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Cellular accumulation and DNA damage induced by liposomal *cis*-bis-neodecanoato-*trans*-R,R-1,2-diaminocyclohexaneplatinum(II) in LoVo and LoVo/PDD cells

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Liposomal *cis*-bis-neodecanato-*trans*-R,R-1,2-diaminocyclohexaneplatinum (11) (L-NDDP) is a liposome-entrapped platinum complex that has shown partial lack of cross-resistance with cisplatin in human colon carcinoma LoVo cells. We studied the drug accumulation and DNA damage induced by L-NDDP and cisplatin in LoVo and LoVo/PDD cells. Our results indicate that the accumulation of L-NDDP in LoVo cells is several-fold higher than that of cisplatin; that the accumulation of L-NDDP is similar in both cell lines, whereas that of cisplatin is reduced by 2- to 3-fold in LoVo/PDD cells; and that the transmembrane transport of cisplatin is highly dependent on temperature while that of L-NDDP is not. We also found that the cytotoxicity of both agents correlates with the extent of DNA–protein cross-link formation, and that DNA interstrand cross-linking does not appear to play a role in the cytotoxicity of L-NDDP, whereas it correlates with cisplatin cytotoxicity.

Key words: Diaminocyclohexane, lipophilic platinum complexes, liposomes.

Introduction

Platinum(II) complexes exert their cytotoxic effects through formation of adducts of their aquated species with DNA.[1–4] Resistance to platinum complexes has been found to be related to decreased drug cellular accumulation[5] and secondarily decreased DNA adduct formation,[6] increased repair of platinum–DNA adducts[7–9] or increased intracellular drug detoxification.[10]

During the past few years, we have developed lipophilic and non-cross-resistant platinum complexes that are completely insoluble in water but ideally suited for liposome delivery, in an attempt to overcome acquired transmembrane mechanisms of resistance to cisplatin and to enhance the therapeutic index through changes in biodistribution.[11–14] The leading compound selected from this research program, L-NDDP, has shown lack of cross-resistance with cisplatin against LoVo/PDD colon[15] and A2780/PDD ovarian carcinoma cells[16] *in vitro*, and L1210/PDD leukemia cells *in vivo*.[13,15] In addition, as a result of the preferential distribution of liposomes to liver and spleen, L-NDDP has shown enhanced anti-tumor activity in tumor models of metastases in these organs.[13] In studies carried out in A2780 human ovarian carcinoma cells, the lack of cross-resistance of L-NDDP was associated with its ability to achieve similar cellular accumulation and DNA platination over time in A2780 and A2780/PDD cells, whereas in the case of cisplatin, the cellular accumulation and DNA platination over time were markedly reduced in A2780/PDD cells.[16] L-NDDP is currently undergoing clinical evaluation. In an initial phase I study, the dose-limiting toxicity was myelosuppression, and no nephrotoxicity was observed.[14]

We attempted to elucidate the mechanisms of partial lack of cross-resistance of L-NDDP in a different tumor cell system, LoVo and LoVo/PDD human colon carcinoma cells. We determined the cellular drug accumulation and DNA crosslinks induced by cisplatin and L-NDDP in this pair of cell lines, and compared the results with those obtained in A2780 and A2780/PDD cells. Our results confirm that the lack of cross-resistance properties of L-NDDP are associated with its ability to achieve similar cellular accumulation in sensitive as well as in resistant cells. Interestingly, our results also confirm the observation made previously in A2780 cells that interstrand DNA crosslink formation does not appear to play a role in the cytotoxicity mediated by L-NDDP.

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Materials and methods

Drugs and cell lines

Cisplatin was purchased from Bristol-Myers Company (Evansville, IN). L-NDDP was prepared as previously described,[13,14] using dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) in a molar ratio of 7:3. DMPC and DMPG were obtained from Avanti Polar Lipids (Birmingham, AL).

LoVo cells are derived from a carcinoembryonic antigen-producing human colon carcinoma cell line, and the biological properties of this cell line have been described previously.[17] LoVo/PDD cells were developed from LoVo cells by stepwise increment of the cisplatin concentration up to 3.0 μg/ml. After the development of resistance, the resistant cells were maintained in drug-containing medium (Ham's F-10 supplement with 5% fetal calf serum plus 3.0 μg/ml of cisplatin) to maintain resistance. We have previously reported that L-NDDP is partially not cross-resistant with cisplatin in this cell line, as assessed by clonogenic assay.[15]

Intracellular drug accumulation

Cells (2×10^6 cells/well) were seeded in 6-well plates, incubated for 48 h and exposed to various concentrations (5–400 μM) of drugs for 30 min. Drug exposure was carried out at 4 or 37°C to assess the role of temperature in the transmembrane drug transport of L-NDDP and cisplatin. After treatment, the cells were detached with trypsin, centrifuged and resuspended in 0.1 ml of saline. After 0.2 ml of 2 N NaOH solution was added, cells were digested at 50°C for 1 h. The cellular-derived protein was determined with a protein assay kit (Sigma Chemical Co., St Louis, MO; catalog no. 5656) and the amount of elemental platinum was measured by atomic absorption spectrophotometry (AAS).

DNA interstrand and DNA–protein crosslink determination

DNA interstrand and DNA–protein crosslinks were determined by the alkaline elution technique as described by Kohn *et al.*[18] The cells (1×10^6 cells/well) were seeded in 6-well plates, incubated overnight and then labeled with 5 μl of [^{14}C]thymidine (Amersham, Arlington Heights, IL; 56 Ci/mol) for 24 h. After being labeled, the cells were chased for 3 h in fresh medium and exposed to various concentrations (5–30 μM) of drugs for 1 h. Subsequently, the cells were washed twice with phosphate-buffered saline (PBS), and the treated and untreated cells were irradiated with 600 rads of X-ray at 0°C. Then, the cells were deposited on polycarbonate filters (Costar, Cambridge, MA; 2 μm) and lysed with 5 ml of lysis buffer containing 2% lauroyl sarcosine, 0.1 M NaCl and 0.025 M EDTA (pH 9.6). Proteinase K (0.5 mg/ml) was added to accomplish proteolytic digestion of the lysate. The eluting solution (pH 12.1) containing 0.1% sodium dodecyl sulfate (SDS), 0.1 M tetrapropyl-ammonium hydroxide and 0.02 M EDTA was then pumped through the filter at 1.5 ml/h. The fractions were collected at 2 h intervals overnight to determine the rate of release of DNA from the filter. Radioactivity of the eluted fractions and filters was determined by liquid scintillation counting. Crosslinking frequencies were calculated by:

$$K_c = \{[(1 - r_0) - (1 - r)]^{1/2} - 1\} \times 600 \quad \text{(rad equivalents)}$$

where r and r_0 are the retention rates of DNA on the filter from the drug-treated and control cells, respectively.

DNA–protein crosslinks were determined by the same method as described for DNA interstrand crosslinks except for two modifications: the cells were irradiated by 3000 rads of X-rays, and no proteolytic digestion using proteinase K was performed. Crosslinking was quantitated by:

$$P_x = [(1 - r)^{-1} - (1 - r_0)^{-1}] \times 3000 \text{ (rads)}$$

where r and r_0 are the retention rates of DNA on the filter from the drug-treated and control cells, respectively.

Results

Intracellular drug accumulation

Figure 1 shows the drug concentration course of the cellular accumulation of L-NDDP and cisplatin in LoVo (A) and LoVo/PDD (B) cells. The cellular accumulation of L-NDDP in LoVo cells was 3- to 5-fold higher than that of cisplatin at 37°C at the different concentrations tested. In addition, the cellular accumulation of L-NDDP in LoVo/PDD cells was only

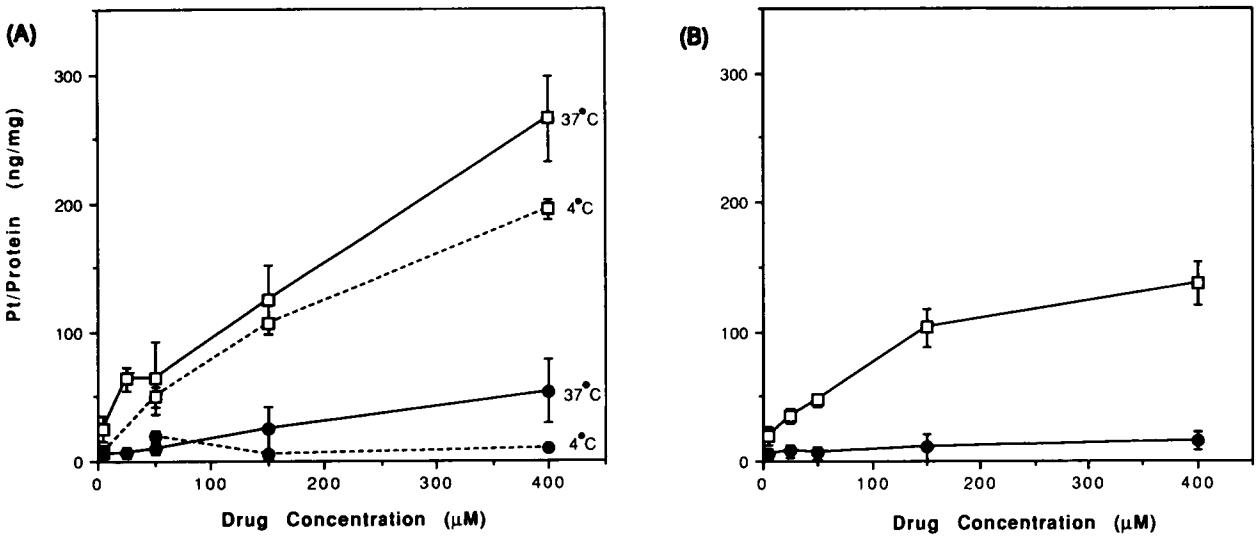

Figure 1. Concentration response of cellular accumulation of L-NDDP and cisplatin in (A) LoVo and (B) LoVo/PDD cells. Both cell lines were exposed to various concentrations of L-NDDP (□) and cisplatin (●) at 4°C (- - -) or 37°C (—) for 30 min. The concentrations of protein and platinum were determined as described in Materials and methods. All points are the mean values from three separate experiments ± SD.

slightly lower than that in sensitive cells, whereas in LoVo/PDD cells, the cellular accumulation of cisplatin was 3- to 5-fold lower than that in sensitive cells.

In order to ascertain whether the drug accumulation of L-NDDP and cisplatin are temperature dependent, we carried out identical concentration course experiments in LoVo cells, but at 4°C. The cellular accumulation of cisplatin was reduced by about 3-fold under these conditions, whereas that of L-NDDP was reduced by only 30%.

Table 1. DNA interstrand and DNA–protein crosslinks induced by L-NDDP and cisplatin in LoVo and LoVo/PDD cells[a]

Drug (μM)		DNA interstrand crosslinks		DNA–protein crosslinks	
		LoVo	LoVo/PDD	LoVo	LoVo/PDD
L-NDDP	5	—	—	288	78
	10	—	—	396	220
	15	13[b]	8	426	204
	30	28	10	—	—
Cisplatin	5	—	—	96	12
	10	—	—	114	24
	15	123	30	362	48
	30	275	32	—	—

[a] Cells were exposed to various concentrations of L-NDDP and cisplatin for 30 min. The determinations of DNA interstrand (K_c) and DNA–protein (P_x) crosslinks were described in Materials and methods.

[b] The data were the mean of two separate experiments expressed in rad equivalents. The standard error was less than 10%.

DNA crosslink formation

Results of DNA interstrand and DNA–protein crosslink formation induced by both agents are shown in Table 1. Cisplatin induced significant DNA interstrand crosslinking in LoVo cells, whereas crosslinking in LoVo/PDD cells was markedly reduced (4- to 8-fold). This result correlates with the 3-fold reduced cytotoxicity of cisplatin against LoVo/PDD cells that we reported previously.[15] In contrast, L-NDDP induced minimal DNA interstrand crosslinking in both cell lines at concentrations that display significant cytotoxicity.

L-NDDP and cisplatin both induced significant DNA–protein crosslinking in LoVo cells, although somewhat less in the case of cisplatin, in good correlation with the slightly increased potency of L-NDDP. In LoVo/PDD cells, DNA–protein crosslinking induced by L-NDDP and cisplatin were about 2- to 3-fold and 5- to 8-fold lower than in LoVo cells, again in good correlation with the observed partial lack of cross-resistance between both agents previously described (resistance indexes: 2 for L-NDDP, 3 for cisplatin).[15]

Discussion

Our results indicate that the lack of cross-resistance properties of L-NDDP is probably mediated by its ability to achieve similar intracellular accumulation in both sensitive and resistant cells, and that both

compounds differ significantly in their transmembrane transport, as well as in their interaction with DNA.

There are several potential explanations for the lack of cross-resistance properties of L-NDDP: (i) the presence of the diaminocyclohexane moiety, which has been previously associated with lack of cross-resistance with cisplatin;[10] (ii) the increased lipophilicity of L-NDDP, which may enhance its cellular transmembrane transport; and (iii) the liposome carrier, which has been shown to be essential in determining the cytotoxicity of the compound through a complex process of intraliposomal drug activation[12] and which could also enhance cellular uptake. Previous cytotoxicity studies in LoVo cells comparing cisplatin, free NDDP in suspension and L-NDDP suggested that the liposome carrier was essential in determining the lack of cross-resistance properties of L-NDDP.[15] Unfortunately, because free NDDP can only be prepared in a gross suspension containing Tween 80, it is difficult to draw a more definite conclusion from those studies. However, it is reasonable to hypothesize that the liposome[5] may markedly enhance the intracellular delivery of NDDP through fusion of the liposome and cell membranes and subsequent exchange of membrane-bound drug.

In previous cellular pharmacology studies of L-NDDP in A2780 and A2780/PDD cells, its lack of cross-resistance was associated with a markedly increased cellular accumulation and DNA platination in resistant cells compared with cisplatin.[16] The current studies in LoVo and LoVo/PDD cells have yielded similar results: the cellular accumulation of L-NDDP in sensitive cells was several-fold higher than that of cisplatin; and the cellular accumulation of cisplatin was reduced by at least 3-fold in resistant cells compared with sensitive cells, whereas that of L-NDDP was only slightly decreased (less than 2-fold). These results correlate with the previously reported cytotoxocity of both agents in these cell lines.[15]

A new and interesting observation from the current study was that the transport system of L-NDDP was only slightly dependent on temperature, in contrast with that of cisplatin, which was highly dependent on temperature. The lack of temperature dependency of the cellular uptake of L-NDDP suggests that the cell membrane transport systems of L-NDDP and cisplatin are qualitatively different. The transport system of L-NDDP is neither saturable nor temperature independent, thus following the pattern of passive diffusion. The transport system of cisplatin is also not saturable at suprapharmacological concentrations but is more temperature dependent, thus suggesting that it has a component of active transport, as suggested by other investigators.[19]

Another important observation already made in the previous studies with A2780 cells is that L-NDDP does not induce significant DNA interstrand crosslinking in either cell line, resistant or sensitive, at cytotoxic concentrations—thus suggesting that this lesion does not play a role in its cytotoxicity. In contrast, in the case of cisplatin, induction of DNA interstrand crosslinking correlated well with its cytotoxicity. There is no explanation available at this point for this surprising finding. The fact that it has been observed in two different cell lines strongly suggests significant differences in the way both drugs interact with DNA. The biological implications of this interaction, in terms of explaining differences in DNA adduct repair for both drugs, remain to be determined. However, L-NDDP and cisplatin were both equally effective in inducing DNA–protein crosslinks in LoVo cells. In resistant cells, L-NDDP was 2- to 3-fold less effective in inducing DNA–protein crosslinks, whereas cisplatin was 5- to 8-fold less effective, in good correlation with the observed resistance indices for both drugs in this cell system.[15]

Our study suggests that L-NDDP is able to overcome cisplatin resistance, probably by bypassing the acquired mechanism of reduced cisplatin accumulation which cells develop upon chronic exposure to cisplatin, since the transmembrane transport systems are significantly different. Differences in the way both drugs interact with DNA have also been detected, although biological implications of these differences remain undefined at this point.

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