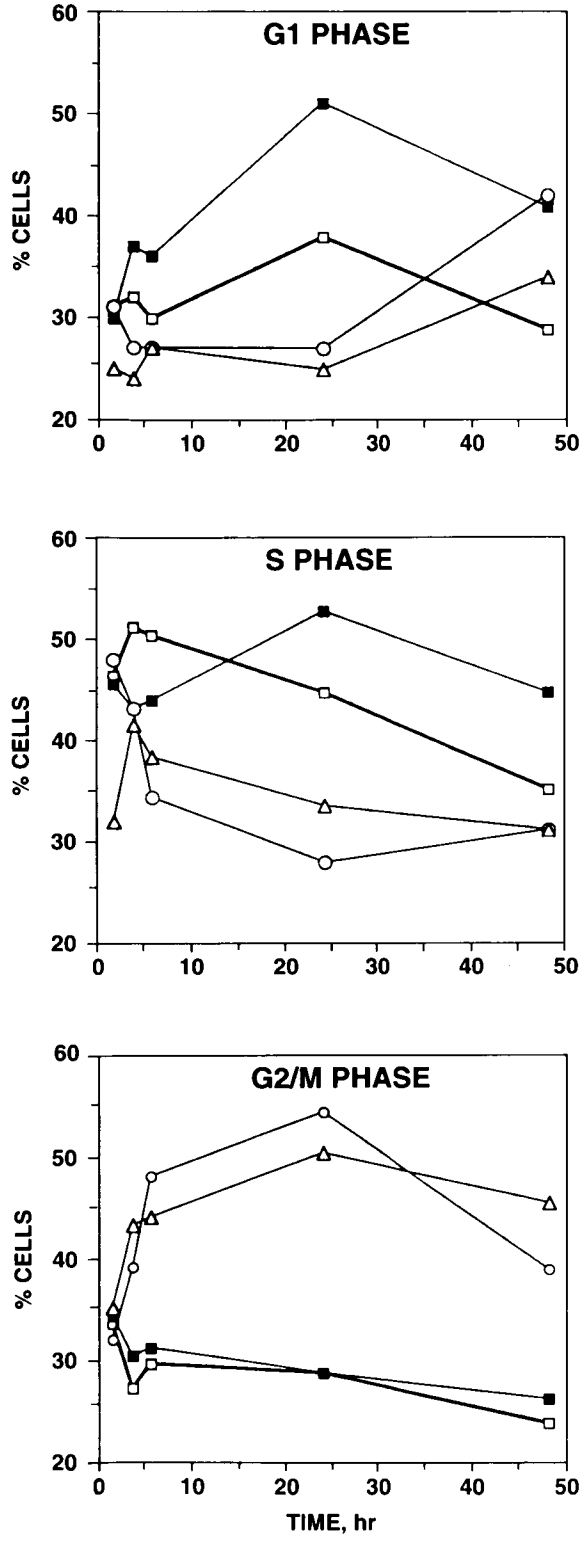

Figure 3. DNA distribution kinetics of CHO cells treated for 1 h with no drug (□), 20 μM cisplatin (■), 2 μM VM (○) or combination (△). Post-treatment incubation for 1–48 h, fixed, stained and analyzed by flow cytometry. The number in each panel shows percentage of G_1, S, G_2/M cells.

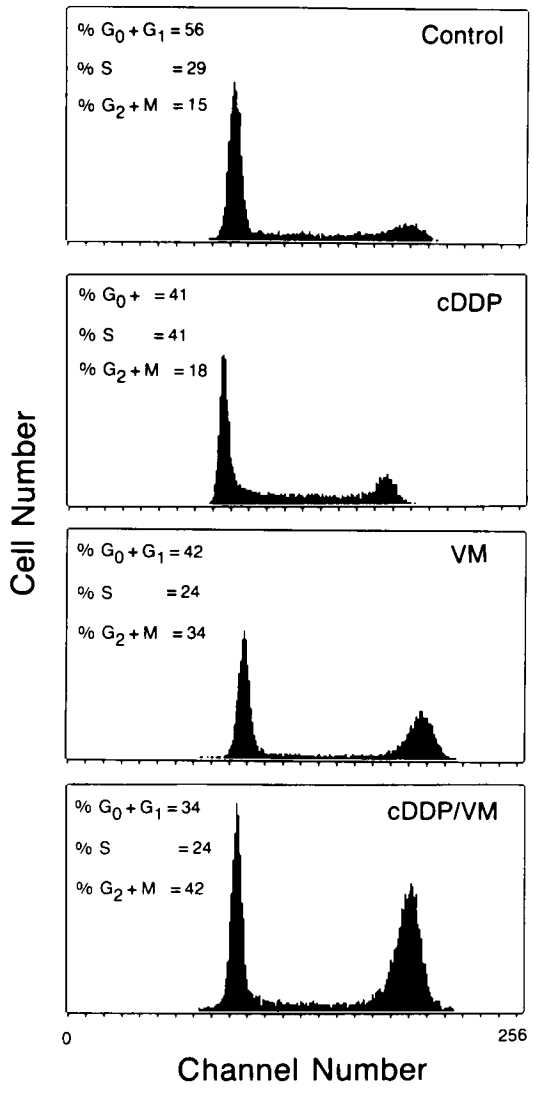

Figure 4. DNA histograms of CHO cells after 48 h of drug-free recovery time. DNA histograms were obtained on a Coulter Epics 751 System using propidium iodide. The percentage of cells in the G_1, S or G_2/M compartments were obtained using the PARA analysis program.

in cellular platinum content.[12] Now, we have established that MLV-VM markedly enhances sensitivity to other clinically important platinum analogs (carboplatin and ormaplatin). The median effect plots indicate that the interaction is truly synergistic for all three platinum drugs over a 90% range of cell kill. However, the extent of drug synergy, as quantitated by the median combination index (CI_{50}), differs significantly for the three analogs with each cell line. For example, with CaOV-3 cells, a strong

Table 2. Effects of cisplatin and VM on PKC activity in CHO cells

Addition	PKC activity (pmol/min/75 μg)	
	cytotosol	membrane
Control	202 ± 23.0	21 ± 3.9
100 nM VM	221 ± 22.0	48 ± 6.5 (2.3)[a]
200 nM VM	245 ± 27.0	57 ± 7.0 (2.7)
100 μM cisplatin	162 ± 19.0	57 ± 6.6 (2.7)
100 nM VM + 100 μM cisplatin	247 ± 27.0	49 ± 4.5 (2.3)
200 nM VM + 100 μM cisplatin	247 ± 22.0	54 ± 2.4 (2.6)

[a]Numbers in parentheses, -fold increase in membrane activity in treated cultures relative to untreated control cultures.
CHO cells were treated with VM (100 or 200 nM), cisplatin (100 μM) and drug combination for 3 h. PKC was isolated and the enzyme activity was determined as described under Materials and methods). Data are mean (± SE) of three to five independent experiments.

synergy is noticed when carboplatin was used comparing to cisplatin or ormoplatin. In contrast, synergy was the strongest for cisplatin when OVCAR-3 cells were used. This difference in the extent of synergy could be attributed to difference in the sensitivity of the ovarian cells towards platinum analogs.[24,25]

The molecular event responsible for the observed synergism between membrane-active agents like VM and DNA-interacting drugs like platinum drugs has not been fully established. Our previous work[12] has indicated that there was a modest (2-fold) increase in cell-associated platinum upon simultaneous treatment with cisplatin and VM for 3 h. This increase in intracellular platinum accumulation seems not to be sufficient to fully explain the cytotoxic synergism we previously observed. However, in the present study, we have also noticed a 2-fold increase in the activity of membrane-associated PKC upon co-treatment with cisplatin and VM for 3 h. This activation is maximal and is mainly due to VM treatment, indicating that the stimulation of PKC at the cellular membrane could be dependent upon the duration and magnitude of the second messengers (such as Ca^{2+} and diacylglycerol) signal.[26] Furthermore, this increase in membrane PKC could also be a result of an activation of a pool of inactive membrane PKC and/or from translocation of cytosolic enzyme. We are currently investigating these possibilities.

Another possibility for the observed synergism between VM and cisplatin is that VM blocks the progression of tumor cells at a certain phase in the cell cycle which is particularly sensitive to cisplatin. The analysis of the cell cycle by flow cytometry suggested that the combination of VM and cisplatin causes an increase the accumulation of CHO cells in the G_2 phase over that caused by treatment of cisplatin alone (Figure 4). Arrest of CHO cells at G_2 phase by cisplatin has been previously shown by several investigators.[27,28] In our present study, we have used 'pulse exposure' to cisplatin at low concentration (20 μM) for 1 h, followed by analysis of cell cycle progression for 48 h. It is clear that CHO cells were arrested at the S–G_2 segment which is still apparent after 48 h (Figure 4). Cells incubated with 2 μM VM, however, showed significant inhibition in the S phase and accumulation in the G_2/M phases. Similar results have also been shown by Kleuser *et al.*[28] to indicate that 3T6 cells were actually arrested in the G_2 phase by VM, i.e. cells are arrested in the G_2 phase which is sensitive for cisplatin action. Thus, when cells were treated with both VM and cisplatin, a remarkable accumulation at the G_2/M phases were observed. In this case, cells became 'recruited' or 'frozen' at the G_2 phase by combined treatment with VM plus cisplatin.

Thus, the major findings of this study are (i) the existence of a marked cytotoxic synergism between liposomal VM and platinum analogs in human ovarian cells and (ii) that the synergism can partly be explained by the activation of membrane-associated PKC and the ability of VM to block the progression of cells at the G_2/M phase in the cell cycle which is the sensitive phase for cisplatin action. Our findings suggest potential usefulness of combining MLV-VM in platinum-based drug therapy for the treatment of ovarian cancer.

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