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Cytotoxic Activity of Two Polyaspartamide-based Monoamineplatinum(II) Conjugates Against the HeLa Cancer Cell Line

Gregg Caldwell,[1] Eberhard W. Neuse[1]* and Constance E. J. van Rensburg[2]
[1]Department of Chemistry, University of the Witwatersrand, WITS 2050, Republic of South Africa
[2]MRC Unit for Inflammation and Immunity, Department of Immunity, University of Pretoria, Pretoria 0001, Republic of South Africa

In this screening study *in vitro*, two polymer-conjugated, square-planar platinum(II) complexes bound to the carrier via a single primary amine ligand were tested for antineoplastic activity against the HeLa human cervical epithelioid carcinoma cell line. In the first of these conjugates, 1-Pt, the spacer connecting the metal complex with the carrier backbone is a short oligo(ethylene oxide) segment, whereas a long poly(ethylene oxide) chain represents the spacer unit in the second conjugate, 2-Pt. IC_{50} data, expressed as conjugate concentration at 50% cell growth inhibition, are 48 μg Pt ml^{-1} for 1-Pt and 120 μg Pt ml^{-1} (estimated) for 2-Pt, the long tether in the latter conjugate presumably causing retarded enzymic release and lysosomal membrane crossing of the monomeric complex. The IC_{50} value of 1-Pt is close to that (44 μg Pt ml^{-1}) of a similar conjugate of an earlier investigation, 3-Pt, in which the metal is chelated by two carrier-attached, *cis*-oriented amino groups in conformance with the ligand arrangement in cisplatin. It thus appears that, in the carrier-bound state, both monoamine- and *cis*-diamine-coordinated platinum(II) complexes of suitable structures may well show similar biological performance patterns. Copyright © 1999 John Wiley & Sons, Ltd.

Keywords: cytotoxicity; Platinum(II) conjugates; polyaspartamide derivatives; HeLa cancer line

* Correspondence to: Eberhard W. Neuse, Department of Chemistry, University of the Witwatersrand, WITS 2050, Republic of South Africa.
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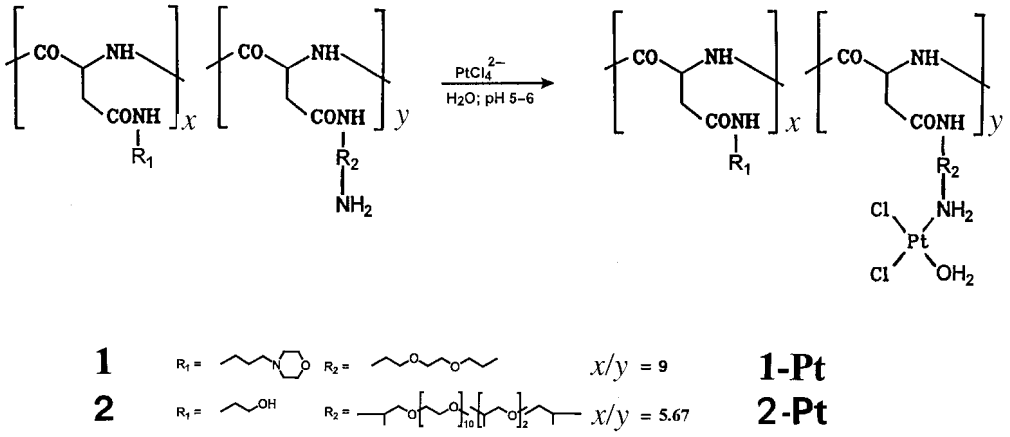
INTRODUCTION

The cytotoxic properties of platinum complexes of interest as antineoplastic agents are commonly considered to be associated with a *cis*-diamineplatinum structural entity. *trans*-Diamineplatinum compounds have generally been found to lack antiproliferative activity, although activity was recently reported[1] for certain members of this class comprising *N*-heterocycles as the amine ligands. In the common belief that the two tightly binding amine ligands as represented in the cisplatin prototype would be required for carcinostatic activity, scant attention has been paid in cancer chemotherapy research to coordination compounds comprising a monoamineplatinum structural unit. Exemplifying monoamine complexes reported to possess antiproliferative properties feature anion structures of the type $[PtCl_3(NH_2R)]^-$ (R = H, alkyl).[2–4] As salt-like compounds, such complexes cannot efficiently penetrate cell membranes by the passive diffusion mechanism generally utilized by nonpolar, neutral compounds. Transmembrane transport is a necessary requirement for antiproliferative effectiveness of most known anticancer drugs, which act upon the nuclear DNA of the affected cell and, for this reason, must cross into intracellular space. The salt-like nature of the typical monoamineplatinum complexes may, therefore, have been considered a convincing argument against their inclusion in major screening projects involving platinum compounds *in vitro* and *in vivo*.

The pharmacokinetic fate of a medicinal agent may be altered profoundly through the expediency of binding (anchoring) the compound reversibly to a water-soluble polymeric carrier.[5,6] The agent so anchored may experience cell entry through



Scheme 1

pinocytosis,[5–8] thereby overcoming internalization problems potentially caused by polar or salt-like structural peculiarities. Agents showing an insufficient degree of antiproliferative activity when administered *per se* may therefore, conceivably, perform with enhanced effectiveness once they have been connected reversibly to water-soluble carrier polymers. Exploiting this concept, we investigated, and herein report on, the antineoplastic activity against human HeLa cancer cells of two representative polymer–platinum conjugates, prepared in our laboratory, in which the metal is conjugated through a single carrier-bound amine ligand.

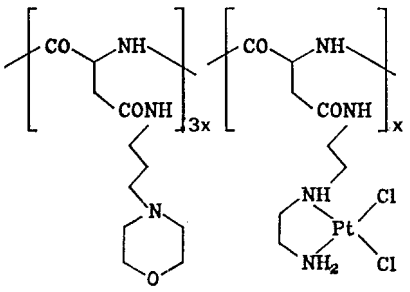
RESULTS AND DISCUSSION

The conjugates investigated, **1-Pt** and **2-Pt**, both derived from a basic polyaspartamide structure, were obtained by platination of the known carrier polymers **1** and **2** (Scheme 1).[9,10] The carriers featured a solubilizing group, R_1, providing water solubility, and a linking segment, R_2, designed to provide flexibility of the tether connecting the polymer backbone with the primary amine function introduced here as the metallation site. The solubilizing groups were of the tertiary amine (carrier **1**) or hydroxyl (carrier **2**) types, and R_2 was represented by a short (carrier **1**) or long (carrier **2**) poly(ethylene oxide) spacer, the latter expected further to enhance the solubilizing capacity and provide such other biomedically desirable features as protection from serum protein binding[11] and increased circulation lifetime,[12] as well as reduced toxicity and immunogenicity.[12,13] Carrier platination was brought about essentially by the method developed in a previous project[14] involving treatment with 1.3 equivalents of tetrachloroplatinate(II) ion in aqueous solution. The work-up included brief exposure of the platinated material to brine in an effort to liberate, and remove, excess platination agent potentially bound either by salt formation with the tertiary amine functions or else by adsorption to the poly(ethylene oxide) chains. The feasibility of platinum binding by either mechanism had been verified in previous experimentation (E. W. Neuse and G. Caldwell, unpublished results). The conjugates, purified by dialysis, were collected as solid, water-soluble compounds upon freeze-drying of their aqueous solutions.

Microanalytical platinum determinations confirmed the complete platination of the amine functions in the carriers. A Cl/OH_2 ligand ratio of 2:1 was assumed for both **1-Pt** and **2-Pt** by analogy with the corresponding ligand arrangement in the monoamineplatinum conjugates of an earlier investigation,[15] the strong chloro ligand *trans* effect favoring chloro/aqua exchange during the aqueous work-up at a position *cis* to the amine ligand. It should be emphasized, however, that this ligand assignment, just as in the preceding work,[14] is speculative. Chlorine analyses were not helpful for confirmation of the postulated structure, as the erratic retention of HCl via tertiary amine protonation or hydrogen bonding to the poly(ethylene oxide) chain[14] led to excessive data scattering. With ^{195}Pt NMR generally ineffective with polymeric platinum complexes for reasons of excessive line

broadening, use of other analytical techniques, including photoelectron spectroscopy, is being considered in an effort to remove the uncertainty at present associated with the proposed structural assignments.

Conjugates **1-Pt** and **2-Pt** were tested *in vitro* for cytotoxic activity against cultured human HeLa cancer cells by the method described previously.[16] The concentrations employed were up to 100 μg Pt ml^{-1}. Cell growth inhibition, as a percentage of untreated control, was determined as a function of conjugate concentration (as μg Pt ml^{-1}). The derived value of IC_{50} (mean drug concentration required to cause 50% cell growth inhibition) is listed for **1-Pt** in Table 1. For comparison, the tabulation also contains the IC_{50} value for a representative *cis*-diamineplatinum conjugate, **3-Pt**, prepared and tested in previous programmes. For the IC_{50} of **2-Pt** only a rough estimate is available (Table 1), as the plot for this conjugate remained just below the 50% inhibition level within the concentration range employed.



3-Pt

Structure **3-Pt**

The poor performance of **2-Pt** in comparison with **1-Pt** may be rationalized in terms of the different lengths of the poly(ethylene oxide) (PEO) chain constituting the 'tails' of the amine ligands of the two compounds. Complex release from the polymer main chain in the lysosomal space presumably proceeds through fission of the aspartamide link, leaving the complex in each case with the PEO 'tail' still attached to the metal center by the strong N–Pt bond. In **2-Pt**, such release will be opposed both by steric crowding and by the known protein repellency of the long PEO chain,[11] retarding enzyme approach and enzymically controlled amide bond fission. In addition, diffusion of the 'long-tailed' complex released from **2-Pt** through the lysosomal membrane, required for the ultimate interaction with nuclear DNA, is likely to be drastically retarded in comparison with the complex released from **1-Pt** with its considerably shorter PEO constituent. Greater bioavailability, and hence potency, within the concentration and time restraints of the cell culture tests is therefore expected for the complex liberated from **1-Pt** than for the one released from **2-Pt**.

Table 1 Cytotoxic activity of conjugates 1-Pt–3-Pt against HeLa cell line

	Pt (wt%)		
Conjugate	Found	Nominal	IC_{50} (μg Pt ml^{-1})[a]
1-Pt	7.6	7.2[b]	48
2-Pt	9.5	9.9	~120[c]
3-Pt	16.1	16.4[b]	44[d]

[a] Mean concentration causing 50% inhibition of cell growth, derived from three parallel experiments for each concentration.
[b] Protonation effects ignored.
[c] Estimate.

It is instructive to observe the nearly identical activity data tabulated for the monoamine complex **1-Pt** and the *cis*-diamine complex **3-Pt**. To illustrate this point further, the percentage inhibition versus concentration relationship has been plotted for both conjugates in Fig. 1. A nearly coincident trend of the two curves can be observed, at least up to a concentration of 60 μg Pt ml^{-1}. (Beyond this limit, the curve for **1-Pt** experiences an as-yet unexplained trend reversal, requiring further investigation.) This shows that structurally dissimilar platinum compounds, which in the unconjugated state tend to follow different pharmacokinetic pathways, and hence display different bioactivity patterns, may well converge to similar activity profiles upon bioreversible attachment to suitable polymeric carriers acting as transport vehicles. The polymer–drug anchoring approach thus offers interesting perspectives in the field of carrier-bound, carcinostatic platinum complexes with different ligands and ligand arrangements.

EXPERIMENTAL

General techniques and procedures

^{1}H NMR spectra were obtained at 200 MHz on D_2O solutions; chemical shifts, δ, are given in ppm relative to internal sodium 3-(trimethylsilyl)-

2,2,3,3-d_4-propionate. The pH was adjusted to 9 immediately before scanning in order to eliminate possible protonation effects. IR spectra (KBr pellets) were recorded over the region 4000–200 cm^{-1}. Inherent viscosities, η_{inh}, were determined with the aid of Cannon–Fenske tubes in deionized H_2O at concentration $c = 0.2$ g/100 ml and are given in units of ml g^{-1}. Spectra/Por 6 wet tubing (25 000 molecular mass cut-off; Spectrum Industries, Los Angeles, CA, USA) was used for redialysis of carrier polymers, whereas Spectra/Por 4 (12 000–14 000 molecular mass cut-off) served to dialyse platinated material as well as the crude carriers. Aqueous polymer solutions were freeze-dried on a Virtis Bench Top 3 freeze-drier operating at −30 °C, 0.08–0.1 Torr. Metal-free polymers were post-dried in a Sartorius Thermo Control Infrared Drying System (heating programme, 2×8 min at 65 °C). Analytical samples were additionally dried in an Abderhalden tube for two days at 65 °C, 20–30 Torr. Platinum analyses were performed in duplicate in the Anglo American Research Laboratories, Crown Mines, Johannesburg, RSA, and the results are given as averages.

Reagents and monomers

Potassium tetrachloroplatinate(II) was prepared by reduction of the hexachloroplatinate(IV) as described.[18] All monomeric reactants were of reagent grade (Fluka AG) and were used as received. The commercial product Jeffamine ED-600 (Fluka AG), a poly(ethylene oxide) terminated at both ends with a primary amino group and catalogued as *O,O′*-bis(2-aminopropyl) poly(ethylene glycol) 500, was used for the preparation of carrier **2**. The nominal molecular mass, as stated by the supplier, was 600. ^{1}H NMR spectroscopy indicated the compound to comprise one propylene oxide in addition to 10 ethylene oxide units in the chain, and the stoichiometric and analytical considerations in this paper were based on this composition.

Polymeric educts, carriers and conjugates

Amounts of polymeric educts and products are given as base moles. For the carriers **1** and **2** and the conjugates **1-Pt** and **2-Pt** these refer to the structures shown, each normalized to $y = 1$. For **3-Pt**, the base mole refers to the structure shown, normalized to $x = 1$.

Poly-D,L-succinimide

This educt for the polyaspartamide carriers was prepared by the procedure of Neri and Antoni.[19] The mass-average molecular mass, determined from viscometric data,[20] was 38 500.

Conjugate 1-Pt

Polyaspartamide **1**, the carrier component of this conjugate, was synthesized as described in a preceding paper.[9] Polysuccinimide (4.85 g; 50 mmol), was dissolved in 80 ml of freshly distilled, anhydrous *N,N*-dimethylformamide (DMF). To the stirred solution was added, over a period of 30 min, 4-(3-aminopropyl)morpholine (6.49 g; 45 mmol), predissolved in 30 ml of DMF. After saturation with N_2, this solution was stirred for 48 h at ambient temperature. Cooling in an ice bath with renewed introduction of N_2 was followed by the rapid addition of ethylenedioxy-*O,O′*-bis (2-ethylamine) (2.223 g; 15 mmol), in 15 ml of DMF. Stirring was continued for 10 h at approx 0 °C and for another 60 h at room temperature. Up to this point, moisture access was strictly precluded. The solution was reduced in volume to about 30 ml by rotory evaporation (bath temperature 60–65 °C), and the product polymer was precipitated with 120 ml of Et_2O–hexane (2:1). The precipitate was washed with hexane, dissolved in water (20 ml) and dialysed for 48 h against several batches of deionized water in Spectra/Por 4 tubing, followed by another 45 h in Spectra/Por 6 tubing. Freeze-drying of the retentate and post-drying on the Sartorius system afforded the carrier (4.84 g; 40.0%), as an off-white, water-soluble solid; η_{inh}, 12 ml g^{-1}. ^{1}H NMR (D_2O)/ppm (expected proton count in parentheses): 4.7–4.5, 11.4H (10H, CH Asp); 3.8–3.5, 45H (44H, CH_2O); 3.4, 1.9H (2H, CONH—CH_2CH_2—O); 3.2, 18.6H (18H, CONH—$CH_2CH_2CH_2$); 3.0–2.3, 77H (76H, $N(CH_2)_3$, CH_2 Asp, NH_2—CH_2); 1.7, 18H (18H, $CH_2CH_2CH_2$).

Of the carrier so obtained, a 1.209 g portion (0.5 mmol) was dissolved in 15 ml of deionized water. To the solution, saturated with N_2, was added K_2PtCl_4 (249 mg; 0.6 mmol), and the mixture was stirred with continued introduction of nitrogen until the platination agent had completely dissolved. Stirring of the solution in the stoppered flask was continued for 30 h at room temperature and for another 48 h at 45 °C with protection from light. During this period, the pH dropped from 8 to 7, and for the last 30 min of the heating period it was adjusted (HCl) further to 5. Addition of NaCl (1.5 g) and stirring for 2 h at ambient temperature was followed by dialysis in Spectra/Por 4 tubing for

2 h against H_2O at pH 7 and for another 40 h against several batches of water acidified to pH5 (HCl). Freeze-drying of the retentate gave 1.23 g (91%) of water-soluble, light-tan solid; η_{inh}, 12 ml g^{-1}. Analysis: Found: Pt, 7.6%. Calcd for $(C_{109}H_{192}Cl_2N_{30}O_{32}Pt)_n$ [mol wt $(2701)_n$] (**1-Pt**): Pt, 7.2%. Protonation effects, although expected at the pH level before freeze-drying, were neglected in this composition.

Conjugate 2-Pt

The carrier polymer **2** used in this experiment was taken from a previous project,[10] where it had been designated **3** (85 : 15); η_{inh}, 14 ml g^{-1}. The carrier was platinated essentially by the method[14] elaborated earlier, except that the period of room-temperature exposure to brine as part of the work-up procedure was increased to 4 h. Yield, 63%; η_{inh}, 15 ml g^{-1}. Analysis. Found: Pt, 9.5%. Calcd for $(C_{70}H_{129.7}Cl_2N_{14.3}O_{33}Pt)_n$ [mol wt $(1966)_n$] (**2-Pt**): Pt, 9.9%.

Conjugate 3-Pt

The polymer was prepared by a procedure described earlier[21] (designated **4**(25)-**Pt** in Ref. 21); η_{inh}, 10 ml g^{-1}. Analysis: Found: Pt, 16.1%. Calcd for $(C_{41}H_{73}Cl_2N_{13}O_{11}Pt)_n$ [mol wt $(1190)_n$] (**3-Pt**): Pt, 16.4%. Again, protonation effects were disregarded.

CYTOTOXICITY TESTING

Conjugates **1-Pt** and **2-Pt** were screened *in vitro* for antiproliferative activity against HeLa human cervical epithelioid carcinoma cells, ATCC CC42. The tests were performed in round-bottomed, 96-well tissue culture plates by the previously published procedure.[16] To each well were added 2500 tissue culture cells, and the volumes were brought to 200 μl with minimum essential (MEM) supplemented with 10% foetal calf serum containing the various drug concentrations or control systems. After incubation for 72 h at 37 °C in 5% CO_2, the plates were fixed with 10% phosphate-buffered formalin, washed with phosphate-buffered saline and stained with 0.02% Crystal Violet. Plates were washed in water and the stain extracted with 10% sodium dodecylsulfate. With the aid of a multiscan plate reader the absorbance was measured at 620 nm. Background values (medium only) were subtracted from each reading. Results are expressed as percentage inhibition and are plotted in Fig. 1 for **1-Pt** together with the curve for the *cis*-diamineplatinum conjugate **3-Pt** investigated previously[17] and included in this study for comparison. The IC_{50} data, i.e. the platinum concentrations required to reduce the absorbance to 50% of control, are listed in Table 1 for **1-Pt, 2-Pt** (rough estimate) and **3-Pt**. The data were derived as means of three experiments conducted for each concentration.

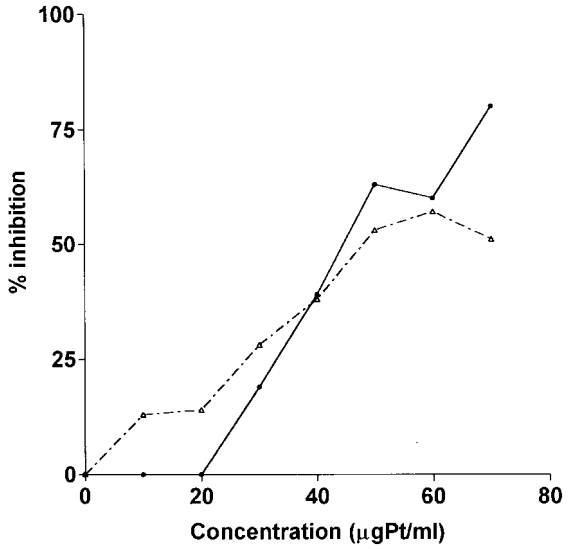


Figure 1 Percentage inhibition of cell growth, relative to control, versus conjugate concentration (μg Pt ml^{-1}): broken line, **1-Pt**; solid line, **3-Pt**.

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