

Studies Exploring the Interaction of the Organophosphorus Compound Paraoxon with Fullerenes

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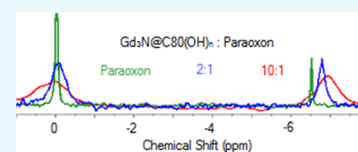
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ABSTRACT: In vitro experiments previously published demonstrated the ability of fullerenes to decrease the capability of organophosphorus (OP) compounds to inhibit acetylcholinesterase. Experiments described herein demonstrate molecular level affinity interactions between fullerenes and the OP test compound paraoxon with NMR spectroscopy. The calculated binding constant of 19 M^{-1} indicates that this binding was not covalent.



INTRODUCTION

Acetylcholinesterase (AChE)-inhibiting organophosphorus (OP) compounds are toxic substances used as insecticides and nerve gases. Cholinergic poisoning associated with AChE inhibition can be rapid and severe.¹ Although protection from OP compounds can be provided by protective clothing, poisonings still occur and rapid treatment with atropine and pralidoxime may be needed for survival. However, situations in which large numbers of people are exposed, such as occurred when an OP compound was released in the subway system of Tokyo in 1995, have prompted the search for additional protectants.^{2,3}

Fullerenes are spherical structures containing 60–80 carbons in their respective molecular structures. They have been proposed for a variety of medical uses, including those based on their antioxidant property and their potential capability to deliver drugs and genes.^{4–6} The C80 metallofullerenes used in this study have also been proposed for use as MRI contrast agents.^{7,8}

Previous results demonstrated protection from OP-induced AChE inhibition in incubates of SH-SY5Y human neuroblastoma cells or hen brain homogenates containing a series of OP compounds plus fullerenes.⁹ This protective effect was demonstrated using a wide range of OP concentrations with a wide variety of derivatized fullerenes. Delays in OP-induced toxic effects were also demonstrated in vivo.^{10–12} The experiments outlined in this article were designed to investigate the mode of action of the OP–fullerene interaction at the molecular level to help explain how derivatized fullerenes decrease OP toxicity. The data generated using nuclear magnetic resonance (NMR) suggest that molecular binding of an OP compound to a fullerene provides a possible mechanism for fullerene reduction of OP-induced AChE inhibition seen in previous studies.^{9–12}

MATERIALS AND METHODS

Test Materials. For these studies, paraoxon-ethyl (97.5% pure, Sigma-Aldrich, St. Louis, MO) and hydroxylated fullerene nanomaterials (Luna NanoWorks, Danville, VA) were used. The fullerenes listed in Table 1 include a C₇₀-OH fullerene and a C80 gadolinium trimetaspere metallofullerene (Gd₃N@C80(OH)_n).

The fullerene compounds were prepared by functionalizing the surface of the cage molecule with water solubilizing small molecule groups. C₆₀(OH)_n has approximately 14 hydroxyl groups on the surface of fullerene C₆₀. C₆₀(COOH) and C₆₀-pyrrolidine have the same number of six carboxylic acid and pyrrolidine groups, respectively. C₇₀(OH)_n has about 20 hydroxyl groups. M3N@C80(OH)_n has 24 hydroxyl groups for both M as Sc and Gd. C₇₀-Bis-malonate has two malonic acid groups attached to the fullerene surface (Figure 1).

Chromatographic Methods for the Examination of OP–Fullerene Interactions. *Thin-Layer Chromatography (TLC).* Initial experiments were done with a mixture of C₇₀-bis-malonate fullerene (C₇₀-OH) and paraoxon using silica as the stationary phase and chloroform/methanol/trifluoroacetic acid as the mobile phase (50:45:5) in molar ratios of 1:1, 4:1, and 10:1.

High-Performance Liquid Chromatography (HPLC). HPLC analysis was also used to examine possible covalent OP–fullerene interactions. The hypothesis was that the fullerene could form a covalent bond with the phosphorus of paraoxon, releasing the leaving group *p*-nitrophenol. For these analyses, each of the six fullerenes listed in Table 1 was tested. The respective fullerene solutions were made up in a 0.1 M phosphate buffer at pH 8.0. Each of the individual solutions

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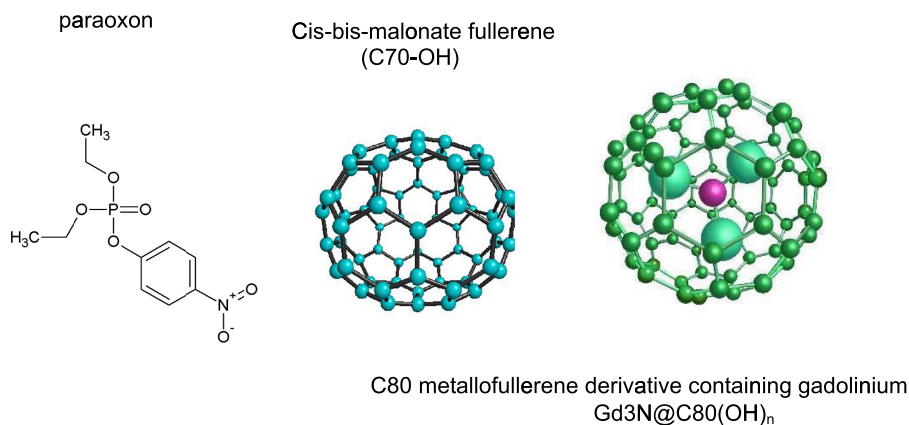


Figure 1. Structures of test products.

Table 1. HPLC Analysis of the Incubation Mixture between the Fullerenes and the Paraoxon^a

sample	paraoxon recovery (%)	<i>p</i> -nitrophenol
paraoxon control	100	not detected
Gd ₃ N@C ₈₀ (OH) _n	99	not detected
C ₆₀ (OH) _n	96	not detected
C ₆₀ (COOH)	102	not detected
C ₇₀ -bis malonate	98	not detected
C ₆₀ -pyrrolidine	97	not detected
Sc ₃ N@C ₆₀ (OH) _n	100	not detected

^aDetection limit for *p*-nitrophenol: <10⁻⁷ M.

was spiked with 100 μL of 1 μM paraoxon made up in the same buffer before incubation for 25 min at 37 °C. A portion of the final sample volumes was aliquotted into 2 mL amber autosampler vials and loaded into the autosampler of an Agilent 1100 Series HPLC. The respective solutions (20 μL) were injected for analysis. The analytes of interest were separated on an Agilent Zorbax XDB C18 column (150 mm × 4.6 mm × 5 μm) using a gradient mobile phase composition that started in the aforementioned phosphate buffer before increasing the concentration of methanol with a constant flow rate of 0.8 mL/min. UV detection and quantification of paraoxon and *p*-nitrophenol were performed at 270 and 312 nm, respectively.

Nuclear Magnetic Resonance Spectroscopy for the Examination of OP–Fullerene Interactions. Additional interactions of OP and fullerene were examined by the complexation of paraoxon with two different hydroxylated fullerenes. The test fullerenes were the C₈₀ derivative containing gadolinium (Gd₃N@C₈₀(OH)_n) and a nonmetallic C₇₀ derivative (C₇₀-OH). All proton (¹H) and phosphorus (³¹P) NMR spectra were collected on a Bruker Advance III 600 NMR instrument (600 MHz, 242 MHz, respectively). Prior to the NMR experiments, the purity of paraoxon was checked in deuterated chloroform (CDCl₃) with TMS by ¹H NMR.

Each of the NMR experiments with paraoxon and the fullerenes C₇₀-OH or Gd₃N@C₈₀(OH)_n was run in deuterium oxide (D₂O) with phosphoric acid (H₃PO₄) 0.04% as the internal reference (chemical shift δ = 0.00 ppm). Proton-decoupled ³¹P NMR spectra were collected for each sample. Increasing quantities of fullerenes were added to 0.5 mL of a fresh solution of paraoxon 0.5 mg/mL (1.8 mM) in D₂O. The mixture was homogenized with a vortex mixer, and 10 μL of H₃PO₄ 2% in D₂O were added. The solution was then

transferred to a clean, dry NMR tube for analysis. NMR spectra data were acquired 15 min after preparing the tube.

RESULTS

TLC. No changes in migration distances among the fullerene alone and the fullerene + paraoxon were observed when analyzed at *t* = 0, 30 min, and 18 h. These results suggest that paraoxon was not forming a covalent bond with the fullerene molecule.

High-Performance Liquid Chromatography (HPLC). No significant changes in paraoxon concentration were observed and the metabolite of paraoxon, *p*-nitrophenol, was not detected in any of the incubated mixtures. The detection limit for *p*-nitrophenol was set at a concentration of <10⁻⁷ M (Table 1 and Figure 2). These results further substantiate TLC results, indicating that paraoxon was not forming a covalent bond with the fullerene molecule.

No major change in the chemical shift was observed after the addition of sodium chloride to the mixture of C₇₀-OH, even after

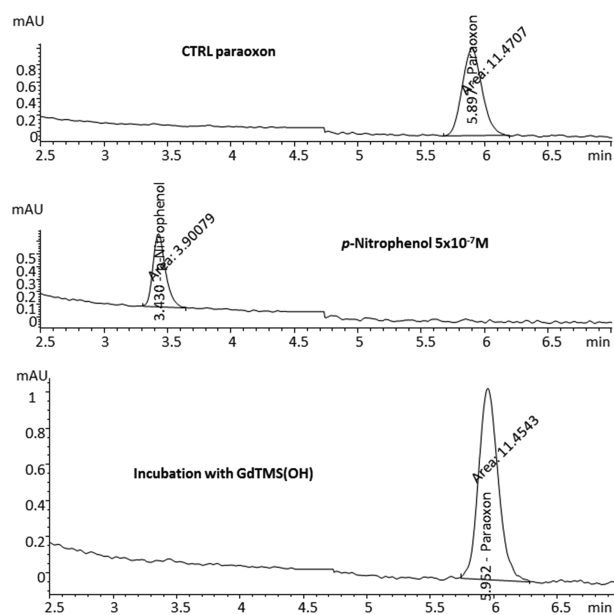


Figure 2. HPLC chromatograms of the paraoxon (270 nm, top) and *p*-nitrophenol (312 nm, middle) control standards and the incubation mixture of paraoxon with Gd₃N@C₈₀(OH)_n (bottom), labeled in the figure as GdTMS(OH).

long incubation times. Sodium chloride may not be the best salt to deaggregate the fullerenes as it was difficult to get a strong enough signal in the presence of sodium chloride. Sodium phosphate buffer has been reported to be a stronger salt to disrupt aggregation.¹⁴

Table 2. ³¹P NMR Data for Paraoxon-Gd₃N@C80(OH)_n Fullerene Interactions^a

[Gd ₃ N@C80(OH) _n] (M)	Gd:paraoxon ratio	δ (ppm)	Δδ (ppm)
0.0 × 10	0	6.49	N/A
3.6 × 10 ⁻³	2:1	6.71	0.22
9.0 × 10 ⁻³	10:1	6.97	0.48

^aΔδ (ppm) represents the observed chemical shift (δ) change for that specific fullerene-containing sample vs the δ observed for the paraoxon control.

NMR Results for Gd₃N@C80(OH)_n. (Table 2 and Figure 3) demonstrated an upfield change in the chemical shift as the concentration of fullerene was increased. Band broadening in the NMR signal was also observed, most likely as a result of strong paramagnetic interferences associated with the increasing inorganic Gd content. Longer acquisition times were used in an attempt to compensate for this observed effect but did not produce ideal peak shapes necessary for the quantitative NMR analysis. As a result, subsequent studies described herein were performed using the C₇₀-OH fullerene. In doing so, higher concentrations of the fullerene were able to be used while maintaining ideal peak shapes, thereby allowing observable chemical shift changes (Δδ) within a reasonable amount of spectral collection time (Table 3 and Figure 4).

Plotting the difference in the ³¹P NMR chemical shift (Δδ) versus fullerene concentration resulted in a binding isotherm curve that reaches a plateau at around 0.03 M (Figure 5). The binding constant (K_b) could be calculated from the double reciprocal plot of the binding isotherm plot (Figure 6) following the Benesi–Hildebrand equation.¹³

Table 3. ³¹P NMR Data for Paraoxon–C₇₀-OH Fullerene Interactions^a

[C ₇₀ -OH] (M)	C ₇₀ :paraoxon ratio	δ (ppm)	Δδ (ppm)	1/[C ₇₀] (M ⁻¹)	1/Δδ (ppm ⁻¹)
6.3 × 10 ⁻²	70:1	9.10	3.03	15.769	0.330
4.5 × 10 ⁻²	50:1	8.96	2.89	22.076	0.346
3.6 × 10 ⁻²	40:1	8.82	2.75	27.595	0.364
2.7 × 10 ⁻²	30:1	8.6	2.53	36.793	0.395
1.8 × 10 ⁻²	20:1	7.69	1.62	55.190	0.617
9.1 × 10 ⁻³	10:1	6.97	0.90	110.380	1.111
4.5 × 10 ⁻³	5:1	6.58	0.51	220.760	1.961
0.00	0	6.07	N/A	N/A	N/A

^aΔδ (ppm) represents the observed chemical shift (δ) change for that specific fullerene-containing sample vs the δ observed for the paraoxon control.

$$\frac{1}{\Delta} = \left(\frac{1}{\Delta^b K^b} \right) \frac{1}{[C_{70}]} + \frac{1}{\Delta^b}$$

where the binding constant (K_b) was calculated by dividing the intercept by the slope from the double reciprocal plot (1/Δδ) versus 1/[C₇₀] and was determined to be 19 (M)⁻¹.

DISCUSSION

The results suggest that derivatized fullerenes interact with OP compounds in a manner much like cyclodextrins that have previously been shown to decrease the OP capability to inhibit AChE.^{15–17} The binding constant of three cyclodextrins incubated with paraoxon ranged from 110 to 182 mole/L in a publication that provided these data.¹³ The OP aggregate with a derivatized fullerene in experiments described here has a binding constant about 10 times lower than cyclodextrin–OP aggregates. This may be because there is no cavity in the fullerene to complex the OP molecule. Most of the complexation studies done with OP compounds have been done with proteins where the hydrophobic and π–π interactions are larger. For example, the binding constant of paraoxon to human bovine albumin was found to be 1.914 × 10⁻⁴ mole/L.¹⁸ The binding constant of paraoxon to the C₇₀-OH is modest if we compare it

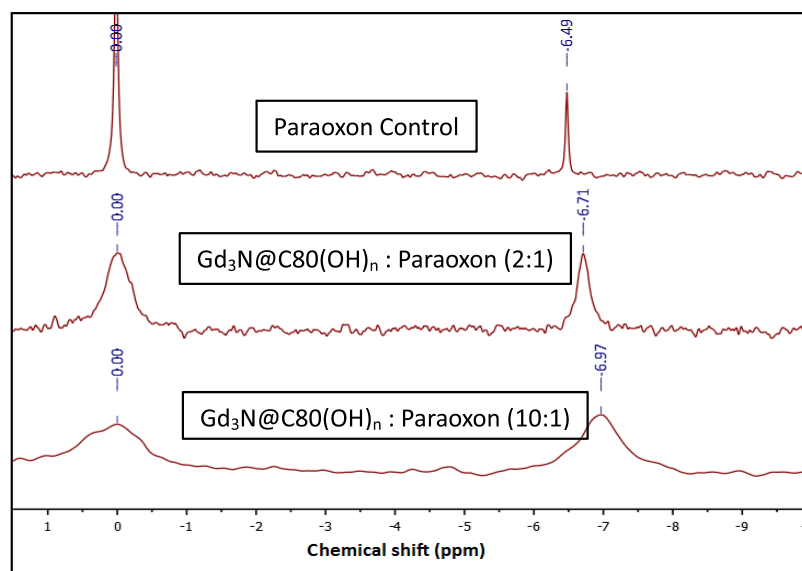


Figure 3. ³¹P NMR spectra of paraoxon with increasing concentrations of the Gd₃N@C80(OH)_n fullerene. The peak for the internal reference standard, H₃PO₄, is shown at 0.00 ppm.

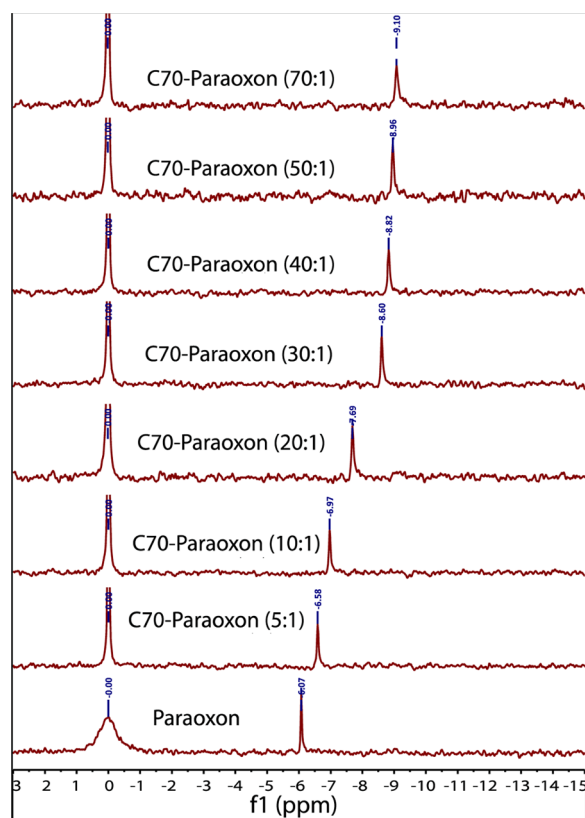


Figure 4. ^{31}P NMR spectra of paraoxon with decreasing concentrations of the $\text{C}_{70}\text{-OH}$ fullerene. The peak for the internal reference standard, H_3PO_4 , is shown at 0.00 ppm.

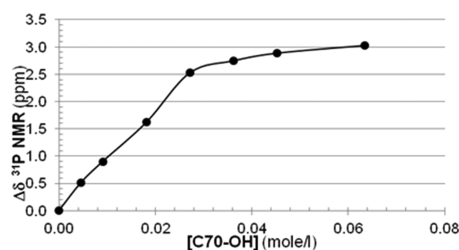


Figure 5. Binding isotherm curve generated by ^{31}P NMR data. Incubation of paraoxon with increasing concentrations of $\text{C}_{70}\text{-OH}$.

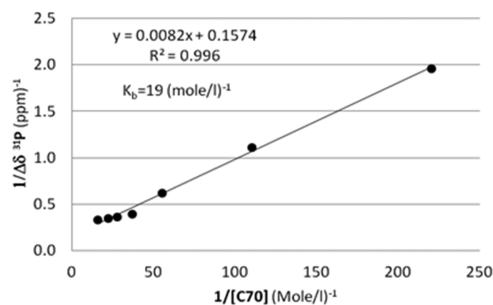


Figure 6. Benesi-Hildebrand plot for the binding of paraoxon with the $\text{C}_{70}\text{-OH}$ fullerene.

to the binding constant of immobilized paraoxon to cholinesterase ($3084\text{--}6327\text{ M}^{-1}$ depending on the enzyme source).¹⁹

Another study examined the interaction of cyclodextrins with two OP nerve gases and published disassociation (K_d) values of

$7\text{--}9\text{ mM}$.²⁰ The K_d of the paraoxon-cyclodextrin aggregates contrasts with the K_d (0.34 mM) reported for paraoxon with human recombinant acetylcholinesterase, a difficult disassociation.²¹

OP compounds interact with solubilized fullerenes, but the difference in binding constants between OP compounds with cholinesterases versus OP compounds with solubilized fullerenes is vast. Also, although not specifically calculated for the compounds used here, the disassociation constant for OP-fullerene is highly likely to be greater than for OP-AChE. This suggests that solubilized fullerenes are likely to only have a minor role for prophylaxis of potentially severe OP toxicity after dermal exposure, including toxicity associated with OP nerve agents. However, further exploration may be warranted to determine if, especially in combination with other protectants, they could be used after dermal exposure to OP compounds occurs, especially if signs of toxicity had not appeared before treatment could commence. Topical exposures to toxic OP compounds are relevant, and time for absorption is longer by this route of exposure, so substances like fullerenes that interact with OP compounds but do not have intrinsic toxicities could have future value in amelioration of OP-induced toxicities especially if applied alone or in combination before potential exposure or after exposure and before onset of symptoms. Further experiments are needed to determine such potentially beneficial possibilities.

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Notes

The authors declare no competing financial interest.

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