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Polymerase chain reaction analysis of cisplatin-induced mitochondrial DNA damage in human ovarian carcinoma cells

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The purpose of this study was to determine whether the observed synergistic interaction between cisplatin and valinomycin (VM) in human ovarian carcinoma is the result of mitochondrial DNA (mtDNA) damage. A polymerase chain reaction (PCR)-based method was used to quantitate the lesion frequencies produced by cisplatin, VM and/or drug combination in a 1.1 kbp segment of mtDNA and a 0.536 kbp segment of the nuclear-located β-globin gene in human ovarian CaOV-3 carcinoma cells. Our data indicates that the nuclear DNA (nDNA) received more cisplatin-induced damage at doses of 25 μM or less than did mtDNA. At higher cisplatin doses (50 μM or more), however, the damage was relatively equal in both segments. VM alone produced little or no damage on mtDNA, yet a significant amount of damage was detected within nDNA. However, when 1 μM VM was used in combination with low doses of cisplatin (0–40 μM), extensive mtDNA damage was detected as compared with the absence of detectable damage on nDNA. In mtDNA, the lesion frequency was 5.45 lesions/10 kb/10 μM cisplatin in the presence of 1 μM VM, whereas no detectable lesions were induced by cisplatin alone. This drug combination produced no detectable damage on DNA, indicating that cisplatin-induced mtDNA damage could be the basis for the observed synergistic interaction with VM. These results also correlate well with our recent *in vivo* study with the nude mice model of human ovarian cancer treated with a cisplatin/liposomal VM drug combination. Furthermore, this report shows evidence for the role of mitochondria and mtDNA as alternative targets for drug action in cancer therapy.

Key words: Cisplatin, mitochondrial DNA, quantitative polymerase chain reaction, ovarian cancer, valinomycin.

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 often accompanied by two notable clinical limitations: its unfavorable toxicity profile (including nephrotoxicity, neurotoxicity, myelotoxicity, severe nausea and vomiting[3]) and a propensity for tumor cells to progress to a cisplatin-resistant state.[4] These undesirable effects prompted us to develop rational platinum-based drug combinations in which the individual agents interact to potentiate the cytotoxic effects on tumor cells in a synergistic manner. We have recently adopted this approach and reported that valinomycin (VM), a membrane-active agent with antitumor activity, when incorporated into liposomes (MLV-VM), enhances the cytotoxic effects of cisplatin against human ovarian carcinoma cells.[5] the cytotoxic interaction was truly synergistic and is associated with the blocking of cell cycle progression in G_2/M phase.[6] More recently, our laboratory has reported that i.p. administration of low doses of MLV-VM in combination with low doses of cisplatin has significant antitumor activity against OVCAR-3 tumors in a nude mouse model of human ovarian cancer.[7] Multiple i.p. administration of low doses of MLV-VM (1 mg/kg; 1.1 μM) greatly enhanced the antitumor activity of low doses (1 mg/kg; 3.3 μM) of cisplatin. In this experiment, 70% of the animals receiving this drug combination appeared to be tumor-free and survived beyond 150 days; this has occurred in the absence of overlapping nephrotoxicity. This observation confirms our earlier finding that low doses of MLV-VM can potentiate the cytotoxicity of cisplatin toward human ovarian cells in a synergistic interaction.

As a membrane-active agent with ionophoric activity, VM can also discharge the mitochondrial membrane potential leading to disruption of the connection between cellular electron transport and the state of oxidative phosphorylation.[8] This in turn could have a major impact on cell growth and viability as a result of the collapse of cellular mitochondrial functions.[9] Thus it is possible to assume that VM enhances cisplatin cytotoxicity in ovarian tumor

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cells by potentiating its *mitochondrial* DNA (mtDNA) damaging effects. A recent study has shown that cisplatin can induce mtDNA damage in murine leukemia L1210 cells.[10] The damaging effect produced, however, was less than that induced on the *nuclear* DNA (nDNA). To test if VM can potentiate cisplatin-induced mtDNA damage, we developed a quantitative polymerase chain reaction (QPCR) assay to detect the extent of such damage on the mtDNA and nDNA in human ovarian CaOV-3 cells. Similar to previously reported QPCR assays,[10,11] this assay is based on the fact that many lesions in the DNA templates can block the Taq polymerase, and thereby result in a decreased amplification of a damaged DNA segment compared with the amplification of the non-damaged segment. Assuming that cisplatin induces damage randomly across the DNA molecule, Poisson analysis can then be used to determine lesion frequency/strand/specific dose.[10]

Materials and methods

Drugs and chemicals

VM was obtained from the Natural Products Branch, National Cancer Institute (Bethesda, MD) and was prepared as concentrated stock solutions by dissolving in absolute alcohol. The final concentration of ethanol in culture was adjusted to 0.1%, which is essentially non-toxic to cells. Cisplatin was obtained from Bristol Myers Squibb (Evansville, IN). Agarose and gel bond paper were obtained from FMC Bioproducts (Rockland, ME). [α-^{32}P]dCTP was purchased from DuPont/NEN (Boston, MA). Unless otherwise indicated, all reagents were obtained from Sigma (St Louis, MO).

Cell cultures

Human ovarian tumor CaOV-3 cells were seeded into 60-mm tissue culture dishes at 37°C in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS) in humidified air containing 5% CO_2. When the cells were subconfluent, the medium was aspirated and replaced with serum-free medium. Cells were then treated with either cisplatin (0–300 μM) and/or VM (0–1000 nM). Following incubation for 3 h at 37°C, cells were washed three times with cold PBS, pelleted and frozen for DNA isolation.

Isolation of DNA

Frozen CaOV-3 cell pellets were thawed at room temperature and resuspended in 2 volumes of lysis buffer (2% SDS, 0.5 M NaCl, 0.05 M EDTA, 0.05 M Tris–HCl) (pH 7.5) containing 200 μg/ml proteinase K. After overnight incubation at 37°C, the lysates were extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and twice with chloroform:isoamyl alcohol (24:1, v/v). The aqueous phase was transferred to a sterile 15 ml Corex tube, adjusted to 500 mM with sodium acetate and the total genomic DNA was precipitated with 2 volumes of ice-cold 95% ethanol. The genomic DNA was washed in 70% ethanol, dried and resuspended in TE buffer. The concentration of DNA was determined using an UltraspecIII spectrophotometer (Pharmacia, Milwaukee, WI).

Amplification of nuclear and mtDNA by PCR

The oligonucleotide primers used in this study, as indicated below, were prepared by Operon Technologies (Alameda, CA). A 1.1 kbp segment of mtDNA (Figure 1) was amplified using Mito A and Mito B primers;[12] a 0.536 kbp segment of the β-globin gene was amplified using the β-globin 1 and

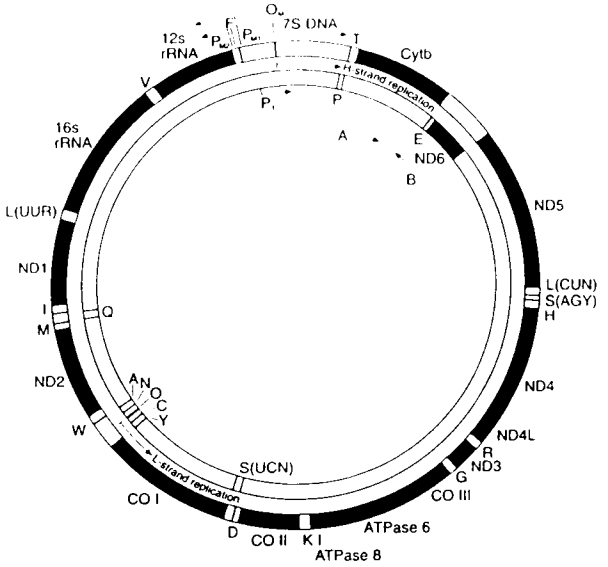

Figure 1. Human mitochondrial DNA function map and locations of the olignucleotide primers (Mito A and B) used for the PCR amplification reaction. The amplified segment of the mitochondrial genome is 1.1 kbp. O_H and O_L, origins of heavy and light strand replication, respectively; P_H and P_L, heavy and light strand transcription promoters, respectively.

β-globin 2 primers.[13] Mito A: 16085 5′-GCGGTTGTTGATGGGTGAGT-3′16066. Mito B: 14947 5′-CCACATCACTCGAGACGTAA-3′14966. β-globin 1: 5′-GGTTGGCCAATCTACTCCCAGG-3′. β-globin 2: 5′-GCTCACTCAGTGTGGCAAAG-3′.

All amplification reactions were carried out in 0.2 ml PCR tubes in a final volume of 50 µl. Each reaction consisted of 50 ng of total genomic DNA, 20 pM of each primer, 100 µM dNTP, 1 mM $MgCl_2$, 2.5 units Replitherm™ Thermostable DNA Polymerase (Epicenter Technologies, Madison, WI), the supplied reaction buffer and 2 µCi [α-^{32}P]dCTP (3000 Ci/mmol). For the amplification of *mtDNA*, samples underwent an initial denaturation at 94°C for 10 min, followed by 18 cycles of template denaturation at 94°C for 1 min, primer–template annealing at 55°C for 1 min and primer extension at 72°C for 1 min. For the amplification of *nDNA*, samples underwent an initial denaturation at 94°C for 5 min, followed by 30 cycles of template denaturation at 94°C for 20 s, primer–template annealing at 55°C for 20 s and primer extension at 72°C for 30 s. At the end of either 18 or 30 cycles, extension was allowed to continue for an additional 7 min. PCR amplification was performed on a programmable thermal cycler (Ericomp, San Diego, CA). The amplified products were separated by agarose gel (1%) electrophoresis. The agarose gels were dried on gel bond paper for quantitative analysis using AMBIS 100 Detector (AMBIS, San Diego, CA). The amount of amplification was represented by the amount of radioactivity (c.p.m.) of the amplification product minus the background radioactivity (c.p.m. = 100–350). In order to measure the effect of DNA damage on amplification, the amount (c.p.m.) of amplification product from DNA isolated from treated cells was divided by the amount (c.p.m.) of amplification product from DNA isolated from non-treated cells. This gave the fraction of non-damaged templates at a given dose of cisplatin and/or VM.

Results and discussion

In this study, we have utilized the PCR to develop a sensitive assay for the detection of DNA damage produced by cisplatin and VM in the mitochondrial genome (Figure 1). We previously reported[5–7] the existence of a marked cytotoxic synergism between MLV-VM and cisplatin on human ovarian carcinoma cells in culture and in nude mice. We selected to study the *mitochondria* as a possible target for the observed synergism, because (i) cisplatin has been reported to produce damage to mtDNA in addition to nDNA[10,14] and (ii) VM can disrupt cell energy metabolism as well as mitochondrial membrane potential.[8,15] With the use of specific primers to mtDNA and a region of the nuclear located β-globin gene, these studies show that it is possible to detect and evaluate damage in mtDNA in the presence of nDNA. Thus it is possible to simultaneously perform this type of experiment without isolating mtDNA from nDNA.

Ideally, in a typical PCR, there is a period in the amplification process where the amount of the amplified product doubles during each cycle. This exponential increase eventually slows and finally levels off, reaching a plateau. This plateau effect can result from limitation of critical components such as template, primer, polymerase and other factors that are well characterized.[16] To accurately quantitate the product generated by PCR, we needed to empirically identify the exponential phase of the reaction for each primer set. To specifically examine the level of lesions within a given template, it is important that the only limiting component in the reaction be the non-damaged DNA. These criteria can be met only during the exponential phase of the reaction. Thus PCR amplifications using 50 ng of total genomic DNA were performed and the amplified product was plotted against cycle number (Figure 2A and B). For the multicopy 1.1 kbp mtDNA segment, the exponential phase was from cycle 14 through 18 (Figure 2A), after which a plateau was observed (not shown). The single copy 0.536 kbp nDNA segment exhibited a similar profile from cycle 15 through 30 (Figure 2B) with a plateau observed after 30 cycles. Based on these results, amplifications of mtDNA were performed at 18 cycles and amplifications of the nDNA segment were performed at 30 cycles.

Next, it was necessary to demonstrate a linear relationship between the amplification signal and amount of non-damaged DNA template at the predetermined cycle. Figure 3(A and B) shows the optimized linear regions for the amplification of the 1.1 kbp mtDNA segment at 18 cycles and the 0.536 kbp nDNA segment at 30 cycles, respectively. The amplifications were generally linear over a range of 3.1–50 ng of non-damaged total DNA template. Thus a starting amount of 50 ng of total genomic DNA template was used in all of our amplification reactions.

Once the condition for QPCR had been optimized, experiments were conducted to detect damage frequency resulting from various doses of cisplatin, VM and or drug combination. DNA was purified from treated and non-treated CaOV-3 cells.

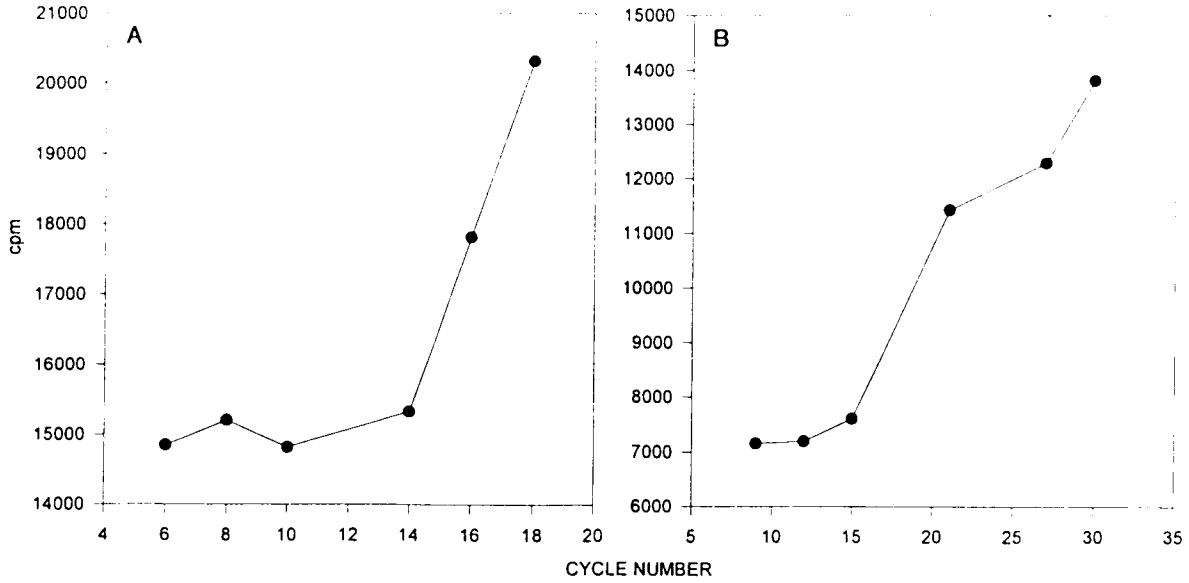

Figure 2. The cycle range for mtDNA segment (A) and a region of nuclear located β-globin gene segment (B). The maximum rate of amplification for mtDNA and the β-globin gene segments has been achieved after 18 and 30 cycles, respectively.

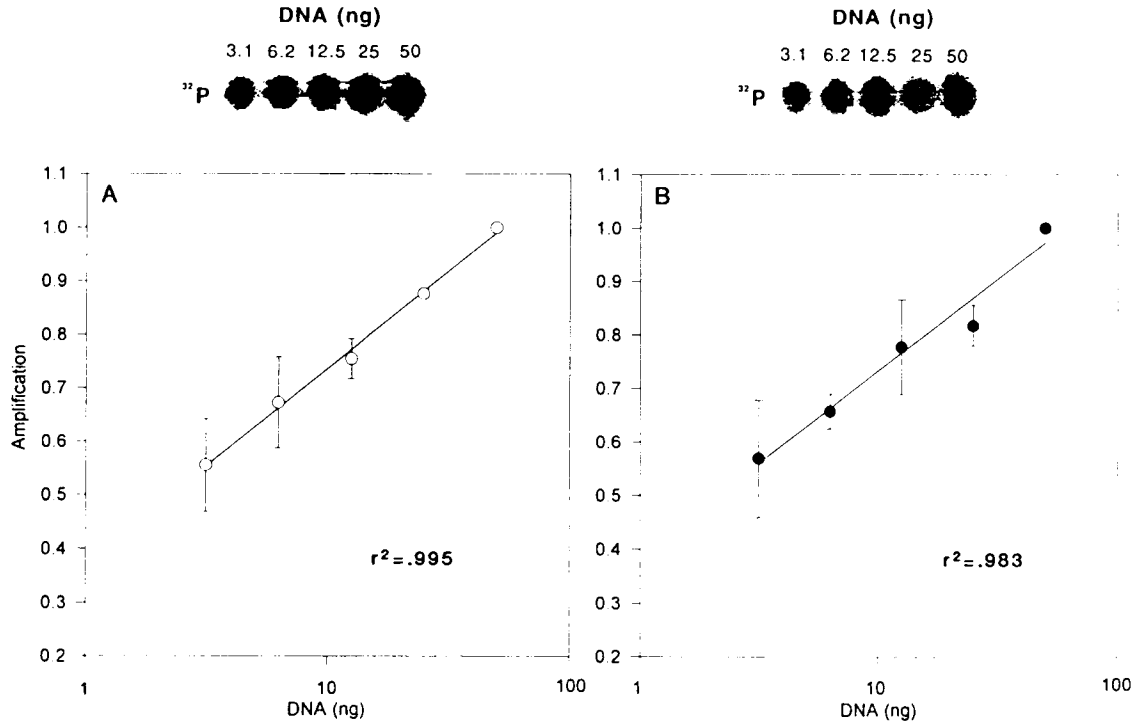

Figure 3. PCR amplification of the mtDNA segment at 18 cycles (A) or the nuclear DNA gene segment at 30 cycles (B) with increasing genomic DNA template. PCR experiments were performed with serial dilution of total genomic DNA corresponding to 3.1, 6.2, 12.5, 25 and 50 ng, and products were separated on 1% agarose gel, as described in Methods. The amount of amplification produced with 50 ng of template DNA = 1.0. The regression curve and coefficient for the mtDNA segment and the nDNA were calculated (r^2 = 0.995 and 0.983, respectively).

and then subjected to PCR using primers spanning 1.1 and 0.536 kbp fragment of mtDNA and nDNA, respectively. A tracer amount of [α-^{32}P]dCTP was included in the reaction mixture and after electrophoresis the radioactivity of the resulting bands was quantified. Figure 4 reports the results obtained from the amplification of the mtDNA and the nDNA segments following treatment with cisplatin and VM. With cisplatin, amplification decreased in a dose-related manner from 0 to 300 μM in both the mtDNA and nDNA segments (Figure 4A). When normalized, for the difference in segment length, cisplatin produced more damage on the nDNA than mtDNA, at doses below 100 μM. Equal DNA damage was observed with 100 μM and more. At 25 μM, for example, the lesion frequency for nDNA = 7.1 lesions/10 kb segment, compared with 1.91 lesions/10 kb for mtDNA (Figure 4C). This result is in close

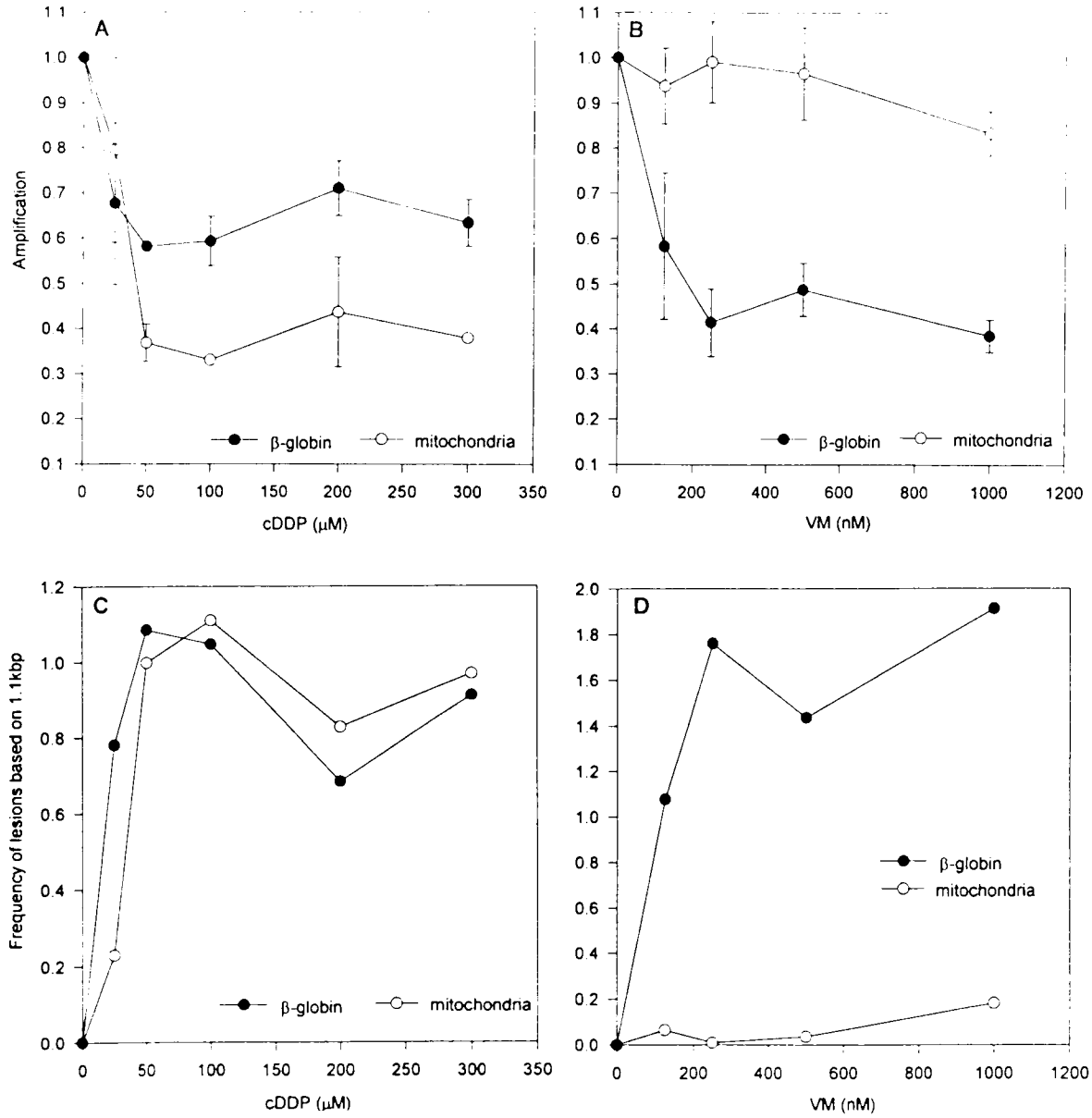

Figure 4. Cisplatin and VM dose–response curves for induction of DNA damage. The amplification level represents the amount of amplification (in c.p.m.) from a damaged DNA template at each cisplatin or VM dose divided by the amount of amplification from a non-damaged template. The data were derived from two or three independent experiments, and represent the mean of three or four PCR amplifications of the mtDNA (A) and the DNA (B) ± SD. Values from (A) and (B) were normalized for the difference in segment length, and data are presented as lesion frequency based on 1.1 kbp segment for mtDNA (C) and nDNA (D).

agreement with the recent finding in murine leukemia L1210 cells which showed that cisplatin induces more damage on nDNA than on mtDNA.[10] By contrast, VM produced little or no damage on mtDNA, whereas a significant amount of damage occurred within the nDNA (Fig. 4B and D). This data does not imply that VM can produce direct damage on nDNA, but rather the level of damage observed could be due to modulation of certain internal signaling pathway(s). Mobile ionophores like VM have been reported to affect a variety of membrane related activities in mammalian cells. This would include actions on ion gradients, pH and potentials in plasma membrane.[17] This may have an indirect damaging effect on the genomic DNA and cell proliferation.[18,19]

Once the dose-effects of cisplatin and VM had been established, experiments were conducted to determine whether the damage induced by cisplatin could be modulated by VM. Figure 5 reports the

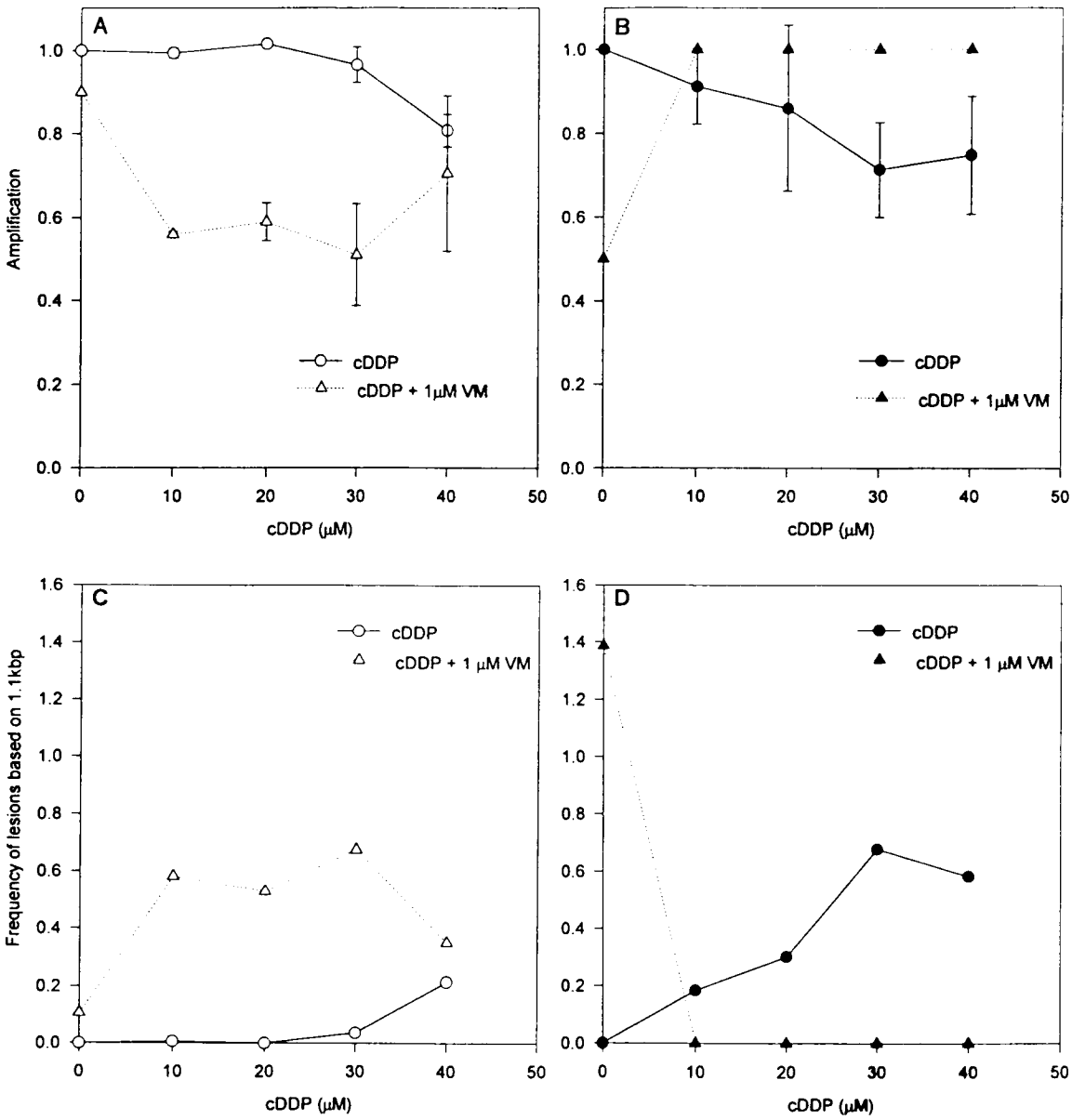

Figure 5. Combination treatment with cisplatin and VM. Amplification of the mtDNA (A) and DNA (B) segments versus low doses of cisplatin (μM) in the presence or absence of 1 μM VM. The data were derived from two independent experiments, and represent the mean of three PCR amplifications of the mtDNA and the nDNA segments ± SD. Lesion frequency in the mtDNA (C) and the nDNA (D) after data has been normalized based on 1.1 kbp segment.

amplification data obtained when CaOV-3 cells were exposed for 3 h to low doses of cisplatin (0–40 μM) in the presence or absence of 1 μM VM. We have selected this dose range to be compatible with those that were used with our *in vivo* drug combination doses.[7] In this study, mice treated with 3 mg/kg cisplatin (10 μM) and 1 mg/kg MLV-VM (1.1 μM) showed 100% survival beyond 150 days. Thus a concentration of 1 μM VM was used in combination with low doses of cisplatin to correlate the DNA damaging effect with our *in vivo* data, i.e. whether the observed synergistic interaction is due to mtDNA damage or nDNA damage. As seen in Figure 5(A), treatment with 1 μM VM resulted in a significant decrease in the amplification of the mtDNA fragment, with an increase in the amplification of the nDNA segment (Figure 5B). This was observed when cells were treated with low doses of cisplatin. When the mtDNA amplification data was normalized (Figure 5C), the lesion frequency was 5.45 lesions/10 kb/10 μM cisplatin in the presence of 1 μM VM; with no detectable lesions in the nDNA (Figure 5D). This data suggests that VM enhances the damaging effects of cisplatin on the mitochondrial genome and not on the DNA. Thus, cisplatin-induced mtDNA damage could be the basis for the observed synergistic interaction with VM.[5–7] Carcinoma cells have been shown to have a higher negative mitochondrial membrane potential than normal cells.[20,21] As an ionophore, VM can depolarize the mitochondrial membrane potential without increasing plasma membrane potassium fluxes.[8,22] Elevated mitochondrial membrane potential in carcinoma cells has been shown to protect tumor cells from cisplatin-induced damage.[9] Thus it is reasonable to assume that collapsing the mitochondrial membrane potential by VM would enhance cisplatin-induced mtDNA damage. Nonetheless, the question remains as to whether damaging the mtDNA by the cisplatin/VM combination will deregulate nucleomitochondrial relationships? Figure 5(B) shows that this drug combination produces an antagonistic effect on the β-globin nDNA that resulted in the reversal of cisplatin VM-induced nDNA damage, i.e. increases nDNA content. This effect is not clear yet, but it may imply that a compensation mechanism(s) has (have) been induced in the DNA as a result of mtDNA damage. Induction of nuclear gene expression has been previously observed as a consequence of the accumulation of mtDNA damage in ischemic hearts.[23] This seems to be a compensatory effect for respiratory deficiency. The mechanism of this observation in CaOV-3 cells is under active investigation in our laboratory.

In conclusion, this study has demonstrated that the mtDNA can be damaged with low doses of cisplatin and VM, and the extent of the damage can be easily detected with a QPCR assay. Further, there is a good correlation between mtDNA damage induced by the cisplatin/VM combination and our recent *in vivo* study in a nude mice model of ovarian cancer. Our study also shows evidence that the mitochondria and mtDNA may play a role as an alternative target for drug action in cancer therapy. However, the question is how are they involved in the phenomenon? Does damaging mtDNA activate nuclear proto-oncogenes? Does it deregulate nucleo-mitochondrial relationships? To what extent will we be able to modify energetic and mitochondrial metabolism in tumor cells and produce selective cell kill? These are questions to be addressed in future investigations. The answers to which will certainly enhance our knowledge of the interaction between anticancer agents and extranuclear DNA.

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