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Abstract: Synthetic organic selenium compounds, such as ebselen, may show glutathione peroxidase-like antioxidant activity and have a neurotrophic effect. We synthesized 1,3-selenazolidin-4-ones, new types of synthetic organic selenium compounds (five-member ring compounds), to study their possible applications as antioxidants or neurotrophic-like molecules. Their superoxide radical scavenging effects were assessed using the quantitative, highly sensitive method of real-time kinetic chemiluminescence. At 166 M, the O_2- scavenging activity of 1,3-selenazolidin-4-ones ranged from 0 to 66.2%. 2-[3-(4-Methoxyphenyl)-4-oxo-1,3-selenazolidin-2-ylidene]malononitrile (compound b) showed the strongest superoxide anion-scavenging activity among the 6 kinds of 2-methylene-1,3-selenazolidin-4-ones examined. Compound b had a 50% inhibitory concentration (IC50) at 92.4 M and acted as an effective and potentially useful O_2- scavenger in vitro. The effect of compound b on rat pheochromocytome cell line PC12 cells was compared with that of ebselen or nerve growth factor (NGF) by use of the MTT [3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay. When ebselen was added at 100 M or more, toxicity toward PC12 cells was evident. On the contrary, compound b suppressed serum deprivation-induced apoptosis in PC12 cells more effectively at a concentration of 100 M. The activity of compound b to phosphorylate mitogen-activated protein kinase/extracellular signal-regulated protein kinase (ERK) 1/2 (MAP kinase) in PC12 cells was higher than that of ebselen, and the former at 100 M induced the phosphorylation of MAP kinase to a degree similar to that induced by NGF. From these results, we conclude that this superoxide anion-scavenger, compound b, suppressed serum deprivation-induced apoptosis by promoting the phosphorylation of MAP kinase.

A superoxide anion-scavenger, 1,3-selenazolidin-4-one suppresses serum deprivation-induced apoptosis in PC12 cells by activating MAP kinase

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ABSTRACT

Synthetic organic selenium compounds, such as ebselen, may show glutathione peroxidase-like antioxidant activity and have a neurotrophic effect. We synthesized 1,3-selenazolidin-4-ones, new types of synthetic organic selenium compounds (five-member ring compounds), to study their possible applications as antioxidants or neurotrophic-like molecules. Their superoxide radical scavenging effects were assessed using the quantitative, highly sensitive method of real-time kinetic chemiluminescence. At 166 μM, the O_2^- scavenging activity of 1,3-selenazolidin-4-ones ranged from 0 to 66.2%. 2-[3-(4-Methoxyphenyl)-4-oxo-1,3-selenazolidin-2-ylidene] malononitrile (compound **b**) showed the strongest superoxide anion-scavenging activity among the 6 kinds of 2-methylene-1,3-selenazolidin-4-ones examined. Compound **b** had a 50% inhibitory concentration (IC_{50}) at 92.4 µM and acted as an effective and potentially useful O_2^- scavenger *in vitro*. The effect of compound **b** on rat pheochromocytome cell line PC12 cells was compared with that of ebselen or nerve growth factor (NGF) by use of the MTT [3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide] assay. When ebselen was added at 100 μM or more, toxicity toward PC12 cells was evident. On the contrary, compound **b** suppressed serum deprivation-induced apoptosis in PC12 cells more effectively at a concentration of 100 μM. The activity of compound **b** to phosphorylate mitogen-activated protein kinase/extracellular signal-regulated protein kinase (ERK)1/2 (MAP kinase) in PC12 cells was higher than that of ebselen, and the former at 100 μM induced the phosphorylation of MAP kinase to a degree similar to that induced by NGF. From these results, we conclude that this superoxide anion-scavenger, compound **b**, suppressed serum deprivation-induced apoptosis by promoting the phosphorylation of MAP kinase.

Key words: selenazolidine, superoxide radicals, scavenging effect, PC 12 sells, MAP kinase, phosphorylation

Introduction

      A relatively large amount of O_2^- is generated in mitochondria of the cardiovascular system, and in phagocytes including polymorphonuclear leukocytes (PMNs), macrophages/monocytes, eosinophils, mast cells, and basophils (Fridovich, 1983). O_2^- reacts not only with biomolecules, but also with other reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and lipid radicals (LOOH) (Meydani *et al.*, 1992; Fridovich, 1998; Balaban *et al.*, 2005). Additionally, ROS derived from leukocytes induce excessive inflammation, leading to cell/tissue injuries (Curnutte *et al.*, 1974; Berridge, 1984). In extreme instances such as endotoxin shock, neutrophils kill the infected host (Ryter *et al.*, 2007), and therefore the generation of ROS must be controlled (Long *et al.*, 1997; Ramirez *et al.*, 2003).

      Superoxide dismutases (SODs), catalase, glutathione peroxidase (GPX), and some vitamins are representative antioxidants, and these molecules act as protectors against ROS-induced toxicity in cells (Fridovich, 1995; Kimura *et al.*, 2005). GPX is believed to be an important antioxidant enzyme, and it effectively reduces the toxicity of H_2O_2 *in vitro* and *in vivo* (Wendel and Tiegs, 1986; Kimura *et al.*, 2005). The active site of GPX contains selenium atoms (Wendel and Tiegs, 1986), and various studies have shown that selenoproteins, including GPX, reduce oxidative stress in cells (Tiano *et al.*, 2000; Jeong *et al.*, 2002). Thus, various organic selenium compounds may be potent candidate scavengers of ROS.

      Ebselen®, a five-member ring selenium-containing heterocyclic compound showing GPX-like activity (Engman, 1989), is a typical synthetic antioxidant for the scavenging of ROS (Zhang *et al.*, 2002). The antioxidative effects are due to selective blockade of leukocyte infiltration and activation, which results in elimination of these ROS (Zhang *et al.*, 2002). Thus, ebselen is a multifunctional antioxidant having a potential chemopreventive effect on inflammation (Nakamura *et al.*, 2002). On the basis of these facts, various types of organic selenium compounds might be applicable for the reduction of oxidative stresses (Takahashi *et al.*, 2004; Takahashi *et al.*, 2005a; Takahashi *et al.*, 2005b).

      Morey et al. (2001) demonstrated that some selenoproteins, such as selenoprotein P,

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regulate the redox potential in cells, resulting in modulation of various phosphorylation pathways including Ras/MAP kinase signaling (Morey *et al.*, 2001). On the other hand, we demonstrated earlier that ebselen regulates the redox in PC12 cells and induces activation of the MAP kinase cascade and neuronal differentiation via regulation of kinases or phosphatases related to intercellular signaling (Nishina *et al.*, 2008). Thus, such selenium compounds may act not only as ROS scavengers, but also as intracellular signal regulators.

We have synthesized various organic selenium compounds, such as selenoamides (Takahashi *et al.*, 2004), selenoureas, thioureas (Takahashi *et al.*, 2005b), and selenocarbamates (Takahashi *et al.*, 2005a), and found that most of these compounds act as effective scavengers of O_2^- *in vitro* (Takahashi *et al.*, 2005a; Takahashi *et al.*, 2005b). In the present study, we newly synthesized five-member ring compounds, 1,3-selenazolidin-4-ones, and tested not only their superoxide radical-scavenging activity (SOSA) by using a real-time kinetic chemiluminescence method, but also their suppressive activity toward serum deprivation-induced apoptosis and their ability to induce MAP kinase phosphorylation in PC12 cells.

MATERIALS AND METHODS

Materials   2-[3-(Aryl)-4-oxo-1,3-selenazolidin-2-ylidene] malononitriles (compound **b**) were prepared according to procedures previously reported (Sommen et al., 2006). A Cypridina luciferin analogue, 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo-[1,2-]pyrazin-3-one hydrochloride (MCLA) was obtained from Tokyo Kasei (Tokyo, Japan) as a chemiluminescent probe for superoxide radicals. MCLA was dissolved in doubly distilled water and stored at -80ºC prior to usage. The concentration of the MCLA solution was determined based on its absorbance at 430 nm and an absorbance coefficient value of $\square = 9,600 M^{-1} cm^{-1}$, as previously described (Kimura and Nakano, 1988). SOD (lyophilized powder, 3400 units/mg protein) and xanthine oxidase (XOD grade III) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hypoxanthine was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and used without further purification. All other chemicals and solvents

were analytical grade and used without further purification.

Methods for the synthesis of 2-[3-(aryl)-4-oxo-1,3-selenazolidin-2-ylidene] malononitriles (a-f)   A 25-mL round-bottom flask equipped with a magnetic stirrer and condenser was charged with a solution of malononitrile (73 mg, 1.1mmol) in DMF (10 mL). Triethylamine (0.15 mL, 1.1 mmol) was then added, and the mixture was stirred for 30 min at room temperature (RT). Next, the appropriate isoselenocyanate (1.1 mmol) was added and the mixture stirred for 1 h at RT. The halogenated compound (1.1 mmol) was then added dropwise, and the mixture was stirred for 4 h before being evaporated to dryness under reduced pressure. The crude product was purified by column chromatography on silica gel with hexane–ethyl acetate (100/0–50/50) as the eluant and recrystallized from ethyl acetate.

2-(4-Oxo-3-phenyl-1,3-selenazolidin-2-ylidene)-malononitrile, Compound **a**. Yield: 235–270 mg (74–85%). Colorless crystals. Mp 265–267°C. IR: 2985w, 2216s, 1733s, 1596w, 1522s, 1493m, 1368m, 1223s, 851w, 758w, 698m. ^{1}H-NMR (300MHz, CDCl$_3$): δ 4.39 (s, CH$_2$), 7.43 (d-like, *J*=7.9Hz, 2 arom. H), 7.50–7.60 (m, 3 arom. H). ^{13}C-NMR (75MHz, CDCl$_3$): δ 29.1, 56.7, 110.1, 115.1, 128.9, 129.4, 130.8, 134.8, 173.3, 173.9. CI-MS: 307 (100, $[M(^{80}Se)+NH_4]^+$); CI-MS (i-butane): 290 (100, $[M(^{80}Se)+1]^+$). Anal. calcd for C$_{12}$H$_7$N$_3$OSe (288.16): C 50.02, H 2.45, N 14.58; found: C 49.98, H 2.60, N 14.34.

2-[3-(4-Methoxyphenyl)-4-oxo-1,3-selenazolidin-2-ylidene]malononitrile, Compound **b**. Yield: 259 mg (74%). Orange crystals. Mp 193–195°C. IR: 2945w, 2216m, 2210m, 1752m, 1606w, 1523s, 1508s, 1369m, 1303w, 1255m, 1212m, 1015w, 822w. ^{1}H-NMR (300MHz, CDCl$_3$): δ 3.83 (s, CH$_3$O), 4.38 (s, CH$_2$), 7.07, 7.35 (AA'BB', *J*$_{AB}$=8.0Hz, 4 arom. H). ^{13}C-NMR (75MHz, CDCl$_3$): δ 28.9, 55.4), 56.6, 110.2, 114.6, 115.1, 127.3, 130.2, 132.4, 160.8, 173.9. CI-MS: 337 (100, $[M(^{80}Se) + NH_4]^+$). Anal. calcd for C$_{13}$H$_9$N$_3$O$_2$Se (318.20): C 49.07, H 2.85, N 13.21; found: C 48.84, H 3.01, N 13.57.

2-[3-(2,6-Dimethylphenyl)-4-oxo-1,3-selenazolidin-2-ylidene]malononitrile, Compound **c**.

Yield: 289 mg (83%). Colorless crystals. Mp 296–298°C. IR: 3000w, 2944w, 2217s, 2206s, 1742s, 1517s, 1474m, 1392w, 1352s, 1217m, 1205s, 1183m, 1151m, 1035w, 837w, 785m, 736w. ^{1}H-NMR (300MHz, CDCl$_3$): δ 2.16 (s, 2CH$_3$), 4.72 (s, CH$_2$), 7.28 (d, J =7.8Hz, 2 arom. H), 7.44 (t, J =7.9Hz, 1 arom. H). ^{13}C-NMR (75MHz, CDCl$_3$): δ 16.7, 28.7, 57.1, 109.3, 114.7, 128.3, 130.9, 132.4, 136.5, 171.5, 173.3. CI-MS: 335 (100, [M(^{80}Se) + NH$_4$]$^+$). Anal. calcd for C$_{14}$H$_{11}$N$_3$OSe (316.22): C 53.18, H 3.51, N 13.29; found: C 53.18, H 3.58, N 13.03.

2-[3-(4-Fluorophenyl)-4-oxo-1,3-selenazolidin-2-ylidene]malononitrile, Compound **d.**
Yield: 280 mg (83%). Colorless crystals. Mp 244–246°C. IR: 2995w, 2982w, 2219s, 2210s, 1737s, 1600w, 1528s, 1516s, 1507s, 1373m, 1222s, 1208s, 1161w, 858w, 826w, 791w. ^{1}H-NMR (300MHz, CDCl$_3$): δ 4.65 (s, CH$_2$), 7.67 (t-like, J =9.0Hz, 2 arom. H), 7.49–7.59 (m, 2 arom. H). ^{13}C-NMR (75MHz, CDCl$_3$): δ 29.0, 56.7, 110.2, 114.9, 116.4 (d, $^2J_{CF}$=23 Hz, 2CH), 131.1, 131.5 (d, $^3J_{CF}$=9 Hz, 2CH), 163.2 (d, $^1J_{CF}$=248 Hz, CF), 173.7, 173.9. CI-MS: 325 (100, [M(^{80}Se) + NH$_4$]$^+$). Anal. calcd for C$_{12}$H$_6$N$_3$OSeF (306.16): C 47.08, H 1.98, N 13.73; found: C 47.01, H 2.21, N 14.02.

2-[3-(4-Chlorophenyl)-4-oxo-1,3-selenazolidin-2-ylidene]malononitrile, Compound **e**.
Yield: 242 mg (68%). Colorless crystals. Mp 245–247°C. IR: 2947w, 2223m, 2213m, 1733s, 1529s, 1485m, 1404w, 1370m, 1219s, 1172w, 1084w, 1015w, 845w, 813w, 722w. ^{1}H-NMR (300MHz, CDCl$_3$): δ 4.33 (s, CH$_2$), 7.45, 7.58 (AA'BB', J_{AB}=8.7Hz, 4 arom. H). ^{13}C-NMR (75MHz, CDCl$_3$): δ 29.1, 56.7, 110.3, 114.9, 129.5, 131.0, 133.7, 135.5, 173.4, 173.8. CI-MS: 341 (100, [M(^{80}Se, ^{35}Cl) + NH$_4$]$^+$). Anal. calcd for C$_{12}$H$_6$N$_3$OSeCl (322.61): C 44.68, H 1.87, N 13.03; found: C 44.75, H 2.10, N 12.92.

2-[3-(4-Bromophenyl)-4-oxo-1,3-selenazolidin-2-ylidene]malononitrile, Compound **f**.
Yield: 351–38 3 mg (87–95%). Colorless crystals. Mp 247–249°C. IR: 2947w, 2222s, 2211s, 1736s, 1585w, 1526s, 1481s, 1399w, 1371m, 1218s, 1171m, 1066w, 1012m, 842m, 809m, 711w. ^{1}H-NMR (300MHz, CDCl$_3$): δ 4.67 (s, CH$_2$), 7.71, 8.05 (AA'BB', J_{AB}=8.7Hz,

4 arom. H). ^{13}C-NMR (75MHz, CDCl$_3$): δ 29.1, 56.8, 110.3, 114.9, 129.5, 131.0, 133.7, 135.6, 173.4, 173.8. CI-MS: 387 (79, [M(^{80}Se, ^{81}Br) + NH$_4$]$^+$), 385 (100, [M(^{80}Se, ^{79}Br) + NH$_4$]$^+$). Anal. calcd for C$_{12}$H$_6$N$_3$OSeBr (367.07): C 39.27, H 1.65, N 11.45; found: C 39.44, H 1.86, N 11.49.

Assay of superoxide anion-scavenging activity (SOSA)   SOSA of 2-[3-(aryl)-4-oxo-1,3-selenazolidin-2-ylidene] malononitriles (a-f) was measured by using a previously reported method (Kimura and Nakano, 1988). Briefly, the reaction mixture contained 5.8 x 10^{-7} M MCLA, 5 x 10^{-5} M hypoxanthine, xanthine oxidase (6.5 U), and 50 mM Tris-HCl buffer containing 0.1 mM EDTA at pH7.8, in the presence or absence of various concentrations of compounds (**a-f**). To evaluate certain SOSA, we added SOD standard solutions (0.6–30 ng/mL) to the reaction mixture instead of compounds (**a-f**). The total volume was 3.0 mL. Compounds (**a-f**) (25 mM) were dissolved in dimethyl sulfoxide and stored at -80°C prior to use. Chemiluminescence was measured by using a luminometer (Aloka, BLR201) at 25°C. The reaction was initiated by the addition of MCLA to the standard incubation mixture excluding XOD, continued for 3 min without XOD, and then for an additional 2 min after the addition of XOD. A representative example of a measurement showing the effect of compound **b** on MCLA-dependent luminescence is shown in Fig. 2. As compound **b** showed strong SOSA at 166 µM, we also measured the activity at 16.6, 41.5, and 83.0 µM. The percent inhibition of MCLA-dependent chemiluminescence was calculated as previously described (Kimura and Nakano, 1988). The 50% inhibitory concentration (IC$_{50}$) was calculated from 4 concentrations of compound **b** (16.6, 41.5, 83.0, and 166 µM). In this study, SOSA was measured twice for each sample and the mean value was used.

Cell culture   PC12 cells were cultured as described previously (Nishina *et al.*, 2008). In brief, the cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% heat-inactivated horse serum (Gibco BRL, Grand Island, NY) and 5% heat-inactivated fetal bovine serum (FBS; Sanko Junyaku, Co.,

Ltd., Tokyo, Japan) or in serum-free medium [DMEM supplemented with 1% bovine serum albumin (BSA)], unless otherwise stated.

Calculation of cell numbers by MTT method   We serially prepared the cell suspensions (10^2 to 10^6 cells/ml) in DMEM supplemented with 10% FBS. Cell number was determined using a hemocytometer and cell viability always exceeded 98%, as determined by trypan blue exclusion. One hundred microliters of each cell suspension (10–10^5 cells/100 µl) was added to collagen-coated 96-well plates (Corning Incorporated Life Sciences, Lowell, MA) and incubated for 4h at 37˚C in an atmosphere of 95% air/ 5% CO_2. After the incubation, 50 µg of MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide] was added to each well and incubated for 2 h at 37˚C in an atmosphere of 95% air/ 5% CO_2. Then, 50 µl of 50% (v/v) dimethyl formamide (DMFA) containing 20% (w/v) SDS was added to each well. Absorbance at 562 nm of the cell lysate in each well was directly measured using a microplate reader (Ultrospec Visible Plate Reader II, Amersham Biosciences, Tokyo, Japan). The calculation formula for determining the cell number was as follows: $Absorbance_{562 nm} = 0.228e^{x/10,000,000}$ (e, natural logarithm; x, cell number). Using the method, we established a standard curve for each assay.

Measurement of cell viability and inhibition of caspase-3 and 7 activities   PC12 cells were seeded at a cell density of 2×10^6 cells/well into collagen-coated 96-well plates (Corning), and precultured in the above serum-containing medium for 2 days at 37˚C in an atmosphere of 95% air/ 5% CO_2. The cells were then cultured with DMEM containing 1% BSA and a given reagent for various numbers of days. The number of viable cells was evaluated by the above-mentioned method. Activities of caspase-3 and 7 were measured by the Caspase-Glo® 3/7 assay kit (Promega, Madison, WI) using Typhoon 9200 (Amersham Biosciences, Tokyo, Japan) in chemiluminescence mode.

Treatment with MAP kinase kinase (MEK1/2) inhibitor   Cells were preincubated with serum-free medium (DMEM supplemented with 1% BSA) containing 10 µM of

U0126 (a MEK1/2 inhibitor) for 15 min, and transferred to and cultured in the serum-free medium supplemented with compound **b** or NGF for the appropriate periods of time. The effect of U0126 on the cell number was evaluated using the MTT assay, and that on morphology was evaluated by optical observation. Photographs were taken under phase-contrast observation at magnification 100x.

Detection of phosphorylated proteins   PC12 cells were cultured in serum-free medium containing a given test compound similarly as described in the previous section. Then the culture plates were placed on ice, and each well was washed with 3 ml of 2 mM Tris-HCl buffer, pH 8.0, (TBS) containing 0.33 M NaF and 6.25 M Na_3VO_4. The cells were subsequently lysed with 150 μl of 20 mM TBS containing 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40 (w/v), 1% sodium deoxycholate (w/v), 0.1% sodium dodecyl sulfate (SDS) (w/v), 50 mM NaF, 0.1% aprotinin (w/v), 0.1% leupeptin (w/v), 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were collected using a cell scraper, transferred to 1.5 ml microcentrifuge tubes, and centrifuged at 15000 x *g* for 30 min at 4˚C. The supernatant was collected and transferred to another tube, and the overall protein concentration was determined using a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL) with BSA as the standard.

Each supernatant containing proteins (20 μg aliquot) was mixed with sample buffer containing 4% SDS for SDS gel electrophoresis (SDS-PAGE) and incubated for 5 min at 80˚C. The protein samples were then subjected to the SDS-PAGE, and the proteins in gel were electroblotted onto polyvinylidene difluoride filters (Fluorotrans membrane W, 0.2 μm; Nihon Genetics, Tokyo, Japan). MAP kinase and its phosphorylated form were visualized by immunoblotting using monoclonal antibodies against p44/42 ERK and phospho p44/42 ERK, respectively (Cell Signaling Technology, Lake Placid, NY). Neurofilament M was similarly reacted with its primary antibody (Sigma). The membranes were then reacted with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody (Promega) as the secondary antibody. The blots were developed by the enhanced chemiluminescence method (Hyperfilm-ECL plus, Amersham Biosciences Corp.,

Piscataway, NJ).

Statistical analysis   The results are expressed as means ± SD. Significant difference from the control value was determined by using analysis of variance (ANOVA), followed by Dunnett's test. In all tests, statistical significance was set as *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

Results

Superoxide radical-scavenging activity (SOSA) of 1,3-selenazolidin-4-one

The structures of 2-[3-(aryl)-4-oxo-1,3-selenazolidin-2-ylidene]malononitriles' (compounds **a-f**) are shown in Fig. 1. Among them, only 2-[3-(4-methoxyphenyl)-4-oxo-1,3-selenazolidin-2-ylidene]malononitrile, compound **b,** had SOSA at 166 µM (66.2%). The other compounds, **a** and **c-f,** showed no activity. SOSA of compound **b** was dose-dependent (Fig. 2A). Compound **b** was sufficiently active to justify further testing after serial dilutions, and the IC_{50} was determined to be 92.4 µM (Fig. 2B). To determine whether compound **b** could eliminate superoxide anions generated by XOD, the amount of uric acid, the by-product of the superoxide anion in the standard reaction mixture, was measured with or without compound **b** (Fig. 3). Generation of uric acid was not significantly affected by adding a relatively high concentration of compound **b** (166 µM), suggesting that this compound **b** acts not as an inhibitor of XOD but as a scavenger of superoxide anions. On the other hand, SOSA at 166 µM of ebselen was 100%, and IC_{50} of ebselen was 20 µM.

*Effect of compound **b** on PC12 cells in serum- deprived culture medium*

In order to evaluate the effect of compound **b** or ebselen on PC12 cells in serum-deprived culture medium, we evaluated cell viability and caspase-3 and 7 activities by using the MTT assay and Caspase-Glo® 3/7 assay kit, respectively. Ebselen supplemented in the

medium at 100 µM or higher showed toxic effects on the cells (Fig. 4 A). On the other hand, cell viability was promoted by the addition of compound **b** or NGF (Fig. 4 B, D). Compound **b** showed the maximum effect at 100 µM. Serum-deprived PC12 cells undergo apoptosis (Nishina *et al.*, 2008), but the addition of NGF protects against this form of cell death (Biswas and Greene, 2002). Our present results thus suggest that compound **b** attenuated serum deprivation-induced apoptosis of PC12 cells. Furthermore, serum deprivation-induced activation of caspase-3 and 7 was also attenuated by the addition of compound **b** at 100 µM or higher; however, neither NGF nor ebselen showed such effect (Fig. 5).

*Effects of compound **b** and U0126 on MAP kinase cascade and morphological changes in PC12 cells*

As it is reported that activation of the MAP kinase cascade represses apoptosis (Wang *et al.*, 2008), the effect of U0126 (a MEK1/2 inhibitor) on the compound **b**- or NGF-facilitated cell viability was examined. The treatment with U0126 diminished the activity of compound **b** and NGF (Fig. 4C, D). Next, to clarify a relationship between cell viability and MAP kinase activation, we evaluated the effects of compound **b** and NGF on ERK1/2 phosphorylation. Compound **b** and NGF markedly stimulated ERK1/2 phosphorylation at 100 µM and 10 ng/ml, respectively, and the phosphorylation evoked by both compound **b** and NGF was significantly inhibited by the treatment with a MEK inhibitor, U0126 (Fig. 6), demonstrating that compound **b** stimulates ERK1/2 phosphorylation via MEK activation as well as NGF. ERK1/2 was significantly activated by the addition of 100 µM of compound **b**, but activation was low with 100 µM of ebselen.

The morphological changes in serum-deprived PC12 cells after treatment with compound **b**, NGF, and U0126 alone or in combination are shown in Fig. 7. After serum-deprivation, the cells with nuclear fragmentation increased in number with the increase of culture period, and the number of cells with normal morphology was severely decreased by 6 days (Fig. 7 A). U0126 alone did not affect the cell morphology (Fig. 7 B).

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NGF induced neurite outgrowth, and suppressed cell growth and nuclear fragmentation (Fig. 7 C), but U0126 treatment completely suppressed these NGF-induced effects (Fig. 7 D). Compound **b** stimulated the proliferation of PC12 cells and suppressed nuclear fragmentation, but U0126 treatment suppressed this effect. No morphological change was seen following the addition of 100 μM of ebselen (data not shown).

Discussion

We newly synthesized 6 organo-selenocompounds (Fig. 1), and as a result of comparing SOSA for each, we clarified that only 2-[3-(4-methoxyphenyl)-4-oxo-1,3-selenazolidin-2-ylidene]malononitrile (compound **b**) exhibited SOSA**.** Furthermore, the inhibition of serum deprivation-induced apoptosis of PC12 cells by compound **b** was observed.

Among the 6 compounds synthesized, only compound **b** had significant SOSA. As a possible reason for the mechanism, compound **b** bears a methoxy group at the para position of the benzene ring. The existence of an electron-donating group at this position of the benzene ring might contribute to SOSA. Previously, we systematically synthesized various organic selenium compounds, including selenocarbamates, selenoureas, thioureas, tertiary selenoamide compounds, 2-amino-1,3-selenazoles, and bis-(2-amino-5-selenazoyl) ketones, and demonstrated their SOSA by using the same chemiluminescence method. The IC_{50} values of these compounds ranged from approximately 0.1 to 100 μM (Takahashi *et al.*, 2004; Sekiguchi *et al.*, 2005; Takahashi *et al.*, 2005a; Takahashi *et al.*, 2005b; Sekiguchi *et al.*, 2006). The SOSA of compound **b** might be relatively low compared with that of other compounds such as selenocarbamates, selenoureas, thioureas, tertiary selenoamide, and ebselen. On the other hand, compound **b** significantly inhibited serum deprivation-induced apoptosis in PC12 cells.

U0126 significantly suppressed compound **b**-facilitated cell survival (Fig. 4C) and ERK1/2 phosphorylation (Fig. 6), suggesting that compound **b** promoted cell survival by the MEK-mediated activation of the MAP kinase pathway. On the other hand, serum deprivation-induced caspase-3 and 7 activation was attenuated by the addition of compound

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b in a concentration-dependent manner. Among 11 caspases so far identified, there are two types of apoptotic caspases: initiator (apical) caspases and effector (executioner) caspases. Initiator caspases (e.g. caspase-2, 8, 9 and 10) cleave inactive pro-forms of effector caspases, thereby activating them. Effector caspases (e.g. caspase-3, 6, and 7) in turn cleave other protein substrates within the cell, to trigger the apoptotic process (Sairanen *et al.*, 2009). From the present results shown in Figs. 5 and 6, MAP kinase activation and suppression of the effector caspases were induced by compound **b** simultaneously. Thus, the inhibition of apoptosis in PC12 cells could be caused by both MAP kinase activation and effector caspase suppression by compound **b**. Because the activation of caspase-3 and 7 was not suppressed by NGF, apoptosis-inhibitory mechanisms of compound **b** were thought to be different from those of NGF.

At 1 mM of compound **b**, although the activities of caspase-3 and 7 were decreased, cell viability was attenuated compared with 100 μM of compound **b**. It is suggested that apoptosis in the PC12 cells is associated with not only these caspase activities but also activation or inactivation of another factor such as one from the Bcl family (Rong *et al.*, 1999). In the present study, since only caspases and ERK1/2 were used as the index, no significant correlations were seen between concentration and effects. Further studies may be needed to confirm the role of the Bcl family in the compound **b**-affected PC12 cells, because relationship between concentration and activity of compound **b** are contradictory.

U0126 suppressed NGF-induced ERK1/2 phosphorylation and neurite outgrowth (Fig. 4D, 6 and 7 D), but not cell survival. These observations were in good agreement with previous reports showing that the phosphorylation of ERK1/2 is necessary for neurite outgrowth from PC12 cells (Williams *et al.*, 2000), and the activation of phosphoinositide 3-kinase for cell survival (Wert and Palfrey, 2000).

Previous reports suggested that sustained activation of ERK1/2 by Crk, and Shp-2 promotes the NGF-induced differentiation of PC12 cells (Matsuda *et al.*, 1994; Wright *et al.*, 1997). In other words, sustained ERK1/2 activation requires activation of Crk and Shp-2. In the present study, because the activation of ERK1/2 due to compound **b** may be transient, compound **b** only led to the inhibition of cell death (Aletta, 1994). Another report has

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suggested that the activation of caspases is associated with cell differentiation in PC12 cells (Vaisid *et al.*, 2005). However, caspase-3 and 7 activities were not inhibited in the NGF-affected cells. Hence, further studies are needed to elucidate the precise mechanism involved in the differentiation of PC 12 cells and the relationship with caspase activities.

Ebselen, a well-known antioxidant containing a selenium atom in its molecule, was used for comparison with compound **b**. Unlike compound **b**, ebselen enhanced serum-deprived cell death at concentrations above 10 μM (Fig. 4A). Ebselen is a candidate therapeutic drug, and the toxicity of compound **b** was found to be lower than that of ebselen. Also, the effects of ebselen on the activation of ERK1/2 and the inactivation of caspases were lower than those of compound **b**. Therefore, in regard to the low toxicity and inhibition of apoptosis, compound **b** might be a more attractive and promising candidate than ebselen.

A low concentration of O_2^- produced in the human body generally plays a beneficial role in biological defenses and intercellular signal transduction (Kimura *et al.*, 2005). Conversely, excessive O_2^- production has a role in the pathogenesis of many disorders, including inflammation, rheumatoid arthritis, and asthma (Vignola *et al.*, 1998; Kato *et al.*, 2005). Oxidative stress may be defined as an imbalance between cellular production of ROS and antioxidant defense mechanisms (Kimura *et al.*, 2005), and ROS is a key component of inflammation and inflammatory disorders. The processes associated with inflammatory responses are complex and often involve ROS, including O_2^-. In this study, we demonstrated that our compounds functioned effectively as O_2^- scavengers *in vitro,* which action suggests that they suppress ROS overproduction through the elimination of excess O_2^-.

Intracellular signaling pathways involving enzyme activities of kinases and phosphatases are regulated by the redox state (Kamata *et al.*, 2005). It is thought that there are two kinds of mechanisms involved in the compound **b**-induced ERK1/2 phosphorylation. One is the activation of cell membrane receptors and the other is regulation of the redox state in cells. Full disclosure of the effects of compound **b** on neuronal cells and the mechanism by which it activates ERK1/2 are expected to be reported in the near future.

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Although in this study only PC12 cells were used for biological evaluation of compound **b,** additional *in vitro* and *in vivo* studies are necessary to clarify whether this compound may be applicable as an antioxidant drug.

References

Aletta, J. M. (1994). Differential effect of NGF and EGF on ERK in neuronally differentiated PC12 cells. *Neuroreport* **5**, 2090-2092.

Balaban, R. S., Nemoto, S., and Finkel, T. (2005). Mitochondria, oxidants, and aging. *Cell* **120**, 483-495.

Berridge, M. J. (1984). Inositol trisphosphate and diacylglycerol as second messengers. *Biochem J* **220**, 345-360.

Biswas, S. C., and Greene, L. A. (2002). Nerve growth factor (NGF) down-regulates the Bcl-2 homology 3 (BH3) domain-only protein Bim and suppresses its proapoptotic activity by phosphorylation. *J Biol Chem* **277**, 49511-49516.

Curnutte, J. T., Whitten, D. M., and Babior, B. M. (1974). Defective superoxide production by granulocytes from patients with chronic granulomatous disease. *N Engl J Med* **290**, 593-597.

D'Alessio, A., Cerchia, L., Amelio, I., Incoronato, M., Condorelli, G., and de Franciscis, V. (2007). Shp2 in PC12 cells: NGF versus EGF signalling. *Cell Signal* **19**, 1193-1200.

Engman, L. (1989). Expedient synthesis of ebselen and related compounds. *J Org Chem* **54**, .2964-2966.

Fridovich, I. (1983). Superoxide radical: an endogenous toxicant. *Annu Rev Pharmacol Toxicol* **23**, 239-257.

Fridovich, I. (1995). Superoxide radical and superoxide dismutases. *Annu Rev Biochem* **64**, 97-112.

Fridovich, I. (1998). Oxygen toxicity: a radical explanation. *J Exp Biol* **201**, 1203-1209.

Jeong, D. W., Kim, T. S., Chung, Y. W., and Kim, I. Y. (2002). Selenoprotein W is a glutathione-dependent antioxidant in vivo. *FEBS Lett* **517**, .225-228.

Kamata, H., Oka, S., Shibukawa, Y., Kakuta, J., and Hirata, H. (2005). Redox regulation of nerve growth factor-induced neuronal differentiation of PC12 cells through modulation of the nerve growth factor receptor, TrkA. *Arch Biochem Biophys* **434**, 16-25.

Kato, M., Hayashi, Y., and Kimura, H. (2005). Oxygen radicals in inflammation and allergy

related to viral infections. *Curr Drug Targets Inflamm Allergy* **4**, 497-501.

Kimura, H., and Nakano, M. (1988). Highly sensitive and reliable chemiluminescence method for the assay of superoxide dismutase in human erythrocytes. *FEBS Lett* **239**, 347-350.

Kimura, H., Sawada, T., Oshima, S., Kozawa, K., Ishioka, T., and Kato, M. (2005). Toxicity and roles of reactive oxygen species. *Curr Drug Targets Inflamm Allergy* **4**, 489-495.

Long, J. F., Dutta, P. K., and Hogg, B. D. (1997). Fluorescence Imaging of Reactive Oxygen Metabolites Generated in Single Macrophage Cells(NR8383) upon Phagocytosis of Natural Zeolite(Erionite) Fibers. *Environ Health Perspect* **105**, .706-711.

Matsuda, M., Hashimoto, Y., Muroya, K., Hasegawa, H., Kurata, T., Tanaka, S., Nakamura, S., and Hattori, S. (1994). CRK protein binds to two guanine nucleotide-releasing proteins for the Ras family and modulates nerve growth factor-induced activation of Ras in PC12 cells. *Mol Cell Biol* **14**, 5495-5500.

Meydani, M., Evans, W., Handelman, G., Fielding, R. A., Meydani, S. N., Fiatarone, M. A., Blumberg, J. B., and Cannon, J. G. (1992). Antioxidant response to exercise-induced oxidative stress and protection by vitamin E. *Ann N Y Acad Sci* **669**, 363-364.

Morey, M., Serras, F., Baguna, J., Hafen, E., and Corominas, M. (2001). Modulation of the Ras/MAPK signalling pathway by the redox function of selenoproteins in Drosophila melanogaster. *Dev Biol* **238**, 145-156.

Nakamura, Y., Feng, Q., Kumagai, T., Torikai, K., Ohigashi, H., Osawa, T., Noguchi, N., Niki, E., and Uchida, K. (2002). Ebselen, a glutathione peroxidase mimetic seleno-organic compound, as a multifunctional antioxidant. Implication for inflammation-associated carcinogenesis. *J Biol Chem* **277**, 2687-2694.

Nishina, A., Sekiguchi, A., He, Y., Koketsu, M., and Furukawa, S. (2008). Ebselen, a redox regulator containing a selenium atom, induces neurofilament M expression in cultured rat pheochromocytoma PC12 cells via activation of mitogen-activated protein kinase. *J Neurosci Res* **86**, 720-725.

Ramirez, R., Carracedo, J., Jimenez, R., Canela, A., Herrera, E., Aljama, P., and Blasco, M.

A. (2003). Massive telomere loss is an early event of DNA damage-induced apoptosis. *J Biol Chem* **278**, 836-842.

Rong, P., Bennie, A. M., Epa, W. R., and Barrett, G. L. (1999). Nerve growth factor determines survival and death of PC12 cells by regulation of the bcl-x, bax, and caspase-3 genes. *J Neurochem* **72**, 2294-2300.

Ryter, S. W., Kim, H. P., Hoetzel, A., Park, J. W., Nakahira, K., Wang, X., and Choi, A. M. (2007). Mechanisms of cell death in oxidative stress. *Antioxid Redox Signal* **9**, 49-89.

Sairanen, T., Szepesi, R., Karjalainen-Lindsberg, M. L., Saksi, J., Paetau, A., and Lindsberg, P. J. (2009). Neuronal caspase-3 and PARP-1 correlate differentially with apoptosis and necrosis in ischemic human stroke. *Acta Neuropathol*.

Sekiguchi, A., Nishina, A., Kimura, H., Fukumoto, R. H., Kanoh, K., Ishihara, H., and Koketsu, M. (2005). Superoxide anion-scavenging effect of 2-amino-1,3-selenazoles. *Chem Pharm Bull (Tokyo)* **53**, 1439-1442.

Sekiguchi, A., Nishina, A., Kimura, H., Fukumoto, R. H., Kogami, M., Ishihara, H., and Koketsu, M. (2006). Bis-(2-amino-5-selenazoyl) ketone as a superoxide anion-scavenger. *Biol Pharm Bull* **29**, 1404-1407.

Sommen, G., Linden, A., and Heimgartner, H. (2006). Selenium-containing heterocycles from isoselenocyanates: synthesis of 2-methylidene-1,3-selenazolidine derivatives. *Tetrahedron* **62**, .3344-3354.

Takahashi, H., Nishina, A., Fukumoto, R. H., Kimura, H., Koketsu, M., and Ishihara, H. (2005a). Selenocarbamates are effective superoxide anion scavengers in vitro. *Eur J Pharm Sci* **24**, 291-295.

Takahashi, H., Nishina, A., Fukumoto, R. H., Kimura, H., Koketsu, M., and Ishihara, H. (2005b). Selenoureas and thioureas are effective superoxide radical scavengers in vitro. *Life Sci* **76**, 2185-2192.

Takahashi, H., Nishina, A., Kimura, H., Motoki, K., Koketsu, M., and Ishihara, H. (2004). Tertiary selenoamide compounds are useful superoxide radical scavengers in vitro. *Eur J Pharm Sci* **23**, 207-211.

Tiano, L., Fedeli, D., Santroni, A. M., and Falcioni, G. (2000). Effect of three diaryl tellurides, and an organoselenium compound in trout erythrocytes exposed to oxidative stress in vitro. *Mutat Res* **464**, .269-277.

Vaisid, T., Kosower, N. S., and Barnoy, S. (2005). Caspase-1 activity is required for neuronal differentiation of PC12 cells: cross-talk between the caspase and calpain systems. *Biochim Biophys Acta* **1743**, 223-230.

Vignola, A. M., Chanez, P., Campbell, A. M., Souques, F., Lebel, B., Enander, I., and Bousquet, J. (1998). Airway inflammation in mild intermittent and in persistent asthma. *Am J Respir Crit Care Med* **157**, 403-409.

Wang, Z., Cao, N., Nantajit, D., Fan, M., Liu, Y., and Li, J. J. (2008). Mitogen-activated protein kinase phosphatase-1 represses c-Jun NH2-terminal kinase-mediated apoptosis via NF-kappaB regulation. *J Biol Chem* **283**, 21011-21023.

Wendel, A., and Tiegs, G. (1986). A novel biologically active seleno-organic compound. VI. Protection by ebselen (PZ 51) against galactosamine/endotoxin-induced hepatitis in mice. *Biochem Pharmacol* **35**, .2115-2118.

Wert, M. M., and Palfrey, H. C. (2000). Divergence in the anti-apoptotic signalling pathways used by nerve growth factor and basic fibroblast growth factor (bFGF) in PC12 cells: rescue by bFGF involves protein kinase C delta. *Biochem J* **352 Pt 1**, 175-182.

Williams, T. M., Ndifor, A. M., Near, J. T., and Reams-Brown, R. R. (2000). Lead enhances NGF-induced neurite outgrowth in PC12 cells by potentiating ERK/MAPK activation. *Neurotoxicology* **21**, 1081-1089.

Wright, J. H., Drueckes, P., Bartoe, J., Zhao, Z., Shen, S. H., and Krebs, E. G. (1997). A role for the SHP-2 tyrosine phosphatase in nerve growth-induced PC12 cell differentiation. *Mol Biol Cell* **8**, 1575-1585.

Zhang, M., Nomura, A., Uchida, Y., Iijima, H., Sakamoto, T., Iishii, Y., Morishima, Y., Mochizuki, M., Masuyama, K., Hirano, K., and Sekizawa, K. (2002). Ebselen suppresses late airway responses and airway inflammation in guinea pigs. *Free Radic Biol Med* **32**, 454-464.

Fig. 1. 2-Methylene-1,3-selenazolidin-4-one derivatives tested in this study.

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Fig. 2. Effect of 2-[3-(4-methoxyphenyl)-4-oxo-1,3-selenazolidin-2-ylidene]malononitrile, compound **b,** on （2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo-[1,2-*a*] pyrazin-3-one hydrochloride (MCLA)-dependent luminescence.

A: Chemiluminescence inhibition curves of compound **b**. Arrows indicate the times at which MCLA or xanthine oxidase (XOD) was added. B: Fifty-percent inhibitory concentration (IC_{50}) of the compound.

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Fig. 3. Uric acid generation in a hypoxanthine-xanthine oxidase system.

2-[3-(4-Methoxyphenyl)-4-oxo-1,3-selenazolidin-2-ylidene]malononitrile **b** (166 μM) was added to the standard reaction mixture, and the rate of generation of uric acid was measured by using a uric acid measurement kit (L type WAKO UA M; Wako Pure Chemical Industries, Ltd. Osaka, Japan). The data are representative of 3 separate experiments and presented as the means ± SE. (-●-: Control, -□-: with **b**)

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Fig. 4. Effects of ebselen, compound **b** or NGF on serum deprivation-induced apoptosis in PC12 cells. PC12 cells were cultured in serum-containing medium for 2 days. The cells were then preincubated with U0126 for 15 min (*C, D*) or not (A,B). Thereafter, the medium was changed to serum-free medium containing ebselen (*A*) , compound **b** (*B, C*), NGF (*D*) or no reagents (*control*); and the cells were cultured for another 11 days. Data are expressed as mean ±SE. Significant differences of the values from the value of the control or no inhibitor at four days after serum deprivation were determined by ANOVA, followed by Dunnett's test. Significance: **,p<0.01; -, no significant difference.

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Fig. 5. Effects of compound **b** on caspase-3 and -7 activation. PC12 cells were cultured in serum-containing medium for 2 days. Thereafter, the medium was changed to serum-free medium containing NGF, compound **b** or no reagents (*control*); and the cells were cultured for another 6 days. Data are expressed as mean $\pm$ SE. Significant differences of the values from the value of the control at four days after serum deprivation were determined by ANOVA. Significance: ***, $p < 0.005$; **, $p < 0.01$.

Fig. 6. Effects of compound **b**, NGF and/or U0126 on ERK1/2 activation. PC12 cells were cultured in serum-containing medium for 2 days. They were then preincubated with U0126 for 15 min or not, and thereafter treated or not with compound **b** or NGF for 10 min. The phosphorylation of ERK1/2 of each sample was evaluated by immunoblotting. The intensity of each band of ERK1/2 was measured densitometrically, and the values are expressed as the mean ± SEM of fold-increase over the control value (treatment without any reagent) of 3 separate experiments. Significant differences of the values from the value of the corresponding control group were determined by ANOVA, followed by Dunnett's test. Control group was set as O. Significance: *, P<0.05; ***, P<0.005; -, no significant difference.

control A
+U0126 B
+NGF C
+NGF,U0126 D
+compound **b** E
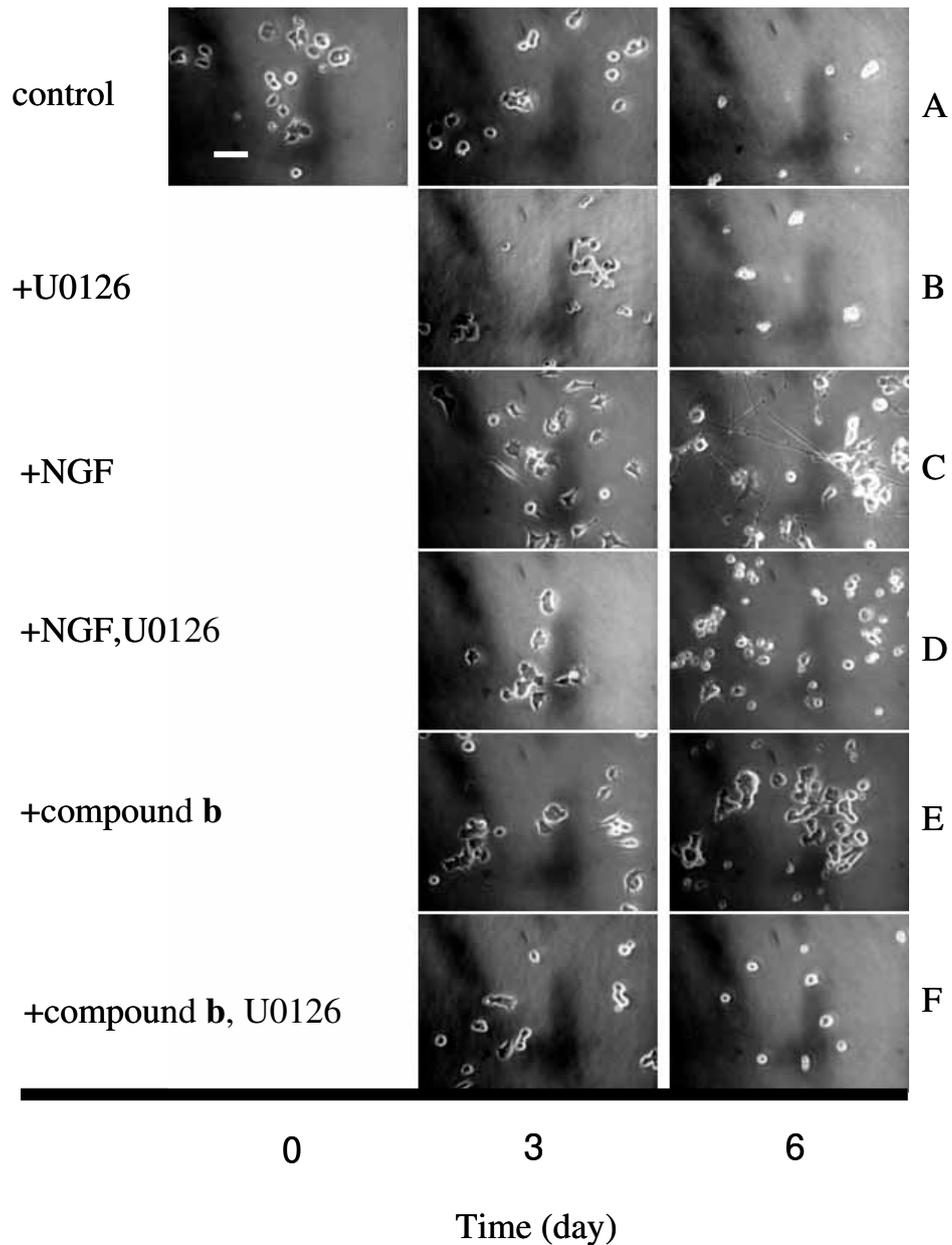+compound **b**, U0126 F

0   3   6

Time (day)

Fig. 7. Effect of U0126, compound **b** or NGF alone on cell survival of serum-deprived PC12 cells, and that of U0126 on NGF- or compound **b** promoted cell survival. PC12 cells were cultured in serum-containing medium for 2 days. The cells were preincubated with U0126 for 15 min (*B, D, F*) or not (A,C,E), and subsequently cultured for another 6 days in the serum-free medium supplemented with no reagents (*A, B*), NGF (10 ng/ml, *C, D*) or compound **b** (100 μM, *E, F*). Photographs were taken under phase-contrast observation at magnification 100 x. Bar indicates 10 μm.