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Neuroprotective effects of furopyrazole derivative of benzylindazole analogs on C2 ceramide-induced apoptosis in cultured cortical neurons

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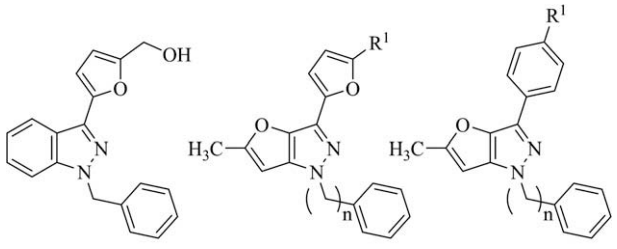
ABSTRACT

Ceramide accumulation in neurons during various disorders is associated with acute and chronic neurodegeneration. Here we investigate the neuroprotective effects of furopyrazole derivative of benzylindazole analogs on C2 ceramide-induced cell death in primary cortical neurons. Among the 12 furopyrazole derivative of benzylindazole analogs tested, carbinol derivatives exhibited strongest neuroprotection against C2 ceramide-induced apoptosis. The results suggest that furopyrazole derivative of benzylindazole analogs can be developed as useful neuroprotectants against neurodegenerative diseases.

Benzylindazole derivative such as 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1), greatly potentiated the response of soluble guanylate cyclase (GC) to nitric oxide (NO).[1–4] YC-1, not only affected the cGMP-forming GC but also inhibited the cGMP-degrading phosphodiesterases such as PDE 5 and PDE 1.[1,5] NO serves as neurotransmitter/neuromodulator in the central and peripheral nervous systems and certain neuronal cells possess cGMP signaling pathway similar to that in vascular smooth muscle cells.[6,7] Although NO at hight concentrations is toxic and thought to participate in neuronal death during stroke and neurodegenerative diseases, at low physiological concentrations, it can act as an antiapoptotic/prosurvival factor in certain neural cells.[6,8,9] The antiapoptotic effects of NO are mediated, in part, by cGMP and downstream target PKG.[6] Nitric oxide-cGMP-PKG signaling pathway was involved in the learning enhancement of YC-1 and appeared to play an essential role in preventing activation of a proapoptotic pathway, thus promoting neural cell survival.[3,6]

Ceramide is a second messenger and its functions range from proliferation, differentiation to growth arrest and apoptosis.[10,11] Ceramide can be generated either from de novo synthesis or by the action of sphingomyelinases (SMases).[12] A variety of apoptosis initiators activated sphingolipid metabolism leading to increased ceramide levels which correlated with subsequent cell death.[13–16] Ceramide also has been implicated in neuronal death during development and in models of acute and chronic neurodegenerative disorders such as cerebral ischemia or Alzheimer's disease.[17,18] In the

Table 1
Chemical structure of compounds **1**–**12**



YC-1 1- 6 7- 12

Compound	*n*	R^1
YC-1	—	—
1	1	$COOCH_3$
2	1	COOH
3	1	CH_2OH
4	0	$COOCH_3$
5	0	COOH
6	0	CH_2OH
7	1	$COOCH_3$
8	1	COOH
9	1	CH_2OH
10	0	$COOCH_3$
11	0	COOH
12	0	CH_2OH

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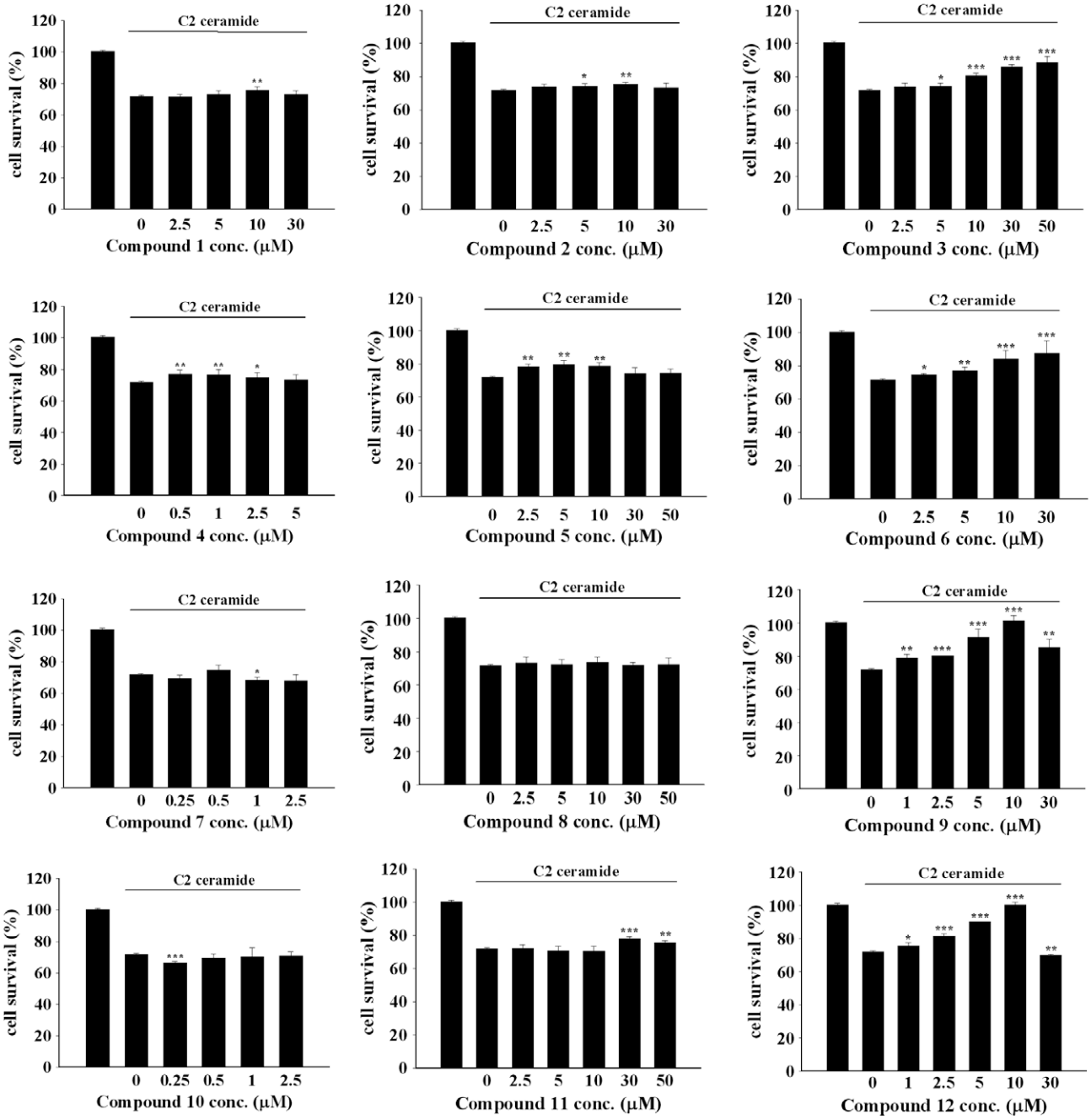

Figure 1. Neuroprotective effects of compounds **1**–**12** against C2 ceramide toxicity in cultured cortical neurons. Cells were exposed to compounds **1**–**12** at indicated concentrations for 2 h and then treated with 20 μM of C2 ceramide for 24 h. Cell viability was determined by MTT assay. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to the cells treated with C2 ceramide alone.

present study, we examine the anti-apoptotic action of furopyrazole derivative of benzylindazole analogs using C2 ceramide as an apoptotic inducer in the primary cortical cultured neurons.

Furopyrazole derivative of benzylindazole analogs were synthesized from the laboratories of Huang and Kuo.[19] After a 4-day maturation period in vitro, cortical neurons were exposed to compounds **1**–**12** (Table 1) for 2 h and then treated with 20 μM C2 ceramide for 24 h. Cell viability was measured using the MTT metabolism assay.[20] As shown in Figure 1, pretreatment with carbinol forms of compounds **3**, **6**, 9 and **12** significantly reduced C2 ceramide-induced neuronal death. The ester forms of compounds **1**, **4**, **7**, 10 and carboxylic acid forms of compounds **2**, **5**, **8**, 11 did not offer protection. Among carbinol derivatives, compounds **9** and **12** exhibited stronger neuroprotection against C2-ceramide-induced apoptosis. Compound **9** increased cell survival to 78.8 ± 2.2%, 80.1 ± 0.2%, 91.4 ± 4.8% and 101.4 ± 3.1% at concentrations of 1, 2.5, 5 and 10 μM, respectively. Compound **12** also increased the cell survival to 75.6 ± 1.8%, 81.5 ± 1.5%, 90.1 ± 0.2%, 100.2 ± 1.7% at concentrations of 1, 2.5, 5, 10 μM, respectively. The maximal effect was at 10 μM and the effect decreased with a higher concentration. Compounds **3** and **6** (5-hydroxymethy-2-furyl) at concentration of 10 μM was also able to inhibit cell death but by far less extent than those of compounds **9** and **12** (*p*-hydromethylphenyl). Overall, the order of potency was carbinol form > car-

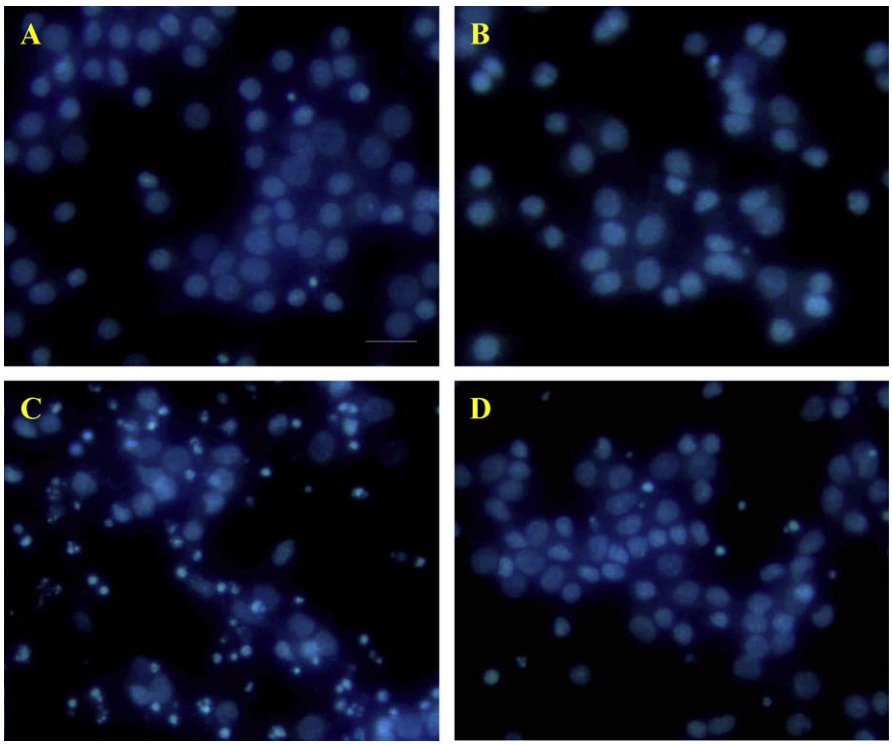

Figure 2. Morphological analysis of cell death induced by C2 ceramide exposure. Cortical neurons were treated with 10 μM Compound **12** (B), 20 μM C2 ceramide (C), or both (D) for 24 h. Control cells were without any treatment (A). Morphological studies were conducted by Hoechst 33258 staining. The arrowheads indicate apoptotic nuclei. Scale bar = 50 μm.

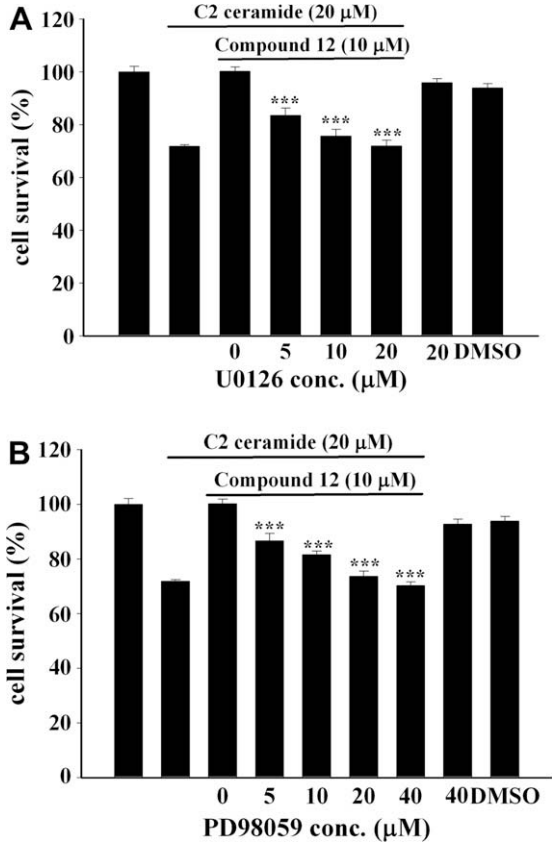

Figure 3. Requirement of ERK activation for the neuroprotective effect of compound 12. Pretreatment with U0126 (A, 20 μM) or PD98059 (B, 40 μM) blocked the neuroprotective effect of compound **12**. $^{***}P < 0.001$ as compared to the cells treated with C2 ceramide + compound **12**.

boxylic acid ≈ ester forms. These results suggest that a hydromethyl group was essential for neuroprotective action against C2 ceramide-induced cell death.

To confirm whether C2 ceramide-induced cell death was due to apoptosis, morphological analysis with Hoechst staining was conducted. The Hoechst staining method was used to identify the apoptotic nuclei in cells.[21] Apoptotic cells that contained the apoptotic bodies showed blue peripherally clumped or fragmented chromatin. The number of fragmented, brightly fluorescent nuclei indicative of apoptosis appeared to be increased in cortical cultures challenged with C2 ceramide alone (Fig. 2C) as compared to those co-treated with compound **12** (Fig. 2D). Compound **12** at 10 μM alone did not result in apparent cell death (Fig. 2B). This result demonstrated that compound **12** attenuated neuronal apoptosis by suppressing C2 ceramide-induced apoptosis.

ERK1/2 is critical for neuronal survival in many nervous systems.[22] To determine the involvement of ERK pathway in cell survival, we pretreated cultured neurons with ERK1/2 inhibitors, U0126 or PD98059, 30 min before adding compound **12**. As shown in Figure 3, pre-treatment with U0126 (20 μM) or PD98059 (40 μM) abolished compound **12**-mediated protection (Fig. 3). Cell viability was significantly decreased from 100.2% to 71.9% (U0126) and from 100.2% to 70.3% (PD98059). These results suggest that ERK pathway is involved in compound **12**-mediated protection of cultured cortical neurons against C2 ceramide-induced cell death.

It has been shown previously that benzylindazole derivative such as YC-1 had protective effects against sodium nitroprusside-mediated apoptosis in vascular smooth muscle cells. YC-1 inhibited cell apoptosis via a cGMP- and phosphatidylinositol 3-kinase-mediated inhibition on Bcl-2 down-regulation/cytochrome *c* release/caspase-3 activation cascades.[23] YC-1 also protected the white matter axons against damage by simulated ischemia.[24] In rat astrocytes, YC-1 attenuated H_2O_2-induced decrease in cell viability in a

dose-dependent way.[19] This is the first study investigating possible beneficial effect of benzylindazole analogs in cortical neurons. Our results demonstrated that furopyrazole derivative of benzylindazole analogs (compounds **3**, **6**, 9 and **12**) possessed neuroprotective effects against cellular injury induced by C2 ceramide. Compounds **3**, **6**, 9 and **12** attenuated C2 ceramide-induced cell death in a dose-dependent manner. The neuroprotective properties of compounds **9** and **12** may be useful in the treatment of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and stroke. However, further studies are necessary to determine the exact mechanism by which carbinol form of furopyrazole derivative confers neuroprotection.

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20. Cortical cultured neurons were prepared from postnal (P0–P2) Sprague-Dawley rats. Briefly, cortices were cleaned from their meninges and blood vessels in Basal BME. Cortices were then minced and dissociated in papain solution at 37 °C for 20 min. This solution was removed after centrifugation (200 rpm, for 5 min), removal of the latter solution and the cells were dissociated in a new BME. After passage through a 5 ml pipette several times, cortical cells were plated at a density of 1×10^5 cells per well in 96-well plates for cell viability assay. All plates and coverslips were coated with poly-D-lysine. The neurons were cultured in BME containing 10% foetal calf serum. After 2 h of incubation, the culture medium was replaced with neurobasal medium supplemented with 2% B27, 0.5 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin and cultured in a humidified incubator at 37 °C (95% air and 5% CO_2). Cytosine arabinoside (4 μM) was added at day 1 in vitro (DIV 1) after plating to prevent the proliferation of non-neuronal cells. All experiments were performed on cultures at 4 days in vitro (DIV 4). Cell survival was evaluated by MTT reduction assays. Cells were incubated with MTT for 2 h at 37 °C. The absorption value at wavelength of 570 nm was determined with an ELISA plate reader. Data were presents as the percentage of survival relative to vehicle-treated control culture. All measurements were performed in triplicate and each experiment was repeated at least three times.
21. For Hoechst staining, cells grown on coverslips were washed 1× PBS and then fixed with 4% paraformaldehyde in PBS for 10 min. After several washes with 1× PBS, 2.5 μg/ml DNA dye Hoechst 33,258 (bis-benzimide, Sigma) was applied to each coverslip. The samples were protected from light and incubated under room temperature for 10 min. After one wash 1× PBS, nuclei were visualized using a fluorescent microscope. Fluorescence of the soluble DNA (apoptotic) fragments was measured in a Varian Fluorometer at excitation wavelength of 365 nm and emission wavelength 460 nm.
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