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ORIGINAL ARTICLE

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The effects of terbium on the cellular accumulation of cisplatin in MDA-MB-231 human breast tumor cells

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Abstract *Purpose*: Cisplatin (DDP) is an effective antitumor agent limited in its efficacy by the development of tumor cell resistance. The defective accumulation of DDP has been shown to be a prominent feature in many DDP-resistant cell lines. In an effort to circumvent this problem, we examined the cellular accumulation of DDP in the presence of terbium (Tb^{3+}). We also examined the effects of verapamil on the cellular accumulation of DDP in order to delineate the specific interaction of Tb^{3+} and DDP. All experiments were performed on DDP-sensitive or DDP-resistant MDA-MB-231 human breast tumor cells. *Methods*: The cellular accumulation of DDP and verapamil were determined by electrothermal atomic absorption spectrophotometry. Time-resolved luminescence spectroscopy was used to obtain equilibrium binding constants for the Tb^{3+}/MDA cell complexes. *Results*: We found that 100 μM Tb^{3+} increased DDP accumulation in the parent MDA cell line, 5.7-fold resistant MDA/A13 and 10-fold resistant MDA/CH cells by 56.2 ± 7.4, 71.9 ± 9.4 and $50.8 \pm 9.4\%$, respectively ($P < 0.0001$ for all MDA cell types). In contrast, 20 μM verapamil had no significant effect on DDP accumulation in the MDA cell lines. In addition, a positive correlation between the membrane binding of Tb^{3+} and the cellular accumulation of DDP was found to exist in the parent cell line and sublines ($r = 0.9$). *Conclusions*: In agreement with earlier studies, the plasma membrane of MDA cell lines contain a specific Tb^{3+}-binding protein. Our findings suggest that the Tb^{3+}-binding protein may be intimately associated with the accumulation of DDP in breast tumor cells.

Key words Terbium · Cisplatin accumulation · Cisplatin resistance · Breast cancer

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Introduction

Each year, breast cancer accounts for more than 175 000 deaths across the globe. In 1992, there were 46 300 deaths in the United States alone [27]. Currently, it is believed to be the fourth leading cause of death due to cancer in women [2].

Cis-diamminedichloroplatinum(II) (DDP), generically referred to as cisplatin, is a broad-spectrum, chemotherapeutic agent which has been proven effective in the treatment of testicular and ovarian cancers [21]. Likewise, it has also demonstrated significant efficacy in breast cancer when administered in high doses [16] or as a second-line treatment in combination chemotherapy [25]. Despite its significance in the treatment of cancer, the clinical use of DDP is hindered by the onset of ototoxicity [16, 23], nephrotoxicity [13, 14], and acquired tumor cell resistance. Cisplatin resistance arises as a result of four possible mechanisms. One of these mechanisms includes decreased cellular accumulation of DDP resulting from impaired transport processes of the drug [5]. Elevated levels of cellular glutathione and metallothioneins are also associated with the development of DDP resistance because of their roles in cellular detoxification [3, 6]. In addition, increased DNA repair also contributes to the development of cellular resistance in DDP chemotherapy [17].

In an attempt to understand the DDP accumulation defect in resistant cells, we examined the cellular accumulation of DDP in the presence of terbium ion (Tb^{3+}), a lanthanide metal. Tb^{3+} has been used extensively as a luminescent probe of calcium binding sites in purified protein, membrane, and cellular systems [9–11, 20, 22]. Other notable studies have led investigators to conclude that Tb^{3+} possesses calcium channel blocking activity [11, 18]. Moreover, Canada et al. have suggested that the calcium channel is the specific terbium-binding protein involved in the transport of DDP across the plasma membrane [10, 12]. Aside from the association of Tb^{3+} with the calcium channel, an interaction between the calcium channel and DDP has also been demonstrated [24–26, 28].

As a result of our investigation, we found that Tb^{3+}, through its membrane binding, can enhance the cellular accumulation of DDP in DDP-sensitive and -resistant human breast tumor cells. Furthermore, we believe that this cellular phenomenon is mediated at the extracellular surface of the calcium channel.

Materials and methods

Chemicals

Platinol was obtained from the Bristol Laboratories of Bristol-Myers U.S. Pharmaceutical and Nutritional Group (Evansville, Ind). Terbium(III) chloride hexahydrate was purchased from Aldrich Chemical Company (Milwaukee, Wis). ($\pm$)-Verapamil hydrochloride was purchased from Sigma Chemical Co (St. Louis, Mo.) as a powder. All chemicals were diluted in a buffer solution containing 145.0 m*M* NaCl, 5.0 m*M* KCl, 1.0 m*M* $MgCl_2 \cdot 6H_2O$, 1.0 m*M* $CaCl_2 \cdot 6H_2O$, 6.0 m*M* glucose and 10.0 m*M* Hepes. The final solution was adjusted to pH 7.2 by the addition of 12 *N* NaOH.

Breast tumor cell lines

The MDA-MB-231 human breast tumor cell line originated from a 51-year-old Caucasian woman who underwent a radical mastectomy for advanced breast cancer [8]. The 5.7-fold acutely-resistant MDA/A13 daughter subline was established by monthly exposures to high concentrations of DDP as previously described [4]. A 10-fold chronically resistant daughter subline, designated MDA/CH, was developed by frequent exposures to low concentrations of DDP [4].

Determination of DDP accumulation in the presence of Tb^{3+} or verapamil

For each experiment, cells were seeded into 60-mm plastic culture dishes and grown to subconfluence as monolayers in improved minimum essential medium supplemented with 10% fetal bovine serum and 50 μg/ml gentamicin at 37 °C in an atmosphere containing 5% CO_2. The cells were incubated with 100 μ*M* DDP and various concentrations of Tb^{3+} (0.0, 10, 50, 100 or 500 μ*M*) for 1 h at 37 °C; or the cells were incubated with 100 μ*M* DDP and various clinically relevant concentrations of verapamil (0.0, 2, 10, 20 or 102 μ*M*) for 1 h at 37 °C. After medium removal, the cells were washed four times with ice-cold phosphate buffered saline. Next, 800 μl Triton-X-100 was added to each dish and the cells were scraped from the bottom surface with a rubber policeman. The detached cells were frozen in snap-cap tubes. Upon thawing, the cells were sonicated with a Vibra Cell Sonicator (Sonics & Material's Danbury, Ct.), and 200 μl of the cell lysate was analyzed for platinum by electrothermal atomic absorption spectrophotometry (Perkin-Elmer, Norwalk, Ct.). DDP accumulation is expressed as picomoles of cisplatin per milligram of protein [7].

Determination of the binding of Tb^{3+} to human breast tumor cells

Cells were grown as monolayers in 75-cm^2 plastic culture flasks in minimum essential medium supplemented with 10% fetal bovine serum, nonessential amino acids, sodium pyruvate and insulin at 37 °C in an atmosphere containing 5% CO_2. The cells were harvested using trypsin/EDTA and counted via trypan blue exclusion. Time-resolved luminescence intensities were measured with a Spex Industries Fluorolog-2 Model F212I spectrofluorometer and phosphorimeter accessory, using 3.0-ml samples of cell suspension at 50 000 cells/ml in 1 × 1-cm quartz cuvettes. The samples were excited by a pulsed xenon lamp (7 W). The excitation wavelength and entrance slit were set at 280 nm and 5 nm, respectively. The emission wavelength and exit slit were set at 543 nm and 10 nm, respectively. The gating of the sample photomultiplier was delayed by 50 μs after the flash of the xenon lamp. This eliminated error due to light scattering and/or interferences due to the intrinsic fluorescence of the cells. All measurements were taken on MDA-MB-231 cells suspended in cell buffer solution, while under gentle stirring at 37 °C. Aliquots of 3.0 m*M* Tb^{3+} (2–10 μl) were automatically dispensed into each cuvette. The final Tb^{3+} concentrations ranged from 2 to 97 μ*M*. The cell concentration was held constant and the total volume change for the titration series did not exceed 5%. Each titration series was repeated three times. Likewise, a set of blank samples containing only buffer solution (no cells) was titrated with Tb^{3+}. Binding data are presented as the intensity of the Tb^{3+}/cell complex plotted against the corresponding Tb^{3+} concentration.

Statistical analysis

Accumulation data are presented as the mean of the percentage change in absolute value (Δ%) of six different experiments $\pm$ the standard error of the mean (SEM). The Δ% value for DDP accumulation was calculated by subtracting the accumulation of DDP without Tb^{3+} from the accumulation of DDP with Tb^{3+}, dividing the difference by the accumulation of DDP without Tb^{3+} and multiplying the quotient by 100. For all of the binding experiments, equilibrium binding constants (ΔI_{max} and K_d) were calculated by nonlinear regression analysis, and are expressed for each cell line as the mean of six to nine experiments $\pm$ SEM. The normalized change in intensity (ΔI) of the Tb^{3+}/cell complex is proportional to the amount of Tb^{3+} bound to the cell, and is equal to the intensity of Tb^{3+} in the absence of cells subtracted from the intensity of Tb^{3+} in the presence of cells, and normalized to the maximum intensity change of the Tb^{3+}/cell complex at 97 μ*M* Tb^{3+}. The percentage of Tb^{3+}, bound to the cells ($\Delta I/\Delta I_{max}$) at a particular Tb^{3+} concentration, i.e. the fraction of cellular-bound Tb^{3+}, is ΔI divided by ΔI_{max}, i.e. the maximum change in intensity when all of the Tb^{3+} binding sites are saturated, and multiplied by 100.

Unpaired Student *t*-tests were performed on all binding data to detect statistically significant differences between the equilibrium binding constants of DDP-sensitive and DDP-resistant cell types. One- and two-way analyses of variance (ANOVA) were performed on all accumulation data to examine the significant effects of Tb^{3+} and/or cell type on the cellular accumulation of DDP. The accepted level of significance for all statistical analyses was set at $P < 0.05$. The Pearson Product-Moment Correlation was used to establish

and estimate the dependence of the percentage change in DDP accumulation on the percentage of Tb^{3+} bound to the cell. The degree of this association is expressed by the product-moment correlation coefficient (r).

Results

Defective accumulation is believed to be a major determinant of DDP resistance. The cellular accumulation of DDP in the cell lines selected for resistance by acute and chronic DDP exposures (at 0.0 μM Tb^{3+}) were only 4.6 $\pm$ 2.8% and 7.9 $\pm$ 6.0%, respectively, less than that in the parent MDA cell line (data not shown). Nonetheless, the cellular accumulation of DDP was increased by Tb^{3+}. Fig. 1 illustrates the enhancement above baseline levels of the cellular accumulation of DDP as a result of Tb^{3+} treatment. All three MDA cell types were treated with 100 μM DDP and various concentrations of Tb^{3+} for 1 h at 37 °C. The Δ% DDP accumulation for the parent MDA cell line increased from 5.6 $\pm$ 4.3% at 10 μM Tb^{3+} to 77.7 $\pm$ 9.6% at 500 μM Tb^{3+} ($P < 0.0001$). In the MDA/A13 cell line, the Δ% cellular accumulation of DDP increased from 9.4 $\pm$ 2.9% at 10 μM Tb^{3+} to 92.9 $\pm$ 3.2% at 500 μM Tb^{3+} ($P < 0.0001$). Likewise, there was a statistically significant increase in Δ% DDP accumulation for the MDA/CH cell line from 8.6 $\pm$ 4.9% at 10 μM Tb^{3+} to 62.9 $\pm$ 4.2% at 500 μM Tb^{3+} ($P < 0.0001$).

A two-way ANOVA revealed that among the three cell types, there was a significant group difference in the enhancement of DDP accumulation with respect to the Tb^{3+} concentration ($P < 0.001$). To determine which group(s) differed from the others in their responsiveness to the stimulatory influences of Tb^{3+}, a multiple comparison procedure was performed. The MDA/CH cell line was found to be less susceptible to the stimulatory effects of Tb^{3+} than either the parent MDA cell line ($P < 0.05$) or the MDA/A13 subline ($P < 0.05$). However, there was no clear significant difference in the cellular accumulation of DDP between the MDA and MDA/A13 cells ($P > 0.05$).

Unlike Tb^{3+}, the cellular accumulation of DDP was not significantly affected by verapamil from 0 to 20 μM, as shown in Fig. 2 ($P > 0.6$). All three MDA cell types were exposed to 100 μM DDP and various concentrations of verapamil for 1 h at 37 °C. However, there was a significant change in the response of the cells to 102 μM verapamil. The Δ% DDP accumulation increased to 18.8 $\pm$ 3.8% for MDA cells ($P < 0.0001$), 13.1 $\pm$ 3.5% for MDA/A13 ($P < 0.001$) and to 17.6 $\pm$ 1.6% for MDA/CH cells ($P < 0.0001$).

The luminescent intensity measurements of the Tb^{3+}/MDA cell complexes increased hyperbolically as a function of the Tb^{3+} concentration (Fig. 3). The maximum luminescent intensity (ΔI_{max}) is the theoretical intensity yielded when all of the Tb^{3+} binding sites are completely saturated, and is proportional to the maximum number of Tb^{3+} binding sites on the plasma membrane. ΔI_{max} values for the parent MDA cell line, the acutely selected MDA/A13 cell line and the chronically selected MDA/CH cell line were 124.8 $\pm$ 8.0, 111.7 $\pm$ 11.2 and 70.8 $\pm$ 11.7, respectively. For the MDA/A13 cell line, ΔI_{max} was 10.5% less, but not significantly different from the ΔI_{max} for the parent MDA cell line ($P > 0.3$). On the other hand, the ΔI_{max}

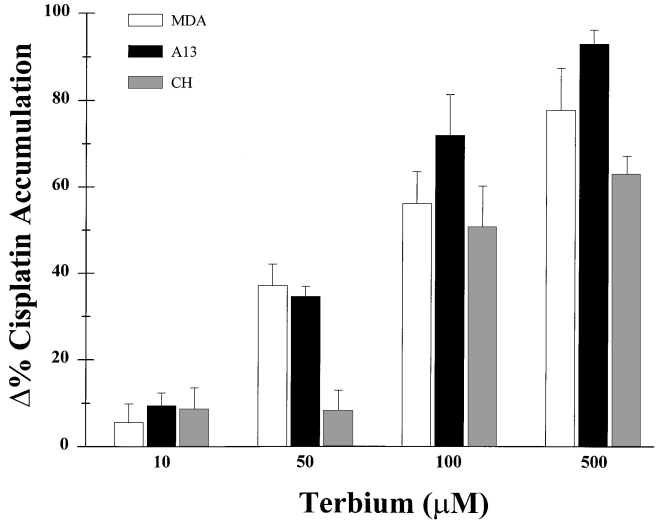

Fig. 1 Percentage change in cisplatin accumulation in cisplatin-sensitive and cisplatin-resistant MDA human breast tumor cells in the presence of Tb^{3+}. All cells were treated with 100 μM cisplatin for 1 h at 37 °C. The baseline levels of accumulation of cisplatin for the MDA, MDA/A13 and MDA/CH cells were 467 $\pm$ 39, 453 $\pm$ 48 and 423 $\pm$ 41 pmol/mg protein, respectively. Each bar represents the mean $\pm$ SEM of six experiments

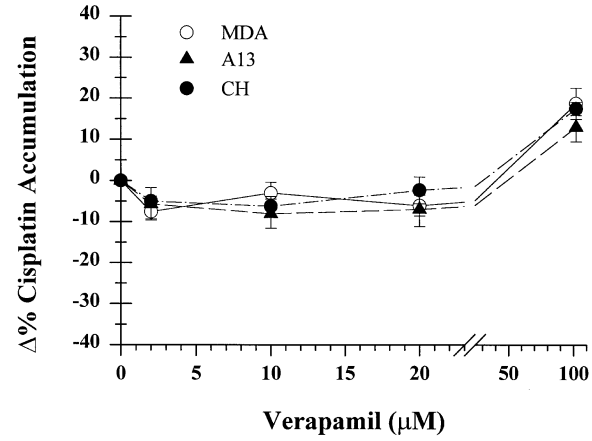

Fig. 2 Percentage change in cisplatin accumulation in cisplatin-sensitive and cisplatin-resistant MDA human breast tumor cells in the presence of verapamil. All cells were treated with 100 μM cisplatin for 1 h at 37 °C. Each value is the mean $\pm$ SEM of six experiments

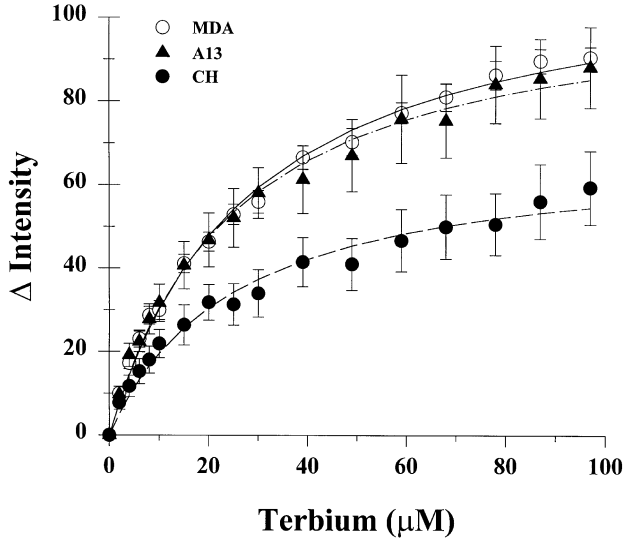

Fig. 3 Binding of Tb^{3+} to cisplatin-sensitive and cisplatin-resistant MDA human breast tumor cells. Each value is the mean ± SEM of 6 to 12 experiments

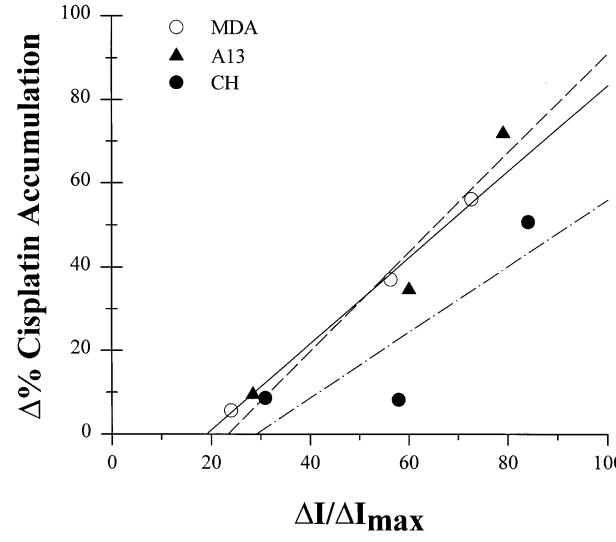

Fig. 4 Association of Tb^{3+} binding with cisplatin accumulation in MDA human breast tumor cells. Each value is the mean of 6 accumulation experiments and 8 to 18 binding experiments

for the chronically resistant MDA/CH subline was significantly less, by 43.3%, than that of the parent MDA cell line ($P < 0.001$). K_d is the theoretical concentration of Tb^{3+} that yields a half-maximal intensity, and represents the affinity of Tb^{3+} for its binding sites on the membrane. The K_d for the receptor binding of Tb^{3+} was similar in all there MDA cell types, averaging 33.8 ± 4.2 μ*M* ($P > 0.6$). The receptor binding of Tb^{3+} as a function of the Tb^{3+} concentration was positively correlated with the cellular accumulation of DDP in the presence of Tb^{3+} for the parent MDA cell line ($r = 0.999$), the DDP-resistant MDA/A13 cell line ($r = 0.968$) and the DDP-resistant MDA/CH ($r = 0.857$) cell line. Fig. 4 illustrates the linear relationship between the amount of bound Tb^{3+} and the accumulation of DDP at corresponding Tb^{3+} concentrations. As the percentage of bound Tb^{3+} ($\Delta I/\Delta I_{max}$) increased, there was an accompanying increase in the cellular accumulation of DDP (Δ%).

Discussion

The precise mechanism whereby breast tumor cells become resistant to cisplatin treatment has not been clearly demonstrated [1]. Improvement in DDP efficacy in breast cancer treatment lies in delineating the mechanism(s) of resistance, whether it is a single mechanism or a combination of the four known mechanisms [1].

Defective accumulation of DDP is believed to be one of the major mechanisms of DDP resistance [1]. In this study, however, no statistically significant reduction in DDP accumulation in the absence of Tb^{3+} was observed for either the acutely or chronically selected resistant breast tumor cell lines. Therefore, it is assumed that DDP resistance in these MDA cell lines may be due to either an increase in the detoxification of DDP via elevated glutathione or metallothionein levels, or an increased repair of DDP-induced DNA damage.

Canada et al. have reported that Tb^{3+} can enhance the cellular accumulation of DDP in human ovarian cancer cell lines [12]. Likewise, our results indicated that Tb^{3+} can stimulate the cellular accumulation of DDP in DDP-sensitive and -resistant MDA human breast tumor cells. There was also a significant difference in the extent to which Tb^{3+} was able to stimulate the accumulation of DDP, depending on the cell type. Our results suggest that MDA/CH cells were the least sensitive to the stimulatory effects of Tb^{3+}, indicating that certain cells may be more or less susceptible to the influences of Tb^{3+}.

Cardinal to the stimulatory action of Tb^{3+} is its receptor binding. The receptor binding of the Tb^{3+} mirrored the Tb^{3+}-induced accumulation of DDP in the MDA cells. The MDA/CH breast tumor cells, with the least Tb^{3+}-induced increase in DDP accumulation, were found to have the lowest value of ΔI_{max}. This suggests that the lower susceptibility of the MDA/CH cells to the stimulatory influences of Tb^{3+} on the cellular accumulation of DDP may have been due to their significantly lower number of Tb^{3+} binding sites. In both DDP-sensitive and DDP-resistant breast tumor cells, a positive correlation was found to exist between the amount of bound Tb^{3+} and the cellular accumulation of DDP. This further implies that the number of Tb^{3+} binding sites on the membrane of breast tumor cells determines their predisposition to the influences of

Tb^{3+}. The apparent dissociation constants for Tb^{3+} were the same in all three cells lines, suggesting that the affinity of Tb^{3+} for its binding site is not altered from parent to daughter cell line. Hence, we believe that the receptor binding of Tb^{3+} may mediate the stimulatory effect of Tb^{3+} on the cellular accumulation of DDP.

The results of this investigation revealed two opposing trends. In the presence of Tb^{3+}, DDP accumulation significantly increased, but, in the presence of verapamil, the cellular accumulation of DDP was not altered. The increase in DDP accumulation at 102 μM verapamil is attributed to an increase in membrane permeability due to the cytotoxic effects of verapamil on the cells. Of the two proposed binding sites associated with the calcium channel, the primary site of action for verapamil and other organic calcium channel blockers, is at the intracellular surface of the plasma membrane near the inner mouth of the calcium channel, where it functions in closing the calcium channel gate [15, 19]. In addition, verapamil and other organic calcium channel blockers act upon L-type calcium channels which are not associated with breast tumor cells. On the other hand, inorganic calcium channel blockers such as Tb^{3+} are believed to interact primarily at the extracellular surface of the membrane. Hence, the primary site of action of Tb^{3+} is assumed to be at the outer mouth of the calcium channel at its selectivity filter. Therefore, we believe that these two calcium channel blockers, which represent different classes of blockers, do not interact with the calcium channel in the same manner and as a result have different effects on DDP accumulation. Moreover, these results suggest that not all calcium channel blockers can influence the cellular accumulation of DDP.

The precise mechanism of action of Tb^{3+} on DDP accumulation in tumorigenic cells remains to be elucidated. Since DDP has been shown to behave as a calcium channel blocker and Tb^{3+} has a higher affinity for the calcium channel than DDP, Tb^{3+} may enhance the cellular influx of DDP by electrostatically repelling DDP through the calcium channel and into the cytoplasm [10, 12, 24, 28]. However, we must also consider that Tb^{3+} may inhibit the efflux of DDP from the cells; or perhaps both mechanisms occur simultaneously. Regardless of the mechanism, the enhanced cellular accumulation of DDP in breast tumor cells centers around the receptor binding of Tb^{3+}. Therefore, because of its potential significance to understanding and reversing DDP accumulation defects, further studies probing the biochemical and biophysical nature of the Tb^{3+}-binding protein are warranted.

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