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Increased DNA-Binding Activity of *cis*-1,1-Cyclobutanedicarboxylatodiammineplatinum(II) (Carboplatin) in the Presence of Nucleophiles and Human Breast Cancer MCF-7 Cell Cytoplasmic Extracts: Activation Theory Revisited

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ABSTRACT. The molecular mechanism of carboplatin [*cis*-1,1-cyclobutanedicarboxylatodiammineplatinum(II)] activation is still unresolved. We studied the binding of carboplatin to calf thymus DNA in the presence of thiourea, glutathione, and human breast cancer MCF-7 cell cytoplasmic extracts by measurement of DNA-dependent ethidium bromide fluorescence and atomic absorption spectroscopy. After a 96-hr period of reaction, the decrease in the DNA-dependent fluorescence yield of ethidium bromide due to the formation of platinum (Pt)-DNA adducts increased significantly in the presence of thiourea (6-fold) and glutathione (3- to 4-fold) as compared to the controls in the absence of the nucleophiles. There was also a marked elevation in the levels of platinum incorporated into DNA, measured by atomic absorption spectroscopy (2- to 3-fold and 5- to 7-fold for thiourea and glutathione, respectively). More remarkably, the Pt-DNA adducts formed in the presence of cytoplasmic extracts of MCF-7 human breast cancer cells also showed similar results in a dose-related fashion. Carboplatin, therefore, displayed a characteristic increase in DNA binding/damaging in the presence of the very same S-containing nucleophiles that showed the expected quenching effects in the case of cisplatin [*cis*-diamminedichloroplatinum (II)]. We propose a nucleophile-facilitated release of the active species of carboplatin prior to binding with DNA. BIOCHEM PHARMACOL **58**;10:1625–1629, 1999. © 1999 Elsevier Science Inc.

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The molecular basis behind the antitumor activity of carboplatin§ is thought to be due to its reaction with DNA [1, 2], quite analogous to that of cisplatin. The major reaction product in the case of cisplatin has been shown to be the GpG intrastrand cross-link [3, 4]. This type of adduct has been reported to be biologically important and relevant to the antitumor activity. Other reaction products include: 1) G-G interstrand cross-links; 2) monofunctional Pt-DNA adducts; 3) adducts and cross-links at other bases; and 4) low levels of DNA–protein cross-links [5–7]. The focus, until now, has been on the damage produced in the DNA, though recent reports on the involvement of high-mobility group proteins have begun to attract considerable attention [8, 9].

Two major mechanisms have been proposed to describe the reaction of carboplatin with DNA: the aquation hypothesis and the activation hypothesis. The former, which is more accepted, holds that the drug complex aquates and reacts with DNA in a similar fashion to cisplatin. The difference in activity has been attributed to the variation in the rates of aquation of the respective platinum complexes [10]. Under *in vitro* conditions, it was well demonstrated that carboplatin and cisplatin aquated to release the active Pt^{2+} species, albeit at very different rates [1] and form the known adducts with DNA. The latter hypothesis assumes carboplatin to be *biologically* activated, giving rise to a still unknown platinum species which then binds to DNA. The requirement of much lower doses of carboplatin in patients and cell lines [11, 12] compared to concentrations in cell-free experiments [1] seems to point to the activation of carboplatin by a cellular constituent not present in the cell-free reaction solution [13]. Due to lack of experimental support at the molecular level, this hypothesis has not gained much credence.

Recently, however, distinctly different DNA-binding properties of carboplatin have been reported. ^{31}P NMR

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§ *Abbreviations:* Carboplatin, *cis*-1,1-cyclobutanedicarboxylatodiammineplatinum(II); cisplatin, *cis*-diamminedichloroplatinum(II); CBDCA-O, 1,1-cyclobutanedicarboxylate; EtBr, ethidium bromide; and Pt, platinum.

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studies indicated that the action of carboplatin may involve a direct attack of nucleotides on platinum [14]. Remarkably, stable ring-opened complexes with methionine [Pt(CBDCA-O) $(NH_3)_2$-(L-methionine-*S*)] were observed. It has been suggested that the monodentated sulfur-containing ligands may play a role in the antitumor mechanism of action of carboplatin [15]. These observations gain significance in the light of the recent evidence [16] showing that DNA adducts of carboplatin in Chinese hamster ovary cells are not reconcilable with those observed in the cell-free systems and for cisplatin.

Keeping in view that sulfur-containing compounds could attack platinum(II) complexes directly [17] without prior aquation, we considered glutathione and thiourea as possible candidates that could activate carboplatin. These reactions are slow, but compete with the slow aquation reaction of carboplatin. In contrast, cisplatin aquates and reacts with DNA much more rapidly. To investigate this activation mechanism, we also included methionine in our investigation as another reactive candidate. Finally, we showed that extracts of cells (in our example human breast cancer MCF-7 cells) which contained the natural nucleophiles were able to activate carboplatin.

MATERIALS AND METHODS

DNA and Platinum(II) Complexes

Lyophilized calf thymus DNA (Pharmacia) was dissolved in sodium 2-(*N*-morpholino)ethanesulfonate buffer (10 mM, pH 5.7) containing 10 mM NaCl. Reactions involving glutathione were carried out in sodium 3-(*N*-morpholino)propanesulfonate buffer (10 mM with 10 mM NaCl, pH 8.0). Carboplatin and cisplatin were kind gifts from Tamilnadu Dadha Pharmaceuticals Ltd; they were prepared as 10 and 2 mM stock solutions, respectively, in fresh distilled water and stored at −20°.

Platination of DNA

Concentrations of DNA were measured spectrophotometrically ($\epsilon = 6600\ M^{-1}\ cm^{-1}$, 260 nm). Stock solutions of the Pt(II) complexes were added at final Pt/nucleotide ratios of one. Glutathione (2 mM), thiourea (2 mM), or MCF-7 cell cytoplasmic extract (20 μL) was added prior to the addition of the drugs. Samples were incubated at 37° for 24 hr and used at time intervals for atomic absorption spectroscopy analyses or in 1-mL aliquots for fluorescence measurements.

Preparation of Cytoplasmic Extracts

MCF-7 breast cancer cells (American Type Culture Collection) were maintained in improved minimal essential medium (Boerhinger Ingelheim) without phenol red, supplemented with 10% fetal bovine serum (GIBCO). Cells were passaged weekly and were plated at a titer of 2×10^4/mL. After 24-hr incubation at 37° at 5% CO_2, the cells were harvested by treatment with trypsin and collected as a pellet. This was resuspended and washed in sodium 2-(morpholino)ethanesulfonate buffer before centrifugation (500 *g*, 5 min). By addition of 250 μL of Tris–HCl (pH 7.8), the cells were again pelletted. Cells were then frozen (−70°, 0.5 min) and thawed (37°). This procedure was repeated several times to ensure the complete rupture of the cells. After centrifugation (12,000 *g*, 5 min), the supernatant was taken as the cytoplasmic extract. It was virtually devoid of DNA.

Fluorescence Studies

Samples were mixed with ethidium bromide (EtBr/nucleotide = 1:3). Fluorescence intensities were recorded (Hitachi F-3000, $\lambda_{excitation}$ = 540 nm, $\lambda_{emission}$ = 590 nm) and expressed as percent values with respect to DNA controls in the absence of platinum complex but otherwise identical sample compositions. Ethidium bromide was added shortly before the measurement at 20° to rule out possible effects by the sulfur-containing compounds on EtBr fluorescence.

Precipitation of DNA-Pt(II) Adducts

Platination of DNA was carried out as above except that the concentration of DNA was adjusted to a minimum of 750 μL (250 μg/mL). After incubation at 37° for 24 hr, aliquots of 200 μL (in duplicate) were mixed with 100 μL of 0.9 M sodium acetate and three volumes of ice-cold ethanol. Samples were frozen in liquid nitrogen and stored at −20° for 30 min. The precipitate was collected by centrifugation (10 min at 5400 *g* and −20°) and resuspended in 300 μL of 0.3 M sodium acetate. After precipitation with ice-cold ethanol, the pellet was washed three times in ice-cold ethanol, resuspended in 500 μL of 0.5% HNO_3, and incubated for 48 hr in a rotary shaker at 70° to dissolve the pellet. Concentrations of the platinum atoms incorporated into the DNA were determined using flameless atomic absorption spectroscopy (atomic absorption spectroscopy with Varian DS-15). Readings were averaged from three experiments. The calibration curves included 5 standards with concentrations ranging from 50 to 600 ng platinum per mL; calibration was repeated for every sixth sample. Nucleotide concentrations in the samples were measured spectrophotometrically using an average molar extinction coefficient of 8900 $M^{-1}\ cm^{-1}$ at 260 nm wavelength assuming the four kinds of free nucleotides. The level of platinum bound to DNA was expressed in picomoles of platinum per nanomole of DNA bases.

RESULTS

Platinum-Induced Changes in DNA-Dependent Ethidium Bromide Fluorescence

The requirement of stacked bases in the DNA for successful intercalation by ethidium bromide was taken as an indicator of the damage caused by the coordination of Pt(II) complexes in the double helix [18]. The changes in the

TABLE 1. Reaction of carboplatin or cisplatin with calf thymus DNA (Pt:P = 1, pH 5.7) in the presence of L-methionine, glutathione, or thiourea (each 2 mM)

	Control		Methionine		Glutathione		Thiourea	
Pt(II) complex	k (sec^{-1})	$t_{1/2}$ (hr)	k (sec^{-1})	$t_{1/2}$ (hr)	k (sec^{-1})	$t_{1/2}$ (hr)	k (sec^{-1})	$t_{1/2}$ (hr)
Carboplatin	7.5×10^{-7}	255	1.9×10^{-6}	96	2.8×10^{-6}	68	6.1×10^{-6}	31
Cisplatin	1.9×10^{-5}	10	1.2×10^{-5}	16	9.9×10^{-6}	19	4.7×10^{-6}	43

Calf thymus DNA (Pt:base = 1, pH 5.7) was treated with carboplatin or cisplatin in the presence of L-methionine, glutathione, or thiourea (2 mM each). The extent of DNA lesions was measured by ethidium bromide-dependent fluorescence. Rate constants (k) and reaction half-lives ($t_{1/2}$) are calculated by fitting the fluorescence intensity data points with a single exponential decay.

quantum yield of EtBr fluorescence (50 μM) in the presence of calf thymus DNA (150 μM) treated with cisplatin or carboplatin (Pt: nucleotide = 1) was followed over an incubation period of 96 hr. The addition of either of these two Pt(II) complexes resulted in a characteristic time-dependent exponential decrease in fluorescence yield. Pseudo first-order rate constants were of the order of the values reported in [1]: 1.6×10^{-5} sec^{-1} and 8.3×10^{-7} sec^{-1} for cisplatin and carboplatin, respectively. Carboplatin caused a 7% decrease in EtBr fluorescence intensity compared to 75% by cisplatin after 96 hr. The difference in reactivity was attributed to the aquation-resistant cyclobutane dicarboxylate ligand in carboplatin.

The effects of sulfur-containing compounds shown in Table 1 represent the changes in rate constants and half-lives of the DNA–platinum(II) reaction for both platinum complexes in the presence of glutathione and thiourea. In the presence of glutathione, the decrease in fluorescence intensity after 96-hr incubation of the reaction mixture with carboplatin corresponded to a 25 ± 1% decrease in the initial EtBr fluorescence yield (platinum absent) compared to a 7% decrease in the drug-treated controls (glutathione absent). This corresponded to a 3- to 4-fold increase in the number of DNA lesions by carboplatin. Greater effects were observed in the case of thiourea, which showed a 41 ± 1% decrease in fluorescence intensity over the same period, i.e. there was a 6-fold increase in the amount of induced damage. Similar effects could also be observed in the case of methionine. In contrast, the number of lesions induced by cisplatin was markedly reduced in the presence of glutathione (57 ± 1%) and thiourea (23 ± 1%). This corresponded to a 24% and 69% inhibition in the activity of cisplatin. Thus, it could clearly be observed that the S-containing nucleophiles decreased the half-lives of the DNA-dependent EtBr fluorescence yield. In other words, the presence of these nucleophiles increased the carboplatin-induced DNA damage, the effect being at its maximum in the case of thiourea. On the other hand, the presence of these very agents had a quenching effect on the activity of cisplatin that was along the expected lines. These results amply demonstrated that carboplatin had an increased DNA-binding activity in the presence of the very same agents that quenched the activity of cisplatin. The sulfur-containing nucleophiles thus had an opposite effect on the DNA-damaging activity by carboplatin compared with cisplatin.

Atomic Absorption Studies

EFFECT OF S-CONTAINING NUCLEOPHILES. The results of the fluorescence analyses using ethidium bromide provided an indirect indicator of the level of damage produced by the Pt(II) complexes in DNA. To examine if these results were also reflected in the amount of platinum incorporated into calf thymus DNA, measurements were carried out using flameless atomic absorption, the results of which are shown in Table 2. The amounts of bound platinum from carboplatin increased by a factor of 6 in the presence of glutathione and by a factor of 2.6 in presence of thiourea. The amount of platinum incorporated from cisplatin decreased by a factor of 1.6 for glutathione and a factor of 36 for thiourea. These results qualitatively confirm the effects recorded by the fluorescence measurements above. The discrepancies observed between the changes in fluorescence intensity and the amounts of DNA-bound platinum are assumed to refer to the different types of lesions. These seemed to vary for the different nucleophiles in an unknown fashion.

EFFECT OF MCF-7 CYTOPLASMIC EXTRACT. To demonstrate that similar agents could be present in cells during treatment with platinum(II) complexes, we studied the incorporation of platinum into DNA in the presence of the cytoplasmic extract of MCF-7 breast cancer cells (Table 2). Here again, the contrasting nature of carboplatin and cisplatin was evident. Whereas the incorporation of carboplatin was promoted in the presence of increasing amounts of extract, that of cisplatin was inhibited.

DISCUSSION

Carboplatin was introduced as a second generation anticancer drug during the mid 1980s [2, 19] in response to the severe side effects exhibited by its predecessor, cisplatin. Since then, this drug has gained wide acceptance in the clinical treatment of different cancers [20–22], the dose-limiting factor being myelosuppression [23]. Carboplatin possesses a slowly aquating, ringlike, bulky side group, i.e.

TABLE 2. Effect of *S*-containing nucleophiles and cytoplasmic extracts of MCF-7 cells on the incorporation of platinum into calf thymus DNA

Pt(II) complexes	Control	Thiourea (2 mM)	Glutathione (2 mM)
Carboplatin	$(14 \pm 5) \times 10^{-4}$	$(36 \pm 1) \times 10^{-3}$	$(86 \pm 6) \times 10^{-4}$
Cisplatin	$(289 \pm 4) \times 10^{-3}$	$(8 \pm 9) \times 10^{-4}$	$(182 \pm 1) \times 10^{-3}$

MCF-7 cytoplasmic extract (μL)	Carboplatin	Cisplatin
None	$(14 \pm 5) \times 10^{-4}$	$(289 \pm 4) \times 10^{-3}$
10	$(16 \pm 5) \times 10^{-4}$	$(250 \pm 4) \times 10^{-3}$
20	$(21 \pm 5) \times 10^{-4}$	$(150 \pm 4) \times 10^{-3}$

The effects of *S*-containing nucleophiles (upper panel) and cytoplasm extracts (lower panel) of MCF-7 cells were measured by atomic absorption spectroscopy after 96-hr incubation of the reaction mixtures. Reaction conditions have been described elsewhere in the text. Nucleotides were measured by ultraviolet light absorbance. Relative binding ratios in picomoles platinum per nanomole nucleotides are indicated.

the 1,1-cyclobutanedicarboxylato group, instead of the readily leaving chloro groups in cisplatin. It is well established that hydrolysis of platinum–chloride coordination bonds is a prerequisite for the expression of the antitumor activity of cisplatin [3]. The transformation of these neutral platinum(II) prodrugs into a reactive bifunctional electrophile results in the coordinate binding of the nucleophilic bases in the DNA. The presence of the very slow-leaving 1,1-butanedicarboxylato group in carboplatin led some initial investigators [13] to believe that other mechanisms such as enzymatic cleavage might also exist for the prodrug activation under *in vivo* conditions.

Cisplatin is a prodrug that is activated by the substitution of the chloro ligands by water. It has to be reckoned here that competing reactions can be expected to change the reactivity of a Pt(II) complex in the cellular environment, e.g. capture of the active Pt^{2+} species by sulfhydryl compounds. It has also been postulated that the active Pt^{2+} species may be captured by such nucleophiles and then released in a sustained fashion [24]. The effects of such competing reactions are not completely understood, and their results could be different for cisplatin and carboplatin.

With regard to the present results, the question as to whether cisplatin and carboplatin react with DNA and form adducts by the same drug activation mechanism may be addressed. The nucleophilic nature of the sulfhydryl-containing biomolecules is generally expected to quench the electrophilic platinum (II) species [17], accounting for the observed reduction in the yields of cisplatin-induced DNA lesions and the relative binding values when either thiourea or glutathione was present in the reaction mixture. A very similar effect was found for the cytoplasmic extract of MCF-7 cells, probably due to sulfur-containing compounds or other nucleophiles as their contents. The difference in prodrug activation is indicated by the finding of an increased DNA-binding activity for carboplatin in the presence of the very same agents that quenched the binding of cisplatin. Because the chloride ligands of cisplatin leave much more rapidly than the 1,1-cyclobutanedicarboxylato ligand of carboplatin, cisplatin rapidly becomes monoaquated and then bisaquated. These "activated" forms of cisplatin can attack the DNA or any other competing nucleophiles such as glutathione or thiourea. Under cellular conditions, this competition may become considerable, leading to a decrease in the yields of DNA-bound platinum. However, if the rate of substitution by water is very slow, as in the case of carboplatin, a direct attack by a competing nucleophile on the platinum complex gains importance. We propose that sulfur-containing compounds such as glutathione or thiourea with their easily polarizable sulfur atoms replace the first arm of the CBDCA ligand. The coordination of the platinum with the remaining second arm could be then labilized via a ligand–*cis*-effect [17], facilitating DNA binding either through a direct attack by a nucleo base or through aquation. Thus, because of the slow dissociation of the CBDCA ligand in carboplatin, the activation of the prodrug will not primarily involve aquation but rather the reaction with a sulfur-containing bionucleophile. In the case of such a mechanism, carboplatin and cisplatin should not display cross-resistance if resistance is based on a detoxification reaction by glutathione, unless the enzyme-calatalyzed ligation of carboplatin with glutathione results in an inactive kind of product.

The proposed activation of carboplatin by nucleophiles not only represents a particular mode of drug activation, but also suggests the formation of DNA adducts which are different from adducts by cisplatin. These adducts might contribute to the antitumor activity by causing cell death [13]. It is interesting to note that stable monodentate ligands with sulfur-containing compounds (methionine) reported to occur *in vitro* may play a role in the mechanism of action of carboplatin [14, 15]. Depending on their kinetics, the adducts of DNA with activated carboplatin may be minor ones. This may explain why they have hitherto escaped detection. Isolation and characterization of such adducts is one of the future promising prospects that would elucidate the mechanism of action of such platinum complexes.

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