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ESTROGEN PLATINUM–DIAMINE COMPLEXES: PREPARATION OF A NON-STEROIDAL ESTROGEN PLATINUM–DIAMINE COMPLEX LABELED WITH PLATINUM-191 AND A STUDY OF ITS BINDING TO THE ESTROGEN RECEPTOR *IN VITRO* AND ITS TISSUE DISTRIBUTION *IN VIVO*

JAMES P. DIZIO,[1] KATHRYN E. CARLSON,[1] CHRISTOPHER J. BANNOCHIE,[2] MICHAEL J. WELCH,[2] ERWIN VON ANGERER[3] and JOHN A. KATZENELLENBOGEN[1]*

[1]Department of Chemistry, University of Illinois, 1209 West California Street, Urbana, IL 61801, [2]Division of Radiation Sciences Research, Mallinckrodt Institute of Radiology, Washington University Medical School, 510 S. Kingshighway, St. Louis, MO 63110, U.S.A. and [3]Institut für Pharmazie, Universität Regensburg, Universitäts-strasse 31, 8400 Regensburg, Germany

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Summary—We have prepared in radiolabeled form (platinum-191) a non-steroidal estrogen platinum–diamine complex (Pt–diamine complex) that is reported to have selective cytostatic activity in estrogen receptor positive mouse mammary tumors. We then studied the interaction of this metal radiolabeled complex with the estrogen receptor *in vitro* and its distribution in immature rats *in vivo*. The radiolabeled complex was prepared by incubation of the non-steroidal estrogen diamine with $[^{191}Pt](II)Cl_4^{-2}$ ($t_{1/2} = 2.96$ days, sp. act. 7.54 Ci/mmol) in dimethylformamide (DMF)/H_2O, followed by purification by HPLC. The final radiolabeled product coeluted with an authentic standard of the unlabeled Pt–diamine complex, with a retention time distinct from those of the precursor diamine and chloroplatinate. In competitive radiometric receptor binding assays with rat uterine estrogen receptor, samples of the unlabeled diamine and Pt–diamine complex have apparent binding affinities of $53 \pm 3\%$ and $32 \pm 11\%$, respectively, relative to estradiol (RBA = 100% as standard). However, attempts to observe the binding of the ^{191}Pt–diamine complex with the estrogen receptor were complicated by a very high level of non-receptor binding, an irreversible binding to proteins in the receptor preparation, and a degradation of the platinum complex that, in part, releases the diamine. As a result, it is difficult to be certain whether the binding affinity measured for the Pt–diamine complex in the competitive binding assays is due to the complex itself, or whether it arises from diamine released upon degradation of the complex. In tissue distribution studies in immature female rats, much of the ^{191}Pt–diamine complex was deposited in the liver; there was no evidence of selective uptake of this compound by estrogen target tissues. Thus, it is not clear, from these studies, that the observed bioactivity of this complex arises from the interaction of the Pt complex or the diamine ligand with the estrogen receptor.

INTRODUCTION

One approach to increasing the selectivity of action of anticancer agents has been to link such cytotoxic species to a hormonal agent [1]. It is hoped, thereby, that the selective distribution of the hormonal portion of this conjugate, mediated by binding to its receptor, would guide the normally non-selective cytotoxic portion selectively to the hormone's target tissue. If this tissue were also the site or origin of the tumor, then antitumor activity with improved selectivity might be achieved.

While simple in concept, the development of hormone-directed cytotoxic conjugates and the definitive evaluation of the efficacy of the hormone receptor-mediated component of their activity have proved to be major challenges for several reasons [2–4]: (1) the chemical conjugation of the hormone and cytotoxic agent may impair the receptor binding affinity and the cytotoxic potency of each unit, respectively; (2) the potency of the cytotoxic agent needs to be sufficient so as to provide an adequate cell kill at the dose that can be delivered by a limited capacity receptor system; and (3) the biological effects of these conjugates that are *receptor-mediated* need to be distinguished from those

*To whom correspondence should be addressed.

that result from the action of the individual components, as the hormone and cytotoxic agents by themselves may have tumoristatic or tumoricidal activity. This latter issue requires the use of carefully developed control experiments and can be confounded by chemical and metabolic instability of the conjugates that might result in the release of the component parts, still in bioactive form [2–4].

A number of estrogen cytotoxic agents have been prepared, in which a steroidal or non-steroidal estrogen has been linked to a chemical function with known cytotoxic activity (nitrogen mustards, aziridines, nitrosoureas and carbamates, vinca alkaloids, platinum diamines, etc.) [3–6]. Some of these have been evaluated for their receptor binding and their activity *in vitro* and *in vivo* as potential selective cytotoxic agents for breast cancer. While in some cases, improved potency or selectivity appears to have been achieved, it was not clear whether the action was actually receptor-mediated.

In this work, we describe a study of the estrogen receptor (ER) interaction and *in vivo* tissue distribution of a non-steroidal estrogen diamine–platinum complex (Pt–diamine complex, see Fig. 1 for structure), previously investigated for its cytotoxic and antitumor activity [7]. As was previously reported, this platinum complex binds to the ER and is effective in suppressing the growth of ER positive breast cancer cells in culture and mammary tumors in mice, but has no effect on ER negative cells and tumors; while the platinum complex and the diamine ligand have equivalent activities in cell culture, the complex appears to be more potent *in vivo* [7]. We have prepared this complex in metal-radiolabeled form, using the gamma emitting isotope Pt-191 ($t_{1/2} = 2.96$ days) and studied its binding *in vitro* and its tissue distribution *in vivo*. It is not clear, from our findings, whether the observed bioactivity of this complex arises from the interaction of the Pt complex or the diamine ligand with the ER.

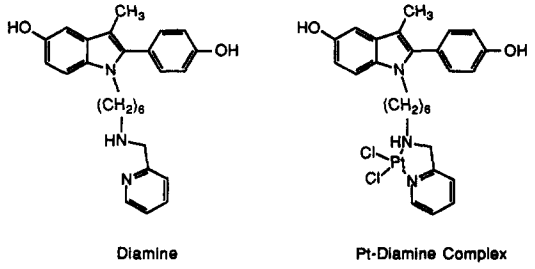

Fig. 1. Structures of the diamine and the Pt–diamine complex.

Metal-labeled ligands for hormone and neurotransmitter receptors would be useful as *in vivo* imaging agents as well as cytotoxic agents. This steroid Pt complex represents a simple model system for metal-labeled ligands. Other agents, labeled with technetium-99m and rhenium-186, are currently under active investigation as *in vivo* imaging agents ([8, 9] and references cited therein).

MATERIALS AND METHODS

Materials

Steroids were obtained from the following sources: radiolabeled estradiol (E_2), [6,7-^{3}H]E_2 (49 Ci/mmol), Amersham Corp. (Arlington Heights, IL). Unlabeled E_2 (1,3,5[10]-estratriene-3,17β-diol), Sigma Chemical Co. (St Louis, MO). The preparation of the diamine and the unlabeled Pt–diamine complex has been described [7]. Other chemicals were obtained from the sources indicated: activated charcoal, and Trizma Base, Sigma; dextran grade C, Schwarz/Mann (Orangeburg, NY); (ethylenedinitrilo)tetraacetic acid tetrasodium salt (EDTA) and sodium azide, Eastman Organic Chemicals (Rochester, NY); dimethylformamide (DMF), Fisher Scientific (Fairlawn, NJ); Triton X-114, Chem. Central-Indianapolis (Indianapolis, IN); 2,5-diphenyloxazole (PPO), Research Products International Corp. (Mt Prospect, IL); 1,4-bis(5-phenyloxazol-2-yl)benzene (POPOP), Aldrich Chemical Co. (Milwaukee, WI); hydroxylapatite, Bio-Gel HT (HAP), Bio-Rad Laboratories (Richmond, CA); G-25 Sephadex and Blue Dextran, Pharmacia Fine Chemicals (Piscataway, NJ).

Preparation of ^{191}Pt–diamine complex

^{191}Pt(IV) ($t_{1/2} = 2.96$ days) in aqua regia was obtained from the University of Missouri Research Reactor. The target consisted of 4.19% enriched Pt-190 bombarded for 150 h with a thermal flux of 3×10^{14} neutrons/cm^2/s. The Pt-191 produced has a specific activity range of 15–55 mCi/mg (2.9–10.5 Ci/mmol). In a typical preparation, 9.93 mCi of ^{191}Pt(IV) in aqua regia (sp. act. 39.5 mCi/mg, 7.5 Ci/mmol) was evaporated with a heat gun to near dryness under N_2 flow. The residue was resolubilized in 3 M HCl (200 μl), reduced with 10% $SnCl_2$ in 6 M HCl (1.0 ml), and the ^{191}Pt(II) extracted into diethyl ether. After removing the ether layer and evaporating to dryness, 8.86 mCi of [$H_2{}^{191}$Pt]Cl_4 remained for the reaction with the diamine.

The $[H_2{}^{191}Pt]Cl_4$ was resolubilized in H_2O, pH 6 (80 μl) and DMF (200 μl). To this solution was added 1.3 equivalents of diamine in 200 μl DMF. The reaction mixture was stirred at 85°C for 21 h. A 100 μl-portion of the reaction solution was diluted with 1.0 ml 60:40 MeOH–KH_2PO_4 20 mM, pH 5.2 and then filtered through a prewetted Acro LC 13 0.2 micron filter. The filter was rinsed with 100 μl MeOH and the total filtrate volume (1.2 mCi) was injected onto an Alltech C18 10 μm semi-prep column (500 × 10 mm i.d.). Elution from the column was done with 60:40 MeOH/KH_2PO_4 20 mM, pH 5.2 mobile phase, using a 3.0 ml/min flow rate. At 40 min, elution was switched to 80:20 MeOH–buffer-H_2O to remove the unreacted diamine from the column. A prominent peak at 31.3 min contained 93% of the activity in the run. Four 1 ml fractions at the center of this peak were combined (781 μCi) and the MeOH evaporated. The remaining solution was extracted twice with diethyl ether (2 ml), and 612 μCi of activity (78%) was obtained in the extract that was evaporated to dryness. The ^{191}Pt–diamine complex had a decay-corrected specific activity of 4.85 Ci/mmol.

In order to analyze the ^{191}Pt–diamine complex, a portion of the ether layer was evaporated to dryness and the activity was taken up in DMF (50 μl). A 25 μl (35.0 μCi) portion was injected onto an Alltech C18 10 μm analytical column (250 × 4.6 mm i.d.) and eluted with the same 60:40 MeOH–buffer mobile phase, using a 1.5 ml/min flow rate. The ^{191}Pt–diamine complex elutes at 7.4 min (DMF, $R_t = 2.2$ min) and accounts for >93% of the activity; a similar run with unlabeled standard Pt–diamine complex in 25 μl DMF gave the same retention time. Coinjection of the labeled and the unlabeled Pt–diamine complexes gives one peak, and both samples show minor impurities and/or decomposition products that elute at roughly 10.9, 15.9, and 18.0 min.

HPLC chromatography and radiochromatography

High performance liquid chromatography (HPLC) was performed on two systems. Unlabeled samples were analyzed on a Varian 5060 system, using a Perkin-Elmer LC-75 Spectrophotometric Detector (at 300 nm). A Varian MCH-10 10 μm (300 × 4 mm) column was eluted isocratically with a 70:30 mixture of MeOH–[KH_2PO_4 (20 mM)-K_2HPO_4]pH 5 with a flow rate of 1.5 ml/min; on this system, the Pt-steroid eluted at 4.32 min and the diamine at 7.57 min. Most of the radiolabeled samples were analyzed on a Spectra-Physics SP8700 solvent delivery system (Spectra-Physics, San Jose, CA), Rheodyne 7000 injector with 2.0 ml loop (Alltech Assoc., Deerfield, IL), Kratos GM770R variable wavelength u.v. detector set at 300 nm (Kratos Analytical Instruments, Arlington Heights, IL), and radioactivity detection with a NaI(Tl) crystal, monitored with a Canberra Series 35 Plus time-mode multichannel analyzer (Canberra, Meriden, CT). Columns, elution conditions and retention times are given in the next section. Final activity levels were measured with a Capintec Radioisotope Calibrator Model 10 with a setting of 353.

The specific activity was determined by HPLC analysis as well as by the competitive radiometric binding assay [10, 11]. The mass of a collected HPLC peak was determined by comparison of the area of the peak to a calibration plot of peak area versus injected quantity. The radioactivity of the collected peak was determined by scintillation counting using a Nuclear Chicago Isocap 300 instrument and a xylene-based cocktail [12]. Comparison of the data from the scintillation counter with that from a γ counter (Beckman γ 8000) showed them to be essentially identical.

Cytosols

Rat and lamb cytosols were prepared and stored as previously reported [13, 14]. All experiments were done in TEA buffer (0.01 M Tris–HCl, 0.0015 M EDTA, 0.02% sodium azide, pH 7.4 at 25°C).

Binding assays

Lamb or rat cytosol containing 2–3 nM ER was incubated with various concentrations (5–1000 nM) of ^{191}Pt–diamine complex in the absence or presence of unlabeled E_2 (3000 nM E_2 was routinely used to block ER; in one experiment, only 30 nM was used). Incubations were at 0°C for 1–2 h. Unbound ligand was generally removed by charcoal-dextran, but also by passage through a column of Sephadex G-25, or by HAP assay.

The charcoal-dextran slurry used to remove unbound ligand was prepared as previously reported [13] and used at 1 part to 10 parts of cytosol solution, at 0°C. The Sephadex G-25 was swollen in TEA buffer, overnight at room temperature. It was then gravity packed into a 0.5 × 6 cm column in a pasteur pipette plugged with glass wool. Samples were eluted at 0°C with TEA. The void volume was determined

by Blue Dextran and the internal volume by $[^3H]H_2O$. Hydroxylapatite was prepared as a slurry and utilized following the procedure of Williams and Gorski [15]. The ethanol extract from this assay represents reversibly bound activity; activity that remains in the pellet after ethanol extraction represents irreversibly bound material.

Relative binding affinity (RBA)

Assays were performed as previously reported [13], using lamb or rat cytosol at ~1.5 nM of ER. Several concentrations of unlabeled competitor or buffer, together with 10 nM $[^3H]E_2$ were incubated with cytosol at 0°C for the times indicated. Unbound ligand was removed with charcoal-dextran. Competitor solutions were prepared in 1:1 DMF–TEA to ensure solubility. A modification of this assay was used to determine effective specific activity [10, 11].

Exchange assays

Exchange assays were conducted as previously reported [16]. ER was allowed to associate with unlabeled ligand at 0°C for 2 h; unbound compound was then removed by charcoal-dextran. $[^3H]E_2$ (30 nM, Hot) alone or together with 3000 nM E_2 (H + C) was then added to the receptor complex and this was incubated either at 0°C for 2 h (to measure empty sites) or at 25°C for 18–20 h (to measure total exchangeable sites). Free was removed by charcoal-dextran.

Covalent attachment assay

Samples were spotted onto filter disks, washed, and counted as previously reported [17].

Animal uptake methods

Immature female Sprague–Dawley rats (25 days, ~50 g) were injected (i.v. femoral vein), under ether anesthesia, with 3 μCi of ^{191}Pt–diamine complex in a 1:1 physiological saline–propylene glycol solution. To ascertain whether the uptake was mediated by a high affinity, limited capacity system, one set of animals was treated with 15 μg unlabeled E_2, coinjected with the radiopharmaceutical. Animals were sacrificed by decapitation, at the times indicated, and samples of tissue and blood were weighed. Radioactivity in organ and standard samples was determined with a Beckman Gamma 8000 automatic well-type γ counter (Beckman Instruments, Fullerton, CA) [18, 19].

RESULTS AND DISCUSSION

Preparation of the ^{191}Pt–diamine complex

The structures of the Pt–diamine complex and the precursor diamine are shown in Fig. 1. ^{191}Pt–chloroplatinic acid was prepared by a $SnCl_2$ reduction of a sample of ^{191}Pt(IV) obtained by neutron bombardment of a ^{190}Pt target. The ^{191}Pt–diamine complex was formed by treatment of $[^{191}Pt]H_2PtCl_4$ with the diamine in DMF and warming, and the complex was purified by HPLC under conditions that separate it from any excess diamine and any remaining H_2PtCl_4. The final material has a radiochemical purity of >93% and a decay-corrected specific activity of 4.85 Ci/mmol.

Binding studies of the diamine, and the Pt- and ^{191}Pt–diamine complexes with ER in vitro

Competitive binding studies with the Pt–diamine complex and free diamine. The RBA of the Pt–diamine complex and the diamine itself for the ER was assayed by a competitive radiometric binding assay, using $[^3H]E_2$ as a tracer, rat or lamb uterine cytosol as a source of ER, and dextran-coated charcoal for removal of free tracer. The assay had a final concentration of 7% DMF (see Materials and Methods). After 18–22 h at 0°C, the apparent RBA (where E_2 is considered to be 100%) is for the diamine 53 ± 3%, whether assayed with rat or lamb ER, and for the Pt–diamine complex somewhat lower, 39% in rat and 24% in lamb (Table 1). These values are considerably higher than those obtained from these compounds in calf uterine cytosol with no DMF (diamine 20% and Pt–diamine complex 2% [7]).

While the RBA for the diamine is somewhat lower in ER from calf uterine cytosol than lamb or rat, the discrepancy for the Pt–diamine complex is much greater (10-fold). This might be due to differences in the extent of non-specific binding to proteins other than ER. Extensive

Table 1. ER binding affinity of the Pt–diamine complex and the diamine[a]

	RBA E_2 = 100%	
Incubation time (h)	Diamine	Pt–diamine complex
1.5	148	56
4.5	132	41
18–22	53 ± 3[b]	32 ± 11[b]
18–22 (+150mM NaCl)	18	14

[a]Determined in a competitive radiometric binding assay using $[^3H]E_2$ as tracer, rat or lamb uterine cytosol as a source of ER, and charcoal dextran to adsorb free ligand. For details, see Materials and Methods.

[b]Given as average of assays in both rat and lamb cytosol ± SD, $n = 3$.

binding to other proteins lowers the effective free concentration of the ligand available to compete with [^{3}H]E_2 for ER sites, thus lowering the measured RBA [22]. This effect would be greater in cytosol preparations with greater total protein concentration relative to ER concentration: in this respect, calf > lamb > rat (calf *ca* 1.7 mg protein/pmol ER [20, 21]; lamb *ca* 1.1 mg/pmol; rat *ca* 0.9 mg/pmol). Binding to non-receptor proteins (i.e. non-specific binding) is also markedly reduced by the presence of low concentrations of DMF [22]. The RBA values for the Pt–diamine complex do, in fact, vary inversely with the protein concentration (calf 2% [7], lamb 24% and rat 39%), and may reflect, as well, the absence of DMF in the calf uterine cytosol binding assay.

The RBA was also assayed in the presence of 150 mM NaCl, since a high concentration of chloride (> 0.1 M) ion has been reported to slow or prevent interaction of the *cis*-Pt complexes in biological systems [23]; only in the low chloride concentrations encountered in cells (4 mM) are they reactive. In buffer containing 150 mM NaCl, the RBA of both the diamine and the Pt complex were lower than in the absence of high chloride (Table 1).

When the RBA was assayed at 1.5, 4.5 and 22 h at 0°C, both ligands showed declining RBAs with time (Table 1). This is often observed with ligands having RBA values $< E_2$, and it represents a shift from an initial, non-equilibrium distribution of competitor and tracer that reflects their relative rates of association, where the competitor and tracer may be quite similar (kinetic distribution), to a final equilibrium distribution that reflects the association and dissociation rate constants of both compounds (thermodynamic distribution) [24]. It is of note that the Pt–diamine complex also shows RBA values that decline with time, because, as will be shown later, about 75% of the added ligand becomes bound irreversibly (covalently?) with cytosol proteins. The fact that the RBA of the Pt–diamine complex drops suggests that this irreversible protein binding is not occurring with ER (see below).

Direct ER binding studies of the ^{191}Pt–diamine complex. The binding of ^{191}Pt–diamine complex to ER was measured both by single point assays (at 30 nM) and by multiple-concentration (5–1000 nM) binding assays [cf. Fig. 2(A)], in both rat and lamb uterine cytosol. The most notable feature of the binding shown in Fig. 2(A) is that it is linear, a characteristic of low affinity,

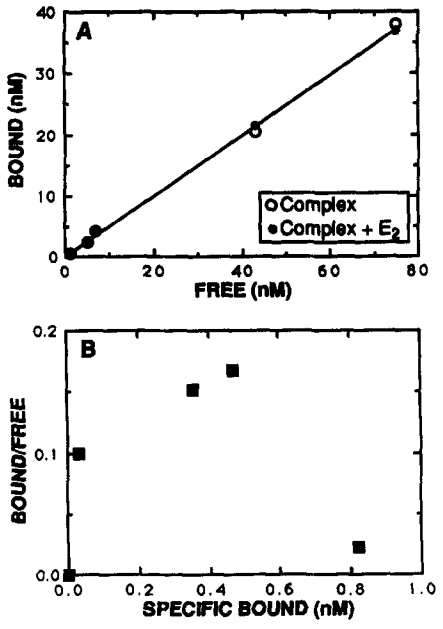

Fig. 2. Interaction of the ^{191}Pt–diamine complex in rat uterine cytosol. Samples of ^{191}Pt–diamine complex were incubated with the cytosol preparation for 1 h at 0°C, either in the absence (○, for total binding) or presence (●, for non-specific binding) of 3000 nM unlabeled E_2, and then treated with charcoal-coated dextran to remove the free tracer. Panel A: direct binding plot. Panel B: Scatchard plot: the difference between the assay points in the absence and presence of 3000 nM E_2 (specific binding) was plotted as a Scatchard plot.

non-saturable (i.e. non-specific) binding. In most cases, however, when the ER was blocked by 3000 nM unlabeled E_2, there is slightly less ^{191}Pt–diamine complex bound to the protein than when the ^{191}Pt–diamine complex alone was added to cytosol. This difference while suggestive of estrogen specific binding, shows no relationship to the ER content in the cytosol, and when the data is converted to a Scatchard plot [Fig. 2(B)], there is no evidence of a high affinity, limited capacity binding component.

Direct binding of the ^{191}Pt–diamine complex to ER was assayed by separation of the bound and free components routinely by the charcoal-dextran adsorption method, but also by the more gentle method of passage through a column of Sephadex G-25. Both of these methods showed that a large fraction (50 to 70%) of the added ligand was bound to protein, with only a very small difference between the total and non-specific components, indicating, at most, only limited estrogen specific binding. However, when the incubations were assayed by hydroxylapatite (HAP), using the normal assay protocol, we observed only 10–30% of the binding seen with the other two methods, for

both the total and the non-specific binding components. Since the usual HAP assay method involves as a final step extraction of the protein-bound radioactive ligand with ethanol and then measurement of the radioactivity in this ethanol extract, but not in the protein–HAP pellet, it would not measure any ligand that is irreversibly (covalently) bound to protein, and remains in the pellet (see below).

To examine further whether this apparent competition observed upon the addition of 3000 nM E_2 represented true estrogen specific binding, we performed a binding experiment with a lamb uterine cytosol preparation that was devoid of ER (certain lamb uterine cytosol preparations do not have detectable ER): a similar reduction in the binding of the ^{191}Pt–diamine complex was observed in the presence of 3000 nM E_2 compared to that seen when no E_2 was present. However, when the competition was done with only 30 nM E_2 (enough to fully saturate the ER, had it been present), the level of binding of ^{191}Pt–diamine complex was not affected. This suggests that what appears to be E_2 specific binding in the presence of 3000 nM E_2 is probably a competition for moderate affinity non-receptor binding sites on other proteins.

Irreversible binding of the ^{191}Pt–diamine complex to proteins. Since Pt(II) is known to interact covalently with macromolecules in biological systems [25], and the HAP assay suggested covalent attachment to cytosol proteins, an experiment was designed to directly measure covalent attachment. Several concentrations of the ^{191}Pt–diamine complex were incubated with cytosol, in the presence or absence of 3000 nM E_2, and then assayed for irreversible protein binding by the covalent attachment assay. At all concentrations, much of the ligand is bound covalently. In fact, the amount of ligand bound covalently as a percent of total ligand bound (both covalent and reversible, measured by charcoal) was inversely related to the concentration of total ligand added to the cytosol: at low concentrations of ^{191}Pt–diamine complex, 100% of the total binding measured by charcoal adsorption was also covalent; this dropped to ~86% at higher concentrations of added ligand. In all cases, 50–70% of the added ligand was bound either covalently or reversibly.

When the ^{191}Pt–diamine complex was added to a solution of 1 mg/ml of bovine serum albumin (BSA), >70% of the added activity again became bound (reversible and covalent) to the protein, but only 57% of this was bound covalently, compared to 91% covalently bound to uterine cytosol at the same concentration. A time course study (Fig. 3) showed that the covalent attachment to the proteins in uterine cytosol proceeds much faster and to a greater extent than that to the pure protein, BSA.

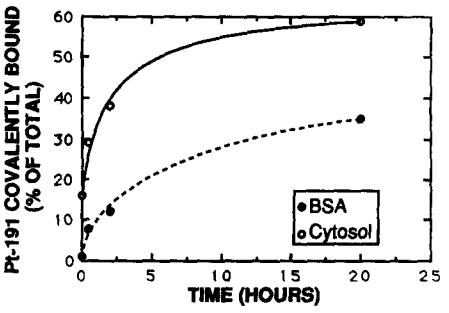

Fig. 3. Covalent attachment of the ^{191}Pt–diamine complex to proteins in rat uterine cytosol and BSA. The ^{191}P–diamine complex (1×10^{-5} M) was incubated with rat uterine cytosol or BSA at 0°C. At the indicated times, the irreversible (covalent) binding to protein was determined by an hydroxylapatite adsorption assay.

Both the attachment and reversible binding to either cytosol or BSA can be compared by measuring the radioactivity in both the HAP assay ethanol extract (reversible) and that which remains with the protein in the pellet. Only ~10% of the radioactivity in the uterine cytosol incubation is released by the ethanol, while in the BSA incubation, 43% is released. The total binding to both proteins is again dependent on the concentration of added ligand suggesting a completely non-specific interaction.

ER exchange assay with the Pt–diamine complex. Since the RBA values for the Pt–diamine complex declined with time (see above, Table 1), suggesting that the binding to ER was not covalent, we assayed the interaction of the unlabeled Pt–diamine complex with ER by an exchange method [16]. Unlabeled Pt–diamine complex with or without 3000 nM E_2, E_2 alone, or no ligand (DMF vehicle only) were incubated with uterine cytosol at 0°C for 2 h. At this point, the cytosol was treated with charcoal-dextran to remove the free ligand, and then incubated with 30 nM [^{3}H]E_2 in the absence or presence of 3000 nM E_2, either at 0°C for 2 h or overnight at room temperature. Incubation for 2 h at 0°C should effect little or no exchange of the ER–ligand complex, allowing the [^{3}H]E_2 to bind only to unfilled receptor, whereas the overnight incubation at 25°C should allow complete exchange. The sample of cytosol which had never been exposed to any ligand during the

Table 2. Extent of ER that undergoes exchange with [^{3}H]E_2 after preincubation with E_2 or the Pt–diamine complex[a]

Preincubation	Percent exchangeable under the conditions below	
	2 h, 0°C	19 h, 25°C
Empty	100%	100%
E_2	12%	116%
Pt–diamine complex	29%	77%

[a]Preincubation involves treatment of ER with buffer, E_2 or Pt–diamine complex for 1 h at 0°C. The exchange assay involves adsorption of free ligand with charcoal-dextran, followed by incubation with [^{3}H]E_2 for the times and at the temperatures indicated. For details, see Materials and Methods.

first incubation and therefore had only empty receptor, filled readily at 0°C with [^{3}H]E_2 and served as the 100% control. The results of this exchange assay are summarized in Table 2.

The sample incubated first with only unlabeled E_2, showed 12% of the sites empty or available to the [^{3}H]E_2 at 0°C, while the sample incubated first with the Pt–diamine complex had 29% of the ER sites available. While one may expect that no sites should be empty when the sample has first been incubated with E_2, about 10–15% of the sites are generally available to the [^{3}H]E_2 at 0°C [16]. (These sites may never have been filled, since the E_2 binding to ER, although >90% complete at 1 h, proceeds slowly to 100%. Alternatively, they may have been filled, and then stripped of ligand by the charcoal, or filled and then undergone exchange at 0°C.) The uterine cytosol sample first incubated with the Pt–diamine complex shows somewhat more empty sites (29%), but certainly *ca* 70% of the ER sites are filled with ligand. (The percentage of sites available to exchange at 0°C is related to the RBA of the ligand; unpublished results).

After overnight exchange at room temperature, 116% of the ER sites in the sample first incubated with E_2 alone had exchanged with the [^{3}H]E_2, compared to 100% in the sample which was originally empty; 77% of the sites in the sample first incubated with Pt–diamine complex were able to exchange with the [^{3}H]E_2. This shows clearly that when the Pt–diamine complex is added to the cytosol preparation, most of the ER sites become filled (71% unexchangeable at 0°C) and that 77% of the ligand in the ER sites is bound *reversibly* (exchangeable at room temperature). The reversible interaction of the Pt–diamine complex with ER is in contrast to its interaction with the proteins in uterine cytosol in general, where the majority of the ^{191}Pt–diamine complex becomes irreversibly (covalently) bound. However, since this exchange experiment must be done with unlabeled Pt–diamine complex (non-specific covalent binding of the ^{191}Pt–diamine complex obscures ER binding measurements, see above), one cannot ascertain in this experiment whether the ER that becomes filled is occupied by the Pt–diamine complex or by some decomposition product (see below).

HPLC analysis of the stability of the Pt–diamine or ^{191}Pt–diamine complex in solution and in cytosol. Assayed by HPLC, the ^{191}Pt–diamine complex coelutes with the unlabeled Pt–diamine complex and is 72% radiochemically pure (Fig. 4, panel A). Recovery from the column is >70%. The most likely degradation products are the diamine and polar complexes of platinum

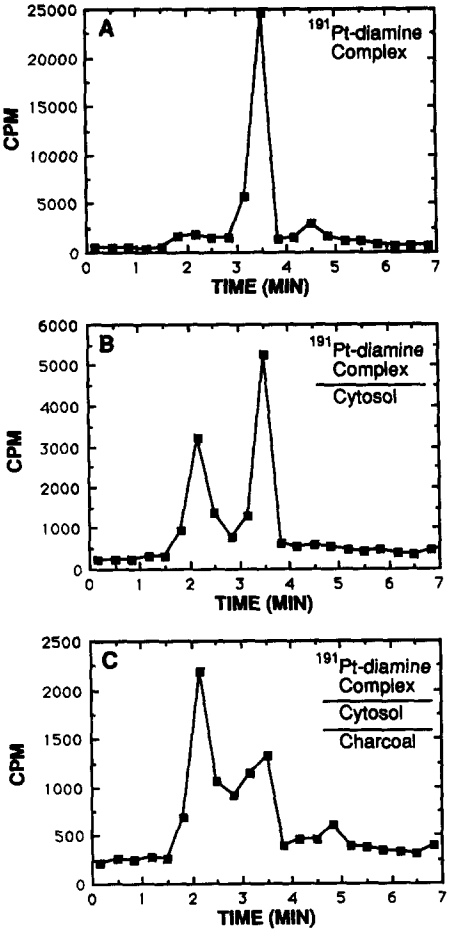

Fig. 4. HPLC analysis of the ^{191}Pt–diamine complex incubated in rat uterine cytosol preparations. All samples were injected onto a Varian MCH-10 10 μm (300 × 4 mm) column and eluted at 1.5 ml/min with an 80:20 mixture of MeOH–[KH_2PO_4 (20 mM)-K_2HPO_4]pH 5. In this system, the Pt–diamine complex elutes at 3.5 min. Samples were collected every 20 s and assayed by liquid scintillation counting. Panel A: HPLC trace of an authentic standard of Pt–diamine complex. Panel B: HPLC trace of ^{191}Pt–diamine complex after incubation in cytosol for 1 h at 0°C. Panel C: HPLC trace of the material in Panel B after treatment with charcoal-dextran.

with low molecular components of the incubation mixture. The diamine elutes later than the Pt–diamine complex, at 5.59 min, but it cannot be seen at these concentrations: it is not radioactive, so it cannot be followed by radioactivity; u.v. assay, which is much less sensitive, precludes detection of the subnanomolar quantities present in the binding assays (see below).

After the ^{191}Pt–diamine complex is incubated with cytosol, only 40% of the ^{191}Pt radioactivity can be recovered by HPLC. This is distributed between an unknown ^{191}Pt-labeled product, that elutes just after the solvent front (2.16 min, 35% of eluted activity), and authentic ^{191}Pt–diamine complex (47% of eluted activity) (Fig. 4, panel B). If the uterine cytosol sample first incubated with the ^{191}Pt–diamine complex is then charcoal treated to remove free ligand, and the protein-bound complex examined by HPLC, only 23% of the radioactivity can be recovered. Of this, 42% is the unknown ^{191}Pt product, and 28% is authentic ^{191}Pt–diamine complex (Fig. 4, panel C). These data again suggest that the ^{191}Pt–diamine complex is binding covalently to the protein, which is then never recovered from the HPLC column. Of the reversibly bound and free ^{191}Pt–diamine complex, only 47% of it is still authentic ligand after 1 h with uterine cytosol. When the sample is treated with charcoal to remove the free ligand, the authentic ^{191}Pt–diamine complex is selectively removed, leaving mostly the ^{191}Pt-labeled unknown material.

The ^{191}Pt–diamine complex in buffer or DMF is completely stable at either 0°C or room temperature for weeks. The percentage of radioactivity recovered from the HPLC column does not change, and no unknown ^{191}Pt material is produced. Since the Pt–diamine complex is likely to bind to proteins through sulfhydryl groups to form S_2–Pt complexes [26], we investigated the effect of sulfhydryl protecting reagents on the ligand. Allowing a mixture of 0.5 M mercaptoethanol and 5×10^{-4} M Pt–diamine (in DMF) to sit for 16 h at 5°C had no effect on the stability of the Pt–diamine complex as assayed by HPLC.

Although stable in solution, it is clear that the Pt–diamine complex is unstable in cytosol incubations. In the incubations with the ^{191}Pt–diamine complex it is not possible to evaluate whether degradation of the complex generates free diamine, since the diamine is not labeled and the concentrations used with the ^{191}Pt-labeled material are below the u.v. detection limit. In order to quantify diamine release through HPLC with u.v. detection, we incubated a large concentration of unlabeled Pt–diamine complex (1×10^{-5} M) with cytosol at 0°C for 1 h. The results are shown in Fig. 5. Under these HPLC conditions, the diamine elutes at 7.3 min [Fig. 5 (B)]. HPLC traces of cytosol and Pt–diamine complexes alone, under the same conditions [Figs 5 (A and C), respectively], give no peak at this retention time. However, the HPLC trace from the sample in which the Pt–diamine complex was incubated with cytosol shows a peak at 7.5 min [Fig. 5(D)]. Thus, under these incubation conditions, *ca* 10% of the unlabeled Pt–diamine complex decomposes to generate diamine. A greater degree of decomposition can be expected when the Pt–diamine

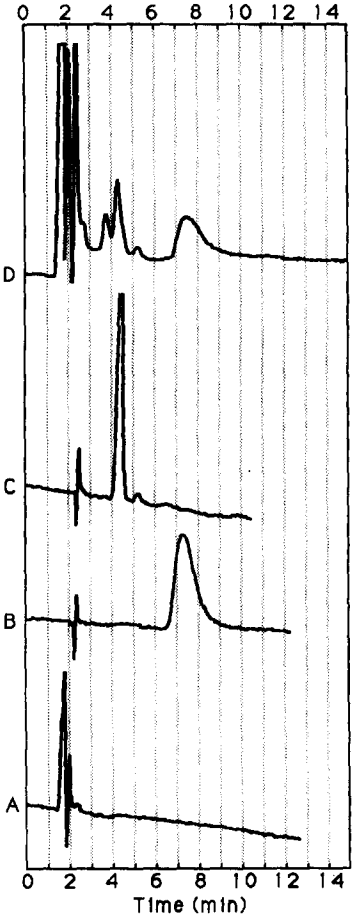

Fig. 5. HPLC analysis of unlabeled Pt–diamine complex incubated in rat uterine cytosol. All samples (in DMF) were injected onto a Varian MCH-10 10 μm (300 × 4 mm) column, eluted at 1.5 ml/min with a 70:30 mixture of MeOH–[KH_2PO_4(20 mM)-K_2HPO_4]pH 5, and detected by u.v. (300 nm). (A) Trace of cytosol alone. (B) Trace of diamine alone. (C) Trace of unlabeled Pt–diamine complex alone. (D) Trace of unlabeled Pt–diamine complex after incubation in cytosol for 1 h at 0°C.

Table 3. Tissue distribution of ^{191}Pt–diamine complex in the immature rat

Tissue	%ID/g[a]		
	1 h	1 h + E_2[b]	3 h
Blood	0.7269 ± 0.1045	0.7695 ± 0.1376	0.5320 ± 0.1058
Liver	11.7780 ± 0.7777	10.6817 ± 4.4475	5.0145 ± 3.3698
Spleen	0.5358 ± 0.0477	0.5186 ± 0.1581	0.3578 ± 0.1207
Kidney	3.3256 ± 0.8188	3.4201 ± 1.1715	1.5889 ± 0.5696
Muscle	0.0971 ± 0.0140	0.0955 ± 0.0352	0.0583 ± 0.0172
Fat	0.1100 ± 0.0378	0.1232 ± 0.0378	0.0765 ± 0.0138
Uterus	0.1846 ± 0.0417	0.2788 ± 0.1891	0.1200 ± 0.0166
Ovaries	0.2088 ± 0.0314	0.2291 ± 0.0870	0.1475 ± 0.0426
Thymus	0.0908 ± 0.0194	0.0816 ± 0.0135	0.0558 ± 0.0283

[a]The data are expressed as percent of the injected dose per gram of tissue (%ID/g). The values are the average ± SD ($n = 5$).
[b]Animals were coinjected with 15 μg of unlabeled E_2.

complex is subjected to binding assay conditions where the concentration of Pt–diamine complex is lower (1×10^{-8} M) and has more of a chance to interact with cytosol proteins.

No attempts were made to characterize the ER–ligand complex, formed in the presence of the ^{191}Pt–diamine complex, by conventional methods such as sucrose gradients or acrylamide gel electrophoresis. This type of characterization is made difficult by the initial low specific activity (4.85 Ci/mmol) and the short 2.96-day half-life of the ^{191}Pt, but mostly by the high non-specific and irreversible protein binding which would obscure any authentic binding to ER.

From the HPLC analysis, we can estimate that there is enough diamine formed to nearly saturate the nM amounts of ER present in cytosol. The diamine would bind rapidly and with reasonable affinity (RBA 53%), and remain a reversibly-binding ligand. Additionally, the ethanol extraction of the HAP assay should be greatly enriched in ligand reversibly bound to the cytosol (the 70–90% which is covalently bound would remain with the pellet). Even this enriched ethanol extract shows no evidence of specific binding of the ^{191}Pt complex to ER. Thus, we propose that a portion of the Pt–diamine complex is being degraded in the cytosol to form diamine and that this diamine itself is the species responsible for the ER competitive binding observed with the Pt–diamine complex. All of this is consistent with the data we have obtained.

In vivo tissue distribution of the ^{191}Pt–diamine complex

Immature female rats were injected i.v. with portions of the ^{191}Pt–diamine complex, and the distribution of radioactivity determined after 1 and 3 h. An additional set of animals was coinjected with a large dose (15 μg) of unlabeled E_2 in order to block the ER sites. The tissue uptake data are presented as percent of injected dose per gram tissue (%ID/g) in Table 3 and as a percent of the injected dose per organ (%ID/organ) in Table 4.

The distribution of the ^{191}Pt–diamine complex is unremarkable. The uptake by the principal estrogen target tissues (uterus and ovaries) is no greater than control tissue (muscle), nor is there evidence of selective depression of target tissue uptake by prior administration of an excess of unlabeled E_2. (Ligands for ER with significant binding affinity are well known to show selective uptake by target tissues and selective displacement by E_2 [11, 27, 29].) The principal site of uptake is the liver (Table 4).

Table 4. Tissue distribution of ^{191}Pt–diamine complex in the immature rat

Tissue	%ID/organ[a]		
	1 h	1 h + E_2[b]	3 h
Blood	2.8908 ± 0.3431	3.0546 ± 0.5229	2.047 ± 0.1611
Liver	29.3753 ± 2.1528	25.5069 ± 9.8690	12.159 ± 8.0214
Spleen	0.1385 ± 0.0229	0.1036 ± 0.0318	0.0942 ± 0.0371
Kidney	1.1628 ± 0.1850	1.1913 ± 0.3426	0.5321 ± 0.1664
Muscle	0.7709 ± 0.0751	0.7551 ± 0.2670	0.4461 ± 0.0842
Fat	0.8636 ± 0.3006	0.9698 ± 0.3120	0.5874 ± 0.1148
Uterus	0.0105 ± 0.0027	0.0144 ± 0.0103	0.0064 ± 0.0010
Ovaries	0.0078 ± 0.0017	0.0082 ± 0.0019	0.0073 ± 0.0011
Thymus	0.0209 ± 0.0055	0.0193 ± 0.0057	0.0133 ± 0.0049

[a]The data are expressed as the percent of the injected dose per organ (%ID/organ). The values are the average ± SD ($n = 5$).
[b]Animals were coinjected with 15 μg of unlabeled E_2.

CONCLUSIONS

We have prepared a non-steroidal estrogen–diamine complex with Pt in radiolabeled form (Pt-191) to investigate the interaction of this species with the ER and its distribution *in vivo*. This complex is reported to show ER-dependent cytostatic activity *in vitro* and *in vivo* [7]. While the unlabeled Pt–diamine complex shows significant apparent binding affinity for ER in competitive binding assays, attempts to demonstrate the binding of the Pt–diamine complex to ER directly using the ^{191}Pt-labeled species gave equivocal results, as non-specific binding is very high and this complex binds irreversibly to proteins (in rat uterine cytosol and to BSA). Furthermore, HPLC analysis indicates that in protein solutions the Pt–diamine complex decomposes, releasing, in part, free diamine, in quantities that could account for the apparent affinity measured for the Pt complex in the competitive binding assay. Furthermore, in tissue distribution studies in immature rats *in vivo*, the ^{191}Pt–diamine complex was deposited largely in the liver and showed no selective and displaceable uptake by estrogen target tissues, a well established characteristic of good ligands for the ER.

Using the metal center as the site of labeling, we have not been able to verify that the Pt–diamine complex is actually binding to the ER in either these *in vitro* or *in vivo* experiments. It is possible that the specific ER binding of the complex could be studied with fewer complications due to the chemically reactive nature of the metal center, by labeling the diamine portion of the complex with the tritium at high specific activity. The chemical nature of the receptor-bound fraction (i.e. whether intact Pt–diamine complex or free diamine) could be ascertained directly by chromatographic analysis of extracts. Without such direct verification of the selective interaction of this Pt–diamine complex with ER, one cannot ascertain that the biological effects observed upon administration of this complex are due to the Pt complex itself or to diamine that is released from this complex. As a model for metal-labeled ligands, as *in vivo* imaging agents for hormone and neurotransmitter receptors, the behavior of this Pt complex demonstrates the importance of achieving chemical and metabolic stability *in vivo* in the design and development of such agents. For diagnostic applications, complexes of technetium-99m and rhenium-186 appear to have higher stability ([8, 9] and references cited therein).

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