The Effect of Benzaldehyde on Terbium Fluorescence Enhancement in Human NHIK 3025 Cells Treated with *cis*-Dichlorodiammineplatinum(II)*

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Abstract

As a measure of platinum–DNA interaction we have examined the enhancement of terbium fluorescence in human cells treated with *cis*-dichlorodiammineplatinum(II) (cisplatin) in the absence and presence of benzaldehyde. While benzaldehyde alone (up to 5 mM) did not affect terbium fluorescence registered from cells, cisplatin alone caused a dose-dependent enhancement of terbium fluorescence. Simultaneous treatment of cells with cisplatin and benzaldehyde, however, suppressed terbium fluorescence enhancement, indicating a reduction in platinum–DNA interaction. Nevertheless, by use of flameless atomic absorption spectroscopy as a measure of cell-associated platinum, it was shown that a considerable amount of platinum was taken up during the simultaneous treatment with benzaldehyde and cisplatin although platinum uptake was even higher during treatment with cisplatin alone. Thus, while benzaldehyde reduces cellular uptake of platinum it also influences platinum binding to DNA.

Introduction

Benzaldehyde, the simplest aromatic aldehyde, has been shown to possess antitumor activity [5]. This aldehyde inhibits cell-cycle progression by having a primary effect on protein synthesis [6, 7]. In studies using cultured human cells treated with the chemotherapeutic drug cisplatin, we have shown that the simultaneous presence of benzaldehyde protects cells from inactivating effects of cisplatin [2]. Furthermore, aldehyde-mediated protection from cisplatin toxicity was shown to involve Schiff base formation with amino groups of the cell cytoplasmic membrane [3]. We have also recently shown that benzaldehyde protects cells against inhibition of cell-cycle progression due to treatment with cisplatin, and that the most likely cause for this protection is a reduction in cellular uptake of cisplatin [4]. In our efforts to determine the mechanisms by which benzaldehyde protects cells against cisplatin toxicity, we here study the amount of cisplatin bound to DNA in the absence and presence of benzaldehyde by recording the enhancement of terbium (Tb^{3+}) fluorescence in treated cells.

The technique of fluorescence enhancement is based on the following findings regarding Tb^{3+}. The lanthanide ion Tb^{3+} has little intrinsic fluorescence; however, on binding to DNA Tb^{3+} fluorescence is enhanced. Topal and Fresco [10] have previously shown that Tb^{3+} interacts with phosphate groups of non-hydrogen-bonded residues of nucleic acids (principally guanine) resulting in substantial enhancement of Tb^{3+} fluorescence. An increase in Tb^{3+} fluorescence was further correlated to the appearance in DNA of single-stranded S_1-nuclease sensitive areas by Ringer *et al.* [8]. Arquilla *et al.* [1] found a 12-fold enhancement of Tb^{3+} fluorescence when cisplatin reacted with double-stranded DNA, and they concluded that cisplatin produces single-stranded regions in DNA which become accessible to Tb^{3+} ions, thus producing fluorescence enhancement. Since cytotoxic drugs, such as cisplatin, may change the secondary structure of DNA, then Tb^{3+} fluorescence may be used as a measure of drug binding.

Experimental

Cells

Cells of the human line NHIK 3025, originating from a cervical carcinoma *in situ*, were grown as monolayers as described previously [6, 7]. Cells were treated with cisplatin alone or cisplatin + benzaldehyde for 2 h in suspension. The cells were then washed in phosphate-buffered saline (PBS) containing 1 mM EDTA, resuspended in PBS +

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5 mM EDTA, and finally fixed by injection into a 4-fold volume of methanol at −20 °C. After 15 min at −20 °C, the cells were collected by centrifuging at 350 g and resuspended in an RNase solution (60 μg RNase/ml PBS + 1 mM EDTA). The cells were then incubated at room temperature overnight in the dark. The next day the cells were centrifuged and washed once in 20 mM Tris-HCl, pH 7.4, then resuspended in 20 mM Tris-HCl to a density of 1×10^6 cells/ml.

Terbium Fluorescence

Tb^{3+} was added to cell suspensions from a 0.01 M $TbCl_3$ stock solution in 20 mM Tris-HCl. The final Tb^{3+} concentration was 100 μM. The cells were incubated 30 min to 1 h in the dark at room temperature before delayed fluorescence spectra of cell samples were recorded as in ref. 9. Emission scans were made in the phosphorescence mode with a delay time of 1.0 ms and a gating time of 1.0 ms. Excitation of samples was at 290 nm. Spectra were recorded, stored and manipulated using a Perkin-Elmer LS-5 fluorescence spectrophotometer controlled by a Perkin-Elmer 3600 data station and PECLS software. All measurements were made at room temperature.

Atomic Absorption Spectroscoy

Cell-associated platinum as measured by flameless atomic absorption spectroscopy of whole cells after cisplatin treatment was used as an estimate of the amount of platinum bound in cells. Aliquots of cells treated with cisplatin alone or cisplatin + benzaldehyde, as indicated above, were dissolved in HNO_3. Cell-associated platinum was analysed using a Varian SpectrAA30 atomic absorption spectrometer equipped with a GTA-96 graphite tube atomizer [4].

Drugs and Chemicals

$TbCl_3$ was purchased from Riedel de Haen, West Germany. Benzaldehyde was from Koch-Light Laboratories Ltd., UK, and was vacuum-distilled before use. *cis*-Dichlorodiammineplatinum(II) and RNase (type I-A) were purchased from Sigma Chemicals, U.S.A.

Results

Figure 1 shows delayed fluorescence emission spectra of three cell samples, a control (panel A) and two cisplatin-treated samples (panels B and C). In all samples cell number, Tb^{3+} concentration and fluorescence scaling are equal. As previously described, Tb^{3+} possesses little intrinsic fluorescence (data not shown); however, on binding to DNA there is an enhancement in Tb^{3+} fluorescence which, from panel A, is detectable even in human cells. The time decay of the fluorescence of Tb^{3+} bound to cellular DNA had a similar lifetime to that of Tb^{3+} bound to calf thymus DNA [9]. Furthermore, treatment of cells with cisplatin caused a dose-dependent further enhancement of Tb^{3+} fluorescence (panels B and C).

Figure 2 shows the enhancement of Tb^{3+} delayed fluorescence in human NHIK 3025 cells treated with

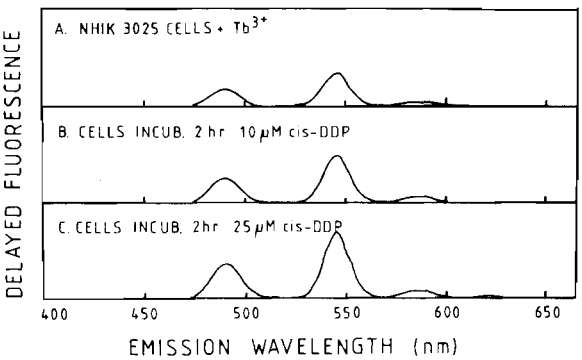

Fig. 1. Tb^{3+} delayed fluorescence intensity (relative units) as a function of the emission wavelength for NHIK 3025 human cells fixed in methanol and treated with RNase. Tb^{3+} was added to (A) 1×10^6 control cells; (B) 1×10^6 cells treated with 10 μM cisplatin, 2 h; or (C) 1×10^6 cells treated with 25 μM cisplatin, 2 h. Samples were excited at 290 nm and emission spectra were recorded at room temperature.

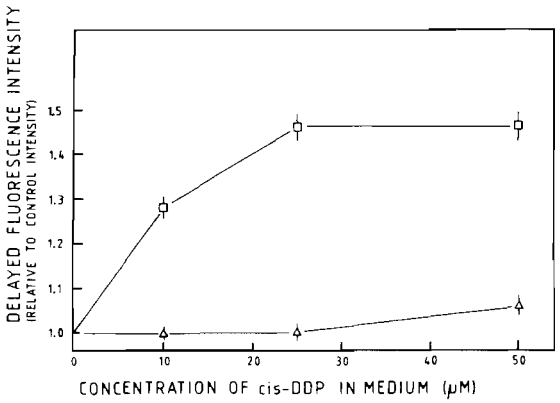

Fig. 2. Delayed fluorescence intensity (relative to control intensity) of NHIK 3025 cells treated for 2 h with cisplatin alone (□) or in simultaneous combination with 5 mM benzaldehyde (△) as a function of the concentration of cisplatin in cell culture medium. NHIK 3025 cells were treated for 2 h with varying concentrations of cisplatin with or without the simultaneous presence of benzaldehyde. Cells were then washed, fixed and digested with RNase as described in 'Experimental'. Tb^{3+} was added to a final concentration of 100 μM and delayed fluorescence intensity of cell samples excited at 290 nm was recorded at an emission wavelength of 544 nm. Vertical bars represent SE of three determinations.

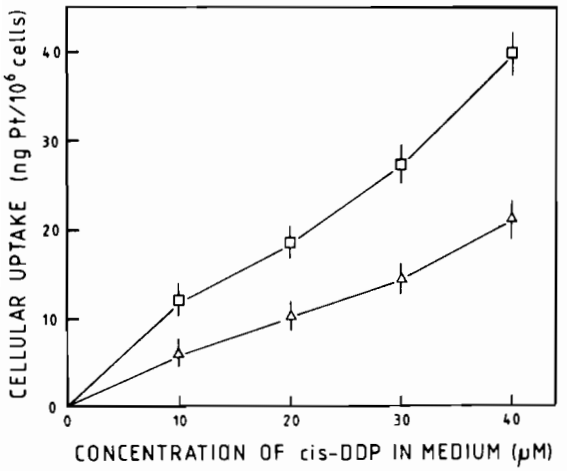

Fig. 3. Cellular uptake of platinum measured by flameless atomic absorption spectroscopy as a function of the concentration of cisplatin in cell culture medium. Cells were treated for 2 h without (□) or with the simultaneous presence of 5 mM benzaldehyde (△). PBS-washed cell samples were dissolved in HNO_3 and the amount of cell-bound platinum was determined in triplicate. Platinum content was analysed at 265.9 nm and background correction with a pulsed deuterium lamp was utilized. SE of three determinations is represented by vertical bars.

cisplatin as a function of the concentration of cisplatin. The RNase digestion step effectively removed all fluorochrome-binding RNA in cells in order to insure that the measured fluorescence intensity was due only to Tb^{3+} inteacting with DNA. From these data there is a significant enhancement of Tb^{3+} fluorescence intensity which increases with increasing concentration of cisplatin up to 25 μM. There is, however, no increase in fluorescence enhancement of Tb^{3+} when cisplatin treatment is given simultaneously with 5 mM benzaldehyde.

The amount of cell-associated platinum as measured by flameless atomic absorption spectroscopy in cells treated with various concentrations of cisplatin in the cell culture medium is shown in Fig. 3. Both in the absence and presence of benzaldehyde the amount of cell-associated platinum increased with increasing concentration of cisplatin in the medium. However, the data clearly indicate that the cellular uptake of platinum is lower in the presence than in the absence of benzaldehyde.

Discussion

In a previous report [9], we showed that delayed fluorescence may be a better measure of Tb^{3+} binding than steady-state fluorescence, since intrinsic fluorescence due to DNA or other cell components would have a shorter lifetime than Tb^{3+} fluorescence. Our results, shown in Fig. 1, show that Tb^{3+} fluorescence can be measured in intact, fixed cells and that the lifetime of fluorescence decay of Tb^{3+} binding to DNA was similar in cells and in DNA solutions [9]. Furthermore, binding of cisplatin induced an enhancement in Tb^{3+} delayed fluorescence (measured at 544 nm) which was large enough to allow estimation of DNA–platinum interaction occurring within treated cells (Figs. 1 and 2).

Tb^{3+} delayed fluorescence was suppressed in cells treated with cisplatin + benzaldehyde (Fig. 2), indicating that with this combined treatment there was little measurable cisplatin–DNA interaction. Nevertheless, from Fig. 3 the uptake of platinum was considerable during the combined treatment (although it was even higher during treatment with cisplatin alone). There are three possible explanations for the differences seen in Figs. 2 and 3. First, cisplatin may possibly not reach the DNA within the cell nucleus. This could be a result of benzaldehyde slowing the uptake process in such a manner that cisplatin becomes fixed (or bound) to structures on the plasma membrane or in the cytoplasm. Secondly, cisplatin may be reaching the cell nucleus, but in a form that does not react with DNA. Thirdly, benzaldehyde itself may alter the secondary structure of DNA such that cisplatin cannot bind to DNA. We are currently investigating each of these possible mechanisms of action.

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