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Impact of intracellular chloride concentration on cisplatin accumulation in sensitive and resistant GLC4 cells

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Abstract Resistance to cisplatin [*cis*-diamminedichloro-platinum(II), CDDP] chemotherapy is a major problem in the clinic. Understanding the molecular basis of the intracellular accumulation of CDDP and other platinum-based anticancer drugs is of importance in delineating the mechanism of resistance to these clinically important therapies. Different molecular mechanisms may coexist, but defective uptake of CDDP is one of the most consistently identified characteristics of cells selected for CDDP resistance. We have studied the impact of intracellular chloride concentration on platinum-based compound accumulation in the human GLC4, GLC4/CDDP, and K562 tumor cell lines. We show that (1) a decrease of intracellular chloride concentration yielded an increase of CDDP accumulation and vice versa and (2) the intracellular chloride concentration in GLC4/CDDP cells is higher than in sensitive cells, whereas CDDP accumulation shows the opposite behavior. The identification of chloride as a critical determinant of CDDP intracellular accumulation and the molecular mechanisms by which CDDP-resistant cells modulate chloride concentration may allow alternative therapeutic approaches. Our findings indicate that increase of intracellular chloride concentration may be a major determinant of CDDP resistance.

Keywords Cisplatin · Resistance · Chloride

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Introduction

cis-Diamminedichloroplatinum(II) (CDDP), or cisplatin, is an important chemotherapeutic agent used in the treatment of a wide variety of solid tumors [1, 2]. It is a key component in the treatment of both testicular and ovarian cancer. Many lung, head, and neck cancer chemotherapy regimens are based on this drug. Unfortunately, certain, types of cancer cells have high intrinsic resistance to the drug or resistance to the drug frequently appears in initially responsive tumors [3–5]. In a given CDDP-resistant cell type, mechanisms from a number of different categories may contribute to resistance, resulting in a great variation and complexity in the CDDP-resistant phenotype [6–8]. However, defective uptake of CDDP has been one of the most consistently identified characteristics of cells selected for CDDP resistance both in vivo and in vitro [9–11].

The mechanisms of resistance to CDDP have received extensive interest because their elucidation will lead to the development of more effective treatment for cancer. In this context, Lee et al. [12] have recently made the very interesting observation that the KCP-4 human epidermoid

cancer cell line, which serves as a model for acquired resistance to CDDP, has virtually no volume-sensitive outwardly rectifying (VSOR) Cl^- channel activity and they concluded that reduction of VSOR Cl^- channel activity contributes to CDDP resistance.

We reasoned that a reduction of Cl^- channel activity should lead to a variation of the intracellular Cl^- concentration and the aim of this study was to determine whether an altered intracellular Cl^- concentration might contribute to modification of CDDP uptake and toxicity in cells. For this purpose we used three different cell lines: the human small cell lung cancer cell line GLC4 and the GLC4/CDDP subline, which serve as a model of acquired resistance of CDDP, and the human erythroleukemia cell line K562. Bumetanide, furosemide, amiloride, and ouabain were used to modulate intracellular Cl^- concentrations. We observed that under conditions in which these modulators decrease the intracellular Cl^- concentration, CDDP incorporation increases and vice versa. In addition, we found that in the GLC4/CDDP cell line the intracellular Cl^- concentration is almost twice that observed in the GLC4 cell line, while CDDP incorporation in these cells is only approximately 50% of that observed in GLC4 cells. Our conclusion is that intracellular Cl^- concentration plays a key role in intracellular CDDP accumulation, toxicity, and resistance.

Materials and methods

Drugs and chemicals

CDDP (1 mg mL^{-1}, approximately 3.3 mM in 154 mM NaCl) was obtained from Dakota Pharm. CDDP in the solid state was provided by Strem Chemicals. Amiloride, furosemide, bumetanide, and ouabain (hereafter named "modulators") were purchased from Sigma. They were dissolved in dimethyl sulfoxide at concentrations such that the amount of dimethyl sulfoxide added to the cells was lower than 0.2%. N-(6-Methoxyquinolyl)acetoethyl ester (MQAE) was purchased from Fluka and was dissolved in water. All the reagents were of the highest quality available and deionized double-distilled water was used throughout the experiments.

Cell lines and cultures

The GLC4 cell line was derived from pleural effusion of a patient with small cell lung carcinoma, in the laboratory of one of us (E. de Vries) [13]. The GLC4/CDDP cells were obtained in the same laboratory by continuous exposure to CDDP. The GLC4 line grew partly floating, partly attached. The GLC4/CDDP cell line grows primarily attached. The doubling time of the latter cell line is longer

(30 h) than that of the former (22 h). There was no clear morphological difference appreciable with light microscopy between the two cell lines. The glutathione (GSH) level was significantly raised in the CDDP-resistant cell line. No amplification of the MDR gene or expression of P-glycoprotein was found in the GLC4/CDDP subline [14]. The K562 cell line is a highly undifferentiated erythroleukemia originally derived from a patient with chronic myelogenous leukemia [15]. The three cell lines were cultured in RPMI 1640 with GlutaMAX^{TM}I (GIBCO) medium supplemented with 10% fetal calf serum (GIBCO) at 37 °C in a humidified incubator with 5% CO_2. Culture initiated at a density of 10^5 cells mL^{-1} grew exponentially to about 10^6 cells mL^{-1} in 3 days. For short-term measurements of platinum accumulation, in order to have enough cells in the exponential growth phase, the culture was initiated at 5×10^5 cells mL^{-1} and used 24 h afterwards, when there were about 8×10^5 cells mL^{-1}. Cultured cells were counted with a Coulter counter before use. The viability of the cells, tested by trypan blue exclusion, was always greater than 95%.

For growth inhibition studies, two different procedures were used: (1) 1×10^5 cells mL^{-1} were cultured for 72 h in the presence of various concentrations of CDDP and (2) 1×10^5 cells mL^{-1} were incubated for 1 h with CDDP, then the cells were washed and resuspended in fresh medium. The sensitivity of the drug was evaluated by the drug concentration that inhibits cell growth by 50% (IC_{50}).

Synthesis of aquated complexes

CDDP (0.3 g, 1 mmol) was mixed with AgNO$_3$ (0.34 g, 2 mmol) in water (10 mL). The mixture was stirred for 15 h under light protection and at 25 °C. The AgCl precipitate formed was separated by filtration. The filtrate was maintained in the dark for 5 h and then filtered. A solution containing different proportions of $[Pt(NH_3)_2(H_2O)_2]^{2+}$, $[Pt(NH_3)_2(H_2O)(OH)]^+$, and $[Pt(NH_3)_2(OH)_2]$ was thus obtained [16]. This mixture is hereafter named "aqua-Pt." It should be noted that small amounts of cis-$[PtCl(H_2O)(NH_3)_2]$ and unreacted CDDP may also be present in this solution [20].

Platinum uptake and retention by cells

The short-term accumulations of CDDP or aqua-Pt were studied in buffers, at 37 °C, under protein-free and amino acid free conditions. For platinum accumulation assays, unless specified, cells were treated with 100 μM of the platinum-based compounds. In most cases, incubation of cells with CDDP was performed at 37 °C in N-(2-hydroxyethyl)piperazine-N'-ethanesulfonic acid (Hepes) isotonic buffer solutions (pH 7.3) with the following

Table 1 Composition (mM) of buffers

Buffer	Na$^+$	K$^+$	Ca^{2+}	Mg^{2+}	Cl$^-$	Gluconate	Methane sulfonate
1	132	3.5	1	0.5	138.5	–	–
2	132	3.5	1	0.5	6.5	–	132
3	132	3.5	1	0.5	3	135.5	–
X	132	3.5	1	0.5	X	$138.5 - X$	–

Solutions contained 5 mM glucose; pH 7.4 was buffered with 20 mM N-(2-hydroxyethyl)piperazine-N'-ethanesulfonic acid. X is 3, 8, 16, 23, 30, 36, 43, 50, 57, 70, 83, and 97 mM, respectively

composition: 20 mM Hepes, 132 mM NaCl, 3.5 mM KCl, 1 mM CaCl$_2$, 0.5 mM MgCl$_2$, and 5 mM glucose (buffer 1, Table 1). Incubation of cells with aqua-Pt was performed in low-Cl$^-$ buffer, in which NaCl was substituted by equimolar concentrations of either sodium gluconate (buffer 3, Table 1) or sodium methanesulfonate (buffer 2, Table 1). Sodium methanesulfonate was made by titration of methanesulfonic acid with NaOH prior to addition to the buffer [17]. In buffers 3 and X, KCl was also substituted by potassium gluconate.

Cells were incubated in buffer either in the absence or in the presence of one of the modulators: 1 mM furosemide, 10 μM bumetanide, 1 mM amiloride, or 200 μM ouabain. After specified time intervals, aliquots containing 10^6 cells were taken and, to eliminate possible noncovalent membrane association, washed twice with phosphate saline buffer. The pellet obtained after centrifugation was digested with nitric acid (65%) and the cellular platinum content was quantified using a Zeeman atomic absorption spectrometer (Varian SpectrAA 220). The local drug concentration inside one cell was calculated taking into account the mean volume of one cell, 10^{-12} L.

Intracellular Cl$^-$ concentration measurements with use of MQAE

The fluorescent dye MQAE was used according to a method developed by Verkman et al. [18]. When excited at 365 nm, MQAE emits light at 460 nm. The fluorescence intensity of this probe changes with changing Cl$^-$ concentration as a result of collisional quenching by halide ions, i.e., the intensity of the emitted light is inversely related to the Cl$^-$ concentration of the MQAE-containing solution. The mathematical description of collisional quenching is given by the Stern–Volmer equation: $F_0/F_X = 1 + K_{SV}^* [quencher]$ where F_0 and F_X denote the fluorescence intensity in the absence and presence of the quencher (Cl$^-$) at concentration X respectively. K_{SV} is the Stern–Volmer constant characteristic for the pair of dye/quencher (MQAE/Cl$^-$). K_{SV} depends—often significantly—on the environment (e.g., in vitro or intracellular).

To determine absolute intracellular quencher (e.g., Cl$^-$) concentrations, K_{SV} has to be determined in the cell of interest. This can be achieved by adding molecules which promote the ion exchange between the interior of the cell and the surrounding solution. MQAE can be passively loaded into cells and loading was accomplished by adding a small aliquot of a stock solution of MQAE in water (final concentration 3 mM) to cells, 10^7 mL^{-1}, suspended in Hepes-Na$^+$ buffer (buffer 1, Table 1). The cell suspension was incubated for 0.5–1 h at 37 °C.

Fluorescence measurements were made with a Perkin-Elmer LS50B spectrofluorometer equipped with a heated cuvette holder set to maintain a temperature of 37 °C. A fluorescence excitation wavelength of 365 nm and an emission wavelength of 460 nm were used.

The steady-state intracellular Cl$^-$ concentration in cells was determined by a null-point measurement technique [19]. Typically, 200 μL of the cell suspension loaded with MQAE was centrifuged, rapidly washed with Cl$^-$-free buffer, and added to 2 mL of gluconate/Cl$^-$ buffer containing different Cl$^-$ concentrations (Table 1). Fluorescence intensity data from cell suspensions were then collected for 100 s (F_i) before the addition of 20 μL Triton X-100 (4%) and for 200 s (F_X) afterward. The Triton X-100 treatment equilibrates the intracellular and extracellular Cl$^-$ concentration by permeabilizing the cell plasma membrane. The values ($F_X - F_i$)/F_i were plotted against the extracellular Cl$^-$ concentration X to calculate the null-point intracellular Cl$^-$ concentration using simple regression analysis.

K_{SV} was experimentally determined for cells from the same set of experiments.

$$F_X = F_0/(1 + K_{SV}X). \tag{1}$$

The F_X values were plotted against X to calculate K_{SV}. F_0 is the fluorescence intensity when $X = 0$

Intracellular Cl$^-$ determination in the presence of modulators

Cells were loaded with MQAE, as described earlier, before chemical treatments. Cells were suspended in buffer 1 containing one of the following modulators: 1 mM furosemide, 10 μM bumetanide, 1 mM amiloride, or 200 μM ouabain and incubated at 37 °C for 0–2 h. For measurements, 200 μL of the cell suspension was centrifuged, washed once with Cl$^-$-free buffer, suspended in 2 mL of buffer 1, and fluorescence F_{Mod} was recorded. The fluorescence intensity F_i of the cell suspension incubated exactly under the same conditions but in the absence of any modulator was recorded simultaneously. The intracellular Cl$^-$ concentration in cells after chemical treatment was determined by the combination of the Stern–Volmer equation written once for cells without the modulator and once for cells with the modulator.

In the absence of modulator

$$F_0/F_i = 1 + K_{SV}[Cl_i^-], \tag{2}$$

where $[Cl_i^-]$ is the intracellular Cl^- concentration.

In the presence of modulator

$$F_0/F_{Mod} = 1 + K_{SV}[Cl_i^-]_{Mod}, \tag{3}$$

where $[Cl_i^-]_{Mod}$ is the intracellular Cl^- concentration in the presence of the modulator. The combination of these two equations yields

$$r = (1 + K_{SV}[Cl_i^-]) / (1 + K_{SV}[Cl_i^-]_{Mod}), \tag{4}$$

where $r = F_{Mod}/F_i$ and

$$[Cl_i^-]_{Mod} = 1/r([Cl_i^-] + 1/K_{SV}) - 1/K_{SV}. \tag{5}$$

Knowing K_{SV} and $[Cl_i^-]$, the measurements of r yielded $[Cl_i^-]_{Mod}$.

Results

Rates of CDDP accumulation in GLC4, GLC4/CDDP, and K562 cells either in the absence or in the presence of modulators

In typical experiments, GLC4 cells (10^6 mL^{-1}) were incubated with 100 μM CDDP in buffer 1. Figure 1 shows
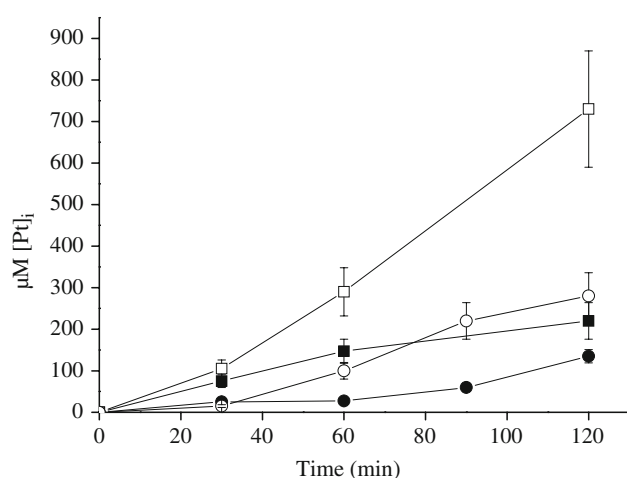

Fig. 1 Time course of *cis*-diamminedichloroplatinum(II) (CDDP) uptake into GLC4 and GLC4/CDDP cells in the absence or presence of furosemide. Cells (10^6 mL^{-1}) were incubated with 1×10^{-4} mol L^{-1} CDDP at 37 °C in the absence or presence of the modulator. After various intervals of time, the intracellular concentrations of platinum were determined (the cell volume equal to 10^{-12} L). GLC4 (*filled squares*), GLC4 plus 1 mM furosemide (*open squares*), GLC4/CDDP (*filled circles*), GLC4/CDDP plus 1 mM furosemide (*open circles*). The values represent the mean ± the standard deviation (SD) of at least three independent experiments performed on different days

the plot of the intracellular concentration of platinum recorded inside the cells as a function of the time of incubation of these cells with the drug. Three independent experiments, at least, were performed on three different days. The uptake of CDDP increased with time. This uptake was modified when cells were incubated with one of the four molecules (modulators) that we used in this study. As can be seen in Fig. 1, platinum accumulation increased when GLC4 cells were incubated with 1 mM furosemide. It also increased when cells were incubated with 10 μM bumetanide or 1 mM amiloride, but decreased when these cells were incubated with 200 μM ouabain. The intracellular platinum concentration measured after 2-h incubation is reported in Table 2. We verified that at these concentrations the modulator toxicity to GLC4 cells was negligible. Similar experiments were performed with GLC4/CDDP cells (Fig. 1) in the absence of modulator; platinum accumulation in GLC4/CDDP is only approximately 50% of that observed in GLC4 cells. The addition of 1 mM furosemide (Fig. 1) or 10 μM bumetanide increased intracellular CDDP accumulation (Table 2). Owing to the toxicity of amiloride and ouabain to GLC4/CDDP cells, the effects of these modulators on CDDP incorporation in these cells could not be analyzed.

To ensure that the effects of modulators observed in GLC4 cells were not specific for the cell type, similar experiments were performed with K562, a completely different cell line. Similar results were obtained (Fig. 2).

Rates of aqua-Pt accumulation in GLC4, GLC4/CDDP, and K562 cells either in the absence or in the presence of modulators

Experiments similar to those described in the previous section were performed with aqua-Pt, with the difference being that incubation was performed in Cl^--depleted buffer (buffer 2 or 3, Table 1). It is known that many buffer components may readily coordinate to Pt(II) [20]. In the present work, gluconate and methanesulfonate were used to substitute Cl^- in Cl^--free buffer. In some cases nitrate was also used to substitute Cl^- (data not shown). In the three cases the data were very similar, suggesting that if these molecules interact with aqua-Pt, the quantities of complexes formed have little impact on the data.

As previously observed [11], the uptake of platinum is much faster when aqua-Pt is used instead of CDDP. Platinum accumulation was very similar in GLC4 and GLC4/CDDP cells and no significant modification of this accumulation was observed in the presence of 1 mM furosemide (Fig. 3) or any of the other modulators (Student's t test, $P > 0.5$). Similar data (not shown) were obtained with K562. Evidence that the aqua-Pt got inside the cell as opposed to simply reacting with the cell surface is given

Table 2 Intracellular Cl^- and platinum concentrations in GLC4, GLC4/cis-diamminedichloroplatinum(II) (CDDP), and K562 cells incubated for 2 h with different modulators

Modulator	GLC4		GLC4/CDDP		K562	
	$[Cl_i^-]$ (mM)	$[Pt_i]$ (μM)	$[Cl_i^-]$ (mM)	$[Pt_i]$ (μM)	$[Cl_i^-]$ (mM)	$[Pt_i]$ (μM)
None[a]	36 ± 5	230 ± 40	60 ± 10	135 ± 40	34 ± 5	105 ± 20
Furosemide[b] (1 mM)	$25 \pm 4^*$	$730 \pm 100^{**}$	$37 \pm 5^*$	$280 \pm 50^*$	$25 \pm 4^*$	$240 \pm 40^{**}$
Bumetanide[b] (10 μM)	29 ± 4	$650 \pm 100^*$	49 ± 5	$310 \pm 50^{**}$	28 ± 4	$170 \pm 25^*$
Amiloride[b] (1 mM)	$22 \pm 4^*$	$740 \pm 120^{**}$	ND	ND	$23 \pm 4^*$	$230 \pm 40^{**}$
Ouabain[b] (200 μM)	$50 \pm 5^*$	$120 \pm 20^*$	ND	ND	$58 \pm 5^{**}$	$32 \pm 5^{**}$

The intracellular Cl^- concentration in the presence of the modulator was calculated using Eq. 4. Cells were incubated with 100 μM CDDP. The values represent means ± the standard deviation of n independent experiments performed on different days. Statistical significance between control cells and modulator-treated cells was determined by Student's t test

ND not determined

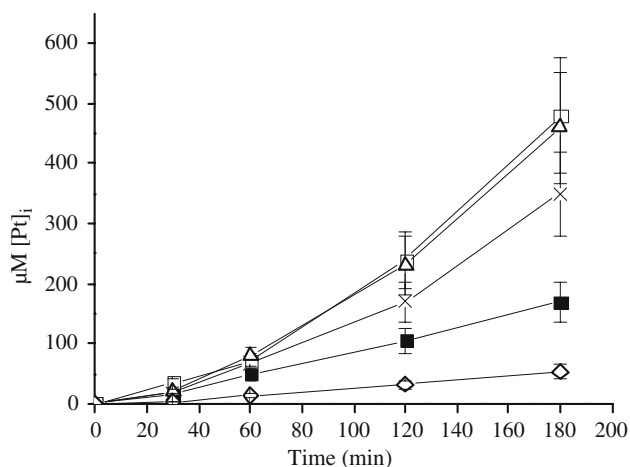$^*P < 0.05$; $^{**}P < 0.01$

[a] $n = 6$

[b] $n = 3$

Fig. 2 Time course of CDDP uptake into K562 cells in the absence or presence of the modulator. Cells (10^6 mL^{-1}) were incubated with 1×10^{-4} mol L^{-1} CDDP at 37 °C in the absence or presence of the modulator. After various intervals of time the intracellular concentrations of platinum were determined (the cell volume equal to 10^{-12} L). No modulator (*filled squares*), plus 1 mM furosemide (*open squares*), plus 10 μM bumetanide (*crosses*), plus 1 mM amiloride (*triangles*), plus 200 μM ouabain (*diamonds*). The values represent the mean ± SD of at least three independent experiments performed on different days

Fig. 3 Time course of uptake of a mixture of $[Pt(NH_3)_2(H_2O)_2]^{2+}$, $[Pt(NH_3)_2(H_2O)(OH)]^+$, and $[Pt(NH_3)_2(OH)_2]$ (aqua-Pt) into GLC4 and GLC4/CDDP cells in the absence or presence of furosemide. Cells (10^6 mL^{-1}) were incubated with 1×10^{-4} mol L^{-1} aqua-Pt at 37 °C in the absence or presence of 1 mM furosemide in a low-Cl^- buffer (3 mM). After various intervals of time the intracellular concentrations of platinum were determined (the cell volume equal to 10^{-12} L). GLC4 (*filled squares*), GLC4 plus 1 mM furosemide (*open squares*), GLC4/CDDP (*filled circles*), GLC4/CDDP plus 1 mM furosemide (*open circles*). The values represent the mean ± SD of at least three independent experiments performed on different days

by previous observations that when cells are incubated with aquated CDDP in the presence of carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), i.e., in the absence of mitochondrial potential, the amount of platinum detected inside the cells decreases from 100% to about 20%. If aqua-Pt was simply reacting with the cell membrane, such impact of FCCP on the amount of platinum recovered in the cells would not be observed [21].
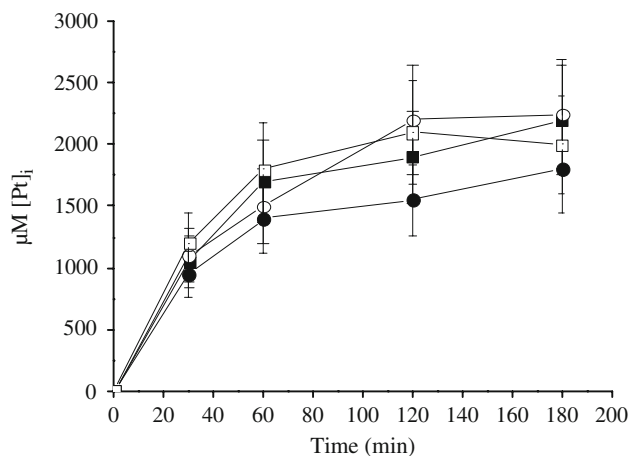
Steady-state intracellular Cl^- concentration and K_{SV} determination

To obtain absolute values for the intracellular Cl^- concentration, MQAE-labeled cells, after one washing, were suspended in Hepes buffer containing various Cl^- concentrations, X, ranging from 3 to 97 mM (Table 1). Triton X-100 treatment (20 μL 4% in 2 mL) rapidly (approximately

1 min) permeabilized the plasma membrane and caused a change in the fluorescence intensity of the cell suspension from F_i to F_X (Fig. 4a). The amplitude and direction of the change was dependent on X. F_X was plotted as a function of X, the data were fitted to the equation $F_X = F_0/(1 + K_{SV}X)$, and K_{SV} was estimated (Fig. 4b). The mean K_{SV} value obtained from six independent experiments was 17 ± 3 and 28 ± 5 mM^{-1} for GLC4 and GLC4/CDDP, respectively. K_{SV} determination was also performed using the traditional method, i.e., MQAE-loaded cells were suspended in buffer where Na$^+$ was substituted by K$^+$ and which contained various Cl$^-$ concentrations. The

fluorescence signal intensity of the cell suspension was recorded after the addition of 10 μM tributyltin (a Cl$^-$–OH$^-$ exchanger) and 10 μM nigericin (a K$^+$–H$^+$ exchanger). The combination of these ionophores has been shown to dissipate Cl$^-$ gradients across the plasma membrane [22]. K_{SV} values obtained using either method were the same and in good agreement with those reported by other workers for other cell types [23, 24]. For K562 cells, K_{SV} was 18 ± 3 mM^{-1}.

The plot of $(F_X - F_i)/F_i$ against X, the extracellular Cl$^-$ concentration, yielded a steady-state intracellular Cl$^-$ concentration of 36 ± 5 mM (six independent experiments) for GLC4 cells, 60 ± 10 mM (six independent experiments) for GL4/CDDP (Fig. 5), and 34 ± 5 mM for K562.

Perturbation of the intracellular Cl$^-$ concentration through incubations of the cells with different modulators

To measure the variation of the intracellular Cl$^-$ concentration, under the effect of various modulators, MQAE-labeled cells were suspended in buffer 1, with or withou modulator. After 2 h, cells were washed once, suspended in buffer 1, and the fluorescence signal intensities of the cell suspension in the presence and in the absence of modulator, F_{Mod} and F_i, respectively, measured. Equation 5 was then used to calculate the intracellular Cl$^-$ concentration in the presence of the modulator. Table 2 shows the values of the
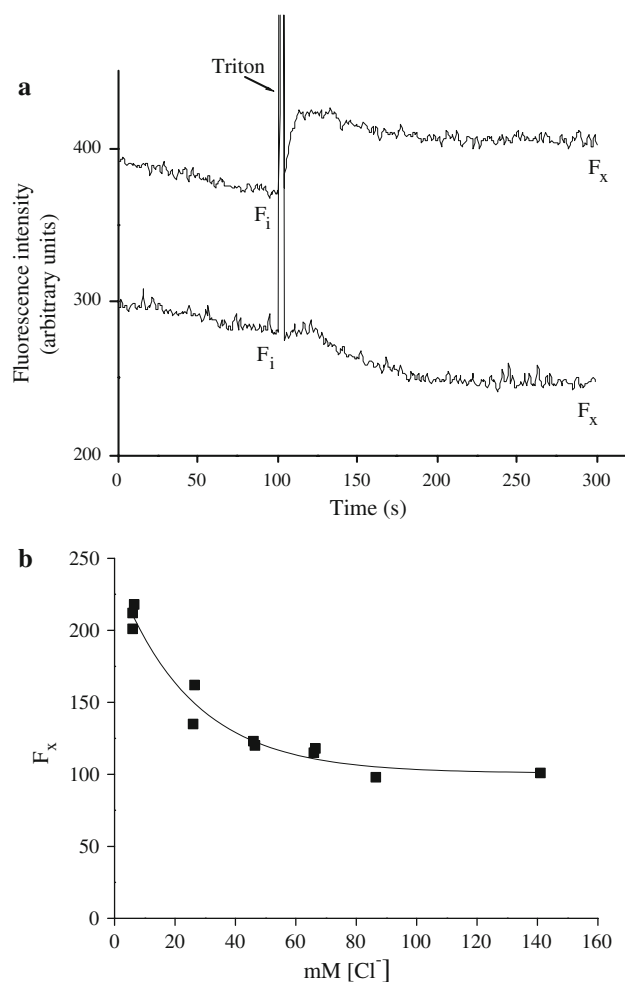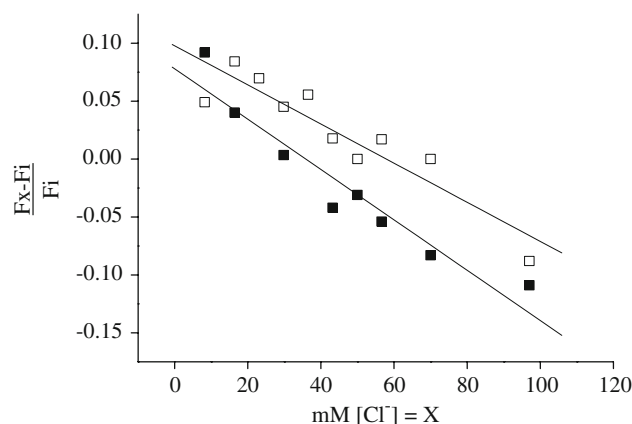

Fig. 4 Stern–Volmer constant determination. GLC4 cells loaded with N-(6-methoxyquinolyl)acetoethyl ester (MQAE) were put in buffer containing various Cl$^-$ concentrations X ranging from 3 to 97 (Table 1). At the 100-s time point, the fluorescence signal intensity was F_i and the cell suspension was treated with Triton X-100 (20 μL 4% in 2 mL). At the 300-s time point the fluorescence signal intensity was F_X. **a** Typical records performed at $X = 8.2$ mM (*upper trace*) and $X = 97$ mM (*lower trace*). The data points are from a representative experiment. **b** F_X as a function of X, the extracellular Cl$^-$ concentration. The data were fitted to the equation $F_X = F_0/(1 + K_{SV}X)$ (*line*) and the value of K_{SV} was estimated

Fig. 5 Null-point determination of intracellular Cl$^-$ concentration using the Cl$^-$-sensitive dye MQAE. The experimental conditions are the same as those for Fig. 4. Data points such as those shown in Fig. 4a were used to calculate $(F_X - F_i)/F_i$, which was plotted as a function of the Cl$^-$ buffer concentration X. This was done for GL4 (*filled squares*) and GLC4/CDDP (*open squares*) cells. The data points are from a representative experiment. A linear regression analysis of $(F_X - F_i)/F_i$ as a function of X yielded the intracellular Cl$^-$ concentration in GLC4 (34 ± 5 mM) and GLC4/CDDP (60 ± 10 mM) cells

intracellular Cl^- concentration thus determined: in the three cell lines, bumetanide and furosemide yielded a decrease of the intracellular Cl^- concentration; in GLC4 and K562 cells, amiloride yielded a decrease of the intracellular Cl^- concentration, whereas ouabain yielded an increase of the intracellular Cl^- concentration.

Cell growth inhibition

When cells were incubated for 72–h in the presence of CDDP, the IC_{50} values obtained for GLC4 and GLC4/CDDP were 1.0 ± 0.3 μM ($n = 3$) and 6.2 ± 0.8 μM ($n = 3$), respectively. When cells were incubated for 1 h in the presence of CDDP and then resuspended in fresh medium, the IC_{50} values obtained for GLC4 and GLC4/CDDP were 6 ± 2 μM ($n = 3$) and 36 ± 7 μM ($n = 3$), respectively.

Discussion

The cytotoxic effects of CDDP are thought to occur via several mechanisms, including inhibition of protein synthesis, mitochondrial injury, and DNA damage [25], leading ultimately to activation of apoptosis in tumor cells [26–30].

In its native form, CDDP is a neutral square-planar complex. Aqueous formulations of CDDP are solutions of the native and aquated forms of the drug in an equilibrium which depends on the pH and Cl^- concentration [31, 32]. At 130 mM extracellular Cl^- concentration and pH 7.3, approximately 90% of the formulation is CDDP, and the other 10% is in the form of cis-$[Pt(NH_3)_2ClH_2O]^+$ and cis-$[Pt(NH_3)_2ClOH]$. CDDP and cis-$[Pt(NH_3)_2ClOH]$ being neutral passively diffuse through the plasma membrane. Once inside the cytosol, the Cl^- concentration being lower than in the extracellular medium, more cis-$[Pt(NH_3)_2ClH_2O]^+$ and cis-$[Pt(NH_3)_2ClOH]$ species are present. Because the H_2O ligand is a better leaving group than the Cl^- ligand, $[Pt(NH_3)_2ClH_2O]^+$ is usually named the "active" species that binds different entities inside the cell such as DNA (approximately 1%) and thiol-containing compounds [25]. It is now well understood that endogenous thiol groups intercept platinum: because the thiolate anion has a high affinity for Pt^{2+}, active species inside the cell may preferentially bind to sulfur atoms rather than the base of DNA, and because GSH is the most abundant, essentially GSH–Pt complexes are formed [33–36]. Binding of CDDP to GSH has been widely studied [36–40]. It is known that the thiol of GSH leads to trans labilization and loss of ammine ligands and further reaction. When CDDP or aqua-Pt enters the cells, it is likely that there is a mixture of product and in the following we will name "GS–Pt" all the complexes containing at least one GSH as a ligand.

Resistance to platinum-based chemotherapy is a major problem and numerous hypothesis have been put forth to explain the phenomenon [4]. Mechanisms of resistance to CDDP are thought to fall into several categories, such as detoxification of CDDP, increased DNA repair, increased tolerance of CDDP–DNA adducts, disruption of apoptosis, and/or reduced intracellular accumulation of CDDP. Though different molecular mechanisms may coexist to give a high net level of resistance [35], defective uptake of CDDP has been one of the most consistently identified characteristics of cells selected for CDDP resistance both in vivo and in vitro [9–11] and although the relationship between the degree of resistance to platinum-based drugs and the level of accumulation in cells is not always direct, 70–90% of the total resistance has been attributed to defective platinum drug accumulation [35].

Recently, using GLC4 and GLC4/CDDP cell lines, we have reported a good correlation between intracellular platinum concentration and cell growth inhibition: when cells are incubated with equitoxic drug concentrations, the same cellular amount of platinum is required to produce similar toxicity, both in sensitive and in resistant cells, irrespective of the drug CDDP or cis-diammine(1,1-cyclobutanedicarboxylato)platinum(II) (CBDCA). Therefore, in the GLC4/CDDP cell line the resistance to CDDP is mainly due to reduced drug accumulation [11]. We have also shown that (1) in GLC4 cells the intracellular accumulation of platinum is much faster when cells are incubated with aqua-Pt than when they are incubated with CDDP and (2) the platinum accumulation is lower in GLC4/CDDP cells than in GLC4 cells when CDDP is used, but is very similar when aqua-Pt or CBDCA are used. The main difference amongst the three compounds is that CBDCA and aqua-Pt have no Cl^- ligand, whereas CDDP has to release Cl^- to generate active species. These data point to the role of Cl^- in intracellular platinum-based-compound accumulation as well as its possible role in the phenomenon of resistance.

The exact role of Cl^- in determining the cytotoxicity of CDDP has been disputed. A study in which the intracellular Cl^- concentration was substantially lowered could not demonstrate any impact on the pharmacodynamics of CDDP [41]. However, Yarbrough et al. [42] have already reasoned that reduction in intracellular accumulation of CDDP, which is believed to be an early change in CDDP-resistant cells, may depend on the concentration of intracellular Cl^- and intracellular pH. Very recently, Lee et al. [12] made a very interesting observation: they reported that the KCP-4 human epidermoid cancer cell line, which serves as a model of acquired resistance to CDDP, has virtually no VSOR Cl^- channel activity. In addition, their results indicate that restoration of the channel functional expression by trichostatin A treatment leads to a decrease in the CDDP resistance of KCP-4 cancer cells.

All these data prompted us to carefully study the impact of intracellular Cl⁻ concentration on platinum-based-compound accumulation and cytotoxicity in sensitive and resistant cells. Our data clearly show that (1) a decrease of intracellular Cl⁻ concentration yields an increase of CDDP accumulation and vice versa and (2) the intracellular Cl⁻ concentration in resistant cells is higher than in sensitive cells, whereas CDDP accumulation shows the opposite behavior.

On the basis of the observation that the intracellular platinum accumulation was energy-dependent, the presence of a transporter, at the level of the uptake and/or at the level of efflux, has been proposed. It has also been suggested that resistance could be related to an impaired functionality of these putative transporters. For instance, it has been proposed that one component of CDDP uptake is mediated by a transport mechanism or channel [10, 41]. More recently, it has been suggested that a copper-uptake transporter, CTR1 and two copper-transporting P-type ATPase, ATP7A and ATP7B involved in copper efflux, may be important in regulating cellular levels of CDDP [43–55]. Altogether the data reported in these papers indicate that Ctr1 and ATP7A/ATP7B are probably involved in resistance to CDDP, but it has not been shown that any of the platinum drugs are substrates for any of the copper transporters [47–49].

Recently, using the GLC4 cell line and several agents to modulate membrane potentials, acid compartment pH, and/or ATP level, we have shown that (1) approximately 70–80% of platinum accumulation in the cells depends on the $\Delta\Psi$ of mitochondrial membrane and on energy [21] and (2) platinum accumulation is decreased in the presence of phenylsuccinic acid, an inhibitor of 2-oxo-glutarate transporter, one of the GSH transporters in membrane mitochondria (M. Salerno and A. Garnier-S uillerot, unpublished data). In addition, as inside the cell most of intracellular platinum is present as GS–Pt complex(es), these data support the proposition that platinum is accumulated inside mitochondria as a GS–Pt complex transported via one of the GSH transporters.

In the following we propose a mechanism which aims to conciliate the different observations made up to now to explain the defective uptake of CDDP in resistant cells. To enter the cell, platinum-based compounds have first to pass the plasma membrane; this occurs by simple diffusion of the neutral species present in the extracellular medium, i.e., essentially CDDP [56]. Once inside the cytosol CDDP undergoes a ligand substitution reaction where Cl⁻ is replaced by a H_2O ligand, yielding the active species cis-$[Pt(NH_3)_2ClH_2O]^+$ in equilibrium with cis-$[Pt(NH_3)_2ClOH]^+$. It should be noted that the rate of cis-$[Pt(NH_3)_2ClH_2O]^+$ formation does not depend on the intracellular Cl⁻ concentration. Then, two main events can happen: the

active species binds Cl⁻ (anation reaction) or binds to any nucleophilic species present inside the cell. Actually, these nucleophilic species are mainly composed of thiol-containing compounds and essentially of GSH. It can be speculated that low intracellular Cl⁻ concentration will promote the reaction of the active species with GSH forming GS–Pt and therefore intracellular platinum accumulation. Figure 6 shows the plot of the intracellular platinum concentration determined after 2-h incubation of cells with CDDP as a function of the reciprocal of the Cl⁻ concentration for GLC4, GLC4/CDDP, and K562 cells. The data points obtained with GLC4 and GLC4/CDDP cell lines follow the same, more or less, linear pattern. The data points obtained with the K562 cell line follow another linear pattern, the intracellular platinum accumulation in K562 cells being about 50% of that observed for GLC4cells. It should be emphasized that when cells are incubated with aqua-Pt, platinum accumulation (1) is very similar in both sensitive and resistant cells and (2) does not depend on the presence of a modulator, i.e., does not depend on the intracellular Cl⁻ concentration.

How can the modulators used in these experiments modulate intracellular Cl⁻ concentration? Cl⁻ flux is regulated by (1) band 3 anion exchangers, Cl⁻/HCO_3^-, (2) the Na^+-dependent Cl⁻/HCO_3^- exchanger, and (3) the Na^+–K^+–2Cl⁻ (NKCC) cotransporter. The anion exchanger is activated by cellular alkalinization and normally acts as a Cl⁻ influx mechanism (the exchange of intracellular HCO_3^- for extracellular Cl⁻). The Na^+-dependent Cl⁻/
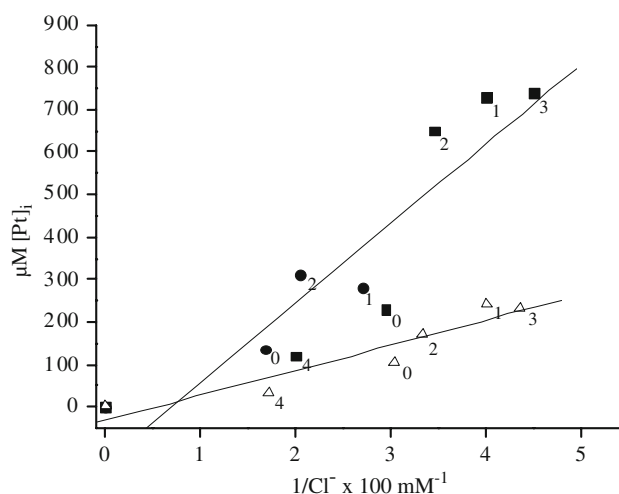

Fig. 6 Relation between intracellular platinum accumulation and intracellular Cl⁻ concentration. The intracellular platinum concentrations measured in GLC4, GLC4/CDDP, and K562 cellaren plotted as a function of the reciprocal of the intracellular Cl⁻ concentration. Cells were incubated for 2 h with 100 μM CDDP in the absence or presence of different modulators. GLC4 (*squares*), GLC4/CDDP (*circles*), K562 (*triangles*) in the absence of modulator (*0*); in the presence of furosemide (*1*); bumetanide (*2*); amiloride (*3*); and ouabain (*4*)

HCO_3^- exchanger mediates a Cl^- efflux (intracellular Cl^- exits the cell in exchange for HCO_3^-). The NKCC cotransporter acts as an influx for one Na^+, one K^+ and two Cl^-. Both bumetanide and furosemide inhibit the NKCC transporter activity, which leads to a decline in the intracellular Cl^- concentration. Ouabain does not inhibit the NKCC transporter, but its effect on this transporter could be indirect: ouabain inhibits the Na^+–K^+ ATPase and then disturbs the Na^+/K^+ chemical gradients. The driving force for the NKCC transporter is the combined chemical gradient of Na^+, K^+, and Cl^- and any change in the intracellular concentrations of the three ions involved would change the net driving force for the cotransporter. There are reports in the literature that an increase in intracellular Na^+ concentration and a decrease in intracellular K^+ concentration inhibit NKCC activity [57]. Amiloride blocks the Na^+/H^+ transporter that exchanges external Na^+ for internal H^+. The available evidence points to the fact that an acidification of the intracellular medium inhibits the NKCC activity [57].

In conclusion, we have shown that there is a very clear correlation between CDDP accumulation and intracellular Cl^- concentration, in both GLC4 and GLC4/CDDP cell lines, as well as in a completely different cell line, K562. All these data, together with the recent proposition of Lee et al. [12] that reduction of VSOR Cl^- channel activity contributes to CDDP resistance, lead us to conclude that resistance, at least in these two cell lines, is due to an increase of the intracellular Cl^- concentration.

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