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Differential cytotoxicity of trace metals in cisplatin-sensitive and -resistant human ovarian cancer cells

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Cellular resistance of cisplatin is related to various factors such as membrane transformations, changes in cellular transport systems, and an increased efflux of cisplatin by the tumor cells. Deficiencies of one or more trace metals can affect normal physiological functions, leading to altered enzymatic activities and a reduction in immune responses. This *in vitro* investigation was undertaken to study and determine the differential cytotoxicity of certain trace metals in human ovarian cancer cells that were sensitive and resistant to cisplatin. Standard cytotoxicity assays were performed using the neutral red assay. In general, the cisplatin-resistant cells exhibited an increased resistance to the externally supplied trace metals. For both cell lines the rank order of cytotoxicity from greatest to least with the non-essential metals was $Cd^{2+} > Bi^{3+}$, and for the macrometals, $Ca^{2+} > K^{+} > Mg^{2+}$. The transition metals and selenium exhibited a slight difference between the two cell lines with respect to the order of cytotoxicity. The cisplatin-sensitive cells had a rank order of $V^{5+} > Se^{6+} > Cu^{2+} > Zn^{2+} > Fe^{3+}$, from greatest to least toxicity. The cisplatin-resistant cells had a rank order of $Cu^{2+} > V^{5+} > Se^{6+} > Zn^{2+} > Fe^{3+}$. Since trace metals have various functions in maintaining normal health, these results provide key baseline cytotoxicity data and show that, in general, cytotoxic resistance to the trace metals tested followed a pattern similar to cellular cisplatin resistance.

Keywords: cisplatin, ctyotoxicity, metals, ovarian cancer

Introduction

Cisplatin, CDDP, is a platinum based alkylated chemotherapeutic agent that has been used to treat a host of tumors including those of the ovaries, testes, head and neck, prostate and bladder (Loehrer & Einhorn 1984, Ortega *et al*. 1996). CDDP works via crosslinking of DNA by hydrolysis of its Cl-groups (Reedijk 1991, Ortega *et al*. 1996). Once the CDDP has become hydrolyzed, it creates stable bifunctional adducts with DNA, yielding different types of inter- and intra-strand crosslinks (Reedijk 1991, Ortega *et al*. 1996). Platinum–DNA crosslinks cause biochemical lesions such as replication and transcription inhibition (Roberts & Thompson 1979, Ortega *et al*. 1996). The inhibition of DNA synthesis has been observed in various cells following platination, which may explain the antitumor activity of CDDP (Roberts & Thompson 1979, Ortega *et al*. 1996).

The effective use of CDDP is limited by such side effects as emetogenesis, nephrotoxicity, ototoxicity and neurotoxicity (Weiss & Christian 1993). Another factor affecting the use of CDDP is the development of resistance of CDDP by cancer cells (Perez *et al*. 1990, Young 1990, Ortega *et al*. 1996). Many investigators have postulated CDDP resistance to be the result of various modes of cellular changes such as alterations in the cell membrane, changes in amino acid transport systems, and an increased efflux of CDDP.

Membrane transformations may play a central role in the cellular uptake of solutes. Such transformations have been shown to occur in cells that were exposed to CDDP. K562 tumor cells that were

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exposed to CDDP displayed secondary changes on the membrane surface following the crosslinking of CDDP to the DNA (Peschke *et al.* 1990). Cell lines derived from squamous human head and neck cancer cells exhibited a decrease in the amount of the membrane protein, SQMI, when treated with chemotherapy and/or radiation, as compared with cancer cells of untreated patients (Bernal *et al.* 1990). A reduced amount of SQMI was also determined in squamous human head and neck cancer cells which developed resistance to CDDP. Furthermore, these CDDP-resistant cells demonstrated reduced membrane transport of CDDP (Bernal *et al.*, 1990). In a series of murine thymic lymphoma cell lines, however, an increased expression of a surface antigen of approximately 200 kDa, later proven to be a plasma membrane glycoprotein, was observed and found to correlate with the degree of resistance to CDDP (Kawai *et al.* 1990). Yet, in a study using CDDP-sensitive and -resistant human ovarian cancer cells, measurements on the major membrane lipid classes revealed that a decreased CDDP accumulation in the resistant cells was not due to the retardation of passive diffusion of CDDP into the cells (Mann *et al.* 1988). Furthermore, the investigators suggest the possibility of a route other than passive diffusion for some of the the CDDP to enter the cells; a route which is attenuated in the resistant cells (Mann *et al.* 1988).

Changes in cellular amino acid transport systems have also been reported in CDDP-treated cells. L1210 cells, 18-fold resistant to CDDP, yielded an 18-fold decrease in exogenous methionine requirement as compared with the parent CDDP-sensitive cells (Gross & Scanlon 1986). Also noted was a decrease in methionine requirement associated with differences in both methionine metabolism and transport. Therefore, changes in amino acid substrate specificities have been postulated to be a mechanism of resistance to chemotherapeutic agents (Gross & Scanlon 1986). Shionoya & Scanlon (1986) reported that CDDP-resistant K562 cells differed in their sodium dependent neutral amino acid transport properties. More specifically, the uptake of neutral amino acids was significantly decreased in the CDDP-resistant cells.

Finally, an increased efflux of CDDP by tumor cells has been considered as another mechanism of CDDP resistance. To demonstrate this mechanism, Vassilev *et al.* (1987) reconstituted Ca^{2+} channels from L1210 leukemic CDDP-sensitive and -resistant cells. It was determined that the mean open time and the open state probability of reconstituted channels were larger for the CDDP-resistant cells. The cause of these effects could be related to an increased Ca^{2+} release from the intracellular Ca^{2+} stores, accompanied by an enhanced intracytoplasmic Ca^{2+} concentration in CDDP-resistant cells. The changes in the Ca^{2+} concentration, yielding increased mean open times and open state probabilities, may be responsible for the increased cellular efflux of CDDP, thereby developing CDDP resistance (Vassilev *et al.* 1987).

Trace metals are vital to humans to maintain a normal and healthy life. Deficiencies of one or more trace metals can affect normal physiological functions and cause various pathological abnormalities. Among these abnormalities are altered enzymatic activities and a reduction in immune responses.

Enzymatic functions can be altered by the lack of certain trace metals. Ribonucleotide reductase, an enzyme essential for the synthesis of DNA and the growth of lymphocytes, is greatly affected by a decrease in intracellular iron, copper and zinc (Oblender & Carpentieri 1991). L1210 murine leukemic lymphocytes were cultured in media depleted of iron, copper and zinc for prolonged periods. The result was decreased ribonucleotide reductase activity and decreased cell growth (Oblender & Carpentieri 1991).

An inadequate dietary supply of specific trace metals has been shown to be related to decreases in immune responses. Beach *et al.* (1982) fed pregnant mice a zinc deficient diet from day seven of gestation until parturition. The first generation offspring displayed depressed immune function through six months of age. Furthermore, second and third filial generations, fed a normal control diet, continued to express reduced immunocompetence, although to a lesser degree (Beach *et al.* 1982). In another study (Prohaska & Lukasewycz 1981), mice that were fed a purified diet low in copper displayed several pathologies including anemia, hypoceruloplasminemia, decreased liver copper concentration, and increased liver iron concentration. The mice also developed an impaired humoral-mediated immune response. The magnitude of the humoral-mediated immune response was highly correlated with the degree of functional copper deficiency (Prohaska & Lukasewycz 1981).

A deficiency of selenium has been associated with various pathologies including cancer. In the United States, a rise in the incidence of stomach, esophageal and rectal cancers has been associated with a low intake of selenium (Pfeiffer 1978). In the Keshan province of China, selenium deficiency in the diet was found to be related to a form of cardiomyopathy mainly affecting children and young women

(Taylor 1988 Braunwald 1992). Hence, the disease became known as Keshan disease. This disease has been known to occur in other countries such a new Zealand and Finland (Taylor 1988), where the soil is deficient in selenium.

Due to the importance of trace metals in many physiological functions, it is necessary to determine if CDDP-resistant cells exhibit any resistance to various externally supplied trace metals. It was earlier reported that CDDP-resistant human ovarian cancer cells, designated 2780CP20, demonstrated resistance to various chelating agents as compared with the CDDP-sensitive parent cell line, A2780 (Nicholson *et al*. 1993). Therefore, it is important to determine the LD_{50} values of various externally supplied trace metals for the A2780 and 2780CP20 cells in an effort to better understand the possible involvement of certain trace metals in the cellular sensitivity and resistance toward CDDP therapies.

Materials and methods

The OVCAR cell lines, A2780 and 2780CP20, were purchased from the Fox Chase Cancer Center (Philadelphia, PA). The cells were cultured as monolayers and maintained in growth medium which consisted of RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% bovine calf serum (HyClone Laboratories, Logan, UT), 50 $\mu g\ ml^{-1}$ gentamicin (Elkin-Sinn, Cherry Hill, NJ), and 0.9 $\mu g\ ml^{-1}$ insulin (Sigma). Stock cultures were harvested from 25 cm^2 disposable tissue culture treated flasks and maintained in an incubator established with a constant humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cells were harvested using trypsin (Life Technologies, Inc., Gaithersburg, MD) every three to five days and reestablished with an initial seeding density of 8×10^4 cells cm^{-2}.

Cells were obtained from stocks after three days of growth and were seeded at a density of 2×10^4 cells per well in 96-well, flat bottom, tissue culture treated plates. Following 24 h of incubation, the medium was removed. Each well then received 100 µl of fresh growth medium followed by the addition of the desired concentration of a metal solution. Growth medium was then added to each well to achieve a total volume of 300 µl and the plates were placed back into the incubator for another 24 h. At the end of this incubation period, the medium containing the metal was removed and the cells were washed with 200 µl of HBSS.

Cytotoxicity was determined by the neutral red assay, which quantifies the number of viable cells following exposure to the appropriate treatment. The neutral red solution was filtered through a sterile 0.22 µm filter and 200 µl were added to each well. The remainder of the assay procedure was followed as described earlier (Borenfreund & Puerner 1984). The neutral red, formal calcium, and acetic acid–ethanol solutions for the neutral red assay were prepared according to an established protocol (Borenfreund & Puerner 1984). Hanks buffered saline solution (HBSS) was prepared according to Life Technologies, Inc. specifications. A Dynatech MR600 MCC/340 plate reader with a 540 µm wavelength filter was used to read the final absorbances.

The Bi^{3+}, Cd^{2+}, Ca^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, K^{+}, Se^{6+}, V^{5+} and Zn^{2+} stock solutions were prepared in distilled water filtered through 0.22 µl bottle top filters (Corning, PES membrane) into sterile glass bottles, and stored in a refrigerator at 8°C. The counter ion for iron, magnesium and zinc was sulfate; for potassium, it was chloride; and for vanadium, it was potassium. The remaining metal stock solutions were made using atomic absorption standards with nitrate as the counter ion. The concentrations of the stock solutions of the metals were determined such that the volume required to achieve the desired final concentration was not more than 10% of the total volume in any well. These stock concentrations were confirmed by inductively coupled plasma atomic emission spectroscopy or flame atomic absorption to ensure that no change in concentration occurred as a result of filtration or precipitation of metal ions. The pH of all metal stock solutions was between 2 and 5 prior to their addition to the test wells and dilution by culture medium. The final pH of the treated medium was 6.8–7.3. At least two experiments were run for each metal tested. First, a wide range of concentrations was tested, with at least one concentration resulting in no toxicity compared with untreated control samples, and at least one concentration killing close to 100% of the cells. In a second experiment, one high and one low concentration were still used, with several concentrations tested within the range projected, based on extrapolation, to yield a 50% kill of the cells relative to the untreated control samples.

Each concentration was tested in 12 replicate wells and each experiment was run in duplicate. Absorbance values were read on the Dynatech MR600 plate reader and collected using a custom Microsoft Windows computer program (H&A Scientific, Inc., Greenville, NC). These data were then imported to Microsoft Excel for further processing. Cell viability as percent of the control was determined for each concentration tested, plotted, and the concentration required to kill 50% of the cells was determined by linear regression.

Results

Overall, as the concentrations of each metal increased, the number of viable cells (as percent of control) decreased for both the CDDP-sensitive and CDDP-resistant cell lines.

For the non-essential metals studied (Table 1), Cd^{2+} was the most toxic of all the metals tested for both cell lines, while Bi^{3+} was the third most toxic to the A2780 cells and fifth most toxic to the 2780CP20

Table 1. Non-essential metals—LD_{50} values (μM)

Metal	Valence	LD_{50} (μM)	
		A2780	2780CP20
Cadmium	Cd^{2+}	9	31
Bismuth	Bi^{3+}	30	83

Values are the concentration (μM) of each metal which reduced the number of viable cells by 50% of control cultures during the 24 h exposure period.

cells. Furthermore, when the cytotoxicities of Cd^{2+} and Bi^{3+} were compared between the two cell lines, the 2780CP20 cells were more resistant to both metals.

The declining order from most toxic to least toxic for the macrometals only was identical for both cell lines. Ca^{2+} was the most toxic followed by K^+ and then Mg^{2+} (Table 2). However, when comparing the LD_{50} values between the two cell lines, the 2780CP20 cells were more resistant to only Ca^{2+} and K^+. The A2780 cells exhibited greater resistance to Mg^{2+} than did the 2780CP20 cells. This was the only example, of all the metals investigated, in which the A2780 cells were more resistant to the specified ion exposure than were the 2780CP20 cells. For both cell lines, K^+ and Mg^{2+} were the least toxic of all the metals tested and Ca^{2+} was the fourth least toxic of the metals tested. It was interesting to note that while Mg^{2+} and Ca^{2+} are chemically similar, there was a marked difference in their relative cytotoxicities.

For the four transition metals studied and selenium (Table 3), the 2780CP20 cells were more resistant to all five metals than were the A2780 cells. The descending order of toxicity from most to least toxic in the A2780 cells was $V^{5+} > Se^{6+} > Cu^{2+} > Zn^{2+} > Fe^{3+}$. However, the descending order of toxicity from most to least toxic in the 2780CP20 cells was $Cu^{2+} > V^{5+} > Se^{6+} > Zn^{2+} > Fe^{3+}$. While the order of Zn^{2+} and Fe^{3+} was identical between the two cell lines, there was a variation in the order of V^{5+}, Se^{6+} and Cu^{2+}. This was the only variation noted when the descending order of toxicity was compared between the two cell lines.

Table 2. Macrometals—LD_{50} values (μM)

Metal	Valence	LD_{50} (μM)	
		A2780	2780CP20
Calcium	Ca^{2+}	2902	3648
Potassium	K^+	72 032	82 917
Magnesium	Mg^{2+}	106 210	104 862

Values are the concentration (μM) of each metal which reduced the number of viable cells by 50% of control cultures during the 24 h exposure period.

Table 3. Transitional metals—LD_{50} values (μM)

Metal	Valence	LD_{50} (μM)	
		A2780	2780CP20
Vanadium	V^{5+}	14	52
Selenium	Se^{6+}	35	70
Copper	Cu^{2+}	45	51
Zinc	Zn^{2+}	120	201
Iron	Fe^{3+}	4467	5657

Values are the concentration (μM) of each metal which reduced the number of viable cells by 50% of control cultures during the 24 h exposure period.

Discussion

In general, 2780CP20 cells demonstrated an increased resistance over the A2780 cells to the externally supplied trace metals. This could be due to numerous factors including: (1) membrane transformations; (2) differential cellular uptake via changes in cellular amino acid transport systems; (3) efflux differentiation; (4) differential metal binding within the various intracellular organelles; and (5) differential intracellular compartmentalization of the metals due to different intracellular organelle membrane permeabilities.

The importance and success of CDDP in treating various cancers is abundant in the medical literature. However, one problem that continues to occur is the development of resistance to CDDP by the tumor cells. This theme of CDDP-resistance is also well documented in the medical literature. Meanwhile, numerous investigators continue to try and determine the mechanism(s) of resistance, and overcome them.

This study establishes baseline data on differential cellular toxicity of three classes of trace metals in CDDP-sensitive and -resistant human ovarian cancer cells. Furthermore, this report corroborates similar cytotoxic results of polyaminocarboxylic acid metal chelating agents investigated with the same CDDP-sensitive and -resistant cell lines (Nicholson *et al.* 1993).

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