An Organobismuth Compound that Exhibits Selective Cytotoxicity to Vascular Endothelial Cells *in Vitro*

Yasuyuki Fujiwara,[a] Makoto Mitani,[a, b] Shuji Yasuike,[b] Jyoji Kurita,[b] and Toshiyuki Kaji*[, a]

[a]*Department of Environmental Health and* [b]*Department of Organic Chemistry, Faculty of Pharmaceutical Sciences, Hokuriku University, Ho-3 Kanagawa-machi, Kanazawa 920–1181, Japan*

(Received February 9, 2005; Accepted February 21, 2005; Published online February 22, 2005)

Selective cytotoxicity of chemical compounds to vascular endothelial cells is often beneficial for clinical applications including antivascular cancer therapy. In this study, the cytotoxicity of 35 organic compounds containing bismuth or antimony was evaluated based on the leakage of lactate dehydrogenase and morphologic observations in cultured bovine aortic endothelial cells and other cell types. The results indicate that only tris[2-(*N*,*N*-dimethylaminomethyl)phenyl]-bismuthane (TDPBi) had potent cytotoxicity to bovine aortic endothelial cells but not to bovine aortic smooth muscle cells and porcine kidney epithelial LLC-PK_1 cells; the compound exhibited moderate cytotoxicity to human fetal lung fibroblastic IMR-90 cells. Neither inorganic bismuth nor tris[2-(*N*,*N*-dimethylaminomethyl)phenyl]stibane, in which antimony substitutes for bismuth with the same organic structure as TDPBi, exhibited cytotoxicity to vascular endothelial cells, suggesting that the entire structure of TDPBi is required for selective cytotoxicity. The present data revealed that TDPBi has selective cytotoxicity to vascular endothelial cells, which may be applicable in antivascular cancer therapy.

Key words —— bismuth, antimony, cytotoxicity, vascular endothelial cell

INTRODUCTION

Since vascular endothelial cell damage is an important event in the progression of inflammation and atherosclerosis,[1,2] prevention of the pathologic changes in vascular endothelial cells has focused on pharmacologic and toxicologic studies. On the other hand, endothelial cells are key targets in cancer therapy because they participate in the growth of tumor tissue through vasculogenesis and angiogenesis. Agents that exhibit selective cytotoxicity to vascular endothelial cells may induce apoptosis in the cells and/or inhibit tumor-associated angiogenesis. Thus the search for such agents will contribute to the development of anticancer drugs.

Metal(loid)-containing compounds have been used historically as medicines for several thousands of years, especially in Chinese traditional medicine. Recently, it has been revealed that several metal-containing compounds exhibit anticancer activities.[3] Arsenic, antimony, and bismuth are classified as metalloids that belong to group V in the periodic table. Arsenic trioxide and antimony trioxide have been used in patients with acute promyerocytic leukemia the past decade.[4] A series of antimony (III) compounds with polydentate carboxylic acids shows antitumor activity in mice inoculated with S180 solid tumors.[5] Treatment with inorganic bismuth results in regression of gastric lymphoma caused by *Helicobacter pylori*[6] and reduction of the toxic side effects of cisplatin by tissue-specific induction of metallothionein.[7]

In the present study, the cell type-dependent cytotoxicity of 35 bismuth- or antimony-containing organic compounds synthesized originally was investigated using a cell culture system to search for novel organometalloid compounds that exhibit selective cytotoxicity to vascular endothelial cells.

*To whom correspondence should be addressed: Department of Environmental Health, Faculty of Pharmaceutical Sciences, Hokuriku University, Ho-3 Kanagawa-machi, Kanazawa 920–1181, Japan. Tel. & Fax: +81-76-229-6208; E-mail: t-kaji@hokuriku-u.ac.jp

MATERIALS AND METHODS

Materials —— Vascular endothelial cells derived from bovine aorta, LLC-PK_1 cells from porcine kid-

Table 1. Organobismuth and Organoantimony Compounds

Compound no.	Molecular formula	
Organobismuth compounds		
01	$C_{18}H_{15}Bi$	Triphenylbismuthane
02	$C_{19}H_{21}BiSi$	1-Phenyl-2-trimethlsilyl-1-benzostilyl-1-benzobismepine
03	$C_{21}H_{21}Bi$	Tris(4-methylphenyl)bismuthane
04	$C_{23}H_{26}BiN$	2-(*N*,*N*-Dimethylaminomethyl)phenylbis(4-methylphenyl)bismuthane
05	$C_{24}H_{27}BiO_3$	Tris[2-(methoxymethyl)phenyl]bismuthane
06	$C_{24}H_{27}BiS_3$	Tris[2-(methysulfanylmethyl)phenyl]bismuthane
07	$C_{27}H_{36}BiN_3$	Tris[2-(*N*,*N*-dimethylaminomethyl)phenyl]bismuthane
Organoantimony compounds		
08	$C_{12}H_9SSb$	1-Phenylthieno[3,4-*b*]stibole
09	$C_{14}H_{11}Br_2Sb$	1,1-Dibromo-1-phenyl-1-benzostibole
10	$C_{14}H_{11}Cl_2Sb$	1,1-Dichloro-1-phenyl-1-benzostibole
11	$C_{14}H_{11}Sb$	1-Phenyl-1-benzostibole
12	$C_{15}H_{17}SSbSi$	1-Phenyl-2-trimethylsilylthieno[2,3-*b*]stibole
13	$C_{17}H_{19}SbSi$	1-Phenyl-2-trimethylsilyl-1-benzostibole
14	$C_{18}H_{12}F_3Sb$	Tris(4-fluorophenyl)stibane
15	$C_{18}H_{13}Sb$	1-Phenyl-1-stibaphenalene
16	$C_{18}H_{15}Sb$	Triphenylstibane
17	$C_{19}H_{15}Sb$	9,10-Dihydro-9-phenyl-9-stibanthracene
18	$C_{19}H_{21}SbSi$	1-Phenyl-2-trimethlsilyl-1-benzostilyl-1-benzostibepine
19	$C_{20}H_{15}Sb$	5-Phenyldibenzo[*b*,*f*]stibepine
20	$C_{21}H_{21}O_3Sb$	Tris(4-methoxyphenyl)stibane
21	$C_{21}H_{21}Sb$	Tris(4-methylphenyl)stibane
22	$C_{22}H_{14}OSb$	8-Phenyldibenzo[*b*,*i*]-1-oxa-6-stibocine
23	$C_{22}H_{23}OSb$	2-(Methoxymethyl)phenylbis(4-methylphenyl)stibane
24	$C_{22}H_{23}SSb$	2-(Methysulfanylmethyl)phenylbis(4-methylphenyl)stibane
25	$C_{23}H_{17}Sb$	Bis(1-phenylethnyl)-*p*-tolylstibane
26	$C_{23}H_{25}SSb$	2-(2-Methylsulfanylethyl)phenylbis(4-methylphenyl)stibane
27	$C_{23}H_{26}NSb$	2-(*N*,*N*-Dimethylaminomethyl)phenylbis(4-methylphenyl)stibane
28	$C_{24}H_{27}O_3Sb$	Tris[2-(methoxymethyl)phenyl]stibane
29	$C_{24}H_{27}S_3Sb$	Tris[2-(methysulfanylmethyl)phenyl]stibane
30	$C_{25}H_{19}Sb$	(1-Phenylethnyl)-α-naphthyl-*p*-tolylstibane
31	$C_{26}H_{26}NSb$	1-[8-(*N*,*N*-Dimethylamino)naphthyl]bis(4-methylphenyl)stibane
32	$C_{27}H_{19}Sb$	7-*p*-Tolyldinaphthp[2,1-*b*;1′,2′-*d*]stibolc
33	$C_{27}H_{28}NSb$	1-[8-(*N*,*N*-Dimethylaminomethyl)naphthyl]bis(4-methylphenyl)stibane
34	$C_{27}H_{36}N_3Sb$	Tris[2-(*N*,*N*-dimethylaminomethyl)phenyl]stibane
35	$C_{48}H_{42}Sb_2$	2,2′-Bis[di(*p*-tolyl)stibano]-1,1′-binaphthyl

ney, and IMR-90 cells from human fetal lung were purchased from Dainihon Pharmaceutical (Osaka, Japan). Vascular smooth muscle cells derived from bovine aorta were a gift from Dr. Yasuo Suda (Kagoshima University Graduate School of Sciences and Engineering, Kagoshima, Japan). Dulbecco's modified Eagle's medium (DMEM) and ASF 301 medium were purchased from Nissui Pharmaceutical (Tokyo, Japan) and Ajinomoto (Tokyo, Japan), respectively. Fetal bovine serum (FBS) was obtained from MP Biomedicals (Irvine, CA, U.S.A.). Tissue culture dishes and plates were obtained from Iwaki (Chiba, Japan). Novel bismuth and antimony compounds were synthesized using the following method. Compounds 05, 06, 28, and 29 were prepared by the reaction of $SbCl_3$ or $BiCl_3$ with the corresponding *ortho*-substituted lithium reagents and 04, 23, 24, and 26 were obtained by treatment of bromodi(*p*-tolyl)stibane with appropriate lithium reagents. λ^5-Stibindoles 09 and 10 were synthesized from 1-phenyl-stibindole by treatment with $SOCl_2$ or Br_2, respectively. Other compounds (see Table 1)

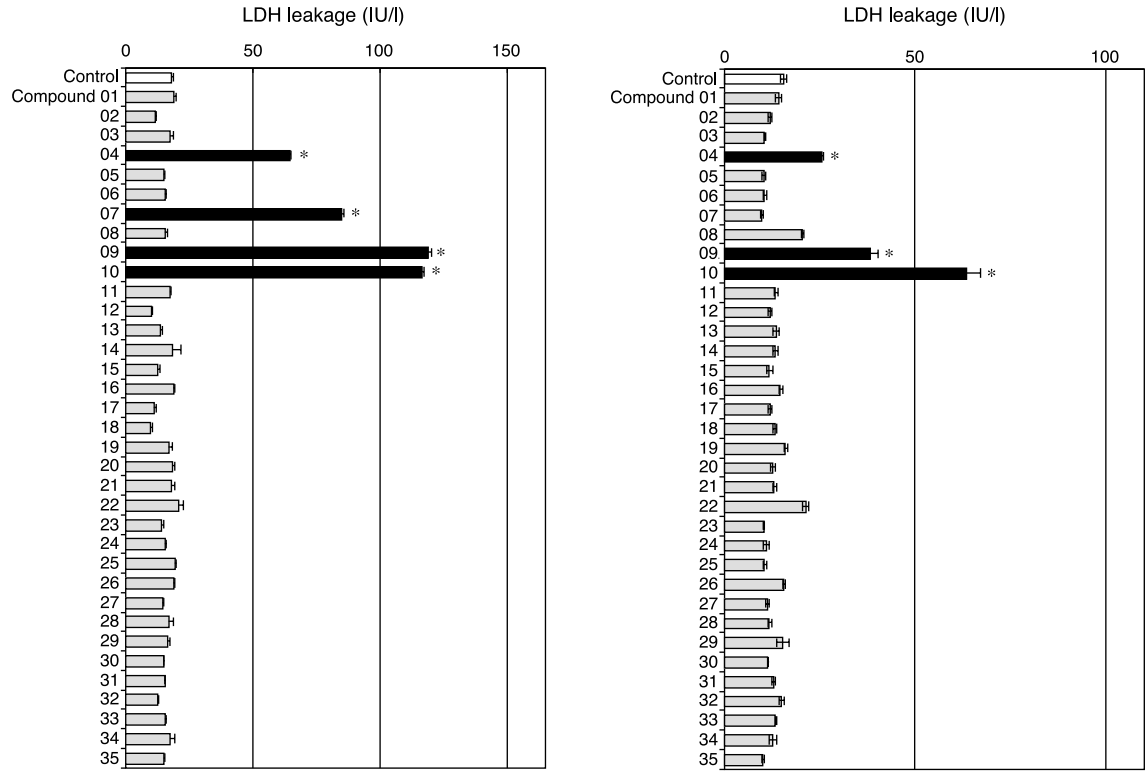

Fig. 1. Leakage of LDH into the Medium from Vascular Endothelial (Left Panel) and Smooth Muscle Cells (Right Panel) after Exposure to Organobismuth and Organoantimony Compounds

Confluent cultures of bovine aortic endothelial and smooth muscle cells were incubated at 37°C for 24 hr in the presence of compounds 01–35 (10 μM each). Values are expressed as mean ± S.E. of four samples. *Significantly different from the corresponding control, $p < 0.01$.

were synthesized as described previously.[8–21] The lactate dehydrogenase (LDH) kit, bismuth nitrate, and other reagents were from Wako Pure Chemical Industries (Osaka, Japan).

Cytotoxicity Assay —— Vascular endothelial cells, vascular smooth muscle cells, LLC-PK_1 cells, and IMR-90 cells were each cultured in DMEM supplemented with 10% FBS in 24-well culture plates at 37°C in a humid atmosphere of 5% CO_2 in air until confluence. The medium was discarded, and the cell layer was washed twice with serum-free ASF 301 medium. The cell layer was then incubated at 37°C for 24 hr in 0.25 ml of fresh serum-free ASF 301 medium with or without bismuth(III) nitrate (10, 20, or 50 μM), organobismuth, and organoantimony compounds (2, 5, 10, or 20 μM). After incubation, the conditioned medium was harvested, and an aliquot of the medium was used for the assay of LDH activity. The cell layer was washed with Ca, Mg-free phosphate-buffered saline and then fixed with methanol and stained with Giemsa.

Statistical Analysis —— Data were analyzed for statistical significance using analysis of variance (ANOVA) and Bonferroni's multiple *t*-test. *p*-Values of less than 0.05 were considered to indicate statistically significant differences.

RESULTS

Table 1 shows the organobismuth and organoantimony compounds of which the cytotoxicity was examined in the present study. There has been no information about the biological activities of these 35 compounds. First, the cytotoxicity of the compounds at 10 μM each to vascular endothelial and smooth muscle cells was investigated because it was reported that the sensitivity of these two cell types to heavy metals is different.[22] As shown in Fig. 1, of the tested compounds, compounds 04, 07, 09, and 10 markedly increased the LDH activity leaked into the medium from vascular endothelial cells after 24-hr treatment, suggesting that these four compounds have strong cytotoxicity to vascular endothelial cells. Interestingly, of these four compounds, only compound 07 did not increase the LDH leakage in vascular smooth muscle cells. Morphologic observations agreed with the results of the LDH leak-

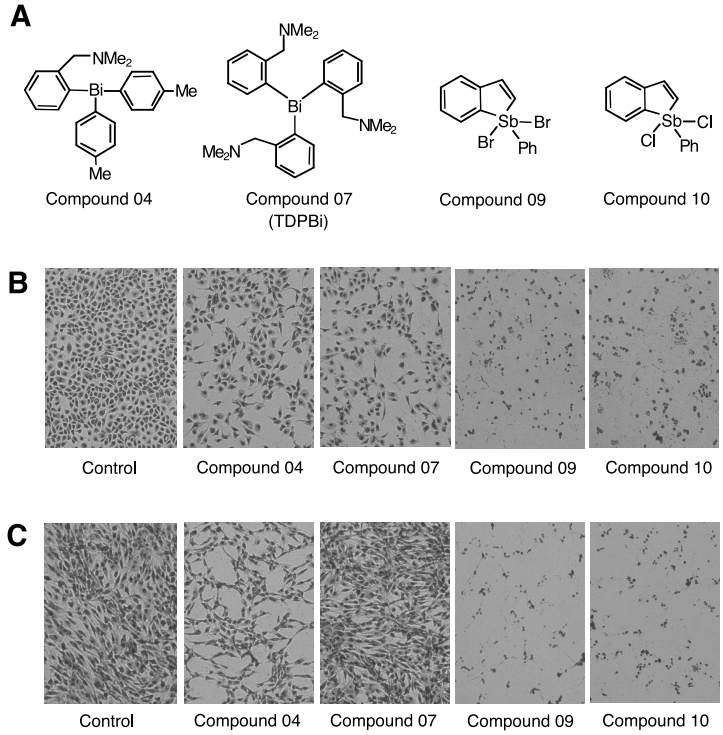

Fig. 2. Structural Formulae of Compounds 04, 07, 09, and 10 (Panel A), and Morphologic Appearance of Vascular Endothelial (Panel B) and Smooth Muscle Cells (Panel C) after Exposure to Compounds 04, 07, 09, or 10 (Original Magnification × 40)

Confluent cultures of bovine aortic endothelial and smooth muscle cells were incubated at 37°C for 24 hr in the presence of compounds 04, 07, 09, or 10 (10 μM each).

age assay. Specifically, compounds 04, 09, and 10 destroyed the monolayer of both endothelial and smooth muscle cell layers, whereas only the endothelial cell layer was damaged by compound 07 (Fig. 2). These results clearly indicate that compound 07, tris[2-(*N*,*N*-dimethylaminomethyl)phenyl]bismuthane (TDPBi), may exhibit selective cytotoxicity to vascular endothelial cells.

To confirm this hypothesis, the cytotoxicity of TDPBi to porcine kidney LLC-PK_1 cells and human fetal lung IMR-90 cells was determined by morphologic observations (Fig. 3). No degenerative change was observed in LLC-PK_1 cells (Fig. 3A), although a decrease in the cell number with necrotic changes was caused by TDPBi 10 μM and more in IMR-90 cells after 24-hr incubation (Fig. 3B). It is suggested that fibroblastic IMR-90 cells as well as vascular endothelial cells are sensitive to TDPBi; however, the destruction of the monolayer in vascular endothelial cells (Fig. 2B) was more marked than that in IMR-90 cells (Fig. 3B).

To compare the sensitivity of vascular endothelial cells to TDPBi with that of other cell types including vascular smooth muscle cells, LLC-PK_1 cells, and IMR-90 cells, the dose–dependent effect of TDPBi on the leakage of LDH was investigated and compared (Fig. 4). TDPBi changed the LDH leakage from neither vascular smooth muscle cells

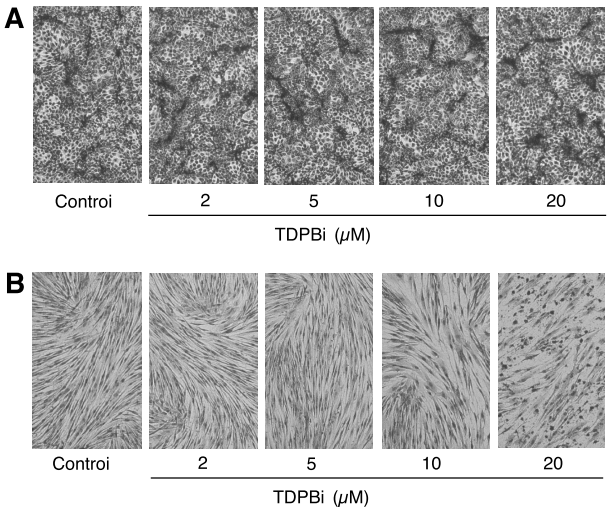

Fig. 3. Morphologic Appearance of LLC-PK_1 Cells (Panel A) and IMR-90 Cells (Panel B) after Exposure to TDPBi (Original Magnification × 40)

Confluent cultures of porcine kidney epithelial LLC-PK_1 cells and human fetal lung fibroblastic IMR-90 cells were each incubated at 37°C for 24 hr in the presence of TDPBi (2, 5, 10, or 20 μM).

nor LLC-PK$_1$ cells. In contrast, the organobismuth compound markedly increased the leakage from vascular endothelial cells after 24-hr treatment. TDPBi also increased the LDH leakage from IMR-90 cells, although the cells were less sensitive to the compound than vascular endothelial cells. The results were consistent with the morphologic observations, supporting the hypothesis that TDPBi exhibits selective cytotoxicity to vascular endothelial cells.

Table 2 shows the effects of inorganic bismuth(III) nitrate on the leakage of LDH from vascular endothelial and smooth muscle cells. The inorganic bismuth did not influence the LDH leakage in either cell type.

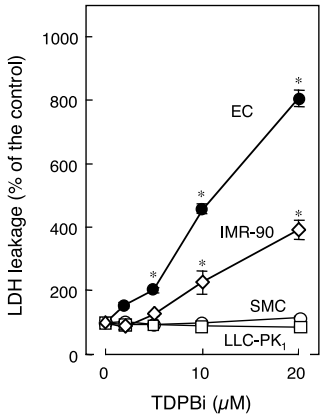

Fig. 4. Leakage of LDH into the Medium from Vascular Endothelial Cells, Smooth Muscle Cells, LLC-PK$_1$ Cells, and IMR-90 Cells after Exposure to TDPBi

Confluent cultures of bovine aortic endothelial cells (EC), smooth muscle cells (SMC), porcine kidney LLC-PK$_1$ cells (LLC-PK$_1$), and human fetal lung IMR-90 cells (IMR-90) were incubated at 37°C for 24 hr in the presence of TDPBi (2, 5, 10, or 20 μM). Values are expressed as mean ± S.E. of four samples. *Significantly different from the corresponding control, $p < 0.01$.

DISCUSSION

In the present study, we searched for bismuth- or antimony-containing compounds that exhibit cell type-dependent cytotoxicity from among 35 organobismuth and organoantimony compounds. It was found that the organobismuth compound TDPBi has potent cytotoxicity particularly to vascular endothelial cells. When bismuth was replaced with antimony (*i.e.*, compound 34), the cytotoxicity to vascular endothelial cells disappeared. In addition, inorganic bismuth had no cytotoxicity to vascular endothelial cells. Taking these results together, the selective cytotoxicity of TDPBi to vascular endothelial cells requires the entire bismuth-containing structure of the molecule.

Sensitivity to chemicals and heavy metals often depends on the cell type. For example, the sensitivity of vascular smooth muscle cells to cadmium cytotoxicity is markedly higher than that of other cell types including vascular endothelial cells, Chang liver cells, and LLC-PK$_1$ cells; the high sensitivity is mainly due to a higher accumulation of cadmium.[22] On the other hand, the present data showed that the sensitivity to TDPBi is markedly high in vascular endothelial cells, moderately high in fibroblastic IMR-90 cells, and very low in vascular smooth muscle cells and LLC-PK$_1$ cells. Although the mechanism by which TDPBi exhibits selective cytotoxicity to vascular endothelial cells is unknown, it is suggested that vascular endothelial cells may lack a protective mechanism against TDPBi and/or have mechanisms that amplify the cytotoxicity of TDPBi. Although the molecules that participate in the protection and the amplification are unclear, the cytotoxicity of tumor necrosis factor (TNF)-α may be a good model of cell type-dependent cytotoxic-

Table 2. Leakage of LDH into the Medium from Vascular Endothelial and Smooth Muscle Cells after Exposure to Inorganic Bismuth

	LDH leakage (IU/l)	
	Vascular endothelial cells	Vascular smooth muscle cells
Control	18.7 ± 1.13	24.1 ± 0.78
Bismuth 10 μM	18.7 ± 1.13	19.4 ± 0.95*
Bismuth 20 μM	20.1 ± 0.23	16.9 ± 0.38*
Bismuth 50 μM	19.7 ± 0.42	16.3 ± 1.07*

Confluent cultures of bovine aortic endothelial cells and bovine aortic smooth muscle cells were incubated at 37°C for 24 hr in the presence of bismuth(III) nitrate (10, 20, or 50 μM). Values are expressed as mean ± S.E. of four samples. Significantly different from the corresponding control, *$p < 0.01$.

ity. Like TDPBi, TNF-α exhibits potent cytotoxicity to vascular endothelial cells, although vascular smooth muscle cells, fibroblasts, and epithelial cells are resistant to cytokine-mediated cytotoxicity.[23–25] Although the mechanisms underlying endothelial cell death induced by TNF-α are not simple, it has been shown that nitric oxide[26,27] and the product(s) of aldose reductase catalysis[28] may mediate the cytotoxicity. It remains to be determined whether TDPBi and TNF-α exhibit cytotoxicity to vascular endothelial cells through a common pathway.

The selective cytotoxicity of TDPBi to vascular endothelial cells appears to be one of the beneficial effects for anticancer drugs,[29] because the cytotoxicity may result in inhibition of angiogenesis and/or vasculogenesis in tumor tissue. In other words, it must be determined whether the vascular tissue in tumors is damaged by TDPBi. At the same time, it should be clarified whether TDPBi can induce tumor cell death and which type of tumor cells is sensitive to TDPBi. On the other hand, it is possible that there are some organobismuth or organoantimony compounds that can control vascular endothelial and smooth muscle cell functions through metallothionein induction. Metallothionein is a low molecular-weight, cysteine-rich protein that detoxifies the toxicity of heavy metals,[30] metabolizes essential trace elements,[31] and scavenges free radicals.[32] Since physiologic factors such as thrombin and cytokines can induce metallothionein in vascular cells,[33,34] it has been hypothesized that the protein may play specific roles in vascular tissue, for example, scavenging endogenous vasodilating nitric oxide. Inorganic bismuth is a potent inducer of metallothionein in vascular endothelial cells.[35] Therefore some organometalloid compounds may induce metallothionein without exhibiting cytotoxicity.

In conclusion, the present data demonstrate that TDPBi is a unique compound that exhibits selective toxicity to vascular endothelial cells. We postulate that the study of this organobismuth compound can be the first step in the development of novel agents that target vascular endothelial cells. Further investigations will be necessary to clarify not only the effects of TDPBi on tumor-associated angionegesis/vasculogenesis and tumor cell survival but also the procedures for controlling TDPBi effects. Such investigations will contribute to the clinical application of organobismuth compounds to vascular tissue.

Acknowledgements We are grateful to Dr. Takashi Tsuchiya for helpful advice on the development of this study. This work was supported by the Specific Research Funds of Hokuriku University (to T. K. and J. K.), a Grant-in-Aid for Young Scientists (B) from the Ministry of Education Culture, Sports, Science and Technology, Japan (to Y. F.), and Mitsubishi Chemical Corporation Fund (to S. Y.).

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