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Short communication

Insight into the reactive form of the anticancer agent iproplatin

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Abstract

The reaction of iproplatin with reduced glutathione at different mole ratios yielded *cis*-di(isopropylamine)chloro-glutathionatoplatinum(II), not the expected *cis*-dichloro- species, indicating a mode of action of this anticancer agent that is different from that of *cis*-diamminedichloro-platinum(II).
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Due to the tremendous success of the treatment of testicular and ovarian cancers by *cis*-diamminedichloroplatinum(II) (cisplatin) [1–3], numerous platinum compounds have been synthesized that show anticancer properties. Among these second generation platinum chemotherapeutics, *cis*,*cis*,*trans*-dichloro-bis(isopropylamine)dihydroxoplatinum(IV) (iproplatin, Fig. 1), although recently discontinued in phase III clinical trials, exhibits improved antitumor activities with reduced toxicity. It is generally accepted that this platinum(IV) compound undergoes reduction in the cellular milieu and generates active platinum(II) metabolites which react with target biomolecules for rendering its antitumor activities. Based on the reduction potential and the concentrations of available reducing agents in and outside extracellular matrices, it is commonly believed that glutathione (GSH, Fig. 1) is the primary reductant for generating platinum(II) metabolites. It is also believed that the platinum(II) compound thus generated upon reduction is cisplatin analog, *cis*-dichloro-bis(isopropylamine)platinum(II) as was obtained from the reduction of iproplatin with ascorbic acid and other reducing agents [4]. In an excellent review, Hall and Hambley [5] have critically analyzed the effectiveness of platinum(IV) compounds as anticancer drugs by considering a number of factors including reduction potentials, rates of reduction to platinum(II), nature of reduced metabolites, ability to bind DNA by both Pt(IV) and reduced platinum(II) species, and their reactions to other biomolecules in the cellular milieu. To date, discussions exclusively have centered around the basic tenet that the platinum(II) metabolite thus formed was *cis*-dichloro-bis(isopropylamine)platinum(II). This conclusion is based on the characterization of platinum(II) product formed by the reduction of ascorbic acid as reported by our laboratory and by other reducing agents [4,5]. Unfortunately, the products of the reaction between iproplatin and glutathione have not been fully characterized. In this communication, we present unequivocal evidence that the major reduced product of iproplatin by glutathione is chloro-bis(isopropylamine) glutathionatoplatinum(II), not the commonly believed *cis*-dichloro-platinum(II) species, under both limiting and excess glutathione concentrations compared to iproplatin. These data clearly point different modes of binding to target molecules including DNA as compared to cisplatin in exhibiting antitumor activities.

The reaction of iproplatin (1.0–3.0 mM) with glutathione (GSH, 3.0–15.0 mM) was monitored by HPLC and proton NMR and the products were characterized by ESI-Mass

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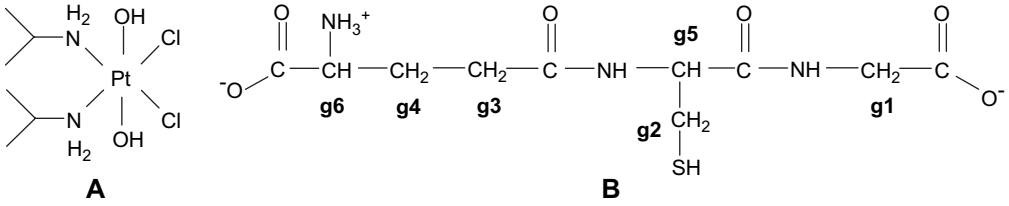

Fig. 1. Structural formulae of iproplatin and reduced glutathione (GSH).

spectrometry and Pt-195 NMR spectroscopy[2]. Based on the HPLC estimations of reduced and oxidized (GSSG) forms of glutathione and iproplatin, 3 mol of glutathione were consumed per mole of Pt(IV) yet only 1 mol of oxidized form of glutathione was produced. These data indicate that 2 mol of glutathione were utilized in the reduction of 1 mol of platinum(IV). Even when the reactions were carried out with 2 equiv of GSH per platinum(IV), only 50% of the platinum(IV) was reduced to platinum(II). To deplete all platinum(IV), 3 equiv of GSH were required. The product contains a platinum-195 NMR signal at −3248 ppm which is indicative of a platinum(II) coordination sphere that contains a sulfur, one chlorine, and two nitrogen donor atoms. Furthermore, no precipitation of the platinum(II) product was observed even when the platinum(IV) concentrations exceeded 1000 times the solubility of *cis*-dichloro-bis(isopropylamine)platinum(II). The ESI-MS spectrometry experiments clearly established the molecular mass of the product (675), corresponding to chloro-bis(isopropylamine)-(glutathionato)platinum(II) (Fig. 2). However, the intense product is seen from the loss an isopropyl group, aquachloro(isopropylamine)-(glutathionato)platinum(II) ($M + H^+$, 615). Several peaks in the Fig. 2 clustered around 615, 637, 659, 681, and 703 represent mono-, di-, tri-, tetra-, and pentasodium adducts ($-H^+ + nNa^+$). The peaks under each cluster are consistent with the predicted isotropic distributions based on the molecular formulae. The aqua complex was formed due to the loss of an isopropylamine group *trans* to the coordinated glutathione moiety. This trans-labilization was accelerated in the electro-spray ionization process. The mass spectrometry, platinum-195 NMR, and HPLC data unambiguously indicate that the third GSH molecule is coordinated with the platinum center. Finally, a thiyl radical[3] was captured by ESR spectroscopy when a radical capturing agent DMPO was used. The EPR signals consist of four lines with the distribution of signal intensities 1:2:2:1 at $g = 2.005$ with a hyperfine coupling constant of 15 G for a DMPO–thyil radical.

Glutathione reduces iproplatin by a second order reaction[4] [6] the first order with respect to each of the reactants, with a second order rate constant of $4.9 \pm 0.2 \times 10^{-1}\ M^{-1}\ s^{-1}$ at 22 °C at pH 7.0 (Fig. 3). The rate constants were evaluated from the NMR signal intensity–time data for both the oxidized and reduced glutathione. This rate constant is larger than that for the substitution of thiol onto *cis*-dichlorodiammineplatinum(II) and other platinum(II) complexes [7–9], implying that the glutathione coordination most likely occurred either at platinum(IV) or at platinum(III). The coordination of GSH to either Pt(III) or Pt(IV) state during the reduction, but not to Pt(II), can also be supported by the fact that when 2 equiv of GSH were used only 0.67 equiv iproplatin and 1.34 equiv GSH were consumed in the redox process and 0.67 equiv GSH was found to be bound to Pt(II). The platinum(III) oxidation state must have generated as a transient species since the reducing agent functions as a one-electron reductant. In fact, the detection of the one-electron oxidized form of glutathione, glutathiyl radical, supports the sequential one-electron transfer implying the existence of transient platinum(III). Although no additional changes in the proton NMR spectra were observed when molar ratio (GSH/Pt) exceeded 3, UV spectra monitored at regular time intervals exhibited slow changes up to several days. These secondary reactions reflect further changes in the platinum(II) coordination sphere including the formation of bis(glutathione) complex and delegation of coordinated amine.

A variety of mechanisms of reduction of platinum(IV) complexes by thilols have appeared in the literature [10–13]. These mechanisms include platinum(II) catalyzed reduction, formation of bridged precursor complexes with the reductant, and conventional outer sphere reactions. Our results indicate yet a new mechanism in which the reducing agent is coordinated to the metal center prior to reaching the reduced form.

[2] The NMR experiments were carried out on a 500 MHz (Bruker) instrument. Iproplatin concentrations for the Pt-195 NMR experiments were at least ten times higher than those for proton NMR and for other experiments listed in the text.

[3] Rate constants were evaluated by an iterative numerical integration with DYNAFIT program (version 3.28) [6] by using the normalized proton NMR signal intensity vs. time data according to the mechanism:

$$\text{iproplatin} + \text{GSH} \xrightarrow{k_2} \text{intermidiate} \quad (1)$$

$$\text{intermediate} + 2\text{GSH} \xrightarrow{\text{fast}} \text{products} \quad (2)$$

which is consistent with the overall reaction:

$$\text{Iproplatin} + 3\text{GSH} \rightarrow \text{Pt(II)} - \text{GS} + \text{GSSG} \quad (3)$$

A minimum value of $20\ M^{-2}\ s^{-1}$ was for the fast step (Eq. (2)) to fit the experimental data. The computer program utilizes non-linear regression using the Levenberg–Marquardt algorithm [6].

[4] Electron paramagnetic resonance experiments were carried out at ambient temperature (22 °C) on an IBM 200D-SRC instrument in a flat quartz cell containing 5.0 mM iproplatin, 15.0 mM glutathione, and 1.0 mM dimethy pyridine-N-oxide (DMPO).High performance liquid chromatography experiments were performed on a Waters system (Model 515) equipped with a diode array detector (Model 996). A mobile phase consists of ammonium formate–formic acid buffer (50 mM, pH 4.0) and methanol with a linear gradient elution for 20 min starting with the formate buffer with a flow rate of 1. 0 ml min^{-1} was used for recording chromatograms.

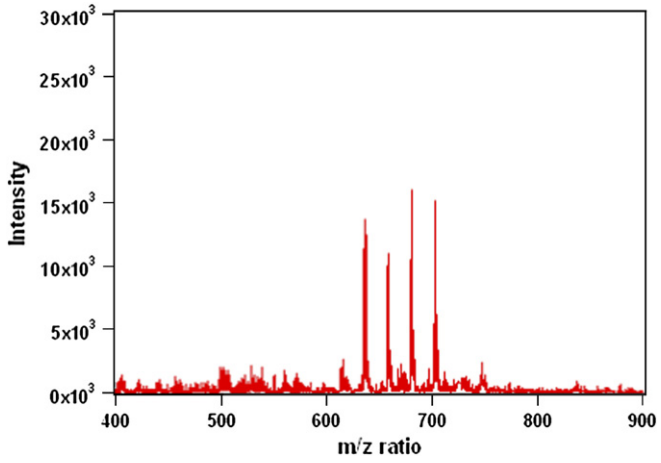

Fig. 2. ESI-MS spectrum (positive mode) of the product formed by the reaction between iproplatin (1.0 mM) and glutathione (3.0 mM).

The characterization of the monochloro(glutathionato)platinum(II) as the primary product has a major implication in the antitumor mechanism of iproplatin. A large abundance (2–10 mM) of glutathione in the cell and its favorable reduction potential make it a highly probable reducing agent in the cellular milieu for reducing iproplatin. The mode of action of this platinum(IV) compound is believed to be due to the binding of *cis*-dichloro-bis(isopropylamine)platinum(II) to DNA [14]. Bifunctional adducts' formations similar to those described for cisplatin are presumed predominantly through the two adjacent guanine and guanine–adenine bases [15]. Since one of the available coordination sites of the platinum(II) product is bound to GSH, such a bifunctional adducts' formation is unlikely. Since it is accepted that transcription [16] but not replication is the main mechanism for cisplatin mediated apoptosis, other targets including binding to cysteine rich cell surface proteins, zinc-finger transcription factors, and

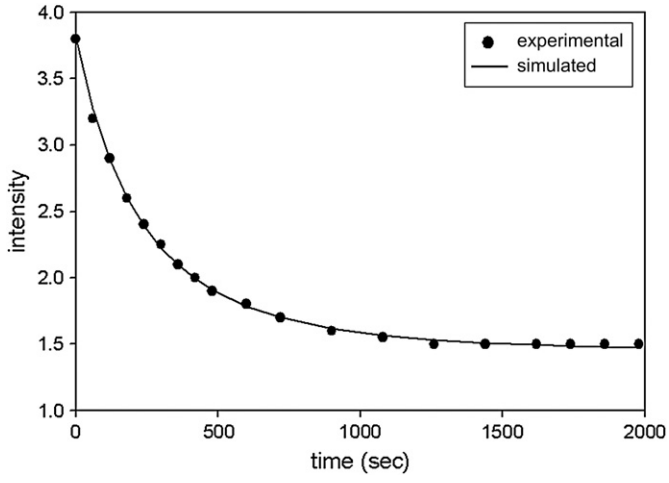

Fig. 3. Simulated and experimental kinetic curves representing normalized signal intensity of the g5 proton at 4.53 ppm of the reduced glutathione over time during the reaction between iproplatin (3.0 mM) and glutathione (9.0 mM) in phosphate buffer at pH 7.0. Experimental data are represented by solid circles and the computer simulated line was generated based on the mechanism indicated in footnote 2. Note that the signal intensity did not deplete to zero due to the fact that coordinated glutathione exhibits g5 signal at 3.53 ppm as well.

disruption of protein–DNA/protein–protein complex formation remain likely possibilities. In fact, Ishikawa and Ali-Osman [17] have shown that glutathione bound platinum complexes can inhibit translation processes. We have also evaluated up- and down-regulation of a number of gene transcripts that suggest that a number of cell surface glycoproteins, DNA binding proteins, and several protein kinases are involved in this apoptosis process [18].

To date, many studies have been carried out to gain the detailed mechanisms of activation and resistance of platinum anticancer drugs by cellular glutathione. These studies did not yield any uniform consensus, rather they opened many new questions. For example, in some cell lines, an increased concentration of GSH seems to delay the apoptosis and decrease the sensitivity of platinum(II) drugs, while in others, such elevated concentrations did not exert significant resistance. Furthermore, in some cases, platinum(II) and platinum(IV) compounds showed correlations between cell survival and GSH in a similar manner [19], whereas in others, platinum(II) and platinum(IV) compounds exhibited either opposite [20] or uncorrelated effects [21,22]. Finally, the effect of D,L-buthionine-*S*,*R*-sulfoximine, a glutathione depleting agent, on cytotoxicity exhibited by platinum compounds was mixed [23–25].

Since many factors are involved in modulating resistance or activation, our results point to the following conclusion with regards to activation of iproplatin by GSH. Since glutathione reduces platinum(IV) to produce glutathione bound Pt(II) compounds in deficient and excess [GSH] and that GSH concentration in cells far exceeds the therapeutic doses of platinum(IV) compounds, these platinum(II)-glutathione compounds are most likely to be the active forms. Furthermore, since mono-glutathione-platinum(II) complex is formed instantly during the reduction and bis-glutathione complex much more slowly, we hypothesize that mono-glutathione-platinum(II) complex is the dominant reactive form for iproplatin. Furthermore, it is conceivable that the bis(glutathione) complex perhaps is the inactive form due to its inability to covalently bind biomolecular targets. Therefore, those cell lines that produce high GSH usually show some deactivation. This glutathione activated mechanism also finds its support from the work of Eastman [26] and Kido et al. [27] who investigated the activation mechanisms of tetrachloro-(D,L-*trans*)1,2-diaminocyclohexaneplatinum(II) (tetraplatin) in the intra and extracellular milieu. For example, intracellular activation of tetraplatin was accomplished by low concentrations of GSH while the same compound was deactivated at higher GSH concentrations in Murine leukemia cells [26]. Kido et al. [27] have also demonstrated that intraperitoneal co-administration of tetraplatin and GSH in mice afforded better absorption of the drug and reduced the platinum concentration in plasma compared to tetraplatin alone. On the other hand, Chaney and et al. [28,29] found that the reduction of tetraplatin in extracellular environment was primarily accomplished by protein sulphydryl groups, but not by GSH, leading mainly to the expected *cis*-dichoro-(D,L-*trans*)1,2-diamineplatinum(II) and other products due to the substitution of chloride ligand. However, a significant portion of tetraplatin was taken up by the cell [28], and

in the intracellular environment, unidentified and unexpected platinum bio-transformation products were formed, although the roles of these compounds remained speculative. Finally, our results also point towards different likely targets other than or in addition to DNA for iproplatin since the corresponding dichloroplatinum(II) metabolite was not produced in the reaction. This notion is supported by the fact that almost undetectable *in vivo* DNA binding was observed for iproplatin while the corresponding dichloroplatinum(II) compound showed time dependent DNA binding [30].

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