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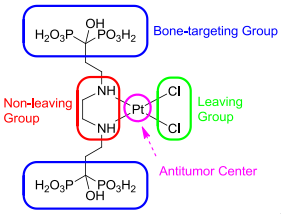

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Bifunctional bisphosphonate derivatives and platinum complexes with high affinity for bone hydroxyapatite

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ABSTRACT

A series of ethylenediamine/1,3-propanediamine derivatives containing bifunctional bisphosphonate substituents and their corresponding dichloroplatinum(II) complexes have been synthesized and characterized by elemental analysis, ^{1}H NMR, ^{13}C NMR, ^{31}P NMR, and HRMS spectra. Based on WST-8 assay with CCK-8, in general, the newly synthesized dichloroplatinum complexes **1-6** showed higher *in vitro* antitumor activity than platinum-free compounds **L1-L6** against three tumor cell lines (especially osteosarcoma MG-63). According to hydroxyapatite binding experiment, complexes **2**, **3**, and **6** showed much higher affinity (K' = 3.7, 4.0, and 3.0, respectively) for bone hydroxyapatite than cisplatin (K' < 0.1), comparable to zoledronate (K' = 2.8). It can be found that representative complex **2** with high cytotoxicity and *in vitro* antiproliferative activity against osteosarcoma cell line, as well as promising hydroxyapatite binding ability has been screened as a potential bone-targeting antitumor agent for subsequent *in vivo* study. In addition, flow cytometry experiment was applied to investigate the mode of action of representative complex **2**.

In clinic, cisplatin is known as one of the most active agents in solid tumor chemotherapy, which was approved for the treatment of testicular and ovarian cancer in 1978.[1,2] However, the clinical applications of cisplatin are confined by its drawbacks including side effects and drug resistance due to the poor tumor-selectivity of cisplatin.[3-5] Therefore, many novel platinum-based complexes with improved target distribution and tumor-selectivity have been designed and prepared in an attempt to overcome these limitations.[6-9]

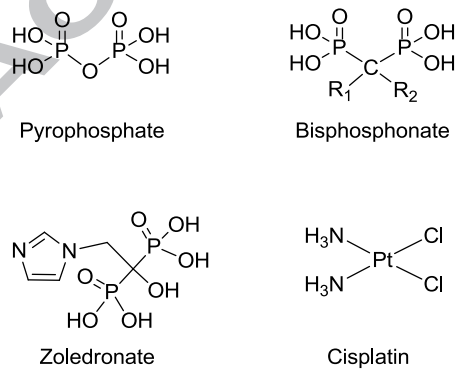

Figure 1. Structures of pyrophosphate, bisphosphonate, zoledronate, and cisplatin.

Cisplatin is also widely used as one of the primary choices for the treatment of osteosarcoma despite its significant side effects.[10] Therefore, it is necessary to develop novel platinum-based drugs with bone-targeting ability in order to reduce the side effects and improve the tumor-selectivity towards osteosarcoma which is considered as one of the most untreatable and painful malignant tumors. And the introduction of bone-targeting group in the non-leaving or leaving moieties of platinum drugs has been exploited as a useful strategy to design novel platinum agents.

Geminal bisphosphonates (Figure 1, BPs), which show high affinity for bone mineral hydroxyapatite (HAP) and other calcified tissues, are known for their bone-targeting properties and are in clinical use for several bone-related diseases including osteoporosis, myeloma, hypercalcemia and Paget's disease.[11-14] BPs has also been reported to exhibit significant inhibition to osteoclastic resorption or antitumor effects.[15] Besides, BPs has been used as a moiety (non-leaving or leaving group) in combination with platinum agents to increase the accumulation of drugs in the bone tissue and consequently reduce the toxicity and improve the biological activity. In the early 1990s, Keppler and co-workers have reported platinum agents linked with functional phosphonates which showed high activity towards transplantable osteosarcoma *in vitro* and *in vivo*.[16] Natile et al. have synthesized a series of platinum complexes by introducing bisphosphonate in the leaving group and found a different

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cytotoxic mechanism from that of cisplatin.[17-19] Bose and co-workers have demonstrated that platinum–pyrophosphato complexes showed non-DNA-binding ability towards human ovarian cancer cells.[20] However, the platinum-phosphato complexes mentioned above may lose the leaving group phosphate moieties in the process of physiological metabolism before reaching the bone tissue or targeting receptor. Therefore, Guo et al. and Liang et al. have designed a series of dichloroplatinum complexes containing pyridine derivative as carrier ligand (non-leaving group) with bisphosphonate and monophosphonate substituent, respectively, and some of these complexes showed cytotoxicity against human osteosarcoma cell line (MG-63).[21-23]

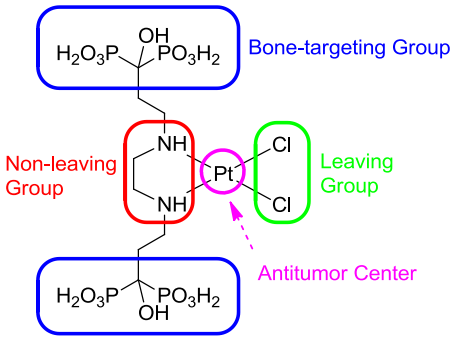
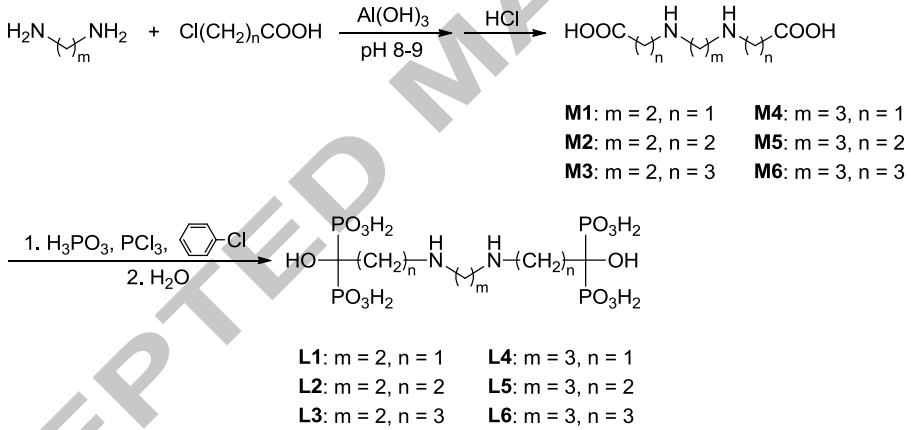


Figure 2. Proposed structure-activity relationship of newly synthesized platinum complexes.



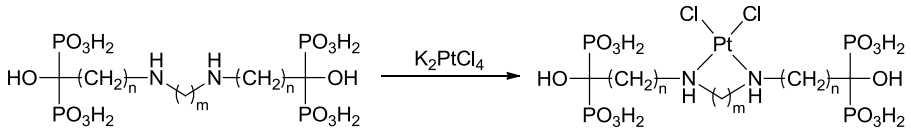
Scheme 1. General synthetic route to ligands **L1-L6**.

In our study, ethylenediamine and monochloroacetic acid have been chosen as starting materials and aluminum hydroxide as catalyst to prepare EDDA (**M1**),[27] followed by the reactions of the carboxyl group with phosphorus trichloride and hydrolyzation to give target compound **L1**.[13,30-32] The other compounds **L2-L6** were synthesized by the similar method. All the compounds (**L1-L6**) were characterized by elemental analysis, ^{1}H NMR, ^{13}C NMR, ^{31}P NMR, and HRMS spectra.

Corresponding platinum(II) complexes **1-6** were obtained by the reaction of potassium tetrachloroplatinate with compounds **L1-L6** in chloroform (Scheme 2) and the resulting complexes were characterized by elemental analysis, ^{1}H NMR, ^{13}C NMR, ^{31}P NMR, and HRMS spectra. NMR spectroscopy of both ligands **L1-L6** and the corresponding platinum(II) complexes **1-6** was performed with d_6-DMSO as the solvent, and the expected signals were observed in ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR spectra. In the ^{1}H NMR spectra of complexes **1-6**, the broad signals of hydrogen atoms belonging to amino groups appear in the range of 4.60-4.79 ppm due to amine coordination with platinum(II), shifting high-field relative to the metal-free ligands (**L1-L6**) in the range of 5.45-5.68 ppm. Besides, the signals of C-H protons connected to the amino groups occur in the range of 2.38-2.70 ppm as a multiplet, shifting high-field relative to the corresponding signals (2.58-2.87 ppm) in the platinum-free ligands (**L1-L6**). These chemical shifts demonstrate the coordination sphere around the metal center as shown in Scheme 2. The coordination of ligands with platinum ion through nitrogen atoms of aminobisphosphonate can also be judged by ^{31}P NMR spectroscopy. In the phosphorus spectra, the free ligand resonates at 17.40-17.45 ppm. After coordination of the bisphosphonate substituted amine ligands, chemical shifts between 21.36-21.62 ppm can be found.

Nevertheless, based on the previous studies by other researchers, it has not been reported that diamine derivatives with two bisphosphonate groups attached to two nitrogen atoms, symmetrically, coordinates with platinum ion to form a novel bifunctional Pt-bisphosphonate complex. In this paper, we report the preparation and cytotoxicity of a series of ethylenediamine/1,3-propanediamine derivatives containing N,N'-dibisphosphonate substituents and their corresponding dichloroplatinum complexes bearing diamine derivatives as non-leaving groups.

The preparation of N,N'-dibisphosphonate substituted ethylenediamine/1,3-propanediamine derivatives (**L1-L6**) was carried out starting from ethylenediamine/1,3-propanediamine and monochloroacetic acid via several synthetic steps (Scheme 1). Several methods have been reported to synthesize ethylenediamine-N,N'-diacetic acid (EDDA, **M1**) in the past decades. In 1930s, Reinhold Fick first reported the preparation of EDDA by the reaction of ethylenediamine with formaldehyde and hydrogen cyanide.[24] Bersworth have applied sodium cyanide in place of hydrogen cyanide in the reaction mentioned above.[25,26] In 1960s, Hoechst reported that ethylenediamine and monochloroacetic acid were used as starting materials and aluminum hydroxide as catalyst.[27] Gorelov have obtained EDDA by decarboxylic reaction of ethylenediamine-N,N'-dimalonic acid.[28] In 1980s, Anton et al. have developed the reaction of ethylene oxide with glycine to synthesize EDDA.[29]



1: m = 2, n = 1 **4**: m = 3, n = 1
2: m = 2, n = 2 **5**: m = 3, n = 2
3: m = 2, n = 3 **6**: m = 3, n = 3

Scheme 2. General synthetic route to platinum(II) complexes **1-6**.

All the platinum(II) complexes show 100% of $[M\text{-}Cl]^+$ peaks in the HRMS spectra, of which have more than three protonated ion peaks owing to the presence of platinum and chlorine isotopes. The structures of the target complexes were further confirmed by elemental analysis.

The *in vitro* cytotoxic potency of free ligands **L1-L6** and corresponding platinum(II) complexes **1-6** has been tested by WST-8 assay with CCK-8 against human tumor cell lines (A549 non-small cell lung cancer, HCT116 colorectal cancer, and MG-63 osteosarcoma) after 48 h incubation. Cisplatin and zoledronate were chosen as positive controls. The cytotoxicity results were in terms of IC_{50} values presented in Table 1. It was notable that ethylenediamine derivatives **L1-L3** (m=2) with N,N'-dibisphosphonate groups showed selective cytotoxic effect against A549 and MG-63 cell lines, which were obviously lower than those of cisplatin and zoledronate. Besides, 1,3-propanediamine derivatives **L4-L6** (m=3) hardly exhibited cytotoxicity towards three tested cell lines. HCT-116 was the least sensitive cell line to all ligands **L1-L6**.

According to the IC_{50} values in Table 1, the cytotoxicity of platinum-based complexes **1-6** seemed to be generally higher than platinum-free ligands **L1-L6** against tested cell lines, indicating that the bifunctional N,N'-dibisphosphonate groups and platinum moiety may have synergic effect on tumor cells. It can be noted from Table 1 that complexes **1-3** (m=2) were more effective *in vitro* than complexes **4-6** (m=3) towards three tumor cell lines, while complexes **1-3** showed lower cytotoxicity than that of cisplatin, with the IC_{50} values of 10.21-54.72 μM, 1.2-7.1-fold less potent than cisplatin (IC_{50} = 6.52-11.83 μM). Especially for MG-63 cell line, complexes **1-3** (m=2) containing ethylenediamine moiety exhibited *in vitro* cytotoxicity (IC_{50} = 10.21-26.53 μM) comparable to that of zoledronate (IC_{50} = 12.91 μM), 1.2-3.1-fold less potent than that of cisplatin (IC_{50} = 8.6 μM). On account of these IC_{50} data, it can be concluded that the ethylenediamine skeleton of resulting platinum-based complexes may have more influence on suppressing tumor cell proliferation *in vitro* than 1,3-propanediamine skeleton.

Table 1. IC_{50} values of compounds **L1-L6** and **1-6** tested by WST-8 assay with Cell Counting Kit-8 against human tumor cell lines.

Compd	m	n	IC_{50} (μM)[a]		
			A549[b]	HCT116[c]	MG-63[d]
L1	2	1	45.92 ± 2.10	> 100	63.84 ± 5.38
L2	2	2	31.94 ± 1.52	> 100	45.45 ± 3.42
L3	2	3	67.66 ± 4.19	> 100	> 100
L4	3	1	> 100	> 100	70.36 ± 4.67
L5	3	2	> 100	> 100	> 100
L6	3	3	> 100	> 100	> 100
1	2	1	35.63 ± 1.82	40.58 ± 2.36	26.53 ± 1.81
2	2	2	18.17 ± 1.04	23.54 ± 2.02	10.21 ± 0.59
3	2	3	46.48 ± 3.27	54.72 ± 3.49	19.67 ± 1.22
4	3	1	> 100	> 100	61.44 ± 4.53
5	3	2	67.20 ± 4.71	44.64 ± 2.88	33.46 ± 2.18
6	3	3	> 100	58.05 ± 5.47	40.39 ± 3.03
Cisplatin			6.52 ± 0.40	11.83 ± 0.82	8.56 ± 0.51
Zoledronate			14.09 ± 1.13	15.57 ± 1.44	12.91 ± 0.77

[a] Values represent the mean ± SD from three independent experiments; IC_{50} is defined as the drug concentration required to inhibit 50% of cell growth determined by WST-8 assay (WST-8 = water-soluble tetrazolium salt) with Cell Counting Kit-8 (CCK-8).

[b] Human non-small cell lung cancer cell line.

[c] Human colorectal cancer cell line.

[d] Human osteosarcoma cell line.

When the length of linear alkyl linking amino and bisphosphonate group was modified from one- to three-carbon (n=1-3), the *in vitro* antitumor activity of complexes **1-6** had a convex parabolic change. Complexes **2** (m=2, n=2) and **5** (m=3, n=2) with two-carbon linker between amino and bisphosphonate group were more active *in vitro* (complexes **2**: IC_{50} = 10.21-23.54 μM, complexes **5**: 33.46-67.20 μM) against three cell lines than other compounds, suggesting that the linear alkyl with moderate-length (n=2) linking ethylenediamine/1,3-propanediamine skeleton and bisphosphonate groups may play a more important role in biological activity than those with one- or three-carbon alkyl linkers.

On basis of the results in Table 1, complex **2** with ethylenediamine skeleton and two-carbon linker between amino and bisphosphonate groups has been screened to be the most effective antitumor agent against three cell lines among the newly synthesized compounds, with the IC_{50} values of 10.21-23.54 μM, which showed promising cytotoxicity comparable to those of zoledronate (IC_{50} = 12.91-15.57 μM), 1.2-2.8-fold less potent than cisplatin in terms of IC_{50} values against three cell lines.

Based on the cytotoxicity results above, MG-63 was selected as the target to test the inhibition of cell growth by all platinum compounds. For comparison, cisplatin and zoledronate were also investigated as positive controls, and the result of cell counting at different concentrations of compounds was shown in Figure 3. It was noted that the inhibition effect of all compounds on the cell proliferation was concentration-dependent. Cisplatin and zoledronate at low concentration of 5 μM completely inhibited cell proliferation in MG-63. Complexes **4-5** had an obvious effect of inhibiting cell growth on MG-63 at the concentration of 10-25 μM within the culture duration of 6 days, while complexes **1** and **3** showed *in vitro* antiproliferative activity at the concentration of 5-10 μM in the same cell line. Among the tested compounds, complex **2** was significantly efficient in inhibiting cell proliferation at the concentration of 1-5 μM comparable to cisplatin and zoledronate, which is consistent with the result of *in vitro* cytotoxicity.

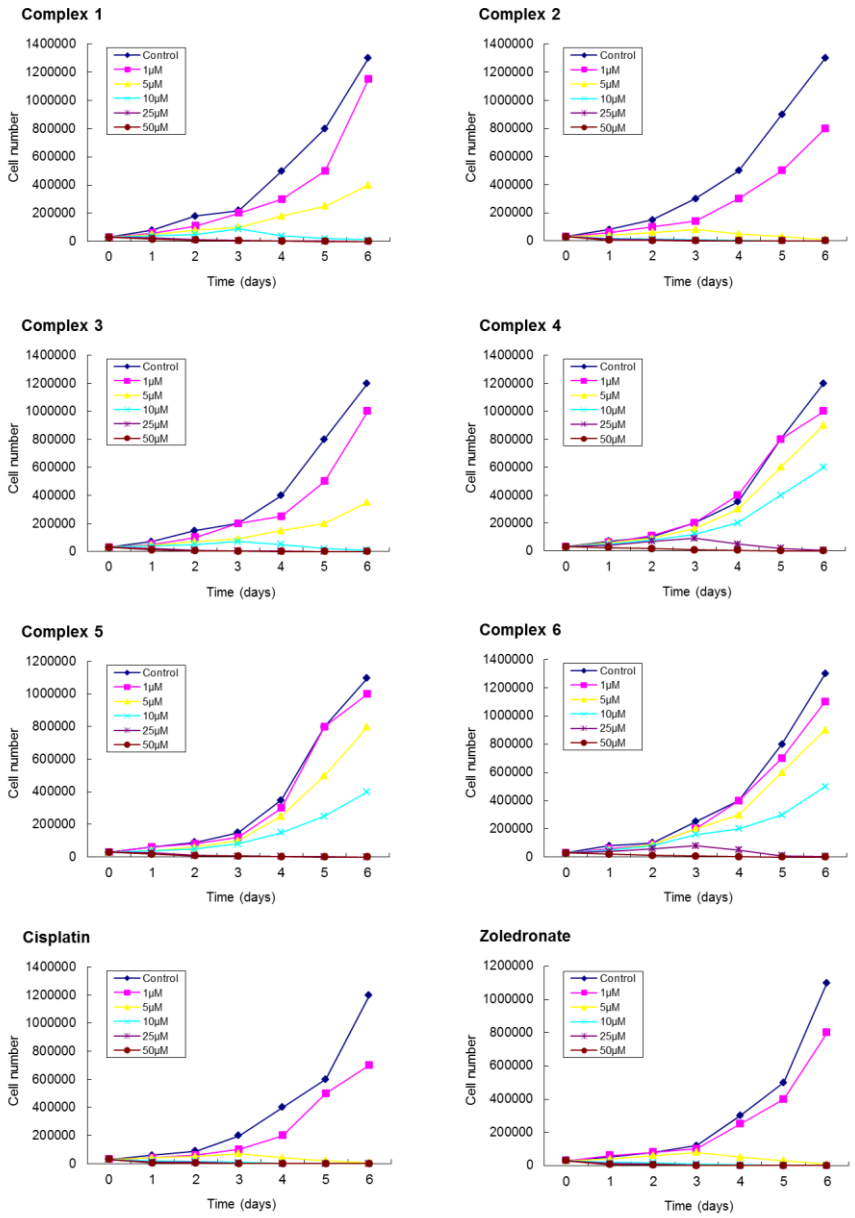


Figure 3. Inhibition of cell proliferation of MG-63 by platinum compounds and positive controls at different concentrations for 6 days of incubation (0-50 μM).

It has been demonstrated that the bisphosphonate moiety shows binding affinity for bone hydroxyapatite, which was used as a significant functional group in a lot of agents used clinically to target bone tissue and treat some bone-related diseases including osteoporosis.[33,34] In this study, the *in vitro* bone affinity of complexes **1-6** was evaluated by developing a bone-binding model as compared to zoledronate and cisplatin. According to the previous report by Bohacek et al., a microcrystalline hydroxyapatite adsorption column with phosphate gradient elution has been applied to test the hydroxyapatite affinity of bisphosphonate group belonging to complexes **1-6** by HPLC assay.[33] The retention time in the chromatography was expressed in terms of capacity factor *K'*.

$$K' = (t_R - t_0)/t_0$$

Where t_R is the measured retention time of tested compound and t_0 is the retention time of void column.

Table 2. *K'* values of complexes **1-6** by hydroxyapatite chromatography.

Compd	IC_{50} (MG-63, μM)	*K'* $((t_R-t_0)/t_0)$[a]
1	26.53 ± 1.81	1.82 ± 0.13
2	10.21 ± 0.59	3.68 ± 0.21
3	19.67 ± 1.22	4.03 ± 0.26
4	61.44 ± 4.53	1.56 ± 0.10
5	33.46 ± 2.18	2.24 ± 0.15
6	40.39 ± 3.03	3.01 ± 0.20
Cisplatin	8.56 ± 0.51	< 0.1
Zoledronate	12.91 ± 0.77	2.77 ± 0.13

[a] Values represent the mean ± SD from three independent experiments; t_R is the measured retention time of tested compound and t_0 is the retention time of void column.

In Table 2, the hydroxyapatite chromatography results revealed that cisplatin barely had affinity for bone hydroxyapatite (*K*' < 0.1). We found that compounds **1** (m=2, n=1), **4** (m=3, n=1), and **5** (m=3, n=2) exhibited slightly lower hydroxyapatite binding efficacy (*K*' = 1.56-2.24) than positive control, zoledronate (*K*' = 2.77). On the other hand, it was worth noting that compounds **2** (m=2, n=2), **3** (m=2, n=3), and **6** (m=3, n=3) were more efficient (*K*' = 3.01-4.03) in binding bone hydroxyapatite than zoledronate (*K*' = 2.77). And the different hydroxyapatite binding behaviors of cisplatin and compounds **1-6** indicated that the bifunctional bisphosphonate moieties of compounds **1-6** were responsible for their affinity for bone hydroxyapatite.

Considering that platinum-based complex **2** as a potential antitumor agent displayed promising *in vitro* cytotoxicity and antiproliferative activity on MG-63 cell line comparable to cisplatin and higher hydroxyapatite affinity than zoledronate, a flow cytometry study was performed for complex **2**, cisplatin and zoledronate to investigate the mechanism action of producing cell death (necrosis or apoptosis). The resulting phase diagrams were shown in Figure 4 and Figure 5 after MG-63 and A549 cells were respectively treated with these compounds at 50 μM for 24 h. Four areas in the diagrams represent four different cell states: necrotic cells (upper left, positive for PI and negative for annexin/FITC), living cells (lower left, negative for annexin and PI), late apoptotic or necrotic cells (upper right, positive for annexin and PI) and apoptotic cells (lower right, negative for PI and positive for annexin).

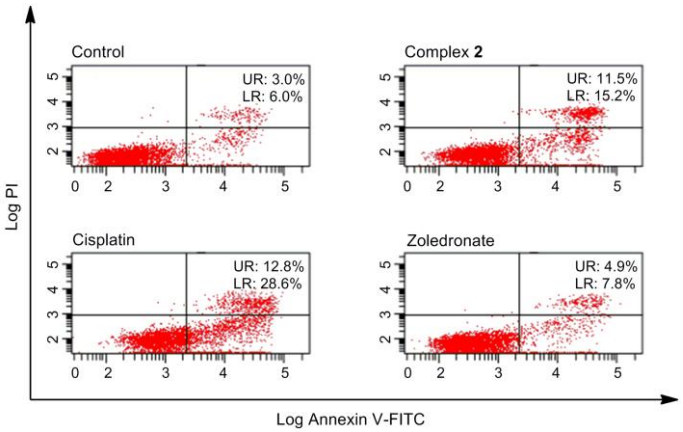


Figure 4. Cisplatin, zoledronate and complex **2** induce apoptosis on MG-63 cells; cells were treated with these compounds at 50 μM for 24 h and then stained with annexin V–FITC and PI, and analyzed by FACScan.

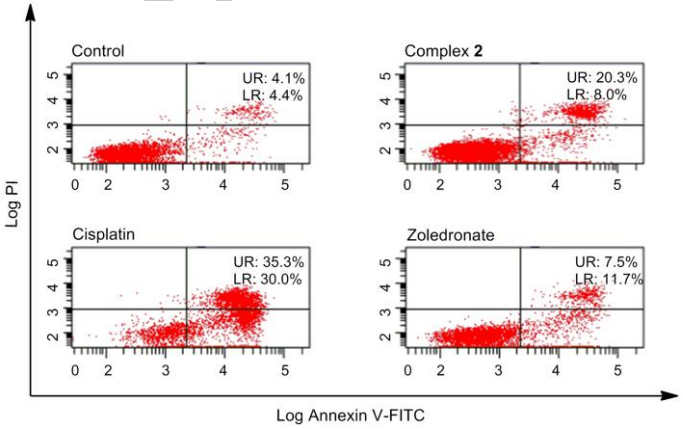


Figure 5. Cisplatin, zoledronate and complex **2** induce apoptosis on A549 cells; cells were treated with these compounds at 50 μM for 24 h and then stained with annexin V–FITC and PI, and analyzed by FACScan.

In Figure 4, it was obvious that complex **2** showed higher apoptosis effect on MG-63 cells than zoledronate after 24 h incubation, with apoptotic population of 26.7%, 2.1-fold more than zoledronate (12.7%). Nevertheless, the apoptosis rate of MG-63 cells produced by complex **2** was lower than positive cisplatin (41.4%) at 50 μM after 24 h incubation, 1.5-fold less than that of cisplatin.

With respect to A549 cell line in Figure 5, complex **2** was more effective in producing cell death by apoptosis than zoledronate at 50 μM after 24 h incubation, with apoptotic population of 28.3%, 1.5-fold more than zoledronate (19.2%), while the apoptosis rate generated by complex **2** was obviously lower than positive cisplatin (41.4%), 1.5-fold less than that of cisplatin.

Based on the results above, both complex **2** and cisplatin produced higher apoptotic cell populations than platinum-free zoledronate at the same concentration, suggesting that platinum-based complex **2** induced cell death by apoptosis effect similar to cisplatin.

In conclusion, six ethylenediamine/1,3-propanediamine derivatives containing bifunctional N,N'-dibisphosphonate substituents and six corresponding dichloroplatinum(II)

complexes bearing diamine derivatives as non-leaving groups have been synthesized and characterized. The result of *in vitro* cytotoxicity assay on A549, HCT116 and MG-63 cell lines showed that the platinum-based complexes **1-6** were generally more potent *in vitro* than platinum-free ligands **L1-L6** against three cell lines, suggesting that the bifunctional bisphosphonate moieties and dichloroplatinum skeleton may have synergistic effect on suppressing tumor cell proliferation. And based on the IC_{50} values and cell growth assay, the resulting platinum-based complexes (**1-3**) with ethylenediamine skeleton revealed higher cytotoxicity and *in vitro* antiproliferative activity than those (**4-6**) with 1,3-propanediamine skeleton, indicating that ethylenediamine moiety may play a more important role in biological activity *in vitro* than 1,3-propanediamine moiety. According to the result of hydroxyapatite binding assay for positive agents and platinum-based complexes, it was testified that complexes **2**, **3**, and **6** showed significant affinity for bone hydroxyapatite superior to zoledronate and cisplatin.

Furthermore, complex **2** has been proved to be a promising antitumor candidate with high cytotoxic efficacy against three tumor cell lines, especially against MG-63, and hydroxyapatite binding efficacy. Flow cytometry assay for complex **2** has been carried out, demonstrating that representative complex **2** produced cell death of A549 and MG-63 by apoptotic effect, similar to cisplatin.

Acknowledgments

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Supplementary Material

Supplementary data include procedures for preparation of ethylenediamine/1,3-propanediamine derivatives with dibisphosphonates, preparation of platinum complexes, cytotoxicity assay (IC_{50} values), cell growth assay, flow cytometry, and hydroxyapatite binding assay.

Highlights

1. Pt(II) complexes with a series of bifunctional bisphosphonate derivatives as ligands.
2. Pt(II) complexes showed cytotoxicity and antiproliferative activity against MG-63.
3. Synergistic effect on tumor cells by bisphosphonate moieties and dichloroplatinum skeleton.
4. Typical complexes showed hydroxyapatite affinity superior to zoledronate and cisplatin
5. Representative compound induced apoptosis analogous to cisplatin.