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ORIGINAL ARTICLE

Teresa L. Fuller · Robert G. Canada

Enhancement of cisplatin cytotoxicity by terbium in cisplatin-resistant MDA/CH human breast cancer cells

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Abstract *Purpose*: The development of cisplatin resistance is a major problem in the treatment of cancer patients with cisplatin chemotherapy. The membrane binding of terbium (Tb^{3+}) has been shown to increase the cellular accumulation of cisplatin in breast cancer cells. Therefore, the ability of Tb^{3+} to modulate the cytotoxicity of cisplatin was investigated in cisplatin-sensitive (MDA) and cisplatin-resistant (MDA/CH) MDA-MB-231 human breast cancer cells. *Methods*: The cytotoxic parameters of cisplatin were determined using live cell microfluorometry and median effect analysis. *Results*: MDA/CH cells (IC_{50} = 142 ± 9 μ*M*) were found to be approximately 3.3-fold more resistant to cisplatin than MDA cells (IC_{50} = 43.5 ± 3.0 μ*M*). In both cell lines, the IC_{50} value for cisplatin was reduced two-fold in the presence of 80 μ*M* Tb^{3+}, thus indicating that the cytotoxicity of cisplatin is increased by Tb^{3+}. The cytotoxic activity of cisplatin alone was observed to be 5.7 and 1.6 times more potent than that of Tb^{3+} alone in MDA and MDA/CH cells, respectively. Combination index analyses revealed that the interaction between cisplatin and Tb^{3+} was only synergistic at very low indices of cell death in MDA cells. However, in MDA/CH cells, the two drugs were synergistic up to intermediate levels of cell death. *Conclusions*: Our results suggest that the enhancement of cisplatin cytotoxicity by Tb^{3+} is more effective in cisplatin-resistant MDA/CH cells than in cisplatin-sensitive MDA cells. Therefore, terbium is potentially useful in cisplatin combination therapy for breast cancer patients, especially for those patients who have developed resistance to the drug.

Key words Terbium · Cisplatin · Cytotoxicity · Resistance · MDA-MB-231

T.L. Fuller · R.G. Canada (✉)
Laboratory of Biophysical Cytochemistry,
Department of Physiology and Biophysics,
Howard University College of Medicine,
Washington, DC 20059, USA
Tel. +1-202-806-4521; Fax +1-202-806-4479

Introduction

Cancer of the breast is the most common malignancy of women in the United States [1, 10, 13]. Cisplatin chemotherapy has been found to be very effective against metastatic breast cancer as first-line therapy, having an overall response rate approaching 50% [17, 19, 20]. Even though cisplatin has shown significant efficacy in many tumors, a major drawback to its use is the development of resistance. Calcium channel blockers have been shown to enhance the cytotoxicity of cisplatin in resistant cancer cells [16, 18]. For example, Onoda et al. [14] have shown that nifedipine significantly enhances the antitumor action of cisplatin against primary Blba-Pt tumors and their pulmonary metastases. The enhancement of cisplatin cytotoxicity by nifedipine was suggested to be due to the interaction of nifedipine with a specific unidentified 'target site' in the plasma membrane, which is independent of the inhibitory effect of nifedipine on the calcium channel [14].

The cellular binding of Tb^{3+} has been shown to enhance the accumulation of cisplatin in cisplatin-sensitive 2008 and cisplatin-resistant C13* human ovarian cancer cells [4]. Mack et al. [11] have demonstrated that Tb^{3+} can increase cisplatin accumulation in cisplatin-sensitive and cisplatin-resistant variants of MDA-MB-231 human breast cancer cells. They found a positive correlation between the binding of Tb^{3+} and the cellular accumulation of cisplatin. We report here that the cytotoxic activity of cisplatin is enhanced by Tb^{3+} in MDA-MB-231 human breast cancer cells.

Materials and methods

Chemicals

Cis-diamminedichloroplatinum(II) ($Pt(NH_3)_2Cl_2$), generically called cisplatin, and terbium trichloride hexahydrate ($TbCl_3{\cdot}6H_2O$) were purchased from Sigma Chemical Company (St. Louis, Mo.). Calcein-AM was purchased from Molecular Probes (Eugene, Ore.).

Tb^{3+} stock solutions were prepared with pure 18 MΩ-cm water. Cisplatin stock solutions were prepared fresh 24 h before each experiment in a HEPES buffered saline solution (HBS) to prevent the hydrolyzation of cisplatin. HBS was composed of 145 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgCl_2 \cdot 6H_2O$, 1 m*M* $CaCl_2 \cdot 6H_2O$, 6 m*M* glucose and 10 m*M* HEPES; the final pH of the solution was set at 7.2 to minimize the precipitation of terbium hydroxides.

Cell lines

The cisplatin-sensitive MDA-MB-231 parent cell line (MDA) and the chronically resistant daughter subline (MDA/CH) were a gift from Dr. Paul A. Andrews (Food and Drug Administration, Rockville, Md.) [11]. The cells were grown in 75-cm^2 flasks with 90% minimal essential medium with Earle's salts and L-glutamine supplemented with 10% fetal bovine serum, nonessential amino acids, sodium pyruvate and 10 μg/ml insulin at 37 °C and in an atmosphere containing 5% CO_2.

Live cell microfluorometry

The live cell microfluorometric assay is a modification of the Live/Dead Viability/Cytotoxicity Assay developed by Molecular Probes as previously described [15]. Briefly, MDA or MDA/CH cancer cells were plated in Corning six-well culture plates at a density of 10 000 cells per well. Cells were washed with HBS, then treated with 0, 10, 20, 30, 40 or 60 μ*M* cisplatin for the MDA cells, or with 0, 20, 40, 80, 160 or 240 μ*M* cisplatin for the MDA/CH cells, for 60 min at 37 °C in an atmosphere containing 5% CO_2 in the absence and presence of 80 μ*M* Tb^{3+} in HBS. A concentration of 80 μ*M* Tb^{3+} was chosen for these studies because, at this concentration, Tb^{3+} does not appreciably affect cell survival. After drug removal, the cells were washed once with HBS and incubated for 3 days in ordinary supplemented medium at 37 °C in an atmosphere containing 5% CO_2. Afterwards, the cells were washed with Dulbecco's phosphate-buffered saline (PBS), and treated for 60 min with 3 μ*M* calcein-AM in PBS at room temperature. Calcein fluorescence was used as a probe to detect live cells. The fluorescence emission intensity of each well was read at 530 nm (λ_{exc} = 485 nm) with a Cambridge Technology Microplate Fluorometer Model 7630 (Watertown, Mass.). The live cell intensity without drug(s) was regarded as the live cell intensity at 100% cell survival. The index of survival at each drug concentration was calculated as the live cell intensity with drug divided by the live cell intensity without drug.

Data were graphically visualized by plotting the index of cell survival against the drug concentration. The theoretical curves were constructed using commercially available software [6]. The concentrations of drug that inhibited cell growth by 20, 50, and 80% (i.e. the IC_{20}, IC_{50}, and IC_{80} values, respectively) were determined by median effect analysis. In addition, combination index analysis was employed to obtain a quantitative expression of the interaction between cisplatin and Tb^{3+} [6]. Data values are reported as the mean ± standard error of the mean (SEM). Student's unpaired *t*-test was used to evaluate differences between pairs of means. Two-way analysis of variance (ANOVA) was used to evaluate differences between pairs of survival curves. The degree of significance of differences in the data is presented as probability (*P*) values; the accepted level of significance is $P < 0.05$. The number of experiments is indicated in each figure legend.

Results

Figure 1 displays the indices of cell survival for cisplatin-sensitive MDA cells with respect to Tb^{3+} alone, and as a function of the cisplatin concentration in the absence and presence of 80 μ*M* Tb^{3+}. Two-way ANOVA revealed that there was a statistically significant difference between the survival curve with cisplatin alone and that with 80 μ*M* Tb^{3+} ($P < 0.001$). Table 1 shows that the IC_{20} and IC_{50} values of cisplatin, when combined with 80 μ*M* Tb^{3+}, were 3.2- and 2.1-fold less than their corresponding control values with 0.0 μ*M* Tb^{3+}, respectively ($P < 0.001$ for both parameters). The cisplatin IC_{80} value did not significantly decrease in the presence of 80 μ*M* Tb^{3+} ($P > 0.2$). The IC_{50} value of Tb^{3+} alone in MDA cells was determined as 250 ± 13 μ*M* (Fig. 1). Thus, cisplatin is approximately 5.7 times more potent than Tb^{3+} in MDA cells ($P < 0.001$). The data suggest that Tb^{3+} can enhance the cytotoxic activity of cisplatin at low to intermediate concentrations of cisplatin, with the greatest enhancement of cisplatin cytotoxicity occurring at higher indices of cell survival.

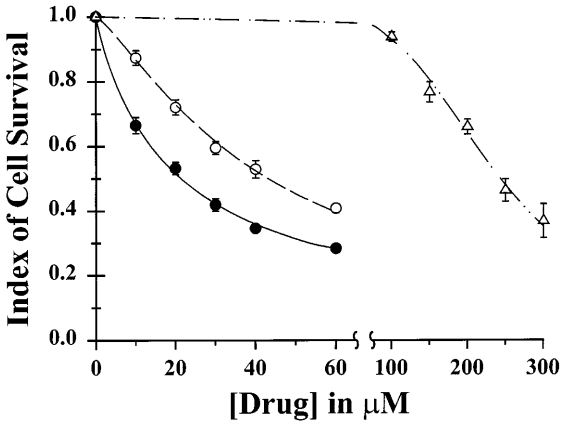

Fig. 1 Index of cell survival of cisplatin-sensitive MDA cells with respects to Tb^{3+} alone, and as a function of the cisplatin concentration with or without 80 μ*M* Tb^{3+}. Each point of the dose-response curve for Tb^{3+} alone (△) represents the mean and SEM of six experiments performed in duplicate. Each point of the dose-response curves for cisplatin alone (○) or with 80 μ*M* Tb^{3+} (●) corresponds to the mean and SEM of 11 or 5 experiments, respectively, performed in duplicate

The effects of Tb^{3+} on the cytotoxicity of cisplatin in MDA/CH cells are displayed in Figure 2. Based on the IC_{50} value for cisplatin, the MDA/CH cells were 3.3-fold more resistant to cisplatin than the MDA cells ($P < 0.001$). However, the cytotoxicity of Tb^{3+} alone in the MDA/CH cells (IC_{50} = 227 ± 13 μ*M*) was similar

Table 1 The effects of Tb^{3+} on the cytotoxicity of cisplatin in MDA and MDA/CH cells. Each value represents the mean ± SEM; n = 5–12

Cell line	$[Tb^{3+}]$ (μ*M*)	Cisplatin cytotoxic parameters (μ*M*)		
		IC_{20}	IC_{50}	IC_{80}
MDA				
	0	15.9 ± 1.7	43.5 ± 3.0	133 ± 20
	80	5.0 ± 0.7	21.2 ± 1.3	95.7 ± 7.8
MDA/CH				
	0	48.2 ± 4.3	142 ± 9	444 ± 41
	80	20.6 ± 7.6	72.6 ± 11.6	318 ± 47

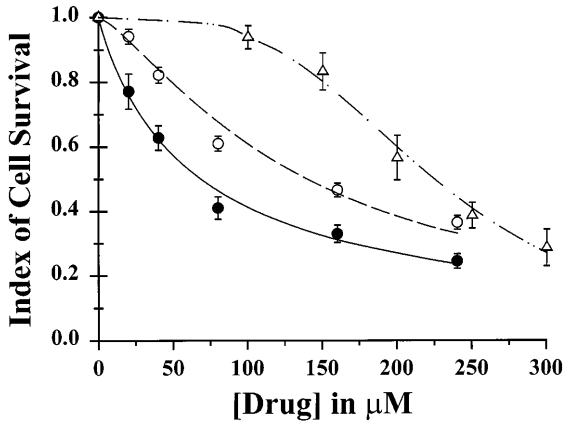

Fig. 2 Index of cell survival of cisplatin-resistant MDA/CH cells with respect to Tb^{3+} alone, and as a function of the cisplatin concentration with or without 80 μM Tb^{3+}. Each point of the dose-response curve for Tb^{3+} alone (△) represents the mean and SEM of six experiments performed in duplicate. Each point of the dose-response curves for cisplatin alone (○) or with 80 μM Tb^{3+} (●) corresponds to the mean and SEM of 12 or 6 experiments, respectively, performed in duplicate

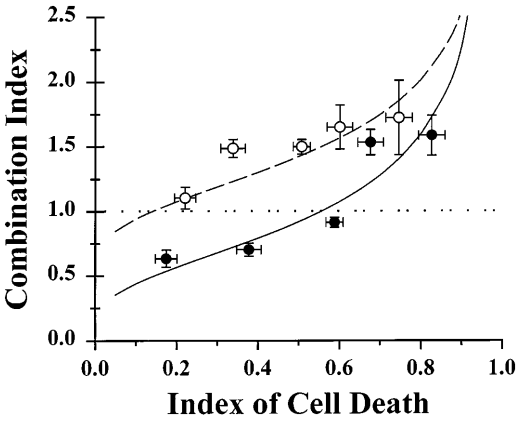

Fig. 3 Combination index analysis of cisplatin combined with Tb^{3+} in a 1:6 ratio for MDA cells (○), and a 1:1.5 ratio for MDA/CH cells (●). Cisplatin and Tb^{3+} were combined in the ratio of their respective IC_{50} values in each cell line. If the combination index at a particular fraction affected is: (a) less than 1, the drugs are synergistic, (b) equal to 1, the drugs are additive, or (c) greater than 1, the drugs are antagonistic. Each point represents the average of six experiments. Horizontal error bars correspond to the SEM for the fraction of cells affected by drugs. Vertical error bars correspond to the SEM for the combination index values

to that in the MDA cells ($P > 0.2$). Consequently, cisplatin was approximately 1.6 times more potent than Tb^{3+} in the MDA/CH cells ($P < 0.001$). The survival curve of cisplatin alone was significantly different from that of cisplatin combined with Tb^{3+} ($P < 0.001$). As shown in Table 1, the IC_{20} and IC_{50} values of cisplatin combined with 80 μM Tb^{3+} were reduced by a factor of 2.4 and 2.0, respectively, when compared with the corresponding values with 0.0 μM Tb^{3+} (IC_{20} $P < 0.004$ and IC_{50} $P < 0.001$). However, no clear significant difference existed between the IC_{80} values of cisplatin alone versus that of cisplatin combined with 80 μM Tb^{3+} ($P > 0.07$). Similar to the findings in cisplatin-sensitive MDA cells, Tb^{3+} significantly enhanced the cytotoxic activity of cisplatin in MDA/CH cells at high to intermediate indices of cell survival.

The nature of the interaction between cisplatin and Tb^{3+} was explored by combination index analysis, as shown in Fig. 3. In the MDA cells, cisplatin and Tb^{3+} interacted nearly additively or slightly synergistically at very low indices of cell death (i.e. below 0.2). The degree of antagonism between the two drugs increased in a monotonic fashion at intermediate levels of cell kill (from approximately 0.3 to 0.7) and rose exponentially as the index of cell death increased beyond 0.7. On the other hand, in MDA/CH cells, cisplatin and Tb^{3+} showed synergism up to an index of cell death of 0.5. The degree of synergism between the two drugs increased monotonically as the level of cell death decreased from 0.4 to 0.05 (note that as the combination index approached zero the level of synergism increased; the synergism between cisplatin and Tb^{3+} varied inversely with cell kill), whereas, at indices above 0.6, the degree of antagonism increased exponentially with the index of cell death. In both cell lines, there was a clear demarcation between synergism and antagonism. Moreover, the synergism between cisplatin and Tb^{3+} in the MDA/CH cell line persisted at a wider range of cell death than in the MDA cell line.

Discussion

Cisplatin is an effective chemotherapeutic agent against breast cancer, but the development of cellular resistance to cisplatin is a hindrance to its use. In this report, we present evidence indicating that Tb^{3+} can circumvent cisplatin resistance in human breast cancer cells. Further, the antitumor activity of Tb^{3+} is considerably less than that of cisplatin in these cells. The mechanism by which Tb^{3+} enhances the cytotoxicity of cisplatin is not known. However, Mack et al. [11] have shown that the accumulation of cisplatin in human breast cancer cells is increased in the presence of Tb^{3+}. They found that 100 μM Tb^{3+} can enhance the cellular accumulation of cisplatin by 60% and 50% in MDA and MDA/CH cells, respectively [11]. The binding of Tb^{3+} and cisplatin to a specific terbium/cisplatin binding protein is suggested to increase the transport of cisplatin across the plasma membrane [2, 3, 8]. The cytotoxicity of cisplatin has been shown to be proportional to the amount of cisplatin accumulated in the cell [9]. Theoretically, this would increase the cellular accumulation of cisplatin and permit more cisplatin to reach its target organelle, thus enhancing cisplatin cytotoxicity.

The MDA/CH subline was found to be approximately threefold more resistant to cisplatin than the parental MDA line. Cellular resistance to cisplatin occurs as a result of impaired transport of cisplatin and/or increased cellular production of glutathione and metallothioneins. In addition, increased DNA repair also

contributes to the development of cisplatin resistance (the repair of damaged DNA is always enhanced and activated first in cisplatin-resistant cells) [5]. Mack et al. [11] have reported that MDA/CH cells do not show an accumulation defect with cisplatin. Therefore, they concluded that the primary mechanism of cisplatin resistance in MDA/CH cells is due to an increased detoxification of cisplatin via elevated levels of glutathione or metallothionein [11].

Paltoo and Canada [15] have demonstrated that Tb^{3+} can enhance the cytotoxicity of cisplatin in FaDu human head and neck cancer cells at low cisplatin concentrations. Likewise, our results demonstrated that the cytotoxicity of cisplatin is enhanced by Tb^{3+} at low and intermediate levels of cisplatin-induced cell death, in both cisplatin-sensitive and cisplatin-resistant breast cancer cells. The two drugs exhibited synergism at a wider range of cell death in the cisplatin-resistant MDA/CH breast cancer cells than in the cisplatin-sensitive MDA cells. These findings suggest that the enhancement of cisplatin cytotoxicity by Tb^{3+} is more effective in cisplatin-resistant MDA/CH cells than in cisplatin-sensitive MDA cells.

Cisplatin-based combination therapy has been shown to be more effective than cisplatin alone [12]. In early clinical trials, the combination of cisplatin and paclitaxel (Taxol), increased the survival of patients with stage III and stage IV ovarian cancer from 24 months to 38 months [12]. Unfortunately, some problems are associated with the clinical use of Taxol including severe toxicity and neutropenia. Taxol is almost 100 times more potent than cisplatin [21]. Tb^{3+} has a relatively low toxicity in animals [7]. The concentration of Tb^{3+} used in our investigation was considerably less than the lethal levels found in animals. The LD_{50} values for intraperitoneally injected $TbCl_3$ are typically in the range of 333–550 mg/kg in mice [7]. In the normal human adult, 80 μM Tb^{3+} in the blood would give levels of 0.3–0.6 mg/kg. The coadministration of cisplatin and Tb^{3+} will permit the treatment of breast cancer patients with lower doses of cisplatin. A desired goal of a high concentration of cisplatin is to kill 100% of the cancer cells. However, concentrations of cisplatin high enough to kill all of the cancer cells would increase the severe side effects of the drug such as ototoxicity and emesis. The antagonism of cisplatin cytotoxicity by Tb^{3+} may reduce the adverse effects of cisplatin at high concentrations. Further, in a heterogeneous cancer cell population, some cells may not be sensitive to cisplatin. The combination of cisplatin with Tb^{3+} may eliminate these resistant cells. Studies attempting to potentiate the effects of Tb^{3+} on cisplatin cytotoxicity are currently under investigation in our laboratory.

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