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A novel kavalactone derivative protects against H_2O_2-induced PC12 cell death via Nrf2/ARE activation

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ABSTRACT

Oxidative stress is involved in the pathogenesis of neurodegenerative disorders such as Parkinson's and Alzheimer's diseases. Natural kavalactones isolated from *Piper methysticum* (Piperaceae) are capable of activating the Nrf2/ARE (antioxidant response element) pathway and thus enhancing the expression of phase II antioxidant enzymes such as heme oxygenase-1 (HO-1). In an attempt to identify kavalactone derivatives that are more potent in Nrf2/ARE activation than natural compounds, we synthesized a series of chemically-modified kavalactones and studied their effects on the ARE enhancer activity in rat pheochromocytoma PC12 cells. Among 81 compounds tested, a kavalactone derivative, 2′,6′-dichloro-5-methoxymethyl-5,6-dehydrokawain [(*E*)-6-(2′,6′-dichlorostyryl)-4-methoxy-5-(methoxymethyl)-2*H*-pyran-2-one] (**1**), exhibited the strongest ARE enhancer activity. The ARE activation and HO-1 protein induction by the compound **1** were higher than those by natural kavalactones. The compound did not affect cell viability and induced expression of various phase II enzymes. Nuclear translocation of Nrf2 after treatment with **1** was preceded by phosphorylation of ERK1/2 and p38. The compound transiently increased intracellular ROS levels. Finally, pretreatment with the compound ameliorated H_2O_2-induced cell death, which was associated with increased expression of HO-1. These results suggest that the compound **1** protects against oxidative stress-induced neuronal cell death via a preconditioning effect on the Nrf2/ARE activation.

1. Introduction

Oxidative stress is associated with a variety of diseases including neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease.[1] Pharmacological or nutritional intervention to reduce oxidative stress could potentially ameliorate oxidative stress-related diseases. Oxidative stress is defined as an imbalance between the cellular antioxidant defense systems and the endogenous or exogenous burden of pro-oxidants. Most of reactive oxygen species (ROS) are generated during respiration in mitochondria as by-products of electron transport and oxidative phosphorylation. At lower levels, ROS, especially H_2O_2, play physiological roles. Excess amounts of ROS, however, damage various bio-molecules including DNA, protein and lipid, thereby leading to cellular dysfunction and apoptosis. ROS are scavenged by endogenous antioxidant enzymes including glutathione peroxidase (GPX) and catalase. The cellular antioxidant, glutathione, scavenges ROS and also acts as a substrate for GPX. Glutathione is synthesized by the consecutive actions of γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase. On the other hand, heme oxygenase-1 (HO-1) catalyzes heme to biliverdin, which is then converted to bilirubin that acts as a potent antioxidant. Thus, induction of phase II enzymes such as GPX, γ-GCS and HO-1, has the potential to enhance the cellular antioxidant capacity and ameliorate oxidative stress-related diseases.

In addition to antioxidant enzymes, the phase II enzymes include detoxifying enzymes such as NADPH quinone oxidoreductase (NQO-1) and glutathione S-transferase (GST). In the promoter region of genes encoding for phase II enzymes, specific DNA enhancer sequences termed antioxidant response element (ARE) are present.[2] Transcription factor Nrf2 (nuclear factor E2-related factor 2) binds to and activates the ARE enhancer. Under normal conditions, Nrf2 associates with cytoplasmic Keap1 (kelch-like ECH-associated protein 1) and undergoes constitutive proteasome-dependent degradation. When cysteine sulfhydryl groups are covalently or oxidatively modified by electrophiles or ROS, Keap1 alters its conformation, resulting in Nrf2 release and its translocation into the nucleus. Phytochemicals such as curcumin, caffeic acid phenethyl ester (CAPE)

Abbreviations: ARE, antioxidant response element; HO-1, heme oxygenase-1; Nrf2, nuclear factor E2-related factor 2; Keap1, kelch-like ECH-associated protein 1; ROS, reactive oxygen species.

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and sulphoraphane activate the Nrf2/ARE system via interacting with Keap1.[3,4] Modification of Nrf2 phosphorylation by protein kinases such as Akt and ERK enhances its translocation into the nucleus.[5]

Piper methysticum popularly known as Kava is a plant that belongs to the pepper family (Piperaceae) and is distributed throughout the South Pacific. Kava beverage produced by extraction of roots with water has been traditionally used in social and ceremonial events for hundreds of years.[6] Kava beverage is still consumed by inhabitants of the South Pacific Islands. Kava extracts have anxiolytic, sedative and analgesic properties and kavalactones isolated from the extracts are responsible for these pharmacological effects. Six kavalactones including yangonin, kawain and methysticin account for most of those contained in kava extracts.[7] Medicines and supplements containing kava extracts had been sold worldwide, but they were withdrawn from European, Canadian and Japanese markets after kava-induced hepatotoxicity was reported.[8,9] The hepatotoxicity may be due to contamination with alkaloids caused by organic extraction of the raw material including stem peelings.[10] A recent randomized, placebo-controlled crossover trial, however, revealed that water extracts of kava produced significant anxiolytic and antidepressant activity and raised no safety concerns.[11]

It has been previously reported that administration of kavalactones ameliorates cerebral infarction in model mice.[12] In rat pheochromocytoma PC12 cells, a model of neuronal cells, natural kavalactones activated the Nrf2/ARE system and attenuated neuronal cell death caused by amyloid β.[13] In the present study, in an attempt to identify kavalactone derivatives that are more potent in Nrf2/ARE activation than natural compounds, we synthesized a series of chemically-modified kavalactones and studied their effects on the ARE enhancer activity in PC12 cells. For the compound that exhibited the strongest ARE enhancer activity, we examined molecular mechanisms underlying Nrf2/ARE activation by the compound and its neuroprotective effects on oxidative stress-induced neuronal cell death.

2. Results

2.1. Identification of kavalactone derivatives that activate the ARE enhancer

First of all, we performed 96-well format luciferase assays and found that 48 out of 81 kavalactone derivatives increase the ARE enhancer activity (data not shown). In 24-well format luciferase assays, the ARE activation was confirmed for 10 out of 48 derivatives (Supplementary Fig. 1). Among 10 derivatives, derivative 32 (hereafter designated as compound **1**) exhibited the most potent ARE enhancer activity. The compound **1** was a kavalactone derivative, which has a methoxymethyl group at the position of 5 and two chlorine groups at the positions of 2′ and 6′ added after dehydrogenation of the positions of 5 and 6 (2′,6′-dichloro-5-methoxymethyl-5,6-dehydrokawain)[(*E*)-6-(2′,6′-dichlorostyryl)-4-methoxy-5-(methoxymethyl)-2*H*-pyran-2-one] (Fig. 1).

2.2. ARE enhancer activation and HO-1 induction by the compound 1 are higher than those by natural kavalactones

Since it has been recently reported that natural kavalactones are capable of activating the ARE enhancer and thus up-regulating expression of phase II enzymes such as HO-1,[13] we compared the ARE enhancer activation and HO-1 induction by **1** (10 μM) with those by yangonin, kawain and methysticin (10 μM) (Fig. 1). The ARE enhancer activity of **1** was higher by about twofold than those of natural kavalactones (Fig. 2A). The ARE enhancer activities of

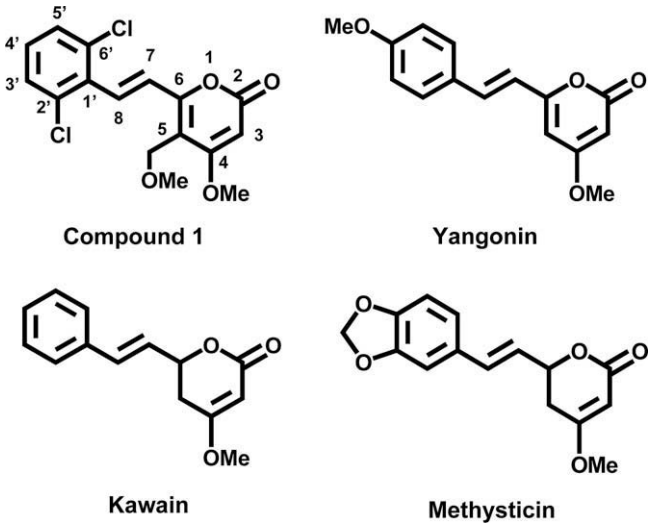

Figure 1. Chemical structures of the compound **1** and natural kavalactones. Structures of **1** and natural kavalactones (yangonin, kawain and methysticin) are shown.

natural kavalactones were relatively lower than those reported by Wruck et al.[13] which may be due to differences in experimental conditions between two studies such as cell sources, cell culture conditions and ARE reporter genes. Induction by the compound of HO-1, chosen as a representative of phase II enzymes, was about threefold higher than those by yangonin, kawain and methysticin (Fig. 2B). These results indicate that the **1** acquired ability to activate the ARE enhancer more strongly than kawain as well as yangonin and methysticin.

2.3. The compound 1 does not affect cell viability even at higher concentrations

We examined effects of **1** on cell viability by the WST-8 method. The compound **1** did not affect cell viability even at 100 μM (Fig. 3). However, morphology of cells treated with **1** at 50 μM or higher looked a little different from that of non-treated cells.

2.4. ARE enhancer activation by the compound 1 is dose-dependent

We determined the ARE enhancer activation by **1** at 2, 5, 10 and 20 μM, at which no apparent cytotoxicity was observed. As shown in Figure 4 and **1** increased the ARE enhancer activity by about twofold at 10 μM and also in a dose-dependent manner. Based on these findings, subsequent experiments were performed at 10 μM of the compound **1**.

2.5. The compound 1 induces expression of various phase II enzymes

We tested the temporal expression of phase II enzymes after treatment with **1**. The compound **1** gradually increased the expression of HO-1 starting at 3 hr after treatment, while induction of γ-GCSc and NQO-1 was first seen 12 h after treatment (Fig. 5). Maximum induction of HO-1, γ-GCSc and NQO-1 was seen 24 h after treatment (2.0-, 2.0- and 1.3-fold, respectively).

2.6. The compound 1 facilitates nuclear translocation of Nrf2

After nuclear translocation, Nrf2 activates the ARE enhancer present in the promoter region of genes encoding phase II

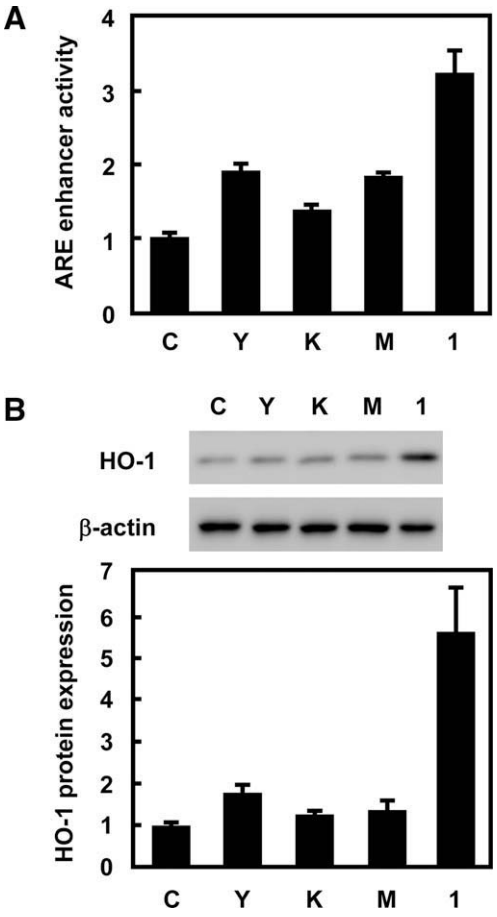

Figure 2. Comparison of the ARE enhancer activation and HO-1 induction by the compound **1** and natural kavalactones. A. PC12 cells transfected with the ARE firefly luciferase reporter vector and Renilla luciferase control vector were treated with 10 μM yangonin (Y), kawain (K), methysticin (M) or **1** for 24 h followed by luciferase assays. The firefly luciferase activity was normalized to the Renilla luciferase activity. The ARE enhancer activity was shown as a ratio to the DMSO control (C). Data are expressed as mean ± SEM (n = 6). B. PC12 cells were treated with 10 μM yangonin, kawain, methysticin or **1** for 24 h. Then, cell lysates were prepared and subjected to Western blot analysis for HO-1 and β-actin proteins. The HO-1 expression was normalized to β-actin expression and shown as a ratio to the DMSO control. Data are expressed as mean ± SEM (n = 4).

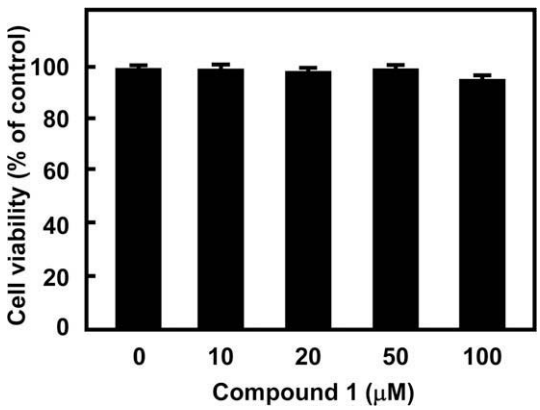

Figure 3. Effects of the compound **1** on cell viability. PC12 cells were treated with **1** at indicated concentrations for 24 h. The number of living cells was determined by the WST-8 method. Cell viability was calculated as a ratio to the DMSO control. Data are expressed as mean ± SEM (n = 12).

enzymes. We isolated nuclear fractions and whole cell extracts after treatment with **1** and determined the Nrf2 levels. Translocation of Nrf2 to the nucleus was first seen 30 min after treatment, which lasted until 24 h (Fig. 6).

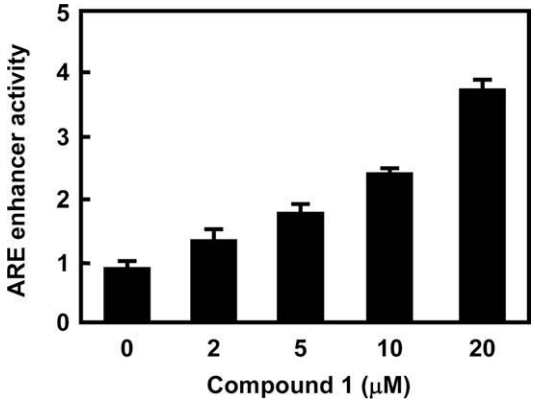

Figure 4. Dose-dependent effects of the compound **1** on the ARE enhancer activation. PC12 cells transfected with the ARE firefly luciferase reporter vector and Renilla luciferase control vector were treated for 24 h with **1** at indicated concentrations followed by luciferase assays. The firefly luciferase activity was normalized to the Renilla luciferase activity. The ARE enhancer activity was shown as a ratio to the DMSO control. Data are expressed as mean ± SEM (n = 5).

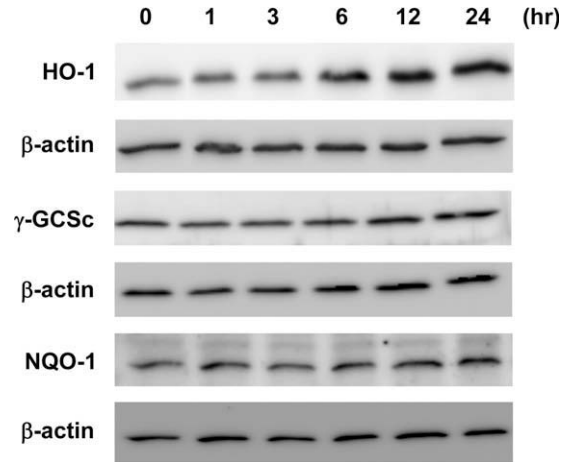

Figure 5. Time-dependent expression of phase II enzymes by the compound **1**. At indicated times after treatment with **1** (10 μM), cell lysates were prepared from PC12 cells and subjected to Western blot analysis for HO-1, γ-GCSc, NQO-1 and β-actin. A representative blot from three independent experiments is shown.

2.7. The compound 1 activates signal transduction

Phosphorylation of Nrf2 potentiates its translocation into the nucleus. The compound **1** increased ERK1/2 phosphorylation

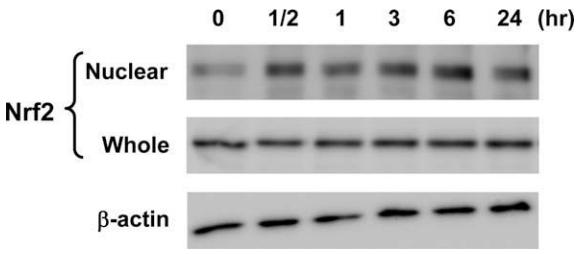

Figure 6. Effects of the compound **1** on nuclear translocation of Nrf2. At indicated times after treatment with **1** (10 μM), nuclear fractions and whole cell extracts were prepared from PC12 cells and subjected to Western blot analysis for Nrf2 and β-actin. A representative blot from three independent experiments is shown.

5 min after treatment, which then gradually diminished until it returned to the basal level 6 h later (Fig. 7A). Activation of p38 was transiently seen at 5 min after treatment. However, the levels of phosphorylated Akt were not changed by **1** (data not shown). In support of these findings, pretreatment for 1 h with MEK1/2 inhibitor, PD98059 (20 μM), and p38 inhibitor, SB203580 (5 μM), attenuated HO-1 protein induction 3 h after treatment with **1** (Fig. 7B), suggesting that transient activation of signal transduction in part contributes to the ARE activation and HO-1 induction by the compound **1**.

2.8. The compound 1 transiently increases the intracellular ROS level

Modification of the cysteine sulfhydryl groups of Keap1 by ROS changes its conformation, thus resulting in Nrf2 release and its translocation into the nucleus. Here we investigated whether **1** might increase the intracellular ROS level. The intracellular ROS level was markedly increased 1 h after treatment with **1**, which returned to the basal level at 3 h (Fig. 8A). In addition, pretreatment for 1 h with *N*-acetylcysteine (NAC; 2.5 mM), which replenishes intracellular glutathione levels, attenuated HO-1 induction by **1** 3 h after treatment, suggesting that transient ROS production is involved in the ARE activation by **1**.

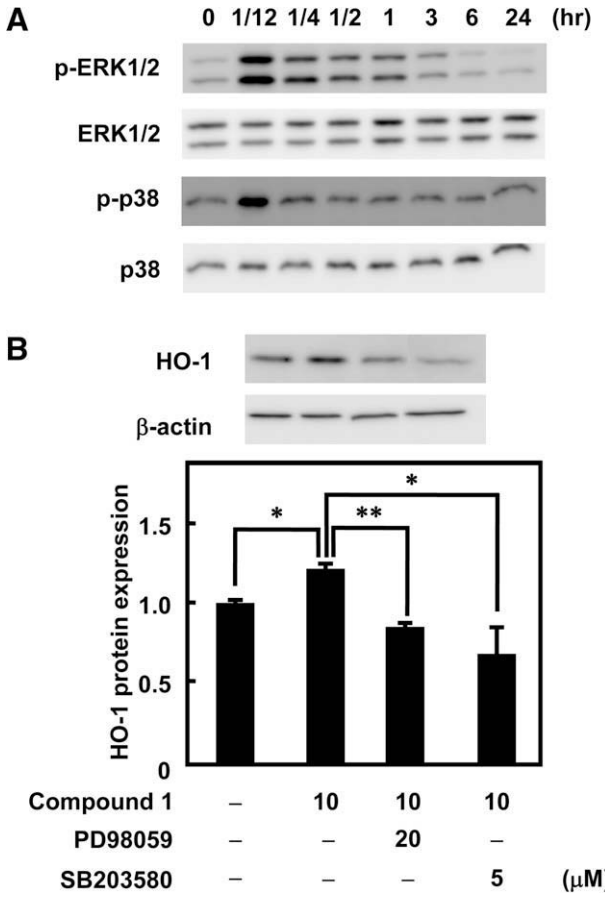

Figure 7. Effects of the compound **1** on signal transduction. (A) At indicated times after treatment with **1** (10 μM), cell lysates were prepared from PC12 cells and subjected to Western blot analysis for phosphorylated and total ERK1/2 and p38. A representative blot from three independent experiments is shown. (B) PC12 cells were pretreated for 1 h with PD98059 (20 μM) or SB203580 (5 μM) and then treated for 3 h with **1** (10 μM). Cell lysates were prepared and subjected to Western blot analysis for HO-1 and β-actin proteins. The HO-1 expression was normalized to β-actin expression and shown as a ratio to the DMSO control. Data are expressed as mean ± SEM ($n = 3$). Differences are statistically significant at $^{*}p < 0.05$, $^{**}p < 0.01$.

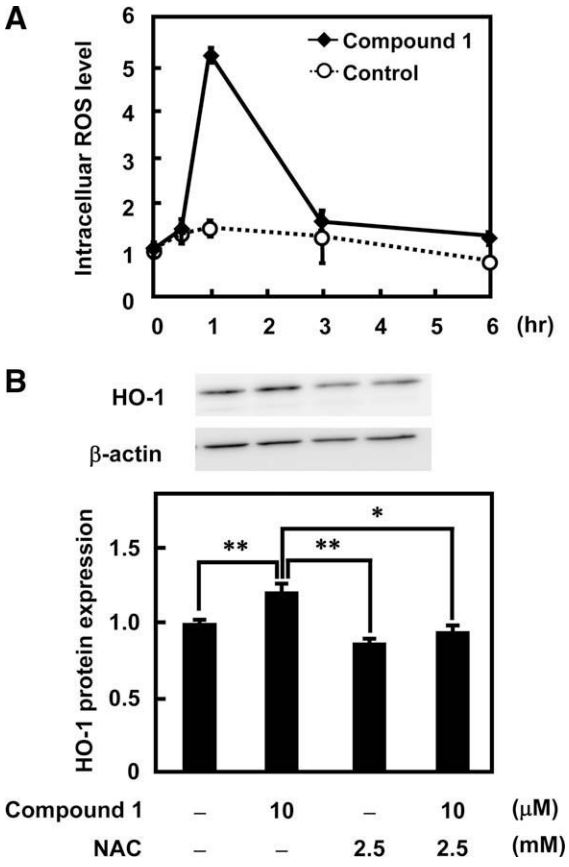

Figure 8. Effects of the compound **1** on the intracellular ROS level. (A) At indicated times after treatment with **1** (10 μM), PC12 cells were subjected to measurement for intracellular ROS levels by using the fluorescent probe CM-H_2DCFDA. The ROS levels were expressed as fluorescence per mg of protein. Data are expressed as mean ± SEM ($n = 6$). (B) PC12 cells were pretreated for 1 h with NAC (2.5 mM) and then treated for 3 h with **1** (10 μM). Cell lysates were prepared and subjected to Western blot analysis for HO-1 and β-actin proteins. The HO-1 expression was normalized to β-actin expression and shown as a ratio to the DMSO control. Data are expressed as mean ± SEM ($n = 3$). Differences are statistically significant at $^{*}p < 0.05$, $^{**}p < 0.01$.

2.9. The compound 1 protects against H_2O_2-induced neuronal cell death via a preconditioning effect on HO-1 induction

Since Nrf2/ARE activation by **1** is likely to enhance the antioxidant capacity, we tested if **1** could ameliorate oxidative stress-induced neuronal cell death. Pretreatment with **1** for 24 h significantly attenuated PC12 cell death induced by treatment with 100 μM H_2O_2 for 24 h (Fig. 9A). At the end of the above experiments, HO-1 expression in the remaining cells was analyzed. As shown in Figure 9B, the HO-1 expression level was higher in cells treated with both **1** and H_2O_2 than in those treated with H_2O_2 alone. These results suggest that **1** protects against oxidative stress-induced PC12 cell death via enhancing HO-1 expression, a preconditioning effect on HO-1 induction.

3. Discussion

In the present study, we first generated 81 distinct chemically-modified kavalactone derivatives by the reactions shown in Supplementary Figure 2. Modifications were made to natural kavalactones with hydroxyl or methoxyl groups including fluorine(s) or chlorine(s) as substituents. Then, we screened them for the ARE enhancer activity in PC12 neuronal cells and identified 10 derivatives as potent Nrf2/ARE activators (Supplementary Fig. 1).

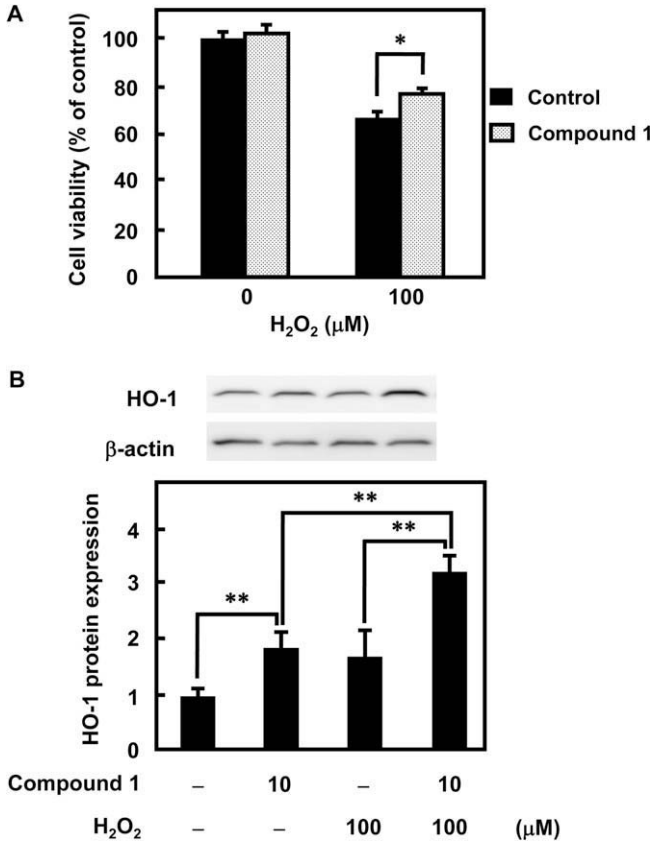

Figure 9. Neuroprotective effects of the compound **1**. (A) PC12 cells were pretreated for 24 h with or without **1** (10 μM) and then treated for 24 h with or without 100 μM H_2O_2. The number of living cells was determined by the WST-8 method. Cell viability was calculated as a ratio to **1**- and H_2O_2-free control. Data are expressed as mean ± SEM ($n = 24$). Differences are statistically significant at $^{*}p < 0.05$. (B) PC12 cells were pretreated for 24 h with or without **1** (10 μM) and then treated for 24 h with or without 100 μM H_2O_2. Cell lysates were prepared and subjected to Western blot analysis for HO-1 and β-actin proteins. The HO-1 expression was normalized to β-actin expression and shown as a ratio to **1**- and H_2O_2-free control. Data are expressed as mean ± SEM ($n = 3$). Differences are statistically significant at $^{**}p < 0.01$.

Intriguingly, they had fluorine or chlorine group(s), which suggests that addition of halogen groups may increase the ARE enhancer activity of kavalactones. Among 10 derivatives, the compound **1** exhibited the most potent ARE enhancer activity. The compound **1** was a kavalactone derivative having a methoxymethyl group at the position of 5 and two chlorine groups at the positions of 2′ and 6′ added after dehydrogenation at the positions of 5 and 6 (Fig. 1). As shown in Supplementary Figure 1, the derivative 81 differs from the compound **1** (which corresponds to derivative 32) by the lack of a methoxymethyl group at the position of 5. The compound **1** showed a higher ARE enhancer activity than derivative 81, suggesting that addition of the methoxymethyl group further increased the ARE enhancer activity. Also, ARE activation and HO-1 protein induction by the compound **1** were higher than those by natural kavalactones such as yangonin, kawain and methysticin (Fig. 2). Thus, we identified a novel kavalactone derivative, compound **1**, from 81 structurally-related kavalactones, which was more potent in Nrf2/ARE activation than natural kavalactones.

Since **1** exhibited little or no DPPH scavenging activity (Supplementary Fig. 3), it was unlikely that the compound *per se* acts as an antioxidant. The compound **1** was not cytotoxic, but a certain morphological changes were observed at higher doses (Fig. 3). At a subtoxic dose (10 μM), **1** increased the ARE enhancer activity by twofold (Fig. 4) and induced expression of various phase II antioxidant/detoxifying enzymes (Fig. 5) through nuclear translocation of Nrf2 (Fig. 6). As potential molecular mechanisms underlying Nrf2/ARE activation by **1**, we identified two mechanisms; activation of signal transduction and transient ROS generation. Nrf2 phosphorylation by protein kinases such as ERK and Akt facilitates its nuclear translocation.[5,14] The compound **1** increased phosphorylation of ERK1/2 and p38 prior to Nrf2 nuclear translocation and pretreatment with MEK1/2 and p38 inhibitors diminished induction of HO-1 by **1** (Fig. 7). On the other hand, oxidative modification of the cysteine sulfhydryl groups of Keap1 by ROS results in its conformational change and Nrf2 release. We thus examined if **1** might increase the intracellular ROS levels. The compound **1** transiently elevated the intracellular ROS levels (Fig. 8). The ROS levels peaked 1 h after treatment with **1**, but immediately decreased thereafter, which was supposed to be due to scavenging of ROS by antioxidant enzymes induced by Nrf2/ARE activation. Consistent with these findings, glutathione replenishment by NAC attenuated HO-1 induction by **1**. These results suggest that **1** activates the Nrf2/ARE system through Nrf2 phosphorylation by activation of signal transduction and oxidative modification of the sulfhydryl groups of Keap1 by transient ROS generation.

In addition to phosphorylation of Nrf2 and oxidative modification of Keap1, covalent modification by electrophiles of the sulfhydryl groups of Keap1 has been proposed as another mechanism of Nrf2/ARE activation. Curcumin, CAPE and zerumbone have 2, 1 and 1 α,β-unsaturated carbonyl groups, respectively. They are supposed to interact with the sulfhydryl groups of Keap1 as electrophiles or Michael reaction acceptors and activate the Nrf2/ARE pathway.[3,4,15] We also reported that sesquiterpene lactones extracted from *Calea urticifolia* (Compositae) and feverfew increase the ARE enhancer activity in a manner dependent on the number of α,β-unsaturated carbonyl groups each compound bears.[16] Since **1** has an α,β-unsaturated carbonyl group, it is possible that it directly interacts with Keap1, thereby releasing Nrf2. However, the higher ARE enhancer activity of compound **1** compared with natural kavalactones cannot be explained by the difference in the number of the groups, because they all have a single α,β-unsaturated carbonyl group (Fig. 1). Taken all these data together, we suggest that Nrf2/ARE activation by **1** may involve Nrf2 phosphorylation and oxidative and covalent modification of Keap1.

Preconditioning is characterized by resistance to insults in response to previous short episodes.[17] For example, when cells are repeatedly exposed to non-lethal heat shock, they acquire a transient resistance to an otherwise lethal heat shock insult.[18] Ischemic preconditioning enhances resistance to cerebral infarction.[19] It has been recently reported that preconditioning by 4-hydroxynonenal (4-HNE), toxic by-products of lipid peroxidation, protects against 6-hydroxydopamine-induced PC12 cell death via Nrf2/ARE activation.[20] We also demonstrated that preconditioning by a sesquiterpene lactone extracted from *Calea urticifolia*, calealactone A, enhances Nrf2/ARE activation caused by oxidative stress in PC12 cells, presumably resulting in increased resistance to oxidative damage.[16] In the present study, we tested if preconditioning by **1** could protect against oxidative stress-induced neuronal cell death through Nrf2/ARE activation. Indeed, pretreatment with **1** ameliorated H_2O_2-induced PC12 cell death (Fig. 9). Furthermore, the HO-1 expression level was higher in cells treated with both **1** and H_2O_2 than in those treated with H_2O_2 alone, indicating that the neuroprotective effect was correlated with increased expression of HO-1. Our findings suggest that **1** protects against oxidative stress-induced neuronal cell death via a preconditioning effect on the Nrf2/ARE activation.

The Nrf2/ARE pathway has been intensively studied in the field of chemoprevention, since phase II enzymes detoxify carcinogens.[21,22] On the other hand, Nrf2/ARE activation has been expected to be an effective therapy to reduce oxidative stress and

ameliorate neurodegenerative disorders, because it induces expression of anti-oxidant and -inflammatory proteins.[23] Thus, it would be of great benefit to identify small molecules that can reach the brain and activate the Nrf2/ARE system. Satoh et al. reported that carnosic acid extracted from Rosemary activates the Nrf2/ARE system through interaction with Keap1 and thus decreases the infarct size in rodent model of brain infarction.[24] Since a previous report demonstrated that kavalactones reached the brain 5 min after intra-peritoneal administration, it is likely that **1** can pass through the blood brain barrier.[25] Nevertheless, it remains to be studied whether **1** could reach the brain and also whether it could ameliorate neurodegenerative disorders such as Alzheimer's and Parkinson's diseases as well as cerebral infarction in animal models.

4. Conclusion

We screened and identified a kavalactone derivative (2′,6′-dichloro-5-methoxymethyl-5,6-dehydrokawain) that is more potent in Nrf2/ARE activation than natural kavalactones. We revealed molecular mechanisms underlying Nrf2/ARE activation by the compound. The compound protected against oxidative stress-induced neuronal cell death, which was considered to be due to its preconditioning effect on the Nrf2/ARE activation. Thus, the compound has a potential to reduce oxidative stress and ameliorate oxidative stress-related diseases in the brain.

5. Experimental

5.1. Cell culture and treatments

Rat PC12 pheochromocytoma cells obtained from RIKEN Cell Bank (Tsukuba, Japan) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% horse serum and 5% fetal bovine serum. In specified experiments, cells were treated with MEK1/2 inhibitor, PD98059,[26] p38 inhibitor, SB203580,[27] (Calbiochem, La Jolla, CA, USA) or *N*-acetylcysteine (NAC) (Merck, Darmstadt, Germany).

5.2. Kavalactone derivatives

Eighty-one kavalactone derivatives were synthesized by the reactions shown in Supplementary Figure 2A. Their chemical structures were confirmed by nuclear magnetic resonance (NMR) and mass spectrometric analyses. Synthesis of the compound **1** (2′,6′-dichloro-5-methoxymethyl-5,6-dehydrokawain) [(*E*)-6-(2′,6′-dichlorostyryl)-4-methoxy-5-(methoxymethyl)-2*H*-pyran-2-one] was as follows (Supplementary Fig. 2B). Condensation of 4-methoxy-6-methyl-2-pyrone (2.8 g, 20 mmol) and 2,6-dichrolobenzaldehyde (3.5 g, 20 mmol) with five equivalent of magnesium methylate in methanol under reflux produced a mixture of **1** and 2′,6′-dichloro-5,6-dehydrokawain. Both compounds were separated with column chromatography on silica gel eluted with an ethylacetate/hexane system to obtain **1**. For further purification, **1** was recrystallized with methanol to give colorless needles as its pure forms (300 mg). The physical and spectroscopic characteristics of **1** were as follows; mp 175–176 °C; HR-EI-MS: $[M]^+$ *m/z* 340.02600 (calcd for $C_{16}H_{14}O_4Cl_2$: 340.02692); MW 340; EIMS *m/z* (rel. int.): 340 (M^+, 100), 309 (58), 281 (14), 273 (11), 245 (14), 199 (34), 171 (27), 141 (28), 135 (24), 109 (8), 99 (16), 69 (15); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.37 (3H, s, 9-OCH_3), 3.89 (3H, s, 4-OCH_3), 4.32 (2H, s H-9), 5.59 (1H, s, H-3), 7.17 (1H, d, J = 8.2 Hz, H-4′), 7.21 (1H, d, J = 16.2 Hz, H-7), 7.35 (2H, d, J = 8.2 Hz, H-3′,5′), 7.36 (2H, d, J = 8.2 Hz, H-3′,5′), 7.63 (1H, d, J = 16.2 Hz, H-8); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 56.5 (4-OCH_3), 58.2 (9-OCH_3), 62.1 (C-9), 89.6 (C-3), 110.1 (C-5), 124.4 (C-7), 128.9 (C-3′,5′), 129.3 (C-4′), 130.6 (C-8), 133.0 (C-1′), 134.9 (C-2′,6′), 157.1 (C-6), 162,7 (C-7), 169.7 (C-3).

5.3. Transfection and luciferase assays

The ARE reporter gene contained two copies of the ARE enhancer sequences found in the promoter region of glutathione S-transferase Ya subunit gene[28] upstream of thymidine kinase minimal promoter fused to a firefly luciferase gene (pGL3-ARE-TK-luc) (Promega, Madison, WI, USA).[16] The ARE firefly luciferase reporter vector and the Renilla luciferase expression vector (phRL; Promega) were transfected into cells seeded onto 6-well plates by using TransIT-LT1 reagent (Mirus, Madison, WI, USA). Transfected cells were re-seeded onto 96-well plates prior to treatment with test compounds. In 24-well format luciferase assays, transfection and treatment were performed on the same plates. Luciferase activities were measured 24 h after treatment using the Dual-Glo luciferase assay system (Promega) and Pikkagene Dual luciferase assay system (TOYO B-Net, Tokyo, Japan) for 96-well and 24-well plates, respectively.

5.4. Western blot analysis

Anti-HO-1 (SPA-896) and -β-actin (A5441) antibodies were purchased from Stressgen Bioreagents (Ann Arbor, MI, USA) and Sigma–Aldrich (St. Louis, MO, USA), respectively. Antibodies recognizing Nrf2 (sc-722), γ-GCSc (sc-22755) and NQO-1 (sc-16464) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against p-ERK1/2 (#9109), p-p38 (#9211), ERK1/2 (#9102) and p38 (#9212) were obtained from Cell Signaling Technology (Beverly, MA, USA). Whole cell extracts were prepared using the RIPA buffer (10 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 0.1% sodium deoxycholate) containing protease inhibitor cocktails and phosphatase inhibitor cocktails 1 and 2 (Sigma–Aldrich). Nuclear and cytoplasmic fractions were isolated using the NE-PER nuclear and cytoplasmic extraction reagents (Pierce Rockford, IL, USA). Extracts were separated by SDS–PAGE and then transferred onto PVDF membranes (Perkin Elmer, Northwalk, CT, USA). After blocking with 5% skim milk for 1 h, membranes were incubated overnight at 4 °C with a primary antibody and then reacted with a HRP-conjugated secondary antibody for 1 h. The immunoreactive proteins were visualized using the ECL Plus Western blotting detection reagents (GE Healthcare, Piscataway, NJ, USA) and the LAS 4000 imaging system (Fuji Film, Tokyo, Japan). Densitometric analysis was performed with the Multi Gage software (Fuji Film).

5.5. Measurement of cell viability

Cell viability was determined by the WST-8 method according to the manufacturer's instruction (Dojin Chemical, Kumamoto, Japan).

5.6. Measurement of ROS levels

The intracellular ROS levels were measured using a ROS-sensitive fluorescent probe, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA; Molecular Probes, Eugene, OR, USA). After incubation with 10 μM CM-H_2DCFDA for 30 min at 37 °C, cells were washed twice with cold PBS and then re-suspended in cold lysis buffer. Cell lysates were subjected to fluorescence measurement at the excitation wavelength of 490 nm and the emission wavelength of 530 nm using the fluorescence microplate reader, MTP-600F (Corona Electric, Ibaragi, Japan).

5.7. Statistical analysis

Statistical significance was determined by the Student's *t*-test. The differences were considered significant at $p < 0.05$.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.03.034.

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