Poly(amidoamine) dendrimer-supported organoplatinum antitumour agents

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While numerous water-soluble biocompatible polymers have been utilized for the construction of drug conjugates that offer significant advantages for drug delivery, poly(amidoamine) (PAMAM) dendrimers are superior in many ways for this purpose. They display nanoscale size, uniform shape, excellent water solubility, low toxicity and high surface functionality. In an attempt to circumvent the toxic side effects associated with the administration of organoplatinum drugs, a polymeric prodrug has been prepared from the treatment of a generation 4.5 PAMAM dendrimer with diaquo(1,2-diaminocyclohexane)platinum(II). A well-defined dendrimer–platinum conjugate containing 40 (1,2-diaminocyclohexane)platinum(II) units coordinated to the dendrimer surface via carboxylate groups is formed. This adduct is well behaved, water soluble, contains a high loading of platinum moieties and displays sustained release of active platinum species over a 24 h period under physiological conditions.

Keywords: sustained release; nanoscale drugs; multivalent antitumour drugs; polymeric prodrugs

1. Introduction

Over the past several years there has been huge progress in the utilization of polymeric carriers for a wide variety of drugs (Batz 1977; Langer 1998, 2001; Duncan 2003, 2006; Allen & Cullis 2004; Hoste et al. 2004; Khandare & Minko 2006; Jagur-Grodzinski 2009). The use of a polymer–drug conjugate may provide a number of advantages over the drug alone. These may include increased water solubility, sustained delivery of drug, reduced toxicity, enhanced biodistribution and preferential permeation of abnormal tissue.

This effort has been afforded a significant boost by the discovery of dendritic polymers (Tomalia et al. 1984, 1985). In particular, the poly(amidoamine) (PAMAM) dendrimers are attractive as drug carriers. They are water soluble, multivalent and, in general, non-toxic (Malik et al. 2000; Tomalia & Frechet 2001; Tomalia 2007). Dendrimers are highly branched macromolecules with precisely controlled size, shape and end-group functionality. They represent unique core–shell structures consisting of three basic architectural components:

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One contribution of 7 to a Special feature ‘Current research trends in dendritic materials’.
a core, an interior of shells (generations) which have repeating branch-cell units and terminal functional groups (the outer shell or periphery). Each generation is built sequentially from a predecessor to form a symmetrical, nearly monodisperse structure or precise molecular weight and nanoscale dimensions (Tomalia 2004). For PAMAM dendrimers half-generation structures are carboxyl terminated. These, in particular, are attractive for drug conjugation (Howell et al. 2008). The carboxyl groups represent useful functionality for the attachment or prodrug units.

The era of organoplatinum drugs was spawned by the discovery of the remarkable biological activity of cis-dichlorodiammineplatinum(II) (cisplatin) (Rosenburg et al. 1965, 1967, 1969; Rosenberg 1980, 1985). Cisplatin is a broad-spectrum cancer drug effective against a wide range of tumours. For many years cisplatin was the most widely used anticancer drug. It is often used in combination with organic antitumour compounds or with carboplatin (1,1-cyclobutanedicarboxylato(diammine)platinum(II); the second platinum anticancer drug to gain widespread commercial use). Carboplatin displays a somewhat different toxicity profile than does cisplatin (figure 1), making it an attractive compliment to cisplatin (Dabrowiak & Bradner 1987; Kelland 1992; McKege & Kelland 1992).

Both of these compounds reflect the structure required for antitumour activity (two inert cis ligands and two labile ligands; chloride displays a near optimum hydrolysis rate under physiological conditions—half-life of about 1 h at 37°C).

The potential of these drugs has been limited because of severe side effects that accompany their administration. Among the most debilitating side effects induced by organoplatinum drugs are severe kidney damage (Jones et al. 1985) and extreme nausea (as a class the platinum compounds are among the most effective nausea-producing agents known—to the point that some patients refuse to complete the treatment regimen) (Rosenberg 1971; Aggrarwal & Menon 1981). In an attempt to identify active but less toxic drugs literally hundreds of platinum compounds in which the structure of the amine ligand has been varied have been synthesized and evaluated for antitumour activity. In the main, this has been a fruitless undertaking. While some ligands impart better solubility, activity or toxicity than similar properties associated with compounds derived from simple ammonia ligands, no compounds with clearly superior performance have been found. Of the hundreds of compounds synthesized, fewer than 30 have entered clinical trials as antitumour agents (Neuse 1988; Lebwohl & Canetta 1998). The nature of the labile ligands has also been varied although the range of options
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is not as great for the inert ligands. These have included carboxylato ligands containing poly(ethylene glycol) units or other water-solubilizing pendants (Spinelli et al. 1992; Ohya et al. 2000; Aronov et al. 2003).

2. Results and discussion

We have, for some time, been using water-soluble polymers as platforms on which a platinum drug or prodrug might be supported and from which it might be slowly released into the extracellular fluid (Howell & Walles 1985, 1986, 1988; Howell et al. 1988a,b,c, 1993; Howell & Richards 1996; Howell & Sastry 1996; Dysterhouse et al. 2000; Saltmarsh et al. 2000). This approach has several major potential advantages over the traditional forced hydration therapy currently practised. First, the solubility of the drug formulation may be dramatically enhanced such that the volume of the fluid required to introduce a satisfactory dose of drug is strongly diminished (cisplatin has a solubility of about $10\text{mg}\text{l}^{-1}$ in aqueous saline). More importantly, if the release rate is optimal, the drug is released into the blood stream at a level that is beneath the toxicity threshold such that side effects may be mitigated (for an excellent review of the development of polymer-based organoplatinum antitumour agents see Siegmann & Carraher 2004). Early attempts involved the formation of non-covalent complexes with poly($N$-vinylpyrrolidone) (Howell & Walles 1985, 1986, 1988; Howell et al. 1988a,b,c, 1993). A strong positive with respect to the use of this polymer is its long history in biological applications and its approval for use in food and drug applications. The platinum compounds used contained (1,2-diaminocyclohexane)platinum(II) units coordinated with catecholato (Howell et al. 1988c), phthalato (Howell et al. 1988b) or salicylato (Howell & Beholz 1990; Howell et al. 1993) ligands bearing a polar functionality for complexation with the polymer. More recently, platform polymers have been poly(acrylamide)s in which the amine portion of the amide is derived from a 1,2-oxazine (Howell et al. 1996; Howell & Southwell 1997). These polymers are versatile materials and may be readily modified in a number of ways for covalent attachment of organoplatinum species (Southwell 1988).

The advent of dendritic polymers, particularly PAMAM dendrimers, has provided a water-soluble, non-toxic base for the preparation of multivalent organoplatinum drugs (Tomalia et al. 1984, 1985; Tomalia & Frechet 2001; Tomalia & Reyna 2007). The first preclinical study of a PAMAM dendrimer–platinate conjugate for the delivery of antitumour agents was reported in 1999 (Malik et al. 1999). A generation 3.5 PAMAM dendrimer containing carboxylate surface groups was treated with cisplatin to generate a dendrimer–platinate (20–25 wt% Pt, about 22 cisplatin units) which was highly water soluble and had a much higher platinum loading than observed for $N$-(2-hydroxypropyl)methacrylamide (HPMA) copolymer platinates (3–8 wt% platinum) (Gianasi et al. 1999). Size exclusion chromatography (gel permeation chromatography) and particle size photon correlation spectroscopy revealed that the PAMAM(G3.5)-cisplatin conjugate consisted of a number of species including those arising from monodentate and bidentate binding to carboxylate groups as well as cross-linked dendrimer via platinum bridges, which caused an increase in particle size from 3–4 nm in the parent dendrimer to 30–40 nm.
diameter of the dendrimer–cisplatin adduct. Thus, a variety of platinum species, including some physically entrapped cisplatin, were present in the polymer–drug conjugate. In vivo the dendrimer–cisplatin and cisplatin administered intraperitoneally (i.p.) were equally active against L1210, and at high dose dendrimer–cisplatin displayed activity against B16F10, whereas cisplatin did not. Additionally, when administered intravenously (i.v.) to treat a palpable squamous cell (s.c.) B16F10 melanoma, the PAMAM(G3.5)-cisplatin adduct displayed antitumour activity whereas cisplatin was inactive. Measurement of platinum levels in blood and tissues after i.v. injection of cisplatin (1 mg kg\(^{-1}\)) or dendrimer–cisplatin (15 mg kg\(^{-1}\))—the maximum tolerated dose of these compounds—showed selective accumulation of the dendrimer–cisplatin in solid tumour tissue by the enhanced permeability and retention (EPR) effect. The improved activity in the s.c. solid tumour model versus the i.p. ascites is indicative of the importance of the EPR effect in tumour targeting. This PAMAM(G3.5)-cisplatin conjugate was also less toxic (3–15-fold) than cisplatin and thus has potential for further investigation as a novel antitumour approach. However, the release of active platinum species was not detected by atomic absorbance spectrometry (less than 1% of the total platinum released) in pH 7.4 and pH 5.5 buffer solutions over a period of 72 h. It should also be noted that the exact proportion of the dendrimer–cisplatin made available as the active diaquo species is not yet known and indeed the time course of platinum liberation is yet to be determined.

Although the activity of the above polymer–platinum conjugate is impressive, the ill-defined nature of the multiple species present as well as the variable release rates for these entities makes this less than an ideal formulation for the treatment of disease. More recently, a generation 4.5 PAMAM dendrimer nanoconjugate containing \((\text{1,2-diaminocyclohexane})\text{platinum(II)}\) \([\text{DACH}])Pt\) moieties covalently bound to surface carboxylate groups has been prepared (Fan et al. 2005; Howell et al. 2008). For the preparation of a useful drug formulation, the PAMAM(G4.5) dendrimer has several positive features. Generally, high-generation symmetrical dendrimers \((G \geq 4)\) have a globular structure with peripheral densely packed terminal groups (Caminati et al. 1990). In the case of PAMAM (G4.5), 128 carboxylate groups are closely packed on the dendrimer surface. This strongly facilitates the interaction of carboxylate groups with platinum species to construct a dendrimer-based multivalent platinum conjugate. In addition, the carboxylate functionality should serve as labile ligands for platinum moieties such that the release of active platinum species \([\text{DACH}])Pt\) should occur at a sustained rate over a period of time. Biological studies using PAMAM dendrimers have also demonstrated that high-generation PAMAM dendrimers are non-immunogenic and display low mammalian toxicity, while anionic PAMAM dendrimers (surface groups with carboxylate or hydroxylic functionalities) are non-toxic \textit{in vitro} (Roberts et al. 1996; Malik et al. 2000; Wiwattanapatapee et al. 2000). These intrinsic properties of PAMAM (G4.5) dendrimer make it an interesting multivalent macromolecule that can serve as an exo-receptor for the construction of novel dendrimer-based platinum anticancer agents. The \textit{trans}-1,2-diaminocyclohexane platinum(II) moiety was selected as the active species for the dendrimer–platinum nanoconjugate since 1,2-diaminocyclohexane is known to serve as a superior inert ligand for the preparation of platinum antitumour compounds and to
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\[
[(\text{DACH})\text{PtCl}_2] \xrightarrow{\text{AgNO}_3/\text{H}_2\text{O}} [(\text{DACH})\text{Pt(OH}_2\text{)}_2]\text{[NO}_3\text{]}_2
\]

\[
[(\text{DACH})\text{Pt(OH}_2\text{)}_2]\text{[NO}_3\text{]}_2 + \text{PAMAM(G4.5)} \rightarrow \text{PAMAM(G4.5)}\text{[(DACH)Pt]}
\]

Scheme 1. Synthesis of PAMAM(G4.5)–[(DACH)Pt] nanoconjugate and a diagram of its structure (other [(DACH)Pt] units omitted for clarity). Filled circle, $\text{−}N(\text{CH}_2\text{CH}_2\text{CONHCH}_2\text{CH}_2)_2$; open circle, COO$^\text{−}$.

contribute to enhanced antitumour activity. Furthermore, the relative bulky size of [(DACH)Pt(OH$_2$)$_2$][NO$_3$]$_2$ and the hydrophobic nature of DACH inhibit the guest drug from penetrating the sterically crowded surface to access the interior of the dendrimer.

The synthesis of the PAMAM(G4.5)–[(DACH)Pt] nanoconjugate and a diagram of a structural model are presented in scheme 1. [(DACH)PtCl$_2$] was prepared from tetrachloroplatinate as previously described (Howell & Richards 1996). This, in turn, was treated with aqueous silver nitrate to generate the corresponding diaquo species. Treatment of this intermediate with a pH 5.0 aqueous solution of PAMAM(G4.5) dendrimer produced the dendrimer-based platinum conjugate with carboxylate groups as the labile ligands at the surface of the dendrimer. The PAMAM(G4.5)–[(DACH)Pt] conjugate was purified by dialysis against deionized water (3500 Da cut-off). The water solubility of the resulting PAMAM(G4.5)–[(DACH)Pt] is extremely good. The resultant sample was checked for purity using thin layer chromatography and dried by lyophilization.

This nanoscale multivalent PAMAM(G4.5)–[(DACH)Pt] conjugate was fully characterized using a variety of spectroscopic, chromatographic and thermal methods. The complexation of surface carboxylate groups by platinum is apparent from the downfield chemical shift of the carboxylate group in the $^{13}$C nuclear magnetic resonance (NMR) spectrum of the PAMAM(G4.5)–[(DACH)Pt], as
shown in figure 2. Generally, the chemical shift of surface carboxylate of a PAMAM half-generation is smaller than that of interior carbonyl groups (Esfand & Tomalia 2001). Here, the strong peak at $\delta$ 175.2 ppm in figure 2a corresponds to the 128 surface carboxylates of PAMAM (G4.5). Upon formation of the PAMAM(G4.5)–[(DACH)Pt] conjugate, this absorption is shifted downfield to 177.7 ppm (figure 2b), reflecting coordination of the surface carboxylate groups with platinum.

The $^1$H NMR spectrum of the conjugate unambiguously shows two characteristic regions representing the ethylene groups ($\delta$ 3.60–2.40) of the PAMAM dendrimer and the cyclohexyl portion of the inert ligand ($\delta$ 1.80–1.20). It is also important to note that the integration of the signal in the two regions indicates that there are about 40 [(DACH)Pt] units coordinated to each dendrimer. That this is a maximum possible loading of [(DACH)Pt] units per dendrimer is apparent from a series of experiments in which the ratio of [(DACH)Pt(H$_2$O)$_2$](NO$_3$)$_2$ to dendrimer was increased well beyond the theoretical maximum of 64 [(DACH)Pt] units. For example, even when the ratio of [(DACH)Pt(H$_2$O)$_2$](NO$_3$)$_2$ to dendrimer was 192 to 1 (well above that required to saturate the carboxylate surface), the dendrimer–Pt conjugate generated contains 39 (DACH)Pt units. The matrix-assisted laser desorption ionization–time of flight

Table 1. MALDI-TOF mass values for six preparations of PAMAM(G4.5)–[(DACH)Pt].

<table>
<thead>
<tr>
<th>batch</th>
<th>molar ratio of G4.5 : [(DACH)Pt]</th>
<th>observed mass value</th>
<th>number of [(DACH)Pt]$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 : 48</td>
<td>29 271</td>
<td>33</td>
</tr>
<tr>
<td>2</td>
<td>1 : 64</td>
<td>30 706</td>
<td>38</td>
</tr>
<tr>
<td>3</td>
<td>1 : 64</td>
<td>31 075</td>
<td>40</td>
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<tr>
<td>4</td>
<td>1 : 72</td>
<td>31 538</td>
<td>41</td>
</tr>
<tr>
<td>5</td>
<td>1 : 128</td>
<td>32 318</td>
<td>44</td>
</tr>
<tr>
<td>6</td>
<td>1 : 192</td>
<td>30 935</td>
<td>39</td>
</tr>
<tr>
<td>pure PAMAM(G4.5)</td>
<td></td>
<td>20 665</td>
<td>—</td>
</tr>
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</table>

$^*$Number of [(DACH)Pt] is calculated on the basis of mass value of pure PAMAM(G4.5).
mass spectrometry (MALDI-TOF MS) data in table 1 support this observation and indicate that an average of 40 \((\text{DACH})\text{Pt}\) moieties per dendrimer are bound to the PAMAM(G4.5) surface. This is in close agreement with the results from \(^1\)H NMR spectra and clearly supports the observation that the maximum loading is approximately 40 \((\text{DACH})\text{Pt}\) moieties per dendrimer molecule. The loading capacity of 40 \((\text{DACH})\text{Pt}\) units onto the nanocarrier PAMAM(G4.5) is consistent with a recent report of a similar limitation for the interaction of PAMAM (G4, amine surface) with an organic ligand (Deng et al. 2005).

The MALDI data also demonstrate that no intermolecular interaction between dendrimer and (diaminocyclohexane)platinum(II) occurred, i.e. no dimmers, trimers, etc., were formed. Thermogravimetric analyses of the PAMAM(G4.5)--\((\text{DACH})\text{Pt}\) conjugate was carried out to further ascertain this value. The conjugate begins to decompose at 173°C and a stable residue of platinum oxide is obtained at 910°C, as shown in figure 3. The mass of the residual oxide corresponds to 24.1 per cent of the initial sample mass. This is in excellent agreement with that expected (24.7%) for a dendrimer–Pt conjugate containing 40 \((\text{DACH})\text{Pt}\) moieties. In poly(acrylamide) gel electrophoresis, the migration difference between PAMAM(G4.5) and \((\text{DACH})\text{Pt}\) conjugate clearly shows single molecules and a few aggregates. Detailed cross-sectional

Figure 3. Thermogravimetric analysis for PAMAM(G4.5)--\((\text{DACH})\text{Pt}\).
measurements on 150 isolated features reflect an average height of 0.40 (±0.16) nm and an average diameter of 7.83 (±1.62) nm. The AFM images document the formation of a novel nanoconjugate of the PAMAM(G4.5) and [(DACH)Pt]. The size of the Pt conjugate is in the nanoscale range as has been observed for other dendrimer species (Jackson et al. 1998). The particles depicted in the AFM image appear to be substantially uniform in size and globular in shape.

The release profile for the active component [(DACH)Pt] was investigated in pH 7.4 phosphate buffer and pH 5.0 phosphate-citrate saline solutions at 37°C by measuring the UV–VIS absorbance of the dialysis saline at 292 nm, which is the characteristic UV–VIS absorbance peak of the [(DACH)Pt] species. The preliminary in vitro release tests showed that most of the active species [(DACH)Pt] is smoothly released from the dendrimer nanocarrier over a period of 24 h. The profile for the release of [(DACH)Pt] from the substrate PAMAM(G4.5) is shown in figure 4. Overall, the release rate is very good with 76 per cent in pH 7.4 buffer and 85 per cent of the available platinum species in pH 5 buffer released in 24 h, respectively. This behaviour suggests that sustainable release should occur inside endosomes at lower pH. The mode of release is probably similar to that observed for other classic platinum drugs in which the labile ligands are carboxylate groups. Generally, under physiological conditions, hydrolysis occurs in a stepwise fashion to form first the monohydrated and subsequently the diaquo platinum species. A study of hydrolysis of oxaliplatin showed that the ring-opening step has a half-life of 16 min and the loss of the oxalate ligand occurs with a half-life of 92 min at 37°C (Jerremalm et al. 2002). In this case, the [(DACH)Pt] units are released from PAMAM(G4.5)–[(DACH)Pt] nanoconjugate with a half-life of 105 min in pH 5 and 310 min in pH 7.4 saline. Therefore, the loss of labile ligand is considerably slower for the PAMAM(G4.5)–[(DACH)Pt] conjugate than that of the similar process for oxaliplatin and suggests a sustainable release of active drug. These observations suggest that the use of PAMAM(G4.5) nanocarrier for [(DACH)Pt] may be used to generate a drug formulation with water solubility, dosage limitations and response characteristics superior to those of classical platinum drugs.

Figure 4. In vitro release profile of [(DACH)Pt] from PAMAM(G4.5)–[(DACH)Pt] in pH 7.4 (filled triangle) and pH 5.0 (filled square) buffer saline.
Clearly, dendritic polymers may be utilized as nanocarriers for the improved delivery of antitumour agents. They offer several advantages over conventional polymers. Most notably, the nanoscale size, uniform shape and high surface functionality of these polymers offer the potential for the generation of ‘multivalent’ drugs which permit the administration of high dosage at low volume and which display enhanced delivery of active agent to the tumour site. In this case, a PAMAM(G4.5) dendrimer has been utilized as a nanocarrier for the generation of a dendrimer–platinum conjugate containing [(DACH)Pt] as the active agent. The dendrimer–platinum conjugate is well defined with approximately 40 [(DACH)Pt] units bound through surface carboxylate groups. This is distinctly unlike an earlier dendrimer–platinum formulation generated from the interaction of a PAMAM(G3.5) dendrimer with cisplatin in which some platinum species are bound at surface groups, some are bound at interior tertiary nitrogen atoms and some are physically trapped as unchanged cisplatin. There are probably at least two reasons for this difference. The first is the more open structure of the smaller generation 3.5 dendrimer. In addition, the large difference in reactivity towards nucleophilic ligands of cisplatin and diaquo(1,2-diaminocyclohexane)platinum(II) probably limits the effectiveness of the interaction of cisplatin with surface groups. The loading of active platinum species is much larger than that achieved for earlier PAMAM(G3.5) dendrimer conjugates and considerably higher than that normally observed for linear polymer platinum conjugates. At the same time, the water solubility and release characteristics are superior to those observed for most polymeric platinum drugs. This conjugate displays the sustained release of active platinum species over a period of 24 h under physiological conditions. The PAMAM(G4.5)–[(DACH)Pt] represents a considerable improvement over previous dendrimer–platinum formulations and offers significant opportunity for clinical use.

3. Conclusions

As in many other areas of drug delivery, polymer–platinum conjugates offer some significant advantages over use of the unmodified drug. Enhanced water solubility, improved dosage protocols, reduced toxicity and more effective utilization of the available active agent are all desirable features provided by the polymer–drug conjugate. In particular, the availability of non-toxic, water-soluble dendritic polymers containing high surface functionality offer great potential for the development of novel, highly effective organoplatinum antitumour formulations of toxicity much lower than that characteristic of simple platinum drugs. For the development of well-behaved dendrimer–platinum conjugates, the dendrimer should be large enough so that surface crowding prevents entry of reactive species into the interior of the molecule and the platinum reagent should be sufficiently reactive so as to be readily bound by ligands at the surface of the dendrimer. These conditions seem to be well met by a generation 4.5 PAMAM dendrimer (128 surface carboxylate groups) and diaquo(1,2-diaminocyclohexane)platinum(II). Presumably, because of the steric crowding, the theoretical maximum number of platinum moieties (64) cannot be attached to the dendrimer surface. In the case of (1,2-diaminocyclohexane)platinum(II) approximately 40 units may be placed at the surface of the dendrimer. This
dendrimer–platinum conjugate displays sustained release of active platinum species over 24 h under physiological conditions. These results offer considerable optimism for the use of dendrimers in the development of more effective but less toxic organoplatinum antitumour agents.

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