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Folacin C_{60} derivative exerts a protective activity against oxidative stress-induced apoptosis in rat pheochromocytoma cells

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ABSTRACT

In the present study, we describe the synthesis and characterization of a novel folacin C_{60} derivative. The compound was analyzed by FT-IR, ^{1}H NMR, ^{13}C NMR, LC–MS and elemental analysis. This water soluble fullerene derivative was able to scavenge both superoxide and hydroxyl radical with biocompatibility. Rat pheochromocytoma (PC12) cells treated with hydrogen peroxide underwent cytotoxicity and apoptotic death determined by MTT assay and flow cytometry analysis. As a novel derivative of C_{60}, the folacin C_{60} derivative self-assembled to form spherical aggregates in H_2O. Because the compound was amphiphilic, it could penetrate the cell membrane and play its distinguished role in protecting PC12 cells against hydrogen peroxide-induced cytotoxicity. The results suggest that folacin C_{60} derivative has the potential to prevent oxidative stress-induced cell death without evident toxicity.

Oxidative stress has been considered as a major cause of cellular injuries in a variety of clinical abnormalities, especially prominent in neural diseases.[1,2] One of the effective ways to prevent the reactive oxygen species (ROS) mediated cellular injury is dietary or pharmaceutical augmentation of some free radical scavenger. Fullerene derivatives have often been used as an effective scavenger for reactive oxygen species (ROS) that behave as a free radical scavenger.[3] Furthermore, there is ample evidence to suggesting that fullerene and its derivatives possess properties which hint at their efficacy in biomedicine, such as inhibition of apoptosis,[4] neuroprotection,[5] and DNA photocleavage.[6] In recent years, covalent modification of C_{60} with water-soluble group has been of significant interest. The modification strategy is not only an effective way to solubilization of C_{60}, but it is also particularly important to the preparation of new compound classes with unprecedented properties.[7]

Folacin is one of the most important elements in the development of adult neural system. Folacin deficiency in pregnant women causes an increased risk of neural tube defects in their babies.[8] Previous studies suggested that folacin deprivation in SH-SY5Y human neuroblastoma cells induced neurodegenerative changes including increased reactive oxygen species, cytosolic calcium and apoptosis.[9] In addition, folacin is a methyl donor in one-carbon metabolism, during which it promotes the remethylation of homocysteine.[10] It is suggested that folacin treatment can lower the level of homocysteine by remethylation process. Thus, C_{60} and folacin share some important properties together. The opportunity of combining C_{60} and folacin appears as a desirable way to develop a new molecule provided with properties that are inherent in both components.

Folacin contains amino and carboxyl groups which offer flexible and efficient routes and make the modification easier and more effective. Furthermore, modifying C_{60} with folacin is also an effective way to solubilize C_{60}. In this letter, we report the first successful covalent modification of C_{60} with water-soluble folacin. We also explore a possible protective role of folacin C_{60} derivative (FFD) against oxidative stress and apoptosis induced by hydrogen peroxide in cultured PC12 cells.

Folacin (10 mmol) and sodium hydroxide (20 mmol) were dissolved in 3 mL water, and then 30 mL ethanol was added, the resulting solution was added to a C_{60} toluene solution (0.1 mmol, 60 mL) dropwise. The solution was stirred at room temperature under nitrogen atmosphere. To make sure the reaction is complete, the solution was stirred for 60 h. The aqueous layer was separated from the maple organic layer, filtered, diluted with 3 mL water. Ethanol 40 mL was then added to cause the precipitation of the product, which was further reprecipitated with H_2O/EtOH for several times. Then the product was further purified by gel exclusion chromatography using a dextran (G-25, pharmacia biotech) column with H_2O. The product was eluted first, and then unreacted folacin and sodium hydroxide were eluted.[11,12] The resulting solution was dried under vacuum to give the product of FFD (83.5 mg, 71.9% based on converted C_{60}).

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Analytic data of the synthesized and purified compound are shown below. ^{1}H NMR (D_2O) δ ppm: 9(s), 7.7(d), 6.8(d), 4.3(s), 3.6(d), 2.3(d), 2.1, 1.2(m,), 1.9(s); ^{13}C NMR (D_2O) δ ppm: 26.0, 31.2, 41.6, 55.6, 106.0, 118.6, 150.7, 156.5, 161.1, 173.7, 174.9, 139.8∼150.8; IR (KBr) v: 3501, 3382, 2976, 1691, 1601, 1563, 1524, 1450, 1387, 1124, 1189, 570, 527, cm^{-1}; ESI-MS m/z (%): 1161.3 (M^+, 38); Anal. Calcd for $C_{79}H_{19}N_7O_6$: C, 81.65; H, 1.64; N, 8.44. Found: C, 79.76; H, 1.87; N, 8.93. The 400 MHz ^{1}H NMR spectrum in D_2O exhibited two peaks at 2.1 and 1.2 ppm, they were probably protons on the C_{60} molecule. In the ^{13}C NMR spectrum, the unsymmetrical broad peaks during 139.8–150.8 were unreacted olefinic carbon of C_{60}. The FT-IR spectrum showed strong broad bands at 3501 and 3383 cm^{-1}, corresponding to –OH and –NH, and three bands at 1189, 570 and 527 cm^{-1}, corresponding to C_{60} core. The reaction on fullerene gives mono-adduct demonstrated by mass spectroscopy and elemental analysis. The chemical structure of FFD is shown in Figure 1.

The preparation of FFD vesicle was done by dissolving FFD (100 mg) in H_2O (100 mL) to form a solution of 1 mg/mL in concentration with ultrasonication for 2 h, followed by centrifugation and decantation. A portion of this solution was diluted to form a solution of 0.1 mg/mL for subsequent transmission electron microscopy (TEM) measurements (Tecnai G2 20, FEI, Holand). Light scattering experiments were performed on a Horiba DLS particle-size analyzer LB550. The data were collected at 25 °C by monitoring the scattered light intensity at a 90° detection angle. Each light scattering measurement was performed at least three times.

The intensity size distribution was measured by DLS particle-size analyzer (Fig. 2A). The average hydrodynamic diameters (Dh's) of 1 mg/mL FFD was 118.1 nm, which was found to have a strong dependence on hydrophobic interaction and the effect of hydrogen bond.[13] The structure of FFD particles was studied by TEM. Figure 2B presents electron micrograph of a colloid particle formed by 0.1 mg/mL FFD in water. The image revealed that FFD associated into stable spherical and ellipsoidal vesicles with average hydrodynamic diameters of about 53 nm.

PC12 cells were maintained in DMEM supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum at 37 °C in a humidified atmosphere of 10% CO_2/ 90% air. All cells were cultured in poly-D-lysine coated culture dishes. The medium was changed every other day and cells were plated at an appropriate density according to each experimental scale. After 48 h incubation, cells were switched into serum-free medium for treatment. In all experiments, cells were preincubated with certain concentrations of FFD for 1 h and then hydrogen peroxide was added to the medium for 24 h.

The scavenging ability of FFD to extracellular ROS was studied by chemiluminescence.[13] The fluorescent probe 2,7-dichlorofluorescein diacetate (DCF-DA) was used to monitor the intracellular accumulation of ROS.[13,14] The scavenging effect of FFD to extracellular ROS is shown in Figure 3. FFD decreased ROS concentration, and the scavenging efficiency of superoxygen anion radical was 84.3% when its concentration was 1 mg/mL, and the 50% inhibition

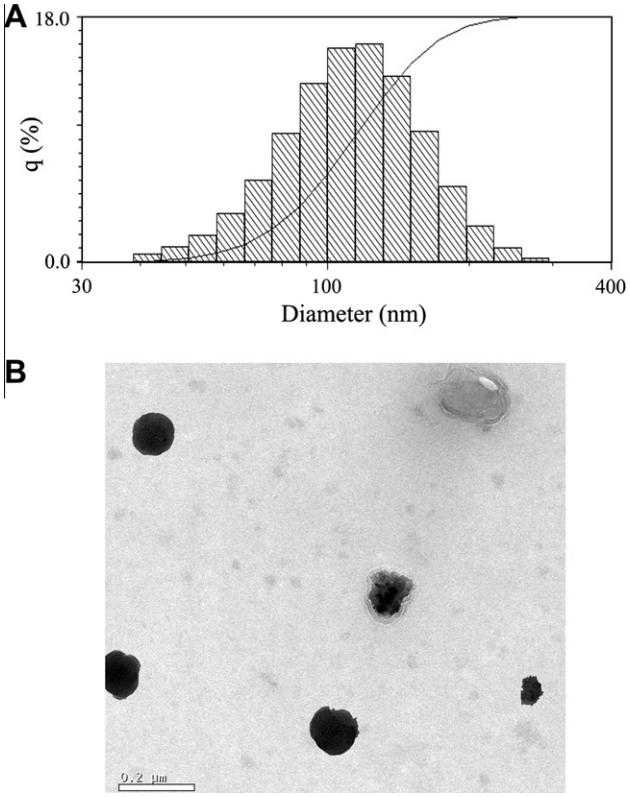

Figure 2. (A) Size distribution functions of the colloidal dispersions formed by FFD in water. The concentration of FFD in the water is 1 mg/mL. (B) TEM micrographs of FFD vesicles prepared at concentration of 0.1 mg/mL.

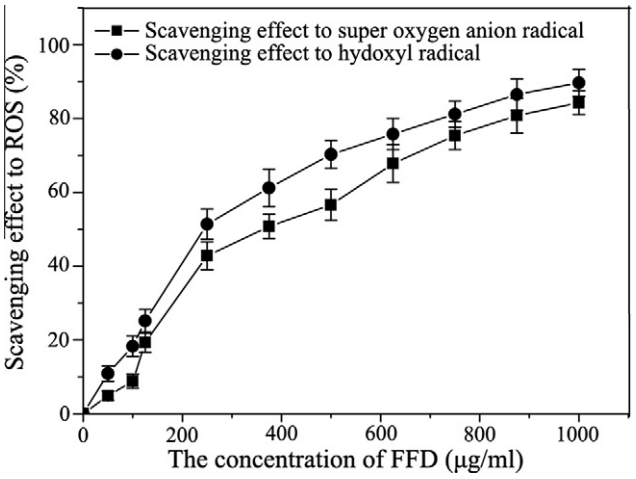

Figure 3. The scavenging effect of extracellular ROS of FFD. Data are presented as mean ± SD (n = 5).

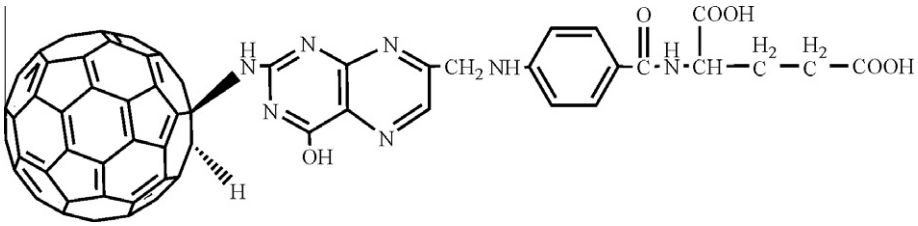

Figure 1. Schematic representation for the chemical structure of folacin C_{60} derivative.

concentration (IC_{50}) was 0.370 mg/mL. Figure 3 also shows the hydroxyl radical scavenging effect of the derivative. The IC_{50} was 0.238 mg/mL, and at the concentration of 1 mg/mL the scavenging efficiency is 89.7%. Accumulation of intracellular ROS was detected by using DCF-DA, which permeated cell membrane freely. PC12 cells treated with 800 μM hydrogen peroxide displayed intense fluorescence inside the cell after staining with DCF dye. The results are shown in Figure 4. When 50, 5, 0.5 μg/mL FFD was added to the media, respectively, intracellular ROS accumulation resulting from hydrogen peroxide treatment was reduced from 467.8 to 223.0, 236.4 and 374.6, respectively, which was determined by the changes in fluorescence intensity. The result in our experiment demonstrated that the derivative was able to scavenge ROS with biocompatibility. The scavenging effect of FFD to ROS was similar to amino acid C_{60} derivatives reported before.[13]

Cell viability was assessed by MTT (3-(4,5-dimethylthiazol-yl)-2,5-diphenyformazan bromide) reduction assay.[12] The protective role of FFD which possess strong anti-oxidation activity was tested in the hydrogen peroxide-mediated cell death assay (Fig. 5). The viabilities of cells exposed to 800 μM hydrogen peroxide for 24 h without FFD pretreatment was 29.7% of the control value. The viabilities of cells pretreated with FFD at 50 μg/mL before exposed to hydrogen peroxide increased significantly to 96.2% of control value, while vitamin E (1 mM) serving as a positive control increased to 65.4%. The result of the MTT assay showed that the hydrogen peroxide treatment significantly reduced the viability of cells, and FFD had excellent protective effect on PC12 cells from 0.5 μg/mL to 100 μg/mL.

To assess whether hydrogen peroxide induced cell death via apoptosis or not, the percentage of apoptotic cell was measured by flow cytometer.[13,15] Staining with propidium iodide revealed that the apoptotic percentage of PC12 cells exposed to hydrogen peroxide for 24 h was 47.69% (Table 1). However, pretreatment with 5 μg/mL FFD was very effective in attenuating hydrogen peroxide-induced apoptotic cell death, reducing the apoptotic cell count to 3.40%. Furthermore, we have investigated anti-oxidative protection of FFD over a long period of time, and the result was shown in Table 1. FFD was very stable in DMEM (without cells) for a long time. In all experiments, cells were switched into serum-free medium for treatment. Culturing PC12 cells in serum-free medium for a long period of time revealed significant apoptosis induced by serum deprivation. The apoptotic percentage of PC12 cells exposed to 800 μM hydrogen peroxide for 72 h was 78.82%. However, cells pretreated with 5 μg/mL FFD were very effective for attenuating hydrogen peroxide-induced apoptotic cell death, reducing the apoptotic cell count to 43.85%. The protective effect of FFD on hydrogen peroxide-induced apoptosis continued over 48, 72 h or even longer. The result also indicated that the FFD could not protect PC12 cells from apoptosis induced by serum deprivation, as FFD played effective protective role on apoptosis via ROS-scavenging.

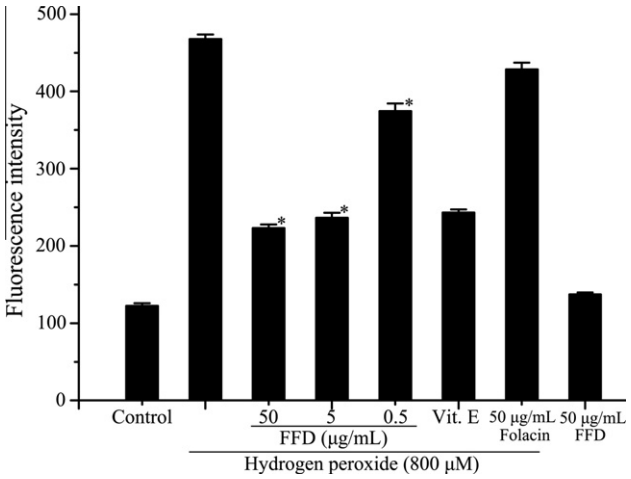

Figure 4. Inhibitory effect of different concentrations of FFD on intracellular ROS. PC12 cells were pre-incubated with FFD (50, 5 and 0.5 μg/mL) for 1 h prior to hydrogen peroxide treatment (final concentration 800 μM, 24 h). Vitamin E (1 mM) served as a positive control. The intracellular ROS accumulation was measured by using a Becton-Dickinson fluorescence-activated cell analyzer. The excitation wavelength is 488 nm and the emission wavelength is 525 nm. Data are expressed as fluorescence intensity and presented as mean ± SD (n = 3). * P <0.05 compared to the group treated with hydrogen peroxide only.

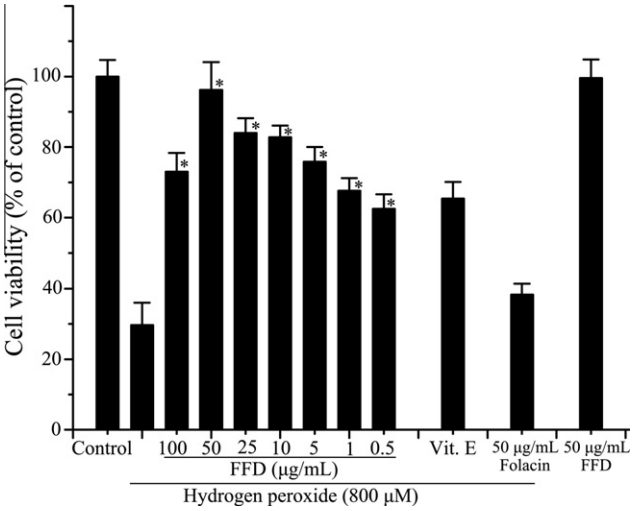

Figure 5. Attenuation of hydrogen peroxide-induced cytotoxicity by FFD in PC12 cells. PC12 cells were pretreated with FFD for 1 h and 800 μM hydrogen peroxide was then added, followed by incubation for 24 h at 37 °C. Vitamin E (1 mM) served as a positive control. The cell viability was determined by the conventional MTT reduction assay. The data are presented as mean ± SD. * P <0.05 compared to the group treated with hydrogen peroxide only. (n = 5).

Table 1
The protective effect of FFD over different period of time in PC12 cells

Chemicals	24 h	48 h	72 h	96 h
Control	1.28 ± 0.37	13.54 ± 1.79	32.47 ± 1.95	38.54 ± 2.73
H_2O_2	47.69 ± 2.87	59.94 ± 2.56	78.82 ± 5.45	83.67 ± 4.38
FFD + H_2O_2	3.40 ± 0.62	19.83 ± 1.69	43.85 ± 2.32	59.67 ± 3.89

Apoptosis in PC12 cells detected by flow cytometry analysis. Cells were pretreated with 5 μg/mL FFD for 1 h, respectively, and then treated with 800 μM hydrogen peroxide for different period of time. Data were presented as mean ± SD (n = 3).

The intracellular and extracellular contents of FFD can be determined by RF spectrum, and the excitation wavelength of fluorescent FFD is 380 nm while the emission wavelength is 458 nm.[11] The relationship between fluorescence intensity and the concentration of FFD is shown in Figure 6A. The intracellular and extracellular FFD contents were calculated according to the fluorescence intensity detected by the RF assay (Fig. 6B). When 50, 5 and 0.5 μg/mL FFD was added into the cell culture with an incubation time of 1 h, and then treated with 800 μM hydrogen peroxide for 24 h, the extracellular contents of FFD was 31.24, 2.55 and 0.23 μg, respectively, while the intracellular content was 11.76, 1.71 and 0.07 μg per 10^6 cells, respectively. The results of our experiments demonstrate that FFD could penetrate through the cell membrane retaining molecular stability, possibly exerting ROS-scavenging effects in both cytoplasm and medium.

In the 1990s, there were many researchers engaged in the study of biological activity of fullerene. However, the number of research papers in the field decreased in recent years. In the papers reported

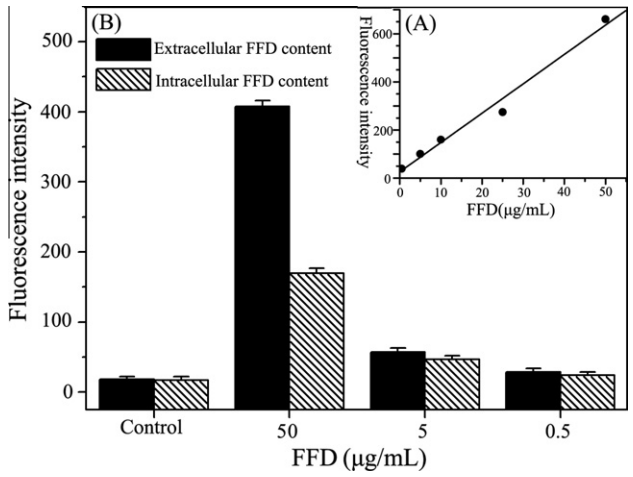

Figure 6. Intracellular and extracellular contents of FFD were quantified by RF method. PC12 cells were pre-incubated with 50, 5 and 0.5 μg/mL FFD for 1 h prior to hydrogen peroxide treatment (final concentration 800 μM, 24 h). The excitation wavelength of fluorescent FFD is 380 nm while the emission wavelength is 458 nm. (A) Relationship between fluorescence intensity and the concentration of FFD. (B) Extracellular and intracellular contents of FFD. Data are presented as mean ± SD (n = 3).

before, the researchers mostly focused on the biological ability of carboxyfullerenes and polyhydroxylated C_{60} derivatives but paid less attention to other water soluble C_{60} derivatives. The functional groups, without biological activities, only make the derivatives soluble in water, without contributing to the biocompatibility. The reason for this phenomenon was that, in recent years, the study of chemical modification of fullerene had undergone a slow progress. It was very difficult to find a fullerene derivative to suit biological research.

In this work, we have focused on the synthesis and characterization of a folacin C_{60} derivative. A notable finding was that combining C_{60} with folacin resulted in a new molecule which can take full advantage of both fullerene and folacin. Toxicity was always a primary concern for the people working with fullerenes. Earlier studies on C_{60} suggested that water soluble substituents could reduce the toxicity of fullerene derivatives observably.[16,17] With available data in hand, we found that the derivative did not show obvious toxicity after combining with folacin. Importantly, modification of C_{60} with folacin produced an amphiphilic molecule, which may pass through the blood–brain barrier, as shown in other C_{60} derivatives.[5,18] For an antioxidant to be effective in reducing the ROS levels especially within cells, it had to enter the cells. Because FFD was amphiphilic, it could permeate through the cell membrane. Meanwhile, because the self-assembled size of FFD was smaller than cystine C_{60} derivative and arginine C_{60} derivative, the cumulative amount of FFD was more than cystine C_{60} derivative and arginine C_{60} derivative in cells.[11,13] The results further proved that the aggregation morphology could affect the cumulative amount of FFD in cells and the scavenging activity of free radical.

Moreover, other researchers have also reported some ROS scavengers on preventing apoptosis. For example, Monti et al., pointed that 10 μM carboxyfullerene reduced apoptotic PBMCs count from 29.4% to 11.0% in the presence of 10 mM dRib for 48 h;[4] Guan et al., pointed that 1.2 mM protocatechuic acid reduced apoptotic PC12 cells count from 11.3% to 8.3% in the presence of 400 μM hydrogen peroxide for 24 h;[19] Hu et al., pointed that 5 μg/mL cystine C_{60} derivative reduced apoptotic PC12 cells count from 44.7% to 7.17% in the presence of 800 μM hydrogen peroxide for 24 h.[11] In this work, 4.31 μM (5 μg/mL) FFD reduced apoptotic PC12 cells count from 47.69% to 3.40% in the presence of 800 μM hydrogen peroxide for 24 h, the protective effect on hydrogen peroxide-induce apoptosis was better than the results reported before. As a novel derivative of C_{60}, the FFD was soluble in H_2O. The confirmation of the folacin-based covalent modification route might lead to further studies aiming for neural protection and counteracting apoptosis.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.05.062.

References and notes

1. Wang, Q.; Tompkins, K. D.; Simonyi, A.; Korthuis, R. J.; Sun, A. Y.; Sun, G. Y. *Brain Res.* **2006**, *1090*, 182.
2. Rayner, B. S.; Duong, T. T. H.; Myers, S. J.; Witting, P. K. *J. Neurochem.* **2006**, *97*, 211.
3. Krusic, P. J.; Wassermann, E.; Keizer, P. N.; Morton, J. R.; Preston, K. F. *Science* **1991**, *254*, 1183.
4. Monti, D.; Moretti, L.; Salvioli, S.; Strafolacince, E.; Malorni, W.; Pellicciari, R.; Schettini, G.; Bisaglia, M.; Pincelli, C.; Fumelli, C.; Bonafe, M.; Franceschi, C. *Biochem. Biophys. Res. Commun.* **2000**, *277*, 711.
5. Dugan, L. L.; Turetsky, D. M.; Du, C.; Lobner, D.; Wheeler, M.; Almli, C. R.; Shen, C. K. F.; Luh, T. Y.; Choi, D. W.; Lin, T. S. *Proc. Natl. Acad.* **1997**, *94*, 9434.
6. Nakamura, E.; Isobe, H. *Acc. Chem. Res.* **2003**, *36*, 807.
7. Hu, Z.; Liu, S.; Wei, Y.; Tong, E.; Cao, F.; Guan, W. *Neurosci. Lett.* **2007**, *429*, 81.
8. Blom, H. J.; Shaw, G. M.; den Heijer, M.; Finnell, R. H. *Nat. Rev. Neurosci.* **2006**, *7*, 724.
9. Ho, P. I.; Ashline, D.; Dhitavat, S.; Ortiz, D.; Collins, S. C.; Shea, T. B.; Rogers, E. *Neurobiol. Dis.* **2003**, *14*, 32.
10. Lucock, M. D.; Daskalakis, I. G.; Wild, J.; Anderson, A.; Schorah, C. J.; Lean, M. E.; Levene, M. I. *Biochem. Mol. Med.* **1996**, *59*, 104.
11. Hu, Z.; Guan, W.; Wang, W.; Huang, L.; Xing, H.; Zhu, Z. *Chem. Biol. Interact.* **2007**, *167*, 135.
12. Hu, Z.; Guan, W.; Wang, W.; Huang, L.; Xing, H.; Zhu, Z. *Cell Biol. Int.* **2007**, *31*, 798.
13. Hu, Z.; Guan, W.; Wang, W.; Huang, L.; Tang, X.; Xu, H.; Zhu, Z.; Xie, X.; Xing, H. *Carbon* **2008**, *46*, 99.
14. Jang, J. H.; Surh, Y. J. *Mutat. Res.* **2001**, *496*, 181.
15. Wu, G. D.; Zhou, H. J.; Wu, X. H. *Vasc. Pharmacol.* **2004**, *41*, 205.
16. Nelson, M. A.; Domann, F. E.; Bowden, G. T.; Hooser, S. B.; Fernando, Q.; Carter, D. E. *Toxicol. Ind. Health* **1993**, *9*, 623.
17. Scrivens, W. A.; Tour, J. M.; Creek, K. E.; Pirisi, L. *J. Am. Chem. Soc.* **1994**, *116*, 4517.
18. Cagle, D. W.; Kennel, S. J.; Mirzadeh, S.; Alford, J. M.; Wilson, L. J. *Proc. Natl. Acad. Sci.* **1999**, *96*, 5182.
19. Guan, S.; Bao, Y. M.; Jiang, B.; An, L. J. *Eur. J. Pharmacol.* **2006**, *538*, 73.