New Active Bisphosphonate (Etidronate) Complexes as Anticancer Agents

Fathy AA¹, Butler IS², Elrahman MA³ and Mostafa SI*¹

¹Chemistry Department, Mansoura University, Mansoura 35516, Egypt
²Department of Chemistry, McGill University, Montreal QC H3A 2K6, Canada
³Oral Biology Department, Mansoura University, Mansoura, Egypt

*Corresponding author: Mostafa SI, Chemistry Department, Faculty of Science, Mansoura University, Egypt, Tel: 002-01008502625; Fax: 0020502246781; E-mail: sihmostafa@gmail.com

Received: November 08, 2017; Accepted: November 27, 2017; Published: December 01, 2017

Abstract
Bisphosphonates (Bps; have P-C-P moieties) are drugs that slow down or prevent bone loss. They inhibit osteoclasts, responsible for bone resorption, and allow osteoblasts, bone building cells, to be more effective; i.e., improve bone mass. Moreover, Bps are considered as anticancer drugs that cause bone damage. New complexes of etidronate disodium (Na₂Etd) with Pd (II) and Pt (II) were synthesized and characterized based on elemental analysis, molar conductivity, thermal and spectral (IR, NMR, UV-visible, mass) measurements. Their anticancer activity was tested against human prostate (DU 145) and breast (MDA-MB231) cancer cell lines compared to Cisplatin, the market drug.

Keywords: Etidronate; Palladium; Platinum; Anticancer; Spectra; Active

Introduction
Bisphosphonates, which containing P-C-P units, are used in the treatment of osteoporosis, Paget's disease of bone and to decrease the high calcium levels in cancer patients [1,2]. They are divided into two categories are: nitrogenous bisphosphonates (palmitronate, alendronate and zoledronate) and non-nitrogenous bisphosphonates (etidronate, clodronate and tiludronate) [3]. They show different mechanism of action in the treatment of osteoclast cells. Many cancers that affect bones are usually started in another organ in the body, followed by spreading to the bone; i.e., secondary bone cancer. These are breast, prostate and lung cancer, and myeloma. Some types of cancer treatment can also affect the bones making them...
weaker, including chemotherapy and hormone therapies. Bisphosphonates are types of drugs that can help in the treatment some types of cancer that cause bone damage [2].

Etidronate disodium (Na$_2$Etd; FIG. 1) is used to prevent osteoporosis which caused by corticosteroids (medicine may cause osteoporosis). Etidronate is also used for treating Paget's disease of bone as well as heterotopic ossification (growth of bone tissue in an area of the body other than the skeleton) in people who have had total hip replacement surgery or an injury to the spinal cord. It works on the base of slowing the breakdown of old bone with the formation of new one [4].

![Etidronate disodium (Na$_2$Etd)](image)

FIG. 1. **Etidronate disodium (Na$_2$Etd).**

Spectrophotometric determination of Etd$^{2-}$ in pharmaceuticals, based on its oxidation with potassium persulfate and reaction of the generated orthophosphate ions with molybdovanadate reagent have been reported [5]. It is known that upon complexation, the pharmacological properties are improved due to the action by combination of chelate and metal ion biological properties [6-8]. The preparation and X-ray structure of some Zn (II), Cd (II), Ca (II), Ba (II), Sr (II) and Mg (II) complexes of Bps have been have been reported [9-21]. This article is dealing with the preparation of Pd (II) and Pt (II) etidronate complexes. Their structure was characterized using IR, NMR, UV-visible, mass spectra, elemental analysis, molar conductivity and thermal measurements. The complexes were tested against human prostate (DU 145) and breast (MDA-MB231) cancer cell lines compared with Cisplatin.

**Experimental**

**Materials**

All reagents and solvents were purchased from Alfa/Aesar and all manipulations were performed under aerobic conditions using materials and solvents as received. All the reagents and solvents purchased commercially were used without further purification. DMSO-d$_6$ was used for the NMR measurements referenced against TMS.

The human prostate cancer (DU 145) and breast cancer (MDA-MB231) cell lines were obtained from the American Type Culture Collection (ATCC catalog number). Cells were maintained in RPMI media from Wisent Bio Products, which was
supplemented with Fetal Bovine Serum (FBS; 10%), HEPES (12 mL), L-glutamine (5 mL), gentamicin sulfate (500 µL), fungisone (250 µL) and ciprofloxacin (170 µL). The bio-products were purchased from Wisent Inc. The cells were grown in corning cell cultured treated polystyrene flasks, placed in an incubator at 37°C and CO₂ level of 5%. The media of each flask were changed when necessary and cell passaging was done between 85% and 95% confluence.

Measurements

Elemental analyses (C, H, N) were performed in Microanalytical Unit, Cairo University. Infrared spectra were recorded on a Nicolet 6700 Diamond ATR spectrometer in the 4000 cm⁻¹-200 cm⁻¹ range. NMR spectra were measured on Varian Mercury 200 MHz, 300 MHz and 500 MHz spectrometer. NMR spectra were recorded on VNMRS 200 and 500 MHz spectrometer in DMSO d₆ using TMS as reference. Mass spectra (ESI-MS) were recorded using LCQ Duo and double focusing MS25RFA instruments, respectively. Electronic spectra were recorded in DMF using a Hewlett-Packard 8453 spectrophotometer. Thermal analysis measurements were made in the 20°C-1000°C range at a heating rate of 20°C min⁻¹ using Ni and NiCo as references, on a on a TA instrument TGA model Q500Analyzer TGA-50. Molar conductivity measurements were carried out at room temperature on a YSI Model 32 conductivity bridge.

Preparations

[Pt (NaEtd)₂].2H₂O: Aqueous solution of K₂PtCl₄ (0.1037 g, 0.25 mmol; 7.5 mL) was added to etidronate disodium (0.125 g, 0.5 mmol). The reaction mixture was heated under reflux with stirred for 18 h. The deep brown solution was reduced in volume and brown precipitate was obtained. It was filtered off, washed with water and air dried. Conductivity data (10⁻³ M in DMF): Λ₉₀=16.0 ohm⁻¹ cm² mol⁻¹. Anal. Calcd. For C₄H₁₆Na₂O₁₆P₄Pt: C, 7.0; H, 2.3, P, 18.1%; Found: C, 6.9, H, 2.4, P, 18.3%. IR (KBr, cm⁻¹); ν(P=O), 1212; ν(P-O), 1122; ν(Pt-O), 555. ¹H NMR (400 MHz, DMSO-d₆): H (ppm); 2.49 (3H, -CH₃), 5.33 (H, -OH). ³¹P NMR (200 MHz, DMSO-d₆): p (ppm); 19.33. ESI-MS (m/z): 648.88 for [Pt (NaEtd)₂]⁺.

[Pd (Etd) (H₂O)₂]: The same procedure of the Pt (II) analogue was applied, using K₂PdCl₄ (0.08 g, 0.25 mmol). Conductivity data (10⁻³ M in DMF): Λ₉₀=20.4 ohm⁻¹ cm² mol⁻¹. Anal. Calcd. For C₂H₁₀O₉P₂Pd: C, 6.9; H, 2.9, P, 17.9; Pd, 30.7%; Found: C, 7.1, H, 3.00, P, 17.6; Pd, 30.9%. IR (KBr, cm⁻¹); ν(P=O), 1225; ν(P-O), 1122; ν(P-O), 1170; ν(Pd-O), 534. ¹H NMR (400 MHz, DMSO-d₆): H (ppm); 2.48 (3H, -CH₃), 5.46 (H, -OH). ³¹P NMR (200 MHz, DMSO-d₆): p (ppm); 18.87. ESI-MS (m/z): 311.5 for [Pd (Etd)]⁺.

Anticancer activity

Cell culture: Human prostate (DU 145) and breast (MDA-MB231) cancer cell lines were maintained in RPMI media from Wisent Bio Products, which was supplemented with Fetal Bovine FBS; 10% Serum, 12 mL HEPES, 5 mL L-glutamine, 500
µL gentamicin sulfate, 250 µL fungisone and 170 µL ciprofloxacin. The cells were grown in corning cell cultured treated polystyrene flasks, which placed in an incubator at 37°C and CO₂ level of 5%. The media of each flask was changed when necessary and cell passaging was done between 85% and 95% confluence.

**Complexes treatment:** In all assays, complexes stocks, [Pd (Etd) (H₂O)₂] and [Pt (NaEtd)₂] 0.3125 × 10⁻² to 100 µM were prepared by dissolving in DMSO to ensure complete dissolution.

**In vitro growth inhibition essays:** The growth inhibiting effects on human prostate cancer (DU 145) and breast cancer (MDA-MB231) cell lines were examined in vitro and the results were compared against negative and positive controls. Both human prostate cancer (DU 145) and breast cancer (MDA-MB231) cell lines were grown to 80% confluence and then incubated in RPMI media onto 96-well plates (corning Inc.) with 3000-5000 cells per well (100 µL medium/well) density. They were allowed to attach for 24 h. treated with a wide range of the complexes concentration range (0.3125 × 10⁻²-100 µM). The treatment was done in triplicate for 5 days in the incubator. The cells were fixed with cold trichloroacetic acid (50 µL, 50%) for 2 h at 4°C. Using flow of water, the wells were rinsed four times, dried well as much as possible and stained with Sulforhodamine B (SRB; 50 µL, 0.4 g/100 mL) for at least 1 h. at RT. Subsequently, the SRB was rinsed with 1% acetic acid and air-dried overnight. Finally, the dye was dissolved in Tris-base (10 mM, pH=10-10.5; 200 µL) for 2-5 min on a shaking platform. The optical density for each well was recorded using a microplate reader (model 2550; Bio-Rad) at 492 nm [22]. Each complex concentration was run in triplicate in at least two independent experiments. The readings were analyzed using the GraphPad Prism program (GraphPad software, Inc.), and a sigmoidal dose-response curve was used to determine the 50% inhibitory concentration (IC₅₀) [22].

**Results and Discussion**

The elemental analyses (C, H, P, Pd) for the complexes are in agreement with the assigned formulae and the molar conductivities (Λₐₘ) in DMF at room temperature suggest the non-electrolytic nature of the complexes [23,24]. The new complexes are stable under normal laboratory conditions and soluble in DMF and DMSO.

**IR spectra**

The IR spectrum of Na₂Etd shows multiple absorptions at 3600-3300 cm⁻¹ can be attributed to v(OH) stretching of P–OH and C–OH units. The bands appear at 1234 cm⁻¹ and 1183 cm⁻¹ attributable to v(P=O) and v(P-O) stretches, respectively [25,26]. Upon complexation, the v(P-O) stretch was shifted to lower wave number (1122 cm⁻¹). The vₐ(P-O) and vₛ(P-O) stretching vibrations are observed at 1055 cm⁻¹ and (1019, 915) cm⁻¹, respectively. The vibrations of P=O and P-O(H) groups show bands in the region below 1320 cm⁻¹. In case of Pt (II) complex, both v(P=O) and v(P-O) stretches were shifted
to lower wave number; i.e., coordination occurs through P=O and deprotonated P-O\(^-\) oxygen atoms [27,28]. Extra band is observed near 540 cm\(^{-1}\) assigned to \(\nu(M-O)\) stretch [23,24,29]. FIG. 2 and 3 show the structure of [Pt (NaEtd)]\(_2\) and [Pd (Etd) (H\(_2\)O)]\(_2\), and the IR spectrum of [Pd (Etd) (H\(_2\)O)]\(_2\), respectively.

FIG. 2. Structure of [Pt (NaEtd)]\(_2\) and [Pd (Etd) (H\(_2\)O)]\(_2\).

FIG. 3. IR spectrum of [Pd (Etd) (H\(_2\)O)]\(_2\).

NMR spectra

The \(^1\)H NMR spectrum of Na\(_2\)Etd exhibits a broad singlet at \(\delta 5.31\) ppm attributed to OH (C-OH), while that at \(\delta 2.23\) ppm, is assigned to CH\(_3\) [30,31]. The later band is shifted to ~ \(\delta 2.50\) ppm upon complex formation (FIG. 4a).

The \(^{31}\)P NMR spectrum of Na\(_2\)Etd exhibits single resonance at 15.69 ppm. In the complexes, this resonance is shifted to lower filed (near \(\delta 19.0\) ppm; FIG. 4b), indicating the participation of P-O oxygen atoms in coordination.
UV-visible spectra

The UV-visible spectrum of the diamagnetic complexes exhibits bands near 470 nm and 325 nm (FIG. 5) due to $^1A_{1g} \rightarrow ^1B_g$ and $^1A_{1g} \rightarrow ^1E_g$ transitions, respectively, showing in a square-planar configuration [24,29].

FIG. 4a. Spectrum of [Pd (Etd) (H$_2$O)$_2$].

FIG. 4b. $^{31}$P NMR spectrum of [Pd (Etd) (H$_2$O)$_2$].
FIG. 5. The UV-visible spectrum of [Pd (NaEtd)₂].

Mass spectra
To confirm the formulation of the complexes, [Pt (NaEtd)₂] and [Pd (Etd) (H₂O)₂], their mass spectra show the including the molecular ions [Pt (NaEtd)₂]⁺ and [Pd (Etd)]⁺ (FIG. 6) at m/z 648.88 and 311.5, respectively [23,24].

TGA studies
The thermal decomposition of both [Pt (NaEtd)₂]₂H₂O and [Pd (Etd) (H₂O)₂] complexes paths (below 130°C) with evaluation of the crystal water content [23,24]. The thermograms of [Pt (NaEtd)₂]₂H₂O shows the first step weight loss corresponds to the loss of hydrated waters molecules (5.3%). The second step (111°C-453°C), due to the release of two C₂H₅O₄Na species (42.8%), leaving a residue with 51.6%. The thermogram of [Pd (Etd)H₂O]₂ shows TG inflection in the ranges 121-299 and 300°C-780°C, assigned to the elimination of coordinated water (10.3%) and (C₂H₄O₂, 2O₂) (31.34) fragments, respectively, leaving residue of 58.9% [23,24,29].

Anticancer activity
Cancers that spread to the bones damage the bones and release proteins that interfere with the normal bone shaping process. The proteins stimulate the cells that break down bone (osteoclasts) and make them overactive. Since bone is destroyed faster than rebuilt and calcium is normally stored in the bones, the breakdown of bone cells releases more calcium than usual into the blood [4].
FIG. 6. Mass Spectra of \([\text{Pd (Etd)}(\text{H}_2\text{O})\text{Cl}])\).

Many studies have clearly demonstrated that \textit{in vitro} bisphosphonates exert cytostatic and pro-apoptotic effects on breast and prostate cancer cell lines similar to those with myeloma cell lines. The viability of breast cancer cell lines cultured \textit{in vitro} with zoledronic acid, as anticancer agent, was more potent than pamidronate, and clodronate. Increasing apoptosis was convincingly based on changing of nuclear morphology, DNA fragmentation, down-regulation of bcl-2, and proteolysis of poly-APD-ribose polymerase [27-32].

\textit{In vitro} anticancer activity of both \([\text{Pt (NaEtd)}_2]\) and \([\text{Pd (Etd)}(\text{H}_2\text{O})_2]\) against human prostate (DU 145) and breast (MDA-MB231) cancer cell lines was examined, with \textit{Cisplatin}; as positive control. The antitumor activities were quantified by the corresponding IC\textsubscript{50} values (FIG. 7; TABLE 1). The complex \([\text{Pt (NaEtd)}_2]\) shows high inhibition against DU 145 and MDA-MB231 human cancer cell lines. The IC\textsubscript{50} values were 2.00 \(\pm\) 0.2 and 1.76 \(\pm\) 0.3 \(\mu\text{M}\), respectively. The IC\textsubscript{50} values of \textit{Cisplatin} were 2.21 \(\pm\) 0.5 and 3.20 \(\pm\) 0.5 \(\mu\text{M}\), respectively.

FIG. 7. IC\textsubscript{50} of \([\text{Pt (NaEtd)}_2]\) against breast MDA-MB231 human cancer cell line.
Since the DNA is the main target of the drug, the anticancer activity is related to the DNA-binding ability. The difference between the anticancer activity of $[\text{Pt (NaEtd)}_2]$ and Cisplatin is based on the binding ability of DNA and cancer cell uptake [24,29]. Moreover, the cell viability of both $[\text{Pt (NaEtd)}_2]$ and $[\text{Pd (Etd) (H}_2\text{O)}_2]$ against human prostate and breast cancer cell lines showed high selectivity of $[\text{Pt (NaEtd)}_2]$ rather than human prostate and breast normal cell lines (TABLE 1).

**TABLE 1. Anticancer activity of Na$_2$Etd and its complexes against the human prostate (DU 145) and breast cancer (MDA-MB231) cell lines**.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC$_{50} \mu$M breast cancer (MDA-MB231) cell line</th>
<th>Selectivity breast normal (MDA-MB231) cell line</th>
<th>IC$_{50} \mu$M human prostate cancer (DU 145) cell line</th>
<th>Selectivity breast normal (DU 145) cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$Etd</td>
<td>45.31 ± 0.3</td>
<td>-</td>
<td>46.99 ± 0.3</td>
<td>-</td>
</tr>
<tr>
<td>$[\text{Pt (NaEtd)}_2]$</td>
<td>1.76 ± 0.3</td>
<td>91.76</td>
<td>2.00 ± 0.2</td>
<td>89.38</td>
</tr>
<tr>
<td>$[\text{Pd (Etd) (H}_2\text{O)}_2]$</td>
<td>4.85 ± 0.4</td>
<td>67.21</td>
<td>5.32 ± 0.3</td>
<td>54.56</td>
</tr>
<tr>
<td><strong>Cisplatin</strong></td>
<td>3.20 ± 0.5</td>
<td></td>
<td>2.21 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

*Selectivity of drugs towards normal cell lines are underlines*

**Conclusion**

Two Pt (II) and Pd (II) etidronate complexes were prepared and characterized using IR, NMR, UV-visible, mass, elemental analysis, molar conductivity and thermal analysis. The complex, $[\text{Pt (NaEtd)}_2]$ shows high anticancer activity against the human prostate (DU 145) and breast (MDA-MB231) cancer cell lines, and higher than the market drug Cisplatin.

**Acknowledgments**

This research is funded by the High Ministry of Education, Post 2014 and BRIDGES-Development-3 Project: Scientific Research Academy, Ministry of Research, Egypt.

**REFERENCES**


