Oxaliplatin Degradation in the Presence of Chloride: Identification and Cytotoxicity of the Monochloro Monooxalato Complex

Elin Jerremalm,[1,5] Mikael Hedeland,[2] Inger Wallin,[1] Ulf Bondesson,[2,3] and Hans Ehrsson[1,4]

Received December 22, 2003; accepted February 5, 2004

Purpose. To study the degradation of oxaliplatin in chloride media and evaluate the cytotoxicity of oxaliplatin in normal and chloride-deficient medium.

Methods. The products of the reaction of oxaliplatin with chloride were separated on a Hypercarb S column with a mobile phase containing 40% methanol in 0.05 M ammonia and subjected to electrospray ionization mass spectrometry. The cytotoxicity of oxaliplatin in normal and chloride-deficient medium was evaluated by 30-min incubations on human colon adenocarcinoma cells (HT-29).

Results. We identified a new intermediate degradation product, the monochloro monooxalato complex ([Pt(dach)oxCl]$^-$) and the final product, the dichloro complex (Pt(dach)Cl_2), by liquid chromatography-mass spectrometry. [Pt(dach)oxCl]$^-$ was found as the negative ion, M$^-$, at *m/z* 431, and the positive ion, [M+2H]$^+$, *m/z* 433. Pt(dach)Cl_2 was found as the negative ion, [M-H]$^-$, *m/z* 377, and the positive ion, [M+NH_4]$^+$, *m/z* 396. The fast initial degradation of oxaliplatin can be coupled to the fast formation of [Pt(dach)oxCl]$^-$. In the cytotoxic assay, the cell survival was not affected by the chloride levels.

Conclusions. [Pt(dach)oxCl]$^-$, a new transformation product of oxaliplatin, has been identified. Its *in vitro* cytotoxic effect does not appear to exceed that of oxaliplatin.

KEY WORDS: biotransformation; HT-29; Pt(dach)Cl_2.

INTRODUCTION

Oxaliplatin ([(1*R*,2*R*)-1,2-cyclohexanediamine-*N*,*N*′]oxalato(2-)-*O*,*O*′platinum), a third-generation platinum drug, is used in combination therapy with 5-fluorouracil against metastatic colorectal cancer. Its proposed principal mechanism of action is the formation of platinum-DNA adducts, resulting in cell death (1).

In vivo, oxaliplatin is biotransformed rapidly and nonenzymatically to various species, including complexes with chloride, water, methionine, and glutathione (2–4). Two hours after the end of an infusion, the dichloro (Pt(dach)Cl_2, dach = cyclohexanediamine) and the monoaqua-monochloro ([Pt(dach)(H_2O)Cl]$^+$) complexes can be found in plasma ultrafiltrate from patients treated with oxaliplatin (4). Pt(dach)Cl_2, [Pt(dach)(H_2O)Cl]$^+$, and the diaqua complex ([Pt(dach)(H_2O)$_2$]$^{2+}$) have been shown to be more cytotoxic than oxaliplatin *in vitro* (5,6).

It is unclear to what extent the parent compound and the different biotransformation products contribute to the cytotoxicity. Because oxaliplatin has a half-life of about 14 min in man (2) and reacts slowly with DNA *in vitro* (7), it seems likely that one or more biotransformation products are responsible for the cytotoxicity. Activation pathways with phosphate and bicarbonate to [Pt(dach)(H_2O)$_2$]$^{2+}$ have been suggested, but they are poor nucleophiles and the reaction with oxaliplatin is slow (1).

For carboplatin, a ring-opened complex has been identified in the presence of chloride. The displacement half-life of the cyclobutane-1,1-dicarboxylic acid from carboplatin with chloride (0.14 M, 37°C) is 250 h (8). Here we have studied the degradation of oxaliplatin in the presence of chloride and used liquid chromatography-mass spectrometry (LC-MS) to identify the fast formation of a new chloride-containing degradation product. Furthermore, the *in vitro* cytotoxic effect of oxaliplatin, in normal and chloride-deficient medium, on human adenocarcinoma cells (HT-29) was evaluated.

MATERIALS AND METHODS

Materials

Oxaliplatin and Pt(dach)Cl_2 were generous gifts from Sanofi-Synthelabo (Malvern, PA, USA). All other chemicals were obtained from commercial suppliers, of analytical grade, and used without further purification.

Apparatus

The LC analysis was carried out using a Valco Model C6W injector (Houston, TX, USA) with a fixed loop volume of 50 μl, a LC-10AD Shimadzu pump (Kyoto, Japan), and a Spectro Monitor 3200 UV detector (Riverbeach, FL, USA) with variable wavelength. The wavelengths monitored were 210 nm for the monochloro monooxalato complex ([Pt(dach)oxCl]$^-$) and Pt(dach)Cl_2 and 254 nm for oxaliplatin. The output signal from the UV detector was processed in a WATREX v 3.1 extra integrating system from DataApex (Praha, Czech Republic).

When performing LC-MS, an Agilent 1100 series liquid chromatograph (Agilent Technologies, Waldbrunn, Germany) with a binary pump, de-gasser, and autosampler was used. The column outlet was coupled to a Quattro LC quadrupole-hexapole-quadrupole mass spectrometer equipped with an electrospray interface (Micromass, Manchester, UK). Both instruments were controlled with the software MassLynx v 3.3. This program was also used for data acquisition and mass spectral simulations.

Chromatographic Conditions

The LC separations were performed at room temperature on a Hypercarb S column (Hypersil, Runcorn, UK), 100

[1] Karolinska Pharmacy, Karolinska Hospital, SE-171 76 Stockholm, Sweden.
[2] Department of Chemistry, National Veterinary Institute (SVA), SE-751 89 Uppsala, Sweden.
[3] Division of Analytical Pharmaceutical Chemistry, Uppsala University, SE-751 23 Uppsala, Sweden.
[4] Department of Oncology-Pathology, Karolinska Institutet, SE-171 76 Stockholm, Sweden.
[5] To whom correspondence should be addressed. (e-mail: Elin.Jerremalm@telia.com)

ABBREVIATIONS: CI, confidence interval; dach, cyclohexanediamine; LC-MS, liquid chromatography-mass spectrometry; Pt(dach)Cl_2, dichloro complex; [Pt(dach)(H_2O)$_2$]$^{2+}$, diaqua complex; [Pt(dach)(H_2O)Cl]$^+$, monoaqua-monochloro complex; [Pt(dach)oxCl]$^-$, monochloro monooxalato complex.

× 4.6 mm i.d., particle size 5 μm. Oxaliplatin was analyzed using a mobile phase containing 60% v/v methanol in 0.01 M HEPES buffer (pH 7.4). $[Pt(dach)oxCl]^-$ and $Pt(dach)Cl_2$ were separated with 30% v/v methanol in 0.01 M sodium hydroxide. For LC-MS, the mobile phase was 40% v/v methanol in 0.05 M ammonia. The flow rate was 0.5 ml/min.

Oxaliplatin Degradation and Formation of the Chloro Complexes

The degradation of oxaliplatin and the formation of $[Pt(dach)oxCl]^-$ and $Pt(dach)Cl_2$ were performed by mixing oxaliplatin with sodium chloride (0.05–0.2 M) in 0.01 M HEPES buffer at pH 7.4 and 37°C, giving a final oxaliplatin concentration of 3.1×10^{-5} M. Samples for LC analysis were directly injected or quenched on ice before injection.

A reaction mixture of 1.5 mM oxaliplatin and 1.0 M sodium chloride was used for collecting fractions of the $[Pt(dach)oxCl]^-$ peak after LC separation. The methanol from the mobile phase was evaporated with nitrogen gas. The concentration of $[Pt(dach)oxCl]^-$ in the fraction was determined by mixing 100 μl of the fraction with 200 μl 0.0125 M HEPES, re-forming oxaliplatin at pH 7, and measuring the LC peak area of oxaliplatin after 2 h. The fraction was used for a colony-forming assay on the HT-29 cells at a concentration of 6 μM. The oxaliplatin content in the fraction was less than 15% when given to cells.

The relative molar absorptivities were calculated with Minitab 10.5 Xtra (State College, PA, USA) to estimate the approximate relative concentrations of the substances, assuming that the sum of the concentrations of oxaliplatin, $[Pt(dach)oxCl]^-$, $Pt(dach)Cl_2$ at all times is 100%.

Mass Spectrometric Conditions

The mass spectrometric parameters were tuned manually by direct infusion of a solution of $Pt(dach)Cl_2$ (~50 μM) with a syringe pump at 5 μl/min through a connecting T, where it was mixed with the LC mobile phase (flow rate, 0.5 ml/min). The electrospray parameters were finally set as follows for the positive mode: capillary voltage 3.43 kV, cone 24 V, extractor 9 V; and for the negative mode: capillary voltage –2.67 kV, cone –24 V, and extractor –9 V. The desolvation temperature was 350°C, and the source block temperature was 130°C. The nebulizer and desolvation gas flows were 104 and 1000 l/h, respectively. When performing MS-MS, the hexapole collision cell was filled with argon at a pressure of 5.5×10^{-4} mbar, and a collision energy of 10 eV was used.

LC-MS-injections were made from a solution of 6.3×10^{-5} M oxaliplatin in 0.1 M sodium chloride incubated for 30 min at 37°C.

Cell Line and Culture Media

The human colon adenocarcinoma cell line (HT-29) (originating from the American Type Culture Collection, Rockville, MD, USA) was cultured in Minimum Essential Medium (MEM), with Earle's salts and L-glutamine, supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (2.5 μg/ml) in a humidified air atmosphere (5% CO_2, 37°C). All culture supplies except the chloride-deficient medium were purchased from Invitrogen, Life Technologies (Grand Island, NY, USA). MEM and chloride-deficient MEM, containing only 1.4 mM chloride (National Veterinary Institute, Uppsala, Sweden) was used for the cytotoxic assay. The cell line was tested negative for *Mycoplasma* infection.

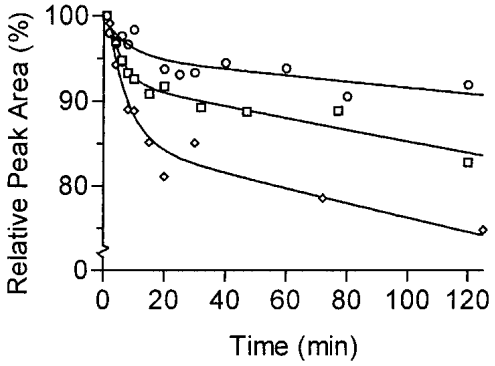

Fig. 1. Oxaliplatin degradation at pH 7.4 and 37°C in 0.05 M (○), 0.125 M (□), and 0.2 M (◊) sodium chloride. The lines represent two-phase exponential decays.

Colony-Forming Assay

HT-29 cells were cultured as monolayers in 75 cm^2 flasks (Corning, Cambridge, MA, USA). The cells in exponential growth were harvested after trypsination for 7 min in 0.25% trypsin and 0.05% EDTA. One hundred cells/culture dish (Becton Dickinson, Oxnard, CA, USA) were incubated with oxaliplatin in serum-free MEM or chloride-deficient serum-free MEM for 30 min. Controls were run in parallel. The cells were incubated with fresh MEM for 14 days (the MEM was changed after 7 days). Colonies of 50 cells or more were counted after being fixed with 10% formaldehyde solution and stained with Giemsa's solution (Merck, Darmstadt, Germany).

Results are expressed as percentage of the controls, with the mean and 95% confidence interval (CI).

RESULTS

The degradation time course of oxaliplatin in sodium chloride solution is shown in Fig. 1. LC analysis of a mixture of oxaliplatin and sodium chloride incubated for 30 min at

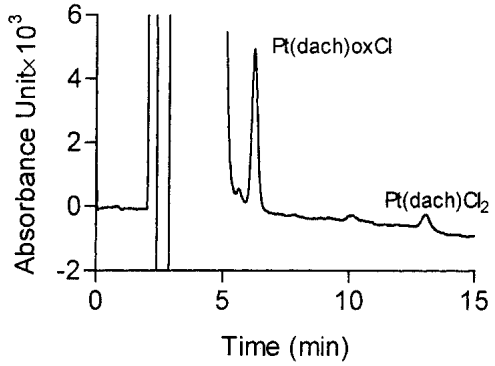

Fig. 2. Liquid chromatographic separation of $[Pt(dach)oxCl]^-$ and $Pt(dach)Cl_2$ after a 28-min degradation of oxaliplatin in 0.1 M sodium chloride at pH 7.4 and 37°C. Mobile phase, 30% v/v methanol in 0.01 M aqueous sodium hydroxide; column, Hypercarb S 100 × 4.6 mm i.d.; particle size, 5 μm; flow rate, 0.5 ml/min; wavelength, 210 nm.

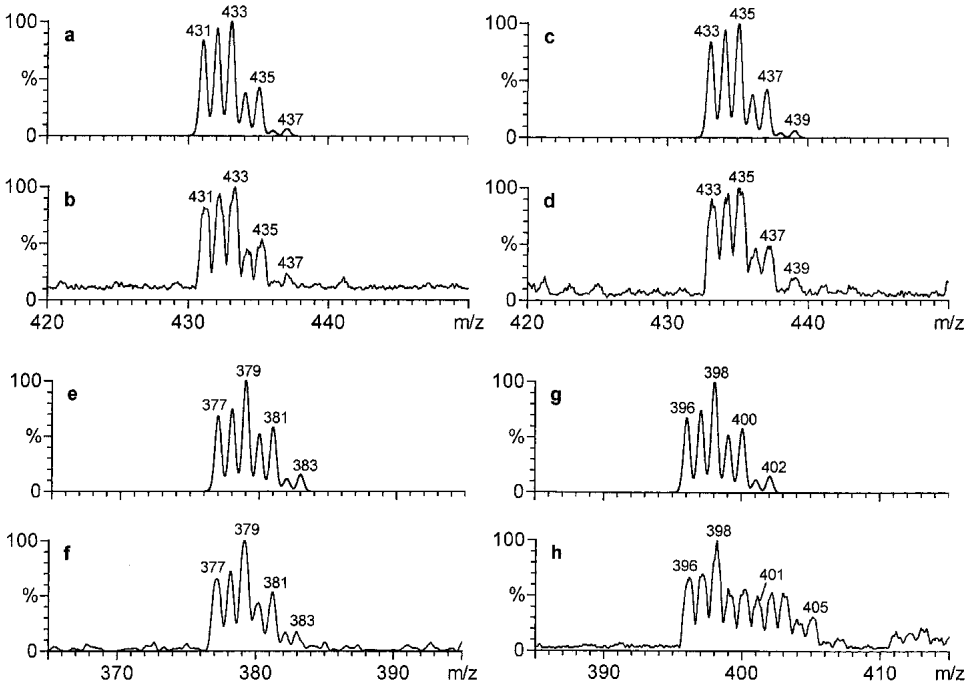

Fig. 3. Electrospray mass spectra of $[Pt(dach)oxCl]^-$, (a) simulation of a negative ion mode spectrum, (b) negative ion mode spectrum, (c) simulation of a positive ion mode spectrum, (d) positive ion mode spectrum, and Pt $(dach)Cl_2$, (e) simulation of a negative ion mode spectrum, (f) negative ion mode spectrum, (g) simulation of an ammonium adduct positive ion mode spectrum, (h) positive ion mode spectrum.

37°C revealed two peaks (Fig. 2), which were subjected to LC-MS. The full scan mass spectra of the first peak matched closely the isotope distribution of simulated spectra of $[Pt(dach)oxCl]^-$ in the negative and positive modes (Figs. 3a–3d). $[Pt(dach)oxCl]^-$ was found as the negative ion, M^-, with the lowest isotope *m/z* 431 and as the positive ion, $[M+2H]^+$, at *m/z* 433. Its identity was further confirmed by collision-induced dissociation (MS-MS) of the isotope *m/z* 432, in the negative mode. Peaks at *m/z* 89 and 342, corresponding to the monoprotonated oxalato residue and $[[Pt(dach)oxCl]^-$-oxalate-$2H]^-$, respectively, were found in the daughter ion spectrum (Fig. 4). The full-scan LC-MS spectrum of the second chromatographic peak (Fig. 2) suggested $Pt(dach)Cl_2$. The negative ion, $[M-H]^-$, was found at *m/z* 377. The positive ammonium ion adduct, $[M+NH_4]^+$ was found at *m/z* 396, probably interfering with a sodium ion adduct, $[M+Na]^+$, at *m/z* 401 (Figs. 3e–3h). The proposed reaction scheme for oxaliplatin with chloride is shown in Fig. 5.

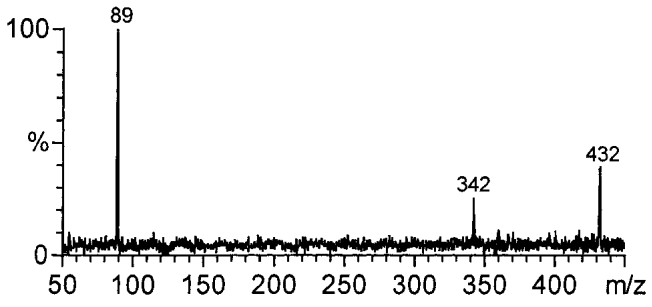

Fig. 4. Daughter ion spectrum of the isotope *m/z* 432 $[Pt(dach)oxCl]^-$ in the negative ion mode.

A plot of the degradation of oxaliplatin and the formation of $[Pt(dach)oxCl]^-$ and $Pt(dach)Cl_2$ vs. time shows a two-phase exponential decay of oxaliplatin, where the initially fast degradation of oxaliplatin (Fig. 1) can be coupled to the formation of $[Pt(dach)oxCl]^-$ (Fig. 6). At 37°C and 0.1 M sodium chloride, $[Pt(dach)oxCl]^-$ reaches a maximum within 30 min.

The cytotoxicity of oxaliplatin in medium with normal and low chloride levels was tested on HT-29 cells. There was no difference in the survival with respect to chloride levels (Fig. 7). An isolated fraction of $[Pt(dach)oxCl]^-$ was evaluated on the cells at a concentration of 6 μM. The cell survival was similar to the controls (data not shown).

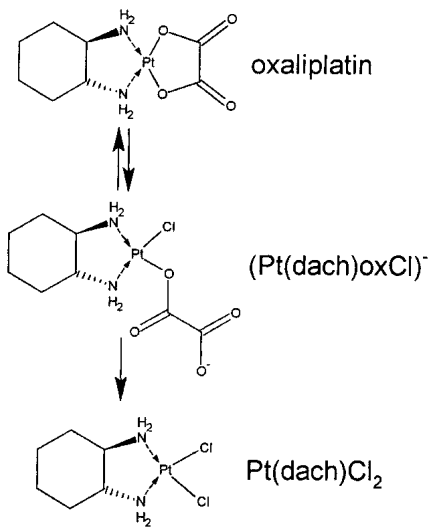

Fig. 5. Proposed reaction mechanism for oxaliplatin and chloride.

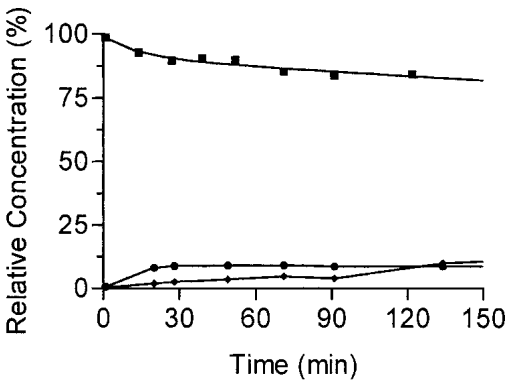

Fig. 6. Degradation of oxaliplatin (■) and formation of $[Pt(dach)oxCl]^-$ (●) and $Pt(dach)Cl_2$ (♦) in 0.1 M sodium chloride at 37°C. The line fitted to the degradation of oxaliplatin represents a two-phase exponential decay.

DISCUSSION

$[Pt(dach)oxCl]^-$, a new transformation product of oxaliplatin, has been identified. $Pt(dach)Cl_2$ has previously been found in plasma ultrafiltrate after the administration of oxaliplatin (3,4). We have shown that in the presence of chloride, oxaliplatin rapidly degrades initially, with a corresponding formation of $[Pt(dach)oxCl]^-$. The slower degradation rate of oxaliplatin seen subsequently must be due to the fact that $[Pt(dach)oxCl]^-$ is converted back to oxaliplatin by an intramolecular reaction (Fig. 5). The rapid initial degradation supports the recommendation that oxaliplatin should not be mixed with solutions containing chloride. A similar degradation pattern of oxaliplatin has been observed in plasma ultrafiltrate (data not shown). The rate of formation of $Pt(dach)Cl_2$ is slow. After 30 min in 0.1 M sodium chloride at 37°C, only about 3% of oxaliplatin has been converted to this complex. With regard to the short elimination half-life (14 min) of oxaliplatin *in vivo* (2), it is highly unlikely that $Pt(dach)Cl_2$ contributes to the cytotoxic effect of oxaliplatin. This is in agreement with the finding that less than 3% of oxaliplatin is converted to $Pt(dach)Cl_2$ *in vivo* (9).

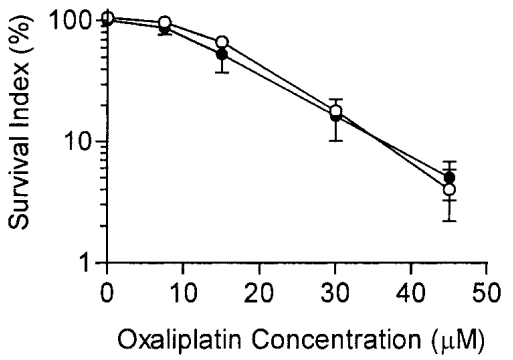

Fig. 7. Cytotoxicity of oxaliplatin in HT-29 cells. Oxaliplatin incubated in normal serum-free MEM (○) and in chloride-deficient MEM (●). Each point represents the mean with a 95% CI from 5 observations.

The cytotoxic assay did not reveal any differences in cell survival where oxaliplatin had been incubated in chloride-deficient or standard medium for 30 min. The incubation time was chosen so that a maximum of $[Pt(dach)oxCl]^-$ and a minimum of $Pt(dach)Cl_2$ is formed. The concentrations of oxaliplatin were selected to give a wide range of the cell-kill. At pH 7.4 and 37°C, $[Pt(dach)oxCl]^-$ is converted back to oxaliplatin within an hour. In spite of this fact, we made an attempt to evaluate the cytotoxicity of an isolated fraction of $[Pt(dach)oxCl]^-$. The result confirmed our other experiments; that is, $[Pt(dach)oxCl]^-$ does not seem to be more cytotoxic than oxaliplatin.

The *in vitro* cytotoxic effect of $[Pt(dach)oxCl]^-$ does not appear to exceed that of oxaliplatin. However, it is not known if the complex formed extracellularly can penetrate the cell membrane. It cannot be ruled out that oxaliplatin could be converted to $[Pt(dach)oxCl]^-$ in the nucleus, where the concentration of chloride is considerably higher than in the cytoplasm (10), and thereby react with DNA.

ACKNOWLEDGMENTS

Ingegerd Hedlöf at Cancer Center Karolinska, Karolinska Institutet, Stockholm, is gratefully acknowledged for helping with the HT-29 cell line. Many thanks to Staffan Eksborg at the Karolinska Pharmacy for help with the Minitab calculations.

REFERENCES

1. S. Mani, M. A. Graham, D. B. Bregman, P. Ivy, and S. G. Chaney. Oxaliplatin: a review of evolving concepts. *Cancer Invest.* **20**:246–263 (2002).
2. H. Ehrsson, I. Wallin. and J. Yachnin. Pharmacokinetics of oxaliplatin in humans. *Med. Oncol.* **19**:261–265 (2002).
3. M. A. Graham, G. F. Lockwood, D. Greenslade, S. Brienza, M. Bayssas, and E. Gamelin. Clinical pharmacokinetics of oxaliplatin: a critical review. *Clin. Cancer Res.* **6**:1205–1218 (2000).
4. P. Allain, O. Heudi, A. Cailleux, A. Le Bouil, F. Larra, M. Boisdron-Celle, and E. Gamelin. Early biotransformations of oxaliplatin after its intravenous administration to cancer patients. *Drug Metab. Dispos.* **28**:1379–1384 (2000).
5. F. R. Luo, S. D. Wyrick, and S. G. Chaney. Cytotoxicity, cellular uptake, and cellular biotransformations of oxaliplatin in human colon carcinoma cells. *Oncol. Res.* **10**:595–603 (1998).
6. P. Videhult, J. Yachnin, E. Jerremalm, R. Lewensohn, and H. Ehrsson. Synthesis and cytotoxicity of the dihydrated complex of oxaliplatin. *Cancer Lett.* **180**:191–194 (2002).
7. J. L. Butour, A. M. Mazard, and J. P. Macquet. Kinetics of the reaction of *cis*-platinum compounds with DNA *in vitro*. *Biochem. Biophys. Res. Commun.* **133**:347–353 (1985).
8. U. Frey, J. D. Ranford, and P. J. Sadler. Ring-opening reactions of the anticancer drug carboplatin: NMR characterization of *cis*-$[Pt(NH_3)_2$(CBDCA-*O*)(5′-GMP-*N7*)] in solution. *Inorg. Chem.* **32**:1333–1340 (1993).
9. S. S. Shord, S. A. Bernard, C. Lindley, A. Blodgett, V. Mehta, M. A. Churchel, M. Poole, S. L. Pescatore, F. R. Luo, and S. G. Chaney. Oxaliplatin biotransformation and pharmacokinetics: a pilot study to determine the possible relationship to neurotoxicity. *Anticancer Res.* **22**:2301–2310 (2002).
10. G. Siebert. The biochemical environment of the mammalian nucleus. *Sub-Cell. Biochem.* **1**:277–292 (1972).