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RELATIONSHIPS BETWEEN THE AQUEOUS CHEMISTRY AND THE *IN VITRO* CYTOTOXIC ACTIVITIES OF MIXED-AMINE CISPLATIN ANALOGUES

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Abstract—The possibility that variations in the cytotoxic activities of cisplatin analogues could be a result of differences in the aqueous chemistry of the compounds was investigated. A series of structurally related mixed-amine dichloroplatinum complexes (*cis*-coordinated with ammine and various diphenylmethylamines and 1,2-diphenylethylamines) was prepared and selected physicochemical properties of the new compounds were characterized. Cytotoxicity was determined in two human breast cancer cell lines (MDA-MB-231 and MCF-7) and one human ovarian cancer cell line (SK-OV-3) by means of a microtiter assay. There is no apparent relationship between the hydrophobicities of the compounds and their cytotoxic potencies. There is no evidence for an inverse relationship between the aqueous stability of the dichloroplatinum complexes and cytotoxic potency, as has been reported for nitrogen mustards and some nitrosoureas. The differences in cytotoxic activity cannot be explained by inter-compound variations in the area under the concentration–time curves (AUC) of the dichloroplatinum complexes in culture medium. Thus, it appears that the differences in the cytotoxic potencies of this series of cisplatin analogues are related to factors other than dissimilarities in these physiochemical properties. Nevertheless, a relationship was found between the AUC of a dichloroplatinum complex in medium and the efficacy of the compound in the MCF-7 cell line. However, the AUC–efficacy relationship does not always hold in the MDA-MB-231 and SK-OV-3 cell lines. In these cells, treatment with a "high" bolus dose of platinum complex over finite exposure times is often less cytotoxic than treatment with lower doses of the same compound but over a continuous exposure time, although the cells are subjected to the same AUC of dichloroplatinum complex.

The inorganic, transition metal complex cisplatin (**1**) is an invaluable chemotherapeutic for the treatment of several types of cancer, having particular activity against carcinomas of the ovaries, testes, bladder, and the head and neck [1]. However, cisplatin has not gained clinical acceptance in the treatment of other common neoplasms; most notable amongst these is breast cancer [2].

The aqueous chemistry of cisplatin is considered important for understanding the pharmacology of the compound [3]. The relatively inert dichloroplatinum complex is a prodrug, requiring a chemical activation step to unmask the antitumor activity. Pharmacoactivation is thought to occur when the Pt-Cl bonds are hydrolysed in the cytosol, where the low chloride concentration favors the formation of the highly electrophilic aquachloroplatinum(II) species. This hydrolysis product reacts readily with bionucleophiles, amongst others the target molecule DNA [4, 5]. The formation of bifunctional DNA adducts are most likely responsible for the cytotoxic and mutagenic activities of cisplatin [6]. Intrastrand cross-links through the N7 positions of neighboring purine bases (GpG and ApG) and the metal atom have been proposed as the critical lesions [7, 8].

Given the central role that Pt-Cl hydrolysis reactions are believed to play in the cytotoxic activity of cisplatin, an understanding of the aqueous chemistry of new analogues might be expected to be an important aspect of rational drug design. Structure–activity relationships of aniline mustard [9], nitrosourea [10–12] and cyclophosphamide [13] analogues have demonstrated the significance of the aqueous chemistry for the cytotoxic activities of these antitumor agents. Considering that the synthesis and testing of thousands of cisplatin analogues have been reported [14], it is surprising that relatively few studies have addressed the relationships between the aqueous chemistry of new platinum compounds and their cytotoxic activities. One study with this objective failed to find a correlation between the binding half-life of platinum complexes to DNA and their *in vitro* cytotoxic potencies on L1210 cells [15]. However, other physicochemical parameters such as the rates of Pt-Cl hydrolysis, hydrophobicity, drug stability in culture medium and the extent of reversible binding to serum protein would also be expected to influence the cytotoxic activities of cisplatin analogues in cell culture.

Valuable insights for the design of new cisplatin analogues might be gained if quantitative relationships could be established between the aqueous chemistry and cytotoxic activities of cisplatin analogues. For this reason, it was considered worthwhile to reinvestigate the relationships between the solution chemistry and the *in vitro* cytotoxic

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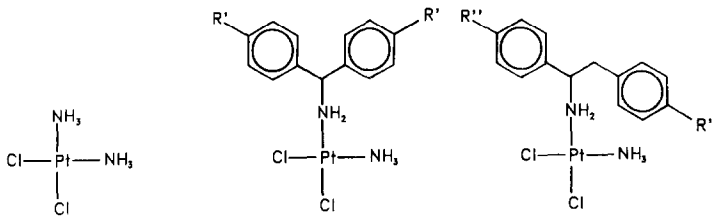

	R'(R'')		
1	H(H):	**2**	**5**
	F(F):	**3**	**6**
	OMe(OMe):	**4**	**7**
	OMe(H):		**8**

Structure 1.

activities of cisplatin analogues having known lipophilicities. Reported herein are the results of experiments designed to identify which (if any) of the above mentioned physicochemical parameters influence the cytotoxic activities of a series of structurally related mixed-amine cisplatin analogues (**2–8**) (see Structure 1) in cell culture.

MATERIALS AND METHODS

Chemicals and equipment. Cisplatin and K_2PtCl_4 were generously supplied by the Degussa AG (Frankfurt a.M., F.R.G.) and cisplatin was recrystallized as described [16]. The syntheses of the mixed-amine platinum complexes *cis*-ammine-[bis(4-methoxyphenyl) methylamine] dichloroplatinum (II) (**4**), *cis*-ammine[1,2-bis(4-methoxyphenyl)ethylamine]dichloroplatinum (II) (**7**) and *cis*-amminedichloro[2-(4-methoxyphenyl)-1-phenylethylamine]platinum(II) (**8**) have previously been reported [17], and the same method was used for the syntheses of *cis* - amminedichloro(diphenylmethylamine)platinum-(II) (**2**), *cis*-ammine[bis(4-fluorophenyl)methylamine]dichloroplatinum(II)(**3**), *cis* - amminedichloro(1,2 - diphenylamine)platinum-(II)(**5**)and*cis*-ammine[1,2-bis(4-fluorophenyl)ethylamine]dichloroplatinum(II) (**6**). Briefly, 1:1 equivalents of amine and $K[PtCl_3(NH_3)]$ were made to react in methanol for several days. The precipitated platinum complexes were collected by suction filtration, washed with methanol and water and dried over P_2O_5 *in vacuo*. The elemental analyses, i.r. spectroscopy, ^{1}H-NMR (250 MHz) and fast atom bombardment-mass spectroscopy of the platinum complexes were all in agreement with the expected structures. All compounds were pure as determined by reversed-phase HPLC and free of the *trans* isomer.

The HPLC analyses were done with an Altex 110A high-pressure mixing system, and fitted with a Rheodyne 7125 sample injector, which had either a 0.5 or 2.0 mL injection loop. A 0.4 × 25 cm Nucleosil-100 RP-18 column (Macherey-Nagel, Düren, F.R.G.) with a 0.4 × 3.0 cm precolumn was used for the chromatography and a Kontron Uvikon 720LC variable wavelength UV spectrophotometer was used for detection (**2** and **5**: 263 nm; **3** and **6**: 269 nm; **4**, **7** and **8**: 272 nm). Eluant flow rate was 0.7 mL/min. Water was deionized by means of a Millipore Milli-Q Water System to a resistivity of 18 MΩ·cm.

Hydrophobicity determinations. The hydrophobicities of the platinum complexes were determined by reversed-phase HPLC according to the Log k_w* concept described by Braumann [18]. According to the equation:

$$\log k' = \mathrm{Log}\, k_w - S\psi \qquad (1)$$

where k' is the HPLC capacity factor, S is a constant and ψ is the volume fraction of methanol in the aqueous eluent, it is possible to estimate a Log k_w value by measuring the log k' at various eluant compositions of methanol. The log k' values for compounds **2–8** were determined on a new Nucleosil-100 RP-18 column (end-capped) with eluant compositions of 35, 40, 45, 50 and 55% (v/v) methanol/20 mM KH_2PO_4 at 37°.

Aqueous chemistry of the platinum complexes. All experiments were done according to a previously described reversed-phase HPLC method [17]. The Pt-Cl hydrolysis rate constants for **2–8** where determined at a concentration of 20 μM platinum complex in dionized water at 37° in the dark. By trapping the various hydrolysis products with Br^- followed by their separations on a C18 column, the concentrations of the various dihaloplatinum species could be quantified by means of their UV absorptions

* Abbreviations: GSH, reduced glutathione; FCS, foetal calf serum; PBS, phosphate-buffered saline; DMF, *N,N*-dimethylformamide; Log k_w, log of the HPLC capacity factor at a 100% aqueous eluant composition; Log P_{oct}, log of the octanol/water partition ratio; f_m, medium free fraction; π, hydrophobicity coefficient; k_{el}, medium elimination rate constant; $\mathbf{PtCl_2}$, free fraction of dichloroplatinum complex in medium; T/C_{corr}, corrected treatment over control ratio multiplied by 100; AUC, area under the concentration–time curve.

as a function of time. (The recoveries of the dihaloplatinum complexes from the HPLC column were quantitative). When the aromatic substituents were *para*-methoxy (**4**, **7** and **8**) or fluorine substituents (**3** and **6**), the absorption differences between Pt-Cl and Pt-Br bonds were small in comparison to the absorptions of the aromatic rings, and the molar absorbances of the various dihaloplatinum complexes were essentially the same at the absorption maxima (λ_{max}) of the aromatic ring. However, when the phenyl ring was unsubstituted (**2** and **5**), the differences in the UV absorptions between the Pt-Cl and Pt-Br bonds became significant at the λ_{max} of the aromatic ring (257 nm). At a wavelength of 263 nm, these chromatic differences between the Pt-Cl and Pt-Br bonds were much less but a correction factor was still needed. By incubating the dichloroplatinum complex overnight in the presence of 0.5 M KBr, a quantitative conversion to the dibromoplatinum complex was achieved. The ratio of the dichloroplatinum versus dibromoplatinum complex peak areas was the correction factor for the dibromoplatinum complex. By incubating the dichloroplatinum complex for several hours with KBr, a mixture of dichloroplatinum, bromochloroplatinum and dibromoplatinum complexes was obtained. After correcting the peak area of the dibromoplatinum complex, a correction factor for the bromochloroplatinum complexes could be calculated. The coupled first-order rate equations were integrated numerically.

To determine the time-dependent concentrations of platinum hydrolysis products in the presence of chloride and GSH (Sigma), the same HPLC assay was used except that incubations were done in a solution of 4 mM KCl, 140 mM $NaNO_3$, 17.5 mM sodium phosphate buffer (pH 7.0), with and without 4 mM GSH.

In determining the time-dependent concentrations of free dichloroplatinum complex in culture medium, 5.0 μM platinum complex was incubated in McCoy's 5A Medium (without Phenol red) (Sigma) with 5% (v/v) FCS (Gibco) under the same conditions as in the cell experiments but without cells. Stability determinations in the presence of cells were done under identical conditions to those used in determining the IC_{50} values (see below). Beginning immediately after the addition of the platinum complex and then at approximately 70-min intervals, an aliquot of medium was removed, cooled on ice and filtered by means of an Amicon MPS-1 micropartition system (Manvers, MA, U.S.A.) through a YMT ultrafiltration membrane (M_w cut-off 30,000) at 1000 *g* and 4° for 15 min. The ultrafiltrate (0.5 mL) was loaded onto the HPLC column and eluted with the following system: a 10-min linear gradient from 3:2 (v/v) phosphate buffer (20 mM, pH 5.0)/MeOH to 2:3 buffer/MeOH, followed by an isocratic elution. The fraction of parent drug remaining after time t was obtained by dividing the peak height of the platinum complex at time t by the peak height at $t = 0$. The retention times were: **2**, 18.0; **3**, 21.5; **4**, 22.2; **5**, 19.5; **6**, 24.3; **7**, 24.3; and **8**, 20.2 min (void volume elution time = 4.0 min).

The free fraction of parent drug in medium (f_m) was estimated by first determining the total dichloroplatinum complex content of the medium. This was found by loading the medium directly onto the HPLC column and eluting as described above. (The recovery of dichloroplatinum complex after such an analysis was quantitative). The free fraction was then determined as follows: after correcting for the losses of the dichloroplatinum complexes to the filter membrane (between 5 and 15% depending on the compound), the peak height of the dichloroplatinum complex from medium ultrafiltrate was divided by the dichloroplatinum complex peak height from the same sample before ultrafiltration.

Cell culture studies. MDA-MB-231, MCF-7 and SK-OV-3 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and the passages 59–67, 180–210, and 25–38 of these cell lines were used for these studies, respectively. The origins of these cells have been described elsewhere [19–21]. The cells were maintained as a monolayer in McCoy's 5A Medium (without Phenol red), supplemented with 10% FCS, 50 mg/L gentamicin sulfate (Sigma) and 2.2 g/L $NaHCO_3$ in 75-cm^2 flasks (3375 Costar, MA, U.S.A.). The cultures were grown at 37° in humidified incubators in an atmosphere of 5% CO_2/air and passaged shortly before the monolayer reached confluence. For cell growth kinetics and cytotoxicity studies, culture medium was supplemented with only 5% FCS.

The growth kinetics of the three cell lines were determined according to a previously described microtiter assay [22]. This assay is based on the crystal violet staining of cellular components, predominately nucleoprotein, and has been shown to correlate well with cell numbers [23]. For the determination of IC_{50} values the same microtiter assay was used. Briefly, 100 μL/well of a cell suspension (6600–7300 cells/mL) were seeded into 96-well microtiter plates. On the second day following plating, which corresponded to the most rapid phase of cell growth, the cells were treated with platinum complex at five concentrations per compound. The complexes **2–8** were serially diluted in DMF to give 10, 5.0, 2.5, 1.25 and 0.63 mM feed solutions, which were then diluted 500× into McCoy's Medium/5% FCS at 37°. (For cisplatin, the feed solutions were 5.0, 2.5, 1.25, 0.63 and 0.31 mM.) At 16 wells/concentration, 100 μL of the platinum complex-containing medium were added to each well, giving a final medium volume of 200 μL/well with a platinum complex concentration 1/1000 of the corresponding feed solutions. As a control, 16 wells received medium with just DMF added. Cells were incubated for either 1 or 5 days, after which time the medium was removed and replaced with a 1% glutardialdehyde/PBS solution for 20 min. Cells were stored at 4° under PBS. Crystal violet staining and the determination of the absorbance were done as described [22].

The corrected T/C (T/C_{corr}) values were calculated as described [24]. The concentrations of Pt complex that gave T/C_{corr} values of less than 90% were used in the determination of the IC_{50} values. Linear least-squares regression of the T/C_{corr} values versus the logarithm of the platinum complex concentrations

Table 1. Log P_{oct} and Log k_w values, and the medium free fraction (f_m) of dichloroplatinum complexes at 37°

Compound	Log P_{oct}	Log k_w*	r†	f_m‡§
1	−2.19‖			
2		2.45	−0.995	0.91
3		2.87	−0.995	0.88
4		3.03	−0.995	0.84
5		2.74	−0.994	0.93
6		3.17	−0.995	0.70
7		3.23	−0.994	0.85
8		3.00	−0.993	0.90
Diphenylmethane	4.14¶			
1,2-Diphenylethane	4.80¶			

* Determined on a new RP18 Nucleosile-100 column with eluant compositions of 35–55% methanol/phosphate buffer.
† Linear regression coefficient from Eqn 1.
‡ Determined in McCoy's Modified 5A medium supplemented with 5% FCS.
§ Mean of two determinations.
‖ Ref. 26.
¶ Ref. 25.

was done to calculate the linear portion of the dose–response curves, from which the IC_{50} values were estimated.

The time-dependent dosing studies were done in essentially the same way as the dose-dependent ones. Concentrations and exposure times are given in the caption to Fig. 5. At the end of the exposure period, the medium was aspirated off and replaced with 200 μL of fresh McCoy Medium and 5% FCS. Cells were incubated an additional 5 days.

RESULTS

Hydrophobicities of the dichloroplatinum complexes

The extrapolated Log k_w values of the platinum complexes **2–8** range over less than one order of magnitude (Table 1). All plots are linear with regression coefficients better than 0.990. The introduction of a CH_2 group between the benzylamine carbon and a phenyl ring leads to an increase in compound hydrophobicity of $\pi = 0.30$ ($\pi = 0.66$ for 1,2-diphenylethane versus diphenylmethane [25]). Likewise, introduction of either *para*-methoxy or fluorine groups results in lipophilicity increases. The π value for *para*-fluorine substituent obtained by means of the Log k_w method ranges between 0.21 and 0.22. This value is comparable to the π value expected for this substituent determined by means of the octanol–water method (0.14 for fluorobenzene versus benzene [25]). However, π values determined by the Log k_w method for the *para*-methoxy group (0.25–0.30) are considerably greater than expected for known π values for this substituent determined by the octanol-water method (−0.04 for anisole

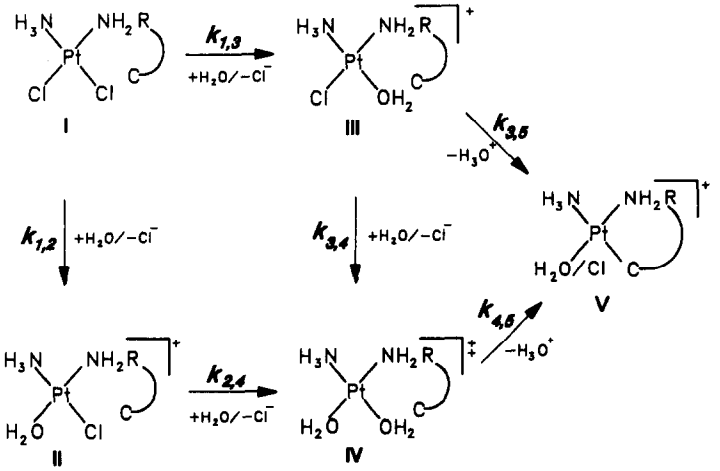

Fig. 1. System of pseudo-first-order hydrolysis and apparent cycloplatination reactions for the mixed-amine dichloroplatinum complexes in deionized water.

Table 2. Apparent rate constants ($k_{1,2} \cdot k_{4,5}$)* in deionized water and medium† elimination rate constants (k_{el}) for compounds **2–8** at 37°

Compound	Rate constants‡ ($\times 10^5$) sec^{-1}						
	$k_{1,2}$	$k_{1,3}$	$k_{2,4}$	$k_{3,4}$	$k_{3,5}$	$k_{4,5}$	k_{el}
2	4.5 ± 0.51	2.6 ± 0.33	5.5 ± 1.6	7.3 ± 1.5	15.5 ± 8.7	2.6 ± 0.59	4.6 ± 0.6
3	4.9 ± 0.26	1.7 ± 0.51	3.4 ± 0.37	5.2 ± 3.7	3.5 ± 2.7	3.6 ± 2.1	3.9 ± 0.4
4	6.3 ± 0.69	2.3 ± 0.34	5.6 ± 0.59	7.1 ± 1.9	9.5 ± 4.6	9.3 ± 0.71	3.9 ± 0.6
5	7.0 ± 0.21	3.7 ± 0.45	10.7 ± 1.0	10.4 ± 9.1	35.5 ± 6.0	25.6 ± 1.47	5.9 ± 0.6
6	6.8 ± 0.90	2.6 ± 0.96	5.9 ± 1.2	1.6 ± 1.2	21.1 ± 10.5	15.0 ± 2.5	5.2 ± 0.5
7	10.9 ± 0.78	2.0 ± 0.53	16.2 ± 1.4	6.5 ± 4.7	16.1 ± 4.0	53 ± 8.6	5.2 ± 0.5
8	7.7 ± 0.40	3.3 ± 1.5	10.5 ± 1.0	6.5 ± 4.3	26.1 ± 10.9	45.9 ± 12.0	5.6 ± 0.8

* See Fig. 1 for a description of these rate constants.
† McCoy's Modified 5A medium supplemented with 5% FCS.
‡ Values are the means of three independent determinations ± 1 SD.

versus benzene [25]). The Log k_w values for **2–8** are between 10^4 and 10^5 times greater than the Log P_{oct} value reported for cisplatin (Table 1) [26]. Interestingly, the sum of the Log P_{oct} for cisplatin (−2.19) and the Log P_{oct} values for either diphenylmethane (4.14) or 1,2-diphenylethane (4.80) gives values that compare reasonably well to the Log k_w values determined for **2** (2.45) and **5** (2.74), respectively.

Determination of the hydrolysis rate constants

Investigations concerning the aqueous chemistry of compounds **2–8** began with a HPLC-based quantification of the Pt-Cl hydrolysis reactions. The system of hydrolysis reactions for the mixed-amine platinum complexes is shown in Fig. 1. The sum of the first two hydrolysis rate constants, $k_{1,2}$ and $k_{1,3}$, remains constant over a platinum concentration of 10–40 μM (results not shown), and shows that released chloride does not alter the observed rate constants. Thus, the assumption that the hydrolysis rate constants are pseudo-first-order, at least at the early times of the incubations, appears to be valid. Since the dichloroplatinum(II) complexes are added to water as concentrated DMF solutions, the possibility that this organic solvent might influence the rates of Pt-Cl hydrolysis was investigated. Over a DMF concentration range of 13–52 mM no changes in the sums of $k_{1,2}$ and $k_{1,3}$ are observed (results not shown).

Two aquachloroplatinum isomers would be expected to form from the hydrolysis of the first Pt-Cl bond in the mixed-amine platinum complexes. The HPLC assay is able to differentiate between these two bromochloroplatinum isomers, trapped from their respective aquachloroplatinum species with Br^- [17]. In accordance with this kinetic scheme, the coupled first-order rate equations are integrated numerically by means of a Gear-Simplex program and the observed hydrolysis rate constants for compounds **2–8** are obtained (Table 2). The algorithm generated concentration–time curves of dihaloplatinum compounds appear to fit well with the experimentally determined data points (Fig. 2A).

In all cases, the hydrolysis of the first Pt-Cl bond

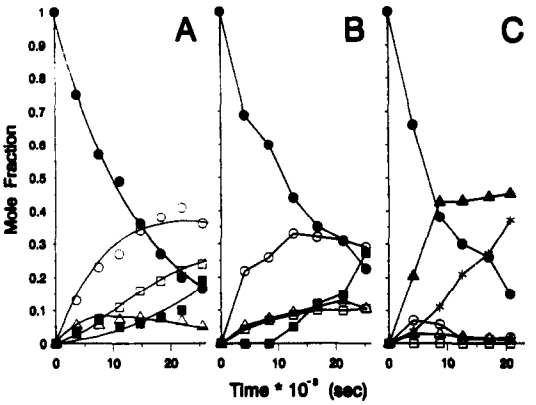

Fig. 2. Representative plots showing the time-dependent, aqueous chemistry of compound **3** (20 μM) at 37°: (●) *cis*-dichloroplatinum complex (**I**); (○) *a*-amine-*b*-chloro-*c*-aqua-*d*-ammineplatinum (**II**); (△) *a*-amine-*b*-aqua-*c*-chloro-*d*-ammineplatinum (**III**); (□) *cis*-diaquaplatinum (**IV**); (■) non-eluting platinum complexes (presumably cycloplatination products) (**V**); (▲) non-eluting platinum complexes (presumably GSH adducts); and (*) amine ligand. (A) Determined in deionized water (pH 6.4). Curves represent the best-fit values determined by the Gear-Simplex algorithm. (B) Determined in the presence of 4 mM chloride (pH 7.0). The chloride/platinum ratio was 200:1. (C) Determined in the presence of 4 mM chloride and 4 mM GSH (pH 7.0). The chloride/glutathione/platinum ratio was 200:200:1.

trans to the coordinated alkylarylamines ($k_{1,2}$) proceeds significantly faster than the hydrolysis of the *cis* Pt-Cl bond ($k_{1,3}$) (Table 2) at 37°. Although the hydrolysis rate constants of this first *cis*-coordinated Pt-Cl bond ($k_{1,3}$) are nearly the same for all compounds, the hydrolysis rate constants of the first Pt-Cl bond *trans* to the coordinated amines ($k_{1,2}$) are dependent on the type of coordinated alkylarylamine. These differences are attributed to the bulkiness of the amine ligand, which hinders the hydrolysis of the *cis* Pt-Cl bond relative to the *trans* one [17]. Furthermore, when the *para*-aromatic

substituents are the same (i.e. **2** and **5**, **3** and **6**, **4** and **7**), the *trans* Pt-Cl bond of the diphenylmethylamines hydrolyses move slowly than that of their respective diphenylethylamine counterparts. The hydrolysis rate constant $k_{1,2}$ is also influenced to some extent by the type of *para*-substituent found in the benzyl aromatic ring. For example, within the class of diphenylethylamines, the *para*-methoxyl-substituent in **7** accelerates slightly the hydrolysis of the Pt-Cl bond in comparison to the unsubstituted (**5**) and the *para*-fluorine-substituted (**6**) analogues. The sums of the Pt-Cl hydrolysis rate constants $k_{1,2}$ and $k_{1,3}$ for compounds **2–8** are all in the same order of magnitude as the hydrolysis rate constant reported for the first Pt-Cl hydrolysis of cisplatin at 37° (11×10^{-5} sec^{-1}) [27].

Following the Pt-Cl hydrolysis reactions, the platinum of the *para*-methoxy-substituted complexes (**4**, **7** and **8**) has been shown to undergo an *ortho*-metalation reaction with one of the aromatic rings [17]. The cycloplatination reaction can be measured indirectly by determining the time-dependent loss of dihaloplatinum complexes from the incubations by mass balance. Based on this criterion, this reaction is apparently operative also for the unsubstituted (**2** and **5**) and *para*-fluorine substituted (**3** and **6**) compounds. Only the platinum hydrolysis species with water coordinated *cis* to the alkylarylamine ligand (species **III** and **IV** in Fig. 1) can *ortho*-platinate an aromatic ring. Quantification of the cycloplatination products and numerical integration according to the kinetic system in Fig. 1 yielded the apparent cycloplatination rate constants for complexes **2–8** (Table 2). The cycloplatination rate constants are independent of the platinum concentration (determined between 10 and 40 μM) but for some of the platinum complexes the presence of the solvent DMF in the aqueous solutions can influence the apparent rates of cycloplatination. It is found that increasing the aqueous concentration of DMF from 13 to 52 mM doubles the rate at which chromatographable substance is lost from aqueous solutions of **4** but has no effect on these same rates for **7** (results not shown). Thus, the cycloplatination rate constants should be viewed with caution.

Effects of chloride and GSH on the time-dependent concentrations of platinum hydrolysis species

The influence of intracellular concentrations of chloride (4 mM) on the stability of the platinum hydrolysis products in the presence and absence of GSH was investigated. GSH was chosen because this nucleophile represents one of the most abundant intracellular sources of non-protein sulfhydryls, being present in concentrations between 0.1 and 10 mM [28]. GSH reacts readily with platinum complexes through its thiol group [29–31], and elevated intracellular concentrations of GSH have been associated with acquired cellular resistance to cisplatin [32, 33].

The addition of 4 mM chloride slows the rates at which dichloroplatinum complex (5 μM) is hydrolysed by about 50% relative to the Pt-Cl hydrolysis rates in deionized water (Fig. 2B). In addition, the accumulation of the diaquaplatinum species is reduced and the reaction of the aquaplatinum species to cycloplatinated products is delayed.

It has been shown recently that GSH reacts directly with chloroplatinum as well as with aquaplatinum species [34]. In the presence of 4 mM chloride and 4 mM GSH, a near quantitative trapping of the platinum hydrolysis species by GSH (Fig. 2C) results, which leads to rates of dichloroplatinum complex loss comparable to the rates of Pt-Cl hydrolysis. The concentrations of aquachloroplatinum species are reduced to only a few percent of the total platinum in the incubations and the diaquaplatinum species are not detected. The appearance of the amine ligand in these incubations is most likely a decomposition product of the GSH–platinum complex adduct; the coordination of a SH-group *trans* to the liganding amine can labilize the Pt-N bond.

The relative stabilities of the hydrolysis products from the various dichloroplatinum(II) complexes appear similar in the presence of 4 mM GSH and chloride; little difference in the time-dependent concentrations of aquachloroplatinum species from **4** and **7** is found in the presence of these two nucleophiles (results not shown). For this reason it is concluded that the aquachloroplatinum species, irrespective of the type of amine ligand, are equally reactive towards sulfhydryl-containing nucleophiles such as GSH.

A sulfhydryl concentration of 4 mM would represent a conservative estimate of the total tissue sulfhydryl content [35]. It seems reasonable to conclude that platinum hydrolysis products do not accumulate to any significant extent intracellularly. In addition, at physiological concentrations, it is likely that sulfur-containing nucleophiles trap the platinum hydrolysis products before they undergo intramolecular cycloplatination reactions to any significant extent. Thus, at least for compounds **2–8**, the *ortho*-platination reactions proceed too slowly relative to reactions with bionucleophiles and it is assumed that cycloplatination does not contribute significantly to the elimination of the aquachloroplatinum species.

Stability of the free fraction of dichloroplatinum complex in cell medium

Irreversible reactions between cisplatin and serum albumin probably involve the reaction of the protein with the aquachloroplatinum species as well as a direct, bimolecular reaction with the parent dichloroplatinum complex [36]. Since the platinum complexes under investigation show differing hydrolysis kinetics, it was considered that the compounds might also have varying stabilities in the culture medium. The time-dependent concentrations of the free fraction of dichloroplatinum complex in cell medium were determined by means of reversed-phase HPLC analysis of the medium ultrafiltrate. Ultrafiltration has been used routinely to determine the extent of drug–plasma protein binding in pharmacokinetics studies [37]. The losses of unbound dichloroplatinum complex from the cell medium follow first-order kinetics, which are accounted to the irreversible reactions of the platinum complexes

Table 3. IC_{50} values for compounds **1–8** determined by means of a microtiter assay

Compound	IC_{50} values (μM)*		
	MDA-MB-231†	MCF-7‡	SK-OV-3§
1	1.7 ± 0.18	0.74 ± 0.01	2.0 ± 0.18
2	2.0 ± 0.21	1.1 ± 0.05	3.3 ± 0.30
3	1.8 ± 0.31	1.6 ± 0.17	5.2 ± 0.54
4	3.6 ± 0.43	2.6 ± 0.26	8.3 ± 0.63
5	4.0 ± 0.33	1.4 ± 0.11	7.2 ± 0.49
6	3.1 ± 0.29	1.5 ± 0.07	—‖
7	4.0 ± 0.54	2.6 ± 0.08	9.1 ± 0.45
8	5.0 ± 0.17	2.3 ± 0.05	6.6 ± 0.24

* Determined by linear least-squares regression analysis. Interexperimental mean ± 1 SE.
† N = 5–9 independent experiments.
‡ N = 3 independent experiments.
§ N = 5–6 independent experiments.
‖ An IC_{50} value could not be determined owing to "basal-toxicity" at 10 μM.

with serum proteins and amino acids of the cell medium.

The type of the liganding alkylarylamine has an influence on the stability of the dichloroplatinum complex in medium; platinum complexes with diphenylmethylamines are more stable than the corresponding 1,2-diphenylethylamines compounds (Table 2). These elimination rates (k_{el}) are approximately 50–65% of the sum of the first two Pt-Cl hydrolyses ($k_{1,2}$ and $k_{1,3}$) of the parent compounds measured in deionized water (Table 2). The stability of the free fraction of dichloroplatinum complex is unaffected by the presence of cells (results not shown).

The free fraction (f_m) represents between 70 and 90% of the total dichloroplatinum complex in medium (Table 1). The f_m values do not change over the 7.5-hr incubations.

In vitro *cytoxicity testing*

The dichloroplatinum compounds were tested for activity in three human cell lines: the MDA-MB-231 and MCF-7 breast cancer cell lines, and the SK-OV-3 ovarian cancer cell line. These cell lines were chosen because they can be grown under identical culture conditions, with minimal doubling times ranging between 24 and 30 hr (MDA-MB-231, 24 hr; MCF-7, 25 hr; SK-OV-3, 30 hr). Identical protocols were followed for the testing of cisplatin and the mixed-amine platinum compounds in all three cell lines.

The dose–response curves for two of the mixed-amine platinum complexes (**2** and **5**) are compared with cisplatin in Fig. 3. For the more potent complexes these curves are of comparable shape to those of cisplatin, while for the less potent ones significant shoulders at the lower concentrations and steeper drops in the curves at the higher concentrations are observed. The IC_{50} values for cisplatin (**1**) range between 0.7 and 2.0 μM in the three cell lines (Table 3). The relative potencies of the mixed-amine platinum compounds vary according to the cell type, with the exception of compound **2**,

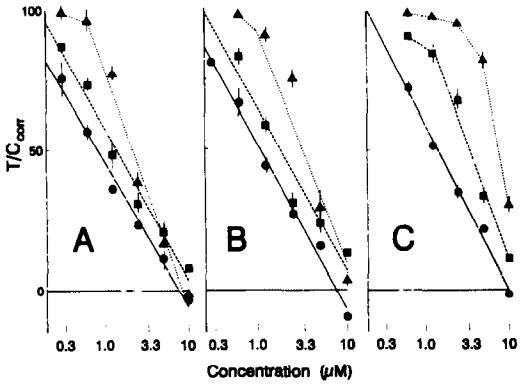

Fig. 3. Dose–response curves of cisplatin (A), **2** (B) and **5** (C) in the (■) MDA-MB-231; (●) MCF-7; and (▲) SK-OV-3 cell lines following a 5-day continuous exposure of cells to platinum complex. Symbols represent the means from 3–9 independent experiments. Bars represent the interexperimental standard errors. Straight lines are results of the interexperimental linear least-squares regression analysis.

which is always one of the most potent, and compound **7**, which is always one of the least potent (Table 3). Only in the MDA-MB-231 cell line do the most potent mixed-amine platinum compounds (**2** and **3**) have comparable activities with cisplatin following a 5-day exposure. In none of the cell lines are the mixed-amine platinum complexes more active than cisplatin. For the MDA-MB-231 and the SK-OV-3 cells, the IC_{50} values range over a factor of three. For the MCF-7 cells, this range is less. However, in spite of the rather tightly grouped IC_{50} values, many of these differences are statistically significant (Student's *t*-test). At 10 μM, the amine ligands show no cytotoxic activity.

As expected, inhibition of cell proliferation increases with increasing drug exposure. An example of the time-dependent onset of biological activity

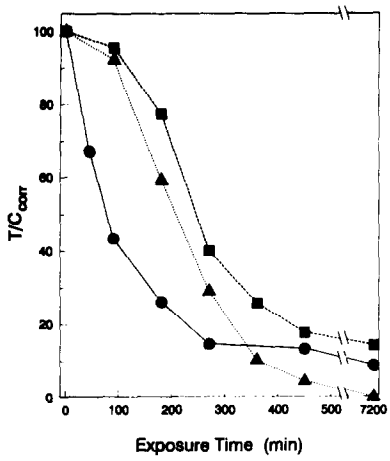

Fig. 4. Representative data showing the time-dependent onset of the cytotoxicity of **2** in the (■) MDA-MB-231 (5 μM); (●) MCF-7 (5 μM); and (▲) SK-OV-3 (10 μM) cell lines. Cells were incubated with platinum complex for the times indicated, the medium was removed and replaced with fresh medium, and the cells were incubated for an additional 5 days.

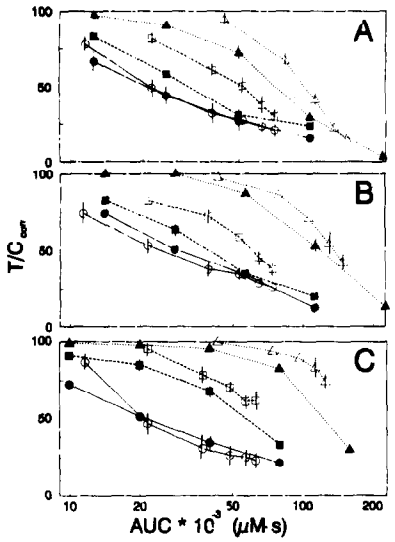

Fig. 5. Cytotoxicity relative to the AUCs of the platinum complexes (A, **2**; B, **3** and C, **5**) in culture medium. (□, ■) MDA-MB-231; (○, ●) MCF-7; (△, ▲) SK-OV-3 cell lines. Closed symbols represent data from the "concentration-dependent" regimen and the AUCs were calculated according to Eqn 4. (Concentrations were 0.63, 1.25, 2.5 and 5.0 μM in the MCF-7 and the MDA-MB-231 cells, and 0.63, 1.25, 2.5, 5.0 and 10.0 μM in the SK-OV-3 cell lines). Open symbols are the "time-dependent" regimen and the AUCs were calculated according to Eqn 3. (MDA-MB-231 cells were treated with 5.0 μM Pt complex for 90, 180, 270, 360 and 450 min. MCF-7 cells were treated with 5.0 μM Pt complex for 45, 90, 180, 27, 360 and 450 min. SK-OV-3 cells were treated with 10 μM Pt complex for 90, 180, 270, 360 and 450 min). Symbols represent means from 3–9 independent experiments. Bars represent the interexperimental standard errors.

for a dichloroplatinum complex (**2**) in the three cell lines is shown in Fig. 4.

AUC and cytotoxic activity

The possibility that the varying pharmacological potencies of the compounds **2–8** could be attributed to differences in the AUCs of the dichloroplatinum complexes was investigated. For these studies, only the concentration of the free fraction of the dichloroplatinum complex is considered, since it is generally believed that protein bound drugs do not cross cell membranes [37]. The extracellular, time-dependent concentration of free dichloroplatinum complex (**$PtCl_2$**) is expressed by the following equation:

$$[\mathbf{PtCl_2}]_t = f_m \cdot [PtCl_2]_0 \cdot \exp(-k_{el} \cdot t) \qquad (2)$$

where k_{el} is the elimination rate constant of the free dichloroplatinum complexes from the cell medium, $[PtCl_2]_0$ is the initial medium concentration of drug and f_m is the free fraction of drug in medium. Since the k_{el} values are the same in the presence and in the absence of cells, it can be assumed that the cellular uptake of **$PtCl_2$** does not contribute significantly to the removal of the parent drug from the culture medium. Integrating Eqn 2 from 0 to the exposure time t gives:

$$AUC^{0\rightarrow t} = (f_m \cdot [PtCl_2]_0/k_{el}) \cdot [1 - \exp(-k_{el} \cdot t)]. \qquad (3)$$

After an infinite drug exposure time Eqn 3 reduces to:

$$AUC^{0\rightarrow\infty} = f_m \cdot [PtCl_2]_0/k_{el}. \qquad (4)$$

According to Eqns 3 and 4, cells can be exposed to the same AUC by either dosing the cells with various initial concentrations of dichloroplatinum complex for an infinite exposure time (Eqn 4) or by dosing the cells with the same concentration of drug for varying finite exposure times (Eqn 3). The AUC–response curves obtained by these two treatment regimens are comparable to one another in the MCF-7 cell line (Fig. 5). However, for the more potent compounds (i.e. **2** and **3**) in the MDA-MB-231 cell line, it is evident that the "dose-dependent" treatment is more toxic to the cells than the "time-dependent" one, although both regimens expose the cells to the same AUC of dichloroplatinum complex (Fig. 5A and B). In the SK-OV-3 cell line the compounds also deviate from the AUC–efficacy relationship (Fig. 5).

To obtain the potencies of the compounds relative to their AUCs, the IC_{50} values for each compound were multiplied by a factor of f_m/k_{el}. The conversion of the IC_{50} values into terms of AUC does not normalize the potencies of the platinum complexes in any of the cell lines; no decrease in the relative differences in the potencies of the compounds is observed.

DISCUSSION

It has long been accepted that the hydrolysis

products of cisplatin and its many analogues are the reactive intermediates responsible for antitumor activity. This assumption is based on the evidence that DNA is the target of cisplatin, and that only the platinum hydrolysis products are sufficiently reactive to covalently bind to nucleic acids under physiological conditions. Recently, efforts have been made to quantify the intracellular concentrations of these platinum hydrolysis species and correlate these levels with biological efficacy. Mauldin *et al.* [38] found that the temporal intracellular concentrations of platinum hydrolysis products from two cisplatin analogues, $PtCl_2$(*trans*-DACH) and Pt(mal)(*trans*-DACH), correlated with their time-dependent onset of cytotoxic activity in the L1210 cell line [38]. Another attempt, however, to quantify intracellular concentrations of hydrolysis product dichloroethylenediamineplatinum(II) ($PtenCl_2$) in the same cell line was unsuccessful [39]. If the platinum hydrolysis products of cisplatin analogues are the reactive intermediates responsible for cytotoxicity, then the rates of Pt-Cl hydrolysis might be expected to have an influence on the activity of such compounds. Moreover, compound lipophilicity, the stability of a platinum complex in cell medium and the extent of reversible binding with serum proteins could influence the apparent cytotoxicity of the compound in cell culture. The purpose of this work was to determine if the variation in the cytotoxicities of a series of structurally related cisplatin analogues could be explained solely on differences in these important physiochemical parameters.

There is no apparent linear correlation between hydrophobicity and cytotoxicity, which is best illustrated by the comparison of **1** with **2**; both of these compounds show comparable cytotoxic activity although **2** is over 10,000 times more lipophilic than **1**. Within the series **2–8** there is also no apparent trend between these two parameters. A similar lack of correlation between the theoretical π values and the cytotoxicities of dichloro(1,2-diphenylethylenediamine)platinum(II) complexes in the MDA-MB-231 cell line has been observed [40].

The substitution of one ammine proton of cisplatin for a bulky organic functionality leads to dissimilar rates of hydrolysis for the first *cis* and *trans* Pt-Cl bonds, which is shown by the differences in the $k_{1,2}$ and $k_{1,3}$ values. A similar phenomenon has recently been reported for the reactions of mixed-amine cisplatin analogues with the dinucleotide r(ApG) [41]. The present results also show that minor changes in the structure of the amine ligand can influence the Pt-Cl hydrolysis kinetics of the dichloroplatinum complexes. For example, the insertion of a methylene group between the phenyl ring and the benzylic carbon of **2** to give **5** leads to a 50% increase in the sum of the Pt-Cl hydrolysis rate constants $k_{1,2}$ and $k_{1,3}$, while decreasing the stability of the dichloroplatinum complex by 30% in culture medium (Table 2). An attempt was made to alter these chemical parameters still further by synthesizing additional analogues with other *para*-aromatic substituents (i.e. methyl- and chloro-). However, these compounds showed high "basal-toxicity" (cell lysis following a 24 hr exposure to substance) in all three cell lines and were not investigated further.

Previous studies have shown that decreases in the aqueous stability of aniline mustards [9] and some 1-(2-chloroethyl)-1-nitrosoureas [12, 42] correlate with increases in antitumor activity of these compounds, inferring that the more rapid the reactive intermediates formed, the more potent the compounds were. For the cisplatin analogues **2–8** this is apparently not the case; there appears to be no correlation between the sum of the first Pt-Cl hydrolysis rate constants ($k_{1,2}$ and $k_{1,3}$) and the cytotoxic potencies of the compounds. In fact, compound **7** which hydrolyses most rapidly is one of the least active in all three cell lines, while compound **2** which shows relatively good stability in water is one of the most potent.

It could be argued that intracellular chloride (*ca.* 4 mM) reacts with the platinum hydrolysis products to reform the dichloroplatinum complex, and for this reason the Pt-Cl hydrolysis rate constants do not accurately depict the rates at which the platinum hydrolysis products form inside the cell. The results from this work suggest, however, that the highly electrophilic aquachloroplatinum species would be rapidly removed from the cellular system through reactions with bionucleophiles (i.e. GSH), and that an equilibrium between dichloroplatinum and aquachloroplatinum species, as is frequently discussed in the literature [3, 43], does not occur. For this reason, the intracellular hydrolysis reactions are assumed to be irreversible.

One possible reason for the lack of a correlation between hydrolysis rate constants and cytotoxic potency could be that the aquachloroplatinum species originating from the various dichloroplatinum complexes are inactivated at differing rates through reactions with such bionucleophiles as GSH. This might then lead to a disproportional amount of platinum binding to DNA than would be expected from the hydrolysis rate constants. In the presence of this nucleophile, however, very little difference in the stabilities of the aquachloroplatinum species, which arose out of either a compound with good activity (**3**) or a compound with poor activity (**7**) are observed.

The AUC is a pharmacokinetic parameter frequently used in evaluating drug efficacy. This relationship between AUC and efficacy holds also for many but not all alkylating agents [44, 45]. In the MCF-7 cell line, cisplatin has been reported to obey this relationship [45]. The results reported here show also that in the MCF-7 line the efficacy of the individual dichloroplatinum complexes is related to their AUCs (Fig. 5). Interestingly, the two most potent compounds (**2** and **3**) in the MDA-MB-231 cell line did not adhere to the AUC–efficacy relationship; the manner of treatment influences the efficacies of these platinum complexes although both treatment regimens should have exposed the cells to the same AUC of drug (Fig. 5). Some of the compounds also deviated from the ideal relationship in the SK-OV-3 cell line. Thus, these cells when exposed to an initial bolus dose of platinum complex for varying times were less sensitive to the same AUC from a continuous "dose-dependent" regimen

that used lower initial concentrations of platinum complex. These results are reminiscent of the effects of melphalan on a melphalan-resistant human melanoma cell line [44]. Parsons reported that although the AUC–efficacy relationship in a melphalan-sensitive melanoma cell line held, the relationship was not valid in a resistant cell line. Specifically, higher doses of melphalan led to a lower efficacy of the drug in culture medium. Since most human breast cancers are resistant to cisplatin, this phenomenon between AUC and efficacy in the MDA-MB-231 cell line warrants further attention.

It was considered that the differences in compound potency might be related through the AUCs of the unbound dichloroplatinum complexes in cell culture medium. The cytotoxic potencies (IC_{50} values) of the series of cisplatin analogues were compared with respect to Eqn 4. If the differences in the pharmacological activities of the platinum complexes were due solely to differing AUCs, then this analysis should normalize the IC_{50} values for all of the compounds relative to the AUC value. In none of the cell lines does this analysis normalize the IC_{50} values of all of the platinum complexes. Thus, the variation in the *in vitro* potencies of these compounds is probably a reflection of factors other than the medium concentrations of dichloroplatinum complex multiplied by the exposure time.

An explanation for the failure to draw correlations between physicochemical parameters of these cisplatin analogues and their cytotoxic potencies could be that there are qualitative differences between the DNA adducts of the various mixed-amine platinum complexes (i.e. the DNA structural alterations or the rates of adduct repair are different for each of the complexes). For example, it has recently been found that although a dichloro(1,2-diaryl-1,2-ethylenediamine)platinum(II) complex was inactive compared to an equimolar dose of cisplatin in MCF-7 cells, both compounds platinated cellular DNA to the same extent [46]. Thus, differences in the nature of the DNA adduct might outweigh other factors such as the rates of Pt-Cl hydrolysis in deciding the cytotoxicity of a compound. If this is true, then it is remarkable that a minor structural change in a part of the amine ligand remote from the DNA-binding dichloroplatinum moiety (i.e. *para*-aromatic position, compare **2** with **4**) can have a greater influence on the lethality of the DNA–platinum adduct than the substitution of an ammine proton for a bulky alkylaryl structure (i.e. compare **1** with **2**). Another reason for the failure to draw correlations might be that the dichloroplatinum complexes are taken up at different rates by the cells. It has been shown that *racemic*- and *meso*-aqua(1,2-diaryl-1,2-ethylenediamine)sulfatoplatinum(II) compounds, which differ only in the configuration of the aromatic rings, are accumulated by MCF-7 cells at varying rates [46].

In summary, the goal to find correlations between the aqueous chemistry of cisplatin analogues and their cytotoxic activities was met with only partial success. While the differences in the physicochemical properties and the cytotoxic activities of the compounds were not great enough to allow for a very extensive structure–activity relationship study, certain trends should have been recognizable. However, there was no evidence that either compound hydrophobicity, the extent of reversible protein binding, the rate of Pt-Cl hydrolysis, or the AUC of the dichloroplatinum complex in culture medium can decide alone the rank of the cytotoxic potencies of a series of closely related cisplatin analogues. Nevertheless, the study did show that what had been reported earlier for the AUC–efficacy relationship of cisplatin in the MCF-7 cell lines holds as well for other *cis*-configured dichloroplatinum compounds in that same cell line. On the other hand, for the compounds in the MDA-MB-231 breast cancer cell line and the SK-OV-3 cell line, the "time-dependent" dosing method is less cytotoxic than the "concentration-dependent" regimen, although the cells should have been exposed to the same AUCs of dichloroplatinum complex. It is tempting to theorize that this phenomenon maybe related to the ineffectiveness of cisplatin in the treatment of certain cancer types.

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