Inhibition of Amino Acid Transport by cis-Diamminedichloroplatinum(II) Derivatives in L1210 Murine Leukemia Cells


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ABSTRACT

The uptake of cis-diamminedichloroplatinum(II) (cisplatin) has been studied in the L1210 murine lymphoid leukemia cell line. Labeled cisplatin and its aquated derivatives were resolved by high-performance liquid chromatography on a strong cationic exchange column. After 10 min of incubation of cisplatin with the cells, the major portion of the non-protein-bound platinum was in the form of cisplatin. However, a portion of this platinum was converted with time to a derivative which coeluted with the monoaquo derivative of cisplatin. With the appearance of this derivative, there was a concomitant inhibition of sodium-dependent amino acid transport as measured by the uptake of amino-isobutyric acid and methionine. Furthermore, the exposure of L1210 cells to a preparation of predominantly aquated product(s) of cisplatin inhibited amino acid uptake following a brief (2-min) incubation, whereas measurable inhibition of amino acid uptake by cisplatin required a longer preincubation period. This inhibition of aminoisobutyric acid and methionine was dependent on the concentration of platinum. Aminoisobutyric acid and methionine were shown to be concentrated in L1210 cells in the presence of sodium ions, and competition experiments suggest similar uptake systems. Since L1210 cells are methionine-auxotrophic leukemic cells, inhibition of essential amino acid transport by cisplatin may be a mechanism of cytotoxic action.

INTRODUCTION

The antineoplastic agent cisplatin is one of a group of platinum coordination complexes that was first shown by Rosenberg et al. (18) to possess antitumor activity against L1210 murine lymphoid leukemia cells. The antitumor effect of cisplatin is believed to involve its binding with DNA bases (17, 29), but the precise mechanism of its cytotoxicity is far from established. Many other sites of cell-drug interaction may well play a role (16). An interaction of cisplatin with plasma membrane constituents is suggested by: (a) the effects of cisplatin on the movement of sodium, chloride, and urea across frog skin epithelium (27); (b) the binding of cisplatin to segments of mammalian tubules (26) and renal tissue slices (19); and (c) inhibition by cisplatin of the membrane-specific enzyme, sodium-potassium ATPase (8). Additional effects of cisplatin on the renal transport of water and urea (20) make it likely that cisplatin may have other cell membrane effects.

Many of the reactions of cisplatin with macromolecules are accelerated if the drug has undergone prior aquation (12). It has been suggested that aquation of cisplatin intracellularly is an important step in its cell toxicity (17). The relative proportions of cisplatin and its derivatives in tumor cells, however, have not been studied.

In the present studies, we have examined the effects of cisplatin on the transport of amino acids in order to assess cisplatin-membrane interaction. The membrane transport systems were analyzed for their sensitivity to cisplatin because of the important consequences of blocking concentrative sodium-dependent methionine transport which may prevent optimal tumor cell growth. L1210 cells were chosen because of their known sensitivity to cisplatin (17, 18) and their established requirement for exogenous methionine (11). Most mammalian cells have at least 3 neutral amino acid transport systems, the A, L, and ASC systems. These systems are responsible for the cellular transport of amino acids. The alanine or A system, which is dependent on extracellular sodium, is specific for the transport of the nonmetabolizable substrate methylaminoisobutyric acid (1). The leucine or L system is characterized by its lack of dependence on extracellular sodium and its specificity for the nonmetabolizable substrate 2-aminoisobicyclo[2,2,1]heptane-2-carboxylic acid (1, 2). The ASC system has been less well characterized. Its properties may vary between different cell types (3, 14). The ASC system has been shown to make a major contribution to concentrative capacity of the neutral amino acid transport systems in mammalian cell lines (25). Recently, surprising differences in substrate specificity for the ASC system have been demonstrated between normal rat hepatocytes and the methionine-auxotrophic hepatoma cell line, HTC (9). L1210 cells and stimulated human lymphocytes are auxotrophic for methionine (11, 13). The uptake of methionine in these cells was inhibited by methotrexate, a 2,4-diamino antagonist (21, 24).

Because of the possible enzymatic or chemical conversions of cisplatin in the cell cytosol, both the intracellular and extracellular forms of platinum were examined by HPLC. Studies were designed to correlate the presence of particular intracellular cisplatin species with inhibition of amino acid transport (23).

MATERIALS AND METHODS

Reagents. L-[1-14C]Methionine (50 mCi/mmole) was purchased from Amersham (Arlington Heights, Ill.). [1-14C]AIB (51.6 μCi/mmole) was purchased from New England Nuclear (Boston, Mass.). Nonradioactive cisplatin was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, Md. [35S]Cisplatin in 0.9% NaCl solution was made available by Dr. J. D. Hoeschele and Dr. F. F. Knapp, Jr., at Oak Ridge National Laboratories, Oak Ridge, Tenn. (10). [35S]Cisplatin was received with a specific activity of approximately 145 mCi/mmole, but due to its short half-life (4.02 days)
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the specific activity declined rapidly. PtAm2Ag2+ was prepared by incubating cisplatin (0.1 mM) in water at pH 4.0 for 48 hr at 24°C with a 10% molar excess concentration of AgNO3 (equivalent to the total chloride in the cisplatin concentration). PtAm2ClAgq+ was prepared by incubating cisplatin (0.1 mM) in water (pH 4.0) for 48 hr at 24°C. The species formed under these conditions were pH dependent, at equilibrium, the resultant mixture contained approximately equal portions of PtAm2ClAgq+ and PtAm2Ag2+ and a small (<10%) amount of cisplatin. These resultant compounds were used as standards against which the platinum-containing compounds in samples of the cell supernatants and medium were compared. Retention times shortened as the column aged, and standards were therefore injected before each analysis.

HPLC was performed on a system constructed from the following components: pump, Eldex B-100S; injector, Altex Model 210; and a Whatman PXS-10 SCX (4 mm x 25 cm) strong cationic exchange column. The column was eluted with 0.03 M ammonium formate (pH 3.5) or 0.03 M potassium phosphate (pH 3.5) buffers at a flow rate of 1 ml/min. Chart 1 gives the chromatographic separation achieved by this system when cisplatin and its aquated forms were injected and eluted. The aquated derivatives were retained on the column longer than was cisplatin. The order in the retention times is presumably a result of the greater positivity and consequent binding to anionic sites on the column by aquated species. Recovery of PtAm2Ag2+ was 70 to 80%, which may have bound irreversibly to the column. Fractions were collected every 0.4 min and counted in 4.5 ml of Budget-Solv (Research Products International Corp., Elk Grove, Ill.) using a Packard (Downers Grove, Ill.) liquid scintillation counter. Incubation media and cell supernatant were analyzed by HPLC. Recovery of 195Pt activity was 95% to 100% for media and about 85% for cell supernatants.

L1210 Cell and Growth Media. Stock suspension cultures of L1210 cells were grown in Fischer’s medium (Grand Island Biological Co.) containing 10% horse serum (Flow Laboratories) and equilibrated with 5% CO2 in air at 37°C. Cultures were maintained in the presence of antibiotics (penicillin G, 62.9 mg/liter; streptomycin, 100 mg/liter). Logarithmic-phase cells, grown in 100 to 200 ml suspension culture, were harvested for transport studies when a cell density of 5 x 10^5/ml was attained. Under such conditions, L1210 displayed an average generation time of 12 hr. Cultures were established from frozen Mycoplasma-mfree stocks at 3-month intervals.

Uptake of Cisplatin. The uptake of [195Pt]cisplatin was determined by incubating L1210 cells (5 x 10^5 cells/ml) in a Cross and Taggart buffer, containing 1 mM CaCl2, 7 mM phosphate (pH 7.4), 10 mM sodium acetate, 36 mM KCl, and 77 mM NaCl (4). [195Pt]Cisplatin was stable in this buffer at 37°C for 60 min. The reaction was stopped by pipeting an aliquot (200 μl) into a 20-fold dilution of ice-cold 0.9% NaCl solution, and the aliquot was centrifuged. The cell pellet was washed 3 times with ice-cold 0.90% NaCl solution, then dissolved in 0.5 ml NCS (Amersham), solubilized overnight at 37°C, and counted.

Incubation Media for Amino Acid Transport Studies. The sodium-dependent amino acid transport was performed in Earle’s balanced salt solution, which contains 116 mM NaCl (IM-1). The sodium-independent transport was studied in NaCl-free Earle’s balanced salt solution containing 150 mM choline chloride (IM-2). The net sodium-dependent amino acid transport was determined by subtracting the sodium-independent transport from the total sodium-dependent transport (IM-1 transport - IM-2 transport = net sodium-dependent transport). Earle’s balanced salt solution (IM-1) was used because it supported maximum rates of amino acid uptake (21). Cisplatin also inhibited amino acid uptake in the Cross and Taggart media.

Amino Acid Transport Measurements. Logarithmic-phase cells were harvested by centrifugation, washed 3 times with each specific incubation medium, and resuspended in the incubation medium to a final density of 5 x 10^6 cells/ml. Cell viability after cisplatin (50 μM) exposure for 30 min was determined by a trypan blue exclusion, using a hemocytometer. For each transport measurement, cells were first incubated in the appropriate medium for 15 min at 37°C with or without cisplatin. At zero time, a portion of the cell suspension was withdrawn and added to the incubation medium with the labeled amino acid. At timed intervals, 200 μl samples were withdrawn. The cells were immediately separated from the incubation medium by centrifugation through a layer of silicone:mineal oil into perchloric acid, as described above. This technique is similar to the method of Wohlhueter et al. (28).

In the present experiments, a series of 400-μl conical polyethylene tubes (Beckman) were prepared by layering 100 μl of silicone (No. 550; Dow-Coming Corp.): mineral oil (8.4:1.6, v/v, respectively) over 50 μl of 7% (v/v) perchloric acid. Each 200-μl aliquot of cells withdrawn from the incubation medium was carefully and rapidly layered on top of the silicone: mineral oil. The tube was immediately centrifuged at 11,600 x g for 5 sec in a Beckman microfuge. At the completion of an uptake experiment, an aliquot from the perchloric acid fraction was placed in 6 ml of Aquasol II (Amersham) and counted for 10 min in a Beckman LS-250 liquid scintillation counter (22). The data presented in the charts are the means of 3 experiments which included all the drug treatments. In these representative experiments, the standard error was less than 10% of the mean.

RESULTS

There was a correlation between increasing concentrations of cisplatin outside and inside the cell as seen in Chart 2. This...
uptake of cisplatin was rapid and reached an apparent steady state during the first 10 to 15 min except at the highest concentrations. To establish the stability of the drug during the incubation period, cisplatin was incubated in the Cross and Taggart buffer, and HPLC analysis revealed very little change in the composition of the platinum-containing species for up to 30 min at 37°C (Chart 3A). Cisplatin was less stable in the Earle’s balanced salt solution; the same intracellular intermediates were formed in both media (results not shown).

Once inside the cell, a portion (25 to 35%) of the cisplatin was converted to a species comigrating with PtAm2ClAq+ (Chart 3B, Peaks 2 and 3). Platinum in the incubation medium was almost completely in the cisplatin form.

The L1210 cells were treated with cisplatin for 30 min and transferred to a drug-free medium after 2 washes. After 60 min, the cells retained 25% of the total labeled cellular platinum. Analysis of the labeled drug released from the cells into the drug-free media during the 60-min incubation revealed only the cisplatin form (Chart 3C). Other forms may have effluxed from the cell; however, in the presence of high concentrations of chloride in the buffer, these forms may have been reconverted back to cisplatin.

**Amino Acid Transport Studies.** Amino acid transport in L1210 cells was measured in Earle’s balanced salt solution, since higher rates of sodium-dependent amino acid transport were achieved in this medium than in the Cross and Taggart buffer. AIB, an amino acid which is transported but not metabolized, and methionine were shown to inhibit the uptake of each other (Chart 4). These competition experiments were measured at one time point where there was primarily unidirectional influx of the substrates AIB (1 min) and methionine (0.5 min).

**Cisplatin Inhibition of Amino Acid Transport.** AIB and methionine were transported by a sodium-dependent and a sodium-independent system (Charts 5 and 6). The uptake of AIB and methionine was greater in sodium than in choline chloride buffer. Cisplatin caused an inhibition of sodium-dependent AIB (500 µM) uptake which was dependent on the concentration of cisplatin (Chart 5). The accumulation of both methionine and AIB was inhibited by concentrations of cisplatin as low as 10 µM (Chart 6). There was little further inhibition of AIB and methionine uptake by cisplatin at concentrations of 25 to 50 µM. The addition of cisplatin did not inhibit amino acid transport immediately. However, the longer the drug was incubated with the cells, the greater was the inhibition of transport. The maximum inhibition of amino acid transport occurred following 10 to 30 min of cisplatin preincubation. This inhibition resulted in a decrease of the sodium-dependent AIB and methionine uptake by about 50%. Sodium-

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**Chart 2.** Uptake of [195mPt]cisplatin (Cpt Pt) into L1210 cells. Cells (5 × 10⁷/ml) were incubated in a Cross and Taggart buffer with several time points over 60 min and with increasing concentrations (µM) of labeled drug for 60 min. Cells were washed in 0.9% NaCl solution and counted as described in "Materials and Methods." The background (zero time) was subtracted from these results.

**Chart 3.** Analysis by HPLC of [195mPt]cisplatin and its aquation products in Cross and Taggart incubation media and L1210 supernatant. HPLC was performed using a 0.03 M KH₂PO₄ elution buffer (pH 3.5) at a flow rate of 1 ml/min. A, chromatogram of cisplatin in Cross and Taggart incubation media for 30 min at 37°C. B, cells incubated for 30 min with cisplatin washed (3 times) in 0.9% NaCl solution and suspended in 0.5 ml of 7% trichloroacetic acid, and the filtered (0.45-µm Milipore filter) supernatant then chromatographed; C, chromatogram of the cell-free media after 60 min. The 3 species present coelute. Peak 1, cisplatin; Peak 2, PtAm2ClAq+; Peak 3, PtAm3Aq2+.

**Chart 4.** Amino acid competition experiments in L1210 cells. Initial (1-min) labeled AIB (500 µM) uptake was measured with increasing concentrations of unlabeled methionine (MET) (1 to 50 mM) (A). Initial (30-sec) labeled methionine (500 µM) uptake was measured with increasing concentrations of unlabeled AIB (1 to 50 mM) (B). AIB (2.5 pmol/10³ cells/min) and methionine (0.3 pmol/10³ cells/min) uptake was measured and counted as described in "Materials and Methods." The transport medium was Earle’s balanced salt solution with 150 mM NaCl.

**Chart 5.** Uptake of AIB in the presence and absence of cisplatin (Cpt Pt) in Earle’s balanced salt solution. Initial AIB (500 µM) uptake was measured in the presence of Earle’s balanced salt solution containing sodium chloride or choline chloride (Ch Cl). Cells were preincubated at 37°C for 15 min in the presence of 1, 10, or 25 µM cisplatin, and AIB uptake was measured during the first 3 min, in the presence of either sodium chloride or choline chloride. See "Materials and Methods" for uptake and counting techniques.
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Taggart buffer where cisplatin was the only species found. Transport of AIB and methionine were observed in Cross and for uptake and counting techniques.

Therefore, the small amount (<10%) of the species of cisplatin that was found in the Earle's balanced salt solution could not completely account for this effect on amino acid transport.

The mechanism of inhibition of methionine transport by cisplatin requires a more prolonged period of incubation to obtain measurable inhibition of amino acid uptake. These results suggest that the inhibition of transport may be due to the formation of the aquated compound in the cell. The aqurated species of cisplatin (<10 μM) can bind irreversibly to the cell and inhibit amino acid transport (Chart 7). Extensive washing of the cells did not reverse this inhibitory effect of cisplatin. This concentration of cisplatin correlates with the pharmacological dose that can be achieved in the plasma (21.8 to 36 μM) (5). However, since cisplatin was the predominant species at both sides of the plasma membrane, a time-dependent direct interaction of cisplatin and some component of the amino acid transport system (see below) cannot be excluded. Trypan blue studies on the cell viability also suggest that cisplatin (>50 μM) with prolonged incubation (>45 min) may contribute to general membrane damage.

The mechanism of inhibition of methionine transport by cisplatin was not examined in these studies, but several possibilities independent transport of AIB or methionine appeared to be less affected by the presence of cisplatin. However, the sodium-independent transport represented a smaller contribution of AIB and methionine transport than did sodium-dependent transport in L1210 cells (Charts 5 and 6).

Similar results with cisplatin inhibition of sodium-dependent transport of AIB and methionine were observed in Cross and Taggart buffer where cisplatin was the only species found. Therefore, the small amount (<10%) of the species(s) of cisplatin that was found in the Earle's balanced salt solution could not completely account for this effect on amino acid transport.

**Aqurated Cisplatin Inhibition of Amino Acid Transport.** Chart 7 summarizes the effect of the aquated cisplatin preparation on AIB and methionine transport. The mixture of aquated cisplatin used for these studies contained 63% PtAm2ClAq+ and equal portions of cisplatin and PtAm2Aq+2+. Pure PtAm2Aq+2+ could not be tested in this system since the nitrate used in its preparation interfered with amino acid uptake. The L1210 cells were exposed to varying concentrations of the aquated cisplatin preparation (1 to 10 μM) for 2 min, and the cells were washed and resuspended in drug-free media. The initial uptake of AIB and methionine was compared in the presence or absence of PtAm2ClAq+. There was about a 30% inhibition of AIB and methionine uptake as a result of a 2-min preincubation with 10 μM aquated cisplatin. This inhibition was most apparent in sodium-dependent transport (data not shown) and was not reversible after washing the cells with drug-free media.

**DISCUSSION**

Cisplatin has been shown to be a highly reactive compound with a potential to bind to most biological molecules (17). This study was initiated in order to determine whether cisplatin interactions were not confined to DNA only. Cisplatin selectively inhibited sodium-dependent amino acid uptake into L1210 cells, which was suggestive of a drug-membrane interaction. This hypothesis is based in part on the inhibition of AIB, a nonmetabolized amino acid analogue. The fact that AIB and methionine are known to share similar transport systems (1–3) was also suggestive for the L1210 cells (Chart 4). Since the concentration component for methionine uptake was blocked by cisplatin in the methionine-auxotrophic L1210 cells, it is possible that this event may contribute to the drug cytotoxicity for L1210 cells. Cisplatin has also been shown to share with other heavy metals and their salts [uranyl nitrate (6), cadmium (7), and p-chloromercuribenzoate (6)] inhibitory effects on amino acid transport.

L1210 cells have been shown previously to require an exogenous source of methionine for optimal cell growth (11). The methionine requirement cannot be eliminated by supplying sufficient quantities of the precursors for methionine biosynthesis, 5-methyltetrahydrofolate and homocysteine (11, 22). Methionine synthetase is present in the cell, and there are sufficient quantities of the enzyme to convert the precursors to methionine (22). The reason for the exogenous methionine requirement in L1210 cells remains unclear. Nevertheless, the inhibition of methionine uptake into L1210 cells by cisplatin could be cytotoxic to these methionine-auxotrophic cells by a mechanism of methionine deprivation.

The only species of platinum detected in the incubation medium during the amino acid uptake inhibition experiments was cisplatin. Once in the cell, however, cisplatin was partially converted to a compound coeluting with PtAm2ClAq+. This conversion would be favored by the low intracellular chloride ion concentration. The formation of this intracellular aquated cisplatin species corresponded in time to the inhibition of amino acid uptake. When aquated derivatives of cisplatin (10 μM) were used, an inhibition of at least 30% of AIB and methionine uptake in the L1210 cells occurred after only 2 min incubation. In contrast, cisplatin required a more prolonged period of incubation to obtain measurable inhibition of amino acid uptake. These results suggest that the inhibition of transport may be due to the formation of the aquated compound in the cell. The aqurated species of cisplatin (<10 μM) can bind irreversibly to the cell and inhibit amino acid transport (Chart 7). Extensive washing of the cells did not reverse this inhibitory effect of cisplatin. This concentration of cisplatin correlates with the pharmacological dose that can be achieved in the plasma (21.8 to 36 μM) (5). However, since cisplatin was the predominant species at both sides of the plasma membrane, a time-dependent direct interaction of cisplatin and some component of the amino acid transport system (see below) cannot be excluded. Trypan blue studies on the cell viability also suggest that cisplatin (>50 μM) with prolonged incubation (>45 min) may contribute to general membrane damage.

The mechanism of inhibition of methionine transport by cisplatin was not examined in these studies, but several possibilities...
may be considered. Inhibition of methionine uptake may be due to (a) drug binding to specific membrane carriers, (b) reduction of the sodium gradient across the plasma membrane driving cellular uptake of amino acids, and (c) effects on intracellular processes which support uptake of amino acids; e.g., glutathione depletion in lymphocytes has been shown to reduce amino acid uptake in these cells (15). Cisplatin can bind to membrane vesicles, and it is conceivable that amino acid carrier molecules could be altered by such binding. Such types of binding might alter the function of these carriers. Regarding the second possibility of a diminished transmembrane sodium electrochemical gradient, it has been reported that cisplatin inhibits the sodium-potassium ATPase isolated from mammalian and amphibian cells (8), the activity of which is essential to maintain and generate transcellular sodium gradients. Indeed, cisplatin had its greatest inhibitory effect on sodium-dependent amino acid uptake. This is a rather discriminating interaction for cisplatin. Its importance is not completely understood at this time, but it is currently the focus of further experiments. Finally, since L1210 cells are methionine-auxotrophic tumor cells, any perturbation of the methionine pools could be cytotoxic.

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REFERENCES

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