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Letter

Cytotoxicity of zinc, copper and rhodium complexes with 1,10-phenanthroline or 2,9-dimethyl-1,10-phenanthroline in cultured vascular endothelial cells

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ABSTRACT — Organic-inorganic hybrid molecules, which have organic structure and metal atoms, can exhibit various biological activities that are distinctly different from their components. Consequently, organic-inorganic hybrid molecules are considered an effective tool to analyze biological systems. Herein, we investigated the cytotoxicity of zinc, copper, and rhodium complexes, with either 1,10-phenanthroline or 2,9-dimethyl-1,10-phenanthroline as a common ligand, in cultured vascular endothelial cells. The copper complexes, that is, dichloro(1,10-phenanthroline) copper and dichloro(2,9-dimethyl-1,10-phenanthroline) copper, exhibited high cytotoxicity accompanied by considerable accumulation inside the cells. Potassium tetrachloro(1,10-phenanthroline) rhodate also exhibited cytotoxicity and considerable accumulation. Thus, it was found that the cytotoxicity of organic-inorganic hybrid molecules to vascular endothelial cells depends on the interaction between the intramolecular metal and ligand, which facilitates their uptake by the cells.

Key words: Bio-organometallics, Coordination compound, 1,10-Phenanthroline, Cytotoxicity, Metal, Vascular endothelial cell

INTRODUCTION

Organic-inorganic hybrid molecules, which have an organic structure and metal atoms, are widely used as synthetic reagents in chemical reactions. Little is known about the biological activities of these compounds. Therefore, it is not clear how the intramolecular metal modifies the biological activities of organic-inorganic hybrid molecules. Recently, we reported that the cell's biological response to organic-inorganic hybrid molecules is different from that toward each of the organic or inorganic components separately; this suggests that a distinct biology for organic-inorganic hybrid molecules should be established (Kohri *et al.*, 2015; Murakami *et al.*, 2015; Fujie *et al*., 2016a; Fujie *et al*., 2016b; Fujie *et al*., 2016c).

The intensity of binding between the intramolecular metal and the organic structure appears to be a defining factor for the biological activities of organic-inorganic hybrid molecules. This binding intensity seems to depend on the type of intramolecular metal or on the polarity or configuration of the organic structure (Kimura *et al*., 2012).

1,10-Phenanthroline (Phen) is a well-known divalent metal chelating agent, selective for Fe(II) but not Fe(III). Based on this characteristic, it has been used as an assay reagent of Fe(II) in environmental water (Hoshi *et al*., 1989). In the present study, we investigated the cytotoxicity of Phen and 2,9-dimethyl-1,10-phenanthroline (DMP) complexes with zinc (Zn-P and Zn-DMP, respectively), copper (Cu-P and Cu-DMP, respectively), or rhodium

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(Rh-P and Rh-DMP, respectively) in vascular endothelial cells.

MATERIALS AND METHODS

Materials

Bovine aortic endothelial cells were purchased from Cell Applications (San Diego, CA, USA). Dulbecco's modified Eagle medium and Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (CMF-PBS) were obtained from Nissui Pharmaceutical (Tokyo, Japan). Fetal bovine serum was purchased from Biosera (Kansas City, MO, USA). Cytotox 96® Non-Radioactive Cytotoxicity Assay, a lactate dehydrogenase (LDH) kit, was obtained from Promega (Madison, WI, USA). The BCA protein assay kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Phen and DMP were sourced from Tokyo Chemical Industry (Tokyo, Japan). Copper (II) chloride ($CuCl_2$) and a CBB protein assay kit were purchased from Nacalai Tesque, (Kyoto, Japan). Cu-P was obtained from Sigma-Aldrich (St. Louis, MO, USA). Zinc chloride ($ZnCl_2$), rhodium (III) chloride ($RhCl_3$), nitric acid, anti-β-actin mouse monoclonal antibody, and other reagents were obtained from Wako Purechemical Industries (Osaka, Japan).

Synthesis of Zn-P, Zn-DMP Rh-P, Cu-DMP, and Rh-DMP

$ZnCl_2$ was dissolved with Phen or DMP in ethanol, stirred for 1 hr at room temperature, and filtered to obtain a white precipitate of Zn-P or Zn-DMP, respectively. These precipitates were recrystallized from acetonitrile to obtain purified Zn-P and Zn-DMP.

A reported procedure was generally followed to synthesize Rh-P and Rh-DMP (Lee *et al.*, 2003). Cu-DMP was synthesized by the reaction of DMP with $CuCl_2{\cdot}2H_2O$ in tetrahydrofuran at room temperature (Wang *et al.*, 2009).

Cell culture and treatment

Vascular endothelial cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and antibiotics (5,000 IU/mL penicillin and 5 mg/mL streptomycin) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The medium was discarded; then the cells were washed twice with Dulbecco's modified Eagle medium and treated for 24 hr with Phen, $ZnCl_2$, Zn-P, Zn-DMP, $CuCl_2$, Cu-P, Cu-DMP, $RhCl_3$, Rh-P or Rh-DMP (at 1, 5, 10, and 20 μM each).

Cytotoxicity assay

Confluent cultures of vascular endothelial cells were incubated with Phen, $ZnCl_2$, Zn-P, Zn-DMP, $CuCl_2$, Cu-P, Cu-DMP, $RhCl_3$, Rh-P, or Rh-DMP for 24 hr. After incubation, the treated medium was collected and used for the determination of LDH activity, as a marker of non-specific cytotoxicity, according to the manufacturer's protocol.

Intracellular accumulation of zinc, copper, and rhodium

Confluent cultures of vascular endothelial cells were incubated with Phen, $ZnCl_2$, Zn-P, Zn-DMP, $CuCl_2$, Cu-P, Cu-DMP, $RhCl_3$, Rh-P, or Rh-DMP (1, 5, 10, and 20 μM each) for 8 hr. After incubation, the medium was discarded and the cell layer was washed and harvested with ice-cold CMF-PBS. The cell suspension was centrifuged at 20,000 × *g* for 2 min and the pellet was re-suspended in 500 μL of CMF-PBS and sonicated. Cell debris was precipitated by centrifugation at 1,000 × *g* for 2 min and the supernatant (100 μL) was added to 4.9 mL of 0.1 M nitric acid; the mixture was used for the detection of intracellular zinc, copper, and rhodium by inductively coupled plasma mass spectrometry (Nexion 300S, PerkinElmer, Waltham, MA, USA) as an indicator of intracellular accumulation of tested compounds. A portion of the supernatant was analyzed for protein concentration using a CBB protein assay kit according to the manufacturer's protocol. The metal content was expressed as pmol/mg protein.

Statistical analysis

Data were tested for statistical significance by analysis of variance and Dunnett's method. *P* values of less than 0.05 were considered to indicate statistically significant differences.

RESULTS AND DISCUSSION

The organic-inorganic hybrid molecules used in this study are shown in Fig. 1. Figure 2 shows the morphological changes and LDH leakage in vascular endothelial cells treated with Phen, $ZnCl_2$, Zn-P, Zn-DMP, $CuCl_2$, Cu-P, Cu-DMP, $RhCl_3$, Rh-P, or Rh-DMP for 24 hr. Morphologically, Cu-P at ≥ 5 μM, Cu-DMP at ≥ 10 μM, and Rh-P at ≥ 10 μM exhibited a significant dose-dependent cytotoxicity. This morphological observation was accompanied with considerable leakage of LDH into the medium. However, no cytotoxicity was observed after exposing the cells to Phen, $ZnCl_2$, Zn-P, Zn-DMP, $CuCl_2$, $RhCl_3$, or Rh-DMP, either morphologically or with regard to LDH leakage. Taking into account the collective results for Phen and its metal complexes, it was found that (1) Phen, $ZnCl_2$, and Zn-P are all nontoxic; (2) Phen and $CuCl_2$ are nontoxic, but Cu-P is toxic; and (3) Phen and

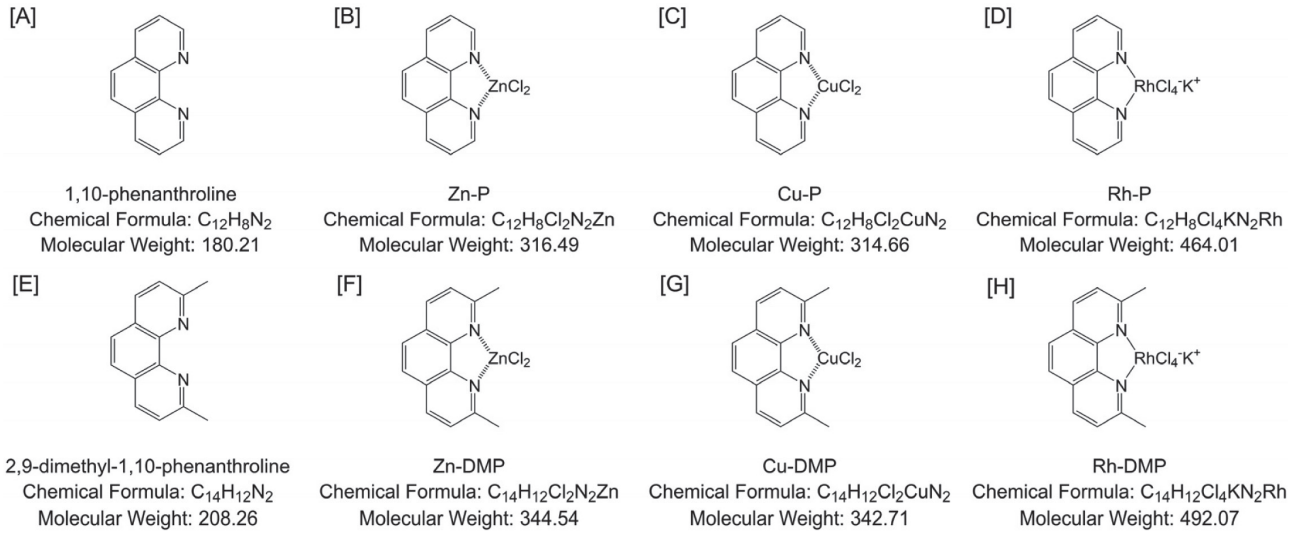

Fig. 1. Structures of organic-inorganic hybrid molecules used in this study. [A] 1,10-Phenanthroline (Phen); [B] dichloro(1,10-phenanthroline)-zinc (Zn-P); [C] dichloro(1,10-phenanthroline)-copper (Cu-P); [D] potassium tetrachloro(1,10-phenanthroline) rhodate (Rh-P); [E] 2,9-dimethyl-1,10-phenanthroline (DMP); [F] dichloro(2,9-dimethyl-1,10-phenanthroline)-zinc (Zn-DMP); [G] dichloro(2,9-dimethyl-1,10-phenanthroline)-copper (Cu-DMP); [H] potassium tetrachloro(2,9-dimethyl-1,10-phenanthroline) rhodate (Rh-DMP).

$RhCl_3$ are nontoxic, but Rh-P is toxic. This indicates that the cytotoxicity of Phen-related metal complexes to vascular endothelial cells was caused by the interaction of Phen with the introduced metal. In other words, the cytotoxicity of Phen-related metal complexes is distinctive from that of their structural components. Furthermore, the cytotoxicity of Cu-DMP and Rh-DMP was significantly lower than that of Cu-P and Rh-P, respectively, suggesting that methylation of Phen reduces the interaction of Cu/Rh with the ligand. This interaction is critical for exhibiting cytotoxicity to vascular endothelial cells. Similarly, it has been reported that intramolecular metal can modify the cytotoxicity of organopnictogen compounds (Kohri *et al.*, 2015; Murakami *et al.*, 2015).

The intracellular accumulation of organic-inorganic hybrid molecules in vascular endothelial cells was used as an indicator of the relationship between their cytotoxicity and the degree of accumulation. As shown in Fig. 3, toxic compounds (Cu-P, Cu-DMP, and Rh-P) significantly accumulated in a dose-dependent manner. On the other hand, nontoxic compounds ($ZnCl_2$, Zn-P, Zn-DMP, $CuCl_2$, $RhCl_3$, and Rh-DMP) did not show any significant accumulation. Specifically, the accumulation of $ZnCl_2$, Zn-P, Zn-DMP, $CuCl_2$, and $RhCl_3$ was almost equal to that in cells treated with Phen. However, the intracellular accumulation of Rh-DMP was slightly higher than that of the other compounds in this group, but was substantially lower than that of Rh-P. Thus, it is hypothesized that the cytotoxicity of the compounds tested in this study relied on the level of intracellular accumulation. This is consistent with our previous study regarding the impact of intracellular accumulation on the modification of the cytotoxicity of organopnictogen compounds (Kohri *et al.*, 2015; Murakami *et al.*, 2015).

In conclusion, it is suggested that cytotoxicity of organic-inorganic hybrid molecules to vascular endothelial cells may result from the interaction of intramolecular metal with the ligand. This interaction can lead to higher accumulation within the cells. Therefore, it is difficult to determine the cytotoxicity of organic-inorganic hybrid molecules based on the cytotoxicity of their structural components individually. Further studies are required to clarify the (1) changes in the characteristics of organic-inorganic hybrid molecules, caused by the metal-ligand interaction, including the electron state; and (2) the mechanisms by which these changes facilitate the entry of the molecules into the cells.

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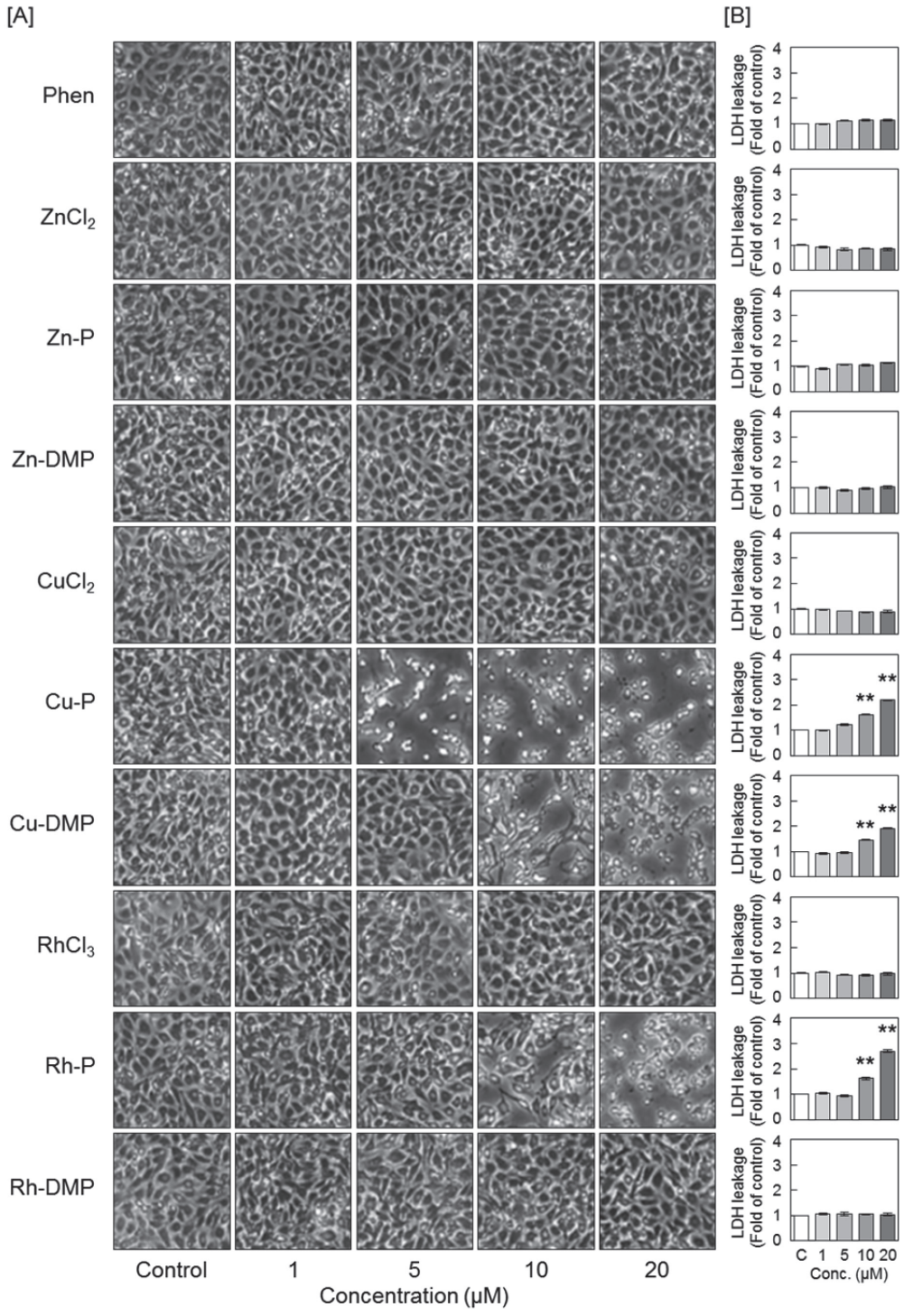

Fig. 2. Cytotoxicity of Phen, $ZnCl_2$, Zn-P, Zn-DMP, $CuCl_2$, Cu-P, Cu-DMP, $RhCl_3$, Rh-P, and Rh-DMP. Confluent cultures of bovine aortic endothelial cells were treated with Phen, $ZnCl_2$, Zn-P, Zn-DMP, $CuCl_2$, Cu-P, Cu-DMP, $RhCl_3$, Rh-P, or Rh-DMP (1, 5, 10, or 20 μM each) for 24 hr. [A] Morphological appearance of vascular endothelial cells. [B] The leakage of lactate dehydrogenase (LDH) from the cells into the medium. Values are mean ± S.E. of four samples. Significantly different from the corresponding control, $^{**}P < 0.01$.

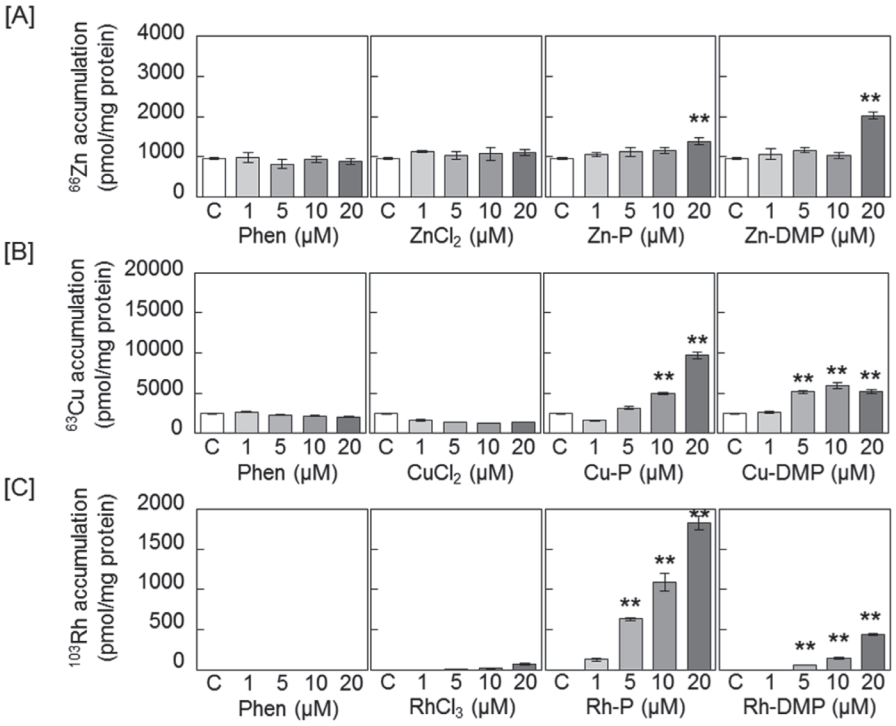

Fig. 3. Intracellular accumulation of [A] ^{66}Zn, [B] ^{63}Cu, and [C] ^{103}Rh in vascular endothelial cells. Confluent cultures of bovine aortic endothelial cells were treated with Phen, $ZnCl_2$, Zn-P, Zn- DMP, $CuCl_2$, Cu-P, Cu-DMP, $RhCl_3$, Rh-P, or Rh-DMP (1, 5, 10, or 20 μM each) for 8 hr. Values are mean ± S.E. of three samples. Significantly different from the corresponding control, $^{**}P < 0.01$.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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