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Synergistic cytotoxic actions of cisplatin and liposomal valinomycin on human ovarian carcinoma cells

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Summary. We have previously shown that the toxicity of valinomycin (VM), a membrane-active agent with antineoplastic activity, can be dramatically reduced with no loss of the antitumor efficacy of the drug by incorporating it into liposomes. In the present study, we investigated the interaction between *cis*-diamminedichloroplatinum(II) (CDDP) and VM in terms of in vitro cytotoxicity to human ovarian tumor cells. Using the MTT assay and analyzing the data using the median-effect principle, we showed that synergistic cytotoxic interactions exist between CDDP and VM in their liposomal form. The degree of cytotoxic synergism was influenced by the duration of drug exposure and the dose ratio. The cellular accumulation of platinum by ovarian cells at 37° C was slightly higher after exposure to VM as compared with controls; however, it is not clear that this accounts for the cytotoxic synergism. These results suggest that the combination of liposomal VM and CDDP may have merit as a form of localized drug delivery for the treatment of ovarian cancer disseminated within the peritoneal space.

Introduction

Most of the existing anticancer drugs act at the DNA level either by direct chemical interaction, as do alkylating agents, or by interference with DNA biosynthesis, as in the case of antimetabolites. The major limitation of these drugs is their lack of *selectivity*, since the structure and biosynthesis of DNA in normal cells is only marginally different from that in tumor cells [48]. As a result, most of the existing antineoplastics have undesirable toxic effects on rapidly dividing normal cells.

The development of agents that act against cellular loci other than the DNA may conceivably lead to drugs that provide effective antitumor therapy and whose toxicities are distinct from those of most currently available chemotherapeutic agents. One of these cellular loci is the *cell membrane*. Some degree of drug selectivity at this site seems possible because numerous subtle differences in composition, structure, organization, dynamics, and function exist between the surfaces of normal and tumor cells; these may be exploited to achieve a degree of selective cytotoxicity (for review, see [4, 24, 47]). Thus, drugs that severely perturb membrane function in tumor cells may represent a valuable adjunct to cancer chemotherapy since their use in multidrug combinations may induce enhanced tumor-cell killing without producing substantial additional toxicity to normal tissues.

We have previously shown that the toxicity of valinomycin (VM), a membrane-active agent with antineoplastic activity, can be dramatically reduced and its antitumor efficacy can be maintained, if not enhanced, by its incorporation into liposomes [11, 12]. The reduced in vivo toxicity obtained using liposomal VM (MLV-VM) was paralleled by a reduction in the drug's toxicity to normal cells in vitro and an enhanced selectivity for *ras* oncogene-transformed cells [13]. Recently, we reported preliminary data showing that VM (or MLV-VM) displays synergistic cytotoxicity when used in combination with cisplatin in vitro [14, 20]. The use of a liposomal carrier can to some extent control the distribution of a drug in vivo [12, 15]; thus, liposomal drugs may be beneficial in local or regional chemotherapy. The ability of MLV-VM to potentiate the cytotoxicity of a major drug such as *cis*-diamminedichloroplatinum(II) (CDDP, cisplatin) could thus conceivably be an asset in chemotherapy of locally confined tumors such as ovarian cancer.

As a preliminary approach to this issue, we conducted an in vitro study on the interaction of CDDP and MLV-VM

Abbreviations: CDDP, cisplatin, *cis*-diamminedichloroplatinum(II); VM, valinomycin; MLV-VM, liposomal valinomycin; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5 diphenyl-tetrazolium bromide (thiazolyl blue); alpha-MEM, alpha minimal essential medium; CHO, Chinese hamster ovary; IC_{50}, concentration causing 50% inhibition of cell growth; IC_{10}, concentration causing 10% inhibition of cell growth; SF, surviving fraction; fa, fraction affected; CI, combination index

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in human ovarian-carcinoma cell line CaOV-3. The objectives of this study were (a) to examine the in vitro sensitivities of CaOV-3 cells to CDDP and to VM or MLV-VM, (b) to analyze the interactions of CDDP and MLV-VM using the multiple-drug-effect analysis of Chou and Talalay [9], and (c) to examine the effects of VM on the cellular accumulation of CDDP as a possible mechanism of synergistic cytotoxicity.

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.); cholesterol, phosphatidylserine, and thiazolyl blue were supplied Sigma Chemical Co. (St. Louis, Mo.); nitric acid and dimethylsulfoxide were obtained from Fisher Scientific (Fair Lawn, N. J.); and dimyristoylphosphatidylcholine was purchased from Avanti Polar Lipids, Inc. (Birmingham, Ala.). All other chemicals were of 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 nontoxic to cells. Immediately before the experiments, cisplatin was reconstituted with 0.9% saline to yield a solution containing 2 mg CDDP/ml; all subsequent dilutions were made in alpha minimum essential medium (alpha-MEM). MLV-VM was prepared as previously described [12, 14]. The preparations used in the present study comprised dimyristoylphosphatidylcholine, cholesterol, and phosphatidylserine (molar ratio, 10:4:1) and included 10% (w/w) VM. The liposomes produced were multilamellar vesicles (MLVs) and were sterilized by passage through a 0.2 µm filter.

Cell and culture conditions. The human ovarian tumor-cell line used in this study, CaOV-3, was obtained from the American Type Culture Collection (Rockville, Md.). It was maintained in monolayer cultures in alpha-MEM (Gibco) supplemented with 10% (v/v) fetal calf serum (Hazelton) at 37° C in humidified air containing 5% CO_2. Under these conditions, the doubling time for these cells was 72 h.

Drug treatment and survival curves. Cytotoxicity studies were initiated by plating 2 ml of cells obtained from exponentially growing cultures in 24-well tissue-culture plates (Corning) in alpha-MEM plus 10% fetal calf serum at about 1×10^4 cells/well and then carrying out cell counts using an Electrozone/Celloscope particle counter (Particle Data, Inc., Elmhurst, Ill.). Following 72 h incubation, the drug was added to quadruplicate wells and incubated for 3 h, the wells were washed twice with prewarmed phosphate-buffered saline (PBS), and 2 ml drug-free medium was added. The plates were then incubated for 6 days. For long-term exposure, cells were continuously treated with drugs for 72 h, washed with PBS, and then incubated with drug-free medium for 3 additional days.

Cell survival was determined using a semiautomated tetrazolium (MTT)-based colorimetric assay [5] as previously described by us [13, 14] and other investigators [1, 38, 39]. The effect of VM on CDDP-induced cytotoxicity was evaluated by exposing cells to graded concentrations of the latter drug in the presence and absence of subtoxic doses of VM. The concentration of drugs causing 50% inhibition of cell growth (IC_{50}) for the drug combination was calculated by logarithmic analysis and was divided by the corresponding value obtained in the absence of VM to give an estimate of the cytotoxicity potentiation effect.

Drug/liposome combinations and data analysis. In this study, cells were exposed to MLV-VM and CDDP either alone or in combination at fixed dose ratios for 3 or 72 h. The surviving cell fraction was determined using the MTT assay as described above. Synergy of activity was analyzed according to the median-effect principle described by Chou and Talalay [9, 10]. This principle is described by the equation

$$fa/fu = (D/D_m)^m,$$

where *fa* is the fraction of CaOV-3 cells affected by the dose D, *fu* is the unaffected fraction, D_m is the dose required for a 50% effect (e.g., 50% inhibition of cell growth at 72 h as compared with the control), and *m* is a Hill-type coefficient signifying the sigmoidicity of the dose-effect curve. A plot of $y = \log(fa/fu)$ vs $x = \log$ (dose), i.e., a median-effect plot, linearizes the dose-effect relationship with the slope *m* and the *x*-intercept at $\log (D_m)$. These values for CDDP and MLV-VM were calculated for each experiment and for each drug combination at a fixed concentration ratio.

Interaction of the effects of the two drugs was quantitatively determined by the combination index (CT) according to the equation

$$CI = D_{MLV\text{-}VM}/D_{MLV\text{-}VMx} + D_{CDDP}/D_{CDDPx} + D_{MLV\text{-}VM} \times D_{CDDP}/D_{MLV\text{-}VMx} \times D_{CDDPx},$$

where $D_{MLV\text{-}VMx}$ and D_{CDDPx} are the doses of each drug required to produce an effect of x% when the drug is used alone and DMLV-VM and DCDDP are the cytotoxic contributions of MLV-VM and CDDP, respectively, in the mixture as calculated from the known dose ratio of the two drugs that also yield an effect of x% in combination. The dose ratios were chosen such that we could conveniently explore a range of toxicities. In general, they were chosen such that the IC_{50} values for the individual drugs would fall in the middle of the range of doses tested. In the case of 72 h treatment with MLV-VM and CDDP, a broader range of doses was examined such that the midrange of doses tested roughly corresponded to the IC_{90} value for each individual drug.

Finally, a CI versus fraction affected (fa-CI) plot was generated for all fa values between 0.01 and 0.99. Thus, when CI<1, synergism was indicated; when CI = 1, an additive effect was indicated; and when CI>1, antagonism was indicated. A computer program [8] based on the above-mentioned equations was used in the present study for automated analysis of the dose-effect data.

Accumulation studies. Duplicate 60-mm tissue-culture dishes were seeded with 5×10^6 CaOV-3 cells and incubated overnight in alpha-MEM medium. Accumulation experiments were then initiated by rinsing the dishes twice with PBS, adding 5 ml alpha-MEM containing various concentrations of cisplatin (10–50 mM) in the presence or absence of 20 nM VM, and incubating the dishes for 3 h at 37° C. Cells were then washed twice with cold PBS, detached, and harvested by scraping the dishes with a rubber policeman into 1 ml phosphate-buffered NaCl solution.

Aliquots of the cell suspension were assayed for protein content using the method of Bradford [3]. Cells from each sample were centrifuged and the pellet was taken up into 100 µl concentrated HNO_3. Following overnight oxidation of organic material, 100 ml H_2O was added to each sample and the platinum content was determined using a Perkin Elmer Zeeman 5100 atomic absorption spectrophotometer with background correction and equipped with an HGA 600 graphite furnace, an automatic sampler (AS-60), and an HGA 600 programmer (Perkin Elmer, Norwalk, Conn.). The following heating program was used: 90° C for 60 s, ramp to 1,500° C in 10 s and hold for 20 s, and run at 2,500° C under maximal power for 7 s. Platinum content was quantitated by running a calibration curve immediately before the samples and was expressed in nanomoles per gram of protein.

Results

Cytotoxicity of VM against human ovarian tumor cells

Figure 1 shows the results of the assay when the cells were exposed to VM or MLV-VM for either 3 (Fig. 1 A) or 72 h (Fig. 1 B). Time-dependent cytotoxic effects were noted. The IC_{10} and IC_{50} values for short-term exposure to VM

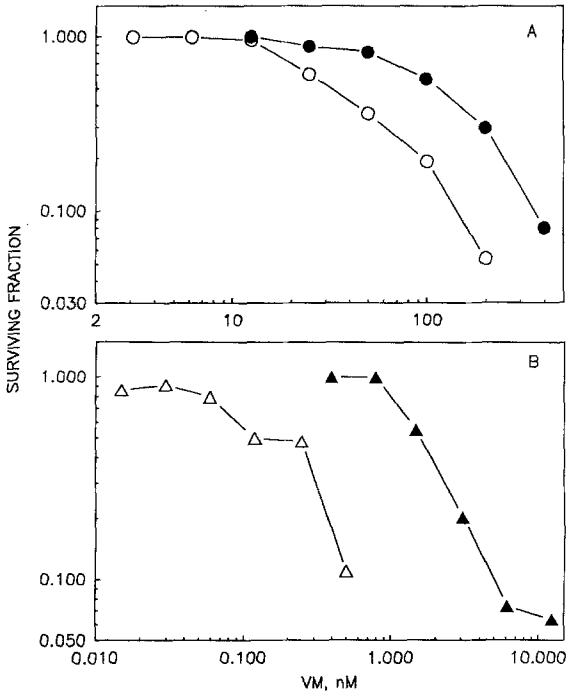

Fig. 1 A, B. Dose-response curves for human CaOV-3 cells following A 3 h and B 72 h exposure to VM and MLV-VM. Exponentially growing cells were seeded in 10% serum-supplemented alpha-MEM medium at 8×10^3 cells/well. After 72 h, the cells were treated with different doses of VM (○, △) and MLV-VM (●, ▲) for 3 and 72 h, respectively. Following drug treatment, the cells were allowed to grow in drug-free medium for 3 additional days and cell survival was determined by the MTT assay, with absorbance being measured at 540 nM with an automated microplate reader. Points represent the means of quadruplicate determinations (SE, <10%) obtained in at least 3 independent experiments

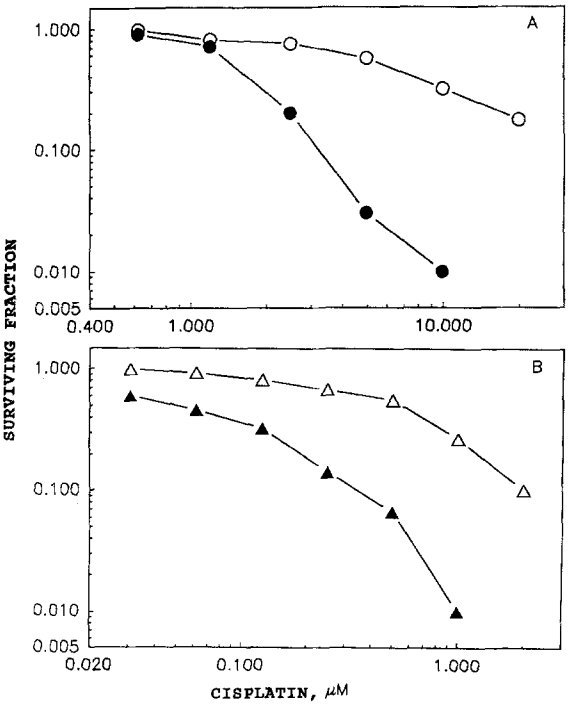

Fig. 2 A, B. Dose-response curves for the exposure of CaOV-3 carcinoma cells to combinations of cisplatin and nontoxic doses of VM. Cells were exposed to various doses of cisplatin A in the absence (○) or presence of 15 nM VM (●) for 3 h or B in the absence (△) or presence of 0.05 nM VM (▲) for 72 h. Following drug treatment, the cells were reincubated in drug-free medium and the surviving cell fraction was determined by the MTT assay. Points represent the means of quadruplicate determinations (SE, <10%) obtained in 2–3 independent experiments

(Fig. 1 A) were 15 and 30 nM, and respectively, and those for the same exposure to MLV-VM were 20 and 100 nM, respectively. For 72 h exposure to VM (Fig. 1 B), the IC_{10} and IC_{50} values were 0.05 and 0.1 nM, respectively, and when MLV-VM was used, the corresponding values were 1 and 2 nM.

Potentiation of cisplatin cytotoxicity by VM

Figure 2 shows the CDDP dose-response curves for the killing of CaOV-3 cells in the presence or absence of sublethal doses (IC_{10}) of VM. During both short- and long-term incubations, the curves were nonlinear. In the 3-h experiments (Fig. 2 A), VM markedly shifted the CDDP dose-response curve to the left. The IC_{50} ratio for CDDP in the presence or absence of 15 nM VM averaged about 3, indicating a 3-fold enhancement of CDDP cytotoxicity on short-term exposure. In the 72-h experiments (Fig. 2 B), the dose-response curves were also nonlinear, and subtoxic doses of VM (0.05 nM) shifted these curves leftward. The IC_{50} ratio for CDDP in the presence or absence of 0.05 nM VM was 10, indicating a 10-fold enhancement of CDDP cytotoxicity on long-term exposure.

The short-term effects of MLV-VM on cisplatin cytotoxicity are shown in Fig. 3 A. Subtoxic doses of MLV-VM (20 nM) produced a modest leftward shift in the dose-response curves for CDDP (the IC_{50} for CDDP shifted from 10 to 5 μM). More profound effects were observed when cells were exposed to MLV-VM for 72 h (Fig. 3 B). CaOV-3 cell growth was inhibited by 50% by 1 μM CDDP, with 1 log cell kill occurring at 2 μM. In the presence of 1 nM MLV-VM, 50% inhibition of growth was achieved using only 10 nM CDDP, with 1 log cell kill occurring at about 0.5 μM.

Synergy between MLV-VM and CDDP

To determine whether the interaction between VM and CDDP was truly synergistic, CaOV-3 cells in culture were exposed to MLV-VM and CDDP either alone or in combination over a wide range of doses but at a fixed dose ratio for 3 or 72 h. In the 3-h experiments (CDDP : MLV-VM molar ratio, 1 : 0.04), the median-effect dose, D*m*, and slope, *m*, for CDDP were 1.803 μM and 2.17 ± 0.07, respectively; the corresponding values for MLV-VM were 229.4 nM and 1.81 ± 0.11. The D*m* and *m* values for 72 h exposure (CDDP : MLV-VM molar ratio, 1 : 0.0063) were 0.439 μM and 2.58 ± 0.4, respectively for CDDP and 2.578 nM and 2.638 ± 0.55, respectively, for MLV-VM.

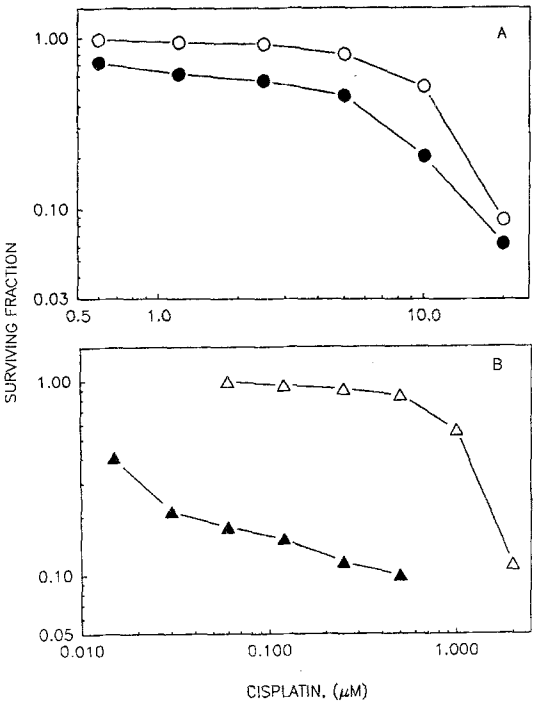

Fig. 3 A, B. Dose-response curves for the exposure of CaOV-3 carcinoma cells to combination of cisplatin and nontoxic doses of MLV-VM. Exponentially growing CaOV-3 cells were exposed to various doses of cisplatin A in the absence (○) or presence of 20 nM MLV-VM (●) for 3 h or B in the absence (△) or presence of 1 nM MLV-VM (▲) for 72 h. The surviving cell fraction was determined by the MTT assay as described in Materials and methods. Points represent the means of quadruplicate determinations

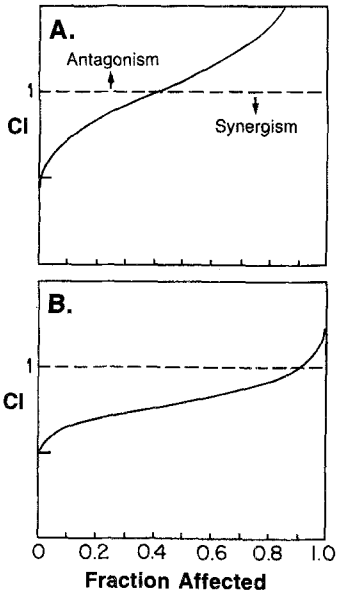

Fig. 4 A, B. Combined effect of 3 and 72 h exposure of CaOV-3 cells to CDDP plus MLV-VM. Computer-generated curves describe the combined effects of CDDP : MLV-VM at fixed ratios of **A** 1 : 0.04 for 3 h and **B** 1 : 0.0063 for 72 h. Results are plotted as a function of the fraction of treated cells affected versus the combination index (fa-CI plot) under a mutually nonexclusive assumption. All points lying above a CI of 1 are antagonistic, those lying below a CI of 1 are synergistic, and those lying at a CI of 1 are additive. Interactions of CDDP and MLV-VM are strongly synergistic (plot below the horizontal dotted line) over nearly the entire range of concentrations tested (**B**), whereas they are antagonistic (plot above the horizontal dotted line) over the higher concentrations of drugs tested and synergistic at lower concentrations (**A**)

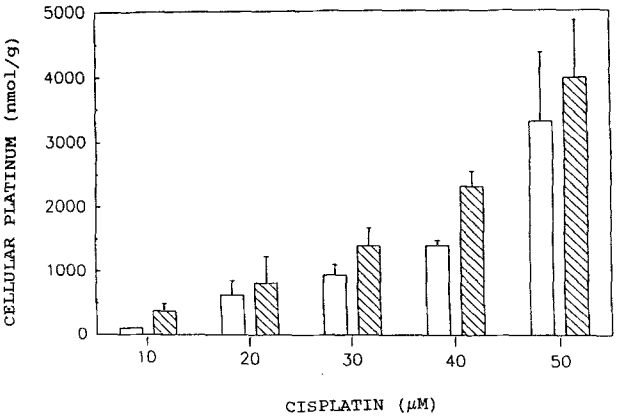

Fig. 5. Accumulation versus concentration of cisplatin. The cell-associated platinum content is shown as measured by flameless atomic absorption spectroscopy of acid extracts of CaOV-3 cells treated for 3 h with either CDDP alone (□) or in combination with 20 nM VM (▨). Cells were treated in monolyers washed in a phosphate-buffered NaCl solution, their protein content was determined, and, finally, organic material in the cell pellet was oxidized with HNO_3. The platinum content in an aliquot was quantitated in quadruplicate for each drug concentration using the 265.9 nM absorbance line of platinum. Background correction was carried out using modulated deuterium lamp. Bars represent the mean (± SE) of 3 experiments

Computed regression coefficients for the linearized dose-effect curves were >0.9; this indicates that the data fulfilled the criteria for computation of the CI according to Chou and Talalay [9, 10]. Such computation yielded values of CI = 1 if activities were additive, CI>1 in case of antagonistic activities, and CI<1, when effects were synergistic. The composite fa-CI plot for the data from one such experiment is presented in Fig. 4. Analysis of the data for CDDP and MLV-VM combined at a ratio of 1 : 0.0063 (Fig. 4 B) suggested that the two drugs acted synergistically over nearly the entire range of concentrations tested (5%–90% cell kill) but were synergistic at lower concentrations (fa<0.5) only following 3 h exposure and when the ratio of CDDP : MLV-VM had been increased to 1 : 0.04 as shown in Fig. 4 A.

The data were analysed under mutually nonexclusive conditions, since we assumed that the two drugs act toward different targets; however, similar results were obtained when the data were analysed under mutually exclusive conditions. This finding suggests that for this particular schedule of administration in this in vitro cellular system, the synergism between cisplatin and MLV-VM is dependent on the relative concentrations of the two drugs in the mixture and on the duration of exposure.

Quantitation of platinum content in treated CaOV-3 cells

Atomic absorption measurements of cellular platinum in treated CaOV-3 cells are summarized in Fig. 5. The amount of cell-associated platinum in CaOV-3 cells exposed to CDDP alone for 3 h increased with increasing concentrations of CDDP in the cell-culture medium. The simultaneous presence of 20 nM VM produced a modest increase in the amount of cell-associated platinum measured.

Discussion

The tumor-cell membrane and the internal membranous organelles may be as important as DNA for chemotherapy [7, 21]. Drugs that perturb the physical stability of membranes or their ability to maintain ion gradients between cellular compartments may well either have profound cytotoxic effects on tumor cells or enhance the effectiveness of other antineoplastic agents that act via DNA mechanisms. In the present study, we concentrated on the latter approach. We recently reported [14] that VM, a membrane-active antitumor agent, significantly enhances the cytotoxicity of cisplatin to chinese hamster ovary (CHO) cells. Our findings led us to use human ovarian carcinoma cells as a model to determine whether the observed enhancement is truly a synergistic interaction.

Cisplatin is an important cytotoxic agent in the treatment of human ovarian, testicular, and head and neck cancers [6, 26, 31, 37]. Cisplatin exerts its effects by DNA adduct formation, leading to intrastrand, interstrand, and DNA-protein cross-linking [17, 42]; both intrastrand and interstrand cross-links have been shown to correlate with its cytotoxicity [19, 51]. However, the clinical effectiveness of CDDP has been limited by its severe side effects, including nephrotoxicity, ototoxicity, gastrointestinal toxicity, and neurotoxicity [30, 34, 43, 50]. Several approaches have been used in attempts to enhance the therapeutic index of cisplatin; these include combination chemotherapy with other cytotoxic agents such as vinblastine, bleomycin, 5-fluorouracil, and/or 1-β-D-arabinofuranosylcytosine [18, 32, 41, 46] as well as with calmodulin antagonists [27] and calcium channel blockers [36]. However, little is known about the ability of membrane-active agents to enhance the therapeutic index of CDDP.

The data presented in Fig. 2 indicate that at relatively nontoxic doses, VM can enhance CDDP-induced cytotoxicity in a time-dependent manner. When this enhancement was assessed according to inhibition of cell growth, the IC_{50} values obtained for CDDP in the presence and absence of VM during long-term exposure were 30 and 300 nM, respectively, whereas those obtained during short-term exposure were 1,500 and 4,800 nM, respectively. Thus, the enhancement of CDDP-induced cytotoxicity was greater during long-term exposure to VM. Time-dependent enhancement of cisplatin-induced cytotoxicity by MLV-VM also occurred (Fig. 3), with the most striking enhancement being seen after 72 h exposure. Over 72 h, an IC_{10} dose of MLV-VM resulted in a 100-fold enhancement of CDDP-induced cytotoxicity as based on cisplatin's IC_{50} values; over 3 h, the same dose of MLV-VM produced only a 2-fold enhancement.

The potentiating effect of MLV-VM on CDDp cytotoxicity raises the question as to whether this phenomenon is additive or synergistic. To address this question, we assessed the outcome of the drug combination using median-effect equations [9]. This approach was necessary because neither the fractional-effect method nor the isobologram method [2, 16] was appropriate due to the sigmoid nature of the dose-response curves and the possible mutually nonexclusive interaction of the two drugs [9, 10]. Using the median-effect principle, we clearly demonstrated synergistic cytotoxic effects for the combination of CDDP and MLV-VM in human tumor cells. Synergism was clearly seen during long-term (72 h) exposure and occurred to a lesser extent during short-term (3 h) exposure.

The mechanism underlying the cytotoxic synergism between CDDP and VM is currently unknown. One possible explanation for the results would be that VM increases the cellular uptake of CDDP. The enhanced uptake would serve to increase the effective intracellular Pt concentration, leading to a consequent increase in the formation of Pt-DNA adducts. From Fig. 5, however, it is clear that only a modest increase in cell-associated Pt occurred on simultaneous treatment with CDDP and VM. Thus, it does not appear that the effects of VM on CDDP accumulation are sufficient to explain fully thy cytotoxic synergism.

Another possibility is that VM blocks the progression of tumor cells at a certain phase in the cell cycle that is particularly sensitive to cisplatin. Interestingly, VM has been shown to arrest transformed cells in the G_2 phase of the cell cycle [29], which is also sensitive to CDDP action [45]. Alternatively, VM may discharge the pH gradient across the membranes of the endosome/lysosome compartment [49]. Since many drugs with titratable moieties can either concentrate in or be excluded from this compartment [25], this could lead to the redistribution of some of the product's CDDP metabolism [33] and thus affect the amount of Pt in the nucleus.

A particularly critical cellular ionic parameter involves the regulation of cytoplasmic pH; two membrane transport systems, the Na^+/H^+ antiport and the $Cl^-/HCO3^-$ exchanger, are important in regulating intracellular pH [23, 35]. Furthermore, the regulation of cytoplasmic pH has been suggested to play a vital role in cell proliferation [40, 44]. Proton ionophores such as nigericin are known to have potent effects on cytoplasmic pH [22, 28]; this may be true for VM as well. Thus, one possible mechanism for the direct antitumor actions of VM that have been observed [12, 13] may be via effects on cytoplasmic pH and, consequently, on tumor-cell proliferation. The modulation of the cytotoxic effects of DNA-targeting drugs such as cisplatin may conceivably be related to changes in intracellular pH, since this could lead to changes in the rate or magnitude of chemical and enzymatic processes that are responsible for drug-induced DNA damage or repair of such damage.

Thus, the major findings of the present study include (a) the existence of a marked cytotoxic synergism between MLV-VM and CDDP, (b) the observation that this synergism is influenced both by the duration of drug exposure and by the dose ratios, and (c) that the synergism can only partly be explained by an increase in intracellular platinum content. These findings should be of value in the design of strategies for in vivo treatment of tumors using combinations of MLV-VM and CDDP. The observation of synergy between MLV-VM and cisplatin may lead to improved intraperitoneal therapy of human ovarian cancer. This prediction is based on the grounds that cisplatin dosing is often limited by renal toxicity, whereas MLV-VM has not shown nephrotoxicity to animals [11, 12]. If agents that do not have overlapping normal organ toxicity were used, full

doses of each drug could theoretically be given clinically. In addition, liposomes would likely be retained in the peritoneum for longer periods than would most free drugs [15], thus increasing the peritoneum/plasma ratio of the drug combination, enhancing the effect on intraperitoneal ovarian tumor cells, and minimizing systemic toxicity.

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