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Characterization of novel furan compounds on the basis of their radical scavenging activity and cytoprotective effects against glutamate- and lipopolysaccharide-induced insults

Keiko Nishio [a], Akiko Fukuhara [a], Yo Omata [a], Yoshiro Saito [a,b,*], Shuuhei Yamaguchi [c], Hisatoyo Kato [d], Yasukazu Yoshida [a], Etsuo Niki [a]

[a] Health Technology Research Center, National Institute of Advanced Industrial Science and Technology (AIST), Ikeda, Osaka 563-8577, Japan
[b] Department of Medical Life Systems, Faculty of Medical and Life Sciences, Doshisha University, 1-3 Miyakodani, Kyotanabe, Kyoto 610-0321, Japan
[c] Productive Technology Department, Toagosei Co., Ltd, Nagoya, Aichi 455-0026, Japan
[d] Base Technology Center, Toagosei Co., Ltd, Nagoya, Aichi 455-0027, Japan

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ABSTRACT

There is increasing evidence indicating that free radicals and oxygenases such as cyclooxygenase (COX) and lipoxygenase (LOX) are related to the onset and development of neurodegenerative diseases. In order to prevent and/or delay these diseases, the use of radical-scavenging antioxidants and inhibitors against oxygenases has received much attention. In the present study, we examined the radical-scavenging activity and cytoprotective effects of some novel furan compounds with potent inhibitory activity against oxygenases such as COX-1, COX-2, and 5-LOX. The radical-scavenging activity was assessed by studying the bleaching of β-carotene by free radicals generated from an azo initiator. In this assay system, the rate constants for scavenging peroxyl radicals by furan S and furan L was estimated to be 2×10^4 and $3 \times 10^4\ M^{-1}\ s^{-1}$, respectively. We also investigated the cytoprotective effects of these compounds against cell death induced by several toxins. We found that the furan compounds exhibited cytoprotective effects against PC12 cell death induced by linoleic acid hydroperoxide, primary neuronal cell death induced by glutamate, and cell death induced by lipopolysaccharide. These results suggest the beneficial effects of the furan compounds against disorders related to glutamate and lipopolysaccharide.

1. Introduction

Among membrane phospholipids, arachidonic acid (AA)—located at the sn-2 position—is released mainly by the action of cytosolic phospholipase A2.[1] AA is reincorporated into membrane phospholipids by reacylation reactions or oxidized to various biologically important oxygenated metabolites by several enzymatic and nonenzymatic mechanisms.[2,3] While cyclooxygenases (COX) metabolize AA to prostaglandins (PG) and thromboxanes (TX), lipoxygenases (LOX) metabolize it to hydroperoxyeicosatetraenoic acid (HpETE) and leukotrienes (LT).[4] Two forms of COX enzymes, designated as COX-1 and COX-2, occur in mammalian tissues:[4,5] COX-1 is constitutively expressed, while inflammatory mediators such as bacterial endotoxins rapidly induce COX-2. The nomenclature of LOX enzymes is based on the position of oxygen insertion (carbon 5, 8, 12 or 15 of the aliphatic chain) and the stereo-configuration (R versus S) of the resulting product, i.e., HpETE.[4,6] It is known that the generation of a peroxyl radical in a regulated manner has several possibilities for the enzymatic reaction of both COX and LOX.[7] Lipid oxidation products generated by these enzymes perform various biological activities and play important roles, not only in regulating signal transduction and gene-transcription processes but also in inducing and maintaining the acute inflammatory responses.[8]

Inflammation is a protective mechanism that isolates the injured tissue from the uninjured area, destroys injured cells, and repairs the extracellular matrix. However, overwhelming chronic or acute inflammations lead to tissue damage. Inflammation is a central feature of neurodegenerative diseases such as Alzheimer's disease, stroke, and neurovascular disease,[9] and it has been known that COX and LOX play a significant role in the onset and

Abbreviations: AA, arachidonic acid; AIPH, 2,2′-azobis[2-(2-imidazolin-2-yl)-propane] dihydrochloride; COX, cyclooxygenase; HpETE, hydroperoxyeicosatetraenoic acid; HpODE, hydroperoxyloctadecadienoic acid; IL, interleukin; LA, linoleic acid; LOX, lipoxygenase; LPS, lipopolysaccharide; LT, leukotriene; MeO-AMVN, 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile); MP, methyl palmitate; MPP^+, 1-methyl-4-phenylpyridinium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PG, prostaglandin; ROS, reactive oxygen species; SDS, Sodium Dodecyl Sulfate; TNF-α, tumor necrosis factor-α; TX, thromboxane; 6-OHDA, 6-hydroxydopamine; 13(*S*)-HpODE, 13*S*-hydroperoxyl-9*Z*, 11*E*-octadecadienoic acid; βC, β-caroten.

* Corresponding author. Address: Department of Medical Life Systems, Faculty of Medical and Life Sciences, Doshisha University, 1-3 Miyakodani, Tatara, Kyotanabe, Kyoto 610-0321, Japan. Tel.: +81 774 65 6258, fax: +81 774 65 6258.
E-mail address: ysaito@mail.doshisha.ac.jp (Y. Saito).

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development of these diseases. It has been reported that in Alzheimer's disease tissue, proinflammatory enzymes such as COX-2 and inducible nitric oxide synthetase are upregulated.[10] Glutamate toxicity is a major contributor to neurodegenerative disease-related pathological neuronal cell death, and it has been reported that several neurotoxic stimuli, including glutamate, can upregulate the cellular activity of oxygenases. Glutamate stimulation has been reported to induce COX-2;[11] it can also activate LOX, particularly 5- and 12-LOX, which are present in the brain tissue and neural cells.[4,12] In fact, it has been reported that COX and LOX inhibitors have beneficial effects on neuronal survival and protect cultured cerebellar granule neurons from glutamate-induced cell death.[13–15] These findings strongly indicate that these oxygenases can mediate the deleterious effects in neurodegenerative disorders.

There is increasing evidence indicating the relationship of peripheral inflammation to the exacerbation of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease.[16] Although the detailed molecular mechanisms have not been fully elucidated, it has been suggested that the proinflammatory cytokines released by activated macrophages in the blood and activated microglia in the brain stimulate a cascade of inflammatory changes related to neurodegenerative diseases.[16,17] It is well known that lipopolysaccharide (LPS), a highly conserved cell wall component of gram-negative bacteria, can initiate a signaling cascade that induces the expression of inflammatory genes, including those that encode cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α).[18] Neural cells such as microglia, astrocytes, neurons, and oligodendrocytes participate in inflammatory reactions. In particular, microglial cells, which show greater COX-2 expression and an enhanced ability to release PGE_2, TXA_2, and TXB_2 on peripheral injection of LPS compared with astrocytes, play important roles in the inflammatory and immune responses.[19] LOX has also been reported to mediate LPS-induced neuroinflammation.[20]

In addition to the abovementioned lipid mediators, COX- and LOX-catalyzed reactions result in the generation of reactive oxygen species (ROS). 15-deoxy-$\Delta^{12,14}$-PGJ_2, which is an electrophilic lipid synthesized via the COX pathway, contains the reactive α,β-unsaturated carbonyl group that forms adducts with proteins containing nucleophilic centers, particularly cysteine residue.[21] LOX activation directly generates lipid hydroperoxides such as HpETE from AA and hydroperoxyloctadecadienoic acid (HpODE) from linoleic acid (LA), which can induce lipid peroxidation leading to oxidative stress.[4,6] At low levels, it is thought that ROS function as signal intermediates in the regulation of fundamental cell activities such as growth, differentiation, and adaptive responses.[22,23] However, at higher concentrations, ROS contribute to pathological disorders when the balance between reducing and oxidizing forces shifts toward oxidative stress.[24] Therefore, the application of radical-scavenging antioxidants, which play an important role against oxidative stress in the defense system in vivo, to COX- and LOX-related pathological conditions have received much attention.

The biological effectiveness of antioxidants has been evaluated in studies on humans as well as on animals. In order to bridge the gap between in vitro and in vivo systems, cultured cells have often been used to study oxidative stress and its inhibition. It is notable that the evaluation of antioxidants using cell culture systems is beneficial in investigating the mechanism behind the biological effectiveness of antioxidants. The protective effects of various compounds, including inhibitors for both COX and LOX, against the cytotoxicity induced by different types of oxidative insults have also been studied extensively.[13–15] As mentioned above, it is thought that ROS are related not only to these enzymatic reactions but also to the secondary cytotoxic effects of the generated products. Some oxygenase inhibitors are known to possess radical-scavenging activity.[25] It has been reported that baicalein, a 12-LOX inhibitor, inhibits glutamate-induced neuronal cell death[15,25] and has radical-scavenging activity.[26] It has also been reported that the lipid-soluble, radical-scavenging antioxidant α-tocotrienol, a vitamin E isoform, has a potent protective effect against glutamate-induced insults and has been predicted to have 12-LOX inhibitory activity.[15] Collectively, these findings suggest that the evaluations of oxygenase inhibitory activity, radical-scavenging activity, and cytoprotective effects is important for the application of novel compounds to pathological conditions related to COX and LOX.

In the present study, we examined both the radical-scavenging activity and the cytoprotective effect of novel furan compounds (Scheme 1) with potent inhibitory activity against lipid oxidation enzymes such as COX-1, COX-2, and 5-LOX (Table 1). In particular, the novel furan compounds have potent inhibitory activity against COX-2 compared with [6]-gingerol, which has also been reported to exhbit COX inhibitory activity.[27] Furthermore, these compounds exhibit stronger inhibitory activity against 5-LOX than [6]-shogaol.[28] Furan S, in particular, exhibits an inhibitory effect similar to nordihydroguairetic acid, which is a well-known non-specific inhibitor for LOX.[29] The radical-scavenging activity of these furan compounds against the peroxyl radical was characterized by studying bleaching action of β-caroten (βC). In the cellular experimental system, these compounds exhibited cytoprotective effects against neurotoxin- and LPS-induced cell death.

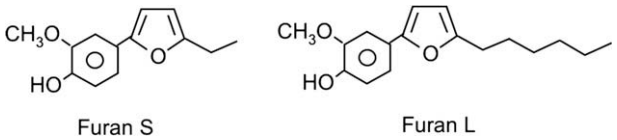
2. Materials and methods

2.1. Materials

Dulbecco's modified eagle medium: nutrient mixture F-12 Ham = 1:1 (D-MEM/F-12) and horse serum were obtained from Gibco BRL, Rockville, MD; fetal bovine serum from JRH Biosciences, Lenexa, KS; RPMI-1640, Sodium Dodecyl Sulfate (SDS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) from nacalai tesque, Kyoto, Japan; trans-β-carotene (βC) from Sigma–Aldrich, St. Louis, MO; 2,2′-azobis[2-(2-imidazolin-2-yl)-propane] dihydrochloride (AIPH), 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN) and methyl palmitate (MP) were obtained from Wako Pure Chemical Industries, Osaka, Japan. Other chemicals were of the highest quality commercially available.

2.2. Oxidation of β-carotene

The oxidation of βC induced by radical initiators was performed in aqueous dispersions and benzene solution at 37 °C in air. In the case of former solution, the emulsions were prepared by vigorously mixing MP (32.3 mM) and βC containing 0.5 M SDS with a vortex mixer for 2 min. The oxidation of βC was followed with a spectrophotometer by absorption at 463 nm.



Scheme 1. Furan compounds used in the study.

Table 1
Inhibitory effect of novel furan compounds against lipid oxidation enzymes.[a]

	COX-1[37] (μM)	COX-2[37] (μM)	5-LOX[38] (μM)
Furan S	13	9.4	0.53
Furan L	62	8.4	2.6
[6]-Gingerol	129	125	nd
[6]-Shogaol	nd	nd	7.4
Nordihydroguiaretic acid	nd	nd	0.38

[a] IC_{50} values against these enzymes are shown. nd, not determined.

2.3. Cell culture and determination of cell viability

Undifferentiated PC12 cells (rat pheochromocytoma cell line) were routinely cultured in D-MEM/F-12 containing 10% heat-inactivated fetal bovine serum and 5% horse serum. In the case of THP-1 (human monocytic leukaemia cell line) were cultured in RPMI-1640 containing 10% heat-inactivated fetal bovine serum. The cells were incubated in humidified atmosphere of 5% carbon dioxide and 95% air at 37 °C. In order to assess the effect of furan compounds, cells were treated with Furan S and Furan L at different concentrations (ranging from 1 to 50 μM) for 24 h, and then stressors such as 13*S*-hydroperoxyl-9*Z*, 11*E*-octadecadienoic acid (13(*S*)-HpODE, Cayman Chemical, Ann Arbor, MI), 6-hydroxydopamine (6-OHDA, Sigma), 1-methyl-4-phenylpyridinium (MPP^+, Sigma) and LPS (Sigma, L2880, serotype Escherichia coli O55: B5; phenol extracted) were added to the medium as an aqueous solution. After each treatment, MTT assay was performed in order to evaluate cell viability, as described previously.[30] Briefly, the cells were incubated with 0.5 mg/ml MTT in fresh medium at 37 °C for 2 h. Isopropyl alcohol containing 0.04 N HCl was added to the culture medium (3:2, by volume), and they were mixed by pipette until the formazan was completely dissolved. The optical density of formazan was measured at 570 nm using a Multiskan Ascent plate reader (Theromo Labsystems, Helsinki, Finland).

2.4. Immature primary cortical neurons

Cells were isolated from the cerebral cortex of rat fetuses (Sprague–Dawley rats, day 17 of gestation, SLC, Sizuoka, Japan) as described previously.[23] Briefly, the cells were gently dissociated with a plastic pipette after digestion with papain (90 U/ml, Worthington Biochemical, Lakewood, NJ) at 37 °C and the cells were then plated on polyethyleneimine-coated plate. Cells were grown in D-MEM/F-12 supplemented with 10% heat-inactivated fetal bovine serum. Over 90% of the cells in the cultures were neurons as determined by immunostaining of the astrocyte-specific marker glial fibrillary acidic protein (Upstate, Lake Placid, NY) and the neuron-specific markers microtubule-associated protein-2 (Sigma–Aldrich, St. Louis, MO) and βIII-tublin (Promega, Madison, WI) (data not shown).

More than 6 h after plating the cultured neurons with serum medium, the cells were treated with furan compounds for 24 h (pretreatment). After treatment, glutamate was added to the medium as an aqueous solution. No change in the medium pH was observed in response to the addition of glutamate. In cotreatment procedure, furan compounds and glutamate were incubated simultaneously for 24 h. For the determination of cell viability using MTT, the cells were incubated with 0.5 mg/ml MTT at 37 °C for 4 h.

2.5. Statistical analysis

All results were expressed as mean ± SD of at least three independent experiments. The statistical significance of difference between determinations was calculated by Student's *t*-test and an analysis of variance (ANOVA) using Dunnett test for multiple comparisons. The calculation method was described in each figure legends.

3. Results and discussion

3.1. Chemical assessment of antioxidant activity of the novel furan compounds

The bleaching of βC by the reaction with free radicals can be easily followed with a spectrophotometer at an absorption wavelength of 463 nm (Fig. 1A and B). Here, AIPH[31] or MeO-AMVN[32] were used for the generation of free radicals at a constant rate, which is essential in the study of kinetics. AIPH was used to examine the reactivity against water-soluble peroxyl radicals, while MeO-AMVN was used for lipid-soluble peroxyl radicals. Both AIPH (data not shown) and MeO-AMVN (Fig. 1) induced the consumption of βC at a constant rate. The consumption of βC by the free radicals was suppressed by the novel furan compounds that competed with βC in scavenging the free radicals from the benzene solution (Fig. 1C and D). Both compounds suppressed the consumption of βC in a dose-dependent manner. The rate of βC consumption in the absence (R_0) or presence (R_{IH}) of the furan compounds was calculated from these results, and it was found that the relative rate of βC consumption was directly proportional to the concentration of the furan compounds (Fig. 2). The rate constant for the furan compounds (k_I) was calculated by the following equation:

$$\frac{R_0}{R_{IH}} = 1 + \frac{k_1[IH]}{k_{\beta C}[\beta C]} \quad (1)$$

where [IH] and [βC] are the concentrations of each compound and $k_{\beta C}$ is the reactivity of βC.[33] The relative reactivity of the furan compounds ($k_I/k_{\beta C}$) against βC in the benzene solution (Fig. 2) as well as in the micelle solution (Supplementary materials) is summarized in Table 2. Using the βC value ($k_{\beta C} = 3.1 \times 10^4\ M^{-1}\ s^{-1}$ [33]), the rate constants for furan S and L were estimated to be 2×10^4 and $3 \times 10^4\ M^{-1}\ s^{-1}$, respectively. These results indicate that the novel furan compounds possess similar reactivity against peroxyl radicals as βC. Therefore, it is considered that the radical-scavenging activity of these furan compounds is relatively low compared with the well-known radical scavenger, α-tocopherol ($k = 1.0 \times 10^6\ M^{-1}\ s^{-1}$ [34]).

3.2. Cytoprotective effect of the furan compounds in neurotoxin-induced PC12 cell death

We first examined the effect of the novel furan compounds against PC12 cell death induced by the neurotoxins 6-OHDA and MPP^+, which are widely used to generate an experimental model of Parkinson's disease.[30,35] In this experiment, the cells were pretreated with the furan compounds for 24 h, and subsequently, 100 μM 6-OHDA or 4 mM MPP^+ was added. Although the furan compounds did not show any significant cytoprotective effects against MPP^+ (data not shown), furan S treatment at 25 or 50 μM exhibited a significant cytoprotective effect against 6-OHDA-induced cell death (Fig. 3A).

We further examined the effects of furan compounds against 13(S)-HpODE-induced PC12 cell death. 13(S)-HpODE is produced on the oxidation of linoleic acid by 15-lipoxygenase in mammals. It is formed also by the free-radical-mediated oxidation. 13(S)-HpODE oxidize lipids in the cellular membrane and induce lipid peroxidation. Both the furan compounds exhibited significant cytoprotective effect against 13(S)-HpODE (Fig. 3B). Both compounds exhibited relatively stronger protective effects against this stressor than against 6-OHDA. We have previously reported that 100 μM 6-OHDA produces both 50 μM hydrogen peroxide (H_2O_2) and 100 μM 2-hydroxy-5-(2-aminoethyl)1,4-benzoquinone (*p*-quinone) and that both compounds exhibit cytotoxicity through different molecular mechanisms.[30] Neurotoxic compounds generated from 6-OHDA are water-soluble, while 13(S)-HpODE is relatively lipid-soluble. These results suggest that the furan compounds are effective against lipid peroxidation-related insults.

3.3. Cytoprotective effect of the furan compounds in glutamate-induced neuronal cell death

Glutamate toxicity is a major cause of pathological cell death within the nervous system. It has been observed that cell death

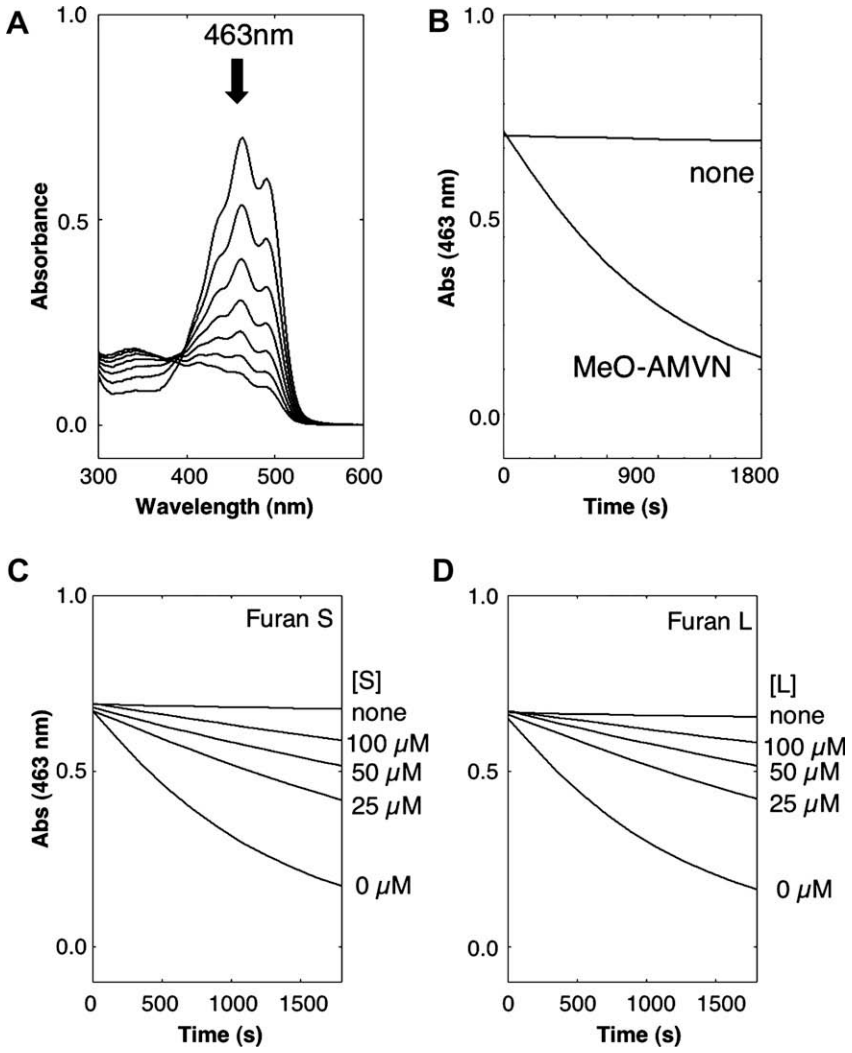


Figure 1. Effect of furan compounds against oxidation of βC induced by MeO-AMVN. (A and B) Oxidation of β-carotene in benzene solution. Oxidation of 8 μM βC by 0 (none) or 0.1 mM MeO-AMVN in benzene solution at 37 °C was measured spectrophotometrically every 5 min, as described under Section 2. (C and D) Oxidation of 8 μM βC in benzene solution induced by 0 (none) or 0.1 mM MeO-AMVN in the presence of indicated concentrations of furan compounds at 37 °C. The oxidation was followed by measuring the absorption at 463 nm.

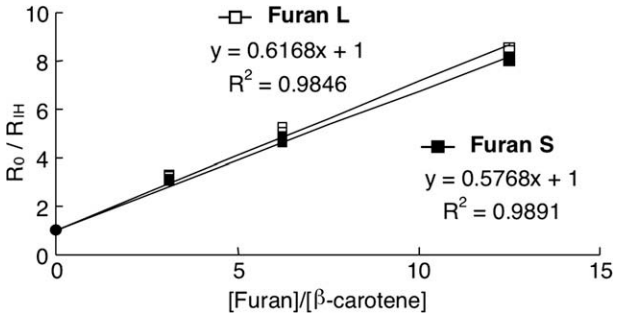


Figure 2. Relative reactivity of furan compounds against oxidation of βC induced by MeO-AMVN. Oxidation of 8 μM βC in benzene solution induced by 0.1 mM MeO-AMVN in the presence of different concentrations of furan compounds at 37 °C. Relative reactivity was calculated by Eq. (1) in Section 3.

in immature cortical neuronal cells is caused by glutamate-induced oxidative stress; and that oxidative glutamate toxicity is initiated by the competition of glutamate with the cystine-uptake system that regulates the synthesis of GSH.[23] Treatment with 10 mM glutamate for 24 h induced significant cell death in cultured cortical neurons (Fig. 4). Next, we examined the protective

Table 2
Relative reactivity of novel furan compounds against peroxyl radical ($k_I/k_{\beta C}$, 37 °C)[a]

	Benzene solution	Micelle solution	
		AIPH	MeO-AMVN
Furan S	0.58	0.69	1.1
Furan L	0.62	1.0	1.3

[a] Relative reactivity was calculated by Eq. 1.

effects of the furan compounds against glutamate-induced cell death. In this experiment, the effects of these compounds were examined using two different protocols, namely, the pretreatment and cotreatment protocols. In the former, the cells were pretreated with the furan compounds for 24 h, and subsequently, 10 mM glutamate was added. In the latter, the furan compounds and 10 mM glutamate were added to the cells simultaneously and coincubated for 24 h. As shown in Figure 4, treatment with the furan compounds prevented the neuronal cell death induced by treatment with 10 mM glutamate. Furan L at 1 μM exhibited cytoprotective effects in both procedures. We observed that pretreatment, not cotreatment, with α-tocopherol, the major isoform of vitamin E, at 2.5 μM resulted in a complete protective effect against

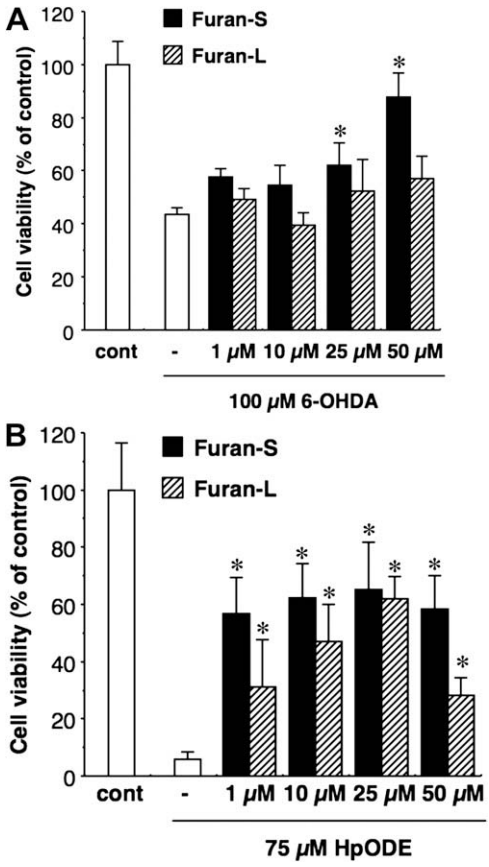


Figure 3. Protective effect of furan compounds against PC12 cell death induced by 6-PHDA and HpODE. (A) Protective effect of furan compounds against 6-OHDA toxicity. PC12 cells were pretreated with different concentrations of furan compounds for 24 h followed by treatment with 100 μM 6-OHDA for an additional 24 h. Cell viability was measured by the MTT assay, as described under Section 2. Mean ± SD of at least three experiments are shown. $^{*}p < 0.05$ (Dunnett, ANOVA) when compared with controls (without antioxidant). (B) Protective effect of furan compounds against HpODE toxicity (MTT assay). $^{*}p < 0.05$ (Dunnett, ANOVA) when compared with controls (without antioxidant).

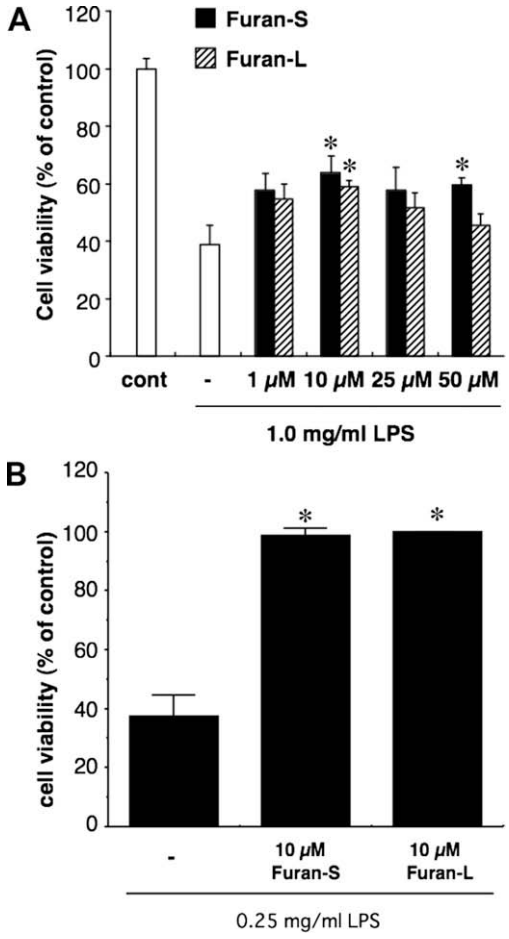


Figure 5. Protective effect of furan compounds against LPS toxicity. (A) PC12 cells were pretreated with different concentrations of furan compounds for 24 h followed by treatment with 1.0 mg/ml LPS for an additional 24 h (MTT assay). (B) THP-1 cells were pretreated with different concentrations of furan compounds for 24 h followed by treatment with 0.25 mg/ml LPS for an additional 24 h (MTT assay). $^{*}p < 0.05$ (Dunnett, ANOVA) when compared with controls (without antioxidant).

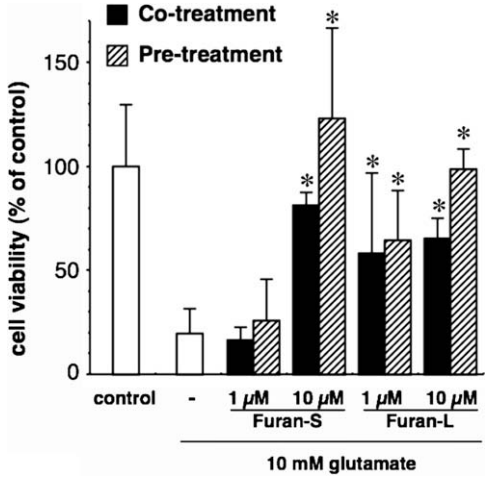


Figure 4. Protective effect of furan compounds against glutamate toxicity. Primary cortical neuronal cells were treated with 10 mM glutamate in the presence of different concentrations of furan compounds according to the pre- or co-treatment protocol described under Results. The viability was determined by MTT assay after 24 h. $^{*}p < 0.05$ (Dunnett, ANOVA) when compared with controls (without furan compounds).

glutamate-induced cell death, while α-tocotrienol, another isoform of vitamin E, exhibited potent cytoprotective effect in both procedures at a concentration of 0.25 μM (data not shown, unpublished observation). These results suggest that furan L possesses similar or more potent protective effects than α-tocopherol against glutamate-induced neuronal injuries.

There are two forms of glutamate toxicity: receptor-initiated excitotoxicity and non-receptor-mediated toxicity. In the present study, we used a model to study non-receptor-mediated, oxidative stress-related neuronal cell death; the model involved the inhibition of cystine uptake, one of the important regulators of the synthesis of GSH, by exposing the cells to high levels of glutamate.[36] The involvement of 12-LOX activation in this model has been previously reported.[15] Furthermore, several papers have reported the activation of 5-LOX and the induction of COX-2 in glutamate-induced neuronal cell death.[13,14,25] These reports indicate the involvement of LOX and COX activity in glutamate-induced cell death. Therefore, it follows that the furan compounds, possessing radical-scavenging activity and inhibitory effect against COX and LOX, may exhibit cytoprotective effects against glutamate-induced cell death. In the present study, we characterized both the radical-scavenging activity and the cytoprotective effect of the novel furan compounds possessing enzyme inhibitory activity. Furan L, possessing lower radical-scavenging activity than α-tocopherol, exhibited similar or more potent cytoprotective effects than

α-tocopherol against glutamate-induced neuronal cell death, suggesting that the enzyme inhibitory activity of furan L is important in inhibiting neuronal cell death induced by glutamate.

3.4. Cytoprotective effect of furan compounds in LPS-induced cell death

LPS, a highly conserved cell wall component in gram-negative bacteria, can initiate a signaling cascade that induces the expression of inflammatory genes, including those that encode cytokines such as IL-1β, IL-6, and TNF-α.[17] It has been reported that several enzymes, including COX and nitric oxide synthase, which are induced during LPS-induced inflammation, contribute to the tissue injuries and cell death induced by LPS.[18,19] In the present study, the effect of the furan compounds against LPS-induced cell death was examined. Treatment with 1.0 mg/ml LPS for 24 h induced significant cell death in PC12 cells (Fig. 5A). The cells were pretreated with the furan compounds for 24 h, and subsequently, LPS was added. It was observed that treatment with the furan compounds prevented the cell death induced by LPS treatment (Fig. 5A). In the case of THP-1 cells, a model of human monocytes, treatment with the furan compounds also prevented the cell death induced by 0.25 mg/ml LPS (Fig. 5B). These results suggest that the furan compounds can also be used to prevent the tissue damage induced by LPS.

In conclusion, we have characterized both the radical-scavenging and cytoprotective properties of the novel furan compounds. The evidence suggests that the furan compounds may be beneficial in disorders related to glutamate and LPS. This study also suggests that free radicals and oxygenases such as COX and LOX play an important role in the cytotoxicity induced by these toxins.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.10.038.

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