Synthesis and characterization of a new bile acid and platinum(II) complex with cytostatic activity

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Abstract With a view to using bile acids as shuttles for delivering platinum-related cytostatic drugs to the liver, a cholylglycine(CG)-derivative of platinum(II) has been synthesized. The complex, named Bamet-H2, was characterized by elemental analysis, FT-IR, NMR, FAB-MS, and UV spectroscopy. The results indicate the following composition: $C_{52}H_{84}N_2O_{12}ClNa$ Pt(II). Conductivity data suggest that the complex behaves as a sodium salt (1:1) of a complex of Pt(II) bound to one Cl^-, one bidentate CG moiety, and another monodentate CG moiety, i.e., Na[Pt(CG-*O*,*N*)(CG-*O*)Cl]. The compound is highly soluble (up to 10 mM) in water, ethanol, methanol, DMF, and DMSO. Bamet-H2 was stable in solution (either water or 150 mM NaCl solution), as measured by HPLC, up to 24 h. At this time, more than 90% of the platinum present in water or saline solutions was found to be Bamet-H2. Cytostatic activity against L1210 murine leukemia cells was found. This characteristic was stronger against rat hepatocytes in primary culture. Isolated in situ rat livers were perfused for 40 min with a recirculating medium containing 1 μM Barnet-H2, CG, or cisplatin. Uptake and excretion into bile were much greater for Bamet-H2 than for cisplatin, but less than for CG. Liver content was higher for Bamet-H2 than for cisplatin or CG. The results point to the potential usefulness of Bamet-H2 in the antitumoral therapy of neoplasias derived from liver parenchymal cells.—**Criado, J. J., M. C. Herrera, M. F. Palomero, M. Medarde, E. Rodriguez, and J. J. G. Marin.** Synthesis and characterization of a new bile acid and platinum(II) complex with cytostatic activity. *J. Lipid Res.* 1997. **38:** 1022–1032.

Supplementary key words cholylglycinate • glycocholic acid • metal • cancer • antitumor

Over the last decades considerable efforts have been devoted to the search for new therapeutic agents with antitumoral activity. There have been important contributions to the field of anticancer chemotherapy but no complete successes have been achieved. This has been mainly due to both the pre-existence or the development of resistance of cancer cells to the cytostatic agents assayed and the appearance of important side effects of most current drugs, deriving from their toxicity towards extratumoral tissues. Several strategies have been envisaged to either circumvent resistance to such drugs (1) or to improve their vectoriality towards tumors (2). In this sense, the marked organotropism of bile acids towards the hepatobiliary system has been proposed as an interesting characteristic in the use of these endogenous compounds or their analogs as shuttles for drugs towards the liver. Examples of these are inhibitors of hydroxymethyl glutaryl CoA (HMG-CoA) reductase (3) or the cytostatic compound chlorambucil (4).

Since their introduction into clinical trials in 1972, the widespread success of *cis*-diamminedichloroplatinum(II) (cisplatin) and its analogs in the treatment of a variety of solid tumors (5) has encouraged the search for new cisplatin derivatives with a view to improving the therapeutic index of the compound, which is reduced due to dose-limiting toxicity, i.e., nephrotoxicity, myelotoxycity, neurotoxicity, nausea, and vomiting. Although many different cisplatin analogs have been synthesized, few of them are currently used in clinical practice (6). This is probably because the side effects are only partially palliated and this situation is often accompanied by a loss of tumoricidal activity. Two interesting second generation cisplatin analogs are *cis*-diammine(1,1-cyclobutanedicarboxylate)-platinum(II) (carboplatin) and 1,2-cyclohexanediammine-platinum(II) (DACH-Pt) and their derivatives. These are not organotropic compounds but changes in the physical–chemical properties of the resulting molecule do facilitate their excretion. They are usually less nephrotoxic than cisplatin although other side effects, such as myelotoxicity or neurotoxicity, have been reported to be similar or even greater than those of their parent drug (7). Owing to the amphipathic properties of these

Abbreviations: CG, cholylglycinate; CMC, critical micellar concentration.

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steroids (8), previous works have aimed at synthesizing DACH-Pt complexes with bile acids in order to obtain derivatives with both lipophilicity and water miscibility (9, 10). However, although promising results have been obtained as regards the antitumoral activity of the complexes, no further investigation in this direction has been carried out. The rationale of the present work was that platinum(II)-containing bile acid derivatives with molecular structures more similar to those of natural bile acids than DACH-Pt–bile acid complexes might be recognized by transmembrane bile acid carrier proteins and hence be efficiently taken up by hepatocytes. The latter cells are responsible for selective bile acid clearance from the systematic blood, where these compounds are maintained at very low concentrations (11). If the above hypothesis were correct, this family of complexes, henceforth designated as Bamet (from Ba-indicating bile acid, and -met, indicating metal), could be envisaged as an interesting strategy for targeting Pt-related cytostatic drugs toward the hepatobiliary system. To check this hypothesis, a cholylglycine derivative of platinum(II) has been synthesized and characterized as described in this paper. Preliminary results on biodistribution and antitumoral activity of this compound, named Bamet-H2, have been described elsewhere (12, 13).

MATERIALS AND METHODS

Chemicals

Sodium tetrachloroplatinate(II) tetrahydrate, $Na_2PtCl_4 \cdot 4H_2O$, was purchased from Fluka Quimica (Madrid, Spain). Sodium cholylglycinate (NaCG, more than 95% pure by thin-layer chromatography) and 3α-hydroxysteroid dehydrogenase were obtained from Sigma Quimica (Madrid, Spain). ^{14}C radiolabeled NaCG was from New England Nuclear (Itisa, Madrid, Spain). All other reagents were of high purity and were used as purchased without any further purification.

Synthesis

The platinum complex named Bamet-H2, Na[Pt (CG-*O,N*) (CG-*O*) Cl], was obtained through the following procedure: an 8 mM $Na_2PtCl_4 \cdot 4H_2O$ solution in water (125 ml) was prepared at room temperature. This was filtered onto paper before adding sodium cholylglycinate, NaCG, which was previously dissolved in water (8 mM, 125 ml). To prevent physicochemical effects due to the presence of bile acid micelles in the reaction mixture, attempts were made to keep the concentrations of free NaCG in the reaction mixture below the critical micellar concentration (CMC) for this bile acid. Therefore, the NaCG solution was added dropwise (0.1 ml/min) to the continuously stirred $Na_2PtCl_4 \cdot 4H_2O$ solution by means of peristaltic pump over about 16 h. The mixture was allowed to react at pH 5.4 under continuous stirring in the dark at room temperature for an additional 8 h. The final product was then evaporated to dryness in a desiccator containing P_4O_{10}, resulting in a grayish-brown solid.

Purification

Reaction products were separated from the excess of unreacted platinum by solid–liquid extraction in octadecylsilane cartridges (C18, Sep-Pak, Waters Cromatografia SA, Madrid, Spain) following a classic procedure (14). The retained compounds were recovered from the cartridges in methanol. The extract was then concentrated for thin-layer chromatography (TLC) on silica gel plates (60 F254, Merck, Darmstadt, Germany) using butyl acetate–methanol 65:35 (v/v) as the solvent system. Two major bands, one corresponding to unreacted CG (R_f = 0.07) and the other corresponding to the complex named Bamet-H2 (R_f = 0.14), were obtained. The latter was scraped off and extracted with methanol. The resulting solution was further purified by semipreparative high performance liquid chromatography (HPLC) in reverse phase using a Waters C18 RCM column (5 μm, 10 mm × 25 cm, Waters, Madrid, Spain) with a gradient pump module (Model 126, Beckman, Madrid, Spain) and a Photo-Diode-Array detector (Model 168, Beckman) set at 250 nm. The system was controlled by an IBM computer (Model 30286, IBM Corp., Portsmouth, UK) using System Gold software from Beckman. The column was equilibrated with 10 mM KH_2PO_4–methanol 25:75 (v/v), pH 7.02, and eluted with a linear gradient from 0% to 80% methanol in 15 min. The solvent rate was 10 ml/min. In this HPLC system, the retention time for CG was 4.8 min and that for Bamet-H2 was 10.1 min. In the semipreparative procedures, 0.5-min fractions were collected automatically. Those corresponding to the Bamet-H2 elution time, considering the time lapse between the detector and the fraction collector, were pooled together and dried. The resulting substance was desalted by methanolic extraction in C18 cartridges and dried again. With this procedure, highly pure (more than 95%) Bamet-H2 was obtained as a reddish-brown solid at a yield of approximately 2% of the starting materials. The results of purity control by HPLC carried out at the end of the purification procedure are shown in **Fig. 1.**

Analytical methods

3α-Hydroxy steroid concentrations were measured by the classic method of Talalay (15). Chemical analyses

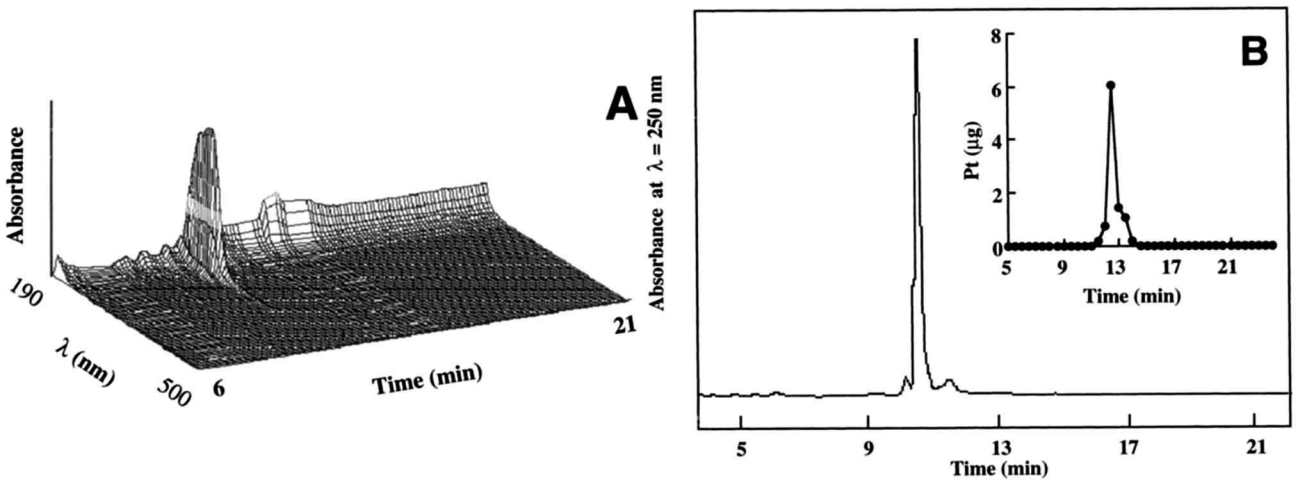

Fig. 1. Reverse phase high performance chromatography (HPLC) of purified Bamet-H2. A) HPLC chromatogram of pure Bamet-H2 as recorded using a photodiode array detector in scan mode from 190 to 500 nm wavelength. B) HPLC chromatogram recorded at 250 nm wavelength. The inset shows the results of flameless atomic absorption atomic spectroscopic measurement of the platinum content in samples collected every 0.5 min during HPLC chromatography of pure Bamet-H2. The difference between both chromatograms corresponds to the time lapse from the detector to the fraction collector.

for C, H, and N were performed on a Perkin-Elmer 2400 elemental analyzer (Perkin-Elmer, Hispania SA, Madrid, Spain). Platinum was determined by atomic absorption in a flameless graphite furnace spectrophotometer (Z-8100 Hitachi, Tokyo, Japan) set at a wavelength of 265.9 nm and using the following temperature program: 90°C (20 s), 100°C (20 s), 800°C (20 s), 1600°C (30 s), 2800°C (5 s), and 3000°C (4 s). Infrared (IR) spectra were recorded in the 4000-200 cm^{-1} range on a Perkin-Elmer FT-IR 17300 instrument coupled to a Perkin-Elmer 3600 Data Station. KBr pellets and spectrophotometric grade Nujol (Fluka Quimica) or polyethylene (Aldrich, Madrid, Spain) discs were used to record spectra above and below 400 cm^{-1}, respectively. Mass spectrometry studies were carried out on a VG-Autospec (Mass Spectrometry Service, Universidad Autónoma, Madrid, Spain), using L-SIMS ionization in the FAB^+ mode (Cs ion emission) and m-NBA as matrix. Electrical conductivity in solution was measured using a CDM2e conductimeter (Radiometer, Copenhagen, Denmark), with a CDC104 immersion cell. Temperature was controlled with a Unitherm water bath (Selecta, Barcelona, Spain) with a precision of ±0.01°C. Electronic spectra were recorded on a Shimadzu UV-2101S (Izasa SA, Barcelona, Spain) coupled to a CPS temperature controller. Nuclear magnetic resonance (NMR) profiles of ^1H (400 MHz) and ^{13}C (102.6 MHz) were obtained in CD_3OD on a Bruker DX400 instrument (Karlsruhe, Germany). The ^{195}Pt NMR spectrum was obtained in D_2O on a Varian (Melbourne, Australia) Unity-300 (64.5 MHz) using K_2PtCl_6 ($\delta = 0$ ppm) as internal standard. Measurement conditions were: 485.000 scans, 2 s relaxation delay, and 48 h acquisition time.

In vitro cytostatic studies

The complex was evaluated for in vitro cytostatic activity against both murine leukemia L1210 cells obtained from the American Type Culture Collection (ATCC, Rockville, MD) and rat hepatocytes in primary culture obtained by an adaptation of the two-step collagenase perfusion method (16). Cells were grown in a humidified atmosphere of 95% air: 5% CO_2 at 37°C using Dulbecco's modified Eagle's medium (Sigma) supplemented with 2 mM glutamine, 26.1 mM $NaHCO_3$ 25 mM HEPES, and 10% horse serum for the culture of L1210 cells and serum-free Williams' Medium E (Sigma) supplemented with 26.1 mM $NaHCO_3$, 25 mM HEPES, 100 nM Na_2SeO_3, 30 nM dexamethasone, 100 nM insulin, 5 nM EGF, 11.1 mM galactose, 1 μM ethanolamine, 5 μg/ml transferrin, 5 μg/ml linoleic acid, 5 μg/ml albumin, 10 mM nicotinamide, and 6 mM ornithine for the primary culture of rat hepatocytes. Based on preliminary studies carried out to investigate the time course of the growth of these particular cell types, the following protocols were followed for testing purposes. L1210 cells were diluted 1/20 from cultures during the exponential growth phase and exposed to the tested compound for 72 h. Hepatocytes were allowed to become attached to the dish and start proliferation before the compounds were added to the culture at 24 h. Liver cells were exposed to the drug up to 72 h of culture. Both types of cells were then separated from the culture medium and cell debris, washed, and di-

gested in Lowry solution (100 mM NaOH and 189 mM Na_2CO_3), after which cellular DNA contents were measured fluorimetrically using Hoechst-33258 (17). Trypan blue test was carried out to check that viability of cells that were used to measure DNA was approximately 100%.

Rat liver perfusion

Non-fasting male Wistar CF rats (approximately 250 g) (Faculty of Pharmacy, Salamanca, Spain) were used as donors. They received humane care as outlined in "Guide for the Care and Use of Laboratory Animals" (NIH Publication no. 80-23, revised 1985). The livers were perfused in situ by a modification (18) of the method of Miller *et al.* (19) in a prewarmed (38°C) cabinet. The recirculating perfusion medium was 150 ml of a modified Krebs-Henseleit solution containing the following in mM: 120 NaCl, 5 KCl, 1.29 $CaCl_2$ 0.65 $MgSO_4$, 25 $NaHCO_3$, 1.17 KH_2PO_4, 5.0 D-glucose, and gentamicin sulfate (100 mg/l). The medium was oxygenated with a mixture of 5% CO_2–95% O_2. Perfused rat liver viability was confirmed by measuring bile flow, oxygen uptake, perfusion pressure, perfusion flow, potassium and lactate dehydrogenase activity released into the perfusate during the experiments, as previously reported (18). Values were similar to those previously obtained in our laboratory for this preparation (18) and were not significantly modified during the experimental period (40 min) (data not shown). Bamet-H2, cisplatin, or [^{14}C]CG was added at the reservoir to reach 1 μM in the medium at the beginning of the perfusion period. The amount of Bamet-H2 or cisplatin in stock solution, perfusate, bile and liver samples was measured by atomic absorption. The amount of ^{14}C-radioactivity present in similar samples collected in experiments carried out using [^{14}C]CG was determined by liquid scintillation counting.

Statistical analysis

Unless otherwise stated, results are expressed as means ± SE. To calculate the statistical significance of differences among the groups, the Bonferroni method of multiple-range testing was used. Analysis of kinetic data was performed using SIMFIT software (Bardsley, 1992, SIMFIT package release 3.2. Department of Obstetrics and Gynaecology at St. Mary's Hospital, University of Manchester, U.K.). Statistical analyses were performed on a Macintosh LC-III computer (Apple Computer, Inc., Cupertino, CA) with programs supplied by Apple Computer, Inc.

RESULTS AND DISCUSSION

Chemical characterization

Elemental analysis of the complex revealed the following results (in %) for $C_{52}H_{84}N_2O_{12}ClNa$ Pt corresponding to the proposed structure shown in **Fig. 2:** C 52.07; H 7.15; N 2.19; Pt 16.08. The calculated values were C 52.81; H 7.24; N 2.36; Pt 16.49, indicating a marked similarity between the results expected and those actually obtained.

The stoichiometry of the bile acid moieties and platinum atoms was assessed in a preliminary step by enzymatically measuring the concentration of 3α-hydroxysteroid groups (3α-HS) in a pure solution of Bamet-H2, using the 3α-hydroxysteroid dehydrogenase assay. Platinum concentrations were measured in the same solution by flameless atomic absorption. The results indicated a 3α-hydroxyl bile acids-to-platinum ratio of 2:1.

The melting point of the substance with decomposition was found to be 185°C. Its solubility in water was high (up to 10 mM). The compound was also soluble in ethanol, methanol, DMF and DMSO. Its stability in solution (either water or 150 mM NaCl saline) as measured by HPLC analysis indicated that Bamet-H2 was stable up to 24 h. At this time, more than 90% of the platinum present in the water or saline solutions was found to be Bamet-H2.

The complex was characterized by a combination of spectroscopic techniques, which allowed us to propose its structure in the absence of X-ray diffraction studies. The latter were not carried out because it was not possible to crystallize the compound under any of a large list of assay conditions.

The IR spectrum of the compound displayed significant degrees of absorption of the bonds involved in the formation of the complex. The main differences with

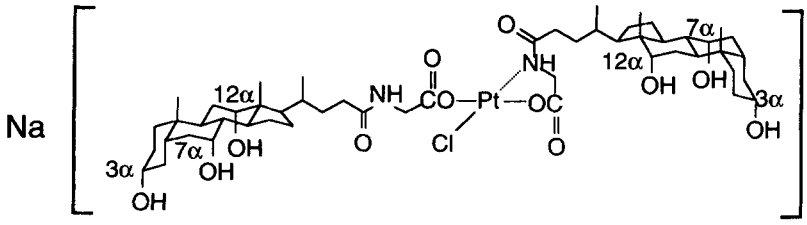

Fig. 2. Schematic representation of proposed structure for the Na[Pt(CG-*O,N*) (CG-*O*) Cl] complex named Bamet-H2.

TABLE 1. ^{13}C and ^{1}H NMR assignments of sodium cholylglycinate (NaCG) and Bamet-H2 in CD_3OD

		Bamet-H2				Bamet-H2	
C	NaCG	L′	L	H	NaCG	L′	L
1	36.5	36.5	36.5	18	0.71 s	0.71 s	0.73 s
2	31.3	31.2	31.2				
3	72.9	72.9	73.0	19	0.92 s	0.91 s	0.91 s
4	40.5	40.5	40.5				
5	43.0	43.0	43.0	21	1.03 d	1.03 d	1.10 d
6	35.9	36.0	36.0				
7	69.1	69.1	69.2	Other	1.30–2.35 m	1.25–2.48 m	
8	41.1	41.1	41.2				
9	27.7	27.7	27.7	3	3.38 m	3.36 m	3.36 m
10	35.9	35.9	35.9				
11	29.6	29.6	29.6	25	3.74 s	3.74 s	3.99; 4.15
12	74.0	74.0	74.2				AB (J = 19)
13	47.5	47.5	47.7				
14	43.2	43.3	43.3	7	3.80 bs	3.65 bs	3.78 bs
15	24.2	24.2	24.4				
16	28.7	28.7	28.9	12	3.95 bs	3.95 bs	3.99 bs
17	48.1	48.1	48.8				
18	13.0	13.0	13.2				
19	23.2	23.2	23.2				
20	37.0	37.0	37.4				
21	17.6	17.7	18.3				
22	34.2	34.7	35.3				
23	33.1	33.1	34.2				
24	176.3	178.9	190.1				
25	44.6	44.6	53.6				
26	176.5	181.1	212.7				

L, bidentate cholylglycinate; L′, monodentate cholylglycinate.

the ligand lay in the glycinate residue, as this contained the carboxylate and amide donor groups. In the free ligand (NaCG) a broad distorted band with a shoulder (1640 cm^{-1}), a peak (1603 cm^{-1}) and another shoulder (1543 cm^{-1}) were observed, whereas in the complex the first shoulder was shifted to higher wavelength values (1730 cm^{-1}); the peak appeared at 1648 cm^{-1}, and the second shoulder was a very well-defined band at 1558 cm^{-1}. These absorptions were assigned to the υ CONH-amide-I, υ_{as} COO^- and υ CONH-amide-II stretching vibrations (20, 21). A band at 322 cm^{-1} was also observed, this was unequivocally assigned to the stretching vibration mode of the Pt-Cl bond (22). These data suggest the existence of a dual behavior for the cholylglycinates; they would act both as monodentate and as bidentate ligands to Pt(II) (21, 23, 24).

The conclusions deduced from the IR spectrum were confirmed by detailed study of ^{1}H, ^{13}C, and ^{195}Pt NMR. **Table 1** compares the ^{1}H and ^{13}C data of the free ligand and the proposed monodentate (L′) and bidentate (L) CG moieties of the complex. In both spectra a duplicate set of signals is observed, consistent with the existence of two types of cholylglycinate ligand in the complex. In the ^{1}H NMR spectrum, the methyl groups 18 and 21 and the protons geminal to the OH groups at 7 and 12 are differentiated. Specially significant is the presence of two signals of the glycinate methylene, one of them appearing as a singlet with the same chemical shift of the free ligand (3.73 ppm) and the other as an AB system (3.99 and 4.15 ppm, J = 19 Hz). This difference is a consequence of the complexation, because in the monodentate ligand the glycinate methylene maintains the same situation as that observed in the free ligand, while in the bidentate ligand the methylene loses the free rotation, thus placing both protons in different environments (25, 26).

In the ^{13}C NMR spectrum, 43 signals are observed, 9 of them of significantly high intensity, thus completing the signals for the 52 carbon atoms. The chemical shifts are nearly identical in the case of carbon atoms distant from the metal (differences < 0.12 ppm), the differences increasing as the carbon atoms become closer to the coordination center and being larger in carbon atoms C15–C18 and C20–C26. A major difference is also observed for the glycine methylene, which is shifted from 44.59 ppm in the monodentate ligand to 53.63 ppm in the bidentate ligand (22, 27).

Study of ^{195}Pt NMR revealed the presence of two signals for the metal at −1296 and −1386 ppm; these were not expected for a single complex. Because the spectrum was obtained from a D_2O solution, substitution of the chloride by a D_2O molecule during the long time required for the spectrum to be obtained accounts for these two signals separated by 90 ppm. This explanation

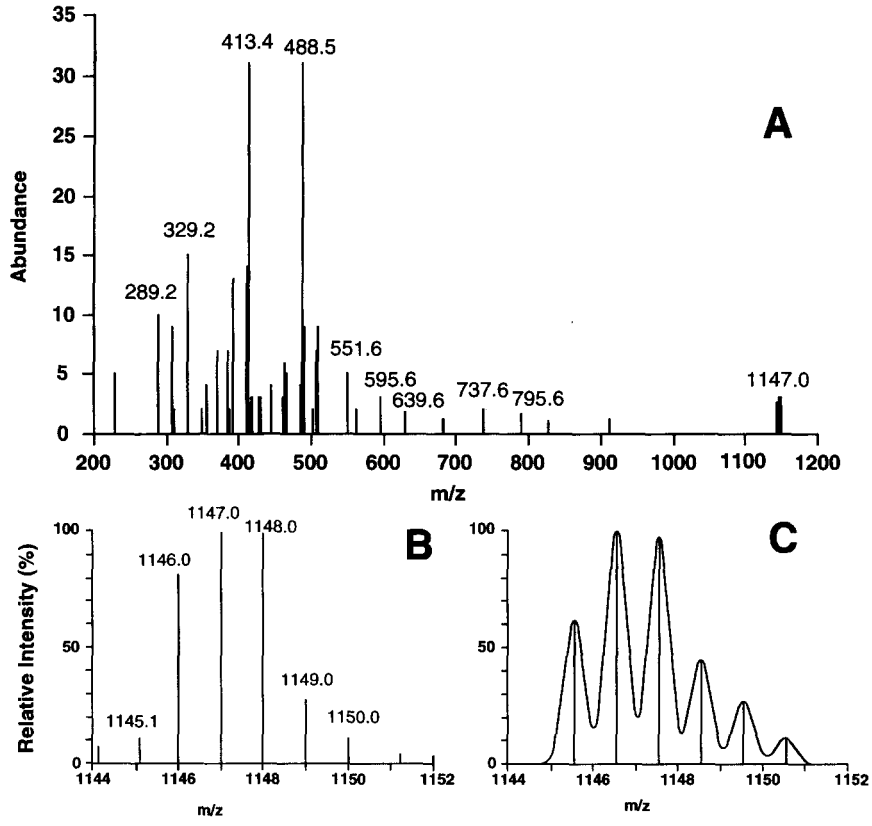

Fig. 3. A) FAB$^+$/MS spectrum of a freshly prepared sample of Bamet-H2. B) Zoom of this spectrum in the m/z region corresponding to the $[M-Cl]^+$ ion. C) Calculated isotopic mass distribution for the $[M-Cl]^+$ ion.

was confirmed in the MS study (with the FAB$^+$/MS technique) of the complex and of a sample of the complex recovered from the D_2O NMR solution (25, 28). All these NMR data are in agreement with the structure proposed for the complex and are similar to those described by Kortes et al. (29) for other Pt(II) complexes with related glycinate moieties.

The MS spectrum (**Fig. 3**) for a freshly prepared sample of the complex showed sets of signals due to the isotopes of the Pt atom, coinciding with the $[M-Cl]^+$ ion at m/z = 1147.0, obtained experimentally (calculated 1147.3), and another at m/z = 1124.8, assigned to the $[Pt(CG)_2]^+$ ion (calculated 1124.3). The loss of a chloride ion is usual for similar species described in the literature (26, 30). In the case of the sample from the D_2O solution (2 days), a new set of peaks was observed at m/z = 1167.8, assigned to a $[M-Cl + D_2O]^+$ ion (calculated 1167.5) and another set at m/z = 1183.5, assigned to a $[M + 1]^+$ ion (calculated 1183.1). Finally, a third sample obtained after a kinetic study, carried out for 12 days in D_2O, had the peak at m/z = 1168 as the 100% peak, thus confirming complete substitution of the chloride ligand by a molecule of the solvent. Owing to the existence of six different isotopic masses for the metal (190, 192, 194, 195, 196, and 198), it was possible to recognize the presence of the metal in the fragmentation peaks of the MS. In order to compare the spectra obtained from these three samples, a common peak belonging to the species $C_{52}H_{84}N_2O_{12}$NaPt (experimental m/z = 1147.0; calculated m/z = 1147.3) was chosen. Comparison of the observed isotopic distribution with that previously calculated revealed considerable consistence between them (Figs. 3B and 3C).

Other peaks in the MS corresponding to the cholylglycinate residue and its fragmentations (31, 32) appeared at m/z = 510 the $[NaCG + Na]^+$ and at m/z = 488 in the case of the $[NaCG]^+$ ions. The usual fragmentation peaks of the cholylglycinate at m/z = 413, 289, 212, and 176 were also found.

All these spectroscopic data support a square-planar structure for the complex, carrying a bidentate and a monodentate cholylglycinate residue and a chloride, with a sodium ion as a counterion, as depicted in Fig. 2. The *trans* stereochemistry between the Cl and the N atoms is that usually observed when a nitrogen ligand and chloride are present in a Pt(II) complex.

Molar conductivity measurements were carried out in

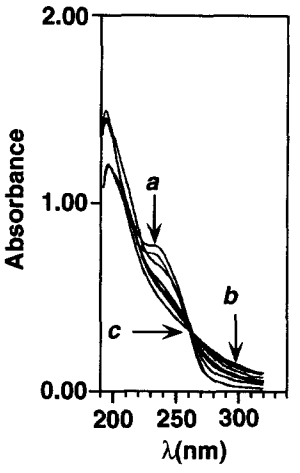

Fig. 4. Absorption spectra of the complex in aqueous solution over 350 h. Arrows indicate first (a) and final (b) spectrum, as well as the presence of an isosbestic point at 260 nm (c).

water and methanol at 25°C using 10^{-3} M Bamet-H2. The values obtained in these solvents were 173 and 134 Ω^{-1} cm^2 mol^{-1}, respectively, in agreement with an 1:1 electrolyte ratio. This allowed us to propose a first step in the dissociation of Bamet-H2 sodium salt in the sodium ion and the platinum complex anion, as is presented in equation 1:

$$Na[PtLL'Cl] \rightarrow Na^+ + [PtLL'Cl]^- \quad \textit{Eq. 1)}$$

$$[PtLL'Cl]^- + H_2O \rightarrow [PtLL'(H_2O)] + Cl^- \quad \textit{Eq. 2)}$$

$$[PtLL'(H_2O)] + H_2O \rightarrow [PtL(H_2O)_2]^+ + (L')^- \quad \textit{Eq. 3)}$$

Where L is bidentate cholylglycinate and L′ is monodentate cholylglycinate.

In the second step, the compound also behaves as an 1:1 electrolyte (equation 2) and its conductivity in water only shows a strong increase (434 Ω^{-1} cm^2 mol^{-1}) after 300 h. This must be due to the displacement of other ligands of the coordination sphere by solvent molecules (equation 3).

As reported above, the FAB$^+$ spectrum of the compound isolated from a D_2O solution showed peaks corresponding to $[PtLL'Cl]^-$ and $[PtLL'(D_2O)]$ species, in agreement with the process proposed for the dissociation of the complex. Furthermore, the ^{195}Pt NMR spectrum in the same solvent also showed two peaks corresponding to these species.

The reaction kinetics were followed by UV spectroscopy. As depicted in **Fig. 4,** the aqueous solution of the compound shows a maximum at 196 nm (long ϵ = 4.15), with a shoulder at 235 nm (log ϵ = 3.85). This shoulder, which can be assigned to the charge transfer of the chloride bonded to the metal (33), decreases with time (**Fig. 5A**) and, simultaneously, the absorption band at 300 nm increases (Fig. 5B) due to the products formed in the dissociation process. For the analysis (34–36) of the displacement of chloride by water (equation 2), the variation in absorbance at 235 and 245 nm over time was plotted, in both cases affording a decay curve (Fig. 5A). This was adjusted to a first order rate equation $A = A_0 \cdot e^{-kt} + C$ where A is absorbance at time t, A_0 is absorbance at zero time, k is the rate constant (h^{-1}) and C term is a constant term introduced to take into account the baseline variations and the absorption of other non-reacting species. For these two wavelengths, the adjustments are satisfactory and the rate constants (±SD) obtained were $2.6 \pm 0.1 \times 10^{-2}$ at 235 nm and $2.8 \pm 0.1 \times 10^{-2}$ at 245 nm. In both cases, the need for a constant C was statistically significant, and if this background term is subtracted in all the absorbance values, the plot shown in Fig. 5C is obtained; this is also well adjusted to a first order rate equation $A = A_0 \cdot e^{-kt}$, with a k value quite similar to those reported above. This kinetic behavior is also seen in the semi-logarithmic plot (Fig. 5D), where the experimental values seem to fit a straight line, as predicted for this reaction order. However, after some time has elapsed, the values deviated from linearity. Therefore, only at times less than 88 h does the reaction seems to follow first-order kinetics. This was probably due to propagation of the logarithmic error of low absorbance values or to the appearance of other reaction products such as those derived from equation 3 or later reaction steps. This notion would be consistent with the increase in the conductivity of these solutions.

Finally, Fig. 5B shows the variation in absorbance at 300 nm with time, corresponding to an increasing curve that fits in well with a first order equation for product release: $A = B(1 - e^{-kt}) + C$, where B represents absorbance at infinite time. In this case, the value obtained for k is $19 \pm 0.2 \times 10^{-2}$, very close to those obtained for the above wavelengths.

After 2 weeks, the kinetic process was concluded and a solution of sodium chloride was added to mimic the concentration in human plasma (150 mM). However, in the UV spectrum the above described shoulder was not observed. This points to the non-reversibility of the process represented in equation 2. The control UV spectrum of a freshly prepared solution of the complex in 150 mM aqueous sodium chloride did not differ appreciably from that found using an aqueous solution of the complex, although absorption at 190–200 nm was more intense due to the higher chloride concentration in the solution. The presence of the shoulder produced by the absorbance of the platinum complex in the UV spectrum of this solution, with no change over time, can

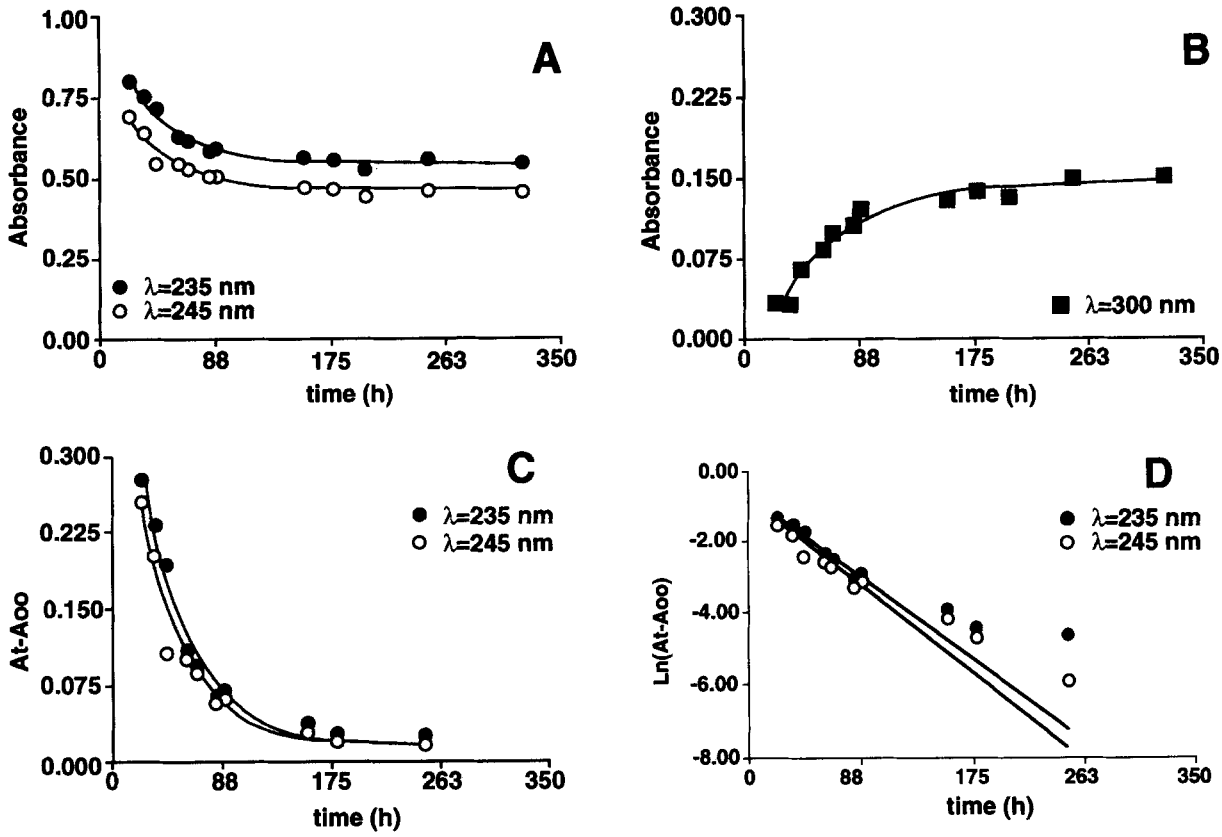

Fig. 5. Kinetics of chloride substitution by acquo ions in Bamet-H2 at 37°C as measured by the time course of the change in absorbance recorded at the following wavelengths: A) 235 nm (closed circles), 245 nm (open circles) and B) 300 nm (closed squares). C) Absorbance results obtained at 235 and 245 nm wavelength as corrected by a constant term (absorbance at infinite time). D) Semilogarithmic plot of the data shown in figure C.

be explained by the persistence of the chloride bound to the metal at plasma concentrations of sodium chloride.

A 10-fold dilution of the saline solution of the complex made up to reach the intracellular concentration revealed decay in the absorbance of the shoulder at 235 nm, very close to that observed for the spectrum of the complex with the chloride ligand substituted by an acquo ligand. This decay would be produced by the same kinetic process discussed previously, which takes into account the disappearance of the characteristic shoulder in the UV spectrum. If saturated sodium chloride is added to the diluted solution, to reach 500 mM NaCl, the spectrum also lacks the shoulder at 235 nm. Accordingly, substitution of the chloride by the acquo ligand in the coordination sphere at these concentrations of complex and chloride ion must be permanent. Even in saturated sodium chloride there is no significant absorbance of this shoulder. These kinetic data are of great interest in the interpretation of the biological activity of this type of complex, because, as is well known, the acquo ligands play a fundamental role in the interaction of different donor groups with the bases of the nucleotides conforming the DNA molecule (37).

Cytostatic activity

We have previously reported from in vivo studies (38) that, unlike the corresponding taurine analog, NaCG, as well as other different glycine-conjugated bile acids, does not seem to be able to affect the rate of liver cell proliferation. The present work confirms this point in rat hepatocytes in primary cultures (**Fig. 6**). Similar results were obtained with L1210 cells. Cisplatin, which presumably enters cells through a nonspecific pathway (probably by diffusion through the plasma membrane), was found to have strong cytostatic activity against both cell types, and culture growth, as measured by the amount of DNA in the plates at the end of the experimental period (72 h), was reduced. Sensitivity to cisplatin was slightly higher for L1210 cells than for rat hepatocytes. Thus, cisplatin-induced inhibitions of culture growth were 90% and 76% as compared with un-

treated control L1210 cells and hepatocytes, respectively. Conversely, hepatocytes were markedly more sensitive to Bamet-H2 (67% inhibition) than L1210 (34% inhibition). This difference was not surprising because hepatocytes, but not L1210 cells, were expected to be able to take up Bamet-H2 more efficiently than cisplatin (if this bile acid-derived compound is indeed carried through the plasma membrane by the specific carrier proteins responsible for liver bile acid uptake (11)) owing to the presence of two bile acid-like moieties in the molecule.

Liver uptake and excretion

The results obtained in experiments on the ability of liver to take up Bamet-H2 indicated that the amount of this complex that was removed from the perfusate after 40 min of perfusion was 68% of that taken up in the case of perfusions using NaCG (**Fig. 7**). On the other hand Bamet-H2 uptake was 203% as compared with that of cisplatin. The difference between Bamet-H2 and NaCG was more marked regarding their output into bile. Bamet-H2 output was only 45% of that found for NaCG. However, Bamet-H2 output was significantly higher (321%) than that of cisplatin. These findings are consistent with the observation that Bamet-H2 liver content was higher than that of either NaCG or cisplatin. Although further investigations are needed in order to evaluate the pharmacokinetic characteristics of Bamet-H2, and hence its potential pharmacological interest, these results, confirming preliminary data (12, 13), suggest the existence of a markedly enhanced hepatobiliary organotropism of Bamet-H2 as compared with the parent cytostatic drug, cisplatin. However, this characteristic is not as strong as for the parent bile acid NaCG Differences between Bamet-H2 and NaCG seem to be more pronounced in the exit from the hepatocytes into bile than in the uptake at the sinusoidal side of the parenchymal liver cells.

In summary, these results indicate that Bamet-H2 is a platinated bile acid derivative with a molecular weight of 1182.68 and a planar structure in the platinum(II) molecular area. One cholylglycinate group is proposed to be bidentately bound to two of the corners of the square in the *cis* position. This is assumed to be important for the action of platinum against DNA replication. An additional cholylglycinate group and Cl^- ion occupy the other two corners of the square as monodentate ligands of the platinum(II) atom and presumably act as leaving groups once inside the cells. The resulting molecule is able to strongly inhibit cell culture growth. This effect is more pronounced in cells, such as hepatocytes, with efficient bile acid carrier systems in their plasma membrane. Our results also point to the existence of an enhanced vectoriality of this compound toward the hepatobiliary system. Taken together these findings

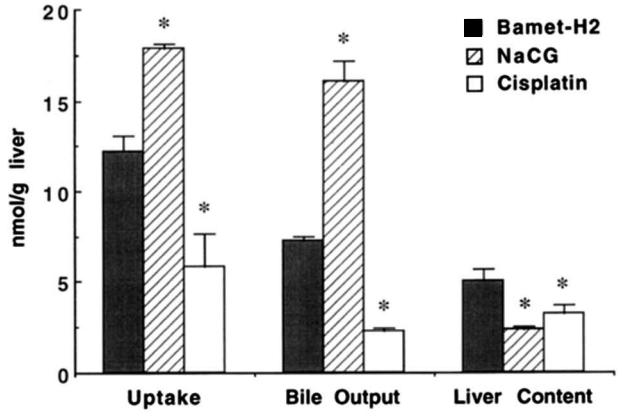

Fig. 7. Total liver uptake, bile output and liver tissue content after 40 min perfusion of isolated in situ rat livers with recirculating Krebs-Henseleit solution containing 1 μM Bamet-H2, sodium cholylglycinate (NaCG) or cisplatin at min 0. Values are means ± SE from (n = 6). *, $P < 0.05$ as compared with Bamet-H2-perfused livers by the Bonferroni method of multiple range testing.

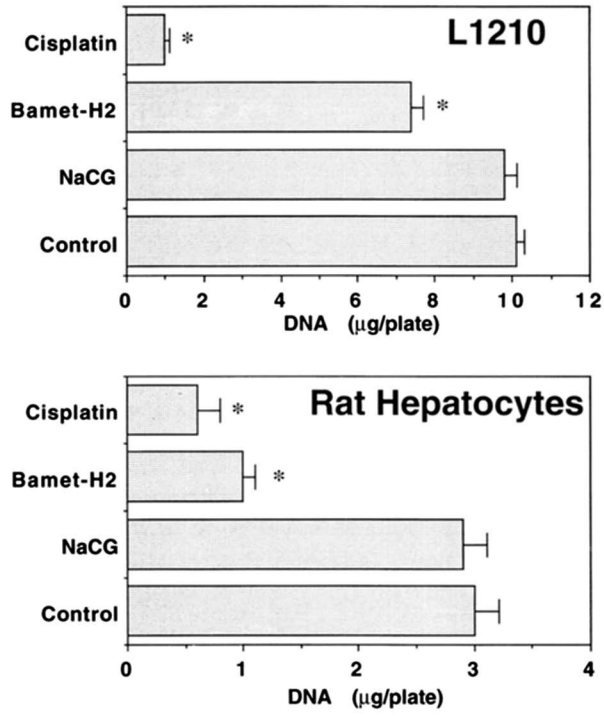

Fig. 6. Effect of sodium cholylglycinate (NaCG), cisplatin and Bamet-H2 on cell growth as assessed by measurement of the amount of DNA in the culture of mouse lymphocytic leukemia L1210 and primary culture of rat hepatocytes at 72 h of culture. Cells were incubated with 100 μM of one of the indicated compounds from the beginning of the culture (72 h) in the case of non-adherent cells (L1210) or after replacing the initial culture medium at 24 h from seeding in the case of rat hepatocytes. Values are means ± SE of three different cultures carried out in triplicate. *, $P < 0.05$ as compared with control cultures by the Bonferroni method of multiple range testing.

suggest a potential usefulness of Bamet-H2 in the antitumoral therapy of neoplasias derived from liver parenchymal cells.

This study was supported in part by the Ministerio de Educacion y Ciencia (Grants SAF94-0693 and SAF96-0146), Spain. The authors thank Dr. F. J. Burguillo for his assistance in the kinetic studies, Ms. M. I. Hernandez Rodriguez for her secretarial help, Mr. M. Fernandez Gutierrez for his technical assistance, and Mr. J. Villoria Terron for caring for the animals. Thanks are also due to Nicholas Skinner for revising the English version of the manuscript.

Manuscript received 10 October 1996 and in revised form 14 January 1997.

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