Fullerene Antioxidants Decrease Organophosphate-induced Acetylcholinesterase Inhibition *in vitro*

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Abstract:

Although organophosphate (OP)-induced acetylcholinesterase (AChE) inhibition is the critical mechanism causing toxicities that follow exposure, other biochemical events, including oxidative stress, have been reported to contribute to OP toxicity. Fullerenes are carbon spheres with antioxidant activity. Thus, we hypothesized that fullerenes could counteract the effects of OP compounds and tested this hypothesis using two *in vitro* test systems, hen brain and human neuroblastoma SH-SY5Y cells. Cells were incubated with eight different derivatized fullerene compounds before challenge with paraoxon (0 = control, 5×10^{-8}, 10^{-7}, 2×10^{-7} or 5×10^{-7} M) or diisopropylphosphorofluoridate (DFP, 0 = control, 5×10^{-6}, 10^{-5}, 2×10^{-5}, and 5×10^{-5} M) and measurement of AChE activities. Activities of brain and SH-SY5Y AChE with OP compounds alone ranged from 55‑83% lower than non-treated controls after paraoxon and from 60‑92% lower than non-treated controls after DFP. Most incubations containing 1 and 10 μM fullerene derivatives brought AChE activity closer to untreated controls, with improvements in AChE activity often >20%. Using dissipation of superoxide anion radicals as an indicator (xanthine oxidation as a positive control), all fullerene derivatives demonstrated significant antioxidant capability in neuroblastoma cells at 1 μM concentrations. No fullerene derivative at 1 μM significantly affected neuroblastoma cell viability, when determined using either Alamar Blue dye retention or a luminescent assay for ATP production. These studies suggest that

derivatized fullerene nanomaterials have potential capability to ameliorate OP-induced AChE inhibition resulting in toxicities.

Keywords: Fullerenes | Organophosphates | Acetylcholinesterase inhibition

Article:

1. Introduction

Organophosphorus (OP) compounds are widely used as insecticides, but they also represent a class of extremely potent chemical warfare agents that can cause incapacitation and death within minutes of exposure (ATSDR, 2006). Their critical mechanism of action is inhibition of acetylcholinesterase (AChE), which is followed by cholinergic poisoning. In addition, rapid AChE inhibition occurring after OP exposure has been reported to result in oxidative stress, as indicated by reduction in glutathione, increases in reactive oxygen species (ROS), and production of stress proteins (Giordano et al., 2006, Jett and Narova, 2000,Pazdernik et al., 2001 and Qiao et al., 2005). Current medical measures against OP toxicoses are atropine, which blocks muscarinic cholinergic receptors, and oxime reactivators, which can remove OP compounds from AChE. The latter compounds are only effective, however, if they are given before the OP compound essentially irreversibly binds to this enzyme. Other treatments are symptomatic and supportive (ATSDR, 2006) and do not specifically target noncholinergic effects associated with OP toxicoses.

Because OP agents can be very toxic and treatments are relatively limited, we initiated experiments to examine a new type of agent that has the potential to improve treatment when such exposures occur. The rationale for these studies was based on two observations. First, OP-induced toxicity can have an oxidative stress component (Giordano et al., 2006, Pazdernik et al., 2001 and Qiao et al., 2005). Second, fullerenes are potent antioxidants that have been referred to as free radical "sponges' (Ali et al., 2008, Dugan et al., 1996, Johnson et al., 2010 and Wang et al., 1999). Since fullerenes without derivation are water insoluble we used addition chemistry to chemically attach various moieties to C_{60}, C_{70}, and C_{80} fullerene cages to make them compatible with biological systems (Iezzi et al., 2002, Johnson et al., 2010, Lee et al., 2002, Olmstead et al., 2001, Wang et al., 1999 and Yin et al., 2006). Whereas native fullerenes produce aggregates in water that are cytotoxic to cultured cells, water soluble fullerenes are generally non-cytotoxic (Isakovic et al., 2006 and Sayes et al., 2004).

The present *in vitro* study was conducted to screen a series of derivatized fullerenes:(1) for their capability to protect AChE derived from two different sources (hen brain and human neuroblastoma cells), (2) to determine if these fullerene derivatives could have added benefit as antioxidants, and (3) to evaluate their safety by examining their ability to affect cell viability. In addition, *in vitro* studies aid selection of doses that could be useful for *in vivo* studies (ICCVAM, 2008). Initial testing *in vitro* is especially valuable when studying effects of OP

compounds, as their primary toxic mechanism (AChE inhibition) can be easily and reliably examined in non-animal systems (Ehrich et al., 1997).

2. Materials and methods

2.1. Fullerenes

A total of eight derivatized empty cage or metallo-fullerene compounds were tested, including two hydroxylated C_{60} (C_{60}-OH) (one commercially available at BuckyUSA Inc., and one made and characterized at Luna nanoWorks), carboxylated C_{60} (C_{60}-COOH), C_{60}-pyrrolidine (C_{60}-PRD), hydroxylated C_{70} (C_{70}-OH), C_{70}-tetraglycolic acid (C_{70}-TGA), hydroxylated scandium Trimetaspheres™ ($Sc_3N@C_{80}$-OH), and hydroxylated gadolinium Trimetaspheres™ ($Gd_3N@C_{80}$-OH). All were provided by Luna Innovations Incorporated, Blacksburg, VA. The C_{60}-OH, C_{60}-COOH, and C_{60}-PRD were purchased from BuckyUSA Inc. (Houston, TX). Those synthesized by Luna nanoWorks, Danville, VA, included a C_{60}-OH, C_{70}-OH, C_{70}-TGA, $Sc_3N@C_{80}$-OH and $Gd_3N@C_{80}$-OH. Hydroxylated fullerenes were synthesized as described (Husebo et al., 2004). In brief, pristine (without moieties added to the carbon cage) empty cage or metallo fullerenes (C_{60}, C_{70}, $Sc_3N@C_{80}$, and $Gd_3N@C_{80}$) were suspended in anhydrous dichloromethane under nitrogen atmosphere and 30 equivalents of potassium superoxide (KO_2) and catalytic amounts of 18-crown-6 were added to the suspension and stirred for two hours at room temperature. After the reaction was completed, derivatized fullerenes were precipitated, the organic layer decanted, and the fullerene materials were washed three times with dichloromethane to remove residual 18-crown-6. A small volume of distilled water was added to dissolve the fullerene materials leading to a dark brown solution of hydroxylated fullerenes, which was then purified via a combination of size exclusion chromatography (Sephadex G-25 column) and dialysis (2000 Da MW cut-off). The purity of hydroxylated fullerenes was evaluated by reverse phase TLC as >95%. The carbon cages in hydroxylated fullerenes were functionalized with hydroxyl groups (~20 hydroxyls per molecule) as demonstrated by Fourier transform infrared (FTIR) spectroscopy and thermogravimetric analysis. C_{70}-TGA (4 glycolic acids attached to the cage with two acids at each pole of the oval-shaped C_{70} molecule) was synthesized through the Bingel-Hirsch reaction as follows: C_{70} was reacted with two equivalents of di(tert-butylglycolate)malonate in the presence of non-nucleophilic weak base DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) and alkylating reagent iodine in toluene under nitrogen atmosphere for four hours at room temperature (Zhou et al., 2003). The product was isolated and purified with silica chromatography to yield the tert-butyl ester precursor of C_{70}-TGA. Trifluoroacetic acid deprotection of the tert-butyl esters in dichloromethane resulted in C_{70}-TGA, which was characterized by nuclear magnetic resonance (NMR) and matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) and its purity (>95%) was assessed by HPLC. The molecular size of the synthesized fullerene compounds is 1-2 nm depending on the side groups attached to the carbon cage. However, these materials form aggregates in aqueous

media and DLS (dynamic light scattering) shows the aggregate size of 20‑50 nm for C_{70}‑TGA in phosphate buffered saline (PBS) and 50‑100 nm for hydroxylated fullerenes at concentrations tested in phosphate buffered saline (PBS). The lipophilicity increases in the following order: hydroxylated fullerenes, carboxylated fullerenes, C_{60}‑PRD and C_{70}‑TGA.

2.2. Organophosphorus test agents

Paraoxon and diisopropylphosphoroflouridate (DFP) were obtained from Chem Services, West Chester, PA, and Aldrich Chemical Co., St. Louis, MO, respectively. Suppliers’ information noted purities >98%. They were prepared in a chemical safety hood as 0.1 M solutions in ethanol vehicle and diluted with phosphate buffer (0.1 M, pH 7.4) immediately before incubations following standard operating procedures of Virginia-Maryland Regional College of Veterinary Medicine for handling of laboratory chemicals.

2.3. Assays for cytotoxicity

Cytotoxicity of the eight fullerene derivative test agents was examined in primary murine neuronal cultures and human neuroblastoma cells using a cellular luminescence assay for ATP production (Promega Cell Titer-Glow® assay kit, San Francisco, CA, USA). The cells were seeded at concentrations of 20,000 (mouse cortex) or 5400 (human neuroblastoma) cells per well in 96-well opaque plates overnight and treated with 0, 1, 10 and 100 μM concentrations of fullerenes for 24 h. Control optical density values (mean $\pm$ SD) were 1285 $\pm$ 60 and 17183 $\pm$ 2975 pixels, respectively. Human neuroblastoma cells were also tested with the same concentrations of fullerene derivatives using the Alamar Blue assay (Ehrich and Sharova, 2000). This assay compares fluorescence (excitation 530 nm; emission 590 nm) of a water soluble dye in cultures of treated cells with fluorescence in cultures of vehicle-treated cells after 24 h of treatment. Optical densities of controls were 4151 $\pm$ 941 pixels. Ethanol (10%) was used as a positive control. Lack of cytotoxicity to neuroblastoma cells of the organophosphorus test compounds used for this study was previously determined and reported (Ehrich et al., 1997).

2.4. Fullerene derivatives antioxidant capability

A xanthine/xanthine oxidase system was used to generate superoxide free radical to measure the superoxide-scavenging activity of fullerene derivatives as described (Li et al., 1998). This well-established *in vitro* system is biologically relevant as it produces the reactive oxygen species involved in cell injury. The reaction mixture, kept at 37 °C, contained 1.0 ml of 50 mM phosphate buffered saline (PBS), pH 7.4, 270 μM xanthine, and 3.27 mU/ml xanthine oxidase in the absence or presence of various concentrations of fullerene derivatives prepared in PBS. Lucigenin (10 μM) was added to the reaction mixture and chemiluminescence was monitored over a 30 min period with a tube luminometer (AutoLumat LB 953, Berthold Technologies, Bad Wildbad, Germany). Integrated area under the curve was calculated and values compared

between reaction mixtures of the positive control (incubates without fullerenes) and the fullerene-containing reaction mixtures. Numbers for positive control incubates over the series of experiments ranged from 41,740 to 70,536. Data from each assay were individually converted to percent of control for depiction in this manuscript. Inability of the fullerene derivatives to affect capability to produce uric acid was used to identify the action of the fullerene derivative as a scavenger of the free radicals rather than a xanthine oxidase enzyme inhibitor (Li et al., 1998).

2.5. AChE enzyme sources and AChE assay

Brains were obtained from adult hens, Department of Animal and Poultry Science, Virginia Tech. Human neuroblastoma cells (SH-SY5Y) were obtained from the American Type Culture Collection (ATCC), Manassas, VA, and cultured to prepare retinoic acid-differentiated cells as reported previously (Ehrich et al., 1997). Embryronic day 18 mouse cerebral cortices were obtained from BrainBits LLC, Springfield, IL, and cultured to create a primary neuronal culture following the supplier's protocol. AChE activity was measured spectrophotometrically using a colorimetric microtiter assay previously described (Correll and Ehrich, 1991). Organophosphorus compound (or an equivalent volume of vehicle) and fullerene derivatives were pre-incubated for 15 min at room temperature before addition to the wells of a 96-well plate that contained 0.048 mg of hen brain homogenate or 500,000 neuroblastoma cells. The reaction was initiated with addition of the acetylthiocholine substrate, and the yellow product of its hydrolysis was measured at 412 nm after 30 min at 28 °C. Optical density changes in controls were 0.921 ± 0.324 (mean ± SD, n = 30) with 500,000 cells/well and 1.494 ± 0.440 (n = 40) with 0.05 mg hen brain. Each fullerene derivative was tested against each concentration of OP compound and each concentration of fullerene with a minimum of three different batches of cells or homogenates.

2.6. Statistical analyses

Quantitative results from cytotoxicity determinations, acetylcholinesterase assays, and antioxidant capability were expressed as mean ± SE) of three or more assays using different batches of cells or hen brain homogenates. Subsequently, for each combination of *in vitro* test system (brain or SH-SY5Y cells), OP compound (paraoxon or DFF), and fullerene (two C_{60}-OH compounds, C_{60}-COOH, C_{60}-PRD, C_{70}-OH, C_{70}-TGA, $Sc_3N@C_{80}$-OH and $Gd_3N@C_{80}$-OH), the fullerene control concentration of 0 M was compared to the other 3 concentrations (1, 10 and 100 μM) using ANOVA followed by Dunnett's procedure for multiple comparisons. Residual plots were inspected to verify model adequacy (i.e., that the errors followed a normal distribution with a constant variance). Statistical significance was set to alpha = 0.05. All analyses were performed using SAS version 9.2 (Cary, NC, USA).

3. Results

3.1. Fullerene cytotoxicity

Primary neuronal cells from mouse brain were more sensitive to fullerene derivative-induced decreases in ATP production detected by change in luminescence than human neuroblastoma cells (Fig. 1A and B). The highest concentration tested (100 μM) of all eight test derivatives, except C_{60}-COOH, significantly affected the primary neuronal cells (Fig. 1A), but not all of derivatives had the same detrimental effect on the neuroblastoma cells at this concentration (Fig. 1B). Also, no notable cytotoxicity to the neuroblastoma cells was seen with 10 μM of any fullerene derivatives, whereas $Sc_3N@C_{80}$-OH significantly decreased ATP production in the primary neuronal cells. When viability was determined using the Alamar Blue assay (Fig. 1C), none of the fullerene-challenged cells were affected with any of the concentrations of the test fullerene derivatives (1, 10 or 100 μM, although not all concentrations were tested with some). The Alamar Blue assay could not be used with the primary murine cells due to interference of their medium with the assay reagents.

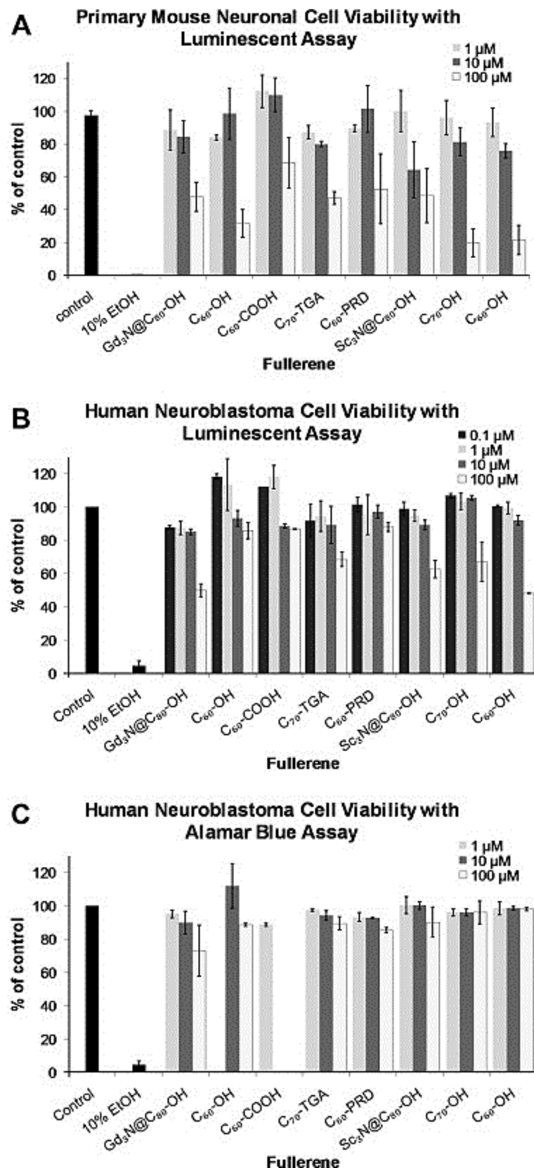

Fig. 1. Fullerene derivatives had limited cytotoxic effects after 24 h of exposure. Viability is expressed as luminescent indication of ATP production in primary murine cells (A) and human neuroblastoma SH-SY5Y cells (B) and as Alamar Blue retention in neuroblastoma calls (C). Each fullerene derivative was tested with a minimum of three different batches of cells, with results expressed as mean ± SE. Viability of vehicle-treated cells (controls) was >94% with every batch of cells with variability too low to illustrate when the neuroblastoma cells were used. Ethanol 10% was used as a positive control, providing viability of <5% in all systems. At 10 μM concentrations, only $Sc_3N@C_{80}$-OH caused any significant detrimental effects, and these effects were only seen with the primary cultures. When 100 μM fullerenes were used, all but C_{60}-COOH caused significant decrease in ATP production in primary murine neuronal cells and five of eight fullerene derivatives had significant effects on the ATP production in human neuroblastoma cells. No fullerene derivative at any test concentration affected Alamar Blue retention (commercial C_{60}-OH and C_{60}-COOH were not tested at all concentrations).

3.2. Antioxidant capability of the fullerenes derivatives

All fullerene derivatives tested at 10 μM were able to scavenge free radicals formed from xanthine by xanthine oxidase. This effect was concentration dependent (Fig. 2). All but the commercial C_{60}-OH had capability to do so at 1 μM also, and C_{70}-TGA, $Sc_3N@C_{80}$-OH, and Luna's C_{60}-OH were effective at 0.1 μM as well. All but C_{70}-OH and Luna's C_{60}-OH of the fullerene derivatives were examined for their effectiveness as antioxidants at 0.01 and 0.001 μM, with no significant protective effect (data not shown). The lack of effects on xanthine oxidase indicated that the effect of the fullerene derivatives was to scavenge free radicals, not to interfere with their production (data not shown).

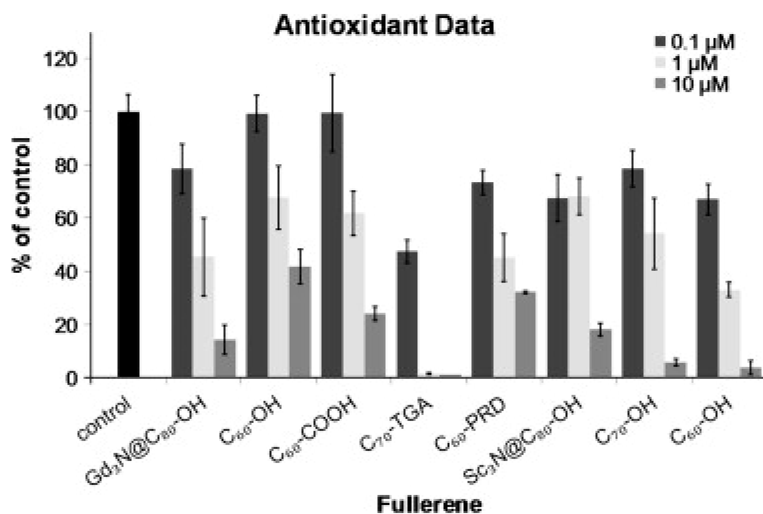

Fig. 2. Free radical scavenging capability of fullerene derivatives. A xanthine/xanthine oxidase system was used as a generating system, with xanthine providing the positive control. Results are presented as mean ± SE, $n > 3$. Antioxidant effects, as indicated by bars lower than that of the xanthine control, were significant ($p < 0.05$) at 1 μm and 10 μM except with the commercial C_{60}-OH at 1 μM (second triplet

of bars). C_{70}-TGA, $Sc_3N@C_{80}$-OH, C_{70}-OH, and Luna's C_{60}-OH (last triplet of bars) were also effective antioxidants at 0.1 μM.

3.3. Decreased OP-induced AChE inhibition in the presence of fullerenes derivatives

Inclusion of the eight derivatized fullerene test compounds in incubates containing paraoxon or DFP with AChE of human neuroblastoma cells and hen brain homogenates generally indicated a protective effect of the fullerene derivatives against OP-induced AChE inhibition (Fig. 3). The fullerene derivatives alone did not affect AChE determinations, with the exception of 100 μM C_{70}-TGA, which reduced activity to 70% of control in neuroblastoma cells and 21% of control in hen brain homogenates (data not shown). The capability of fullerene derivatives to decrease OP-induced AChE inhibition was indicated by higher AChE activities in incubates containing the fullerene derivative than in incubates in which they were lacking. Fig. 3 shows results with only one of the four concentrations of OP compounds used for testing and the lowest concentration (1 μM) of fullerene derivative used for routine screening. Ten- and 100-fold higher concentrations of the fullerene derivatives were not significantly better protectants than 1 μM (Fig. 4A). The effectiveness of the fullerenes, as indicated by differences in AChE activities in their presence, was generally greater with AChE in hen brain homogenates and generally extended over all but the highest OP concentrations tested (5×10^{-7} M for paraoxon and 5×10^{-5} M for DFP, Fig. 4B) for this enzyme source. For example, using both $p < 0.05$ and >20% improvement in AChE activity as indicators of effectiveness, all fullerene derivatives at concentrations of 1 and 10 μM resulted in increased hen brain AChE activity after 5×10^{-8} M paraoxon or 5×10^{-6} M DFP whereas none met this criteria against 5×10^{-7} M paraoxon or 5×10^{-5} M DFP. At these higher OP concentrations, AChE activity in the absence of the fullerene derivatives was <20% of control. All but C_{70}-TGA provided significant improvement of AChE activity against multiple concentrations of both paraoxon and DFP when hen brain homogenates were used. Fullerene derivatives that demonstrated the most effectiveness against paraoxon and DFP in neuroblastoma cells were C_{60}-COOH, $Sc_3N@C_{80}$-OH, and C_{70}-OH.

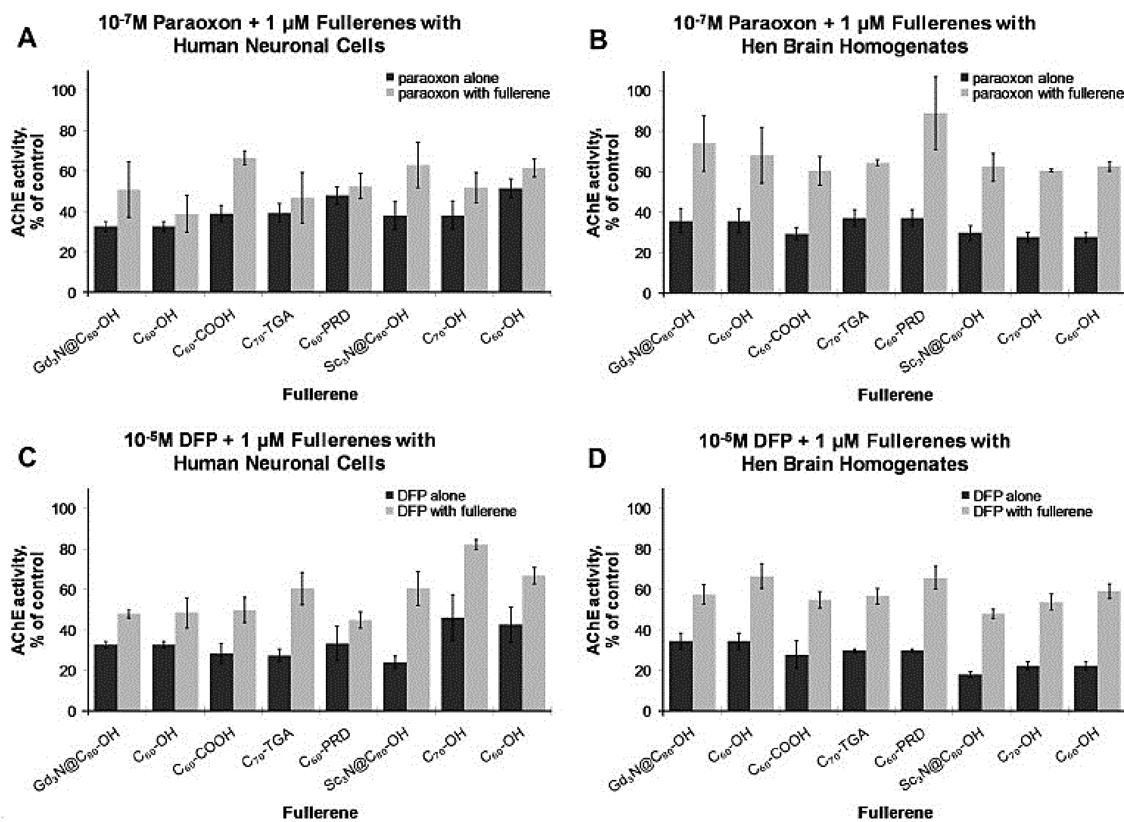

Fig. 3. Comparison of AChE activity in human neuroblastoma SH-SY5Y cells and hen brain homogenates treated with paraoxon or DFP in the absence and presence of 1 μM fullerene derivatives. Paraoxon or DFP were incubated with the fullerene derivative (or vehicle) for 15 min before addition to incubates containing the neuronal cells or brain homogenates. Results are presented as mean ± SE of >3 different assays. Results with commercial C_{60}-OH are in the second triplet of columns and results with C_{60}-OH made by Luna nanoWorks are provided in the last triplet of columns. For (A) (neuronal cells), significant differences ($p < 0.05$) between cell AChE activity after paraoxon in the absence and presence of fullerenes were noted for $Gd_3N@C_{80}$-OH, C_{60}-COOH, and $Sc_3N@C_{80}$-OH. For (B), and (D) (brain homogenates), differences in brain AChE after paraoxon were noted when all fullerene derivatives were included in the incubates. For (C) (neuronal cells), differences in cell AChE after DFP were noted for all but C_{60}-PRD.

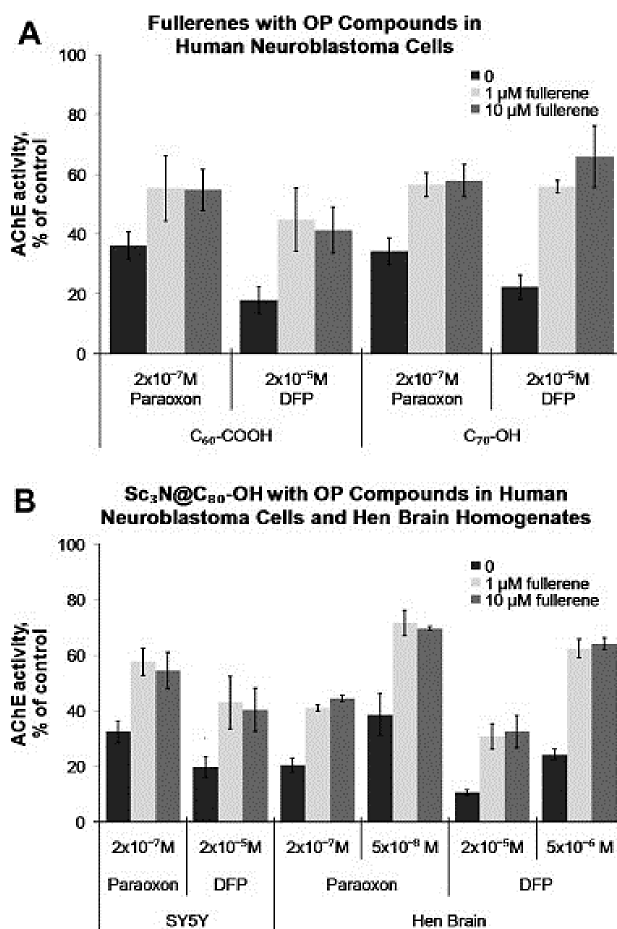

Fig. 4. Comparison of the effectiveness of 1 and 10 μM fullerene derivative concentrations when protecting human neuroblastoma cells and hen brain homogenates from C_{60}-COOH, $Sc_3N@C_{80}$-OH, and C_{70}-OH. (A) demonstrates that increasing the fullerene derivative concentration from 1 to 10 μM did not significantly affect changes in AChE activity. (B) demonstrates differences in fullerene derivative effectiveness against paraoxon and DFP-induced AChE inhibition in neuroblastoma cells and hen brain homogenates, noting that fullerenes had similar effects in cells and brain homogenates, and that greater benefit was seen when concentrations of the OP compounds were lower. Improvement was significant ($p < 0.05$) for all cases demonstrated in Fig. 4.

As 1 μM fullerenes appeared effective against 10^{-7} and 5×10^{-8} M paraoxon-induced AChE inhibition in hen brain homogenates, further testing with these paraoxon concentrations with lower concentrations of $Gd_3N@C_{80}$-OH, commercial C_{60}-OH, and C_{70}-TGA was done. Capability to keep AChE activity >20% higher than in incubates with 5×10^{-8} M paraoxon alone was noted with all three of these fullerene derivatives when their concentrations were lowered to 0.001 μM, and, with $Gd_3N@C_{80}$-OH, at concentrations as low as 0.001 nM (data not shown). These concentrations are sufficiently lower than the paraoxon concentration that adsorption of the OP compound appears unlikely to be responsible for the fullerene effect.

4. Discussion

The present study screened eight derivatized fullerenes for their ability to protect human neuroblastoma cells and hen brain homogenates from paraoxon- and DFP-induced AChE inhibition *in vitro*. All showed protective capability, being more effective when paraoxon and DFP concentrations were not at the highest concentrations tested. Given that the fullerene derivatives tested were effective, although at lower concentrations of paraoxon and DFP, and that they had no detrimental effects on cells, they may serve as a platform for developing next generation compounds that protect against OP exposure.

The *in vitro* studies described here suggest that the derivatized fullerenes would be safe as no cytotoxicity was observed at concentrations that were protective. As expected (because they are not hardy cancer cells), the primary neuronal cells were more sensitive to a detrimental effect of the fullerene derivatives (reduced ATP production) than the neuroblastoma cells. Our results are consistent with those that demonstrated no toxicity to human leukocytes, keratinocytes, or bovine alveolar cells using water soluble, well characterized and purified fullerene preparations (Kolosnjaj et al., 2007). Water solubility, particle size, and surface charge all affect cytotoxicity (Johnson et al., 2010).

Derivatized fullerenes have previously been reported to have free radical scavenging (antioxidant) capability, including restorative effects on protective enzymes and glutathione (Iezzi et al., 2002, Johnson et al., 2010,Lee et al., 2002, Olmstead et al., 2001, Wang et al., 1999 and Yin et al., 2006). This observation was confirmed with the eight test compounds used in these studies, as all demonstrated concentration-related capability to scavenge free radicals produced in a xanthine/xanthine oxidase system. This *in vitro* system used here is biologically relevant as subsequent production of reactive oxygen species, including peroxides and nitric oxides, can lead to cell injury. Given that evidence of oxidative stress induced by exposure to OP compounds (e.g., depletion of extracelluar redox substances such glutathione) has appeared in the literature (Jett and Narova, 2000, Pazdernik et al., 2001, Pena-Llopis et al., 2005 and Qiao et al., 2005), fullerenes could scavenge the OP products that deplete these substances. This property could reduce the deleterious effects of OP compounds that extend beyond acetylcholinesterase inhibition. Therefore, fullerene derivatives are potentially an attractive tool for counteracting some of the deleterious effect of these toxicants.

Although direct association between AChE inhibition and free radical production cannot be made with the test system used here, the derivatized fullerene test compounds were protective. Their ability to scavenge the OP compound itself, as reported for another type of nanomaterial (Newman et al., 2007) could have been a contributor, as this would limit both OP-induced AChE inhibition and production of substances associated with oxidative stress.

The possible effectiveness of derivatized fullerenes in protecting from toxicity that follows significant exposure to AChE-inhibiting OP compounds is of interest. Reports suggest fullerene derivatives similar to those used in the present experiments can be administered to rodents, protecting them from damage to the nervous system, without evidence of detrimental effects

(Dugan et al., 1997, Dugan et al., 2001, Gharbi et al., 2005, Johnson et al., 2010, Quick et al., 2008, Wang et al., 2006 and Yamago et al., 1995). Like oximes (Shrot et al., 2009), the protective effect on the nervous system occurs even though the brain is not the organ receiving the highest concentrations of fullerenes after systemic administration (Wang et al., 2006 and Yamago et al., 1995). We have also demonstrated that fullerene derivatives are potent anti-inflammatories and the efficacy critically depends on the moieties added to the carbon cage (Dellinger et al., 2009 and Ryan et al., 2007). Potential usefulness of derivatized fullerenes in reducing the risk of OP-induced toxicities deserves investigation. This is because the most effective treatment of the cholinergic poisoning OP compounds induce (atropine) is not given before clinical evidence appears because the treatment itself can be toxic at its antidotal dosage (Taylor, 2006). In contrast, the apparent safety of derivatized fullerene derivatives may allow them to be used after exposure but before clinical signs of toxicity appear.

It is not appropriate to extrapolate these *in vitro* results to situations in which humans and animals may need protection from potent, direct-acting AChE inhibitors such as the potent OP agents that provide chemical threats or their surrogates used here (Ehrich and Dorman, 2004). Pharmacokinetics parameters relevant to whole animal exposures are difficult to impossible to study using cultured cells or tissue homogenates. Absorption, metabolism and excretion of toxic agents such as the OP compounds takes place *in vivo*, but not in the incubates used here. Of particular concern is the ability of proposed amelioration agents to reach the OP compound before it gets into the nervous system, or the ability to interact with the enzymatic target of the OP compound within the nervous system. Lipophilicity may be a contributing factor as derivatized fullerenes cross the blood‐brain barrier; fullerenes examined here have partition coefficients similar to those used in published distribution studies (Yamago et al., 1995). For the present studies, the fullerene derivatives were incubated with the paraoxon or DFP before the cells were exposed to these AChE inhibitors because direct exposure results in extremely rapid covalent binding of the OP compound to the enzyme (Taylor, 2006). Such *in vitro* studies allowed for examination of multiple compounds and multiple parameters with high efficiency. These studies provide proof-of-concept that derivatized fullerenes may be used as new agents to counteract the effects of OP exposure. Further studies on next generation fullerene derivatives are warranted to identify lead candidates for further *in vivo* studies.

Conflict of interest

None declared.

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