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Cell-penetration by Co(III)cyclen-based peptide-cleaving catalysts selective for pathogenic proteins of amyloidoses

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ABSTRACT

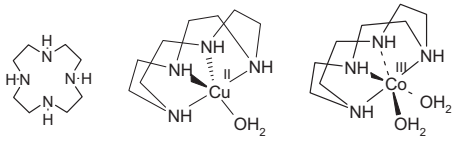
Derivatives of the Co(III) complex of 1,4,7,10-tetraazacyclododecane (cyclen) with various organic pendants have been reported as target-selective peptide-cleaving catalysts, which can be exploited as catalytic drugs. In order to provide a firm basis for the catalytic drugs based on Co(III)cyclen, the ability of the Co(III)cyclen-containing peptide-cleaving catalysts to penetrate animal cells such as mouse fibroblast NIH-3T 3 or human embryonic kidney (HEK) 293 cells is demonstrated in the present study. Since the catalysts destroy pathogenic proteins for amyloidoses, results of the present study are expected to initiate extensive efforts to obtain therapeutically safe catalytic drugs for amyloidoses such as Alzheimer's disease, type 2 diabetes mellitus, Parkinson's disease, Huntington's disease, mad cow disease, and so on.

1. Introduction

We have proposed catalytic drugs based on target-selective peptide-cleaving catalysts as a new paradigm in drug design.[1–4] As the catalytic group for peptide hydrolysis, metal complexes such as the Co(III) complex of 1,4,7,10-tetraazacyclododecane (cyclen) have been used.[3,4] A small synthetic molecule that can recognize a disease-related protein or oligopeptides and cleave the peptide backbone of the target selectively can remove the bioactivity of the target as illustrated in Scheme 1. This may cure the disease related to the target.

Conventional drugs targeting enzymes or receptors occupy the active site of the target protein, requiring at least a stoichiometric amount of the drug molecule. On the other hand, a catalytic amount can be used for the catalytic drug, reducing the drug dosage and the side effects. Conventional drugs require the presence of the active site, and, therefore, cannot be applied to proteins lacking active sites. On the other hand, peptide-cleaving catalyst can recognize any part of the target, not necessarily the active site, and, therefore, can be obtained even for pathogenic proteins without active sites.[3,4]

The first target-selective peptide-cleaving catalysts have been obtained by using myoglobin as the target and by adopting the Cu(II) or Co(III) complex of cyclen as the catalytic site and oligomers of peptide nucleic acids as the recognition site.[5,6] Subsequently, a peptide-cleaving catalyst selective for a disease-related enzyme has been discovered from a chemical library of Co(III)cyclen derivatives containing various organic pendants by using peptide deformylase as the target enzyme.[7]



The first peptide-cleaving catalysts selective for proteins lacking active sites have been designed[8–10] by using Co(III)cyclen as the catalytic site and by using oligomers of amyloidogenic oligopeptides or proteins as the targets, providing a new therapeutic option for amyloidoses[11–21] such as Alzheimer's disease, type 2 diabetes mellitus, and Parkinson's disease. Since conventional drugs targeting active sites of disease-related proteins cannot be designed for amyloidogenic oligopeptides or proteins, clinical test and therapeutic application of peptide-cleaving catalysts are important in curing amyloidoses.

Both Cu(II)cyclen and Co(III)cyclen have been found to be effective in catalyzing peptide hydrolysis in the discovery of the first target-selective peptide-cleaving catalysts.[5,6] In subsequent studies, however, Co(III)cyclen has been mainly used[7–10] as the catalytic site in view of the exchange-inertness[22] of Co(III) complexes. The metal ions in exchange labile complexes such as Cu(II)cyclen may be easily

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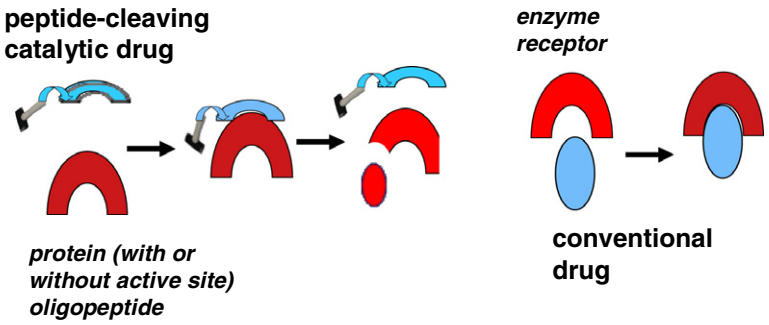


Scheme 1. Comparison of a peptide-cleaving catalytic drug with a conventional drug targeting an enzyme or a receptor.

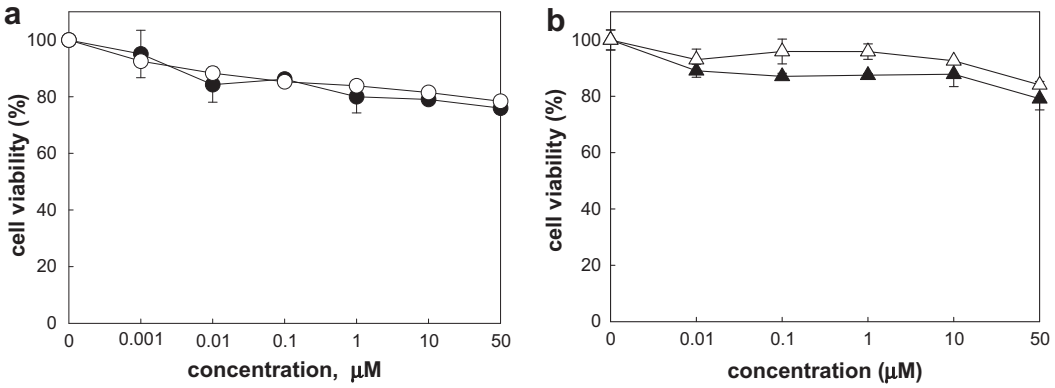


Figure 1. Relative cell viability measured for **A** (a; ●), **B** (a; ○), **C** (b; ▲), and **D** (b; △) using HEK 293 cells.

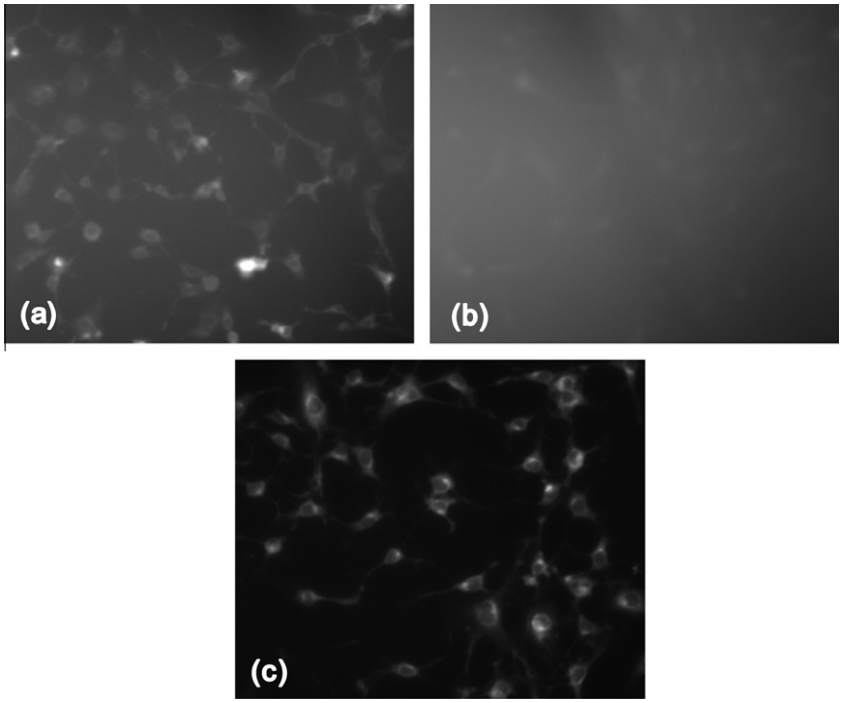


Figure 2. Fluorescence microscope images taken with a DAPI filter obtained for NIH-3T 3 cells incubated with 50 μM **A** (a), **B** (b), or **D** (c) for 1 h at 37 °C and pH 7.4.

extracted by metal-sequestering agents present in biotic environments, leading to loss of the catalytic site and exposure of cyclen. Cyclen has very high affinity for various metal ions[23] and may exert toxic effects under biotic conditions. In Co(III)cyclen, cyclen provides peptide-hydrolyzing ability to Co(III) ion and Co(III) ion masks potential toxicity of cyclen. In order to provide a firm basis for clinical applicability of the peptide-cleaving catalysts containing Co(III)-cyclen and to initiate extensive search for therapeutically safe drugs, it is necessary to demonstrate that the catalysts can readily penetrate animal cells. In the present study, we have observed that Co(III)cyclen derivatives containing organic pendants indeed penetrate animal cells.

2. Materials and methods

2.1. Synthesis of Co(III) complexes

Synthesis of 2-[4-{2-[(4-benzothiazol-2-yl-phenyl)-methyl-amino]-ethoxy}-6-(4-chloro-benzylamino)-[1,3,5]triazin-2-ylamino]-*N*-[3-(1,4,7,10-tetraaza-cyclododec-1-yl)-propyl]-(*S*)-propionamide and its Co(III) complex (**A**), synthesis of 3-(4-{2-[(4-benzothiazol-2-yl-phenyl)-methyl-amino]-ethoxy}-6-cyclododecylamino-[1,3,5]triazin-2-ylamino)-*N*-[3-(1,4,7,10-tetraaza-cyclododec-1-yl)-propyl]-propionamide and its Co(III) complex (**B**), synthesis of 2-((*S*)-4-{2-[(4-benzothiazol-2-yl-phenyl)-methyl-amino]-ethoxy}-6-piperidin-1-yl-[1,3,5]triazin-2-ylamino)-4-methyl-pentanoic acid [3-(1,4,7,10-tetraaza-cyclododec-1-yl)-propyl]-amide and its Co(III) complex (**C**), and synthesis of 2-((*S*)-4-{2-[(4-benzothiazol-2-yl-phenyl)-methyl-amino]-ethoxy}-6-cyclododecylamino-[1,3,5]-triazin-2-ylamino)-4-methyl-pentanoic acid [3-(1,4,7,10-tetraaza-cyclododec-1-yl)-propyl]-amide and its Co(III) complex (**D**) are described in the literature.[8,9]

2.2. Cell culture preparation

HEK 293 fibroblasts and Mouse fibroblast NIH-3T 3 cell line were purchased from American Type Culture Collection and then

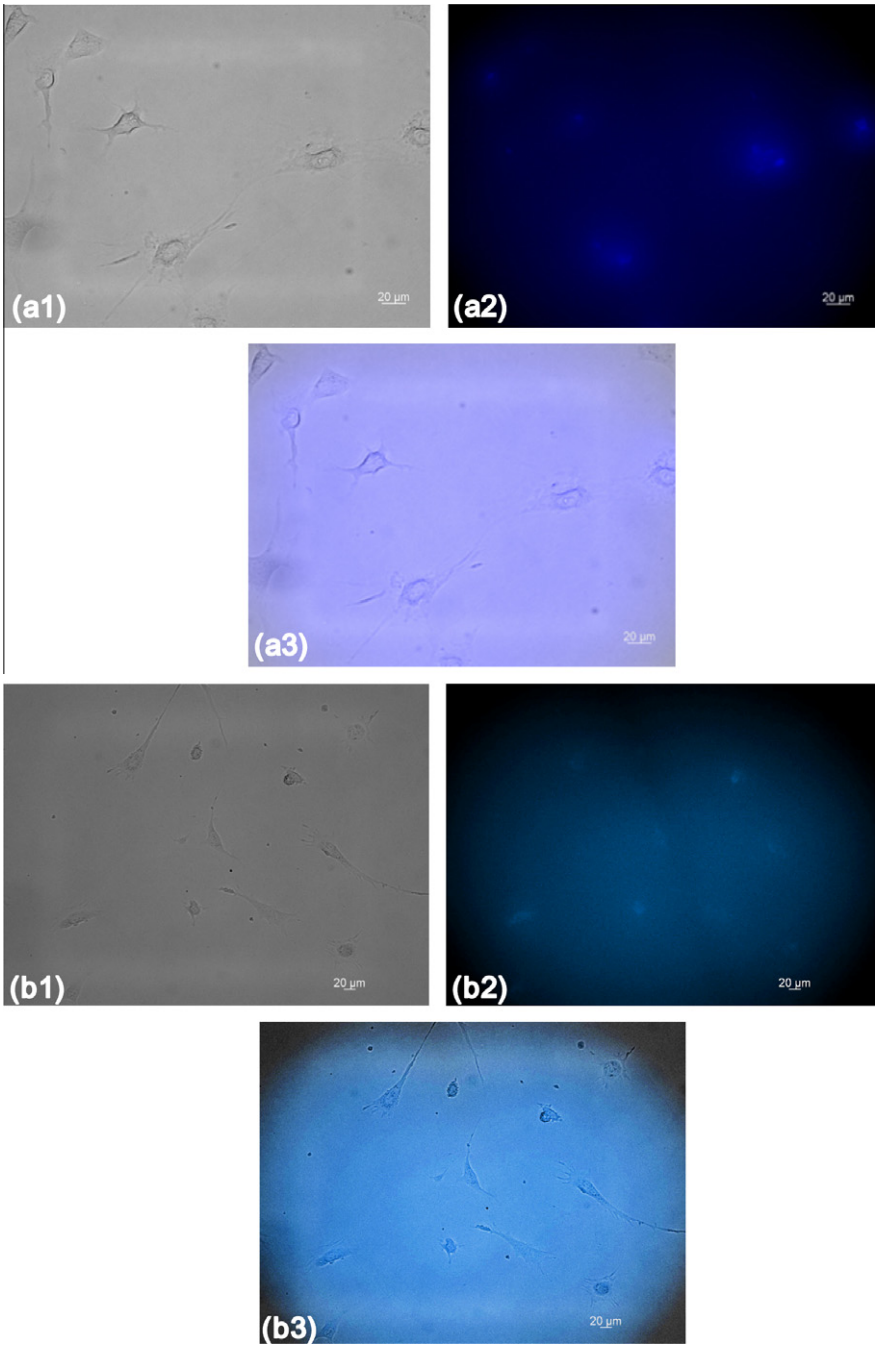


Figure 3. Fluorescence image and fluorescence-UV image obtained for HEK 293 cells after incubation with 10 μM of **A** (a1; UV image, a2; fluorescence image, a3; merge (fluorescence-UV) image), **B** (b1; UV image, b2; fluorescence image, b3; merge image), **C** (c1; UV image, c2; fluorescence image, c3; merge image), or **D** (d1; UV image, d2; fluorescence image, d3; merge image) for 1 h at 37 °C and pH 7.4.

bred and cultured in this laboratory. All media were supplemented with 100 units/mL of penicillin and 100 mg/mL of streptomycin. HEK 293 cells were grown in Dulbecco's Modified Eagle Medium (Hyclone) supplemented with 10% fetal bovine serum (Hyclone). Cells were kept at 37 °C in a humidified atmosphere containing 5% CO_2 for three days.

2.3. Cytoxicity measurement

To measure the cytotoxicity, cell counting kit-8 (CCK-8) (Dojindo Molecular Technologies) was employed. CCK-8 uses a highly water-soluble tetrazolium salt, which produces a water-soluble formazan dye upon reduction by the dehydrogenase activity of the cells directly indicating the proportion of the living cells. After addition of 10 μL of CCK-8 solution to each well and the subsequent incubation of the plate for 1 h in the incubator, the absorbance at 450 nm was measured by using a microplate reader TECAN infinite M200 (Tecan).

2.4. Cell-penetration measurement

After a Co(III) complex was added to the cell line media, the media was incubated for 1 h at 37 °C, and washed at least for three times with PBS. The cell images were then examined by a fluorescence microscope (Zeiss, Axio observer Z1). For the NIH-3T3 cells, a 4′,6-diamidino-2-phenylindole (DAPI) filter was used and the excitation wavelength of 400–418 nm and the emission wavelength of 435–470 nm were adopted. For the HEK 293 cells, the excitation

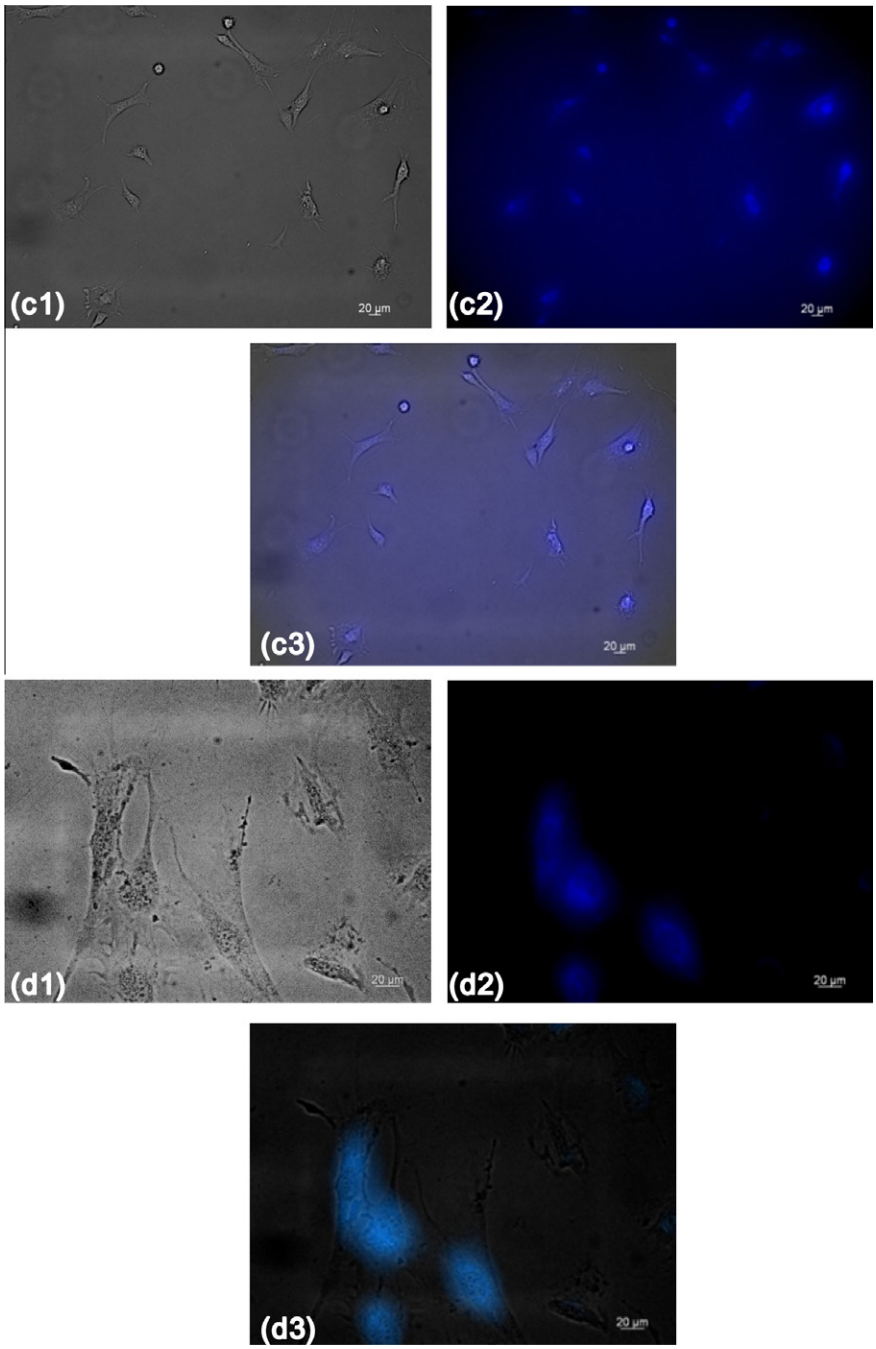
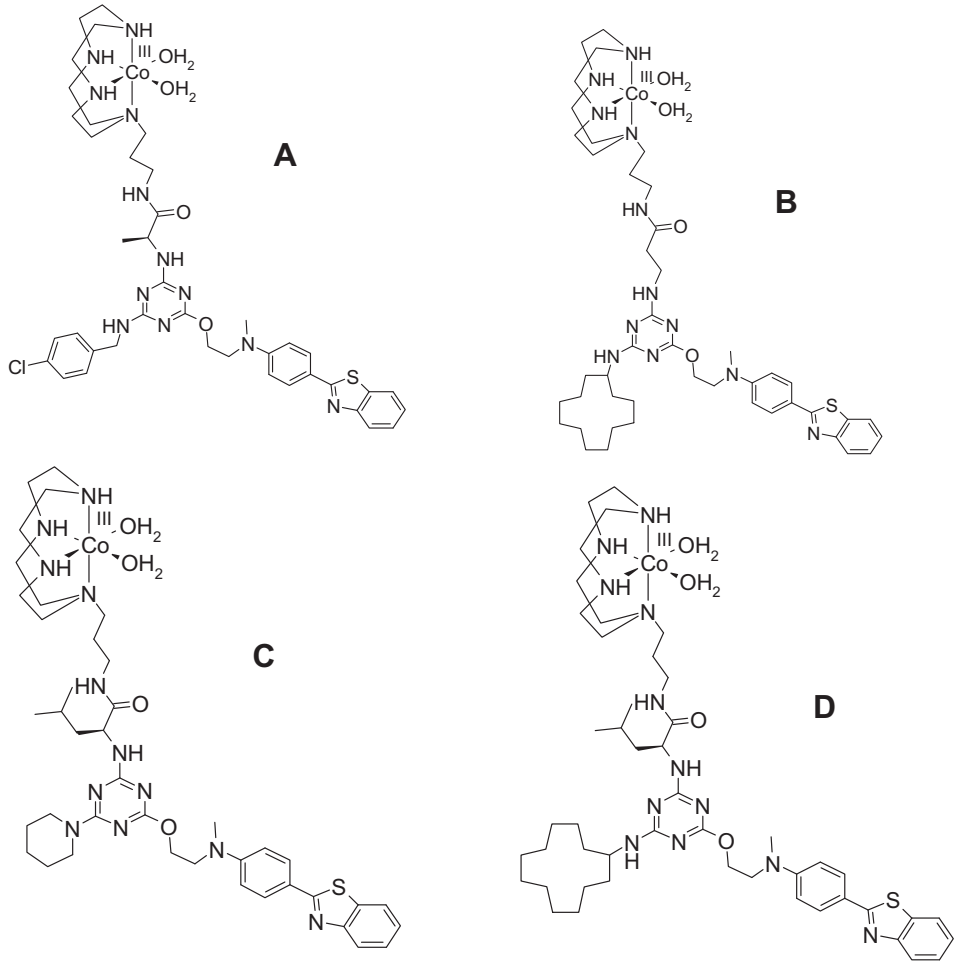


Figure 3. (*continued*)

wavelength of 359 nm and the emission wavelength of 461 nm were adopted.

3. Results and discussion

To examine whether Co(III)cyclen conjugated with organic pendants penetrate animal cells readily, compounds **A**–**D** were selected since their organic pendants are fluorescent. Compounds **A**–**D** have been discovered[8–10] to have activity in hydrolyzing the pathogenic proteins for Alzheimer's disease, type 2 diabetes mellitus, and/or Parkinson's disease.



Cytotoxicity of **A**–**D** was examined by incubating with HEK 293 cells for 24 h at 37 °C. As summarized in Figure 1, only minor cytotoxicity on HEK 293 cells was observed with **A**–**D**.

Cell-penetration experiments were first carried out with the mouse fibroblast NIH-3T3 cell line. After incubating **A**–**D** (50 μM) with the cells in a PBS buffer for 1 h at 37 °C, the medium was washed three times with the PBS buffer and the cells were examined with a fluorescence microscope by using a DAPI filter. By this method, the fluorescent molecules appear as bright white regions in the fluorescence image. The results summarized in Figure 2 reveal that **A**, **B**, and **D** penetrate the cells and the cells are alive after exposure to **A**, **B**, and **D**, although the quality of the image taken for **B** is not good. For **C** (50 μM), cytotoxicity on NIH-3T 3 hampered the measurement.

To obtain further information on cell-penetration of **A**–**D**, additional experiments were carried out with the HEK 293 cell line, by employing a lower concentration of **A**–**D** to obtain clearer fluorescence images. After incubating **A**–**D** (10 μM) with the cells in a PBS buffer for 1 h at 37 °C, the medium was washed three times with the PBS buffer and the cells were examined with a fluorescence microscope. With HEK 293 cells, no toxicity was observed for **C** at 50 μM (Fig. 1(b)). In this experiment, the DAPI filter was not adopted in obtaining the fluorescence image in order to obtain the UV image as well as the fluorescence image. When the UV image is obtained together with the fluorescence image, it is easier to confirm positions of the cells. The results are summarized in Figure 3.

In Figure 3, the blue region indicates the fluorescence. In Figure 3(a3), (b3), (c3), and (d3), the UV images are combined with the fluorescence image. The blue backgrounds shown in Figure 3(a3), (b2), (b3), and (c3) are not due to incomplete washing of the fluorescent catalysts but due to the increased sensitivity of the pictures. The results summarized in Figure 3 reveal that the fluorescent materials penetrate HEK 293 cells and spread around cytoplasm. The exact subcellular localization of these fluorescent materials, however, needs further elucidation.

In Co(III)cyclen, the Co(III) ion is embedded in an egg-like structure formed by its ligands. The shape of Co(III)cyclen derivatives is further affected by the organic pendants which act as the binding site for recognition of the target proteins. The ability of cell-penetration by the Co(III)cyclen derivatives would be, therefore, distinctly different from that of other metal complexes containing simple ligands.

The ability of a drug molecule to penetrate cellular and nuclear membranes depends on several factors including lipophilicity, hydrophilicity, and size. Thus, the degree of penetration into animal cells as well as cytotoxicity would be different for Co(III) com-

plexes **A**–**D** to some extent due to the differences in the structure of the organic pendants. The results of the present study demonstrate that Co(III)cyclen derivatives containing some organic pendants can readily penetrate animal cells.

Compounds **A**–**D** effectively cleaved[8–10] the soluble oligomers of amyloid β-24, human islet amyloid polypeptide, and/or synuclein which are believed to be the pathogenic species[11–21] for Alzheimer's disease, type 2 diabetes mellitus, and Parkinson's disease, respectively. Since those pathogenic species have no active sites, peptide-cleaving catalysts such as **A**–**D** selectively cleaving the soluble oligomers of amyloidogenic oligopeptides or proteins may cure amyloidoses. To discover drugs for the amyloidoses, it is necessary to obtain a large number of derivatives of Co(III)cyclen or other potential catalytic groups and perform biological and clinical tests. One of the major obstacles in this quest is whether derivatives of coordination complexes such as Co(III)cyclen can readily penetrate the animal cells.[24] The present study revealed that the Co(III)cyclen-based peptide-cleaving catalysts can penetrate animal cells readily. This is expected to initiate extensive efforts to obtain therapeutically safe catalytic drugs for amyloidoses such as Alzheimer's disease, type 2 diabetes mellitus, Parkinson's disease, Huntington's disease, mad cow disease, and so on.

Acknowledgments

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