The molecular mechanisms underlying the regulation of Pin1 in the induction and maintenance of pluripotency are likely to be highly complex given that Pin1 interacts with multiple substrates in pluripotent stem cells, as revealed by our proteomics analysis. However, our current findings also indicate that Pin1 is involved in the growth and maintenance of pluripotency in stem cells through its phosphorylation-dependent prolyl isomerization of substrates such as Oct4. In this regard, a recent report by Moretto-Zita *et al.* (30) has demonstrated that Pin1 can also associate with another pluripotent transcription factor, Nanog, in murine ES cells and sustain the self-renewal and teratoma formation of these cells in immunodeficient mice. These results indicate that Pin1 is a crucial modulator of the transcription factor network governing cellular stemness. It is possible also that Pin1 could regulate this process by modulating the function of other substrates. Further studies of Pin1 function in stem cells at various stages might shed new light on the underlying molecular pathways and factors that control self-renewal and multipotency.

It has been demonstrated that Pin1 knock-out mice develop normally but display some proliferation abnormalities, including a decreased body weight, retinal degeneration, and impaired mammary gland development (31, 32). Pin1 knock-out mice also exhibit testicular atrophy with a significantly impaired proliferation of primordial germ cells and the progressive loss of spermatogenic cells (33). These phenotypes can now be attributed to the impaired maintenance and proliferation of germ-related stem cells due to the loss of Pin1 function.

In many circumstances, Pin1 acts as either a repressor or an enhancer of the degradation of substrate proteins (15–17, 34). Our current data now additionally demonstrate that Pin1 can also prolong the protein half-life of Oct4, thereby enhancing its transcriptional activity. Oct4 has been shown to be regulated by post-translational modifications such as SUMOlylation (35). Our current findings reveal that Oct4 is also regulated by phosphorylation and subsequent prolyl isomerization. Identification of the kinase(s) responsible for the association of Pin1 and Oct4 will enhance our understanding of the regulatory pathways that operate during and after the induction of pluripotency.

It is desirable to utilize pluripotent stem cells such as iPS cells for future regenerative medicine applications. However, there are already concerns surrounding the use of iPS cells in a clinical setting because prior studies have suggested that they are likely to develop cancers (4, 36). Our current findings suggest, however, that the Pin1 inhibition could effectively block the proliferation of iPS cells in an undifferentiated state. Pin1 could therefore act as a molecular switch that can reversibly control the proliferation and survival of iPS cells, thereby reducing the risk of cell transformation and tumor formation.

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# 3-(2,6-Dimethylphenyl)-2-Selenoxo-1,3-Thiazolidin-4-One Suppresses Hydrogen Peroxide–Induced Cytotoxicity on PC12 Cells Via Activation of MAPK

**Atsuyoshi Nishina[1], Hirokazu Kimura[2], Kunihisa Kozawa[2], Geoffroy Sommen[3], Francesco Favero[4], Heinz Heimgartner[5], Mamoru Koketsu[6], and Shoei Furukawa[7]**

## Abstract

We newly synthesized organic selenium compounds (5-membered ring compounds) including 2-selenoxo-1,3-thiazolidin-4-ones (compounds A) and 3-alkoxy-4,5-dihydro-5-selenoxo-*1H*-1,2,4-triazole-1-carboxylates (compounds B). To address whether these compounds show antioxidative effects, we also examined their superoxide radical ($O_2^-$)-scavenging effects. Moreover, we examined the effects of compound Aa on the activation of mitogen-activated protein kinase/extracellular signal-regulated protein kinases (MAPK/ERK1/2) and suppression of hydrogen peroxide-induced cytotoxicity in rat pheochromocytoma cells (PC12 cells). We evaluated the $O_2^-$-scavenging activities of the compounds by a chemiluminescence method, and activation of ERK1/2 in PC12 cells was evaluated by Western blot analysis. At 166 μmol/L, the $O_2^-$-scavenging activities were markedly different among compounds A and B. 3-(2,6-Dimethylphenyl)-2-selenoxo-1,3-thiazolidin-4-one (compound Aa) exhibited the strongest superoxide anion-scavenging activity among compounds A and B. The concentration necessary for 50% inhibition of the activity ($IC_{50}$) of compound Aa was 25.9 μmol/L. Compound Aa activated ERK1/2 of the PC12 cell, as did ebselen, and suppressed hydrogen peroxide-induced cytotoxicity more potently than ebselen. In addition, the toxicity of compound Aa was less than that of ebselen. From these results, it is assumed that compound Aa is a candidate drug to prevent oxidative stress-induced cell death.

## Keywords

3-(2, 6-dimethylphenyl)-2-selenoxo-1, 3-thiazolidin-4-one, superoxide anion-scavenging activities, PC12 cells, MAPK, phosphorylation

Reactive oxygen species[1] including superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals (·OH) are generated in aerobic cells.[2] Reactive oxygen species may react with various pivotal biomolecules such as DNA and proteins, and the excessive reactive oxygen species generation in the cells may lead to denaturation of the molecules.[3] In addition, endogenous reactive oxygen species generators such as granulocytes, monocytes, macrophages, eosinophils, and basophils produce reactive oxygen species and damage the tissue/organ due to abnormal inflammation.[4–6] To avoid toxicity of reactive oxygen species under the normal physiological conditions, the reactive oxygen species production may be regulated.[7] As another effect of reactive oxygen species, recent reports suggest that the low concentration of reactive oxygen species in the cell may regulate signaling pathways.[8]

Superoxide dismutases (SODs), catalase, glutathione peroxidases (GPXs), and some vitamins are representative antioxidants that protect against reactive oxygen species toxicity in cells.[4,5] Glutathione peroxidases are believed to be an important antioxidant enzyme and effectively reduce the toxicity of $H_2O_2$ in vitro and in vivo.[5,9] Furthermore, the active domain of GPX essentially contains selenium atoms.[9] Various studies have reported that selenoproteins, including GPX, reduce oxidative

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stress in cells.[10,11] Thus, various organic selenium compounds may be candidates for reactive oxygen species scavengers.

Ebselen is a 5-membered ring selenium-containing heterocyclic compound showing GPX-like activity[12] and is a synthetic antioxidant with reactive oxygen species scavenger effects.[13] The antioxidant effects of ebselen are due to its selective blockade of leukocyte infiltration and activation, leading to the elimination of $H_2O_2$.[13] This compound is a multifunctional antioxidant and a potential chemopreventive agent in inflammation-associated carcinogenesis.[14] As such, various types of organic selenium compounds may be applicable to the reduction of oxidative stress[15–17] and may be used as antioxidative/anti-inflammatory substances for therapeutic drugs against inflammatory diseases (ie, atopic dermatitis) and stroke.[13] Morey et al demonstrated that some selenoproteins, such as selenoprotein P, regulate the redox potential in cells, resulting in the modulation of various phosphorylation pathways including Ras/mitogen-activated protein kinase (MAPK) signaling.[18] On the other hand, we demonstrated that ebselen also influenced the redox potential in rat pheochromocytoma cells (PC12 cells) and induced activation of MAPK/extracellular signal-regulated protein kinases ([ERK1/2], MAPK/ERK1/2) and neural differentiation via regulation of kinases or phosphatases involved in intracellular signaling.[19] Thus, such selenium compounds are thought to play roles not only as reactive oxygen species scavengers but also as modulators of intracellular signaling.

Against this research background, we sought to synthesize various organic selenium compounds, such as selenoamides,[17] thio- and selenoureas,[16] and selenocarbamates,[15] in order to evaluate their biological potential. It is already known that most of these compounds are also highly efficient in vitro $O_2^-$ scavengers.[15,16] Selenoureas in particular showed pronounced anti-inflammatory effects with low toxicity in various human cell lines.[20] In this study, we newly synthesized 5-membered ring compounds such as 2-selenoxo-1,3-thiazolidin-4-one (compound A and its analogues: compounds **Aa-d**) and 3-alkoxy-4,5-dihydro-5-selenoxo-*1H*-1,2,4-triazole-1-carboxylate (compound B and its analogues: compounds **Be-j**), and their superoxide radical ($O_2^-$)-scavenging effects, as antioxidants were evaluated by a highly sensitive and quantitative real-time kinetic chemiluminescence method. Moreover, to evaluate the biological effects such as cytotoxicity, cell death inhibition, and cell death/cell proliferation-related signaling pathways of 3-(2,6-dimethylphenyl)-2-selenoxo-1,3-thiazolidin-4-one (compound **Aa**) and ebselen, we examined whether they could activate MAPK/ERK1/2 and suppress $H_2O_2$-induced cytotoxicity in PC12 cells.

## Materials and Methods

### Materials

Compounds **Aa-d** and **Be-j** were prepared according to previously reported procedures.[21,22] A cypridina luciferin analog, 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo-[1,2-] pyrazin-3-one hydrochloride (MCLA), obtained from Tokyo Kasei (Tokyo, Japan), was used as a chemiluminescent probe for superoxide radicals. 2-Methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo-[1,2-] pyrazin-3-one hydrochloride was dissolved in twice-distilled water and stored at −80°C prior to use. The concentration of 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo-[1,2-] pyrazin-3-one hydrochloride solution was determined by absorbance at 430 nm using an absorbance coefficient value of $\varepsilon = 9600\ M^{-1}\ cm^{-1}$, as described.[23] Superoxide dismutase (lyophilized powder, 3400 units/mg protein) and xanthine oxidase (grade III) were purchased from Sigma Chemical Co (St Louis, Missouri). Hypoxanthine was purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan) and used without further purification. All other chemicals and solvents were of analytical grade and used without further purification.

*Synthetic methods for the preparation of compounds Aa-d.* A round-bottom flask equipped with a condenser and a magnetic stirrer was charged with an aryl isoselenocyanate (1.0 mmol) in 20 mL of a mixture of ethyl alcohol and water (2:1). An equimolar amount of the respective mercapto carboxylic acid was then added dropwise. The mixture was stirred at room temperature for several hours before being poured into 50 mL of cold water. After 2 hours of stirring, the precipitate was filtered and purified, if necessary, by recrystallization from ethyl alcohol.

*3-(2,6-Dimethylphenyl)-2-selenoxo-1,3-thiazolidin-4-one (compound Aa).* Yield 56%. Orange powder. Mp: 123°C to 125°C. $^1$H-NMR (300 MHz, $CDCl_3$): δ 2.10 (*s*, 2 Me), 3.95 (*s*, $CH_2$), 7.18–7.33 (*m*, 3 arom. H). $^{13}$C-NMR (75 MHz, $CDCl_3$): δ 17.5, 37.3, 127.5, 127.9, 130.0, 136.1, 173.2, 200.2. CI-MS: 303 (60, $[M+NH_4]^+$), 286 (100, $[M+H]^+$). Theoretically calculated values were for $C_{11}H_{11}NOSSe$: C, 46.48; H, 3.90; N, 4.93; S, 11.28. Found: C, 46.33; H, 4.12; N, 5.00; S, 11.32.

*3-(4-Methylphenyl)-2-selenoxo-1,3-thiazolidin-4-one (compound Ab).* Yield 91%. Mp: 201°C to 203°C (ethyl alcohol). $^1$H-NMR (300 MHz, $CDCl_3$): δ 2.42 (*s*, Me), 3.90 (*s*, $CH_2$), 7.10, 7.35 (*AA'BB'*, $J = 8.2$, 4 arom. H). $^{13}$C-NMR (75 MHz, $CDCl_3$): δ 21.3, 37.4, 128.0, 130.3, 133.2, 140.0, 173.2, 203.3. CI-MS: 289 (31, $[M+NH_4]^+$), 272 (100, $[M+H]^+$). Theoretically calculated values were for $C_{10}H_9NOSSe$: C, 44.45; H, 3.36; N, 5.18; S, 11.87. Found: C, 44.30; H, 3.21; N, 5.03; S, 11.78.

*3-(4-Methoxyphenyl)-2-selenoxo-1,3-thiazolidin-4-one (compound Ac).* Yield 93%. Mp: 182°C to 184°C (ethyl alcohol).$^1$H-NMR (300 MHz, $CDCl_3$): δ 3.85 (*s*, MeO), 3.89 (*s*, $CH_2$), 7.03, 7.14 (*AA'BB'*, $J = 8.2$, 4 arom. H). $^{13}$C-NMR (75 MHz, $CDCl_3$): δ 37.3, 55.4, 114.9, 128.2 (1 arom. C), 129.4, 160.3, 173.3, 203.6. CI-MS: 305 (32, $[M+NH_4]^+$), 288 (100, $[M+H]^+$). Theoretically calculated values were for $C_{10}H_9NO_2SSe$: C, 41.96; H, 3.17; N, 4.89; S, 11.20. Found: C, 42.23; H, 3.33; N, 4.88; S, 11.12.

*3-(4-Bromophenyl)-2-selenoxo-1,3-thiazolidin-4-one (compound Ad).* Yield 89%. Mp: 111°C to 113°C (ethyl alcohol). $^1$H-NMR (300 MHz, $CDCl_3$): δ 3.92 (*s*, $CH_2$), 7.11, 7.67 (*AA'BB'*,

$J$ = 8.1, 4 arom. H). $^{13}$C-NMR (75 MHz, $CDCl_3$): δ 37.4, 123.3, 130.0, 132.9, 138.5, 171.8, 203.8. CI-MS: 353 (35, $[M+NH_4]^+$), 336 (100, $[M+H]^+$). Theoretically calculated values were for $C_9H_6NOSSeBr$: C, 32.26; H, 1.80; N, 4.18; S,9.57. Found: C, 32.41; H, 2.02; N, 3.98; S, 10.00.

*Synthetic methods for the preparation of compounds Be-j.* A 25-mL round-bottom flask equipped with magnetic stirrer and condenser was charged with a mixture of diethyl or bis(tert-butyl) azodicarboxylate (0.92 mL, 2.0 mmol) and $Ph_3P$ (524 mg, 2.0 mmol) in dichloromethane (20 mL). The mixture was stirred under $N_2$ atmosphere at 0°C (ice bath) for 30 minutes. The appropriate amount of isoselenocyanate (2.0 mmol) was added in 1 portion. The mixture was stirred for 15 hours at room temperature and then evaporated to dryness under reduced pressure. The crude product was purified by column chromatography on $SiO_2$ using hexane/ethyl acetate (100/0 to 50/50) as the eluent and recrystallized in ethyl acetate.

*Ethyl 3-ethoxy-4,5-dihydro-4-phenyl-5-selenoxo-1H-1,2,4-triazole-1-carboxylate (compound Be).* Yield: 552 mg (81%). Yellowish crystals. Mp: 142°C to 144°C (ethyl acetate). IR ($cm^{-1}$): 3422w (br), 2981w, 2931w, 1773s, 1619s, 1595w, 1501w, 1451m, 1386m, 1368m, 1328s, 1308s, 1220s, 1175w, 1155w, 1109w, 1088w, 1066w, 1028m, 1005m, 979w, 902w, 861w, 847w, 776w, 710w, 689w. $^1$H-NMR (300 MHz, $CDCl_3$): δ 1.36 (*t*, $J$ = 7.1, $CH_3$), 1.49 (*t*, $J$ = 7.1, $CH_3$), 4.49 to 4.62 (*m*, 2 $CH_2$), 7.35 (*d*, $J$ = 8.1, 2 arom. H), 7.52 to 7.59 (*m*, 3 arom. H). $^{13}$C-NMR (75 MHz, $CDCl_3$): δ 14.0, 14.1, 65.0, 68.5, 128.1, 129.4, 130.0, 132.6, 148.2, 156.3, 167.8. ESI-MS: 360 (14), 361 (13), 362 (52), 363 (3), 364 (100, $[M+Na]^+$), 365 (11), 366 (15). Theoretically calculated values were for $C_{13}H_{15}N_3O_3Se$: C, 45.89; H, 4.44; N, 11.86. Found: C, 45.58; H, 4.58; N, 12.64.

*Ethyl 3-ethoxy-4,5-dihydro-4-(4-methylphenyl)-5-selenoxo-1H-1,2,4-triazole-1-carboxylate (compound Bf).* Yield: 554 mg (78%). Yellowish crystals. Mp 120°C to 122°C (ethyl acetate). IR ($cm^{-1}$): 3442w (br), 2979w, 2956w, 1768s, 1619s, 1514m, 1477w, 1454m, 1389m, 1368m, 1327s, 1307s, 1294s, 1220s, 1173w, 1152w, 1109w, 1099w, 1064w, 1029m, 981w, 905w, 850w, 817w, 753w, 712w, 622w. $^1$H-NMR (300 MHz, $CDCl_3$): δ 1.37 (*t*, $J$ = 7.1, $CH_3$), 1.49 (*t*, $J$ = 7.1, $CH_3$), 2.43 (*s*, $CH_3$), 4.48 to 4.60 (*m*, 2 $CH_2$), 7.22, 7.34 (*AA'BB'*, $J$ = 8, 4 arom. H). $^{13}$C-NMR (75 MHz, $CDCl_3$): δ 14.0, 14.3, 21.3, 65.0, 68.4, 127.7, 130.0, 127.3, 140.2, 149.0, 156.0, 167.1. ESI-MS: 374 (12), 375 (14), 376 (48), 377 (4), 378 (100, $[M+Na]^+$), 379 (11), 380 (13), 733 (3). Theoretically calculated values were for $C_{14}H_{17}N_3O_3Se$: C, 47.46; H, 4.84; N, 11.86. Found: C, 47.25; H, 4.85; N, 11.85.

*Ethyl 3-ethoxy-4-(4-fluorophenyl)-4,5-dihydro-5-selenoxo-1H-1,2,4-triazole-1-carboxylate (compound Bg).* Yield: 603 mg (84%). Yellowish crystals. Mp 151°C to 153°C (ethyl acetate). IR ($cm^{-1}$): 3442w (br), 3085w, 2982w, 1769s, 1621s, 1512s, 1475w, 1454m, 1386m, 1367m, 1311s, 1290s, 1216s, 1171w, 1150w, 1112w, 1088w, 1063m, 1024m, 1004w, 977m, 905w, 853m, 821w, 759w, 725w, 711w, 635w, 622m. $^1$H-NMR (300 MHz, $CDCl_3$): δ 1.35 (*t*, $J$ = 7.1, $CH_3$), 1.49 (*t*, $J$ = 7.1, $CH_3$), 4.41 to 4.51 (*m*, 2 $CH_2$), 6.95, 7.19 (*AA'BB'*, $J$ = 8, 4 arom. H). $^{13}$C-NMR (75 MHz, $CDCl_3$): δ 14.0, 14.3, 65.1, 68.6, 116.4, 130.1, 128.4, 148.9, 155.8, 162.3, 167.1. ESI-MS: 378 (15), 379 (12), 380 (55), 381 (4), 382 (100, $[M+Na]^+$), 383 (15), 384 (17). Theoretically calculated values were for $C_{13}H_{14}N_3O_3SeF$: C, 43.59; H, 3.94; N, 11.73. Found: C, 43.37; H, 4.00; N, 11.88.

*Ethyl 4-(4-chlorophenyl)-3-ethoxy-4,5-dihydro-5-selenoxo-1H-1,2,4-triazole-1-carboxylate (compound Bh).* Yield: 726 mg (97%). Yellowish crystals. Mp 142°C to 144°C (ethyl acetate). IR ($cm^{-1}$): 3442w (br), 2982w, 1765s, 1625s, 1495m, 1469w, 1442w, 1373m, 1310s, 1289s, 1218s, 1173w, 1148w, 1089m, 1025m, 977m, 907w, 836w, 753w, 703w. $^1$H-NMR (300 MHz, $CDCl_3$): δ 1.31 (*t*, $J$ = 7.1 Hz, $CH_3$), 1.41 (*t*, $J$ = 7.1, $CH_3$), 4.39 to 4.51 (*m*, 2 $CH_2$), 7.24, 7.43 (*AA'BB'*, $J$ = 8, 2H, 4 arom. H). $^{13}$C-NMR (75 MHz, $CDCl_3$): δ 14.1, 14.3, 65.1, 68.7, 124.6, 129.5, 129.7, 131.0, 148.9, 155.6, 166.9. ESI-MS: 394 (13), 395 (14), 396 (51), 397 (4), 398 (100, $[M+Na]^+$), 399 (18), 400 (49). Theoretically calculated values were for $C_{13}H_{14}N_3O_3SeCl$: C, 41.67; H, 3.77; N, 11.21. Found: C, 41.63; H, 3.84; N, 11.34.

*Ethyl 4-(4-bromophenyl)-3-ethoxy-4,5-dihydro-5-selenoxo-1H-1,2,4-triazole-1-carboxylate (compound Bi).* Yield: 686 mg (82%). Yellowish crystals. Mp 147°C to 149°C (ethyl acetate). IR ($cm^{-1}$): 3444w (br), 2982w, 2935w, 1755s, 1718m, 1638s, 1591w, 1493m, 1451m, 1392m, 1366m, 1327s, 1303s, 1286s, 1215w, 1170w, 1154w, 1104w, 1068w, 1030m, 1011m, 988w, 909w, 850w, 754w, 711w. $^1$H-NMR (300 MHz, $CDCl_3$): δ 1.31 (*t*, $J$ = 7.1, $CH_3$), 1.41 (*t*, $J$ = 7.1, $CH_3$), 4.41 to 4.51 (*m*, 2 $CH_2$), 7.20, 7.58 (*AA'BB'*, $J$ = 8, 4 arom. H). $^{13}$C-NMR (75 MHz, $CDCl_3$): δ 14.1, 14.3, 65.1, 68.7, 124.2, 129.8, 132.7, 131.5, 148.9, 155.6, 166.8. ESI-MS: 438 (15), 439 (12), 440 (55), 441 (4), 442 (100, $[M+Na]^+$), 443 (14), 444 (12). Theoretically calculated values were for $C_{13}H_{14}N_3O_3SeBr$: C, 37.25; H, 3.37; N, 10.03. Found: C, 37.12; H, 3.23; N, 9.99.

*Tert-Butyl-3-(tert-butoxy)-4-(4-bromophenyl)-4,5-dihydro-5-selenoxo-1H-1,2,4-triazole-1-carboxylate (compound Bj).* Yield: 855 mg (90%). Yellowish crystals. Mp 118°C to 120°C (ethyl acetate). IR ($cm^{-1}$): 3443w (br), 2985w, 2931w, 1764s, 1701w, 1612s, 1488m, 1453w, 1408w, 1372m, 1337m, 1320s, 1297m, 1278s, 1223m, 1143s, 1065w, 1000m, 895w, 848m, 836m, 804w, 761w, 655w.$^1$H-NMR (300 MHz, $CDCl_3$): δ 1.53 (*s*, 3 $CH_3$), 1.67 (*s*, 3 $CH_3$), 7.21, 7.62 (*AA'BB'*, $J$ = 8, 4 arom. H). $^{13}$C-NMR: δ 27.6, 27.8, 86.6, 88.1, 123.8, 129.8, 132.5, 132.1, 147.1, 153.5, 165.1. ESI-MS: 494 (17), 495 (15), 496 (55), 497 (25), 498 (100, $[M+Na]^+$), 499 (18), 500 (75), 501 (12), 502 (10). Theoretically calculated values were for $C_{17}H_{22}N_3O_3$-SeBr: C, 42.96; H, 4.67; N, 8.83. Found: C, 43.22; H, 4.95; N, 9.15.

*Assay of superoxide anion-scavenging activities.* The superoxide anion-scavenging activities of compounds **Aa-d** and **Be-j** were

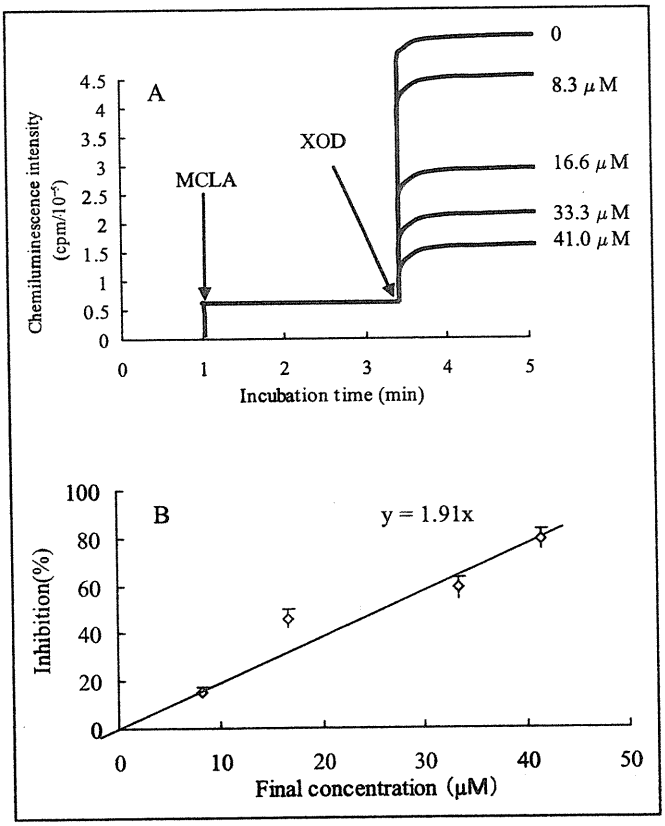



**Figure 1.** Effect of compound Aa on 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo-[1,2-] pyrazin-3-one hydrochloride (MCLA)–dependent luminescence. A, Chemiluminescence inhibition curves by compound Aa. Arrows indicate the addition of MCLA or xanthine oxidase (XOD). B, Concentration-dependent change in superoxide anion-scavenging activity to determine the $IC_{50}$ value of compound Aa.

measured by a previously reported method.[23] In brief, hypoxanthine ($5 \times 10^{-5}$ mol/L) and EDTA (0.1 mmol/L) were prepared in 3 mL of 50 mmol/L tris(hydroxymethyl)aminomethane-HCl (Tris-HCl) buffer, pH 7.8 (the standard solution), with or without various concentrations of compounds Aa-d and Be-j. To evaluate certain superoxide anion-scavenging activities, we prepared a calibration curve using standard solutions of SOD (0.6-30 ng/mL). Compounds Aa-d and Be-j (25 mmol/L) were dissolved in dimethyl sulfoxide and stored at −80°C prior to use. Chemiluminescence was measured with a luminometer (Aloka, BLR201) at 25°C. The reaction was initiated by the addition of 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo-[1,2-] pyrazin-3-one hydrochloride ($5.8 \times 10^{-7}$ mol/L) to the standard in solution excluding xanthine oxidase, continued for 2.5 minutes and then for a further 2 minutes after the addition of xanthine oxidase (6.5 U). A typical result for compound Aa is shown in Figure 1. Superoxide anion-scavenging activity was found to be too strong when the compounds were tested primarily at 166 μmol/L; thus, the activity was measured at 8.3, 16.6, 33.3, or 41.0 μmol/L. The percentage inhibition of 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo-[1,2-] pyrazin-3-one hydrochloride–dependent chemiluminescence was calculated as previously described.[23] The concentration for 50% inhibition ($IC_{50}$) was calculated from the values obtained at 4 different concentrations of compound Aa (8.3, 16.6, 33.3, and 41.0 μmol/L).

## Measurement of Uric Acid Generation in Hypoxanthine–Xanthine Oxidase System

The amounts of uric acid were measured using a uric acid measurement kit (L type WAKO UA M; Wako Pure Chemical Industries, Ltd).

## Cell Culture

In the present study, we used rat PC12 cells because previous reports suggested that this cell line is a useful tool as various models of neurological dysfunctions.[24] Pheochromocytoma cells were cultured as described previously.[25] In brief, the cells were maintained in Dulbecco modified Eagle medium (DMEM; Sigma) supplemented with 10% heat-inactivated horse serum ([HS] Gibco BRL, Grand Island, New York) and 5% heat-inactivated fetal bovine serum ([FBS] Sanko Junyaku, Co, Ltd, Tokyo, Japan; serum-containing medium) or in DMEM supplemented with 1% bovine serum albumin ([BSA] serum-free medium).

## Detection of Phosphorylated Proteins

Each compound was suspended in the serum-free medium and sonicated until fully emulsified. Pheochromocytoma cells were seeded at $2 \times 10^6$ cells per well onto collagen-coated 6-well plates (Corning Incorporated Life Sciences, Lowell, Massachusetts) in the serum-containing medium and precultured for 2 days at 37°C in an atmosphere of 95% air/5% $CO_2$. The cells were then washed with phosphate-buffered saline (PBS) and incubated with the above-mentioned culture medium containing the various agents shown in Figure 2 for 10 minutes at 37°C. The culture plates were then placed on ice and each well was washed with 3 mL of 2 mmol/L Tris-HCl buffer (pH 8.0) containing 0.33 mol/L NaF and 6.25 mol/L $Na_3VO_4$ and subsequently lysed with 150 μL of 20 mmol/L Tris-HCl buffer (pH 8.0) containing 150 mmol/L NaCl, 2 mmol/L EDTA, 1% Nonidet P-40 (w/v), 1% sodium deoxycholate (w/v), 0.1% sodium dodecyl sulfate (SDS; w/v), 50 mmol/L NaF, 0.1% aprotinin (w/v), 0.1% leupeptin (w/v), 1 mmol/L $Na_3VO_4$, and 1 mmol/L phenylmethylsulfonylfluoride (PMSF). Cell lysates were collected using a cell scraper and centrifuged at 15 000*g* for 30 minutes at 4°C. The supernatant was collected, and the overall protein concentration was determined by a BCA Protein Assay Reagent Kit (Pierce, Rockford, Illinois) with BSA as the standard.

Supernatant fluids containing proteins (20 μg) were mixed with lithium dodecyl sulfate (LDS) sample buffer (Invitrogen Corp, Carlsbad, California) and incubated for 5 minutes at 80°C. Proteins in samples were separated on SDS-polyacrylamide gel electrophoresis, and the proteins in gels

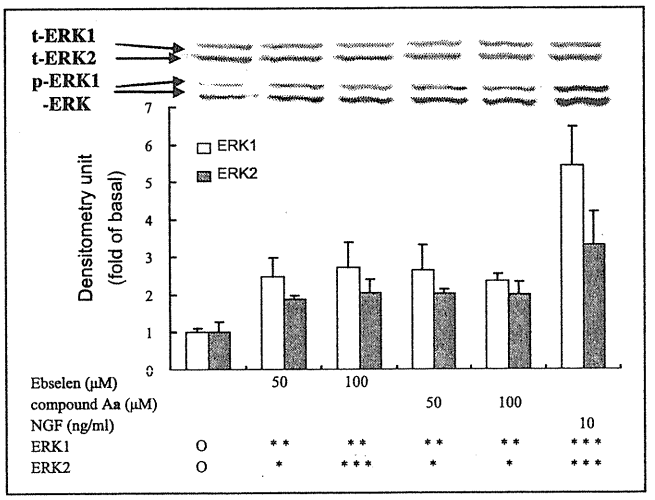



**Figure 2.** Effects of compound **Aa**, ebselen, or NGF on the activation of ERK1/2. The final concentration of each reagent used for the culture is shown. The phosphorylated ERK1/2 and ERK1/2 proteins were visualized by Western blot, and the intensity of each band was densitometrically calculated. Ratios of the relative intensity of the phosphorylated and nonphosphorylated ERK1 or 2 bands are expressed as the means ± SD of 3 independent determinations. The control group is indicated as O. Levels of significance, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$. NGF indicates nerve growth factor; ERK1/2, extracellular signal-regulated protein kinase; SD, standard deviation.

were electroblotted onto polyvinylidene fluoride (PVDF) filters (Fluorotrans membrane W, 0.2 μm; Nihon Genetics, Tokyo, Japan). Immunoblotting analysis was performed using monoclonal antibodies against p44/42 ERK or phospho p44/42 ERK (Cell Signaling Technology, Lake Placid, New York) as primary antibodies, followed by reaction with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (IgG) antibodies from Promega Co (Fitchburg, Wisconsin) as the secondary antibody. The blots were developed by the enhanced chemiluminescence method (Hyperfilm-ECL plus; Amersham Biosciences Corp, Piscataway, New Jersey).

### The Measurement of Cytotoxicity by 5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl-2-Htetrazolium Bromide Assay

Pheochromocytoma cells were cultured onto collagen-coated 96-well plates ($2 \times 10^6$ cells per well) in serum-containing medium for 2 days at 37°C in an atmosphere of 95% air/5% $CO_2$. Culture medium was replaced with 50 μL of the serum-free medium containing each test agent after washing with PBS, and the cells were cultured with each test agent for a range of days. The cytotoxicity was determined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) reduction assay.[26] The cells were incubated with 0.25 ng of MTT/mL (final concentration) for 2 hours, and the reaction was stopped by adding 50 μL of 50% (volume/volume [v/v]) dimethylformamide (DMFA) containing 20% (w/v) SDS. The amount of MTT formazan product was determined photometrically using a microplate reader (Model Ultrospec Visible Plate Reader II of Amersham Biosciences, Tokyo, Japan) by measuring the absorbance at 562 nm with a reference wavelength of 630 nm. The relationships between absorbance and cell numbers were clarified, and the number of cells in each well was calculated.

### Measurement of $H_2O_2$ Concentrations

The concentration of $H_2O_2$ was determined by the horseradish peroxidase-catalyzed oxidation of fluorescent scopoletin method with minor modifications.[27] Briefly, compound **Aa** or ebselen (100 μmol/L) and $H_2O_2$ (400 μmol/L) were added to 100 μL of distilled water or serum-free medium in a 96-well plate followed by 2-hour incubation at 37°C. Scopoletin (10 μmol/L) and horseradish peroxidase (50 μg) were added, and the decrease in scopoletin fluorescence was measured using a microplate fluorometer (1420 Multilabel counter, ARVO; excitation and emission at 335 and 460 nm, respectively; Wallac, Turuk, Finland).

### Statistical Analysis

The results were expressed as means ± standard deviation (SD). The significant differences between the groups compared were determined using analysis of variance (ANOVA) followed by Dunnett test.

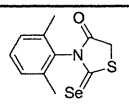
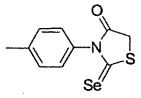
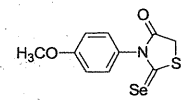
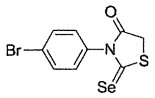
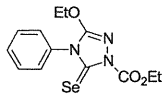
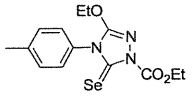
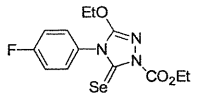
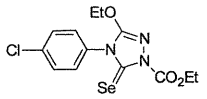
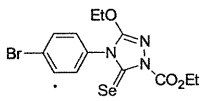
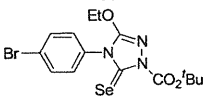
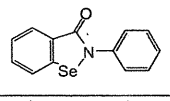
## Results

### Superoxide Anion-Scavenging Activities of Compounds A and B and Ebselen

The chemical structures, superoxide anion-scavenging activities, and $IC_{50}$ values of compounds **Aa-d** and **Be-j** and ebselen are shown in Table 1. The superoxide anion-scavenging activities were nearly dose-dependent for all compounds; and **Aa**, **Ac**, and **Ad** showed the highest level of superoxide anion-scavenging activity at 166 μmol/L. As compound **Aa** had the lowest $IC_{50}$ value at 25.9 μmol/L (Table 1), it was selected and used for further experiments. The superoxide anion-scavenging activities of these compounds did not change by the addition of 50 μmol/L of U0126, a selective mitogen-activated protein kinase kinase (MEK) inhibitor (data not shown).

We investigated whether compounds **Aa-d** and **Be-j** could eliminate superoxide anions generated by xanthine oxidase. For this purpose, the amount of uric acid, which is the by-product of superoxide anions in the mixture for superoxide anion-scavenging activity assay, was measured with or without compound **Aa** (data not shown). The generation of uric acid was not affected by the addition of a relatively high concentration of compound **Aa** (41.0 μmol/L), suggesting that this compound did not inhibit xanthine oxidase. In addition, the effects of compound **Aa** on cultured PC12 cells were investigated.

**Table 1.** Chemical Structures, Superoxide Anion-Scavenging Activities, and $IC_{50}$ Values of 2-Selenoxo-1,3-Thiazolidin-4-Ones 1, 3-Alkoxy-4,5-Dihydro-5-Selenoxo-1H-1,2,4-Triazole-1-Carboxylates and Ebselen[a]

| Compound | | Inhibition (%) at 166 μM | $IC_{50}$ (μM) |
|---|---|---|---|
|  | Aa | 100.0 | 25.9 |
|  | Ab | 44.0 | 192 |
|  | Ac | 100.0 | 52 |
|  | Ad | 100.0 | 47.3 |
|  | Be | 38.3 | 134 |
|  | Bf | 40.0 | 287 |
|  | Bg | 58.9 | 160 |
|  | Bh | 0.0 | - |
|  | Bi | 39.7 | 299 |
|  | Bj | 0.0 | - |
|  | EB | 100.0 | 20 |

[a] $IC_{50}$ values were determined using linear regression analysis of the dose–response curves of the inhibition of chemiluminescence by the compounds. The superoxide anion-scavenging activities were calculated by a rate of decrease in chemiluminescence value after addition of xanthine oxidase. The superoxide anion-scavenging activities of each compound were expressed as a percentage of that of compound Aa.

## *Effects of Compound Aa, Ebselen, or Nerve Growth Factor on the Activation of ERK1/2*

The activation of ERK1/2 is one of the checkpoints to assess the activation of the classical Ras/MAPK cascade,[28] which is triggered by an engaged tyrosine kinase receptor or G-protein-coupled receptor and results in proliferation and/or differentiation. We previously confirmed that ebselen is an activator of the Ras/MAPK cascade and an initiator of neuronal differentiation.[25] Therefore, we examined whether compounds A and B could activate ERK1/2 and found that ERK1/2 was activated only by compound Aa (data not shown). Thereafter, we focused on the biological activity of compound Aa.

The effect of ebselen, compound Aa, or nerve growth factor (NGF) on the phosphorylation of ERK1/2 was examined (Figure 2). Nerve growth factor markedly induced phosphorylation of ERK1/2. Compound Aa and ebselen similarly activated ERK1/2; however, they had a much weaker effect than NGF. On the other hand, phosphorylation of ERK1/2 was not induced in the presence of 400 μmol/L of $H_2O_2$ (data not shown).

## *Effects of Compound Aa or Ebselen on Survival of PC12 Cells Exposed to $H_2O_2$*

The oxidative stress-induced cytotoxicity was evaluated by determining the viability of PC12 cells exposed to $H_2O_2$ (Figure 3). First, we studied the relationship between cell death and $H_2O_2$ concentration. Two hours after the addition of 200, 400, 1000, or 2000 μmol/L $H_2O_2$, the cell viability was 100%, 36%, 8%, or 0%, respectively. At 400 μmol/L of $H_2O_2$, a significant decrease in cell viability was observed, suggesting the potent oxidative stress-induced cytotoxicity of $H_2O_2$.

Pretreatment of PC12 cells with ebselen or compound Aa attenuated this oxidative stress-induced cytotoxicity. However, NGF did not show this effect. Compound Aa was more potent than ebselen, and a concentration of even 50 μmol/L was enough to suppress $H_2O_2$-induced cell death completely. However, such protective effects of both compound Aa and ebselen disappeared on treatment with U0126.

## *Effects of Cell Viability of Compound Aa, Ebselen, or NGF on PC12 Cells*

Ebselen prevents ischemia- or lead-induced cell death,[1,2] so we compared the effects of compound Aa, ebselen, and NGF on serum deprivation-induced cytotoxicity (Figure 4). Cytotoxicity occurred on addition of more than 100 μmol/L of ebselen, when tested over 6 days after exposure. Compound Aa was nontoxic over 6 days at a concentration of 100 μmol/L but toxic at 1 mmol/L in PC12 cells. These results demonstrated that the toxicity of compound Aa was weaker than that of ebselen. Contrary to these results, NGF increased the cell viability after serum deprivation from culture medium, as described previously (Figure 4C).[29]

## Discussion

In the present study, we have systematically synthesized various organic selenium compounds including selenocarbamates, selenoureas, thioureas, tertiary selenoamides, and 2-amino-1, 3-selenazoles and bis-(2-amino-5-selenazoyl) ketones and found that these compounds have superoxide anion-

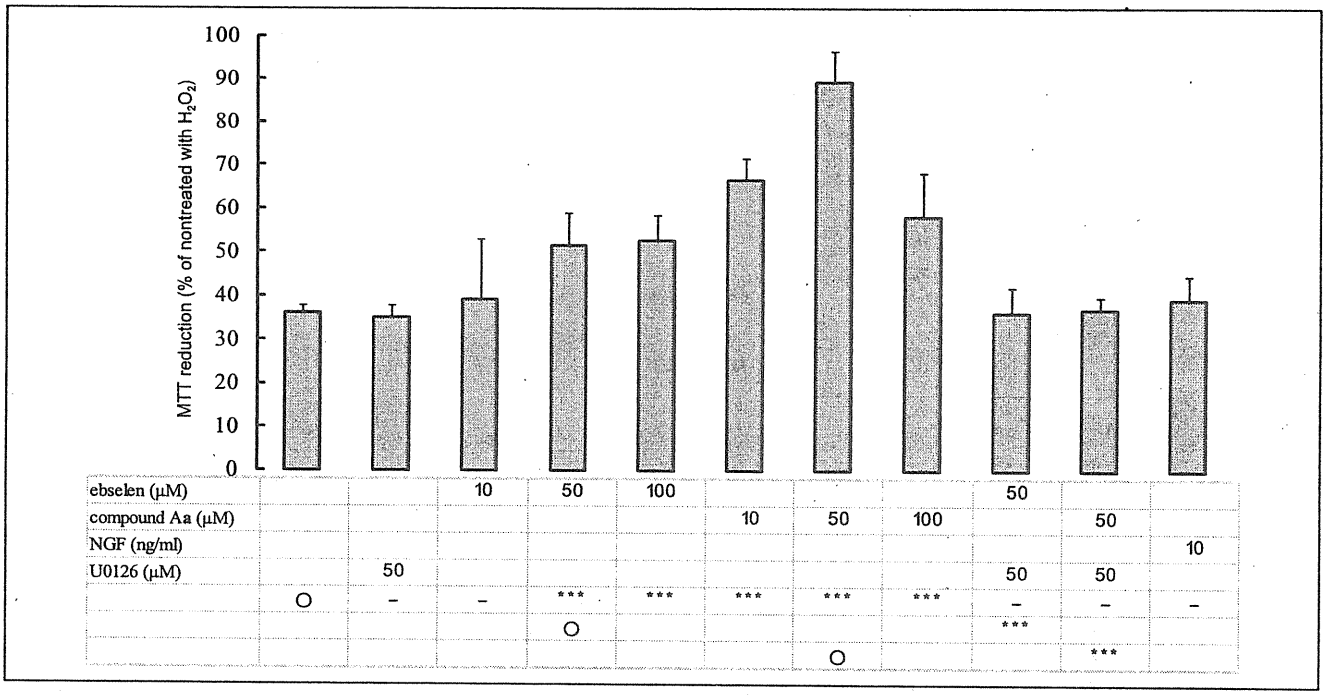



**Figure 3.** Effects of compound Aa or ebselen on survival of PC12 cells exposed to $H_2O_2$. Pheochromocytoma cells were treated with compound Aa, ebselen, or NGF for 10 minutes, and then the cells were washed with PBS. The cells were then exposed to 400 μmol/L of $H_2O_2$ for 2 hours. In some experiments, the cells were pretreated for 4 hours with U0126 (50 μmol/L) before treatment with the reagents. The amount of surviving cells was measured by MTT assay as described in the text. Results shown are means ± SD (n = 3). The significance of differences between the groups compared was determined using the analysis of variance (ANOVA) followed by Dunnett test. The control group is indicated as O. Levels of significance, ***$P < 0.005$; -, no significant difference. NGF indicates nerve growth factor; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; SD, standard deviation.

scavenging activities, and their $IC_{50}$ values[15–17,30,31] range approximately from 0.1 to 100 μmol/L. From the results shown in Table 1, it was confirmed that the superoxide anion-scavenging activities of these newly synthesized compounds, 2-selenoxo-1,3-thiazolidin-4-ones compounds Aa-d and some of the 3-alkoxy-4,5-dihydro-5-selenoxo-*1H*-1,2,4-triazole-1-carboxylates compounds Be-j were similar to those of other previously synthesized organic selenium compounds such as selenocarbamates and selenoureas. The results suggest that compound Aa and some of the B compounds are organic selenium compounds and useful reactive oxygen species scavengers. The generation of uric acid was not significantly affected by the addition of a relatively high concentration of compound Aa (41.0 μmol/L), suggesting that our compounds act as scavengers of superoxide anions rather than inhibitors of xanthine oxidase.

A low concentration of $O_2^-$ in the human body generally plays a beneficial role in biological defenses and intercellular signal transduction.[5] On the other hand, excessive $O_2^-$ production is involved in the pathogenesis of a number of disorders, including inflammation, rheumatoid arthritis, and asthma.[32,33] Oxidative stress may be defined as an imbalance between the cellular production of reactive oxygen species, a key component of inflammation and inflammatory disorders, and antioxidant defense mechanisms.[5] The processes associated with inflammatory responses are complex and often involve the production of reactive oxygen species including $O_2^-$. In this study, our newly synthesized compounds acted as effective $O_2^-$ scavengers in vitro. Thus, these compounds may eliminate excessive $O_2^-$ and lead to suppression of reactive oxygen species overproduction.

Compound Aa and ebselen activated ERK1/2, although their effect was much weaker than that of NGF (Figure 2). Both compound Aa and ebselen suppressed $H_2O_2$-induced cytotoxicity (Figure 3), but this effect was inhibited by pretreatment with U0126. It was confirmed that activation of the MAPK cascade is required for suppression of cytotoxicity by compound Aa and ebselen. However, $H_2O_2$-induced cytotoxicity was not inhibited by NGF. As NGF is known to activate the MAPK cascade strongly,[34,35] it was suggested that activation of only the MAPK cascade was insufficient for suppression of $H_2O_2$-induced rapid cytotoxicity and another unknown factor is required.

It is known that serum deprivation induces apoptosis in PC12 cells.[25] Although NGF increased cell viability under serum-free cultivation, compound Aa and ebselen did not suppress serum deprivation-induced cell death. Recent studies suggested that upregulation of nuclear factor κB[36] or inhibition of p38 MAPK and Jun-amino-terminal kinase (JNK) activation[37] suppressed apoptosis induced by $H_2O_2$ in PC12 cells. Therefore, it might be that both compound Aa and ebselen can inhibit

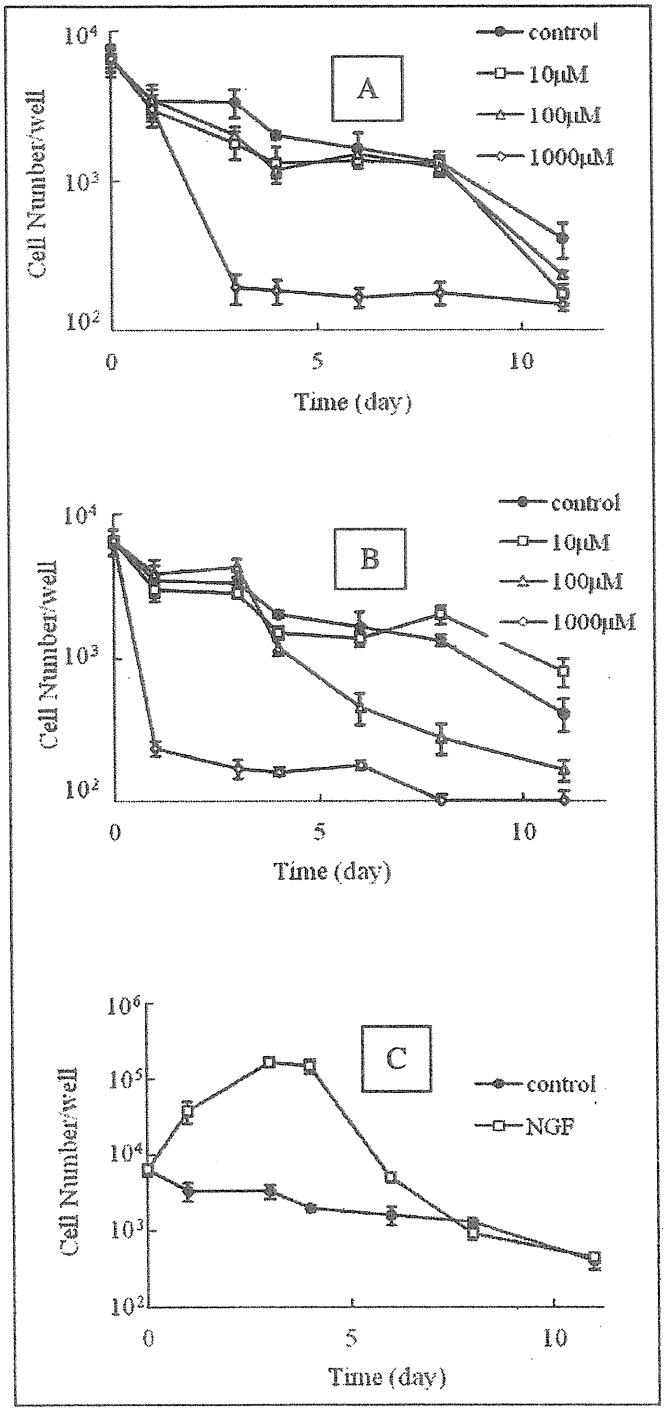



**Figure 4.** . Effects of cell viability of compound Aa, ebselen, or NGF on PC12 cells. The PC12 cells were cultured for several days in DMEM containing 1% BSA and various concentrations of compound Aa (A), ebselen (B), or NGF (C). The amount of cells in the well was measured by MTT assay as described in the text. Results shown are means ± SD (n = 3). NGF indicates nerve growth factor; DMEM, Dulbecco modified Eagle medium; BSA, bovine serum albumin; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; SD, standard deviation.

rapid apoptosis, such as that induced by $H_2O_2$. On the other hand, although NGF induced phosphorylation of Akt, one of a serine/threonine protein kinase that plays a key role in apoptosis inhibition, and JNK 2/3, the addition of compound Aa was irrelevant to the phosphorylation of Akt or JNK 2/3 (data not shown). From this, it was supposed that Aa suppressed cytotoxicity by a mechanism different to that of NGF.

It was suggested that $H_2O_2$ can induce cell death by apoptosis in PC12 cells.[38] In the present study, vesicles were formed and nucleus fragmentation was observed by the addition of Hoechst 33342 solution in the dead cells; therefore, $H_2O_2$ (400 μmol/L)-induced cell death in the cells may be apoptosis. On the other hand, serum deprivation induced apoptosis in PC12 cells.[25] Because $H_2O_2$-induced cell death was suppressed by Aa but serum deprivation-induced cell death was not, of the several types of apoptosis, Aa may suppress $H_2O_2$-induced apoptosis in PC12cells.

We examined whether Aa or ebselen (100 μmol/L) could react with $H_2O_2$ (400 μmol/L). After a 2-hour reaction, the concentration of $H_2O_2$ did not change, demonstrating Aa or ebselen did not directly react with $H_2O_2$. Next, we examined whether Aa or ebselen could react with $H_2O_2$ (400 μmol/L) in culture medium with PC12 cells. Pheochromocytoma cells were treated for 10 minutes with 100 μmol/L of Aa following which the cells were removed and washed with PBS. The cells were then exposed to 400 μmol/L of $H_2O_2$ for 2 hours. The $H_2O_2$ concentration of the supernatant was similar with or without Aa. To exclude the effects of serum in these experiments, a control group utilizing 1% BSA instead of serum was added to the DMEM. $H_2O_2$ was expected to decrease if Aa or ebselen showed GPX-like activity or if GPX was induced by the addition of Aa or ebselen. Since the presence or absence of Aa or ebselen did not affect the concentration of $H_2O_2$, Aa or ebselen was believed to be independent of GPX concentration or GPX-like activity in this study.

Oxidative stress is one of the common causal factors of neuronal cell death.[39] $H_2O_2$-induced cytotoxicity was clearly suppressed by compound Aa and the effect was more potent than that of ebselen. From the results of cytotoxicity measurements (Figure 4), it was confirmed that the toxicity of compound Aa was lower than that of ebselen. It appears that the toxicity of compound Aa will not hinder its usage as a drug, and its clinical application against oxidative stress is hopeful.

Antioxidants such as ebselen regulate intercellular signaling via regulation of kinase and phosphatase.[19] In this study, we found that compound Aa showed superoxide anion-scavenging activity, induction of ERK1/2 phosphorylation, and suppression of $H_2O_2$-induced cell death. It has been suggested that $H_2O_2$-induced cell death is partially associated with ERK phosphorylation.[40–42] However, this mechanism may not be exactly known at present. To address these relationships, further studies may be needed.

On the other hand, NGF did not suppress $H_2O_2$-induced cell death, although ERK1/2 proteins in the PC12 cells were phosphorylated, and serum deprivation-induced apoptosis

was suppressed. Thus, it was assumed that compound Aa activated another pathway in addition to the MAPK cascade. Both compound Aa and NGF inhibited cell death. However, since the mechanisms were different, complementary activity could be expected using both together. Moreover, Aa might be able to cross the blood–brain barrier because of its low molecular weight compared with NGF. However, the potential of compound Aa to prevent and treat some neurological disorders seems to be high enough to warrant future evaluation because the promotion of morphological differentiation was not observed in PC12 cells with the addition of Aa.

In this study, we found that 3-(2,6-dimethylphenyl)-2-selenoxo-1,3-thiazolidin-4-one, compound Aa, had the highest superoxide anion-scavenging activity among the related compounds tested. Furthermore, it was clarified that compound Aa activated ERK1/2 of cultured PC12 cells and suppressed $H_2O_2$-induced cytotoxicity. However, the cytotoxicity of compound Aa was lower than that of ebselen, an analogous active selenium-containing compound. Compound Aa has interesting medical potential to protect against and treat some neurological disorders and may even be effective in treating depression induced by stress.

## Authors Note

Abbreviations used: NMR, nuclear magnetic resonance; CI, chemical ionization; Ph3P, triphenylphosphane; IR, infrared spectroscopy; ESI, electro spray ionization.

## Declaration of Conflicting Interests

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Research Article

# Effects of respiratory syncytial virus infection and major basic protein derived from eosinophils in pulmonary alveolar epithelial cells (A549)

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## Abstract

RSV (respiratory syncytial virus)-induced pneumonia and bronchiolitis may be associated with hyperresponsive conditions, including asthma. Eosinophilic proteins such as MBP (major basic protein) may also be associated with the pathophysiology of asthma. To elucidate the roles of RSV infection and MBP in the pathogenesis of pneumonia with hyperresponsiveness, we investigated the effects of RSV infection and MBP on A549 (alveolar epithelial) cells. CPE (cytopathic effects) in A549 cells were observed by microscopy. Apoptosis and cell death was evaluated by flow cytometric analysis and modified MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. We also measured 15 types of cytokines and chemokines in A549 cell supernatants. Although RSV alone did not affect the CPE of A549, high concentrations of MBP resulted in cell death within 24 h. Combinations of RSV and MBP synergistically induced cell death. In A549 cells infected with RSV alone, the release of GM-CSF (granulocyte-macrophage colony-stimulating factor) was significantly enhanced compared with control cells (no infection). In the cells treated with MBP alone, the production of IL (interleukin)-2, 4, 5, 7, 10, 12, 13, 17, IFN (interferon)-$\gamma$, GM-CSF, G-CSF (granulocyte colony-stimulating factor) and MIP (macrophage inflammatory protein)-1$\beta$ was significantly increased compared with control cells. Notably, the levels of GM-CSF and IL-17 in RSV/MBP-treated cells were significantly higher than those treated with MBP alone. These results suggest that MBP synergistically enhanced the release of various cytokines/chemokines and the cell death of RSV-infected A549 cells, indicating that MBP may be closely associated with the pathophysiology of allergic reactions in bronchiolitis/pneumonia due to RSV.

Keywords: cytokine; eosinophil; hyperresponsiveness; major basic protein; respiratory syncytial virus

## 1. Introduction

RSV (respiratory syncytial virus) of the genus *Pneumovirus* and the family Paramyxoviridae is a major agent of lower respiratory tract infections, including bronchiolitis and bronchopneumonia, in children (Domachowske and Rosenberg, 1999). RSV-induced bronchiolitis and bronchopneumonia may also involve hyperresponsiveness of the airway (Larouch et al., 2000). Furthermore, epidemiological studies suggest that RSV infections may act as a trigger of asthma or may exacerbate symptoms in already asthmatic children (Stein et al., 1999; Sigurs et al., 2000).

EOSs (eosinophils) are proinflammatory granulocytes, as are neutrophils and macrophages (Savani and Sharma, 2002). In addition, it is thought that EOSs are strongly related to allergic diseases such as asthma, allergic rhinitis and atopic dermatitis (Schwarze et al., 1999; Gleich, 2000). EOSs release toxic granules and ROS (reactive oxygen species) when activated by immunoglobulins, cytokines (Ide et al., 1994) and lipid mediators (Kato et al., 2002; Busse and Kraft, 2005). The granule proteins in EOS mainly consist of MBP (major basic protein), ECP (eosinophil cationic protein), EDN (eosinophil-derived neurotoxin) and EPO (eosinophil peroxidase). In particular, MBP and EPO are reportedly toxic in airway epithelial cells, which can lead to airway injury (Ayars et al., 1985; Motojima et al., 1989; Kita et al., 1995). At present, the roles of these proteins in RSV airway infection are not fully understood.

In general, virus-stimulated immunological cells (T cells and macrophages) and virus-infected airway cells/tissues produce

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**Abbreviations:** A549, alveolar epithelial; ATCC, American Type Culture Collection; CPE, cytopathic effects; DMEM, Dulbecco's modified Eagle's medium; EOSs, eosinophils; EPO, eosinophil peroxidase; F, fusion; FBS, fetal bovine serum; G, glycoprotein; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; MBP, major basic protein; MIP, macrophage inflammatory protein; MOI, multiplicities of infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; ROS, reactive oxygen species; RSV, respiratory syncytial virus; SH, small hydrophobic; TNF, tumour necrosis factor; UV-RSV, UV irradiation of RSV.

many types of cytokines/chemokines, and these immunological responders may induce inflammatory responses *in vivo* (Kimpen et al., 1996; Schwarze et al., 1997; Olszewska-Pazdrak et al., 1998; Kato et al., 2005). The surface of RSV particles contains some major antigens, such as F (fusion) protein, G (glycoprotein) and SH (small hydrophobic) proteins (Collins and Crowe, 2007). Interestingly, G and SH proteins can activate Th2 immunity, while F protein activates Th1 immunity (Hussell et al., 1997a; Tripp et al., 1999). Indeed, G protein is associated with the production of Th2 cytokines such as IL (interleukin)-4 and IL-5 (Hancock et al., 1996; Hussell et al., 1997a, 1997b). Dominant Th2 immunity may induce allergic responses (Mosmann and Coffman, 1989; Romagnani et al., 2005). Thus, RSV infection might induce allergic reactions in the respiratory system in humans (Stein et al., 1999; Sigurs et al., 2000). Given these findings, it is important to investigate RSV infection in relation to toxic proteins (particularly MBP) in EOSs and cytokines/chemokines, since their effects in airway epithelial cells are as yet unknown. Therefore, to elucidate the roles of RSV, MBP and cytokines/chemokines in the pathogenesis of airway epithelial cells, we investigated the effects of MBP on RSV-infected pulmonary A549 (alveolar epithelial) cells.

# 2. Materials and methods

## 2.1. Cell line and cell culture

The human type II pulmonary alveolar epithelial cell line A549 [ATCC (American Type Culture Collection) CCL-185] was obtained from the ATCC. The cells were maintained in DMEM (Dulbecco's modified Eagle's medium) (Nissui Pharmaceutical) containing 10% FBS (fetal bovine serum), 10 mM L-glutamine and 40 μg/ml gentamicin. The medium was adjusted to pH 7.6 using Meylon (7.5% $NaHCO_3$ solution, Otsuka Pharmaceuticals). The A549 cells were cultured on 96-well microtitre culture plates (Corning) at 37°C in a 5% $CO_2$ in air atmosphere.

## 2.2. Purification of RSV

RSV (long strain, ATCC VR-26) obtained from the ATCC, was propagated in monolayers of HEp-2 cells (ATCC CCL-23TM). HEp-2 cells were maintained in DMEM supplemented with 10% FBS. At maximum CPE (cytopathic effect), the suspension was centrifuged at 1400 *g* for 30 min at 4°C to remove debris. The supernatants were layered over 50% sucrose in PBS (Sigma) and centrifuged at 65000 *g* for 2 h at 4°C, as previously reported with slight modifications (Ueba, 1978). Aliquots of purified RSV were stored at −80°C until required. The viral titre of the pooled purified RSV was $2.0 \times 10^8$ $TCID_{50}$ (50% of tissue culture infectious dose)/0.1 ml. Full inactivation of RSV was achieved by exposing a sterile glass vial containing viral suspension to an UV light source (302 nm) for 16 h at 4°C.

## 2.3. Purification of MBP

EOSs were obtained from patients with marked eosinophilia by cytapheresis, lysed in 0.25 M sucrose and the granules were isolated by centrifugation, as previously described (Slifman et al., 1986; Abu-Ghazaleh et al., 1992; Gleich, 2000). Written informed consent was obtained from the patient with regard to the usage of eosinophil-derived proteins in *in vitro* studies. Briefly, following cytapheresis, cells were centrifuged at 600 *g* for 10 min, and the pellet was washed once in PBS. Erythrocytes were lysed by suspending the cells in 0.14 M $NH_4Cl$, 10 mM Tris/HCl, pH 7.2 for 10 min at 37°C. EOSs were recovered by centrifugation and lysed in 0.25 M sucrose containing 250 units/ml heparin by repeated passage through a needle attached to a syringe. EOS lysates were sedimented at 600 *g* for 10 min to remove unbroken cells and aggregated materials, and granules were pelleted by centrifugation at 10000 *g* for 10 min. MBP-containing fractions from the third peak from a Sephadex G-50 column (Pharmacia) were pooled and concentrated using an Amicon YM-5 Diaflow membrane (Amicon), then applied to a second Sephadex G-50 column equilibrated with 0.025 M sodium acetate and 0.15 M NaCl, pH 4.3 (acetate buffer) at 4°C, as previously described (Slifman et al., 1986). The eluate was monitored for MBP concentration by absorbance at 277 nm, and fractions were frozen individually at −80°C (Ten et al., 1989). The protein purity was judged by its banding pattern on SDS/PAGE after staining with Coomassie Brilliant Blue (NuPAGE 12% Bis-Tris and Colloidal Blue, Invitrogen) (Kita et al., 1995; Shenoy et al. 2003). The banding pattern was single, and the concentration was 2.4 mg/ml.

## 2.4. RSV infection and treatment of MBP in A549 cells

A549 cells were seeded into 96-well microplates at $2.0 \times 10^4$ cells/well (100 μl/well). The plates were cultured at 37°C in an air atmosphere containing 5% $CO_2$. After 48 h of incubation, the cells were rinsed with PBS and then infected with purified RSV at 0.1, 1.0 and 10 MOI (multiplicities of infection). Cells were incubated for 24 h at 37°C with 5% $CO_2$ in the presence or absence (medium alone) of each concentration of RSV. The RSV-infected cells or medium-treated cells were further treated with MBP or control medium for 24 h. The concentrations of MBP were prepared in the range of 0.4 to 50 μg/ml by the 2-fold dilution method using DMEM with 2% FBS. After treatment with MBP, the cells were cultured at 37°C at 5% $CO_2$, and morphological changes and CPE in the cells were observed by light microscopy.

## 2.5. Cell viability

Cell viability was determined using the Cell Counting Kit-8 (Dojin Laboratories) based on a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Briefly, A549 cells were cultured according to the abovementioned method. The cells were infected with RSV at MOI of 0.1, 1.0 and 10 or were treated with medium alone (control). After 24 h, the cells were further treated with MBP at the abovementioned concentrations or with control medium and cultured for 24 h. Control cells were incubated with medium alone for 48 h. After incubation, 10 μl of Cell Counting Kit-8 solution was added to each well of the microtitre plates. Plates were incubated for up to 4 h under the aforementioned conditions. Absorption of

the samples was measured using the microplate reader SpectraMax 190 (Molecular Devices) at a wavelength of 450 nm, reference 650 nm.

## 2.6. Flow cytometric analysis

Using cell culture six-well cluster plates, 2 ml of A549 cells were suspended into each well ($2.0 \times 10^4$ cells/0.1 ml). Cells were cultured at 37°C in an atmosphere containing 5% $CO_2$. After 72 h of incubation, cells were rinsed with PBS and infected with RSV at 1.0 MOI. As before, both RSV-infected cells and non-infected cells were incubated for 24 h and then treated with MBP (4 μg/ml) or control medium. After 24 h of incubation, the cells were treated with 0.1% trypsin in PBS. Detached cells were centrifuged at 700 ***g*** for 5 min at 4°C,and then suspended with a binding buffer adjusted to between $5.0 \times 10^5$ and $5.0 \times 10^6$ cells/ml in an ice bath. Aliquots of 5 μl of FITC-labelled annexin V solution and 5 μl of PI (propidium iodide) solution were added to each 490 μl of cell suspension and incubated on ice in the dark for 10 min. Sample tubes were kept on ice for flow cytometric analysis (Epics Excel II, Beckman Coulter) (Tachibana et al., 2002).

## 2.7. Profiles of cytokines and chemokines

The concentrations of 15 types of cytokines and chemokines in the cultured supernatants of A549 cells were measured. Cytokine and chemokine concentrations of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 (p70), IL-13, IL-17, G-CSF (granulocyte colony-stimulating factor), GM-CSF (granulocyte–macrophage colony-stimulating factor), IFN (interferon)-γ, MIP (macrophage inflammatory protein)-1β and TNF (tumour necrosis factor)-α were determined using the premixed Bio-Plex multiplex human cytokine assay and Bio-Plex cytokine reagent kits (Bio-Rad Laboratories), according to the manufacturer's protocols. Both RSV-infected cells at 1.0 MOI and non-infected cells were incubated for 24 h and treated with 50 μg/ml MBP or control medium. The cell culture supernatant (50 μl) was measured for the cytokines and chemokines using a highly sensitive fluorescence microsphere system (Biosource) (Fulton et al., 1997). Data from the reaction were then acquired and analysed using the Bio-Plex Suspension Array system and the Bio-Plex Manager software (Bio-Rad Laboratories). The Bio-Plex suspension array system detects and measures molecules bound to the surfaces of fluorescent microspheres, providing highly accurate, real-time digital analysis of serum or culture media samples. We calculated the lower limit of detection for each cytokine/chemokine as 0.20 to 2.6 (pg/ml) using the software (Okazaki et al., 2008).

## 2.8. Statistical analysis

Data from the modified MTT assay and cytokine and chemokine assay were expressed as means ± S.E.M. The modified MTT assay was analysed using Student's *t* test. Non-parametric Mann–Whitney statistical analysis was used to test for significant differences in the cytokine and chemokine concentrations within each group. A *P*-value of $<0.05$ was considered statistically significant.

# 3. Results

## 3.1. Morphological changes in A549 cells

We observed morphological changes or CPE in the A549 cells infected with RSV and/or treated with MBP at several concentrations by light microscopy. RSV-infected cells alone at 0.1 or 1.0 MOI showed no obvious morphological changes or CPE compared with control cells within 48 h of incubation (Figures 1A–1C). In contrast, RSV-infected cells at 10 MOI showed CPE within 48 h of incubation (Figure 1D). Furthermore, we observed morphological changes, in the form of cell-free spaces, in cells treated with MBP alone. These changes were clearly apparent at high concentrations (>25 μg/ml) within 24 h of incubation and after the addition of MBP (Figure 1E). Cells treated with a combination of RSV and MBP also showed morphological changes within 48 h of RSV infection (Figure 1F). This effect was abolished by UV-RSV (UV irradiation of RSV) (Figures 1G–1I). The results suggested that the morphological changes were induced by infective RSV and/or MBP.

## 3.2. Analysis of cell viability

We assessed the viability of A549 cells infected with RSV and/or treated with MBP using the modified MTT method. The viability of A549 cells as the control for each treatment regimen: MBP-treated alone, combined RSV-infected and MBP-treated and combined UV-RSV-infected and MBP-treated, are shown (Figure 2). There were no significant decreases in cell viability for both RSV infection alone and UV-RSV alone at 0.1, 1.0 and 10 MOI, with viability greater than 90% (data not shown). In contrast, viability was significantly decreased in the cells treated with MBP alone, compared with control cells (Figure 2,A). Further, the viability of RSV-infected cells in the presence of MBP at each concentration decreased more significantly than in MBP-treated alone cells, in an RSV dose-dependent manner (Figures 2,B–D). The decrease in viability of UV-RSV-treated cells in the presence of MBP was not significantly different from that of the cells treated with MBP alone (Figure 2,E). Under the present experimental conditions, a high dose (50 μg/ml) of MBP resulted in toxicity in the cells, and RSV-infected cells were more vulnerable.

## 3.3. Flow cytometric analysis

We confirmed apoptosis in A549 cells exposed to RSV infection and/or MBP treatment by FITC-annexin V and PI flow cytometric analysis. The upper right or lower right quadrant mainly represents the necrotic/late-stage apoptotic cells or early stage of apoptotic cells, respectively, which are positive for annexin V binding and PI staining (Figure 3). Although control cells represented 2.89% of apoptotic cells (Figure 3A), in the presence of RSV alone (Figure 3B) or MBP alone (Figure 3C), there was almost a 2-fold increase in apoptotic and/or necrotic cells (5.31 and 5.27%, respectively). In contrast, combined RSV-infected and MBP-treated cells (Figure 3D) showed a marked increase in the number of late-stage apoptotic cells (14.7%). No significant

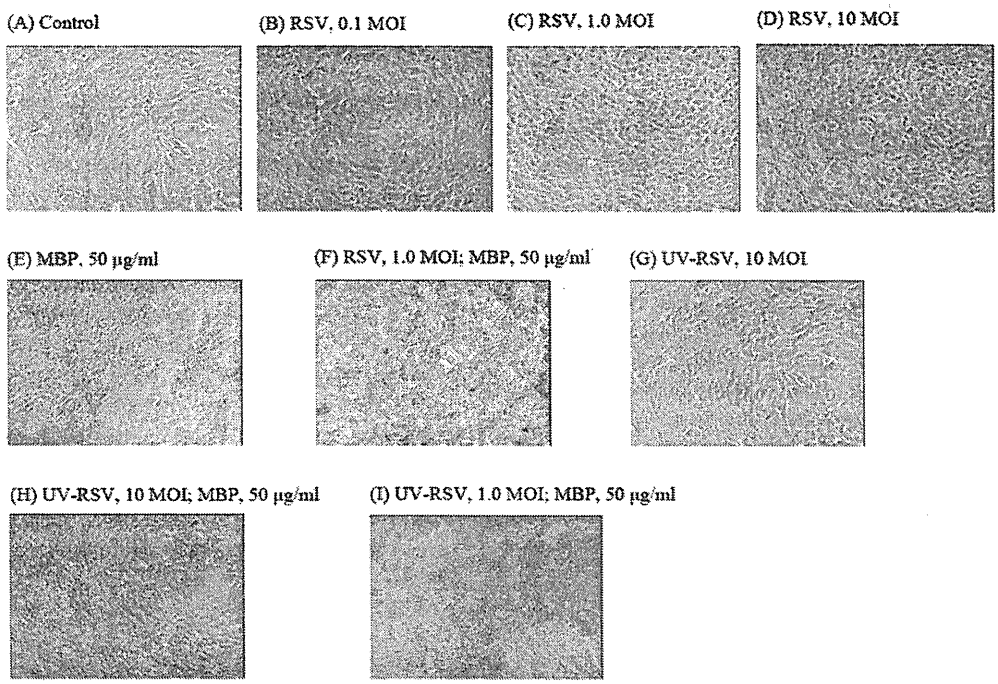


**Figure 1 Effect of MBP on RSV-infected A549 cells**

Neither RSV-infected nor MBP-treated control cells (**A**). RSV-infected cells at 0.1 (**B**), 1.0 (**C**) and 10 (**D**) MOI incubated for 48 h. Neither RSV-infected nor MBP-treated cells incubated by maintenance medium for 24 h, then treated with 50 μg/ml MBP for 24 h (**E**). RSV-infected cells at 1.0 MOI incubated for 24 h, then treated with 50 μg/ml MBP for 24 h (**F**). UV-RSV at 10 MOI was incubated for 24 h, then treated without (**G**) or with (**H**) 50 μg/ml MBP for 24 h; UV-RSV at 1 MOI was incubated for 24 h, then treated with 50 μg/ml MBP for 24 h (**I**). Experiments were performed on four separate days. Scale bar represents 200 μm.

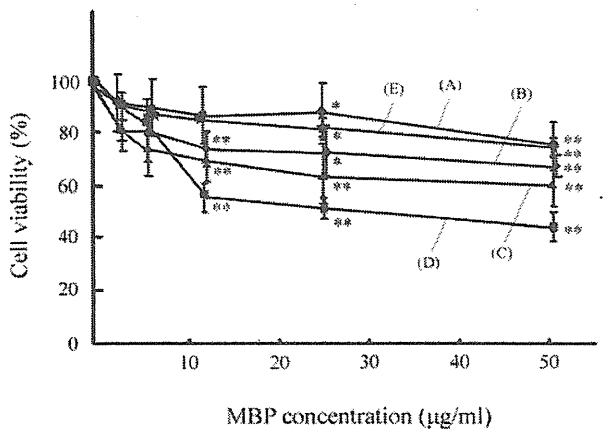


**Figure 2 Effects of RSV and/or MBP on A549 cell viability by modified MTT method**

A combination of RSV infection and MBP treatment markedly decreased the viability of A549 cells. MBP-treated alone at 0.4, 0.8, 1.6, 3.1, 6.3, 12.5, 25, 50 μg/ml (A). RSV-infected cells at 0.1, 1.0 and 10 MOI for 24 h, followed by MBP-treated at 0.4, 0.8, 1.6, 3.1, 6.3, 12.5, 25, 50 μg/ml for 24 h (B, C and D, respectively); UV-RSV-infected cells at 10 MOI for 24 h, followed by MBP treatment at 0.4, 0.8, 1.6, 3.1, 6.3, 12.5, 25, 50 μg/ml for 24 h (E). Graphic data of MBP concentration at 0.4, 0.8 and 1.6 μg/ml were omitted due to no significant differences. Data are expressed as means ± S.E.M. ($n$=4). Significant differences compared with controls are indicated by *$P$<0.05, **$P$<0.01.

differences in the percentages of early apoptotic cells were found in these experiments. These results suggested that RSV and MBP induced cell death by apoptosis, and their effects were synergistic.

## 3.4. Cytokine and chemokine release profiles of A549 cells with RSV and/or MBP

To investigate the inflammatory response induced by RSV infection and/or MBP treatment in A549 cells, we examined 15 types of cytokines and chemokines secreted from the A549 cells using the Bio-Plex Multiplex Cytokine Assay Kit. The cytokine/chemokine concentrations in the supernatant from A549 cells are shown (Figure 4). The release of GM-CSF from cells infected by RSV alone was significantly greater compared with that of control cells (neither RSV infection nor MBP treatment), and the release of GM-CSF and IL-17 from the combination-treated cells was significantly greater than from cells treated with MBP alone. The release of many types of cytokines (IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-17, IFN-γ, GM-CSF and G-CSF) and a chemokine (MIP-1β) from cells treated with MBP alone was significantly greater compared with that of control cells. On the other hand, the release of IL-6 from cells infected with RSV alone, MBP alone and from RSV-infected/MBP-treated cells was significantly lower than from control cells. These findings suggested that MBP synergistically enhanced the release of various cytokines/chemokines in RSV-infected A549 cells.

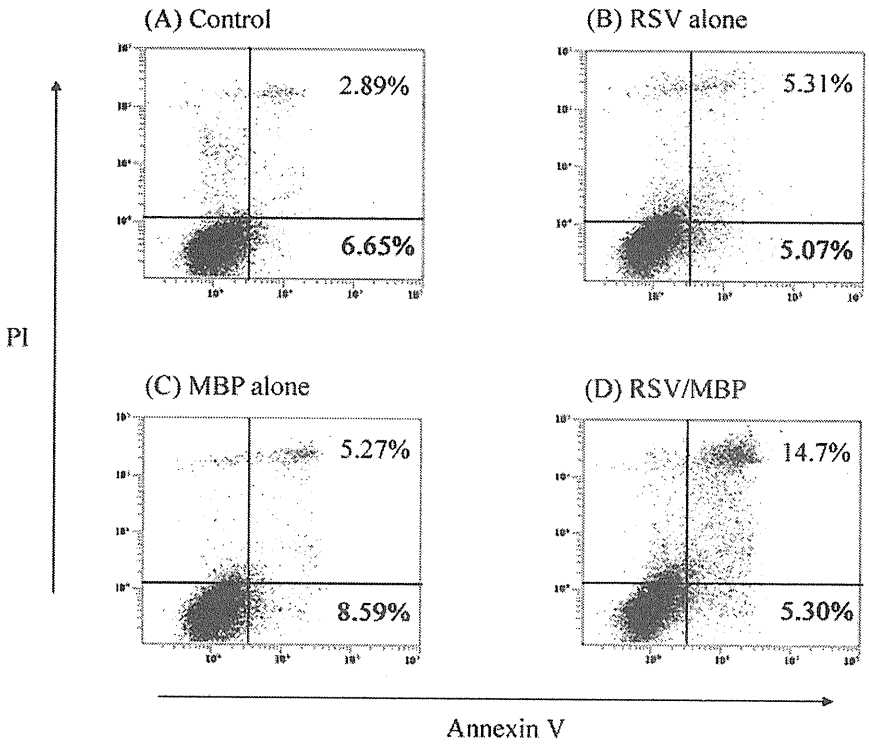


**Figure 3** **RSV enhances MBP-induced apoptosis in A549 cells revealed by flow cytometry**
Neither RSV-infected nor MBP-treated control cells incubated by maintenance medium for 48 h (**A**). RSV-infected cells at 1.0 MOI incubated for 48 h (**B**). Neither RSV-infected nor MBP-treated cells incubated by maintenance medium for 24 h, then MBP-treated at 4 μg/ml for 24 h (**C**). RSV-infected cells at 1.0 MOI for 24 h, then MBP-treated at 4 μg/ml for 24 h (**D**). For each sample, 10000 cells were analysed ($n$=2). Bold letters indicate the percentages of cells in the early stages of apoptosis.

# 4. Discussion

RSV-infected and MBP-treated cells showed remarkable morphological changes compared with those treated with MBP alone, whereas RSV-infected alone did not show obvious morphological changes (Kotelkin et al., 2003) (Figure 1). The analysis of cell death and apoptosis indicated that synergistic effects occurred in combined RSV-infected and MBP-treated cells. The release of GM-CSF (a Th2 cytokine) from cells infected with RSV alone was significantly greater than from control cells (neither RSV infection nor treatment of MBP), whereas the release of many types of cytokines (IL-2, IL-4, IL-5, IL-7, IL-10, IL-12, IL-13, IL-17, IFN-γ, GM-CSF and G-CSF) and a chemokine (MIP-1β) from cells treated with MBP alone was significantly greater than from control cells. Furthermore, the release of GM-CSF and IL-17 from the combination-treated cells (RSV-infected and MBP-treated) was significantly greater than from cells treated with MBP alone (Figure 4). These results indicate that MBP synergistically enhanced the release of various cytokines/chemokines and apoptosis in RSV-infected A549 cells.

Pneumonia or bronchopneumonia due to RSV manifests not only as symptoms of common viral pneumonia, but also as asthmatic symptoms such as wheezing (Domachowske and Rosenberg, 1999; Larouch et al., 2000; Yang et al., 2008). RSV infections may be associated with the trigger for and/or exacerbation of airway hyperresponsiveness, including asthma (Stein et al., 1999; Sigurs et al., 2000). Such disease is called 'infective asthma', and it is recognized by paediatricians as a refractory disease (McIntosh et al., 1973). Although the pathophysiology of RSV-related lower respiratory infections with hyperresponsiveness is not exactly known, properties of RSV antigens (Hussell et al., 1997a; Tripp et al., 1999), RSV infection-induced cytokines/chemokines (Patel et al., 1998; Arnold and Konig, 2006) and EOS functions (Kimpen et al., 1996; Kato et al., 2005) may be involved in the pathogenesis. Thus, we conducted the present study to address these relationships.

RSV contains some major antigens, including F, G and SH proteins (Collins and Crowe, 2007). Interestingly, G and SH proteins directly stimulate Th2 immunity and produce several allergy-related cytokines such as IL-4 and IL-5 in mice (Hancock et al., 1996; Hussell et al., 1997a, 1997b; Tripp et al., 1999). It is suggested that allergy-related cytokines such as IL-4, IL-5, IL-13, IL-17 and GM-CSF are closely related to the pathophysiology of various allergic diseases including asthma (Soloperto et al., 1991; Broide et al., 1992; Oh et al., 1999). In particular, IL-17 is a newly described cytokine that was first identified as the human homologue of cytotoxic T lymphocyte-associated antigen 8 (Rouvier et al., 1993; Yao et al., 1995). IL-17 activates fibroblasts and macrophages for the secretion of GM-CSF, TNF-α, IL-1β and

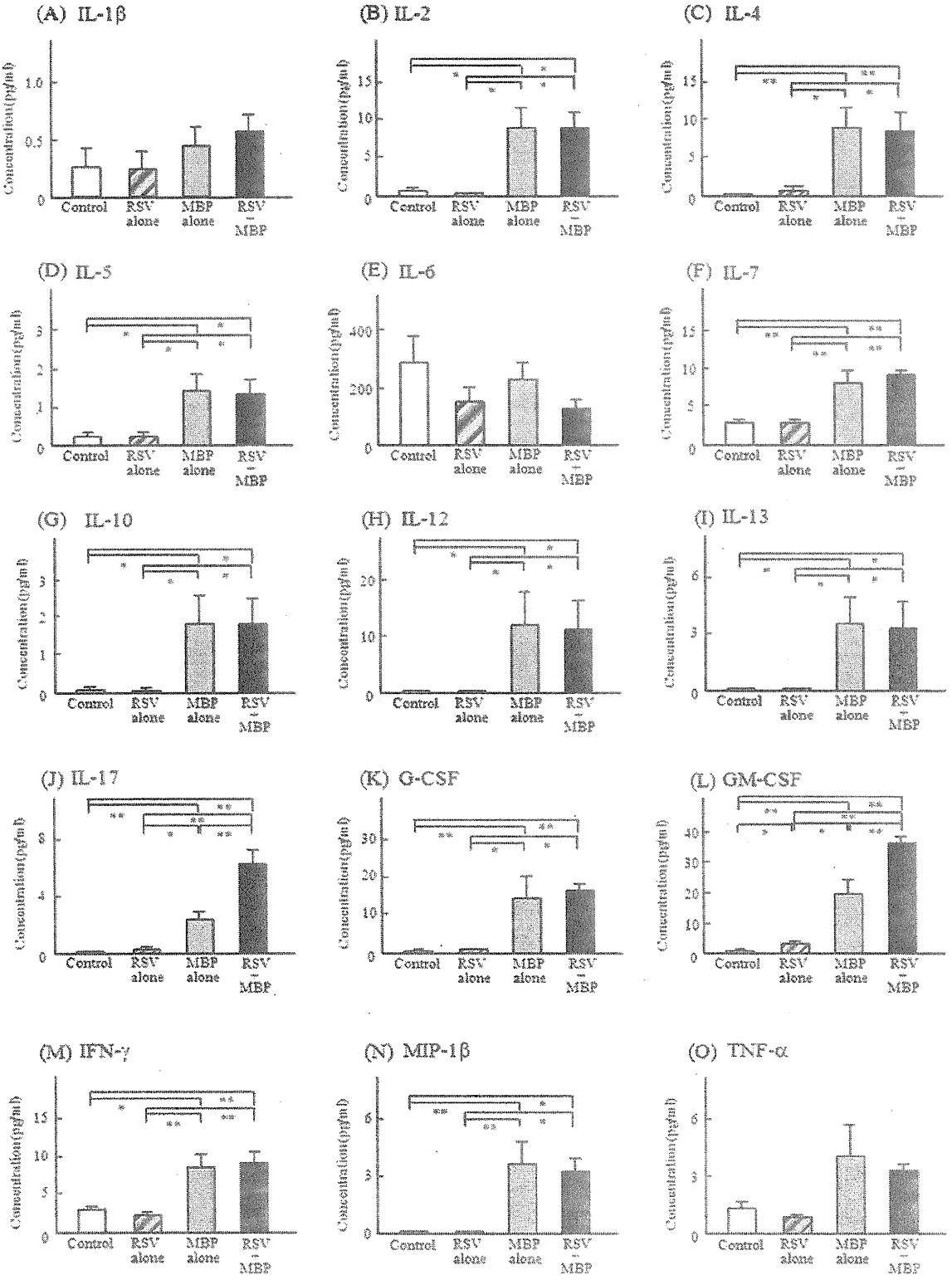


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**Figure 4** **Cytokine and chemokine production in RSV-infected and/or MBP-treated A549 cells**
Neither RSV-infected nor MBP-treated control cells incubated by maintenance medium for 48 h (□). RSV-infected cells at 1.0 MOI incubated for 48 h (▒). Neither RSV-infected nor MBP-treated cells incubated by maintenance medium for 24 h, then MBP-treated at 50 μg/ml for 24 h (■). RSV-infected cells at 1.0 MOI for 24 h, then MBP-treated at 50 μg/ml for 24 h (■). Data are expressed as means ± S.E.M. ($n$=6). Statistical significance is indicated by *$P$<0.05, **$P$<0.01.

IL-6 and many types of cytokines that are involved in the airway remodelling observed within the lungs of asthmatic individuals (Molet et al., 2001). Thus, IL-17 may be an important cytokine for the progress of allergic diseases (Molet et al., 2001; Yang et al., 2008). GM-CSF can activate EOS and enhances effector functions such as degranulation and superoxide production (Motojima et al., 1996; Rothenberg et al., 1988; Yamaguchi et al., 2008). In the present study, we evaluated the release of various cytokines from A549 cells treated with MBP and/or infected with RSV. The results showed that GM-CSF was extensively released from A 549 cells, even in those infected with RSV alone. In addition, treatment with MBP promoted a greater release of inflammatory- and/or allergy-related cytokines in RSV-infected A549 cells, suggesting that RSV infection and treatment with MBP led to dominant production of allergy-related cytokines from pulmonary A549 cells. In general, a dominant production of allergy-related cytokines is associated with various allergic diseases, including asthma (Soloperto et al., 1991; Ide et al., 1994; Oh et al., 1999). Based on these results, MBP may be associated with allergic reactions in patients with pneumonia due to RSV infection.

EOS is an important factor in the pathogenesis of various allergic diseases, such as atopic dermatitis and asthma (Gleich, 2000). Effector functions of EOS (degranulation and ROS generation) are reported to be strongly linked to the pathogenesis of airway hyperresponsiveness, including asthma (Ayars et al., 1985; Motojima et al., 1989; Kita et al., 1995). In fact, RSV infection in mice during allergic provocation aggravates the allergic Th2 immune response and inflammatory infiltrates and also increases the influx of EOS to lung tissue (Barends et al., 2004). These functions of EOS are activated by IL-5 and GM-CSF (Lemanske et al., 1989; Savani and Sharma, 2002). MBP (molecular weight; 13,800) is a major EOS granule protein and is toxic in various cells (Gleich et al., 1973). MBP, a cationic protein, adheres to negatively charged sites on the cell membrane, leading to disturbance of the structural arrangement of the phospholipid bilayer of the cells and resulting in cellular damage in various cells (Abu-Ghazaleh et al., 1992). Thus, MBP may be associated with the pathophysiology of airway hyperresponsiveness, including asthma. We first evaluated the cytotoxic effects of RSV and MBP to address these complicated factors. MBP induced excessive cell death in RSV-infected A549 cells. These responses of EOS and RSV-infected epithelial cells to each other might enhance airway inflammation.

In conclusion, RSV-infected pulmonary A549 cells were more vulnerable to MBP treatment, and MBP was synergistically inducible for some types of allergy-related cytokines, such as IL-4, IL-5, IL-13, IL-17 and GM-CSF. These cytokines may also activate EOS and induce the release of toxic proteins, such as MBP, leading to allergic inflammation in the airway. Thus, the pathophysiology of hyperresponsiveness in bronchiolitis or pneumonia due to RSV infection may be partially responsible.

## Author contribution

Taisei Ishioka, Hirokazu Kimura, Hiroo Hoshino, Kunihisa Kozawa and Masahiko Kato designed the research. Taisei Ishioka, Hirokazu Kimura, Hirohito Kita, Masatsugu Obuchi, Masahiro Noda and Masahiko Kato performed the research. Taisei Ishioka, Hirokazu Kimura, Hiroo Hoshino, Atsuyoshi Nishina and Masahiko Kato analysed the data. Taisei Ishioka, Hirokazu Kimura and Masahiko Kato wrote the paper.

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