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ORIGINAL PAPER

Preferential energy- and potential-dependent accumulation of cisplatin–gutathione complexes in human cancer cell lines (GLC4 and K562): A likely role of mitochondria

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Abstract *cis*-Diamminedichloroplatinum(II) (CDDP) is an important chemotherapeutic agent used in the treatment of a wide variety of solid tumors. We have recently shown that aquated forms of cisplatin (aqua-Pt) rapidly accumulate in K562 and GLC4 cultured cells, in comparison to CDDP. Thus, when cells are incubated with aquated forms of cisplatin a gradient of concentration is observed after a short time, approximately 40 min, with an intracellular concentration of aqua-Pt of 20–30 times higher than that of extracellular aqua-Pt. The same gradient of concentration is observed when cells are incubated with CDDP but it takes a longer time, i.e., about 24 h. Therefore, the question arises as to the identity of the intracellular sites of accumulation of aqua-Pt. Using several agents to modulate membrane potential, acidic compartment pH and/or ATP level, we obtained evidence that aqua-Pt may accumulate rapidly inside mitochondria as this accumulation is energy- and membrane-potential-dependent. However, aqua-Pt complexes are not characterized by a delocalized charge and a lipophilic character that would permit their movement through the inner membrane. Therefore, it is suggested that intracellular aqua-Pt reacts rapidly with glutathione with the resultant complex being transported inside the mitochondria via one of the known glutathione transporters, i.e., dicarboxylate and/or 2-oxoglutarate transporters present in the inner membrane.

Keywords Cisplatin · Mitochondria · Glutathione · Accumulation

Abbreviations

CDDP	*cis*-diammine-dichloro-platinum(II) or cisplatin
aqua-Pt	*cis*-diammine-diaquo-platinum(II)
TDDP	*trans*-diammine-dichloro-platinum(II)
TMR	tetramethylrosamine
GSH	glutathione
GS−	once deprotonated glutathione
mGSH	mitochondrial glutathione
MS	methanesulfonate

Introduction

cis-Diamminedichloroplatinum(II) (cisplatin, Fig. 1) is an important chemotherapeutic agent used in the treatment of a wide variety of solid tumors (Brenner et al., 1982; Rosencweig et al., 1977). The cytotoxic effects of cisplatin are thought to occur via several mechanisms, including inhibition of protein synthesis, mitochondrial injury, and DNA damage (Huang et al., 1995), leading ultimately to activation of programmed cell death pathways in tumor cells (Jiang et al., 1999) as well as in renal tubule cells (Lieberthal et al., 1996; Okuda et al., 2000; Ueda et al., 2000; Van de Water et al., 2000).

Although there is evidence that the mechanism of tumor killing involves the formation of DNA crosslinks (Roberts et al., 1986), there are also significant data showing that the sensitivity of cells to platinum drugs does not always correlate with the formation of DNA adducts (Rawlings and

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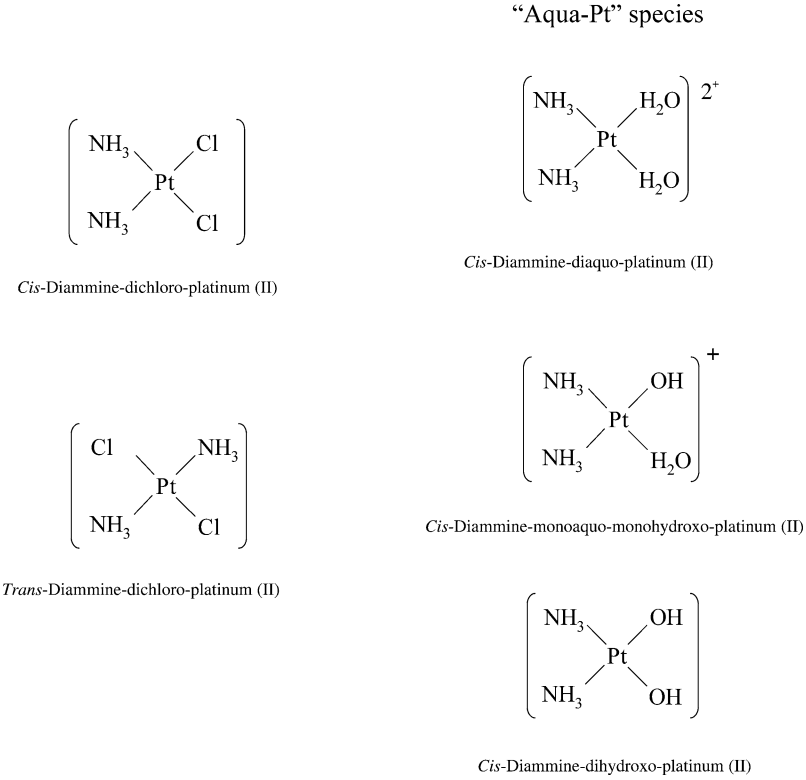
Fig. 1 Chemical structure of platinum complexes



Roberts, 1986; Strandberg et al., 1982). Thus, recent studies have proved that cisplatin and its analogues produce alterations in the structure and function of isolated mitochondria (Rosen et al., 1992). For instance, the results of Park et al. (2002) indicate that cisplatin induces apoptosis in LLC–PK1 cells via activation of mitochondrial signaling pathways. In other cells, the posttreatment concentration of platinum in mitochondria was higher than in other subcellular compartments (Sharma and Edwards, 1983).

The efficiency of cisplatin is dose-dependent. The risk of nephrotoxicity (Leibbrandt et al., 1995) frequently hinders the use of higher doses to maximize its antineoplastic effects. Therefore, a major limiting factor in the usefulness of cisplatin has been the acute toxic effects of the agent, which are not completely explained.

We have recently shown (Pereira-Maia and Garnier-Suillerot, 2003) that in cultured cells, the aquated forms of cisplatin (Fig. 1) rapidly accumulate into cultured cells, compared to CDDP. Within approximately 40 min, a concentration gradient was observed when cells were incubated with aquated forms of cisplatin. The intracellular concentration of Pt was 20–30 times higher than the extracellular one. Cell incubation with CDDP for a longer time (24 h) was needed to obtain the same concentration gradient. Therefore, the question arises: what are the sites of accumulation of Pt inside the cells?

In this study, we have used several agents to modulate membrane potentials, acidic compartment pH, and/or ATP level. Our data show that Pt rapidly accumulates inside mitochondria. This accumulation is energy- and potential-dependent. It strongly suggests that after Pt binding to glutathione (GSH), the Pt–GS complex is transported inside the mitochondria via one of the GSH transporters, i.e., dicarboxylate and/or 2-oxoglutarate carriers present in the inner membrane of mitochondria (Chen and Lash, 1998; Fernandez-Checa and Kaplowitz, 2005).

Materials and methods

Drugs and chemicals

CDDP (1 mg/mL, ~3.3 mM in 154 mM NaCl) was obtained from Dakota Pharm. *trans*-Diamminochloroplatinum(II) (TDDP) was a gift from Dr E. Franck (University of Paris 6). Valinomycin, FCCP, concanamycin, and ouabain were purchased from Sigma. They were dissolved in ethanol. Tetramethylrosamine (TMR) was from Molecular Probe and stock solution, 10^{-3} M, was prepared in ethanol. 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

Prof. E. G. E. de Vries (Department of Internal Medicine, University Hospital, Groningen, The Netherlands). The K562 cell line is a highly undifferentiated erythroleukemia, originally derived from a patient with chronic myelogenous leukemia (Lozzio and Lozzio, 1975). The two cell lines were cultured in RPMI 1640 Medium with GlutaMAX^{TM}I (GIBCO) medium supplemented with 10% fetal calf serum (GIBCO) at 37°C in a humidified incubator with 5% CO_2. Cultures, initiated at a density of 10^5 cells/mL, grew exponentially to about 10^6 cells/mL in 3 days. To have enough cells in the exponential growth phase for assay, culture was initiated at 5×10^5 cells/mL and allowed to grow for 24 h until use; cultured cells were then counted with a Coulter counter immediately before use. The viability of the cells, tested by Trypan Blue exclusion, was always greater than 95%.

Synthesis of aquated complexes

$[Pt(NH_3)_2Cl_2]$ was mixed with $AgNO_3$ at a 1:2 molar ratio. The mixture was stirred for 15 h under light protection and at 25°C. The formed AgCl precipitate was separated by filtration. The filtrate was maintained in the dark for 5 h and then filtered. A solution of $[Pt(NH_3)_2(H_2O)_2]^{2+}$ was thus obtained (Lim and Martin, 1976). This compound is hereafter named aqua-Pt.

Platinum uptake and retention by cells

The short-term accumulation and transport of CDDP, TDDP, or aqua-Pt were studied in buffers, at 37°C, under protein and amino-free conditions. For platinum accumulation assays, cells were treated with various concentrations of the platinum-based compounds. Unless specified, the concentration used was 100 μM. The incubation was performed, at 37°C, in Hepes isotonic buffer solutions (pH 7.3). The buffer composition is (i) Hepes–NaCl buffer: 20 mM Hepes, 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, plus 5 mM glucose; (ii) Hepes–KCl buffer: 20 mM Hepes, 132 mM KCl, 3.5 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, plus 5 mM glucose; (iii) Hepes–MSNa buffer (respectively Hepes–MSK buffer) is low Cl^- buffer, in which NaCl (respectively KCl) is substituted by equimolar concentration of sodium (respectively potassium) methanesulfonate. Sodium (respectively potassium) methanesulfonate was made by titration of methane sulfonic acid with NaOH (respectively KOH) or prior to addition to buffer (Piwnica-Worms et al., 1985).

After specified time intervals, aliquots containing 10^6 cells were taken and washed two times with phosphate saline buffer at 4°C. The pellets obtained after centrifugation were digested with nitric acid (65%), and the platinum was measured using atomic absorption spectrometer. The local Pt intracellular concentration, $[Pt]_i$ was calculated by taking into account the mean volume of one cell, 10^{-12}L.

Absorption spectroscopy and fluorescence spectroscopy

Absorption spectra were recorded on a Varian Cary 300 spectrometer. Fluorescence spectra were recorded using a Perkin Elmer LS50B spectrophotometer.

Atomic absorption spectroscopy

Cellular platinum contents were quantified using a Zeeman atomic absorption spectrometer (Varian SpectrAA 220).

Oxygen consumption

The rate of oxygen consumption by cells suspended in Hepes–MSNa buffer was determined at 37°C with a YSI 5331 oxygen monitoring system either in the absence or in the presence of aqua-Pt. The rate of oxygen consumption was calculated from a value of 199 μM at 37°C for the total dissolved oxygen content of the reaction mixture.

Results

In its native form, CDDP is a neutral square-planar complex (Fig. 1). 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 (Miller and House, 1991). At the 130 mM Cl^- concentration found in Hepes–NaCl and Hepes–KCl buffer, at pH 7.3, CDDP would be about 16% aquated at equilibrium. The aquated Pt species were prepared under conditions where the two Cl^- anions were totally exchanged with water molecules and/or with hydroxyl groups, depending on the pH value. Aqua-Pt is a weak base with pK_1 and pK_2 values equal to 5.4 and 7.2, respectively, for the first and the second water molecule deprotonation (Berners-Price et al., 1996). Consequently, depending on the pH, one finds the following complexes: $[Pt(NH_3)_2(H_2O)_2]^{2+}$, $[Pt(NH_3)_2(H_2O)(OH)]^+$, and $[Pt(NH_3)_2(OH)_2]$. These three entities will be hereafter called aqua-Pt. The aqua-Pt was then diluted to 100 μM platinum in Cl^- deficient buffer at pH 7.3, i.e., Hepes–MSNa or Hepes–MSK.

The cytotoxicity of Pt drugs is proportional to the number of cellular DNA–Pt adducts. The amount of drug available to bind DNA is limited by cellular thiol containing molecules such as GSH and metallothionein. Binding of platinum to GSH inside the cells has been clearly demonstrated by Berners-Price and Kuchel (1990). They studied the reactions of *cis*- and *trans*-$[PtCl_2(NH_3)_2]$ with GSH inside intact red blood cells. Using conditions that mimic those in the

cells, Dabrowiak et al. (2002) have recently reported on this reaction.

Under physiological conditions, the Pt drugs react with cellular GSH at a relatively slow rate (Hagrman et al., 2004). The limiting rate is the release of Cl^-. Once Cl^- has been substituted by H_2O, the reaction with thiol groups is very fast. However, the substitution of hydroxyl groups by thiols is much slower.

CDDP, TDDP, and aqua-Pt reactions with GSH as monitored by UV

We have monitored the reaction of CDDP, TDDP, and aqua-Pt with GSH under intracellular-like conditions: 500 μM CDDP, TDDP, or aqua-Pt were incubated at 37° with 2 mM GSH in Hepes–KCl buffer in the case of CDDP and TDDP and in Hepes–MSK buffer in the case of aqua-Pt. The absorbance at 260 nm, which reflects Pt–sulfur and disulfide bond formation, was measured with respect to time (Fig. 2). As can be seen, the reactions of GSH with TDDP (respectively aqua-Pt) were fast and completed after approximately 30 min (respectively 1 h). With TDDP, Cl^- is a good leaving group (Basolo and Pearson, 1967), it is rapidly substituted by H_2O and therefore the reaction of Pt with GSH is fast. These experiments were also performed using 10 mM GSH and the results were quite similar.

Comparison of the time course of CDDP, TDDP, and aqua-Pt uptake in K562 cells

Incubation of cultured cells in buffers containing CDDP, TDDP, or aqua-Pt resulted in a time-dependent accumulation of the Pt agent. Figure 3 shows the data obtained when

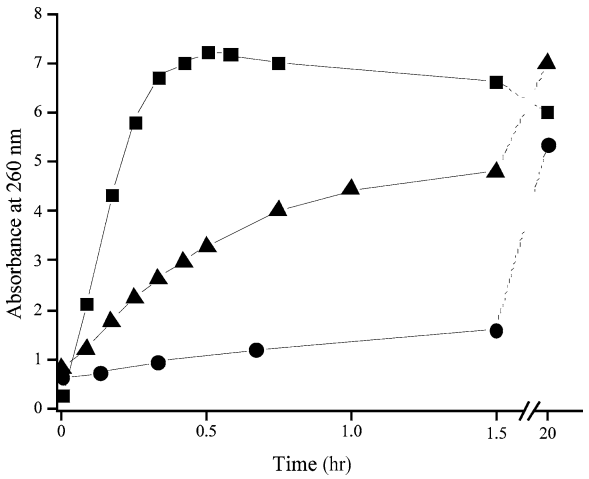


Fig. 2 Glutathione reaction with CDDP, TDDP, and aqua-Pt in Hepes buffer as monitored by UV absorbance. Absorbance at 260 nm is shown as a function of time for 20 h incubation at 37°C of GSH 2 mM with 500 μM of (a) CDDP in Hepes–NaCl buffer (●); (b) TDDP in Hepes–NaCl buffer (■); (c) aqua-Pt in Hepes–MSNa buffer (▲)

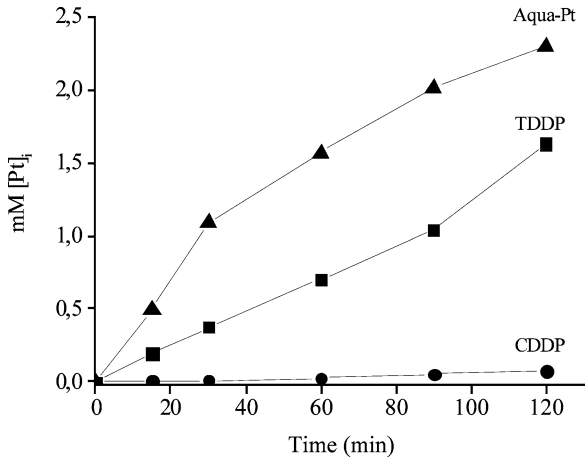


Fig. 3 Time variation of CDDP, aqua-Pt, and TDDP uptake in K562 cells. Cells (10^6/mL) were incubated at 37°C with 100 μM CDDP (●) or TDDP (■) in Hepes–NaCl buffer, or aqua-Pt (▲) in Hepes–MSNa buffer. After various time intervals, the intracellular concentration of Pt was determined (the cell volume was taken equal to 10^{-12} L). The data are from a representative experiment

100 μM CDDP were incubated with K562 cells (10^6/mL), during 2–3 h without reaching the steady state. Because of the high concentration used, it was not possible to incubate a longer time without injuring cells. However, we have already measured that when concentrations close to the IC_{50} are used and, after 1 day, the intracellular Pt concentration $[Pt]_i$ upon the extracellular Pt concentration $[Pt]_e$ ratio ranged from 20 to 30. The incubation of cells in Hepes–NaCl buffer containing TDDP or in Hepes–MSNa buffer containing aqua-Pt resulted in a time-dependent accumulation of the agent toward an asymptote. In both cases nominal intracellular-to-extracellular equilibrium concentration ratios of Pt, $[Pt]_i/[Pt]_e$, range from 20 to 30. The extracellular drug concentration, $[Pt]_e$, can always be considered as equal to the total drug concentration, C_T, added to the cells, the Pt incorporated by the cells being only about 1% of C_T. Similar data were obtained with GLC4 cells.

Collapse of ΔH^+

We have assessed intracellular Pt accumulation after inhibiting the acidification of the acidic vacuolar compartments such as lysosomes. In fact, accumulation of weakly basic substances in intracellular acidic compartments can be prevented by eliminating the pH gradient between the cytosol and the lysosome compartment. The pH gradient collapse is obtained by inhibiting the action of vacuolar H^+-translocating ATPases with concanamycin (Drose et al., 1993). When cells, either GLC4 or K562, were incubated with 20 nM concanamycin, no modification of Pt accumulation was observed either with CDDP or with aqua-Pt. Under these experimental conditions, the pH gradient was correctly eliminated (see Control Experiments section).

Accumulation of aqua-Pt and CDDP inside the cells in the absence of energy

To estimate energy-independent aqua-Pt (respectively CDDP) accumulation, cells were incubated in Hepes–MSNa (respectively Hepes–NaCl) buffer in the absence of glucose and in the presence of 5 mM sodium azide and, in some case, the presence of 5 mM 2-deoxyglucose. Under these conditions, the intracellular Pt concentration decreased down to 30–50% of the control values (Tables 1–3). In the three tables, under the "control conditions," 100% Pt accumulation corresponds to the values obtained after 1 h incubation in the presence of glucose and in the absence of sodium azide. In the case of aqua-Pt, a steady state was reached and the intracellular Pt concentration was within the range of 2.0–2.5 mM. In the case of CDDP, no steady state was reached and the intracellular Pt concentration was within the range of 0.05–0.15 mM.

Collapse of the plasma membrane potential ($\Delta\psi_{plasma}$)

The equalization of the intra- and extracellular concentrations of potassium was performed by incubating cells in Hepes–MSK and Hepes–KCl in the case of aqua-Pt and CDDP respectively. Such experimental conditions almost completely depolarized plasma membrane. This was confirmed with experiments performed with TMR (see Control Experiments section) or Tc-MIBI (Vergotte et al., 1998).

When cells, 10^6/mL, either GLC4 or K562, were incubated with 100 μM aqua-Pt in Hepes–MSK buffer, the uptake was similar to what is observed in Hepes–MSNa buffer (Tables 1 and 2). The addition of 10 nM or 1 μM valinomycin decreased the uptake down to approximately 70% of the initial value. However, it should be noticed that as valinomycin also partially depolarized the mitochondrial membrane, these data can be taken as a first indication of mitochondrial accumulation of Pt. Since the Na^+, K^+-ATPase is the primary generator of the membrane potential, one wonders whether the inhibitor ouabain could inhibit aqua-Pt uptake. No effect was observed when the uptake of aqua-Pt was studied in Hepes–MSNa buffer in the presence of 200 μM ouabain. This agrees with the observation that the aqua-Pt accumulation was the same in Hepes–MSK/Hepes–MSNa buffer (Tables 1 and 2). This first set of experiments shows that aqua-Pt uptake is not driven by $\Delta\psi_{plasma}$.

Similar experiments were performed using CDDP (Table 3). For both cell lines it was observed that (i) the uptake was about 0.03 mM for K562 and 0.09 mM for GLC4 (in Table 3, these values are indicated as 100% respectively), (ii) the addition of valinomycin decreased the uptake down to approximately 50% of the control value, (iii) the addition of ouabain decreased the uptake down to approximately 50% compared to what was observed with aqua-Pt.

Collapse of mitochondrial membrane potential ($\Delta\psi_{mito}$)

Evidence supporting mitochondrial participation in cellular accumulation of Pt was derived from the effect of protonophores. Cells were allowed to accumulate aqua-Pt or CDDP in the presence of 1–10 μM FCCP. The protonophore FCCP is known to rapidly to depolarize mitochondrial membrane.

Incubation of GLC4 cells with aqua-Pt either in Hepes–MSNa or Hepes–MSK buffer also yielded a high decrease of Pt uptake from $[Pt]_i$ approximately 2000 to 300–600 μM at the steady state (Fig. 4). Similar data were obtained with K562 cells (Fig. 4).

Table 1 Uptake of aqua-Pt in GLC4 cells under different experimental conditions

Conditions	Buffer[a]	Glucose (mM)	Deoxyglucose (mM)	N_3^- (mM)	FCCP (μM)	Valinomycin	Ouabain (μM)	$\%[Pt]_i$[b]
Control	Na^+	5	—	—	—	—	—	100
−Energy	Na^+	—	5	10	—	—	—	30 ± 6
$\Delta\psi_{plasma} = 0$	K^+	5	—	—	—	—	—	100
	K^+	5	—	—	—	1 μM	—	70 ± 15
	Na^+	5	—	—	—	—	200	100
$\Delta\psi_{mito} = 0$	Na^+ or K^+	5	—	—	—	—	—	30 ± 6
−Energy, $\Delta\psi_{mito} = 0$	Na^+ or K^+	—	—	10	1–5	—	—	26 ± 5
−Energy, $\Delta\psi_{mito} = 0$	Na^+ or K^+	—	5	10	1	—	—	23 ± 5
−Energy, $\Delta\psi_{plasma} = 0$ $\Delta\psi_{mito} = 0$	K^+	—	5	10	1	10 nM	—	24 ± 5

Note. ATP depletion was performed either in the absence or in the presence of 2-deoxyglucose. Data are means ± SEM of three determinations.

[a] Buffer was Hepes–MSNa or Hepes–MSK, the presence of high concentration of Na^+ or K^+ only was indicated.

[b] The intracellular concentrations were measured after 1 h incubation of cells with aqua-Pt. The value of 100% corresponds to about 2200 ± 300 μM.

Table 2 Uptake of aqua-Pt in K562 cells under different experimental conditions

Conditions	Buffer[a]	Glucose (mM)	Deoxyglucose (mM)	N_3^- (mM)	FCCP (μM)	Valinomycin	Ouabain (μM)	%$[Pt]_i$[b]
Control	Na^+	5	—	—	—	—	—	100
−Energy	Na^+	—	—	10	—	—	—	30 ± 6
$\Delta\psi_{plasma} = 0$	K^+	5	—	—	—	—	—	100
	K^+	5	—	—	—	1–10 μM	—	60 ± 12
	Na^+	5	—	—	—	—	200	100
$\Delta\psi_{mito} = 0$	Na^+ or K^+	5	—	—	1–10	—	—	15 ± 5
−Energy, $\Delta\psi_{mito} = 0$	Na^+ or K^+	—	—	10	—	—	—	15 ± 5
−Energy, $\Delta\psi_{plasma} = 0$ $\Delta\psi$mito = 0	K^+	—	5	10	1	10 nM	—	10 ± 3

Note. ATP depletion was performed either in the absence or in the presence of 2-deoxyglucose. Data are means ± SEM of three determinations.

[a] Buffer was Hepes–MSNa or Hepes–MSK, the presence of high concentration of Na^+ or K^+ only was indicated.

[b] The intracellular concentrations were measured after 1 h incubation of cells with aqua-Pt. The value of 100% corresponds to about 2400 ± 500 μM.

Accumulation of aqua-Pt inside the cells under conditions of decreased membrane potential and absence of energy

The experiments were performed in the presence of 5 μM FCCP and in the absence of energy. As can be seen, similar Pt accumulations were observed with cells either energy deprived, either with $\Delta\psi_{mito}$ closed to 0, or with both energy deprived and with $\Delta\psi_{mito}$ closed to 0 (Fig. 4). Similar experiments were performed using CDDP and the data reported in Table 3 can be compared to those obtained using aqua-Pt.

Is Pt released from cells?

Cells, GLC4 or K562, were incubated in Hepes–MSNa buffer with 100 μM aqua-Pt. After 40 min, 5 μM FCCP was added yielding an elimination of mitochondrial membrane potential. No release of Pt was observed. Figure 5 shows the results obtained with both cell lines.

Effect of aqua-Pt and CDDP on cellular TMR accumulation

TMR is a fluorescent lipophilic cation that accumulates inside the cells under the effect of plasma and mitochondrial membrane potentials. We have investigated the effect of concentrations of CDDP and aqua-Pt, from 10 to 150 μM, on the TMR fluorescence intensity of K562 cells.

TMR accumulation inside the mitochondria due to membrane potential yields to a quenching of its fluorescence (Loetchutinat et al., 2003; Saengkhae et al., 2003). Fluorescence measurements were carried out using a spectrofluorometer equipped with a temperature-controlled sample compartment. Typically, 0.5 μM TMR was quickly added

Table 3 Uptake of CDDP in GLC4 and K562 cells under different experimental conditions

Conditions	Buffer[a]	Glucose (mM)	N_3^- (mM)	FCCP (μM)	Valinomycin	Ouabain (μM)	%$[Pt]_i$
				GLC4			
Control	Na^+	5	—	—	—	—	100
−Energy	Na^+	—	10	—	—	—	30 ± 6
$\Delta\psi_{plasma} = 0$	Na^+	5	—	—	10 nM–1 μM	—	52 ± 10
	Na^+	5	—	—	—	200	60 ± 10
$\Delta\psi_{mito} = 0$	Na^+	5	—	1–10	—	—	37 ± 8
				K562			
Control	Na^+	5	—	—	—	—	100
−Energy	Na^+	—	10	—	—	—	45 ± 9
$\Delta\psi_{plasma} = 0$	Na^+	5	—	—	10 nM–1 μM	—	50 ± 10
	Na^+	5	—	—	—	200	40 ± 8
$\Delta\psi_{mito} = 0$	Na^+	5	—	1–10	—	—	47 ± 9

Note. Data are means ± SEM of three determinations.

[a] Buffer was Hepes–NaCl or Hepes–KCl, the presence of high concentration of Na^+ or K^+ only was indicated.

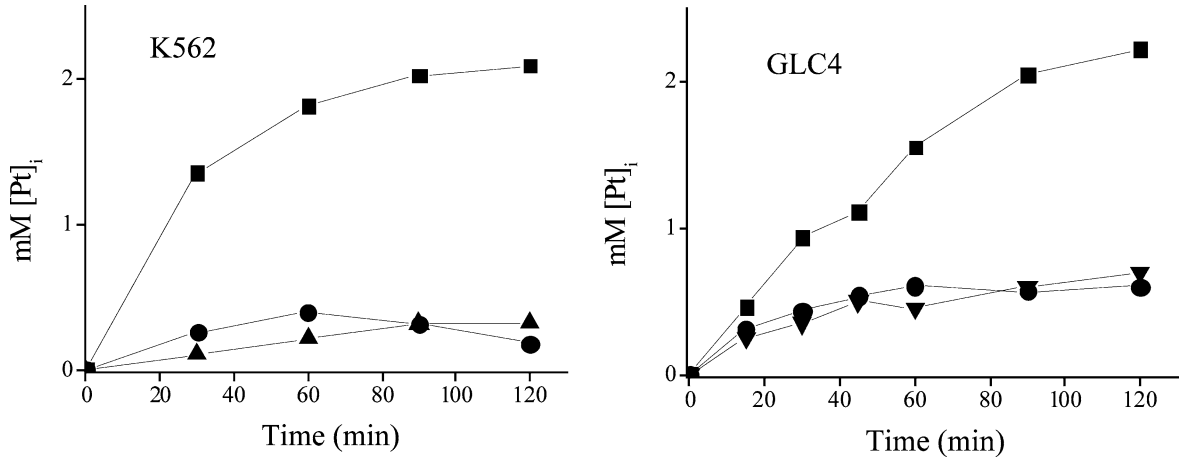


Fig. 4 Time variation of aqua-Pt uptake in K562 and GLC4 cells. Cells (10^6/mL) were incubated at 37°C with 100 μM aqua-Pt in Hepes–MSNa buffer: (■) control experiment; (▼) in the absence of energy, i.e., in the absence of glucose and presence of 10 mM azide and 5 mM 2-deoxyglucose; (▲) in the presence of 1 μM FCCP; (●) in the absence of energy and presence of 1 μM FCCP. The data are from a representative experiment

in a cuvette with magnetic stir containing cells (2×10^6) suspended in 2 mL of buffer. Fluorescence intensity of TMR was measured continuously until steady state was reached (excitation, 502 nm, and emission, 527 nm). Figure 6 shows the decrease of the fluorescence signal during the incubation of cells as a function of time.

In the first set of experiments, GLC4 cells were incubated with 0.5 μM TMR in different buffers: in Hepes–NaCl (respectively Hepes–MSNa) and Hepes–KCl (respectively Hepes–MSK) buffer. The accumulation was always lower in K^+ (versus Na^+) rich buffers indicating a role of plasma membrane potential on TMR uptake. The addition of valinomycin in Hepes–KCl or Hepes–MSK buffer always decreases the uptake. The addition of 5 μM FCCP cancels the uptake (Fig. 6).

In the second set of experiments, cells were first incubated for 2 h with CDDP 50–150 μM and then added to the TMR-containing buffer. The decrease of the fluorescence signal of TMR (100% in the absence of CDDP) became 35 and 20% in the presence of 50 μM and 100–150 μM CDDP, respectively, indicating that less TMR was accumulated in the mitochondria and therefore a decrease in the $\Delta\Psi_{mito}$.

In the third set of experiments, cells were incubated for 40 min in Hepes–MSNa buffer with aqua-Pt at various concentrations ranging from 0 to 150 μM. TMR, 0.5 μM, was then added to the cell suspension. The decrease of the fluorescent signal was dependent on the aqua-Pt concentration (Fig. 7) becoming approximately 25% in the presence of 150–180 μM aqua-Pt.

In the fourth set of experiments, cells were incubated together with 100 μM aqua-Pt and 0.5 μM TMR and the TMR fluorescence was recorded as a function of time (Fig. 8). A first decrease of the fluorescence signal first corresponded to what is observed when cells are incubated with TMR only. As time elapsed, one observed an increase of the fluorescence signal indicating a release of TMR from mitochondria. When

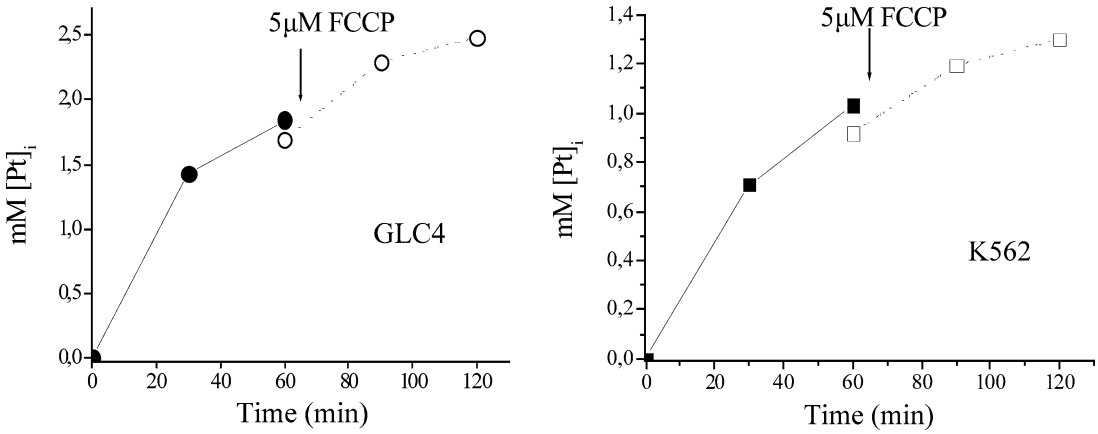


Fig. 5 Is Pt released from the cells? Time variation of aqua-Pt uptake by GLC4 (*left*) and K562 (*right*) cells was followed for 40 min, then 5 μM FCCP was added and the intracellular Pt concentration was measured during the following hour. The data are from a representative experiment

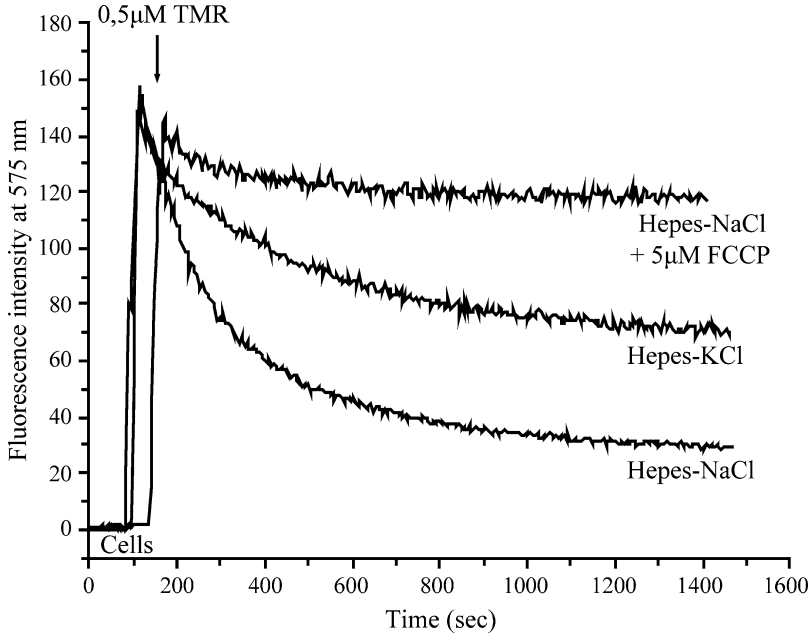


Fig. 6 Time variation of TMR uptake in GLC4 cells. Cells (10^6/mL) were incubated at 37°C with 0.5 μM TMR in (a) Hepes–NaCl buffer; (b) Hepes–KCl buffer; (c) Hepes–NaCl buffer in the presence of 5 μM FCCP. The data are from a representative experiment

the steady state was reached, the intensity of the fluorescence signal was the same as observed when cells were first incubated for 40 min with aqua-Pt and then with TMR (Fig. 7).

Effect of aqua-Pt on the rate of oxygen consumption by cells

Cells (2×10^6/mL) were placed in the oxymeter and the oxygen consumption was measured. Then, 100 μM aqua-Pt was added. A strong decrease of the oxygen consumption, to 30% of the control, could be observed after 20 min of incubation of cells with aqua-Pt, and the decrease was approximately to 10% of the control after 45 min incubation.

Control experiments

A set of control experiments was performed in order to validate the experimental conditions used to investigate the mechanism of the accumulation of Pt inside the cells.

We have verified that 20 nM concanamycin blocked H^+-ATPase by measuring the incorporation of an anthracycline derivative, WP900, which is the enantiomer of daunorubicin and can accumulate only in lysosomes. In the absence of concanamycin one observes a high accumulation of WP900 in lysosomes which does not occur in presence of 20 nM concanamycin (Loetchutinat et al., 2001).

Further, to test whether the variation of aqua-Pt intracellular concentration could be due to ATP depletion, we have

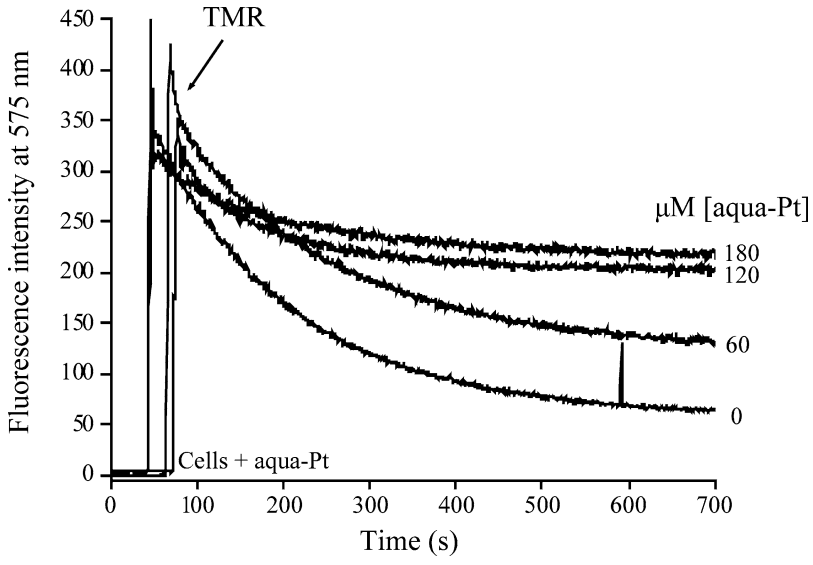


Fig. 7 Time variation of TMR uptake in K562 cells in the presence of aqua-Pt at different concentrations. A total of 0.5 μM TMR was added to cells (10^6/mL), preliminary incubated for 40 min at 37°C in Hepes–MSNa buffer with aqua-Pt at various concentrations. The data are from a representative experiment

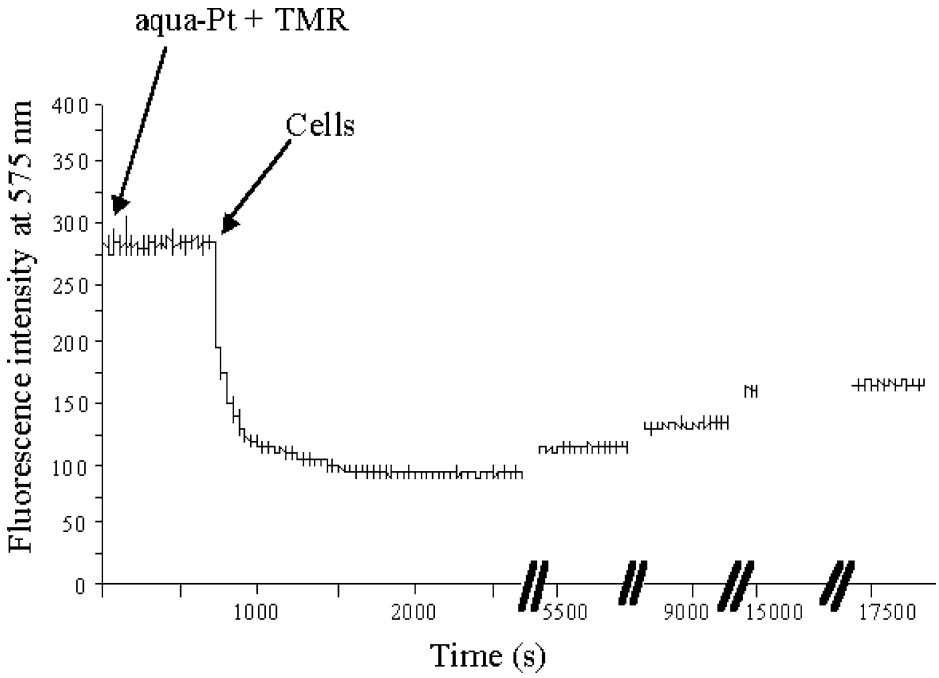


Fig. 8 Time variation of TMR uptake in GLC4 cells in the presence of aqua-Pt. Cells (10^6/mL) were added to Hepes–MSNa buffer containing 0.5 μM TMR and 100 μM aqua-Pt. The data are from a representative experiment

measured the intracellular ATP concentration. When cells were incubated in a glucose-containing buffer, no modification of the cellular ATP concentration was observed after 60 min, and no significant modification of the ATP concentration was observed after addition of 2–10 μM FCCP. However, when cells were incubated in a glucose-depleted buffer, the cellular ATP concentration, measured after 30 min, was 80% (respectively 20%) of the initial value when the incubation was performed in the absence (respectively presence of 2 μM FCCP). This can indicate that, in our cells and at this concentration, FCCP has uncoupling properties.

Discussion

Platinum coordination complexes are an important class of chemotherapeutics in the treatment of testicular, ovarian, head and neck, bladder, and small lung cell cancers (Dwyer et al., 1999). Finding that the ultimate cellular target of cisplatin is DNA has led to intense investigations into the mechanism of action of these drugs. Now, much is known about the molecular interactions of cisplatin with DNA in living cells (Jamieson and Lippard, 1999). However, much of the mechanism of action of platinum antitumor drugs remains unknown. Of special interest is the cellular distribution of the drug and how the drug eventually reaches genomic DNA. Inside the cell, proteins and peptides—through coordination to sulfur donor atoms of the cysteine and methionine amino acids—are thought to play an important role in the mechanisms of cisplatin toxicity. Thus, it has been recently shown that platinum is not exclusively targeted to the nucleus, as shown by measurements of Pt-DNA adducts (Giurgiovich et al., 1997; Olivero et al., 1995) and it is now well understood that endogenous thiol groups intercept cellular cisplatin. Thiol (sulfhydryl) groups, such as those of glutathione and metallothionein protect the cell against cisplatin (Bose et al., 1997). Since the thiolate anion has a high affinity for Pt^{2+}, Pt ions entering the cell may preferentially bind to sulfur atoms rather than the bases of DNA. Previous studies by Sharma and Edwards (1983) have indicated that cisplatin can accumulate in the mitochondrial compartment of cells. It was also reported that CDDP alters the properties of isolated mitochondria (Rosen et al., 1992). Differences in the ATP metabolism of cisplatin-sensitive cells in response to drug treatment had also suggested that cisplatin might alter mitochondrial function (Berghmans et al., 1992). Other studies showed that in drug sensitive living cells, cisplatin transiently affected mitochondrial function.

The major findings of this study is that approximately 70–80% of Pt accumulation in the cells depends on the $\Delta\psi$ of mitochondria and on energy, strongly suggesting there is an energy-dependent and potential-dependent accumulation of Pt in the mitochondria. Our experiments showed that the incubation of cells with either CDDP or aqua-Pt decreased the accumulation of TMR in mitochondria. This indicates the decrease in mitochondrial membrane potential. Kruidering et al. (1997) already showed that exposure of freshly isolated porcine proximal tubular cells in suspension to CDDP resulted in loss of mitochondrial membrane potential. Moreover, the observation that the addition of aqua-Pt to cells decreases the rate of oxygen consumption is in favor of Pt interaction with mitochondria. However, the poor

lipophilicity of Pt-derivatives combined with a localized positive charge, makes it unlikely that Pt complexes enter mitochondrial membranes. Consequently, it follows that if Pt-derivatives accumulate inside mitochondria, they must use transporter(s).

Volckova et al. (2002) have shown that at neutral pH and equimolar concentrations of DNA and thiol groups, only a very small amount of Pt (<5%) was coordinated to DNA, and most of the Pt was coordinated to the thiol groups. The observation that the reaction of aqua-Pt with GSH is fast (Fig. 2) and that inside the cells 70% of Pt is bound to GSH lead us to think that Pt could be accumulated inside the mitochondria as a GS–Pt complex, using for this purpose the carriers used by GSH to enter into mitochondria.

Despite its exclusive synthesis in the cytosol, GSH is distributed in intracellular organelles, including endoplasmic reticulum and mitochondria. In mitochondria, GSH is mainly found in reduced form and represents a minor fraction of the total GSH pool (10–15%). Considering the volume of the mitochondrial matrix, the concentration of mitochondrial GSH (mGSH) may be similar to that of cytosol (10–14 mM). However, unlike cytosol, mitochondria do not contain the enzymatic machinery to synthesize GSH from its constituent amino acids. mGSH arises by the activity of a mitochondrial carrier located in the mitochondrial inner membrane. Because GSH is an organic anion, its transport into mitochondrial matrix occurs against an electrochemical gradient. Early studies indicated that GSH uptake into mitochondria from rat liver is an active, energy- and potential-dependent process, because it is stimulated by ATP, whereas FCCP, which collapses the mitochondrial proton gradient and depolarizes mitochondria, inhibits GSH transport (Martensson et al., 1990). Furthermore, findings in rat kidney mitochondria provided evidence for the mitochondrial uptake of GSH by dicarboxylate and 2-oxoglutarate carriers in exchange with inorganic phosphate or dicarboxylates (Anania et al., 1996; Lluis et al., 2003; Miao et al., 1997).

In addition, Chen et al. (2000) have studied the importance of the free sulfhydryl group on the GSH molecules transport. The competition studies with several S-alkyl GSH analogs and the inhibition by the GSH analog ophtalmic acid, which has an α-aminobutyryl residue replacing the cysteinyl residue, suggested that the thiol group is not critical for transport. From these results, we can infer that complexation of the thiol group to Pt will not prevent the transport of GS–Pt by GSH carriers through the mitochondria membrane.

To conclude, our data suggest that most of intracellular Pt is rapidly bound to GSH in the cytosol, and that the GS–Pt complex thus formed is transported inside the mitochondria by dicarboxylate and/or 2-oxoglutarate transporters.

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