Biochemical Pharmacology, Vol. 35, No. 19, pp. 3365–3369, 1986.
Printed in Great Britain.

0006–2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

INTRACELLULAR DISTRIBUTION OF A PLATINUM–RHODAMINE 123 COMPLEX IN *cis*-PLATINUM SENSITIVE AND RESISTANT HUMAN SQUAMOUS CARCINOMA CELL LINES

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(*Received* 9 *December* 1985; *accepted* 27 *February* 1986)

Abstract—The platinum(II) tetrachlorodianion and two molecules of rhodamine-123 associate to form a neutral tight ion pair. To examine the intracellular fate of this ionic complex, the levels of uptake after a 1-hr exposure to a 100 μM concentration of each component of the complex, the complex itself and *cis*-diamminedichloroplatinum(II) (CDDP) were measured in SCC-25 cells. The uptake of Pt(Rh-123)$_2$ was measured by two independent methods: fluorescence and ^{195m}Pt gamma-counting. There was excellent agreement between these two methods as to the amount of Pt(Rh-123)$_2$ which was taken up by the cells, indicating that the Pt(Rh-123)$_2$ is probably entering cell intact. Association with Rh-123 increased the amount of platinum which entered the cells by about 70-fold compared to CDDP and increased by about 700-fold the amount of platinum which entered the cells compared to K_2PtCl_4. The subcellular distributions of Pt(Rh-123)$_2$, Rh-123, CDDP and K_2PtCl_4 were also examined. When measured by fluorescence or ^{195m}Pt gamma-counting, 40–54% of the Pt(Rh-123)$_2$ was in the nuclei of the SCC-25 or SCC-25/CP cells and 27–35% was in the cytosol of the cells. There was excellent agreement between the findings of fluorescence and ^{195m}Pt gamma-counting regarding the amount of Pt(Rh-123)$_2$ in each of the subcellular fractions immediately after incubation with the drug and over the time course of observation after drug removal, indicating that the Pt(Rh-123)$_2$ is probably remaining largely intact intracellularly. On a per mg protein basis, there was about a 55-fold greater amount of platinum in the nuclei of the SCC-25 cells exposed to Pt(Rh-123)$_2$ compared to cells exposed to CDDP. In the SCC-25/CP cells, there was about 258-fold greater platinum in the nuclei of cells exposed to Pt(Rh-123)$_2$ than those exposed to CDDP because CDDP was taken up to a much lesser extent by the SCC-25/CP cells. Association of Rh-123 with potassium tetrachlorodianion forms a tight ion pair, which enters cells in relatively high amounts and is selectively concentrated in the nuclei of the cells.

In an effort to develop platinum complexes for use as antineoplastic agents and radiosensitizers [1–4], we prepared a series of complexes of tetrachloroplatinate with positively charged dyes [5, ‖, ¶]. These complexes have demonstrated good anticancer activity¶ and are effective radiosensitizing agents both *in vitro* and *in vivo*‖¶. A human squamous carcinoma cell line (SCC-25/CP), which is resistant to *cis*-diamminedichloroplatinum(II) (CDDP) and to the second generation platinum complexes, carboplatin (JM-8, CBDCA) and iproplatin (JM-9, CHIP), is as sensitive to the platinum-positively charged dye complexes as is the parent (SCC-25) cell line [5, ¶]. The complex of tetrachloroplatinate with two molecules of rhodamine-123 [Pt(Rh-123)$_2$] was selected for more detailed biological analysis because the low level of whole animal toxicity and high level of anticancer and radiosensitizing activity made it the most likely candidate for further development.

As determined by X-ray crystallography, the structure of Pt(Rh-123)$_2$ is a tight ion pair with two molecules of rhodamine-123 around the platinum tetrachlorodianion.** These experiments were carried out to determine if Pt(Rh-123)$_2$ entered cells intact, if the molecular components of Pt(Rh-123)$_2$ move together or separately intracellularly, and where each component of Pt(Rh-123)$_2$ localizes within cells. Two cell lines, SCC-25 human squamous cell carcinoma and SCC-25/CP, a subline resistant to CDDP [6–8], were examined.

MATERIALS AND METHODS

Drugs. Rhodamine-123 (Rh-123) was purchased from Kodak Laboratory Chemicals (Rochester, NY). Potassium tetrachloroplatinate (K_2PtCl_4) and *cis*-diamminedichloroplatinium(II) (CDDP) were gifts from Johnson Matthey Inc. (West Chester, PA).

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‖ B. A. Teicher and S. A. Holden, *Int. J. Radiat. Oncol. Biol. Phys.*, submitted, 1985.

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$Pt(Rh\text{-}123)_2$ was prepared in our laboratory by reaction of appropriate molar equivalents of potassium tetrachloroplatinate and rhodamine-123 in water. [^{195m}Pt]-*cis*-diamminedichloroplatinum(II) in isotonic saline and [^{195m}Pt]-potassium tetrachloroplatinate were made available by Dr. Hoeschele and Dr. F. F. Knapp, Jr. at Oak Ridge National Laboratories, Oak Ridge, TN [8, 9]. [^{195m}Pt]-CDDP and [^{195m}Pt]-K_2PtCl_4 were received with specific activities of 145–165 mCi/mmole and 203–227 mCi/mmole respectively. The half-life of ^{195m}Pt is 4.02 days; therefore, experiments were carried out immediately upon receipt of the materials. $Pt(Rh\text{-}123)_2$ was prepared by reaction of 2.1 molar equivalents of Rh-123 with [^{195m}Pt]-K_2PtCl_4 plus non-radioactive K_2PtCl_4 to allow preparation of sufficient material to use.

Cell lines. SCC-25 and SCC-25/CP human squamous carcinoma of the head and neck cells grow as monolayers in Dulbecco's modification of Eagle's medium (Flow Laboratories, McLean, VA) supplemented with antibiotics (Gibco, Grand Island, NY) and 5% fetal bovine serum (Hyclone Laboratories, Logan, VT) [7]. These cell lines have a doubling time of 48–50 hr *in vitro* [6].

Cellular fractionation. Two million SCC-25 and SCC-25/CP cells per plate were seeded in DME medium containing 5% fetal bovine serum overnight. Cells were incubated with 100 μM Rh-123, $Pt(Rh\text{-}123)_2$, CDDP or K_2PtCl_4 for 60 min at 37° in humidified air with 8% CO_2. Plates were washed six times with phosphate-buffered 0.9% saline, fresh medium containing serum was added, and plates were replaced in a 37°, 8% CO_2 incubator for 0, 3, 6 or 24 hr. At the end of each time period, cells were suspended using 0.05% EDTA/0.125% trypsin, centrifuged at 500 *g*, resuspended in water, and lysed by sonication on ice.

The fractionation procedure of Sharma and Edwards [10] was performed at 4° and the final pellets were resuspended in water. Lysed cells were centrifuged at 1000 *g* for 10 min. The pellet was resuspended in 0.25 M sucrose/1.8 mM $CaCl_2$/1% Triton X-100 solution. An equal volume of 0.34 M sucrose/0.18 mM $CaCl_2$ solution was added to the bottom of the tube, pushing the lighter solution up. After centrifugation at 600 *g* for 10 min, the nuclear pellet was obtained. The supernatant fraction from the first 1000 *g* spin was centrifuged at 3500 *g* for 10 min to obtain the mitochondrial pellet. This supernatant fraction was centrifuged at 16,000 *g* for 20 min to obtain the lysosomal pellet. This supernatant fraction was, in turn, spun at 10,000 *g* for 60 min to obtain the microsomal pellet. The final supernatant fraction contained the cytosol.

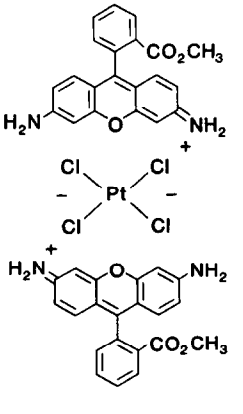
Fluorescence measurements. All resuspended pellets were sonicated to release the contents of the organelle into the solution. The fractions were then centrifuged again at 100,000 *g* for 60 min. The amount of Rh-123 or $Pt(Rh\text{-}123)_2$ in the final supernatant fractions was determined using a Perkin–Elmer MPF-4 fluorescence spectrophotometer. The excitation maximum used for both Rh-123 and $Pt(Rh\text{-}123)_2$ was 522 nm. The amounts of Rh-123 and $Pt(Rh\text{-}123)_2$ were determined by comparison to standard curves prepared for each drug in the same solvent. Each point represents the mean of five independent determinations.

Gamma emission measurements. ^{195m}Pt content was determined using a Beckman Gamma 4000 Counter. The amounts of [^{195m}Pt]-CDDP, [^{195m}Pt]-K_2PtCl_4 and [^{195m}Pt]-$Pt(Rh\text{-}123)_2$ were determined by comparison to standards. For the drug exposure to the cells, 100 μM CDDP was 14.04 μCi/ml, 100 μM $Pt(Rh\text{-}123)_2$ was 10.86 μCi/ml and 100 μM K_2PtCl_4 was 10.64 μCi/ml. Each point represents duplicate determinations in two independent experiments.

Protein determinations. Protein content of the cellular fractions was measured using a modified biuret assay (Sigma Diagnostics, Sigma Chemical Co., St. Louis, MO).

RESULTS

The structure of $Pt(Rh\text{-}123)_2$ is shown in Fig. 1. This complex is a tight ion pair formed between the platinum(II) tetrachlorodianion and two molecules of Rh-123 to give a neutral species. To examine the intracellular fate of this ionic complex, first the levels of uptake after 1 hr of exposure to a 100 μM concentration of each component of the complex (K_2PtCl_4 and Rh-123), the complex itself [$Pt(Rh\text{-}123_2$], and CDDP were measured in SCC-25 cells (Table 1). Rhodamine-123 entered cells quite readily. Fluorescence measurements showed that 9.25 nmoles was taken up per 10^6 cells. The uptake of $Pt(Rh\text{-}123)_2$ was measured by two independent methods. Fluorescence allowed measurement of the Rh-123 portion of the $Pt(Rh\text{-}123_2$ and ^{195m}Pt gamma-counting allowed measurement of the $PtCl_4$ portion of $Pt(Rh\text{-}123)_2$. There was excellent agreement between these two methods of measurement as to the amount of $Pt(Rh\text{-}123)_2$ which entered the cells, 2.13 nmoles/10^6 cells by fluorescence and 2.27 nmoles/10^6 cells by ^{195m}Pt gamma-counting. The agreement of these determinations indicates that the complex is probably entering cells intact. Association with the $PtCl_4^{2-}$ reduced by about 2-fold the amount of Rh-123 which was taken up by the cells. Thirty picomoles of CDDP and only 3 picomoles of K_2PtCl_4



$Pt(Rh\text{-}123)_2$

Fig. 1. Structure of $Pt(Rh\text{-}123)_2$, a complex formed by the tetrachloroplatinate dianion and two molecules of rhodamine-123.

Table 1. Intracellular concentration of platinum complexes and rhodamine-123*

Drug	Measurement method	Concn (nmoles/10^6 cells)
Rh-123	Fluorescence†	9.25 ± 0.35‡
$Pt(Rh\text{-}123)_2$	Fluorescence†	2.13 ± 0.28
$Pt(Rh\text{-}123)_2$§	^{195m}Pt gamma-counting	2.27 ± 0.17
$Pt(NH_3)_2Cl_2$§	^{195m}Pt gamma-counting	0.03 ± 0.007
K_2PtCl_4	^{195m}Pt gamma-counting	0.003 ± 0.0005

* SCC-25 cells were exposed to 100 μM drug for 1 hr.
† Fluorescence emission was 525 nm for Rh-123 and 522 nm for $Pt(Rh\text{-}123)_2$.
‡ Values are means for three experiments ± S.E.M.
§ ^{195m}Pt concentration was approximately 1.8 μCi/μmole. $Pt(NH_3)_2Cl_2$ is CDDP.

were taken up by the SCC-25 cells after 1 hr of exposure to each of these drugs. Therefore, association with Rh-123 increased the amount of platinum which entered the cells by about 70-fold compared to CDDP and increased by about 700-fold the amount of platinum which entered the cells compared to K_2PtCl_4.

The subcellular distribution of $Pt(Rh\text{-}123)_2$ as measured by ^{195m}Pt gamma-counting is shown in Fig. 2. About 48% of the drug in the SCC-25 cells was in the nucleus at time 0 hr and about 54% of the drug was in the nuclei of the SCC-25/CP cells. The cytosol of both cell lines contained 27–35% of the drug and the remainder was in the other cellular components. Overall, after 24 hr about 10% of the drug was retained by the cells. When measured by fluorescence, about 45% of the $Pt(Rh\text{-}123)_2$ was in the nuclei of the SCC-25 cells and about 47% was on the nuclei of the SCC-25/CP cells (Fig. 3). There was excellent agreement between the findings by fluorescence and ^{195m}Pt gamma-counting regarding the amount of $Pt(Rh\text{-}123)_2$ in each of the subcellular fractions immediately after incubation with the drug and over the time course of observation after drug removal. This indicates that the complex $Pt(Rh\text{-}123)_2$ was probably remaining largely intact intracellularly.

Rhodamine-123 cellular distribution is shown in Fig. 4. Most of the drug was in the cytosol of the cells (81–86%). It appears that Rh-123 is largely excluded from the nuclei since only 0.3–0.7% of the drug was found in the nuclear fractions. The mitochondria contained 6–7% of the drug. After 24 hr, 4–5% of the drug was retained by the cells. Potassium tetrachloroplatinate was also largely found in the cytosol (76–77%) (Fig. 5). The nuclear concentrations of this drug were low with 3.3–8.5% of K_2PtCl_4 being found in the nuclei. By complexing Rh-123 to $PtCl_4^{2-}$, a new molecular species was formed which, unlike the components from which it was made, enters the nuclei of cells to a greater degree than expected.

Protein determinations were made on each of the cellular fractions of the SCC-25 and SCC-25/CP cells (Table 2). These measurements may be used as an approximation of the cellular volume occupied by each of the cellular components. Therefore, the nucleus was about 13–15% of the cellular volume; mitochondria, lysosomes and microsomes were 5, 6–7 and 5–7% of the cellular volume respectively; and the cytosol was 67–69% of the cellular volume in both cell lines. Using this information, the amount of each drug present in the cellular fractions was calculated on a per mg protein basis (Table 3). Of the drugs examined, CDDP and $Pt(Rh\text{-}123)_2$ were concentrated intracellularly. CDDP and $Pt(Rh\text{-}123)_2$

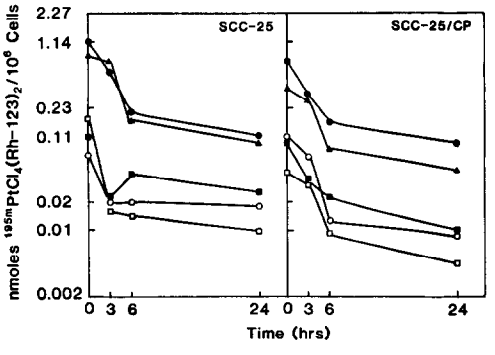


Fig. 2. $Pt(Rh\text{-}123)_2$ content of various subcellular fractions of SCC-25 and SCC-25/CP cells as measured by ^{195m}Pt gamma-counting. Cells were exposed to 100 μM drug for 1 hr. Zero time was immediately after removal of the drug. The symbols are: cytosol (▲), nuclei (●), mitochondria (○), lysosomes (■) and microsomes (□).

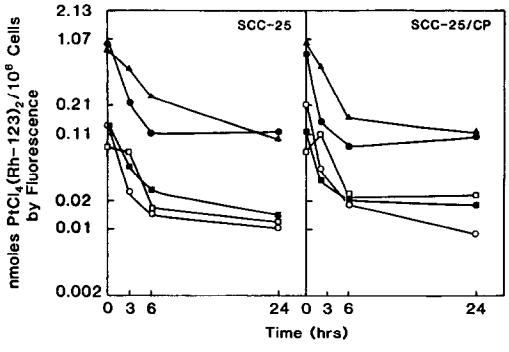


Fig. 3. $Pt(Rh\text{-}123)_2$ content of various subcellular fractions of SCC-25 and SCC-25/CP cells as measured by fluorescence emission at 522 nm. Cells were exposed to 100 μM drug for 1 hr. Zero time was immediately after removal of the drug. The symbols are: cytosol (▲), nuclei (●), mitochondria (○), lysosomes (■) and microsomes (□).

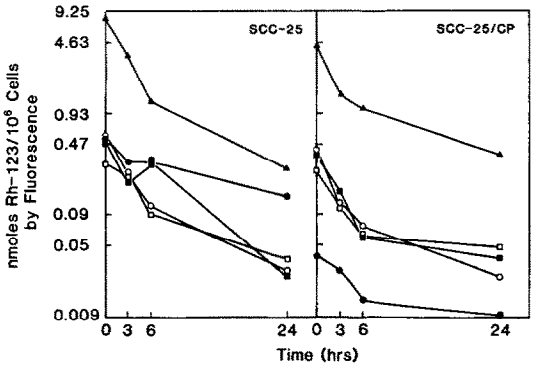


Fig. 4. Rhodamine-123 content of various subcellular fractions of SCC-25 and SCC-25/CP cells as measured by fluorescence emission at 522 nm. Cells were exposed to 100 μM drug for 1 hr. Zero time was immediately after removal of the drug. The symbols are: cytosol (▲), nuclei (●), mitochondria (○), lysosomes (■) and microsomes (□).

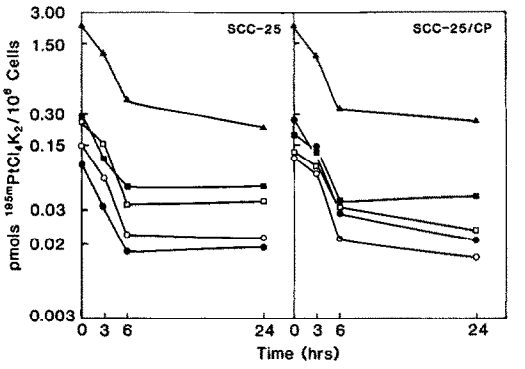


Fig. 5. Potassium tetrachloroplatinate content of various subcellular fractions of SCC-25 and SCC-25/CP cells as measured by ^{195m}Pt counting. Cells were exposed to 100 μM drug for 1 hr. Zero time was immediately after removal of the drug. The symbols are: cytosol (▲), nuclei (●), mitochondria (○), lysosomes (■) and microsomes (□).

were relatively concentrated in the nuclei. There was about a 55-fold greater amount of platinum in the nuclei of SCC-25 cells exposed to $Pt(Rh\text{-}123)_2$ compared to cells exposed to CDDP. In the SCC-25/CP cells, however, there was about 258-fold greater platinum in the nuclei of cells exposed to $Pt(Rh\text{-}123)_2$ than those exposed to CDDP because CDDP was taken up to a much lesser extent by the SCC-25/CP cells. The relatively small amount of Rh-123 which entered the nuclei of the cells was retained there to a greater extent (37–47%) after 24 hr. Although less CDDP was taken up by the SCC-25/CP cells than the SCC-25 cells, all of the other drugs

Table 2. Protein content of subcellular fractions

Cellular fraction	Protein content (mg/10⁶ cells) SCC-25	SCC-25/CP
Nuclei	0.058 ± 0.001	0.058 ± 0.005
Mitochondria	0.023 ± 0.005	0.0183 ± 0.007
Lysosomes	0.0378 ± 0.002	0.027 ± 0.001
Microsomes	0.030 ± 0.005	0.018 ± 0.053
Cytosol	0.308 ± 0.072	0.271 ± 0.053
Whole cells	0.457 ± 0.050	0.391 ± 0.030

Measurements are the means of three determinations ±S.E.M.

Table 3. Subcellular content of platinum complexes and rhodamine-123 at various times post-treatment

Drug	Cellular fraction	Drug (pmoles/mg protein) SCC-25 0 hr	SCC-25 24 hr	SCC-25/CP 0 hr	SCC-25/CP 24 hr
$Pt(Rh\text{-}123)_2$*	Cells	4,773	596	3,448	438
	Nuclei	19,256	1,956	12,524	1,644
	Cytosol	2,575	317	1,343	180
$Pt(Rh\text{-}123)_2$†	Cells	4,679	527	5,453	621
	Nuclei	16,526	1,836	12,486	1,689
	Cytosol	2,697	297	3,701	338
Rh-123	Cells	20,326	787	13,721	1,251
	Nuclei	510	239	638	239
	Cytosol	25,828	856	16,072	946
K_2PtCl_4*	Cells	6.80	0.80	7.64	0.94
	Nuclei	1.71	0.26	4.39	0.31
	Cytosol	7.44	0.75	8.54	0.98
CDDP*	Cells	65.7	8.1	17.0	2.9
	Nuclei	331	25.9	46.5	5.5
	Cytosol	20.8	3.4	8.1	1.5

* $Pt(Rh\text{-}123)_2$, K_2PtCl_4 and CDDP as measured by ^{195m}Pt gamma-counting.
† $Pt(Rh\text{-}123)_2$ and Rh-123 as measured by fluorescence emission at 522 and 525 nm respectively.

examined were taken up in equal levels by both cell lines.

DISCUSSION

In has been known for some time that platinum complexes can act as radiosensitizers of hypoxic tumor cells [2–4]. This phenomenon depends upon the quantity of platinum(II) present in the cells at the time X-rays are delivered [3]. The examination of the radiosensitizing potential of platinum complexes was hindered by the potent cytotoxicity of CDDP. The advent of less cytotoxic platinum complexes such as carboplatin and iproplatin has enabled investigators to take greater advantage of radiosensitization by platinum in addition to direct antitumor activity by the drugs [11]. We have found that the new platinum complex $Pt(Rh\text{-}123)_2$, which is much less toxic than CDDP, enters cells more readily than does CDDP and, like CDDP, concentrates in the nuclei of cells.

The distribution of platinum in cells treated with CDDP has been studied previously using scanning transmission electron microscopy in conjunction with X-ray probe microanalysis. By this method, Kahn and Sandler [12] noted high concentrations of platinum in the nucleolus and inner side of the nuclear double membrane. Using fluorescent probes, it was shown that DNA and histone #3 are the major targets of platinum(II) complexes in nucleosomes [13]. Intracellular platinum levels in cells from tissues of animals treated with CDDP, especially liver and kidney, have been examined [10, 14]. Berry *et al.* [14], using electron microscopy techniques, found that platinum concentrates in the lysosomes of kidney tubules, from which it can be re-released into the cytoplasm and thus cause necrosis in the region. Sharma and Edwards [10] found that about 25% of platinum is bound to the metallothionein-like protein in the liver as well as the kidneys. It may be possible that in tissues which are especially developed for dealing with xenobiotics that platinum may be metabolized before reaching the nucleus.

Rh-123 has been described as mitochondrial poison [14–26]. Carcinoma cells, when treated with Rh-123, specifically accumulate and retain the drug in their mitochondria [19, 24, 25]. Non-tumorigenic cells accumulate less Rh-123 in their mitochondria and lose the dye very quickly [18]. Recently, it was shown that, in cells sensitive and resistant to Rh-123, cytotoxicity does not correlate with inhibition of oxidative phosphorylation in mitochondria isolated from each cell line [27]. In our studies, Rh-123 was found in highest amounts in the cytosol and in relatively low amounts in the nuclei of both cell lines.

Association of Rh-123 with platinum tetrachlorodianion forms a tight ion pair which enters cells in relatively high amounts and is selectively concentrated in the nuclei of cells. This property of $Pt(Rh\text{-}123)_2$ makes it a very favorable candidate for development both as an antitumor agent and a hypoxic cell radiosensitizer. The ionic complex $Pt(Rh\text{-}123)_2$ appears to stay intact intracellularly, and studies of the interaction of this drug with cellular DNA are in progress.

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