Characterization of Intact Hemoglobin and Oxaliplatin Interaction by Nanoelectrospray Ionization Tandem Mass Spectrometry

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Background: Mass spectrometric (MS) detection of intact hemoglobin (Hb) adducts presents considerable analytical challenges because of the noncovalent association of the 4 subunits of Hb, and MS characterization of the interaction of intact Hb with platinum drugs has not been reported. We developed a technique for detecting intact Hb and its drug adduct and studied the interactions between intact Hb and oxaliplatin.

Methods: We incubated a series of mixtures of Hb and oxaliplatin at 37 °C for 24 h or 5 days to investigate adduct formation. Blood samples from colorectal cancer patients undergoing oxaliplatin treatment were analyzed for novel adducts of intact Hb–oxaliplatin, which were characterized with nanoelectrospray ionization quadrupole time-of-flight MS.

Results: Two intact Hb adducts, one with the whole oxaliplatin molecule and the other with oxaliplatin losing the oxalate ligand, were identified. Analysis of erythrocytes from the cancer patients provided direct evidence that oxaliplatin accumulated as Hb adducts in erythrocytes. A higher fraction (~70%) of Hb was bound to oxaliplatin in erythrocytes from a patient who could not tolerate oxaliplatin treatment than in erythrocytes from another patient who benefited from this treatment.

Conclusions: The nanoelectrospray tandem MS technique enabled determination of the intact Hb tetramer and its association with oxaliplatin. Hb–oxaliplatin adducts in erythrocytes may serve as a clinical biomarker for toxic response and treatment efficacy.

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Oxaliplatin (see Scheme 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue12) shows activity against cancerous cell lines, including those resistant to cisplatin and carboplatin (1–4). The trans-1-(R,R)-1,2-diaminocyclohexane (DACH)3-platinum moiety is more active than other stereochemical conformations (5, 6). Oxaliplatin, which is used in combination with 5-fluorouracil or related drugs, is the only platinum drug that is effective against colorectal cancer (7). Unfortunately, oxaliplatin also has several toxic effects, including progressive peripheral sensory neuropathy, diarrhea, vomiting, and hematologic suppression.

The mechanisms of oxaliplatin action and toxicity are related to the formation of DNA adducts. Similarly to cisplatin, oxaliplatin can form intra- and interstrand platinum–DNA cross-links, which inhibit DNA replication and transcription. Unlike cisplatin, however, oxaliplatin has a nonhydrolyzable DACH carrier ligand, and its DNA adduct formation occurs through the displacement of the oxalate ligand. The intrinsic chemical and steric characteristics of the DACH carrier ligand are thought to contribute to the differences between oxaliplatin and cisplatin (8, 9).

After oxaliplatin infusion, up to 40% of the blood platinum is found in erythrocytes (10, 11), and a large percentage of oxaliplatin (almost 85%–88%) in blood plasma is bound to plasma proteins (mostly albumin) (10, 12–14). The extensive binding of oxaliplatin to serum albumin is suspected to reduce its anticancer potential...
(12), and the accumulation of oxaliplatin in cells may be associated with toxic effects such as anemia and cumulative dose-related neurologic toxicity (11, 13, 15). Although the formation of oxaliplatin-protein adducts has been proposed to play an important role in the mechanism of action and toxicity of oxaliplatin (11–13, 15–18), little information is available about the interaction of oxaliplatin with specific proteins and the characteristics of protein-oxaliplatin adducts.

The first aim of this study was to determine the chemical species of oxaliplatin that bound to hemoglobin (Hb). Previous pharmacokinetic studies of oxaliplatin were generally conducted by measuring platinum (13, 16, 19), which does not provide structural information of the platinum-containing compounds. A size-exclusion liquid chromatography–inductively coupled plasma mass spectrometry study of the transformation of oxaliplatin during the first 3 h after infusion showed 3 platinum-containing protein adducts in plasma with molecular masses of 200, 160, and 60 kDa, and a small molecule of <2 kDa (14). The 200- and 160-kDa platinum peaks were identified as platinum adducts of γ-globulins, the 60-kDa peak as platinum–albumin, and the 2-kDa peak was suggested to be free drug or adducts of glutathione, l-methionine, or l-cysteine. Two platinum peaks of 60 kDa and <2 kDa were detected in erythrocytes. The peak at ~60 kDa was assumed to be hemoglobin–platinum adducts because it also contained a high quantity of iron. No information was available on whether oxaliplatin in erythrocytes was present as the parent molecule or as a metabolite.

The second aim of this study was to determine the form of Hb that bound to oxaliplatin. The quaternary structure of Hb is important for its function (20). The principal human adult hemoglobin (Hb A0) consists of 2 α (141 amino acids; 15 126.4 Da) and 2 β (146 amino acids; 15 867.2 Da) polypeptide chains; a heme (h) group is bound to each of the chains through noncovalent interaction (21). The noncovalent complex of the Hb tetramer can reversibly dissociate to dimers and then to monomers (22). Detection of the intact Hb tetramer and its adducts rather than the dimer or monomer is important because intact Hb–drug adducts may serve as biomarkers indicating the nature of the accumulation of the drug in erythrocytes as well as its metabolism and potential toxicologic effects. However, the noncovalent association of the Hb subunits presents considerable analytical challenges in detecting the intact Hb tetramer and its drug adducts by mass spectrometry (MS) (23–25).

Several MS studies have reported binding of platinum drugs to glutathione (26) and proteins, including albumin (27, 28), ubiquitin (29, 30), transferrin (31), and Hb (32, 33). We previously reported nanoelectrospray ionization MS (nanoESI-MS) characterization of cisplatin, carboplatin, and oxaliplatin adducts with subunits of Hb and the release of a heme group as a result of drug binding (33). However, no published reports have described the characterization of the binding between the intact Hb tetramer and platinum drugs. We report here the development of a nanoESI-MS technique and its application to study the formation of intact Hb and oxaliplatin adducts.

**Materials and Methods**

**REAGENTS AND MATERIALS**

A stock solution (5 mmol/L) of oxaliplatin (99.999% purity; Sigma-Aldrich) was prepared in HPLC-grade water and was stored for up to 2 days. Human Hb A0 and horse heart myoglobin were purchased from Sigma. The purity of the Hb was determined by gel electrophoresis and nanoelectrospray MS, as described previously (32, 33). The myoglobin was used as received without further purification. A stock solution (0.2 mmol/L) of Hb was prepared in 100 mmol/L ammonium acetate buffer. All stock solutions were kept at 4 °C in the dark until use. Formic acid, methanol, ammonium acetate, and water (Fisher Scientific) were HPLC-grade.

Microcon YM-10 centrifugal filters (membrane nominal molecular cutoff, 10 kDa; Millipore) were used to desalt the protein samples according to the manufacturer’s instruction manual. All samples were desalted with these filters, and the retained proteins were dissolved in 5 mmol/L ammonium acetate buffer before nanoESI-MS analysis.

**INSTRUMENTATION**

MS experiments were performed with a QSTAR Pulsar i and a QSTAR XL hybrid quadrupole time-of-flight mass spectrometer (Applied Biosystems/MDS SCIEX), equipped with a nanoESI source. Analyte solutions were introduced into the source by nanoES capillaries (long and medium; Proxeon). The mass spectrometer was operated in the positive ion mode for the detection of intact Hb and its drug adducts. Analyst QS software (Applied Biosystems) was used for spectrum acquisition and deconvolution of the raw mass spectrum. Igor Pro software (WaveMetrics) was used to plot the spectra.

Full-scan mass spectra were obtained with the time-of-flight section of the instrument, and the 2 quadrupoles (Q1 and Q2) were operated in radiofrequency-only mode. The instrument sensitivity and mass accuracy were externally calibrated by use of myoglobin (16 592 Da). The optimized instrumental conditions included a nanoelectrospray voltage of 1100 V, a first declustering potential (DP1) of 30 V, a second declustering potential (DP2) of 15 V, a focusing potential of 130 V, collision-activated dissociation (CAD) of 7 (arbitrary units), and collision energy (CE) of 3 eV.

The QSTAR Pulsar i quadrupole time-of-flight mass spectrometer has an m/z limitation of 3000 for Q1. To obtain tandem MS (MS/MS) spectra for the ions of intact Hb–oxaliplatin adducts, the QSTAR XL with Q1 mass limit up to m/z 6000 was used. The specific settings included mass resolution of 1 Da, CE of 15 and 30 eV, and...
INCUBATION OF Hb WITH OXALIPLATIN
The test solutions were prepared to contain a constant concentration of Hb (10 μmol/L) and various concentrations of oxaliplatin (0–100 μmol/L) with molar ratios of oxaliplatin to Hb of 0:1, 1:1, 5:1, and 10:1. Excess amounts of oxaliplatin were used to drive the formation of the complex with Hb to the maximum. These solutions in 100 mmol/L ammonium acetate (pH 7.0) were then incubated at 37 °C for 24 h and for 5 days. Parallel incubation of the Hb solution without oxaliplatin was used as a control.

PREPARATION OF ERYTHROCYTE SAMPLES FROM PATIENTS AND HEALTHY VOLUNTEERS
Blood samples from 10 colorectal cancer patients were collected 1 and 48 h after the first infusion of oxaliplatin. The dosage of oxaliplatin was 100 mg/m² in 500 mL of 50 g/L dextrose in water, infused over 120 min. Written informed consent was obtained from the patients. The protocol was reviewed and approved by the Research Ethics Board of the Alberta Cancer Board. In addition to the samples from cancer patients, blood samples from healthy volunteers were also collected as controls. Whole-blood samples from both patients and volunteers were collected in lithium heparin–coated tubes. All samples were centrifuged at 2500g for 15 min. The erythrocyte layer was separated from the plasma layer according to the published method (34). The erythrocyte and plasma samples were placed separately in cryovials and then stored at −35 °C until analysis. The frozen erythrocytes were thawed on ice, diluted 1000-fold with water, and desalted 4 times immediately before nanoESI-MS analysis to completely remove the salts and small molecules.

RESULTS
OPTIMIZATION OF nanoESI-MS DETECTION OF INTACT Hb TETRAMER
We first established a nanoESI-MS technique for detection of the intact Hb, (αhβh)₂, by optimizing both the solution and the instrumental conditions. Typical nanoESI-MS spectra of 10 μmol/L Hb in a mixed solution of 100 mL/L methanol with 0.1 mL/L formic acid in water (pH 4) and in a 5 mmol/L aqueous ammonium acetate solution (pH 7) are shown in panels A and B, respectively, of Fig. 1. Both methanol and acid induced dissociation of the Hb, which produced mainly monomers of α and β, consistent with previous studies of Hb (23–25, 32, 33, 35). Aqueous ammonium acetate solution was found to significantly improve the detection of intact Hb, as shown in Fig. 1B. A typical ion spectrum of Hb in 5 mmol/L aqueous ammonium acetate solution (Fig. 1B) showed intact Hb ions with charge states of 19⁺, 18⁺, and 17⁺ as the predominant species, along with Hb dimers (αhβh) with charge states of 12⁺ and 13⁺. Identification of these species was based on the accurate mass measurements.

It is also necessary to optimize instrumental settings such as the DP, CE, and collision gas (CAD) to minimize dissociation of the intact tetrameric ions while allowing sufficient declustering to produce ions for detection. As expected, declustering and fragmentation increased when the DP was increased from 30 to 65 V (data not shown). A DP of 30 V was found to be optimum for sufficient declustering and minimum fragmentation. Likewise, the CE was optimized at 3 eV for the highest intensity of Hb tetramer ions. In addition, increasing the collision gas from CAD 1 to 7 (arbitrary units) effectively enhanced the ion signals of the Hb tetramer, demonstrating that detection of large intact protein ions benefits from the additional collision focusing in the pressurized collision cell, as reported previously (36–38).

CHARACTERIZATION OF INTACT Hb-OXALIPLATIN ADDUCTS
Having established a technique that allowed detection of the intact Hb, we first applied this technique to study the interaction between the intact Hb tetramer and oxaliplatin in an in vitro system. The nanoESI mass spectra of the reaction mixtures of Hb (10 μmol/L) and oxaliplatin (0, 10, 50, and 100 μmol/L) after 24 h of incubation at 37 °C are shown in Fig. 2. Without oxaliplatin, the intact Hb control showed 3 distinctive peaks with charge states of 19⁺, 18⁺, and 17⁺ (Fig. 2A), whereas incubation of equal moles of Hb and oxaliplatin produced a new peak in addition to the Hb peaks (Fig. 2B). After further increases of oxaliplatin to 5- and 10-fold excess over Hb, 2 and 3 new peaks were detected (Fig. 2, C and D, respectively). These new peaks corresponded to 1, 2, and 3 oxaliplatin molecules bound to Hb. As can also be seen in panels C
and D of Fig. 2, the relative intensities of the Hb–oxaliplatin adducts increased relative to that of the unbound Hb, from 52% to 95% for peak 1 and from 10% to 30% for peak 2, with 5- to 10-fold increases in oxaliplatin concentrations relative to Hb.

Prolonged incubation for 5 days of the same mixtures of Hb and oxaliplatin solutions (as shown in Fig. 2) led to the formation of additional Hb–oxaliplatin adducts; up to 5 oxaliplatin molecules bound to the Hb (see Fig. 1 in the online Data Supplement). In addition, when the oxaliplatin concentration was increased while Hb was kept constant, the relative intensities of the Hb–oxaliplatin adducts also increased and the intensity of the Hb tetramer decreased. These results provide evidence supporting the formation of the Hb–oxaliplatin adducts in the solution phase and not during the nanoESI process. Furthermore, the fact that before nanoESI-MS analysis the excess amount of oxaliplatin in the incubation solutions was removed by use of centrifugal filters with a molecular cutoff of 10 kDa confirms that the adducts were formed in the solutions.

We used MS/MS to further characterize the chemical species of oxaliplatin that bound to the Hb. The tandem mass spectra of the precursor ion of m/z 3603.75, corresponding to the intact Hb–oxaliplatin adduct (with charge 18+), are shown in Fig. 3. To monitor both the precursor ion (charge 18+; m/z 3603.75) and the fragment ions, a moderate CE (15 eV) was used to obtain the tandem mass spectra. The fragments corresponding to monomers, monomer complexes, and the heme group can be clearly seen in Fig. 3A. To further characterize the fragment ions of the intact adduct, a higher CE of 30 eV was used to enhance the fragmentation, as shown in Fig. 3B. Panels C and D show expanded regions of the spectrum in Fig. 3B. The spectrum in Fig. 3C shows the fragments of monomers and the corresponding adducts of oxaliplatin.

Species of \( \alpha^* \) [Pt(DACH)], \( \alpha^{**} \) [Pt(DACH) (oxalate)], \( \beta^* \) [Pt(DACH)], and \( \beta^{**} \) [Pt(DACH)(oxalate)] were identified based on the accurate mass measurements (see Table 1 in the online Data Supplement). In addition, a fragment ion corresponding to oxaliplatin (m/z 397.2) was also detected, as shown in the expanded spectrum in Fig. 3D, confirming that the parent oxaliplatin can form adducts with intact Hb. The identification of [Pt(DACH)] and [Pt(DACH)] suggests that the hydrated species of oxaliplatin [Pt(DACH)(H2O)] may form adducts with Hb with loss of 2 H2O molecules. The hydrated species is the major species of the nonenzymatic transformation products of oxaliplatin, as reported previously (5, 7, 12, 14).

**Hb–oxaliplatin adducts in erythrocytes of cancer patients undergoing oxaliplatin treatment**

We further investigated the formation of intact Hb and oxaliplatin adducts by analyzing erythrocyte samples from cancer patients undergoing oxaliplatin treatment and from healthy volunteers. Shown in Fig. 4 are typical nanoESI mass spectra of erythrocyte samples from a healthy volunteer (Fig. 4A) and from a patient, obtained 1 h (Fig. 4B) and 48 h (Fig. 4C) after infusion of oxaliplatin. Analysis of the erythrocyte sample from a healthy volunteer showed only the intact Hb and no adduct with oxaliplatin (Fig. 4A). Erythrocyte samples from a cancer patient undergoing oxaliplatin treatment clearly showed the intact Hb–oxaliplatin adducts, primarily with a binding stoichiometry of 1:1. The peak intensities of the (1:1) intact Hb–oxaliplatin adducts relative to that of the intact Hb in the erythrocyte samples obtained at 1 and 48 h after infusion of oxaliplatin were ~56% and 53%, respectively. The persistence of the Hb–oxaliplatin adducts over time suggests that oxaliplatin accumulates in erythrocytes predominantly as Hb adducts. Our results are consistent with the previous observation that platinum in erythrocytes was not released after incubation of oxaliplatin in whole blood (10, 13).

The intact Hb–oxaliplatin adducts in erythrocyte samples were also characterized with nanoelectrospray MS/MS (see Fig. 2 in the online Data Supplement) and were identical to those in Fig. 3. The mass accuracy of the fragment ions was 11 to −93 ppm (see Table 2 in the online Data Supplement). The mass differences between
the fragment ions of the Hb tetramer (e.g., $\alpha^{\theta+}$ and $\beta^{\theta+}$) and the corresponding adduct ions (e.g., $\alpha^{*+}$, $\beta^{*+}$, $\alpha^{*\theta+}$, and $\beta^{*\theta+}$) were 307 and 397 Da, respectively, identifying the parent oxaliplatin [Pt(DACH)(oxalate)] and a species [Pt(DACH)], as described above (Fig. 3). The fragment ion of $m/z$ 397 and isotopic pattern (see Fig. 2D in the online Data Supplement) confirm the presence of the parent molecule of oxaliplatin in these adducts.

Blood sample analysis revealed interpatient differences in the Hb–oxaliplatin complex. A mass spectrum of erythrocytes collected 1 h after infusion is shown in Fig. 5. Interestingly, most of the Hb in the erythrocyte samples of this patient was in the form of Hb–oxaliplatin adducts. The binding stoichiometry of the major Hb–oxaliplatin adducts was 1:1, 1:2, 1:3, and 1:4, similar to those in Fig. 2 and in Fig. 1 of the online Data Supplement. The MS/MS spectra of the Hb–oxaliplatin adducts were similar to those in Fig. 3. The mass spectrum of the erythrocyte sample collected at 48 h after the first infusion closely resembles that in Fig. 5.

The intensities of the mass spectral peaks of the unbound Hb and the Hb–oxaliplatin adducts in erythrocyte samples from patient 1 (Fig. 4, B and C) show that approximately one third of the total Hb is bound to
oxaliplatin. However, a significantly higher concentration (~70%) of Hb–oxaliplatin adducts was found in the erythrocyte samples from patient 2 (Fig. 5). Both patients were infused with a similar dose of oxaliplatin (157 mg for patient 1 and 160 mg for patient 2). Examination of the clinical records showed that the 2 patients demonstrated marked differences in tolerance of the treatment. Patient 1 was able to tolerate oxaliplatin, and his cancer was stabilized after 9 cycles of the oxaliplatin and 5-fluorouracil treatment. Unlike patient 1, patient 2 had to stop treatment after the second infusion of oxaliplatin because of severe side effects, and his cancer was characterized as “in progress”. Examples of distinct differences between the 2 patients are shown in Fig. 4B and Fig. 5. Subsequent analysis of erythrocyte samples from additional patients further confirmed this difference between 6 patients who benefited from the oxaliplatin treatment (25%–40% adducts) and 4 patients who showed severe side effects (72%–82% adducts). Taken together, the results of this study suggest that the amount of Hb–oxaliplatin adduct formation may serve as a biomarker of side effects and treatment efficacy.

Discussion

Our results demonstrate that nanoESI MS/MS can be used to directly characterize the interactions between the noncovalent Hb tetramer and oxaliplatin in incubated solutions and in patient blood samples. This is the first time that the structures of the intact Hb–oxaliplatin adducts in erythrocytes have been identified as [(αbβh)2-Pt(DACH)(oxalate)] and [(αbβh)2-Pt(DACH)]. In neutral solutions (ammonium acetate buffer, pH 7) and with optimized soft nanoESI conditions, the noncovalent associations between the 4 subunits of the Hb molecule are maintained, which is evident from the detection of the tetrameric Hb. The conditions suitable for monitoring noncovalent associations enable the detection of noncovalent binding that would not otherwise be observed. Thus, binding of the whole oxaliplatin molecule to the tetrameric Hb can be detected. When Hb was dissociated to α and β subunits, only the Pt(DACH) moiety of the oxaliplatin (loss of oxalate group) was found to be bound to the α and β subunits, as shown in the tandem mass spectra (Fig. 3; see also Fig. 2 in the online Data Supplement). Noncovalent bindings could not be observed from the dissociated subunits. In contrast, the technique demonstrated here for studying interactions between the intact Hb tetramer and oxaliplatin enables the monitoring of both covalent and noncovalent bindings.

The formation of Hb–oxaliplatin adducts may lead to release of heme, as our previous in vitro studies demonstrated (33). Because Hb binding to oxygen requires the specific coordination of the 4 subunits and the heme group, it is conceivable that formation of Hb–oxaliplatin adducts would reduce the oxygen-binding capacity of Hb, which could produce some side effects in patients.

Oxaliplatin also formed protein adducts in plasma. However, the plasma protein adducts of oxaliplatin in all patients were reduced by more than 50% at 48 h after the infusion of oxaliplatin compared with 1 h after the infusion (39). The concentrations of Hb–oxaliplatin adducts in the same patient were similar in a comparison of samples collected at 1 and 48 h after infusion. Thus, the concentrations of Hb–oxaliplatin adducts in erythrocytes could be used as a biomarker of the effective dose of oxaliplatin. Previous studies have shown that the binding of platinum to erythrocyte proteins is irreversible (10, 13); therefore,

![Fig. 4. Analysis of erythrocyte samples from patient 1, who was undergoing oxaliplatin treatment, showing in vivo formation of intact Hb–oxaliplatin adducts.](image)

Spectra are obtained from erythrocytes of a healthy volunteer (A), and erythrocytes from blood samples of patient 1 collected at 1 h (B) and 48 h (C) after the first infusion of oxaliplatin.

![Fig. 5. Mass spectrum of erythrocyte samples from patient 2, showing extensive formation of Hb–oxaliplatin adducts.](image)

The blood sample was collected at 1 h after the first infusion of oxaliplatin (160 mg in 500 mL of solution). Peaks 1–4 correspond to the number of oxaliplatin molecules in the Hb complexes. The ions in the spectra are of charges 19+, 18+, and 17+.
the extensive Hb–oxaliplatin adduct formation should be expected to reduce the effective dose of oxaliplatin for DNA binding. The Pt(DACH) moiety has been identified as being bound to DNA, and its DNA adduct formation causes inhibition of transcription and cell replication (40). Our MS/MS identification of the parent oxaliplatin and the Pt(DACH) moiety in the Hb adducts supports the suggestion that an extensive amount of oxaliplatin bound to the Hb in erythrocytes may lead to a reduced dose of oxaliplatin for DNA binding.

The mechanism of transport of oxaliplatin into erythrocytes to form Hb adducts is, however, not understood. Further studies are necessary to investigate how oxaliplatin is transported into erythrocytes to form Hb adducts, to examine why different patients have variable Hb adduct formation, and to examine whether and how such binding may affect the function of Hb, which exerts some of the side effects of platinum drugs in cancer patients. A large number of patient samples should be analyzed to validate the potential association of the Hb–oxaliplatin adduct formation with side effects and efficacy of treatment. This study demonstrates the potential application of the technique described here for further clinical investigations, and preliminary results suggest that the extent of Hb–oxaliplatin adduct formation may serve as a biomarker of biologically effective doses of oxaliplatin.

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