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Uptake and metabolism of iproplatin in murine L1210 cells

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Summary. Iproplatin is structurally unique among the platinum (Pt) agents in the clinic because it is a quadrivalent complex. On the basis of the redox parameters for the Pt(IV) and Pt(II) oxidation states in a chloride system, it has been suggested that Pt(IV) complexes will be reduced to Pt(II) complexes in a biological environment [7]. To test this hypothesis, uptake and metabolism studies of [^{14}C]-iproplatin were carried out in L1210 cells. The L1210 cells raised in DBA2/J mice were incubated in vitro with 50 and 100 μM [^{14}C]-iproplatin at 37° C in Hanks' balanced salt solution, and total uptake and radioactivity associated with acid-insoluble fractions were measured for up to 3 h. Under these conditions, the uptake of iproplatin was linear with time and increased with increasing concentrations of iproplatin in the medium. At all times measured, >35% of radioactivity was associated with the acid-insoluble fraction, suggesting binding to macromolecules. The [^{14}C]-labelled compounds in neutralized acid extracts of cells were separated by reverse-phase high-performance liquid chromatography (HPLC). Three labelled compounds were detected; based on chromatographic elution times, they appeared to be iproplatin, *cis*-dichloro-*bis*-isopropylamine platinum(II) (CIP), the reduction product of iproplatin, and a third compound more polar than iproplatin and CIP. The finding of free CIP and the macromolecular binding of radioactivity in the cells suggests that iproplatin is reduced intracellularly.

Introduction

Iproplatin [*Cis*-dichloro-*trans*-dihydroxy-*bis*-isopropylamine platinum(IV)] is a second-generation platinum (Pt) complex that has shown activity in a number of tumor types [1, 2, 6, 11–13]. The three-dimensional structure of this drug is different from that of the other Pt complexes being tested clinically, in that it is a quadrivalent complex with an octahedral configuration, with two hydroxy ligands projecting from a square plane, whereas the other complexes are divalent and have a square planar structure. The Pt(IV) complexes are kinetically inert [8]. Additionally, in the octahedral configuration, steric hindrance may interfere with the interaction of these complexes with biological nucleophiles, whereas in the square planar configuration they interact readily [20]. It has been suggested that the quadrivalent species would be reduced to the divalent state in a biological milieu [8–10]. We have previously demonstrated the presence of divalent reduction products of iproplatin in plasma and urine of patients receiving the drug [17, 18]. Our studies have indicated that such reduction does not take place in plasma but must occur intracellularly [18]. The present study was aimed at determining whether iproplatin is taken up and reduced in a tumor model system (L1210).

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Materials and methods

Drugs. Analytical grade iproplatin was kindly supplied by Bristol-Myers Co. [^{14}C]-iproplatin (sp. act., 24.6 mCi/mmol; 99% pure) was synthesized at Roswell Park Memorial Institute [18].

High-performance liquid chromatography. The high-performance liquid chromatographic (HPLC) system consisted of a Waters Associates (Milford, Mass) model M6000A pump, a model 710B WISP, a model 441 fixed-wavelength UV detector equipped with a zinc lamp, and a Varian 402 data system. The separation of [^{14}C]-iproplatin, [^{14}C]-CIP, and other [^{14}C]-labelled metabolites in cell extracts was accomplished by isocratic reverse-phase chromatography on a μBondapak phenyl (10 μm) radial compression cartridge, with 3% methanol in H_2O as the mobile phase, at a flow rate of 1.5 ml/min [15]. Fractions eluting from the column were collected and counted for radioactivity in a Searle Mark III liquid scintillation counter (Des Plaines, Ill) for 10 min each. Automatic background subtraction was programmed into the instrument during radioactivity counts. An external standard method was used for quench correction. The elution times for iproplatin and CIP under these conditions are approximately 6 and 15 min, respectively. Iproplatin and CIP standards were used during the chromatography of radioactive extracts to control for any deviation in retention times.

Stability of [^{14}C]-iproplatin. The stability of iproplatin was tested in plasma ultrafiltrate (PUF) prepared by centrifugal ultrafiltration of plasma using Amicon CF-25 membranes (Danvers, Mass), Earle's balanced salt solution, Hanks' balanced salt solution, Eagle's basal minimal essential medium (BME) and RPMI 1640 medium.

[^{14}C]-Iproplatin ws added to each of the media to a final concentration of 20 μg/ml and incubated at 37° C. At zero time and at 3 h, 40-μl aliquots were injected onto the HPLC column and 1-min fractions of the eluate were collected and counted for radioactivity.

Cells. The L1210 cells used in these studies were grown in DBA2/J mice. Log-phase cells were obtained and the number of viable cells was determined by trypan blue exclusion with a hemocytometer. The cells were resuspended in appropriate media to a final density of 1×10^7 viable cells/ml and were used immediately in studies of the uptake and cellular metabolism of iproplatin.

Uptake studies. [^{14}C]-Iproplatin (50 and 100 μ*M*) was added to L1210 cells incubated at 37° C for 3 h. Aliquots in duplicate (1 ml) were removed at fixed time intervals for measurement of total radioactivity as well as radioactivity in the acid-insoluble fraction. The samples were centrifuged for 5 min at 2,000 rpm at 4° C in an IEC CRU 5000 centrifuge (Needham Heights, Mass). The supernatant was removed and the cell pellet was washed twice with 10 ml 0.9% ice-cold saline. For measurements of total uptake, the cell pellet was directly counted in 10 ml Liquiscint 2. For measurements of the acid-insoluble fraction, the cell pellet was extracted twice with 100 μl ice-cold 6% perchloric acid (PCA) prior to the measurement of radioactivity.

Intracellular metabolism of iproplatin. Intracellular metabolism of iproplatin was evaluated in three separate experiments using L1210 cells suspended in Hanks' balanced salt solution (2×10^8 cells/10 ml). The cell suspension was incubated at 37° C with [^{14}C]-iproplatin (100 μ*M*) for periods of 1 and 2 h. At the end of each incubation period, 10-ml samples were centrifuged for 5 min at 2,000 rpm. The supernatant was discarded and the cell pellet was washed three times with 20 ml ice-cold 0.9% saline, extracted with 200 μl ice-cold 6% PCA, and centrifuged again for 5 min. The acid extract was neutralized to pH 7.0 with 2 *N* potassium hydroxide. After removal of the precipitated potassium perchlorate, the supernatant was injected onto the HPLC system to separate the iproplatin from its metabolites. Fractions of the eluate were collected at 5-min intervals and counted for radioactivity.

To ensure that acid extraction did not introduce artifacts into the metabolic profile of iproplatin, cell extracts were also generated in alternate experiments by sonic disruption of cells placed on ice for 45 s using a Model 150 sonic dismembrator (Fisher Scientific, Farmingdale, NY). Total cell lysis was confirmed by microscopic examination of an aliquot. The sonicate was ultrafiltered for 60 min at 2,000 rpm using an Amicon Centrifree micropartition system, and the ultrafiltrate was analyzed by HPLC as described above.

Results

Stability of [^{14}C]-iproplatin in growth media. Because it is known that iproplatin can be chemically reduced to CIP in vitro with reducing agents [17], the stability of [^{14}C]-iproplatin in the growth media was tested prior to the studies on cellular uptake and metabolism. [^{14}C]-Iproplatin added to Eagle's BME and RPMI 1640 media showed an immediate degradation of approximately 35% and 50%, respectively, giving rise to CIP and another radioactivity-containing peak that eluted close to the solvent front (data not shown). A 3-h incubation of iproplatin in each of these media resulted in a further small decline in radioactivity under the iproplatin peak. In contrast, [^{14}C]-iproplatin added to human PUF, Earle's balanced salt solution, and Hanks' balanced salt solution remained intact even after 3 h of incubation, with >90% recovery in all cases. On the basis of these results and the fact that Hanks' balanced salt solution does not change its pH with oxygenation, the latter medium was chosen for use in uptake studies.

Uptake of [^{14}C]-iproplatin into cells. The uptake of [^{14}C]-iproplatin and the incorporation of radioactivity into the acid-insoluble fraction by L1210 cells at the 50 and 100 μ*M* concentrations tested is shown in Figs. 1 and 2. As evident from these figures, both the uptake of iproplatin and the radioactivity associated with the acid-insoluble fraction increased linearly with time. The total uptake and the label incorporated into the acid-insoluble fraction also increased with increasing concentrations of the drug (Figs. 1, 2). However, the extent of uptake fluctuated markedly between experiments, presumably due to variations in the age of cells when they were obtained. The data from three separate experiments indicated that the total uptake of iproplatin at 3 h was 56.2 ± 39.0 and 120 ± 53.4 pmol iproplatin equivalents/10^7 cells at 50 and 100 μ*M* concentrations, respectively. In all experiments at all sampling times, >35% of the radioactivity was associated with the acid-insoluble fraction, indicating binding to macromolecules.

Cellular metabolism of [^{14}C]-iproplatin. The intracellular radioactivity profile of the acid-soluble fraction of cells incubated for 2 h with 100 μ*M* [^{14}C]-iproplatin was evaluated in L1210 cells. After the HPLC separation, three peaks containing radioactivity were detected in these samples (Fig. 3). The retention characteristics of peak *b* correspond to that of iproplatin, and that of peak *c*, to CIP (Fig. 3).

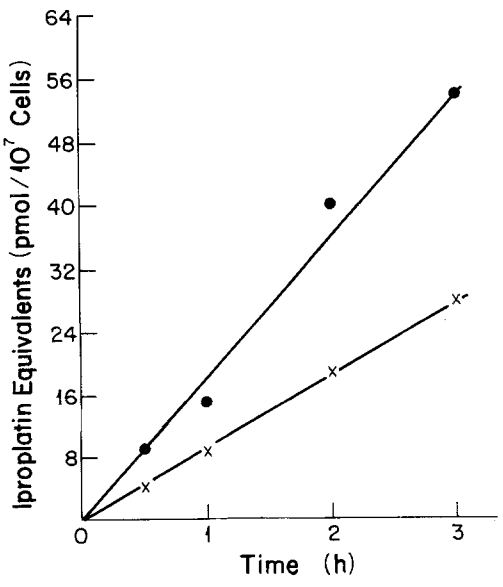

Fig. 1. Uptake of [^{14}C]-iproplatin (50 μ*M*) by L1210 cells (−0− total radioactivity; −X− radioactivity in acid-insoluble fraction). Data shown is from one experiment.

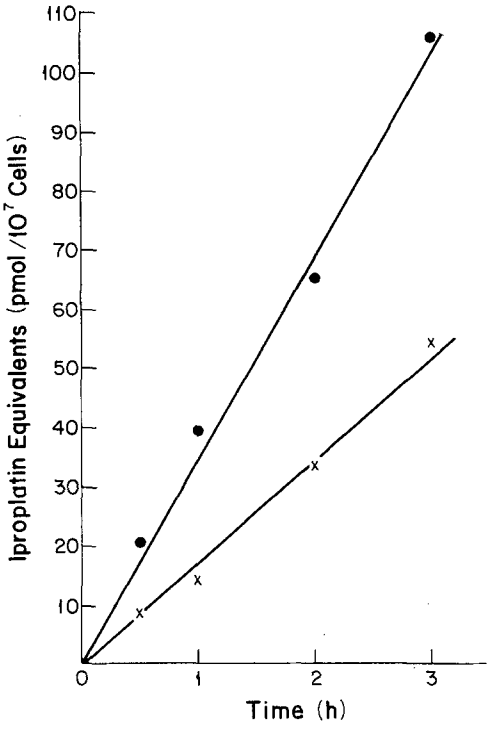

Fig. 2. Uptake of [^{14}C]-iproplatin (100 μ*M*) by L1210 cells (−0− total radioactivity; −X− radioactivity in acid-insoluble fraction). Data shown is from one experiment

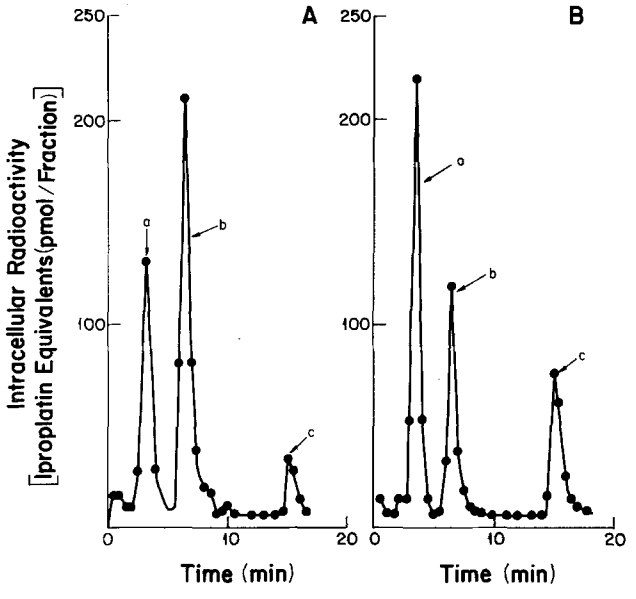

Fig. 3. HPLC separation of radioactive compounds in acid extracts of cells incubated for **A** 1 h and **B** 2 h with [^{14}C]-iproplatin (100 μ*M*). The peaks are as follows: *a*, unknown; *b*, iproplatin; *c*, CIP

The identity of peak *a*, which is more polar than iproplatin and CIP, is not known. This figure also illustrates the decline in iproplatin with time, concomitant with an increase in the proportion of Pt-containing metabolites. The same HPLC profiles were observed when cell extracts were generated by sonic disruption of cells rather than acid extraction (Fig. 4).

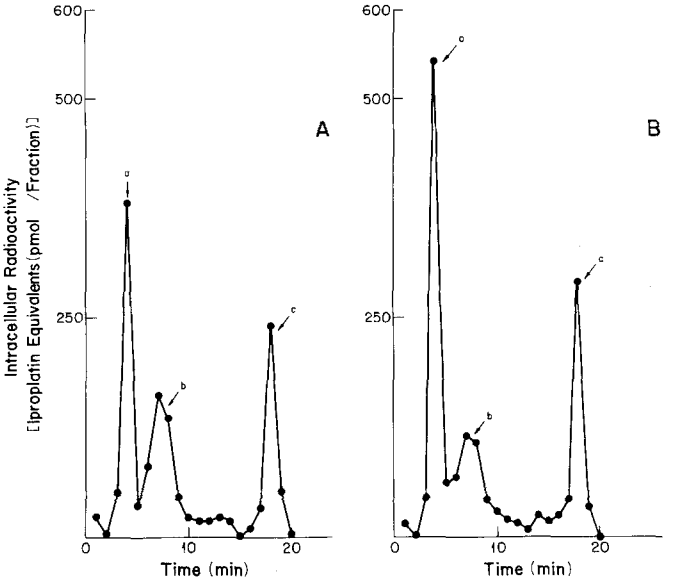

Fig. 4. HPLC separation of radioactive compounds in sonicates of cells incubated for **A** 1 h and **B** 2 h with [^{14}C]-iproplatin (100 μ*M*). The peaks are as follows: *a*, unknown; *b*, iproplatin; *c*, CIP

Discussion

Some studies have implicated CIP, the divalent counterpart of iproplatin, as being the DNA-interactive and therefore cytotoxic species after iproplatin administration [3, 4, 9]. Using mass spectrometry and ^{195}Pt-NMR (nuclear magnetic reasonance), we have previously demonstrated the presence of CIP and other divalent Pt metabolites in the urine of patients receiving the drug [17, 18]. We have also demonstrated the presence of CIP in the plasma of patients receiving iproplatin [17]. In vitro incubation of CIP in plasma results in protein binding, whereas that with the parent drug, iproplatin, does not [18]. Since the majority of Pt in plasma of patients receiving the drug is protein-bound [14, 15], we concluded that a derivative of CIP may be the protein-bound species and that the reduction of iproplatin to CIP must take place in cells [18]. The present studies were therefore focussed on the cellular metabolism of iproplatin. We chose murine L1210 cells for the present study because of the demonstrated cytotoxicity of Pt complexes to these cells [5]. To simulate human pharmacologic plasma concentrations, experiments were carried out at drug concentrations of 50 and 100 μ*M* [14, 16].

In the present studies, we showed the intracellular formation of CIP from iproplatin. In addition, these studies indicated that there is an increase with time in (a) the amount of drug taken up by the cells, (b) the amount of radioactivity associated with the acid-insoluble fraction, and (c) the amount of radioactivity associated with the metabolite fraction in the acid-soluble pool. The rate of uptake increased with the external iproplatin concentration and remained linear with time for a 3-h period at both the 50 and 100 μ*M* concentrations. The same was true for the incorporation of [^{14}C]-iproplatin into the acid-insoluble fraction of the cell. The significant incorporation of radioactivity into the acid-insoluble fractions at all times measured indicates that much of the intracellular radioactivity is bound to macromolecules. Although the identity of these macromolecules is not known, they may possibly include

both proteins and nucleic acids. Since CIP, in contrast to iproplatin, has been shown to give rise to plasma protein [18] and DNA binding [19], the acid-insoluble fraction is likely to represent the reduction products of iproplatin as well as free CIP. This study demonstrated that the intracellular environment is much more conducive to the reduction of iproplatin than is plasma [18].

The free radioactivity in the cell was associated with CIP as well as another metabolite, which eluted close to the solvent front. Urine from a patient treated with iproplatin has been shown to contain a material eluting in a similar position. ^{195}NMR studies of this urine have suggested that this material may be a monochloro derivative of CIP [18], indicating that it also is a divalent Pt species; however, its identity remains to be established.

In conclusion, this study demonstrated that iproplatin is indeed intracellularly reduced to CIP. If all of the intracellularly bound Pt is the divalent species, as we deduced on the basis of our previous studies, the reduction of iproplatin to CIP occurs rapidly.

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