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Binding of an Antitumor Platinum Compound to Cells as Influenced by Physical Factors and Pharmacologically Active Agents[1]

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SUMMARY

cis-Dichloro(dipyridine)platinum(II)-^{3}H (*cis*-PPC-^{3}H) binds to intact Ehrlich ascites tumor cells at 37° *in vitro* and remains associated with the acid-insoluble fraction of the cells. Binding is virtually unimpeded at 0° but is enhanced markedly at 60°. The maximum number of binding sites per cell at this latter temperature is approximately 7 billion. The extent of binding is also increased when the hydrogen ion concentration of the suspending medium is increased. Double-reciprocal plots of pg of platinum bound per cell as a function of extracellular *cis*-PPC-^{3}H concentration extrapolate to infinity on the ordinate, indicating no rate-limiting factor other than *cis*-PPC-^{3}H concentration. Of 49 chemicals and drugs tested, none reduces appreciably the rate or extent of binding, while certain heavy metals and compounds that compromise membrane permeability enhance considerably the amount of platinum bound per cell. The binding characteristics of *cis*-PPC-^{3}H are substantially the same with human and bovine lymphocytes as with Ehrlich ascites tumor cells; however, both types of lymphocytes bind much more platinum per unit of cell volume. Entry of *cis*-PPC-^{3}H into all three cell types appears to proceed by passive diffusion..

INTRODUCTION

The antitumor effectiveness of *cis*-PtII[2] against a variety of animal neoplasms is well established (8, 12, 13, 16, 17, 19, 21), and clinical trials have been conducted at several institutions (2, 7, 14, 20). The unavailability of a long-lived radioisotopically labeled form of this totally inorganic compound prompted an earlier study of an organoplatinum congener, *cis*-PPC, which was shown to have pharmacological properties similar to those of *cis*-PtII but which was somewhat less potent (3). Resynthesis of *cis*-PPC with pyridine-^{3}H yielded *cis*-PPC-^{3}H, which was used in a subsequent study of its binding to tumor cells and selected macromolecules (9). *cis*-PPC-^{3}H bound to intact EATC, and only a portion of that which was bound was dissociable when washed with 0.9% NaCl solution or when precipitated and washed with TCA. Dialysis experiments in distilled water revealed an avid binding of the compound to calf thymus DNA, high-molecular-weight yeast RNA, and bacterial and yeast tRNA, but not to bovine serum albumin, dextran, or purified erythrocyte membranes. Of the 4 polyribonucleotides, the greatest extent of binding was observed with polyguanylate and polyuridylate. Except in experiments with intact, viable cells, the presence of chloride ion at concentrations greater than 10^{-4} M almost totally suppressed the binding, indicating that dissociation of chloride from *cis*-PPC-^{3}H is a prerequisite for binding. Certain other anions were virtually equally effective in suppressing binding (Ref 9; unpublished data).

Aquation of *cis*-PPC occurs by a mechanism similar to that of other neutral platinum complexes by the scheme

$$Pt(py)_2Cl_2 + H_2O + H^+ \rightarrow Pt(py)_2Cl(H_2O)^+ + HCl$$
$$Pt(py)_2Cl(H_2O)^+ + H_2O + H^+ \rightarrow Pt(py)_2(H_2O)^{2+} + HCl$$

The doubly positive aquo complex presumably interacts with critical nucleophilic groups within the cell. Intramolecular spatial considerations would suggest principally intrastrand binding to nucleic acids; however, some degree of interstrand cross-linking of DNA has been detected (15).

The apparent paradox of suppression of *cis*-PPC-^{3}H binding by 10^{-4} M chloride ion in a nonliving (dialysis) model, in spite of extensive binding to intact cells in the presence of an extracellular chloride ion concentration of almost 0.14 M, led to speculation that binding to viable cells may be mediated enzymatically (8, 10). Enzymatic participation in the expression of the action of HN2 via its active transport into certain tumor cells has been documented, and failure of the transport mechanism may be a contributory factor to HN2 resistance (4, 5, 18).

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[2] The abbreviations used are: *cis*-PtII, *cis*-dichlorodiammineplatinum(II) (NSC 119875); *cis*-PPC, *cis*-dichloro(dipyridine)-platinum (II); EATC, Ehrlich ascites tumor cells; TCA, trichloroacetic acid; HN2, nitrogen mustard; DMSO, dimethyl sulfoxide; MEM, Eagle's minimum essential medium with Hanks' balanced salt solution; NaCl-Glu, 0.9% NaCl solution, 0.2% with respect to glucose; PO_4, 0.067 M sodium-potassium phosphate buffer; PO_4-Glu, PO_4, 0.2% with respect to glucose; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (NSC 79037).

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The following work was initiated to detect any enzymatic involvement in the binding of *cis*-PPC-^{3}H to cells and to determine the ways in which the rate and/or extent of binding may be influenced by various physical factors and pharmacologically active agents.

MATERIALS AND METHODS

cis-PPC-^{3}H was synthesized by the method of Kauffman (11), with the use of potassium tetrachloroplatinate (a generous gift from Matthey Bishop, Inc., Malvern, Pa.) and pyridine-^{3}H (Amersham/Searle Corp., Arlington Heights, Ill.) diluted with pyridine-^{1}H to a specific activity of 2.5 mCi/mmole. Elemental analysis yielded data that agreed with theoretical values within 0.1%, and the product had a specific activity of 4.27 mCi/mmole. Immediately prior to each experiment, it was dissolved in DMSO, and the final DMSO concentration in the reaction mixture was 0.5%. All drugs and chemicals were from various commercial sources. EATC were maintained in BALB/c mice (Flow Laboratory, Dublin, Va.) and were used 6 to 10 days after transfer.

Tumor cells were harvested just prior to use, washed free of ascitic fluid, and suspended to approximately 1.0% (v/v) in MEM, 0.9% NaCl solution, NaCl-Glu, PO_4, or PO_4-Glu. Absolute cell counts were done with a Coulter Model B electronic particle counter. Following incubation in a Dubnoff metabolic shaker for varying periods of time, 4-ml aliquots of the cell suspensions were either washed 3 times with fresh suspending medium and solubilized in 2.0 ml of 1.0 M hydroxide of Hyamine (Rohm and Haas Co., Philadelphia, Pa.) in methanol or were sedimented, resuspended in, and washed 3 times with 5% TCA and then solubilized in Hyamine.

Cultured human lymphocytes (Associated Biomedic Systems, Inc., Buffalo, N. Y.) and bovine thoracic duct lymphocytes (obtained through the courtesy of Dr. Robert Sharbaugh, Department of Surgery, Medical University of South Carolina, Charleston, S. C.) were treated in substantially the same fashion as EATC, except that the cell populations were adjusted to approximately 10^6 cells/ml.

The mean cell radius was determined for each cell type, and the mean cell volume was calculated with the assumption that the cells were spherical. Binding of *cis*-PPC-^{3}H to the cells was usually expressed as pg of *platinum* per cell as a function of time of incubation under various conditions and was computed on the basis of the volume of aliquot sampled, the number of cells per ml, the mean cell volume, the specific activity of *cis*-PPC-^{3}H, the percentage of the molecular weight of the compound contributed by platinum, and the scintillation counting efficiency (about 35%) in a Nuclear-Chicago Mark I spectrometer.

Moderate differences were noted in mean cell diameter from day to day, which conferred rather large daily differences in the calculated mean cell volume. As a consequence, the position of the broken horizontal line used in certain of the charts, which indicates the pg of platinum in a volume of suspending medium equivalent to 1 mean cell volume, shows variation among experiments. In any given experiment, however, duplicate reaction vessels generally agreed within 5%, and any experiment in which this did not occur was totally rejected. Except as indicated otherwise, all experiments were done in duplicate, and each was done a minimum of 3 times. Due to the daily cell volume variations, expression of data as mean values of 3 experiments would imply a disproportionate variability which actually did not occur between duplicate control and experimental vessels in any 1 experiment. Data presented in each chart thus represent in each case a single experiment which is substantially identical to at least 2 other experiments, with the sole exception of the absolute ordinate values.

RESULTS

Both the rate and extent of binding of platinum to EATC were influenced by the composition of the suspending medium (Chart 1). In each of the 5 media tested, binding was nonlinear, and the most rapid rate occurred within the 1st 30 min after addition of *cis*-PPC-^{3}H to the cell suspensions. Following this interval, the rate slowed appreciably and, after 1 to 2 hr, virtually reached a plateau in PO_4 and PO_4-Glu media. The greatest extent of binding within the 4-hr period of observation occurred in cells suspended in NaCl-Glu, while the least amount was bound to those cells suspended in the more physiological MEM.

The effect of varying pH on the amount of platinum bound to EATC is shown in Chart 2, which reveals a direct relationship between hydrogen ion concentration and amount bound. At pH 7.4, the amount accumulated was just over twice the amount present in an equivalent volume of supernatant solution while, at the lowest pH tested, the degree of accumulation was over 4-fold.

One of the most critical indications of enzymatic participation in the binding of *cis*-PPC-^{3}H would probably be a measurement of the effects of temperature on the process. If mediated enzymatically, accumulation would be expected to show some temperature optimum, with marked suppression occurring at temperatures approaching 60 and 0° (5). The effects of these incubation temperatures on binding of platinum to EATC are shown in Chart 3, and they reveal a modest but reproducible suppression of binding at 0° but show a greatly enhanced degree of binding at 60°.

A further indication of a nonenzymatic binding mechanism in EATC was obtained in experiments in which the concentration of *cis*-PPC-^{3}H was varied over a 100-fold range from 10^{-6} to 10^{-4} M. This upper limit was imposed by the fact that, at concentrations over 10^{-4} M, the compound was not completely soluble in the 0.5% DMSO, with the result that solid *cis*-PPC-^{3}H in the medium was sedimented along with the cells upon centrifugation and gave erroneously high values. Below 10^{-4} M, the extent of binding was closely related to the *cis*-PPC-^{3}H concentration when measured after either 10 min or 4 hr. Double-reciprocal plots of *cis*-PPC-^{3}H concentration against pg of platinum bound per cell yielded satisfactorily linear functions and intercepted the ordinate at the origin, indicating no rate-limiting factor other than the concentration

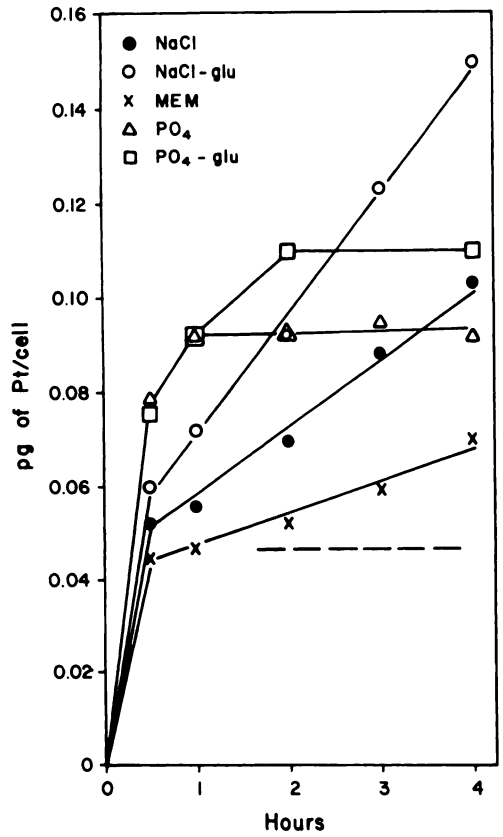

Chart 1. Binding of *cis*-PPC-^{3}H (3×10^{-5} M) to EATC as influenced by the composition of the suspending medium. At the intervals indicated, aliquots of each suspension were sedimented and washed 3 times with fresh suspending medium. The incubation temperature was 37°, and PO_4 and PO_4-Glu were at pH 7.4. - - - -, pg of platinum in a volume of suspending medium equivalent to 1 mean cell volume.

of *cis*-PPC-^{3}H (Chart 4). The intracellular/extracellular ratio of platinum showed virtually no dependence upon the extracellular concentration in these experiments and, over the 100-fold concentration range used, it remained substantially constant at about 1.9 when measured after 10 min of incubation and remained at approximately 4.5 when measured after 4 hr.

To determine the maximum mean number of binding sites per cell, we performed experiments in which *cis*-PPC-^{3}H was used at the highest concentration feasible in terms of solubility (10^{-4} M); incubation was at 60°, and the course of binding was monitored until the curve reached a plateau. When the latter event occurred, after 6 hr of incubation, calculation showed that each cell had bound a mean value of 2.334 pg of platinum, which corresponds to 7.2×10^9 atoms of platinum per cell.

In all of the experiments reported thus far, aliquots of cell suspensions were sedimented by centrifugation and the cells were washed rapidly 3 times by resuspension in and resedimentation from the suspending medium, devoid of *cis*-PPC-^{3}H. In order to assess the tenacity of binding under certain other conditions, we incubated EATC suspensions in the presence of *cis*-PPC-^{3}H for 2 hr, following which the entire cell suspension was rapidly sedimented, the cells were

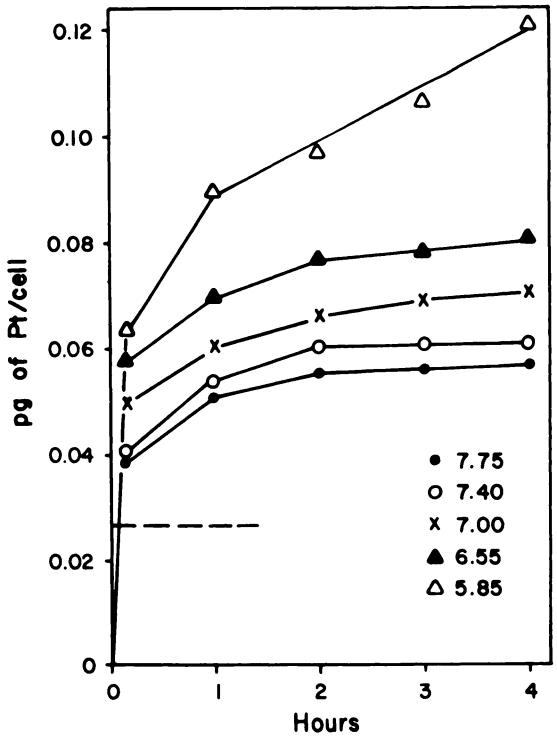

Chart 2. Binding of *cis*-PPC-^{3}H (3×10^{-5} M) to EATC in PO_4-Glu medium at various hydrogen ion concentrations. - - - -, same as Chart 1.

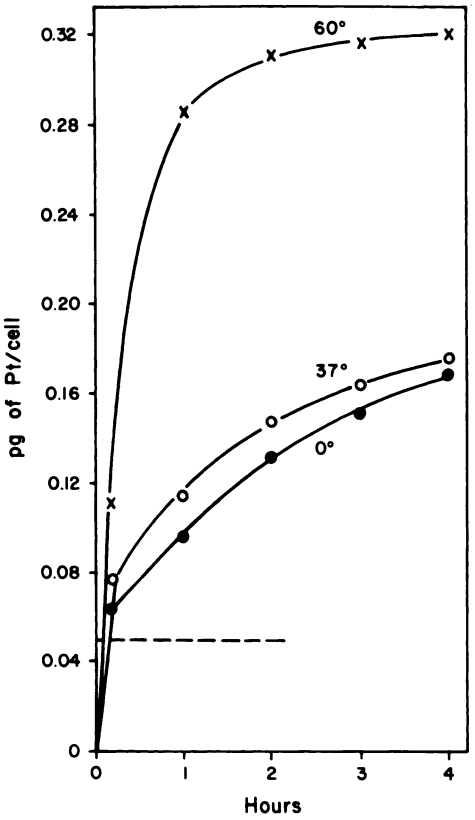

Chart 3. Binding of *cis*-PPC-^{3}H (3×10^{-5} M) to EATC in NaCl-Glu medium as influenced by temperature of incubation. For the washing procedure and - - - -, see the legend of Chart 1.

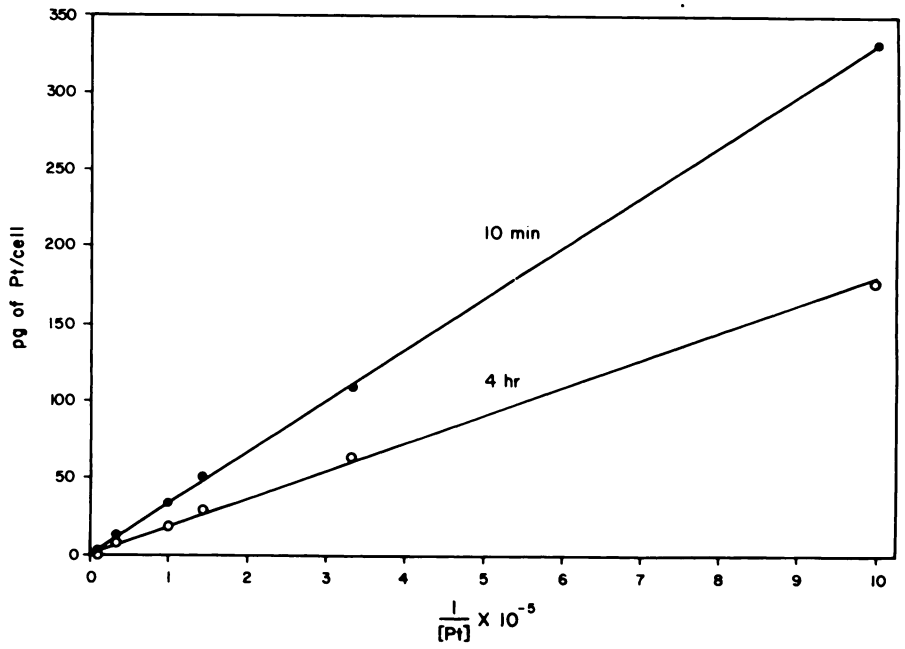

Chart 4. Double-reciprocal plot of the binding of *cis*-PPC-^{3}H to EATC in NaCl-Glu medium as measured after 10 min and 4 hr of incubation at 37°. The washing procedure was as described for Chart 1.

immediately resuspended to the original volume of fresh medium devoid of *cis*-PPC-^{3}H, and the subsequent loss of platinum from the cells was monitored. Approximately one-half of the platinum originally bound was lost upon 4 hr of additional incubation in the absence of *cis*-PPC-^{3}H, while one-half was firmly retained by the cells.

A further indication of a remarkably firm bond was obtained in experiments in which parallel aliquots of a cell suspension were removed from the incubation flask. Then 1 sample was sedimented and washed 3 times with the suspending medium without *cis*-PPC-^{3}H; the other sample was sedimented, the medium was removed, and the cells were resuspended in and washed 3 times with 5% TCA. There was no detectable removal whatsoever of the bound platinum by this rather rigorous treatment.

A comparison of the binding of *cis*-PPC-^{3}H to bovine and human lymphocytes as well as EATC in various suspending media is shown in Table 1. The data are expressed on the basis of the mean pg of platinum bound per cu μm of cell volume, and they represent the averages of 6 to 8 individual experiments. The compound bound to human lymphocytes only to a slightly greater extent than to bovine lymphocytes (1- to 3-fold) in each of the 4 media tested, and this ratio was the same whether measured after 1 or 3 hr of incubation. The most striking difference was the binding of platinum to lymphocytes, as compared with that bound to EATC. In NaCl-Glu, 32- and 24-fold more platinum was bound to human lymphocytes than to EATC at 1 and 3 hr, respectively. In the more physiological MEM, the corresponding respective values were 21- and 14-fold. The greatest difference in binding was noted in the acidic (pH 5.8) PO_4-Glu medium, where the differences were 32- to 38-fold. The effects of temperature variations on the binding of platinum to both types of lymphocytes were substantially the same as those observed with EATC.

Forty-nine diverse chemical compounds were assessed for possible effects on the rate and/or extent of binding of platinum to EATC. In this phase of the study, over 200 individual experiments were performed. Parameters that varied in tests of each compound included the composition of the suspending medium, the concentration of the compound being tested, and the duration of incubation with *cis*-PPC-^{3}H. Generalizations that were apparent from these experiments were that (*a*) no compound tested lowered appreciably the

Table 1

Binding of cis-PPC-^{3}H (3×10^{-5} M) to lymphocytes and EATC in various media

	NaCl-Glu		MEM		PO_4-Glu, pH 7.4		PO_4-Glu, pH 5.8	
	1 hr	3 hr	1 hr	3 hr	1 hr	3 hr	1 hr	3 hr
Bovine lymphocytes	2.72[a]	4.27	1.27	0.94	1.22	1.44	4.27	7.77
Human lymphocytes	6.18	7.96	2.69	2.22	2.53	3.08	7.67	10.87
Ascites tumor cells	0.19	0.33	0.13	0.16	0.25	0.29	0.24	0.29

[a] Values are expressed as (pg of platinum per cu μm of cell volume) $\times 10^4$.

rate or extent of binding; (*b*) the general pattern of response was similar in each of the suspending media; and (*c*) compounds known to compromise membrane integrity (polymyxin B, benzalkonium chloride, cyanogen iodide, and deoxycholate) and compounds containing heavy metals generally increased both the rate and extent of binding. In addition, each compound that was found to promote the binding of platinum to EATC was tested for a similar effect on both cultured human lymphocytes and bovine thoracic duct lymphocytes. These latter 2 cell types did not differ appreciably from EATC with regards to increased uptake of platinum in the presence of each compound tested.

DISCUSSION

Recent studies of the kinetics of the interactions of certain alkylating agents with intact cells and isolated macromolecules have yielded varying patterns. Goldenberg *et al.* (4, 5) studied extensively the binding of HN2 to HN2-sensitive and -resistant L5178Y lymphoblasts and found that, in sensitive cells, the uptake was linear from zero time, proceeded according to simple Michaelis-Menten kinetics, and demonstrated chemical specificity. Hydrolyzed HN2 and the monofunctional congener dimethyl 2-chloroethylamine were competitive inhibitors of HN2 uptake, while chlorambucil, melphalan, and cyclophosphamide were not inhibitory. Those authors demonstrated transport against a concentration gradient as high as 35-fold, with virtually total inhibition at 4° and partial inhibition upon the addition of ouabain or 2,4-dinitrophenol. The intracellular/extracellular HN2 ratio was closely related to the extracellular HN2 concentration, and a 20-fold increase of HN2 concentration resulted in a reduction of over 50% of the final ratio attained.

In a study of chlorambucil uptake by Yoshida ascites sarcoma cells, Hill (6) found that this drug was rapidly taken up by passive diffusion, and the rate was linear from zero time. The rate and extent of uptake were independent of temperature and were not depressed by fluoride, cyanide, 2,4-dinitrophenol, iodoacetate, or ouabain, and the uptake did not proceed against a concentration gradient.

Cheng *et al.* (1) studied the macromolecular binding of radioactivity from CCNU labeled with ^{14}C in either the cyclohexyl moiety or the ethylene residue. Using L1210 leukemia cells *in vitro* and *in vivo*, they showed that the time course of binding of each to the acid-insoluble fraction was nonlinear over a period of 4 hr. Radioactivity from the cyclohexyl-^{14}C-labeled portion of the molecule was detected largely in association with proteins, while radioactivity from the ethylene-^{14}C-labeled drug was bound primarily to nucleic acids. Those authors presented no data concerning effects of temperature or drugs on the course of binding, nor did they report the intracellular/extracellular ratios attained.

The data obtained in this study clearly contrast with those of Goldenberg *et al.* (4, 5) in their studies of HN2 with regard to the effects of temperature and drugs, the kinetics of the binding, and the influence of concentration on the intracellular/extracellular ratios of each agent. Double-reciprocal plots failed to reveal any rate-limiting factor in binding other than *cis*-PPC-^{3}H concentration, since K_m equated virtually to infinity. It must be concluded that this compound enters the cells by passive diffusion, the rate of which is enhanced by several compounds that increase membrane permeability. The time course of the uptake of *cis*-PPC-^{3}H most closely resembles that demonstrated with CCNU (1) but is much slower than the binding of chlorambucil (6).

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