Water Soluble DACH–Pt(II) Complexes: Problems of Purification; Stability of Complexes with Nitrogen-containing Ligands

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Abstract

Two previously reported water soluble 1,2-diaminocyclohexaneplatinum(II) antitumor complexes with nitrogen-containing dicarboxylato ligands (N-substituted iminodiacetato(1,2-diaminocyclohexane)platinum(II) and aminomalonato(1,2-diaminocyclohexane)platinum(II)) were discovered to have significant residual impurities in the chemical formulations. Upon further purification each complex was found to be significantly less active in biological systems than previously reported. Each complex is stable in aqueous solution. This experience suggests that commonly accepted criteria for chemical identification and purity are inadequate for this type of complex. We hypothesize that tridentate bonding between the nitrogen-containing dicarboxylato group and platinum renders these complexes chemically stable and biologically inert.

Introduction

Cisplatin (*cis*-diaminodichloroplatinum(II)) is an effective cancer chemotherapeutic agent. Clinical limitations of cisplatin include a narrow spectrum of antitumor activity, the phenomenon of acquired resistance, significant toxic effects, and marginal water solubility. Several complexes in which platinum is bonded to 1,2-diaminocyclohexane (DACH–Pt(II)) are remarkable for activity against murine L1210 leukemia rendered resistant to cisplatin [1, 2]. A variety of problems have limited the clinical utility of these complexes. DACH–Pt–$(Cl)_2$ is virtually insoluble in water. In phase I trials DACH–Pt–SO_4 was found to have unpredictable, potentially lethal, toxicities. The structure of a proposed DACH–Pt–isocitrate could not be established, and the complex was found to be relatively unstable in solution [4].

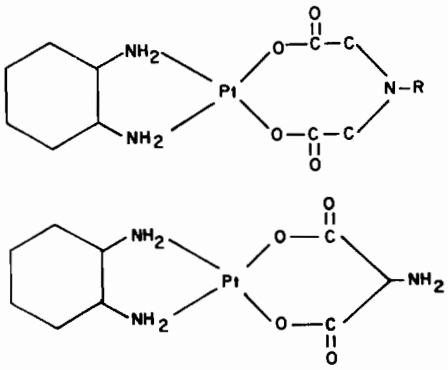

Fig. 1. Structures (as originally proposed) of DACH–Pt–IDA (top) and DACH–Pt–AM (bottom).

To date, no DACH–Pt(II) compound has undergone adequate clinical evaluation, and the *in vivo* activity of such complexes in human tumors with natural or acquired resistance to cisplatin is unknown [5].

One strategy used in the attempt to synthesize new, water soluble DACH–Pt(II) complexes suitable for clinical use has been to substitute as the coordinated anionic group a dicarboxylato ligand containing a nitrogen. We have published early results with a series of such complexes in which the ligand is an N-substituted iminodiacetate (DACH–Pt–IDA) (Fig. 1) [5]. Subsequently, Gandolphi *et al.* reported a series of Pt(II) malonato complexes, the most active of which was the DACH–Pt(II)–aminomalonate (DACH–Pt–AM) (Fig. 1) [6].

In the course of further work we have encountered problems with product purity and purification previously unappreciated by both groups. In each case apparently active complexes have been found, upon further purification, to be stable in aqueous solution and relatively inactive in biological systems. We hypothesize that in these complexes there may be tridentate bonding of the ligand with Pt resulting in a biologically inert species.

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Experimental

Synthesis of the DACH–Pt–IDAs has been reported previously [5]. Briefly, in a three step synthesis K_2PtCl_4 first is reacted with DACH in aqueous solution to yield as a precipitate DACH–Pt–$(Cl)_2$; this is reacted in aqueous solution with Ag_2SO_4, precipitated AgCl is filtered off, and DACH–Pt–SO_4 remains in solution; this is reacted in stoichiometric proportion with BaIDA (prepared *in situ* by reaction of $Ba(OH)_2$ with an IDA) and a precipitate of $BaSO_4$ is filtered off to yield DACH–Pt–IDA which is recovered from solution by evaporation. Product identification and structural assignment was based upon elemental analysis for carbon, hydrogen, nitrogen, and platinum, and IR spectroscopy.

We initially proposed the structure to be coordination of the two carboxylato groups to platinum with the imino group free to function as an amphoteric center. As DACH has two chiral centers, this proposed structure is consistent with three stereoisomeric configurations. Whereas in the experiments previously reported no effort was made to synthesize a pure stereoisomer, in the experiments reported here *trans-R,R*-DACH–Pt–SO_4 (gift of James Hoeschele, Warner-Lambert Pharmaceuticals, Inc., Ann Arbor, Mich., U.S.A.) was used as starting material in order to produce an isomerically pure product.

Synthesis of DACH–Pt–AM was similar with the salient difference being recovery of the final product by ethanol precipitation of the concentrated filtrate from the third step [6]. The product was characterized by elemental analysis for carbon, hydrogen, and nitrogen, IR, and ^{13}C NMR. A structure similar to that proposed for the DACH–Pt–IDAs was proposed with bidentate coordination of the carboxylato groups and a free amine. In experiments reported here authentic DACH–Pt–AM synthesized by Gandolphi was used (gift of John J. McCormack, University of Vermont).

HPLC was used to assess the purity and stability of the synthetic products as well as for purification of DACH–Pt–AM. Analytic HPLC of DACH–Pt–IDAs was performed in reverse phase with an octadecylsilane column (Microsorb™ 4.6 mm × 25 cm), isocratic elution with 50 mM KH_2PO_4/CH_3CN (92.5/7.5), and UV detection at 260 or 220 nm. Analytic and preparative HPLC of DACH–Pt–AM also was performed in reverse phase with a trimethylsilane column (ZORBAX™ 4.6 mm × 15 cm), isocratic elution with water, and UV detection at 220 nm. For preparative separations the major UV peak was collected and lyophilized. In order to assess stability, compounds in solution were subjected to repeated HPLC analysis and mass changes estimated by changes in peak area over time.

An HPLC system with a volatile mobile phase capable of separating components of the DACH–Pt–IDAs could not be discovered, and so preparative HPLC was not done. Purification of the chemical product was attempted with conventional column chromatography utilizing either silica gel or Chelex™ (a chelating ion exchange resin consisting of iminodiacetate ions bonded to a styrene divinylbenzene copolymer; Bio-Rad Laboratories, Richmond, Calif., U.S.A.).

Biologic activity studies against L1210 leukemia *in vitro* and *in vivo* have been described previously [5]. In brief, for estimation of *in vitro* activity, cells in log growth phase in liquid suspension were exposed to varying concentrations of a test complex for 48 or 72 h; from cell concentrations a drug concentration causing 50% inhibition of proliferation (ID_{50}) in comparison with control was calculated. In *in vivo* studies, BDF_1 mice were inoculated intraperitoneally (ip) with 10^6 cells and ip treatment was initiated the following day. Activity ($\%T/C$) was calculated as the ratio of the median survival times of treated and control animals; animals surviving 60 days ('cures') were excluded from this calculation and noted separately. Acute lethal toxicity was assessed on the basis of a single ip administration of test complex in varied amounts to groups of CD_1 mice which were then observed for survival duration. Amounts causing lethal toxicity to a percentage of animal ($LD\%$) were calculated in a probit analysis.

Results and Discussion

The chemical synthesis and biological properties of the DACH–Pt–IDA series and of DACH–Pt-AM have been reported previously [5, 6]. In each case routine elemental analysis for carbon, hydrogen, and nitrogen was reported, but elemental analysis for sulfur or chloride, present in intermediates in each synthesis, was not. Such an analysis of various lots of DACH–Pt–*N*-benzyl-IDA revealed the chemical product to contain from 0% to 0.46% sulfur and from 0.95% to 1.52% chloride. Similarly, an analysis of DACH–Pt–AM revealed no sulfur but 2.22% chloride. Considerations of mass balance had not indicated the presence of these contaminating elements because earlier elemental analysis results were rationalized by assumptions concerning hydration of DACH–Pt(II) complexes in the usual solid form. As DACH–Pt–$(Cl)_2$ and DACH–Pt–SO_4 are active complexes in preclinical systems, albeit unsuitable for clinical use, these results suggested the potential for active contaminants within the chemical formulations of these new DACH–Pt(II) complexes.

Reverse phase HPLC was used to assess further the purity of DACH–Pt(II) complexes after chemical synthesis. HPLC of the DACH–Pt–IDAs and of DACH–Pt–AM freshly dissolved in water revealed each to contain multiple components of varying

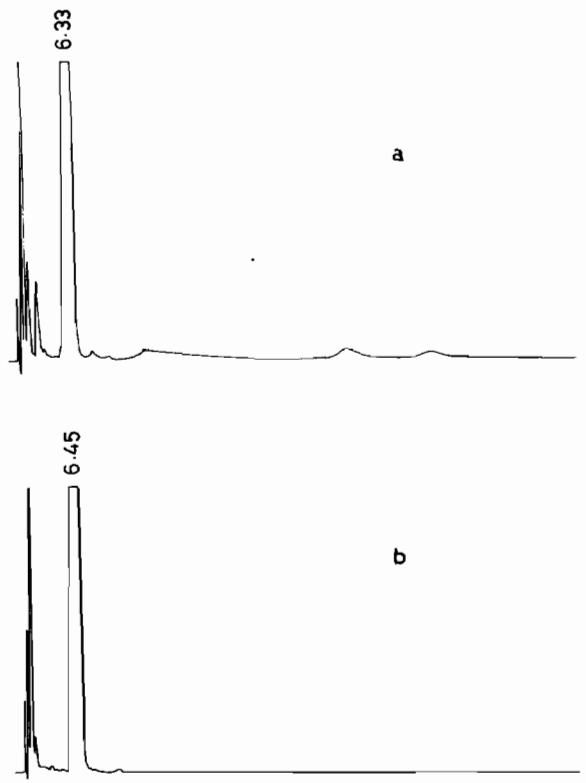

Fig. 2. (a) Reverse phase HPLC of DACH–Pt–*N*-benzyl-IDA (chemical formulation). The first two peaks are attributable to the solute front. (b) Reverse phase HPLC of DACH–Pt–*N*-benzyl-IDA after Chelex™ chromatography.

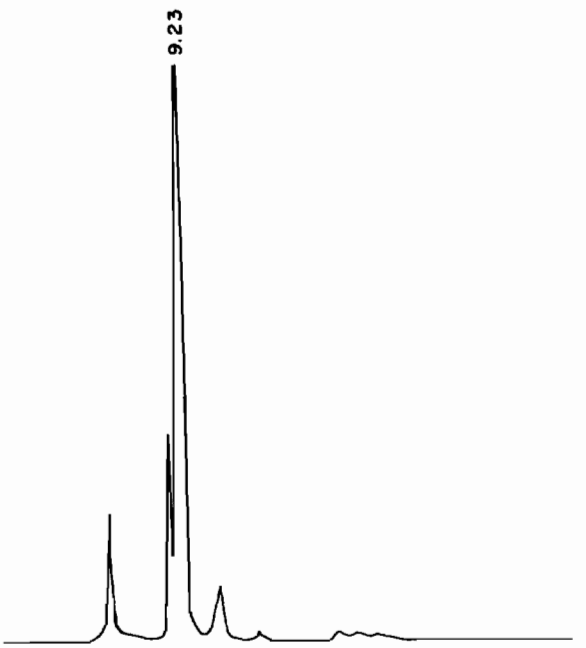

Fig. 3. Reverse phase HPLC of DAC–Pt–AM (chemical formulation).

retention time and relative amount. HPLC of DACH–Pt–*N*-benzyl-IDA with UV detection at 220 nm revealed a single major component, three minor components, and five or more trace components (Fig. 2a). On the basis of the retention time in comparison with authentic standard, one of the minor components was identified as free *N*-benzyl-IDA. None of the minor components coeluted with DACH–Pt–SO_4.

Conventional chromatographic methods were used in an attempt to further purify DACH–Pt–*N*-benzyl-IDA. HPLC after Chelex™ chromatography revealed persistence of the major component and free *N*-benzyl-IDA, loss of a least one minor component and loss of several trace components (Fig. 2b). Elimination of chromatographic peaks after Chelex™ indicates that some components resolved by HPLC are not merely rapidly formed hydrolysis products of the proposed complex but rather separate species within the original formulation. HPLC after silica gel chromatography (analyzed only at 260 nm which resulted in a less complex chromatogram than that at 220 nm) revealed loss of one minor component with persistence of the major component and of free *N*-benzyl-IDA. ^{1}H NMR of DACH–Pt–*N*-benzyl-IDA after silica gel chromatography continued to show protons of the benzyl and diaminocylhexane groups in a 1:1 ratio; upon this basis it was concluded that the further purified formulation contained the proposed product.

HPLC of DACH–Pt–AM revealed one major and three minor components (Fig. 3). As no effort was made in the synthesis of DACH–Pt–AM to isolate an isomerically pure compound, one presumes that the chemical product was a racemic mixture. Although theoretically such a mixture might be resolved by a chromatographic procedure, this seems to us to be unlikely with the system used, and we believe that the chromatogram is evidence for a mixture of (nonisomeric) substances.

DACH–Pt–AM was partially purified by preparative HPLC on an analytic scale. Subsequent HPLC analysis revealed this procedure to be only partially effective with persistence of one minor as well as the major component in the purified product. IR of the partially purified product revealed broad absorption bands between 3260–3080 cm^{-1} attributed to the coordinated neutral amine ligands and absorption at 1670 cm^{-1} attributed to the coordinated carboxylato groups; upon this basis it was concluded that the partially purified product contained the proposed chemical product.

Although efforts at purification of these new DACH–Pt(II) complexes were only partially successful as assessed by HPLC criteria, effects of purification upon biological activity were significant. Comparison of activities of the chemical and partially purified products against L1210 leukemia

TABLE I. L1210 *In Vitro* Cytotoxicity: ID_{50} (μg/ml)

DACH–Pt–benzyl-IDA	
Chemical product	0.6
After Chelex™	6.8
After silica gel chromatography	>10
DACH–Pt–AM	
Chemical product	0.5
After HPLC	1.9

TABLE II. L1210 *In Vivo* Activity

	Dose (mg/kg)	Schedule	T/C (%)	Longterm survivors
DACH–Pt–*N*-benzyl-IDA				
Chemical product	25	d1,5,9	237	1/6
After silica gel	25	d1,5,9	108	0/6
Chemical product	10	d1,5,9	152	0/6
After Chelex™	10	d1,5,9	111	0/6
DACH–Pt–AM				
Chemical product	25	d1	264	0/6
After HPLC	25	d1	136	0/6

in vitro revealed significant loss of biologic effect for each complex with DACH–Pt–*N*-benzyl-IDA now inactive and DACH–Pt–AM only moderately active (Table I). A similar change was seen in the L1210 leukemia *in vivo* assay in which each purified complex failed to show significant activity (Table II).

Acute lethality studies performed only with DACH–Pt–*N*-benzyl-IDA also revealed a remarkable loss of biologic activity after partial purification: whereas the LD_{10}, LD_{50}, and LD_{90} values for the chemical product were 56, 81, and 135 mg/kg, respectively, the LD_{10} for the purified product was observed to be greater than 200 mg/kg.

As Pt(II) complexes are thought to undergo spontaneous activation via hydrolysis yielding reactive intermediates which bind covalently to biomolecules [7], it was of interest to evaluate the stability of the new complexes in solution. Stability of the major components of these complexes in aqueous solution at room temperature was analyzed using HPLC. No loss of the major component of either the DACH–Pt–*N*-benzyl-IDA or of DACH–Pt–AM could be detected over several days time.

From these observations we draw the following conclusions.

Water soluble DACH–Pt(II) complexes synthesized by this commonly-used method must be subjected to more rigorous analysis than simple elemental analysis, IR, ^{13}C NMR, and ^{1}H NMR in order to establish product purity. In investigations reported here HPLC has been a useful analytical tool.

DACH–Pt–*N*-benzyl-IDA, further purified from the chemical preparation previously reported, is biologically inactive. DACH–Pt–AM, further purified from the chemical preparation previously reported, is significantly less active than previously reported.

Each of these complexes is essentially stable in aqueous solution.

These observations lead us to hypothesize that when nitrogen-containing dicarboxylato groups are liganded to DACH–Pt(II), tridentate bonding, with one axial and two planar bonds, may result. Such tridentate bonding may confer upon these complexes considerable stability in solution and render them biologically inert. Recent chemical observations are consistent with this hypothesis [8].

We propose that the active species in the chemical formulations of the complexes as previously described actually are DACH–Pt(II) complexes containing chloride ligands. This proposal raises the question of how presumably insoluble chloride-containing complexes could be carried through the aqueous synthesis as described. Recently, bis-diamminePt(II) complexes bridged by chloride and hydroxide have been identified in solution [9]. It is possible, then, that similar bridged, chloride-containing bis-DACH–Pt(II) species carry chloride through the syntheses described here to yield active, chloride-containing complexes in the chemical products of DACH–Pt–IDA and DACH–Pt–AM. If one attributes to such hypothetical intermediates the potency of DACH–Pt–$(Cl)_2$ in *in vitro* and *in vivo* systems, one can explain the activity of the chemical formulations of DACH–Pt–bIDA and DACH–Pt–AM entirely upon the basis of the small amounts of chloride observed upon elemental analysis.

An alternate hypothesis would be that the 'purification' methods used in these studies actually facilitated further chemical conversion and inactivation of an active (non-chloride-containing) component within the chemical formulations. Specifically, it may be that the previously proposed structures for these complexes with coordination of the dicarboxylato groups and a free imine or amine group exists in the chemical formulations, and that the nitrogen-containing group bonds to Pt, forming a stable complex as hypothesized above, during further passage in aqueous solution in dilute concentration during 'purification'. If this is the case, however, purification of the active but unstable intermediate will be a formidable task. Further, it is likely that conversion to a stable and inactive product will occur in any conventional pharmaceutical formulation comprised of a dilute concentration in aqueous solution.

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