Synthesis, characterization, and cytotoxicity of dinuclear platinum-bisphosphonate complexes to be used as prodrugs in the local treatment of bone tumours

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Received 22nd September 2009, Accepted 29th October 2009
First published as an Advance Article on the web 16th November 2009
DOI: 10.1039/b919721d

For over 30 years cisplatin has been one of the most active antitumour agents in clinical use, nevertheless research for overcoming cisplatin toxicity and resistance or for improving its efficacy has never ceased. In this context we have recently proposed dinuclear Pt complexes with bridging geminal bisphosphonates as novel Pt-prodrugs with potential activity at the bone surface after embedment in inorganic matrices and implantation at the tumour site. In the present paper we report the synthesis and full characterization of four new platinum complexes having a dinuclear structure with a bisphosphonate (2-ammonium-1-hydroxyethane-1,1-diyl-bisphosphonate or 3-ammonium-1-hydroxypropane-1,1-diyl-bisphosphonate, AHBP-H and PAM-H, respectively) acting as a bridging ligand between two platinum moieties (cis-[Pt(NH3)2]+, directly related to cisplatin, and [Pt(cis-1,4-DACH)]2+, known to be able to overcome the cisplatin resistance). Moreover, as a preliminary investigation, the in vitro cytotoxicity of the new complexes has been evaluated on a panel of 13 human tumour cell lines including cisplatin- and multidrug-resistant sublines.

Introduction

Cisplatin is one of the most active antitumour agents in current clinical use; apart from being curative against testicular tumours, it is effective against several other cancers such as ovarian, head and neck, cervical, and bladder. Despite its success, treatment with cisplatin is limited by undesirable side effects such as nephrotoxicity, nausea, vomiting, myelosuppression, ototoxicity and neurotoxicity. Moreover, tumour resistance to cisplatin (acquired during cycles of therapy or intrinsic in some types of cancers) is another factor that limits its use.

Since the serendipitous discovery of the antitumoural activity of cisplatin, an intensive research has led to the synthesis of thousands of new platinum compounds with the aim of overcoming cisplatin toxicity and resistance or to improve its efficacy. The use of carrier ligands able to promote the specific accumulation of the drug in target organs or cells has also been exploited as a strategy to overcome the side effects of cisplatin. For instance, platinum complexes containing (aminoalkyl)phosphonic acids have been exploited by the group of Keppler for their potential use as selective platinum drugs for bone tumours. Those authors were inspired by the analogy between these ligands and geminal bisphosphonates (BPs), commercial drugs, which show affinity for bones and other calcified tissues and are in clinical use for the treatment of hypercalcaemia and skeletal metastases.

The combination of the bisphosphonic functionality with the platinum moiety could promote the specific accumulation of the antitumour drug in the bone with consequent significant improvement of the biological effect and reduction of the systemic toxicity. Pharmacological investigations performed on Pt(II) complexes with amino-bis/tris(methylene)phosphonate ligands were a proof of concept demonstrating their superior activity as compared to cisplatin in an orthotopically transplanted rat osteosarcoma model closely resembling the human osteosarcoma. A further lowering of the systemic side effects of a drug could be obtained by local administration directly at the site of the tumour and, in this context, we have recently proposed dinuclear Pt complexes with bridging geminal bisphosphonates (Chart 1) as novel Pt-prodrugs with potentiated activity at the...
bone surface after embedment in inorganic matrices capable to activate them.\(^{22-24}\)

The dinuclear Pt-bisphosphonate complexes were loaded either onto silica xerogels, obtained with the sol–gel method, or onto synthetic biomimetic hydroxyapatite (HA) nanocrystals in order to obtain biocompatible inorganic composite materials capable to act as bone filler materials and to release an active antitumoural Pt-species after local implant at the tumour site. The drug-releasing kinetic from the two inorganic composites was modulated by the presence of Ca\(^{2+}\) ions, which rendered the matrices able to retain the bisphosphonic ligand (this latter has a very high affinity for calcium ions) so that only the antitumoural Pt(en)\(^{2+}\) residue (en = ethylenediamine) was released from the silica xerogels or from the biomimetic HA. Moreover, in a recent paper the cytotoxicity of the Pt-complexes released from the HA were tested towards human cervix carcinoma cells and they resulted to be more cytotoxic than the unmodified complexes\(^{29}\) and active at a concentration very similar to that of pure [PtCl\(_2\)(en)] (the reference compound). It was also demonstrated that the released species from the matrices was [PtCl\(_2\)(en)] or related solvato-species.

The obtained results stimulated us to extend the investigation to the prototype platinum complex effectively used in therapy (cisplatin) and to another complex which is known to be able to overcome the cisplatin-resistance ([PtCl\(_2\)(cis-1,4-DACH)], DACH = diaminecyclohexane). Therefore, we have synthesized four new dinuclear platinum(II) complexes (Chart 2) carrying the am(m)ine ligand(s) mentioned above and two types of aminoalkyl-hydroxybisphosphonate bridging ligands (either 2-ammonium-1-hyroxyethane-1,1-diyl-bisphosphonic (AHBP-H\(_4\)) or 3-ammonium-1-hydroxy-propane-1,1-bisphosphonic acid (pamidronate, PAM-H\(_4\)).

Moreover, as a preliminary investigation, the in vitro cytotoxicity of the new dinuclear complexes has been evaluated on a set of 13 human tumor cell lines including cisplatin- and multidrug-resistant (MDR) sublines.

**Materials and methods**

**Chemicals**

Commercial reagent grade chemicals and solvents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and used without further purification.

**Instrumental measurements**

Mass spectrometry: electrospray ionisation mass spectrometry (ESI-MS) was performed with an electrospray interface and an ion trap mass spectrometer (1100 Series LC/MSD Trap system Agilent, Palo Alto, CA) using H\(_2\)O as solvent.

NMR spectra were recorded on Bruker Avance DPX instruments operating at 300 or 600 MHz \((^1H)\). Standard pulse sequences were used for \(^1H\), \(^{31}P\)\(^(^1H)\) (121.5 MHz), and \(^{195}Pt\)\(^(^1H)\) (64.5 MHz) 1D spectra. Chemical shifts are given in ppm. \(^1H\) chemical shifts were referenced to internal sodium 3-(trimethylsilyl)propionate (TSP), \(^{31}P\) chemical shifts were referenced to external H\(_3\)PO\(_4\) (85% w/w), and \(^{195}Pt\) chemical shifts were referenced to external K\(_2\)[PtCl\(_4\)] in D\(_2\)O fixed at \(-1628\) ppm. Elemental analyses were carried out with a Hewlett Packard 185 C, H, and N analyzer.

A Crison Micro-pH meter Model 2002 equipped with Crison micro-combination electrodes (5 and 3 diameter) and calibrated with Crison standard buffer solution at pH 2.00, 4.01, 7.02, and 9.26 was used for pH measurements. The pH readings for D\(_2\)O solution are indicated as pH* values and are uncorrected for the effect of deuterium on glass electrodes.\(^{26}\)

**Synthesis of the bisphosphonic acids**

Neutral 2-ammonium-1-hyroxyethane-1,1-diyl-bisphosphonic acid (AHBP-H\(_4\))\(^{23}\) and 3-ammonium-1-hydroxy-propane-1,1-bisphosphonic acid (pamidronate, PAM-H\(_4\))\(^{27}\) were prepared following procedures reported in previous papers. The elemental analyses and the spectroscopic characteristics of the synthesized complexes were in agreement with those of the starting bisphosphonic acid.

**Chart 2** Sketches of the new dinuclear Pt-bisphosphonate complexes. AHBP-H = 2-ammonium-1-hyroxyethane-1,1-diyl-bisphosphonic; PAM-H = 3-ammonium-1-hydroxy-propane-1,1-bisphosphonate (pamidronate).
geminal bisphosphonates were consistent with the data reported in the literature.

**Synthesis of the platinum compounds**

\[ [\text{PtCl}_2(cis-1,4-DACH)]_2^{2+}, [\text{Pt(OSO}_3)(cis-1,4-DACH)(OH)_2]_2^{2+} \] and \( cis-[\text{Pt} (\text{NH}_3)_2]^{2+} \) were prepared as already reported. The elemental analyses and the spectroscopic features of the Pt precursor complexes were consistent with the data reported in the literature.

**Preparation of \( cis-[\text{Pt} (\text{OSO}_3)(\text{NH}_3)_2(\text{OH})_2] \).** \( cis-[\text{Pt} (\text{NH}_3)_2] \) (0.550 g, 1.14 mmol) was suspended in water (20 mL) and treated with a solution of Ag\(_2\)SO\(_4\) (0.356 g, 1.140 mmol in 5 mL H\(_2\)O). The obtained suspension was stirred for 24 h in the dark meanwhile a yellow solid (AgI) precipitated from the solution. The precipitate was removed by filtration through Celite and the solution was evaporated to dryness under reduced pressure at 50 °C. The yellow residue was the desired \( cis-[\text{Pt} (\text{OSO}_3)(\text{NH}_3)_2(\text{OH})_2] \) compound (0.344 g, 88% yield). Anal. calcd for \( (\text{H}_8\text{N}_2\text{O}_5\text{Pt})_2 \): H 2.34%, N 6.96%. Found: H 2.30%, N 6.85%.

**Preparation of \( [\text{cis-Pt}(\text{NH}_3)_2]_2(\text{AHBP-H})\text{HSO}_4 \) (1A).** 2-Ammonium-1-hydroxyethane-1,1-diy-bisphosphonic acid (AHBP-H\(_2\)) (0.75 g, 0.339 mmol) was dissolved in H\(_2\)O (15 mL) and the resulting solution was first partially neutralized with Ba(OH)\(_2\):8H\(_2\)O (0.315 g, 0.339 mmol) and then treated with a solution of \( cis-[\text{Pt} (\text{OSO}_3)(\text{NH}_3)_2(\text{OH})_2] \) (0.236 g, 0.678 mmol, in 15 mL of H\(_2\)O). The resulting suspension was kept under stirring at room temperature for 40 h meanwhile a white solid (BaSO\(_4\)) separated. After removal of the solid, the filtered solution was concentrated to ca. 2 mL and then treated with methanol which caused the precipitation of a yellow-greenish solid which was the desired product having HSO\(_4\) as counter-ion. The precipitate was left standing at 4 °C for 2 h and then isolated by filtration of the solution, washed with methanol (2.0 mL), and dried under vacuum. Obtained 0.162 g (62% yield). Anal. calcd for \( (\text{C}_3\text{H}_20\text{N}_5\text{O}_7\text{P}_2\text{Pt}_2\text{S})_2 \): C 4.19, H 0.24, N 3.26%. Found: C 4.09, H 0.24, N 3.25%.

**Preparation of \( [\text{cis-Pt}(\text{NH}_3)_2]_2(\text{AHBP-H})\text{HSO}_4 \) (1B).** AHBP-H\(_2\) (0.026 g, 0.118 mmol) was dissolved in H\(_2\)O (15 mL) and treated with Ba(OH)\(_2\):8H\(_2\)O (0.037 g, 0.118 mmol) and then with a solution of \( cis-[\text{Pt} (\text{OSO}_3)(1,4-DACH)(OH)_2] \) (0.109 g, 0.464 mmol) was added to lyophilized Pt(OH)\(_2\):8H\(_2\)O (0.146 g, 0.464 mmol) and then with a solution of \( cis-[\text{Pt} (\text{OSO}_3)(\text{NH}_3)_2(\text{OH})_2] \) (0.318 g, 0.928 mmol in 15 mL of H\(_2\)O). The resulting suspension was kept under stirring at room temperature for 24 h. The solid was isolated by filtration of the solution and dried under vacuum. Obtained 0.143 g (40% yield). Anal. calcd for \( (\text{C}_14\text{H}_34\text{N}_5\text{O}_7\text{P}_2\text{Pt}_2\text{S})_2 \): C 16.72, H 3.97, N 6.96%. Found: C 16.61, H 3.98, N 6.85%.

**Fig. 1** 1H and 31P (\( H \))-NMR spectra of compound 1A in D\(_2\)O (\( pH^* = 7 \)). The asterisk indicates the residual solvent peak. Numbering of protons is reported in italics.

(2 H, t, C(2)H\(_2\)), 3.13 ppm (4 H, s, C(a)H), 1.17 ppm (16 H, C(b)H\(_2\)). 31P (\( H \))-NMR (D\(_2\)O, \( pH^* = 7 \))): 37.20 ppm. ESI-MS: calcd for \( \text{C}_4\text{H}_8\text{N}_2\text{O}_5\text{Pt}_2 \): 676.9 ppm. Found, m/z (% relative to the base peak): 675.9 (100) [M\(^+\)].

**Preparation of \( [\text{cis-Pt}(\text{NH}_3)_2]_2(\text{PAM-H})\text{HSO}_4 \) (2A).** 3-Ammonium-1-hydroxy-propane-1,1-bisphosphonic acid (PAM-H\(_2\)) (0.109 g, 0.464 mmol) was dissolved in H\(_2\)O (15 mL) and treated first with Ba(OH)\(_2\):8H\(_2\)O (0.146 g, 0.464 mmol) and then with a solution of \( cis-[\text{Pt} (\text{OSO}_3)(\text{NH}_3)_2(\text{OH})_2] \) (0.318 g, 0.928 mmol in 15 mL of H\(_2\)O). After stirring for 48 h at room temperature the obtained white solid (BaSO\(_4\)) was removed and the filtered solution was concentrated to a volume of ca. 2 mL. The pH of the concentrated solution was lowered to 1 by addition of H\(_2\)SO\(_4\) (1 M) and then acetone was added to induce precipitation of a yellow-greenish solid which was left standing at 4 °C for 2 h. The solid was isolated by filtration of the solution and dried under vacuum. Obtained 0.143 g (40% yield). Anal. calcd for \( (\text{C}_14\text{H}_34\text{N}_5\text{O}_7\text{P}_2\text{Pt}_2\text{S})_2 \): C 16.72, H 3.97, N 6.96%. Found: C 16.41, H 4.27, N 6.85%.

\[ 31P (\text{H})\text{-NMR (D}_2\text{O, pH}^* = 5): 39.81 \text{ ppm. ESI-MS: calcd for C}_8\text{H}_9\text{N}_2\text{O}_5\text{Pt}_2\text{P}_2\text{H}^+: 690.32. \text{ Found, m/z (% relative to the base peak): 690.0 (100) [M}^+\].


Preparation of \[[\text{Pt}(\text{cis}-1,4\text{-DACH})]_2(\text{PAM}-\text{H})]\text{HSO}_4\ (2B). A solution of \[\text{Pt}([\text{OSO}_3])(\text{cis}-1,4\text{-DACH})(\text{OH}_2)\] (0.111 g, 0.233 mmol in 15 mL of H2O) was added to a white suspension obtained by mixing PAM-H4 (0.025 g, 0.106 mmol) with Ba(OH)2·8H2O (0.033 g, 0.106 mmol) in 25 mL of H2O. The final suspension was kept under stirring for 48 h at room temperature meanwhile a white solid (BaSO4) separated. This latter was removed by filtration of the solution and the filtrate was concentrated to ca. 3 mL and acidified to pH = 1.0 by addition of H2SO4 (1 M). The resulting acidic solution was treated with acetone which caused the precipitation of the desired product, having HSO4- as counter-ion, as a white solid. The reaction mixture was kept at 4 °C for 2 h, then the solid was isolated by filtration of the solution, washed with acetone and dried under vacuum. Obtained 0.065 g (65.1% yield). Anal. calcd for \[[\text{Pt}(\text{cis}-1,4\text{-DACH})]_2(\text{PAM}-\text{H})]\text{HSO}_4\cdot6\text{H}_2\text{O}\ (\text{C}_{15}\text{H}_{49}\text{N}_{5}\text{O}_{17}\text{P}_{2}\text{Pt}_{2}\text{S})\): C 17.06, H 4.68, N 6.63%. Found: C 16.98, H 4.68, N 6.47%.

NMR experiment at different pH and calculation of the pK_a values

\[
\text{PAM-H}_4\ (\text{ca.} 0.006 \text{ mmol}), \ [\{\text{cis-Pt(NH}_3)_2\}(\text{AHBP-H})]\text{HSO}_4\ (1\text{A}, \text{ca.} 0.0018 \text{ mmol}), \ [\{\text{Pt}(\text{cis}-1,4\text{-DACH})\}(\text{AHBP-H})]\text{HSO}_4\ (1\text{B}, \text{ca.} 0.0015 \text{ mmol}), \text{ or } [\{\text{Pt}(\text{cis}-1,4\text{-DACH})\}(\text{PAM-H})]\text{HSO}_4\ (2\text{B}, \text{ca.} 0.0015 \text{ mmol}) \text{ were dissolved in 0.7 mL of D}_2\text{O and placed into an NMR tube. Before recording the } ^{31}\text{P}\{^1\text{H}\}-\text{NMR spectra...}
\]
Fig. 5. 1H (top), 31P{1H} (middle), and 195Pt (bottom) NMR spectra of compound 2B in D2O (pH* = 3). The asterisk indicates the residual solvent peak. Numbering of protons is reported in italics.

the pH* of the samples (measured by using a 3 mm diameter electrode for NMR tubes) was adjusted to the desired value by addition of either D2SO4 (0.9 M) or NaOD (2.0 or 0.5 M). No control of the ionic strength was performed. The 31P chemical shifts were plotted against pH (Fig. 3) and different sections of the pH titration curve were fitted to the Henderson–Hasselbach equation using the program Kaleidagraph.

In vitro cytotoxicity assays

The cytotoxicity studies have been performed using cisplatin as reference compound (Sigma Chemical Co., St Louis, MO, USA). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and doxorubicin were obtained from Sigma. The dinuclear platinum complexes were dissolved in physiological solution just before the experiment.

Cell cultures

Human lung (A549), ovarian (A2780, A2780 ADR) and colon (HT-29) carcinoma along with melanoma (A375) cell lines were obtained from ATCC, Rockville, MD, USA. Human ovarian carcinoma cell lines 2008, derived from an untreated patient with an ovaric adenocarcinoma, and C13*, cisplatin-resistant subline of the 2008 cell line, were kindly provided by Prof. G. Marverti (Dept. of Biomedical Science of Modena University, Italy). A431 and A431/Pt are cisplatin-sensitive and -resistant human cervical carcinoma cells, respectively, and were kindly supplied by Prof. F. Zunino (Division of Experimental Oncology B, Istituto Nazionale dei Tumori, Milan, Italy). LoVo human colon-carcinoma cell line and its multidrug-resistant sub-line (LoVo MDR) were kindly provided by Prof. F. Majone (Dept. of Biology of Padova University, Italy). MCF-7 and its multidrug-resistant phenotype, MCF-7 ADR, are human breast carcinoma cell lines kindly provided by Prof. N. Colabufo (Dept. Farmaco-Chimico of Bari University, Italy). Cell lines were maintained in the logarithmic phase at 37 °C in a 5% carbon dioxide atmosphere using the following three culture media containing, in addition to 10% foetal calf serum (Euroclone, Milan, Italy), antibiotics (50 units mL\(^{-1}\) penicillin and 50 μg mL\(^{-1}\) streptomycin, Euroclone) and 2 mM l-glutamine (Euroclone): (i) RPMI-1640 medium (Euroclone) with 25 mM HEPES buffer for 2008, C13*, A431, A431/Pt, MCF-7, MCF-7 ADR, A2780 and A2780 ADR cells; (ii) F-12 HAM’S (Sigma Chemical Co.) for A549, LoVo and LoVo MDR cells; (iii) D-MEM medium (Euroclone) for HT-29 and A375 cells. LoVo MDR, MCF-7 ADR, LoVo MDR, and A2780 ADR culture medium also contained 0.1 μg mL\(^{-1}\) doxorubicin.

MTT test

The growth inhibitory effect towards tumour cell lines was evaluated by means of MTT (tetrazolium salt reduction) assay. Briefly, 3–5 × 10\(^{3}\) cells per well, depending upon the growth characteristics of the cell line, were seeded in 96-well microplates in growth medium (100 μL) and then incubated at 37 °C in a 5% carbon dioxide atmosphere. After 24 h, the medium was removed and replaced by a fresh one containing the compound to be studied at the appropriate concentration. Triplicate cultures were established for each treatment. After 48 or 72 h, each well was treated with 10 μL of a 5 mg mL\(^{-1}\) MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) saline solution, and after 5 h of incubation, 100 μL of a sodium dodecylsulfate (SDS) solution in HCl 0.01 M was added. Following overnight incubation, the inhibition of cell growth induced by the tested complexes was evaluated by measuring the absorbance of each well at 570 nm using a Bio-Rad 680 microplate reader. Mean absorbance for each drug dose was expressed as a percentage of the control untreated well absorbance and plotted vs. drug concentration. IC\(_{50}\) values represent the drug concentrations that reduced the mean absorbance at 570 nm to 50% of those in the untreated control wells.

Results and discussion

Synthesis and characterization of dinuclear platinum complexes with AHBP-H

The syntheses of the dinuclear platinum complexes \([\{\text{cis-Pt(NH}_3)_2\}\{\text{AHBP-H}\}]^+ (1A) and \([\text{Pt(cis-1,4-DACH)}]\{\text{AHBP-H}\}]^+ (1B), both having the bridging 2-ammonium-1-hydroxyethane-1,1-diyl-bisphosphonate (AHBP-H), were very similar. 1A was prepared starting from the barium salt Ba[AHBP-H\(_2\)] and two equivalents of cis-[Pt(OSO\(_3\)](NH\(_3\))\(_2\)(OH\(_2\))]\(^{35,36}\). The
elemental analysis of 1A was in accordance with the presence of one aminobisphosphonate and two [cis-Pt(NH₃)₂]⁺ residues. The ¹H-NMR spectrum (Fig. 1) shows a multiplet centered at 3.24 ppm assigned to the BP methylene protons. This resonance is slightly downfield (Δδ = 0.06 ppm) with respect to that of the free ligand and is the AA’ portion of a AA’XX’ spin system where AA’ represent the methylenic protons and XX’ are the two P atoms. In our experimental conditions the determined coupling constants were: JH₃P = JH₅P = 20.28 Hz and JH₃P⁺ = JH₅P⁺ = 1.65 Hz.

The ³¹P{¹H}-NMR spectrum (Fig. 1) shows a singlet with unresolved platinum satellites (platinum satellites usually broaden beyond detection by chemical shift anisotropy relaxation of Pt especially at high magnetic field strengths, as those used in our experiment) falling at 37.09 ppm assigned to the two phosphorus atoms of the bisphosphonic group, which are magnetically equivalent and shifted at lower field (Δδ = 19.98 ppm) with respect to that of the free AHBP ligand at the same pH*. Such a downfield shift of the phosphorus nuclei is characteristic of phosphonate that of the free AHBP ligand at the same pH*. This downfield shift is due to the lack of symmetry with respect to the coordination plane. The two platinum coordinated oxygen atoms cannot undergo neither protonation nor deprotonation steps by changes of pH* and therefore the ³¹P chemical shift remains constant. Unfortunately, compound 1A underwent structural changes at pH* higher than 9.0 with formation of new product(s) having non-equivalent phosphorus atoms and by chemical shift anisotropy relaxation of ¹⁹⁵Pt. The ³¹P{¹H}-NMR spectrum (Fig. 1) shows also a triplet centered at 3.13 ppm assigned to the aminic protons in water solution.

The synthesis of [(Pt(cis-1,4-DACH))₂(AHBP-H)]HSO₄ (1B) was performed similarly, starting from the barium salt Ba[AHBP-H₄]₂ and two equivalents of [Pt(OSO₃)(cis-1,4-DACH)(OH₂)]⁺. The elementary analysis of 1B was in accordance with one aminobisphosphonate per two [Pt(cis-1,4-DACH)]₂⁺ moieties. The ¹H-NMR spectrum (Fig. 2) shows two multiplets placed at 5.38 and 5.02 ppm assigned to the amine protons of coordinated cis-1,4-DACH (geminal protons of each amine group are made non-equivalent by the lack of symmetry with respect to the coordination plane). The acidity conditions (pH⁺ = 3) slow down the exchange of the amine protons with the deuterium of the solvent, allowing detection of the amine protons in water solution.

The ¹H-NMR spectrum shows also a triplet centered at 3.37 ppm assigned to the methylenic protons of the bisphosphonic. These protons are shifted at higher field (Δδ = -0.08 ppm) as compared to the free ligand at pH⁺ = 3. The signals at 3.13 ppm and 1.77 ppm were assigned to the methine and methylenic protons of coordinated cis-1,4-DACH, respectively. The ³¹P{¹H}-NMR spectrum shows a signal at 39.70 ppm assigned to the two phosphorus nuclei which are magnetically equivalent and shifted at lower field (Δδ = 20.74 ppm) with respect to the free AHBP ligand at the same pH*.

For a full characterization of complex 1B, the ¹⁹⁵Pt-NMR spectrum was also recorded (Fig. 2). Two broad signals at ~1582 and ~1595 ppm were observed. These signals are broadened by the quadrupolar effects of ¹⁴N (99.6% abundance) from the amine ligands and by chemical shift anisotropy relaxation of ¹⁹⁵Pt. The ¹⁹⁵Pt signals are shifted at lower field with respect to the starting [Pt(cis-1,4-DACH)(H₂O)]₂⁺ substrate. The same trend has already been observed in platinum complexes with a diamine and a dicarboxylic ligand with respect to the species with a diamine and two aqua ligands.³⁹,⁴⁰,⁴¹

The presence of only one signal in the ³¹P{¹H} spectrum and of two signals in the ¹⁹⁵Pt spectrum fully supports the structure in which one bisphosphonate unit bridges two platinum atoms in a W conformation. The plane of symmetry passing through the two platinum atoms and perpendicular to the coordination plane renders the two phosphorus atoms chemically equivalent. In contrast, the absence of a plane of symmetry passing through the P–C(1)–P atoms and perpendicular to the coordination plane (presence of two different substituents, –OH and –CH₃NH₃⁺, on the C(1) carbon atom), renders the two platinum atoms non-equivalent. Considering that the protonated amino group should shift at lower field the nearby Pt atom, the signal falling at ~1595 ppm could be tentatively assigned to the Pt atom on the side of the –OH group. The two aminic groups and the bifunctional bisphosphonate saturate completely the coordination of each platinum center excluding the hydroxylic group from coordination. This is not the case for complexes with other metal ions in which the bisphosphonate can be tricoordinated contributing two oxygen atoms from the phosphate groups and one from deprotonated hydroxyl group to the same metal ion.⁴²

Also for 1B we performed ³¹P{¹H}-NMR experiments at different pH*. As already observed for compound 1A, the ³¹P chemical shift remains constant in the pH* range from 1 to 9, indicating that all the bisphosphonate hydroxyk oxygen atoms are involved in coordination to the platinum atom. As for complex 1A, the titration was stopped at pH* 9 since 1B started to rearrange. This is probably due to coordination to platinum of the deprotonated amine group.

Synthesis and characterization of dinuclear platinum complexes with PAM-H

Pamidronate (3-ammonium-1-hydroxy-propane-1,1-bisphosphonic acid, PAM-H₄) was synthesized according to a procedure already reported by some of us.⁴⁷ We have measured the pKₐ values of the various deprotonation steps of pamidronate by recording ³¹P{¹H}-NMR spectra at different pH*. The plot of the ³¹P chemical shift as a function of pH* is given in Fig. 3.

The titration curve shows four inflexion points corresponding to the various deprotonation steps (Scheme 1). We did not determine the constant related to the first deprotonation step (pKₐ₁ in Scheme 1) because this requires a hydrogen ion concentration beyond reach in our experimental conditions. As the pH* was increased from ca. 0 to 3 we observed a shift to higher field of the ³¹P chemical shift with an inflexion point (calculated by fitting the points between pH* 0 and 3 to the Henderson–Hasselbach equation) falling at pH* 1.69. This pH value corresponds to the equilibrium constant of the second deprotonation step (pKₐ₂, Scheme 1). As already found for the ligand AHBP-H₄, ²²
deprotonation of a phosphonate –OH shields the phosphorus nucleus directly attached to it and deshields the distal phosphonate group present in the molecule. The former is called “direct effect” while the latter is called “remote effect”. Similarly, we observed the second deprotonation step and a shielding of the 31P nuclei with an inflexion point falling at pH* 6.23. This value corresponds to pK_{a3} and represents the second deprotonation constant of one phosphonate group. The deshielding of the 31P nuclei indicates that in this case the remote effect prevails over the direct effect. By further increasing the pH* from 3 to 7, we observed a deshielding of the phosphorus nuclei with an inflexion point falling at pH* 10.1 (corresponding to pK_{a4}, Scheme 1) and then a deshielding of the phosphonate nuclei with an inflexion point occurring at pH* 12.5 (corresponding to the deprotonation of the amine group, pK_{a5}, Scheme 1). As for AHBP-H₂ and analogous aminobisphosphonates, the deprotonation of the amine group of PAM-H₂, and hence the loss of the zwitterionic character, induces a great deshielding of the phosphorous nuclei.⁴⁺⁴³

Pamidronate was then used for the synthesis of the dinuclear complexes [{(cis-Pt(NH₃)₂}₂(PAM-H)]⁺ (2A) and [{(cis-1,4-DACH)}₂(PAM-H)]⁺ (2B) in conditions very similar to those used for the syntheses of compounds 1A and 1B.

The complex [{(cis-Pt(NH₃)₂}₂(PAM-H)]⁺ (2A) was characterized via elemental analysis and ¹H and ³¹P{¹H}-NMR. The ¹H-NMR spectrum (Fig. 4) shows a broad signal at 4.35 ppm assigned to the ammine protons of the platinum moieties. The triplet at 3.29 ppm, which is slightly shielded (Δδ = -0.06) with respect to the analogous signal of the free PAM at the same pH*, was assigned to the methyllic protons in position 3 of coordinated PAM-H. The multiplet falling in the range from 2.25 to 2.17 ppm was assigned to the methyllic protons in position 2 of the coordinated PAM-H.

The ³¹P{¹H}-NMR spectrum (Fig. 4) shows a singlet with unresolved Pt satellites falling at 39.81 ppm and assigned to the phosphorous atoms of the two bisphosphonic groups which are shifted at lower field (Δδ = 21.11) with respect to the analogous signal of free PAM at the same pH*. Because of the low solubility in water of this complex it has not been possible to record a ¹⁹⁵Pt-NMR spectrum as well as to perform ³¹P{¹H}-NMR experiments at different pH*.

Also, the synthesis of [{(cis-1,4-DACH)}₂(PAM-H)]⁺ (2B) was performed starting from the barium salt Ba[PAM-H₂] and two equivalents of [Pt(OSO₃)(cis-1,4-DACH)H₂O]. The elemental analysis of 2B confirmed the formation of a dinuclear species containing one bisphosphonate per two [Pt(cis-1,4-DACH)]⁺ residues. The characterization of 2B was performed by ¹H, ³¹P{¹H}, and ¹⁹⁵Pt-NMR. The ¹H-NMR spectrum (Fig. 5) shows two signals at 5.32 and 4.95 ppm assigned to the amninic protons of coordinated cis-1,4-DACH. The triplet at 3.31 ppm was assigned to the protons of the methylenic group in position 3 of coordinated PAM-H. These protons are shifted at higher field with respect to the free ligand (Δδ = -0.04 ppm). The broad singlet at 3.14 ppm was assigned to the methylic protons of coordinated cis-1,4-DACH. The multiplet (a triplet of triplets) falling in the range 2.27–2.16 ppm was attributed to the methylic protons in position 2 of coordinated PAM-H. Finally, the broad signal falling at 1.85 ppm was assigned to the methylic protons of the cis-1,4-DACH ligands.

The ³¹P{¹H}-NMR spectrum (Fig. 5) shows a signal at 39.90 ppm assigned to the two P atoms of coordinated PAM-H, which are magnetically equivalent and shifted at lower field (Δδ = 21.2) with respect to the free ligand at the same pH*. The ¹⁹⁵Pt-NMR spectrum (Fig. 5) shows only one broad signal at -1599 ppm (a chemical shift characteristic of a Pt atom in a PtN₂O₂ coordination environment).⁴⁴ Differently from complex 1B, 2B showed only one ¹⁹⁵Pt signal, most probably because the longer chain of the amninic substituent prevents this group from exerting its deshielding effect on the proximal Pt atom.

For compound 2B, ³¹P{¹H}-NMR spectra in the pH* range from 0 to 13 were recorded. Differently from the case of free PAM ligand, a constant ³¹P chemical shift (data not shown) was observed confirming the involvement of all phosphonate hydroxyl oxygen atoms in coordination to platinum. Differently from complexes 1A and 1B, compound 2B was found to be stable even at pH*
values higher than 9. Most probably, the amonic group of PAM-H, even after deprotonation, is unable to coordinate to platinum because of the long alkyl chain. This explanation is supported by the observation of a single resonance for platinum while in the analogous complex 1B, with a shorter alkyl chain, two distinct platinum resonances were observed. Extending the $^{31}$P[$^1$H] NMR titration above pH$^+$ 9, an inflexion point, corresponding to the deprotonation of the amonic group of coordinated PAM-H, was observed at pH$^+$ ca. 11. It is to be noted that coordination of PAM-H to platinum increases the acidity of the amonic group even if this latter group is not able to bind to the metal.

**Cytotoxic activity**

The *in vitro* cytotoxicity of the four new dinuclear complexes has been evaluated in 13 human tumour cell lines of multiple origins. The panel includes: LoVo and HT-29 (colorectal adenocarcinoma cells), LoVo-MDR (Multi Drug Resistant colorectal adenocarcinoma cells), A549 (non small lung adenocarcinoma cells), 2008 and A2780 (ovarian adenocarcinoma cells), A2780-ADR (Multi Drug Resistant ovarian adenocarcinoma cells), C13* (cisplatin resistant ovarian adenocarcinoma cells), MCF-7 (mammary carcinoma cells), MCF-7-ADR (Multi Drug Resistant mammary adenocarcinoma cells), A375 (malignant melanoma cells), A431 (cervical carcinoma cells), and A431/Pt (cisplatin resistant cervical carcinoma cells). Under the same experimental conditions, cisplatin was also evaluated as reference metallodrug.

Cytotoxic activity

The IC$_{50}$ values, calculated from the dose–survival curves obtained after 72 h of drug treatment from the MTT test, are reported in Table 1.

The less active complexes are those containing the [cis-Pt(NH$_3$)$_2$]$^2+$ residues (1A and 2A), which show mean IC$_{50}$ values of 50.34 (29.52–79.71) and 30.75 (14.83–49.83) μM, respectively, which are about 6 and 3.5 times higher than that of cisplatin (8.32 μM). In contrast, the two complexes containing [Pt(cis-1,4-DACH)]$^+$ (1B and 2B) show a better cytotoxic activity with mean IC$_{50}$ values of 22.46 (6.25–66.65) and 13.57 (5.01–26.19) μM.

In the panel, two pairs of cell lines have been selected for their sensitivity and resistance to cisplatin: A431 and A431/Pt respectively. In the panel, two pairs of cell lines have been selected for their sensitivity and resistance to cisplatin: A431 and A431/Pt respectively. In the panel, two pairs of cell lines have been selected for their sensitivity and resistance to cisplatin: A431 and A431/Pt respectively. In the panel, two pairs of cell lines have been selected for their sensitivity and resistance to cisplatin: A431 and A431/Pt respectively. In the panel, two pairs of cell lines have been selected for their sensitivity and resistance to cisplatin: A431 and A431/Pt respectively. In the panel, two pairs of cell lines have been selected for their sensitivity and resistance to cisplatin: A431 and A431/Pt respectively. In the panel, two pairs of cell lines have been selected for their sensitivity and resistance to cisplatin: A431 and A431/Pt respectively.

Although cisplatin resistance is multifactorial, the main molecular mechanisms involved in Pt resistance of C13* and A431/Pt have been identified. In particular, in C13* cells resistance is correlated to reduced cellular drug uptake, high cellular glutathione and thioredoxin reductase levels, and enhanced repair of DNA damage. In A431/Pt cells, resistance is due to defect in drug uptake and to decreased levels of proteins involved in DNA mismatch repair (MSH2), which causes an increased tolerance to cisplatin-induced DNA damage. By comparing the RF values (where RF is the resistance factor and is defined as the ratio between IC$_{50}$ values calculated for the resistant cells and those arising from the sensitive ones), it appears that all new compounds with bisphosphonate ligands are able to overcome the cisplatin-resistance (mean RF values of 1.25 (1A and 2A), 0.92 (1B and 2B), and 1.73 (cisplatin) for A431 and A431/Pt cells, and of 1.12 (1A and 2A), 0.93 (1B and 2B), and 10.46 (cisplatin) for 2008 and C13* cells). The two complexes containing [Pt(cis-1,4-DACH)]$^+$ (1B and 2B) are slightly better than those containing [cis-Pt(NH$_3$)$_2$]$^2+$ in overcoming the cisplatin resistance. This feature becomes much

**Table 1. In vitro antitumour activity**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ μM ± S.D.</th>
<th>A431</th>
<th>A431/Pt</th>
<th>2008</th>
<th>C13*</th>
<th>HT-29</th>
<th>A431</th>
<th>A431/Pt</th>
<th>2008</th>
<th>C13*</th>
<th>HT-29</th>
<th>A431</th>
<th>A431/Pt</th>
</tr>
</thead>
<tbody>
<tr>
<td>1B</td>
<td>28.43 ± 0.04</td>
<td>42.04 ± 0.04</td>
<td>14.20 ± 0.04</td>
<td>42.04 ± 0.04</td>
<td>14.20 ± 0.04</td>
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<td>14.20 ± 0.04</td>
<td>42.04 ± 0.04</td>
<td>14.20 ± 0.04</td>
</tr>
<tr>
<td>2B</td>
<td>19.34 ± 0.40</td>
<td>23.87 ± 0.40</td>
<td>19.34 ± 0.40</td>
<td>23.87 ± 0.40</td>
<td>19.34 ± 0.40</td>
<td>23.87 ± 0.40</td>
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<td>19.34 ± 0.40</td>
<td>23.87 ± 0.40</td>
<td>19.34 ± 0.40</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>1.17 ± 0.39</td>
<td>1.59 ± 0.39</td>
<td>1.17 ± 0.39</td>
<td>1.59 ± 0.39</td>
<td>1.17 ± 0.39</td>
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<td>1.17 ± 0.39</td>
</tr>
</tbody>
</table>

Antitumour activity via MTT test. IC$_{50}$ values have been evaluated by probit analysis ($p < 0.05$, test of $Z$). Cells have been treated for 72 h with increasing concentration of tested compound. Resistant factor (RF) is defined as IC$_{50}$ resistant/parent line.
Table 2  Cisplatin cross-resistance profiles

<table>
<thead>
<tr>
<th>Compound</th>
<th>A431</th>
<th>A431/Pt</th>
<th>RF</th>
<th>2008</th>
<th>C13*</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>53.23±1.23</td>
<td>98.56±2.38</td>
<td>1.85</td>
<td>87.41±1.99</td>
<td>169.42±1.59</td>
<td>1.93</td>
</tr>
<tr>
<td>2A</td>
<td>40.43±1.04</td>
<td>71.31±2.27</td>
<td>1.76</td>
<td>54.11±1.46</td>
<td>98.12±2.48</td>
<td>1.81</td>
</tr>
<tr>
<td>1B</td>
<td>32.13±2.03</td>
<td>30.13±1.49</td>
<td>0.96</td>
<td>29.24±1.73</td>
<td>33.63±2.14</td>
<td>1.15</td>
</tr>
<tr>
<td>2B</td>
<td>15.62±1.56</td>
<td>14.38±1.42</td>
<td>0.85</td>
<td>16.34±1.32</td>
<td>17.80±1.37</td>
<td>1.08</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>22.06±2.21</td>
<td>57.76±3.94</td>
<td>2.61</td>
<td>5.23±1.43</td>
<td>89.18±2.08</td>
<td>17.05</td>
</tr>
</tbody>
</table>

Antitumour activity via MTT test. IC₅₀ values have been evaluated by probit analysis (p < 0.05, test of χ²). Cells have been treated for 48 h with increasing concentration of tested compounds. RF (Resistant factor) is defined as IC₅₀ resistant/parent line.

more evident by performing chemosensitivity tests at 48 h drug exposure (Table 2). Indeed, mean RF values for 1B and 2B appear roughly 3 and 15 times lower that those calculated for cisplatin in cervical and ovarian carcinoma cells, respectively.

The human tumour cell line panel also includes three pairs of sensitive and multi-drug resistant cell lines (LoVo and LoVo MDR, MCF7 and MCF-7 ADR, and A2780 and A2780-ADR; the last three pairs in Table 1). In LoVo/MDR cells, resistance to doxorubicin is associated with an over expression of multispecific drug transporters, such as the 170 kDa P-glycoprotein.

The multi-drug resistance of breast MCF-7 ADR and ovarian A2780 ADR cancer cells is associated to an over expression of different species of specific ATP-binding cassette transporters and membrane proteins. It appears that, while phosphate compounds 1A and 2A are able to overcome multi-drug resistance in all MDR sublines [mean RF values of 1.09 (LoVo and LoVo-MDR), 0.82 (MCF7 and MCF7-ADR), and 1.37 (A2780 and A2780-ADR)], compounds 1B and 2B circumvent MDR only in the colorectal cell line pair, being the RF value 1.25 in LoVo/LoVo-MDR, 2.11 in MCF7/MCF7-ADR, and 3.09 in A2780/A2780-ADR cells. This behaviour could be due to a different expression of MDR-related proteins in colorectal cancer in comparison with breast and ovarian cancers, the latter notoriously possessing similar genetic patterns.

Furthermore, our preliminary pharmacological investigation indicates that the dinuclear complexes with pamidronate (second and fourth row in Table 1) are more active than those with AHBP-H. This could be due to the greater stability of the PAM complexes at higher pH and the scarce tendency of the amine group in PAM to coordinate to platinum by displacing a phosphate oxygen or a carrier amine ligand. The slightly greater activity of compounds B, containing [Pt(cis-1,4-DACH)]²⁺ with respect to compounds A containing [cis-Pt(NH₃)²⁺] is due to the involvement of the organic cation transporters (OCT) 1 and 2. These transporters have been recently invoked to explain the different activity of cisplatin and oxaliplatin towards colo-rectal cancers. Oxaliplatin contains 1,2-diaminocyclohexane as non-leaving ligand and appears to be a good substrate for OCT1 and OCT2, while cisplatin is a poor substrate for these transporters.

Conclusion

Four new platinum complexes having a dinuclear structure with a bisphosphonate (AHBP-II or PAM-H) bridging the two platinum moieties [cis-Pt(NH₃)²⁺] or [Pt(cis-1,4-DACH)]²⁺) in a W conformation have been synthesized and fully characterized. Although we have not been able to obtain crystals suitable for X-ray crystallography investigations for any of the four new compounds to confirm their structure, all analytical and spectroscopic measurements support the involvement in coordination of all phosphate hydroxyl oxygen atoms (each phosphate contributing one oxygen per platinum atom) resulting in a W-conformation of the type already reported and fully characterized by X-ray diffraction for [(Pt(R,R-DACH))₂(medronate)]³⁻. ¹H and ¹³P{¹H}-NMR experiments at different pH values have allowed the determination of the acidity constants for free pamidronate (those for free AHBP had already been reported) and an estimation of the stability of complexes 1A, 1B, and 2B (2A was not sufficiently soluble in water). Complexes 1A and 1B are stable at pH values not exceeding 9.0, which was compatible with carrying on the pharmacological investigations, while complex 2B (and presumably also 2A) is stable also at pH higher than 9. This has been found to be the result of the longer alkyk chain of PAM (as compared to AHBP) holding the NH₂ group farer from platinum and therefore less keen to coordination. This explanation was also supported by the equivalence of the platinum nuclei in 2B (one resonance) but not in 1B (two separate resonances).

The in vitro cytotoxicity of the new complexes has been evaluated on a set of 13 human tumour cell lines comprising two pairs of cisplatin-sensitive and -resistant cell lines and 3 pairs of multi-drug-sensitive and -resistant cell lines. On the average, compounds A containing [cis-Pt(NH₃)²⁺] are less active than B containing [Pt(cis-1,4-DACH)]²⁺ and this could be due to an intrinsic feature of the [Pt(cis-1,4-DACH)]²⁺ moiety, which could have a better pharmacokinetic and/or give more effective adducts with the target DNA. This latter hypothesis appears to be supported by the better non-cross resistance of complexes B towards cisplatin-resistant cell lines. On the other hand compounds B appear to be less effective than compounds A against some multi-drug-resistant cell lines. This latter aspect is worth of further investigation since, once more, platinum-DACH derivatives do not conform to the behaviour of cisplatin analogues.

We also found that, on the average, compounds 2 are more cytotoxic than compounds 1. It is possible that this is the result of a greater stability of compounds 2 with respect to compounds 1, particularly at high pH.

None of the four complexes has shown better activity than cisplatin. However, we believe that carboplatin, which unlike cisplatin but similarly to 1A, 1B, 2A, and 2B contains an O-donor chelate leaving ligand, could have been a better reference compound. In fact preliminary data do indicate that several of the new compounds possess similar or better activity than carboplatin.
We want to recall that the new complexes are soluble in water and are cationic under normal pH conditions, therefore they could take advantage of the organic cation transporters and could accumulate more easily into the tumour cells.

The high solubility in water of complexes 1A, 1B and 2B renders these compounds ideal candidates for loading into inorganic matrices to be used as bone-fillers and reservoir of Pt-based drugs. We have recently reported that the complex \([\text{Pt(en)}]_2(\text{AHBP-H})\)\(^{25}\) (Chart 1), embedded in inorganic composites (synthetic hydroxyapatite nanocrystals or silica xerogels-based matrices), was able, after activation, to release \([\text{PtCl}_2(\text{en})]\) at a cytotoxic concentration. The new complexes reported in this work should have similar adsorption/release properties with respect to the inorganic matrices. The new complexes reported in this work should have similar adsorption/release properties with respect to the inorganic matrices. The new complexes reported in this work should have similar adsorption/release properties with respect to the inorganic matrices. The new complexes reported in this work should have similar adsorption/release properties with respect to the inorganic matrices. The new complexes reported in this work should have similar adsorption/release properties with respect to the inorganic matrices.

Acknowledgements

The authors thank the Universities of Bari and Padova, the Italian “Ministero dell’Università e della Ricerca (MUR)” (PRIN 2007EWK9B), and the EC (COST Chemistry project D39/0004/06), the Inter-University Consortium for Research on the Chemistry of Metal Ions in Biological Systems (C.I.R.C.M.S.B.) for support.

References