# Dialkyl Bisphosphonate Platinum(II) Complex as a Potential Drug for Metastatic Bone Tumor

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**Bisphosphonates have high affinity for hydroxyapatite (HA), which is abundantly present in bone. Also, platinum complexes are known that have a wide spectrum of antitumor activities. The conjugate of bisphosphonate and a platinum complex might have HA affinity and antitumor activity, and become a drug for metastatic bone tumor. In this study, the authors synthesized platinum complexes that had dialkyl bisphosphonic acid as a ligand, and evaluated the possibility of the synthesized complexes as a drug for metastatic bone tumor. The synthesized dialkyl bisphosphonate platinum(II) complex was characterized, and its stability in an aqueous solution was also confirmed. The synthesized platinum complex showed higher HA affinity than other platinum complexes such as cisplatin and carboplatin in an experiment of adsorption to HA. *In vitro*, the platinum complex showed tumor growth inhibitory effect stronger than or equal to cisplatin, which is the most commonly used antitumor agent. Moreover, the platinum complex showed a bone absorption inhibitory effect on the osteoclast. These results suggest potential of dialkyl bisphosphonate platinum(II) complexes as a drug for metastatic bone tumor.**

**Key words** bisphosphonate; platinum complex; metastatic bone tumor; hydroxyapatite affinity

Unlike other tissues, there is abundant hydroxyapatite (HA) in bone tissues. Due to this feature, it is attractive to use HA as a target for bone selective drug delivery. Since bisphosphonates have high affinity for HA, HA affinity will be conferred on compounds, which originally do not have HA affinity, by conjugation to bisphosphonates. This approach has been proposed for bone selective drug delivery such as prostaglandin $E_2$, estradiol, gemcitabine, osteoprotegerin.[1—4] Our interest was to develop bone selective antitumor agents. Bone is tissue to which tumors easily metastasize, and metastatic bone tumor causes pain, pathologic fractures, compression of nerve syndrome, and hypercalcemia, all of which are factors of patient morbidity and mortality. In order to develop an effective drug for metastatic bone tumor, some conjugates of bisphosphonate and platinum(II) complexes, such as cisplatin,[5—8] which has a wide spectrum of cancer treatment, have been synthesized.[9] In most cases, bisphosphonic acids that all phosphonate groups are not substituted were used as a ligand.[10] In contrast, we designed and synthesized platinum(II) complexes with dialkylated bisphosphonic acid as a ligand (Fig. 1), and evaluated its possibility as a drug for metastatic bone tumor.

*In vivo*, dialkyl bisphosphonate platinum complexes are expected to effect bone tumor when the platinum gradually dissociates after adsorption to HA, the main component in bone. By using the dialkylated bisphosphonic acid, we expected changes of some properties of the bisphosphonate platinum complexes as compared with those of non-alkylated bisphosphonate platinum complexes as follows; non-alkylated bisphosphonate platinum complexes have high hygroscopicity. Hygroscopicity will be decreased and stability will be improved in the solid state in dialkylated bisphosphonate platinum complexes due to the hydrophobic properties of dialkylated bisphosphonic acid. Non-alkylated bisphosphonate platinum complexes show an acidic pH in an aqueous solution and pH adjustment is necessary for animal dosing, which might cause dissociation of the platinum complex. On the other hand, an aqueous solution of the dialkylated bisphosphonate platinum complexes may be near a neutral pH so pH adjustment could be omitted.

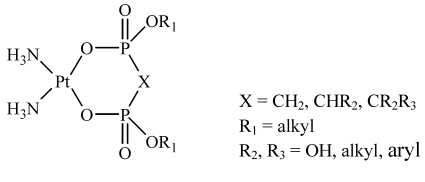


Fig. 1. Sturcture of Dialkyl Bisphosphonate Platinum(II) Complex

## Results

We synthesized various dialkyl bisphosphonate platinum(II) complexes.[11] Among them, we intensively evaluated HA affinity, effect on the tumor cells and osteoclast of *cis*-diammine (*P*,*P*′-diethyl methylenebisphosphonato) platinum(II) (DEBP-Pt) that was synthesized efficiently and showed better properties and stability.

**Synthesis** *P*,*P*′-Diethyl methylenebisphosphonic acid (DEBP) used as a ligand was synthesized through the following process. Tetraethyl methylenediphosphonate was refluxed in morpholine, and dialkylated bisphoaphonic acid morpholinium salt was obtained. Treatment of the salt with cation exchange resin in methanol gave DEBP.[12] The obtained DEBP and cisplatin were reacted with silver oxide in *N*,*N*-dimethylformamide (DMF), the precipitated silver chloride was removed, and DEBP-Pt was given in a high yield by recrystallization from methanol (total yield 65% through three reactions, Chart 1).[13] The resulting DEBP-Pt was a crystalline solid with little hygroscopicity. It is highly soluble in water as well as organic solvents such as methanol and dimethyl sulfoxide (DMSO). An aqueous solution of DEBP-Pt was slightly acidic (between pH 6 and 7).

**Stability** We assumed that DEBP-Pt selectively adsorbs

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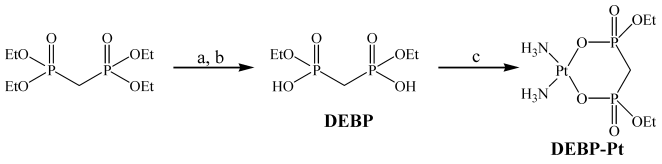


Chart 1. Preparation of DEBP-Pt

(a) Morpholine, reflux, 6 h; (b) amberlite IR-120, MeOH, 16 h; (c) cisplatin, $Ag_2O$, DMF, 16 h.

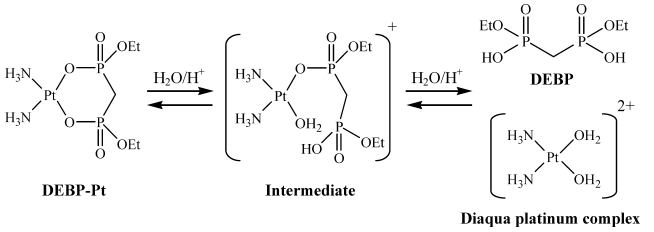


Chart 2. Proposed Mode of DEBP-Pt in an Aqueous Solution

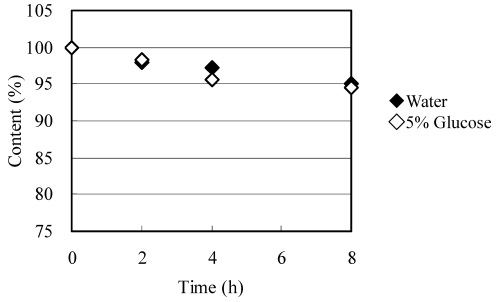


Fig. 2. Stability of DEBP-Pt in an Aqueous Solution

Sample: 20 mM DEBP-Pt in water or 5% glucose solution; stored condition: sealed in the dark at room temperature.

to the bone and then is hydrolyzed gradually. Also, diaqua platinum complex (see Chart 2) dissociates gradually. The dissociated diaqua platinum complex showed an effect on bone tumor. When DEBP-Pt was administrated to the animal, an intravenous drip was assumed. Therefore, some stability in an aqueous solution was required. Then, the stability of DEBP-Pt in water or a 5% glucose solution was confirmed at room temperature. The content of DEBP-Pt in solution was measured by HPLC after being dissolved in water or a 5% glucose solution (Fig. 2). Content of DEBP-Pt was approximately 95% after 8 h, which suggests that DEBP-Pt was stable enough for use in an intravenous drip. Furthermore, the content of DEBP-Pt did not change after 24 h (approximately 95%).

**Hydroxyapatite Affinity** HA affinity of the synthesized DEBP-Pt was evaluated and compared with those of cisplatin and carboplatin.[14,15] The HA was swelled one night in water or human serum and was incubated with the platinum complex at 37 °C. The aliquots of the incubated sample solution were separated from the suspension, and the concentration of platinum in the solution was quantified with inductively coupled plasma mass spectrometer (ICP-MS). Also, the rate of adsorption to HA was calculated. Adsorption of DEBP-Pt for the indicated amount of HA was assessed after 2 h incubation in water (Fig. 3). The rate of HA adsorption of DEBP-Pt increased when the amount of additive HA increased; Rate of HA adsorption of 29.5% was shown with

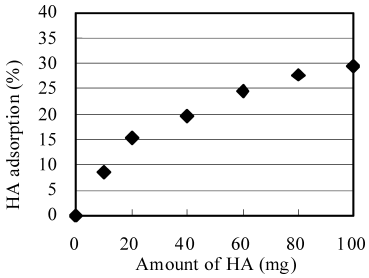


Fig. 3. Adsorption of DEBP-Pt to HA as a Function of HA Amount

Initial sample solution: DEBP-Pt 1 mM; solvent: water 4 ml; incubation: 2 h, 37 °C.

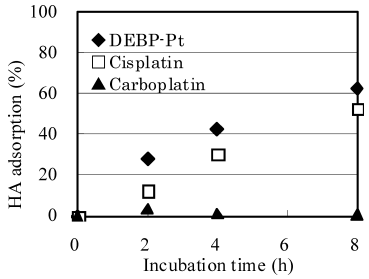


Fig. 4. Adsorption of Each the Platinum Complexes to HA as a Function of Incubation Time

Initial sample solution: platinum complexes 1 mM; amount of HA: 100 mg; solvent: water 4 ml; incubation: 37 °C.

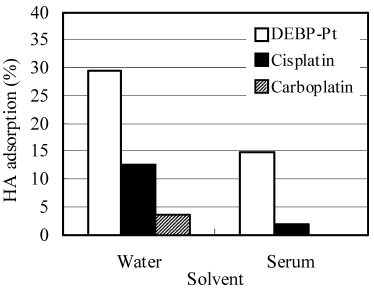


Fig. 5. Adsorption of Platinum Complexes to HA in Water and Serum

Initial sample solution: platinum complexes 1 mM; amount of HA: 100 mg; solvent: water, 100% human serum, 4 ml; incubation time: 2 h.

HA 100 mg. Next, the HA adsorption of each platinum complex in water was examined (Fig. 4). HA adsorption of DEBP-Pt is higher than that of cisplatin after 2, 4, 8 h. DEBP-Pt, showed 62.6% of adsorption after 8 h, DEBP-Pt and cisplatin showed more than 80% after 24 h. DEBP-Pt showed a faster adsorption speed to HA than cisplatin, especially in the early stage. Carboplatin was hardly adsorbed.

Preferential adsorption of compounds to bone rather than other tissues before hydrolysis is critical to exert a bone specific effect. We assessed adsorption of each platinum complex to HA in water and human blood serum with HA 100 mg after 2 h incubation (Fig. 5). DEBP-Pt revealed the highest adsorption to HA; 2.3 times in water and 7.5 times in human blood serum compared with cisplatin. Carbopoatin was adsorbed only a little in water. In the human blood serum which assumed *in vivo* conditions, DEBP-Pt showed higher affinity for HA than the other platinum complexes.

***In Vitro* Assay** We examined the cell growth inhibitory effect of DEBP-Pt against 4 tumor cell lines by CCK-8 assay.[16] The $IC_{50}$ values of DEBP-Pt against the tumor cell

Table 1. Cytotoxicity of the DEBP-Pt against Various Human Tumor Cell Lines

| Cell lines | $IC_{50}$ (mol/l) |
|---|---|
| HLC-2 | $3.6\times10^{-5}$ |
| HCC1954 | $2.8\times10^{-5}$ |
| MCF-7 | $2.6\times10^{-5}$ |
| K562 | $4.7\times10^{-6}$ |

Table 2. Effect on Bone Resorption Activity of Osteoclast

| Substance | $IC_{50}$ (mol/l) |
|---|---|
| DEBP-Pt | $2.8\times10^{-6}$ |
| Cisplatin | $1.1\times10^{-5}$ |

lines are shown in Table 1. The $IC_{50}$ value from $10^{-6}$ to $10^{-4}$ M suggest that DEBP-Pt has a similar antitumor activity to cisplatin.[17] In addition, we evaluated the effect of DEBP-Pt on bone resorption activity,[18] since inhibition of bone resorption by osteoclast leads to a cell growth inhibitory effect of bone tumors. The osteoclast was cultured on dentine that the platinum complexes had been applied. The area of the resorption pit formed by the osteoclast was measured, and the bone resorption inhibitory effect of DEBP-Pt on the osteoclast was compared with cisplatin. DEBP-Pt showed a greater inhibitory effect of bone resorption than cisplatin as shown in Table 2.

## Discussion

When platinum complexes with non-alkylate bisphosphonic acid were synthesized, purification of bisphosphonated platinum complexes is difficult because of solubility, crystallinity and stability in solvents. In many cases, bisphosphonate platinum complexes are purified by recrystallization from heating water.[10] Under these conditions, complexes dissociate and the yield lowers. However, our synthesized DEBP-Pt had highly crystallinity, and was easily crystallized in methanol. The recrystallization was possible without using an aqueous solvent and that lead to high yield synthesis. Moreover, DEBP-Pt is a crystalline solid with little hygroscopicity and highly water solubility, and the pH of aqueous solution was slightly acidic. Stability of aqueous solution, which is necessary for animal administration, was also confirmed. In evaluation of HA affinity, DEBP-Pt showed higher HA affinity and faster HA adsorption compared with other platinum complexes. Even in human blood serum, which assumed *in vivo* conditions, DEBP-Pt demonstrated high HA affinity. As platinum complexes dissociated gradually in the presence of water and competitive ligands, adsorption of platinum complexes to HA in bone before dissociation is a critical factor to exert bone specific effect of the complex.

In general, adsorption of bisphosphonic acid to HA has been ascribed to adsorption of free phosphonate groups to Ca cation in HA. DEBP-Pt, however, has no free phosphate group. We anticipated that an intermediate was formed by partial hydrolysis of DEBP-Pt and equilibrium between DEBP-Pt and an intermediate exists in an aqueous solution of DEBP-Pt similarly to carboxylate platinum complexes such as carboplatin[19] and oxaliplatin[20] (Chart 2). The phosphate group of the intermediate might be responsible for DEBP-Pt adsorption to HA. Diaqua platinum complex dissociates after adsorption of an intermediate to HA. Additionally, $[Pt(I)–OH_2]^+$ of the intermediate might also adsorb to anion of phosphates in HA.

Cytotoxicity of DEBP-Pt might be attributed to binding of the intermediate or the diaqua platinum complex to DNA similarly to other platinum complexes. According to the literature,[12] the estimated bond energy between platinum and phosphate is lower than that between chloride and platinum, and DEBP-Pt is thought to be converted to its active form (intermediate or diaqua platinum complex) more easily compared to cisplatin. Hence, DEBP-Pt was expected to exert equivalent or greater antitumor activity than cisplatin. In fact, our experiments showed equivalent or greater antitumor activity of DEBP-Pt to tumor cells than cisplatin. Moreover, DEBP-Pt showed a greater bone resorption inhibitory effect on the osteoclast that was cultured on dentine compared with cisplatin. This fact suggests that DEBP-Pt has superior HA affinity to cisplatin, and that DEBP-Pt is present in sufficiently higher concentration on dentine, and tends to exert the effect on osteoclast.

## Conclusion

DEBP-Pt can be easily synthesized and purified, demonstrating the suitable properties and stability for administration to animals. In addition, DEBP-Pt with high HA affinity showed antitumor activity and a bone resorption inhibitory effect. Those results suggest the potential of DEBP-Pt as a drug for metastatic bone tumor.

In future, elucidation of the DEBP-Pt adsorption mode to HA and examination of the effects and pharmacokinetics in animals might lead to development of medicine for metastatic bone tumor.

## Experimental

**Chemistry. Instrumental Measurements** The $^1$H-NMR spectra were recorded with a Varian UNITY INOVA (400 MHz) instrument using tetramethylsilane (TMS) as an internal standard. The $^{31}$P-NMR spectra were recorded with a Varian UNITY INOVA (400 MHz) instrument using 85% $H_3PO_4$ as external standard. The FAB-MS spectra were recorded with a JEOL JMS-SX102 mass spectrometer. The IR spectra were recorded Nicolet Avatar-320KYD spectrometer. Concentration of platinum were mesured with ICP-MS Agilent 7500C. The Elemental analysis were performed with Yanaco CHN recorder (MT-5). The HPLC system (HITACHI LaChrom Elite) consisted of HPLC pump (HITACHI L-2130) and UV detector (HITACHI L-2455).

**Preparation of *P*,*P*′-Diethyl Methylenebisphosphonic Acid (DEBP)** Tetraethyl methylenediphosphonate (14.4 g, 50.0 mmol) was added to morpholine (109 g, 1.25 mol) at room temperature, stirred at reflux for 6 h. Cooled to room temperature, the reaction mixture was concentrated *in vacuo*. The residue was recrystallized from acetonitrile to give morpholinium salt as a white powder (19.2 g, 94.5%). $^1$H-NMR ($CDCl_3$) $\delta$: 3.87 (12H, m), 3.14 (8H, t, $J$=4.9 Hz), 2.08 (2H, t, $J_{P,H}$=19.7 Hz), 1.26 (6H, t, $J$=7.0 Hz).

Amberlite IR-120 resign (200 g) was added to morpholinium salt (17.5 g, 43.0 mmol) in methanol (300 ml), stirred for 16 h at room temperature. Filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (methanol : chloroform=1 : 4) to give DEBP as a colorless oil (9.25 g, 92.7%). $^1$H-NMR ($CD_3OD$) $\delta$: 4.11 (4H, dq, $J_{P,H}$=8.0 Hz, $J$=7.0 Hz), 2.46 (2H, t, $J_{P,H}$=21.1 Hz), 1.32 (6H, t, $J$=7.0 Hz). $^{31}$P-NMR ($CD_3OD$) $\delta$: 18.20 (s). IR (KBr) cm$^{-1}$: 979, 2902, 2350, 1647, 1206, 1043, 998, 951, 823, 506, 456. FAB-MS *m*/*z*: 233 $[M+H]^+$.

**Preparation of *cis*-Diammine (*P*,*P*′-Diethyl Methylenebisphosphonato) Platinum (II) (DEBP-Pt)** Cisplatin (10.8 g, 36.0 mmol) was dissolved in DMF (600 ml), and silver oxide (8.34 g, 36.0 mmol) and DEBP (8.77 g, 37.8 mmol) were added, stirred for 16 h at room temperature. The reaction mixture was centrifuged, the residue was washed with DMF (280 ml).

The residue was dissolved in water (480 ml), and centrifuged. The supernatant was filtered and concentrated *in vacuo*. The residue was recrystallized from methanol to give DEBP-Pt (12.3 g, 74.4%) as a white powder. $^1$H-NMR ($CD_3OD$) $\delta$: 4.04 (4H, m), 2.28 (1H, t, $J_{P,H}$=19.3 Hz), 2.26 (1H, t, $J_{P,H}$=19.6 Hz), 1.30 (6H, t, $J$=7.1 Hz). $^{31}$P-NMR ($CD_3OD$) $\delta$: 27.89 (s). IR (KBr) cm$^{-1}$: 3234, 3069, 1180, 1034, 944, 803, 568. FAB-MS *m/z*: 460.0354 (Calcd for $C_5H_{19}O_6N_2P_2Pt$: 460.0366 $[M+H]^+$). *Anal.* Calcd for $C_5H_{18}N_2O_6P_2Pt$: C, 13.08; H, 3.95; N, 6.10. Found: C, 12.98; H, 3.99; N, 5.82.

**Stability Test** The 20 mM sample solutions (5 ml) that dissolved DEBP-Pt in purified water or a 5% glucose solution were made. Sample solutions were incubated at room temperature. At a scheduled time, aliquots (0.2 ml) of the sample solutions were analyzed with HPLC following the previously stated conditions. Concentration of DEBP-Pt was calculated by the absolute calibration curve method.

The chromatographic conditions were used in the determination of DEBP-Pt, SHODEX OHpak SB802.5 HQ (300×8 mm, 2 columns) column with 20 mM formic acid and methanol (7 : 3, v/v) as the mobile phase, a flow rate of 1.0 ml/min, a 20 $\mu$l injection, and temperature of 25 °C, UV detection at 256 nm.

**HA-Adsorbing Experiment** DEBP-Pt, cisplatin (Wako) and carboplatin (Wako) were used for the sample. HA (10—100 mg; Bio-Rad, Hydroxyapatite, type I, 40 $\mu$m) in solvent (2 ml ultra pure water or 100% human serum) was stirred overnight at 37 °C. After a 2 mM sample solution (2 ml) was added to the above sample, the resulting mixture was stirred vigorously at 37 °C. At a schedule time, aliquots (0.2 ml) of the supernatants were well separated from the suspension by centrifugation and filtered. The concentration of platinum in the supernatant was quantified with ICP-MS, and the rate of adsorption to HA was calculated by the following formula:

$$\text{HA adsorption (\%)} = 100 \times [(X - Y)/X]$$

$X$ shows the concentration of platinum in the blank solution without adding HA. $Y$ shows the concentration of platinum in the incubated sample solution.

**Pharmacology. Cytotoxicity Analysis** In the present study we examined the cytotoxic effects of DEBP-Pt against four different human tumor cell lines: lung cancer, HLC-2; breast cancer, MCF-7 and HCC1954; chronic myeloid leukemia, K562. The cytotoxic effects of DEBP-Pt on these cell lines were detected using a CCK-8 assay (cell counting kit 8, DOJINDO).

The cells (5000 cells/well) were plated into 96-well plates in RPMI 1640 with 10% fetal bovine serum and cultured for 24 h at 37 °C in a 5% $CO_2$ incubator. A DEBP-Pt solution in medium was then added to the wells to achieve final concentrations ranging from $10^{-7}$ to $10^{-3}$ M. Control wells were prepared by addition of a culture medium. The plates were incubated at 37 °C in a 5% $CO_2$ incubator for 48 h. Ten microliters of the CCK-8 solution were added to each well of the plate. After incubation for 2 h, the absorbance at 450 nm was measured using a microplate reader. The inhibition rate of cell growth was calculated as follows:

$$\text{inhibition rate (\%)} = 100 \times (T/C)$$

$T$ shows the absorbance of test well. $C$ shows the absorbance of control well. The $IC_{50}$ value was analyzed from the plot of inhibition rates against each concentration.

**Effect on the Bone Resorption by Osteoclast** In this study we used an Osteoclast culture kit (OSC01, COSMO BIO). Dentine slice and medium 180 $\mu$l were plated into 96-well plates. 20 $\mu$l of the sample solution (DEBP-Pt in 5% glucose solution, cisplatin in normal saline solution) were added to each well to achieve final concentrations ranging from $10^{-5}$ to $10^{-3}$ M. Control wells were prepared by sample solution not being added. After 2 h, all the medium was removed. One hundred microliters medium and osteoclast culture medium 100 $\mu$l were to each well ($10^5$ cells/well). The plates were incubated at 37 °C in a 5% $CO_2$ incubator for 10 d, changed the 100 $\mu$l medium at day 3 and day 6. Cells were disrupted (after being cultured on the dentine slice for 10 d) in 5 ml of 1 M ammonia by sonication. Dentine slices were removed from the ammonia and stained with Mayer's (Gill) hematoxylin solution for 1 min, then washed with water and dried. The total area of the resorption pit formed by the osteoclast was measured. The inhibition rate of bone resorption was calculated as follows:

$$\text{inhibition rate (\%)} = 100 \times [(C - T)/C]$$

$T$ shows the area of resorption pit in the test well. $C$ shows the area of resorption pit in the control well. The $IC_{50}$ value was analyzed from the plotted inhibition rates against each sample's concentration.

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