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Biochemical and Biophysical Research Communications 357 (2007) 200–205
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Reversible AChE inhibitors in *C. elegans* vs. rats, mice

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Received 19 March 2007
Available online 28 March 2007

Abstract

We are investigating whether *Caenorhabditis elegans* could be used as a screen for vertebrates by comparing the responses of components of its cholinergic system to well-characterized toxicants. We assessed whether *C. elegans* displays similar toxicity as rats and mice to reversible acetylcholinesterase (AChE) inhibitors, and sought to corroborate that the toxicity mechanism is the same. To determine relative potencies, movement–concentration curves were generated, 50th percentiles for movement were located, ranked and compared statistically to rat and mouse oral acute LD50s. The ranking was significantly correlated to rat and mouse rankings ($\alpha = 0.05$). We measured a concentration-dependent decrease in AChE activity correlating to a decrease in movement for each carbamate, suggesting that the mechanism of toxicity is the same. Finally, as seen in mammals, inhibition of AChE activity occurred before a movement decrease. The response of *C. elegans* to carbamate exposure shows significant correlation to rat and mouse data.

Keywords: *Caenorhabditis elegans*; Carbamate; Pesticide; Screen; Toxicity; Acetylcholinesterase

We are exploring the feasibility of *Caenorhabditis elegans* to serve as a screen for predicting relative vertebrate neurotoxicity. A sensitive, quantifiable, easily-reproduced, fast, and inexpensive screen is needed to enable rapid prioritization of compounds most detrimental to the nervous system, as well as to serve as an environmental indicator of exposure to neurotoxicants. There are logistical reasons and advantages of the physiology of *C. elegans* that show promise towards its use as a screening organism for at least some aspects of vertebrate neurotoxicology. *C. elegans* reproduces exponentially, completes its life cycle in approximately 3 days and is well-characterized. In addition, large-scale worm culturing and automated high-volume worm sorting techniques such as the Union Biometrica COPAS Biosort (Harvard Biosciences, Boston, MA) have lead to much investigation of *C. elegans* as a potential high-throughput organism by pharmaceutical companies [1–3] and the National Toxicology Program (http://ntp.niehs.nih.gov). In addition to the practical advantages of using the simple nematode, our current knowledge of the connections from gene to neural circuit to behavior in *C. elegans* makes it an indispensable behavioral model [4,5]. In this experiment, we continued our analysis of toxicants affecting the acetylcholinesterase (AChE) enzymes of the cholinergic nervous system of *C. elegans* by examining the endpoints of movement and AChE activity. Our intent is to explore correlations between the effects of well-characterized toxicants to components of the cholinergic nervous system of *C. elegans* to existing vertebrate data in the hope of defining the strengths and weaknesses of comparison between them.

Past studies have shown significant correlations between the relative toxicity of AChE inhibitors predicted by *C. elegans* and existing mammalian data [6,7]. However, both studies were conducted using irreversible AChE inhibitors, organophosphates (OPs). Evidence in the literature suggests reversibility of both the OP and carbamate classes of pesticides in nematodes that is greater than normally seen in vertebrates [8–11]. In view of the reversibility of common AChE inhibitors, coupled with the short lifespan of *C. elegans*, this study addresses the following questions:

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0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.03.122

can a change in *C. elegans*' movement be accurately measured after exposure to a class of reversible AChE inhibitors, the carbamate pesticides? Do potencies correlate to vertebrate data for this class? And, finally, can we corroborate that the mechanism of toxicity of the reversible carbamates is the same in *C. elegans* and vertebrates?

Materials and methods

Culture of nematodes. We obtained *C. elegans*, wild-type strain N2 from the *Caenorhabditis* Genetics Center (Minneapolis, MN, USA). We raised all developmental stages of all strains of *C. elegans* in 115 mm Petri dishes with K-agar (0.032 M KCl, 0.051 M NaCl, 0.1 M $CaCl_2$, 0.1 M $MgSO_4$, 2.5% Bacto-peptone, 0.17% Bacto-agar, and 0.01% cholesterol), a modification of Williams and Dusenbery [12]. We seeded the plates with *Escherichia coli* strain OP50 as a food source and incubated at 20 °C for 24 h [13]. To age-synchronize the populations, we harvested eggs from adult populations. Two-day juvenile populations were transferred to a plate containing a fresh lawn of OP50 to maintain high nutritional status before testing on day 3 [14]. All exposures were carried out using 3-day-old adults.

Chemicals tested and exposure conditions. We ordered all compounds from Sigma–Aldrich (St. Louis, MO, USA) as reagent-grade chemicals. For each replicate, we dissolved a known amount of each chemical in K-medium to create a stock solution with further dilutions carried out by adding additional K-medium (0.032 M KCl, 0.051 M NaCl in dH_2O) [15]. Exposures for assessment of movement used a 12-well sterile tissue culture plate, where we loaded 5 µl (approximately 100 worms) into a single 1-ml well containing K-medium for the controls or a carbamate. We prepared a control population for each exposure. The same exposure routine was used to assess AChE activity with the exception that 40 µl of worm pellet (gravity-settled worms) were distributed into 2 wells containing K-medium with or without a carbamate. In accordance with previous studies, we placed all exposure plates in an incubator at 20 °C for 4 h in the absence of food [6,16].

To control for any daily variation between worm populations collected from different egg populations and raised in separate cultures, we normalized all replicates to their control by dividing the movement or AChE activity of a single exposure concentration by its control's measured value, creating a percent of control.

Movement tracking. We patterned the movement tracking after Boyd et al. [17]. Immediately following the 4 h exposure, we transferred the worms with a Pasteur pipette into 2 ml glass centrifuge tubes. They were washed by allowing the worms to gravity settle into a pellet, removing the supernatant, adding ~1.5 ml of fresh K-medium, gently mixing the worms by creating bubbles with a Pasteur pipette, and repeating the process for a total of three washes. We then transferred 5 µl of the settled pellet (~50–80 worms) to a cooled, 2 ml 1% agar pad on a clear glass slide measuring 100 mm × 200 mm. The worms were allowed to disperse on the agar pad inverted over a Petri dish filled with water to avoid desiccation. We began movement tracking at exactly 1 h after the end of exposure for each replicate. We placed the individual glass slides in a tracking chamber with a gentle humidified air stream. Using a video camera interfaced with a Macintosh® computer that contains a modification of the NIH tracking software [18], the individual worm movements were tracked and recorded to an Excel® spreadsheet. We used a macro to calculate the average micrometer of movement per worm per second.

Acetylcholinesterase activity assay. After we constructed a movement–concentration response curve for each carbamate, acetylcholinesterase activity was measured at three concentrations along the curve in order to construct an acetylcholinesterase activity–concentration response curve for each chemical. We chose concentrations representing the EC50 for movement (concentration required to reduce movement by 50% relative to controls), a value approximating an EC80, and a value termed a 'no-observable-effect-concentration for movement' (NOEC) because the worm population was exposed, but we measured no significant change in movement compared to controls. We measured AChE activity using an adaptation of the Ellman assay most closely resembling Moulton et al., to measure AChE activity in tissues with low AChE activity [19,20]. After a 4-h exposure, we washed the worms as detailed above, transferred them to 1.5 ml plastic centrifuge tubes and centrifuged at 4 °C for 10 min. After the diluent was removed, the worms were flash-frozen in liquid nitrogen and placed in a −80 °C freezer until analyzed. During analysis, we added phosphate buffer 0.05 M (pH 8.0, 0.1 M potassium phosphate monobasic, 0.1 M potassium phosphate dibasic) to each sample, and then homogenized the sample using a Teflon pestle and a vortexor for 20 s. The worms were again centrifuged at 4 °C for 10 min and the supernatant was transferred to new 1.5 ml centrifuge tubes. We incubated each sample for 5 min in a disposable 1.5 ml cuvette containing 0.25 mM dithiobis (2-nitrobenzoic acid). Acetylthiocholine-iodide 156 mM was added; it was inverted to mix and placed immediately into a Shimadzu UV-1601 spectrophotometer (Shimadzu Scientific Instruments; Columbia, MD, USA), where we measured the change in absorbance at 405 nm over 90 s. We quantified protein concentrations for each sample using the Bio-Rad protein assay kit II (Bradford assay) (Bio-Rad Laboratories; Hercules, CA, USA). We used bovine serum albumin as the protein standard, and analyzed the samples at an absorbance of 595 nm.

pH measurement. As pH has been shown to affect movement, we measured the pH values for the highest and lowest concentrations of each compound using an Orion Z20A pH meter at room temperature (22–24 °C) [7,21] (Orion research; Beverly, MA, USA). No effects of pH on *C. elegans*' movement have been observed within the pH range of those observed for the 11 carbamates used in this study [7]. Because of this, we did not use buffers or exclude any carbamates from analysis due to confounding pH effects. Measured pH values of all concentrations ranged from 5.8, K-medium, to 3.9.

Statistical analysis. We used the χ^2 and the Shapiro–Wilk tests for normality (SAS Inc.; Cary, NC, USA). We modeled movement and concentration for each compound using nonlinear regression, PROC NLIN (SAS Inc.; Cary, NC, USA) and generated EC50 values with 95% confidence intervals. We ranked the EC50 values from most to least potent. We assessed the correlation between the rankings of *C. elegans* and rat or mouse by using Spearman's correlation coefficient. In the event of overlapping confidence intervals for *C. elegans*' EC50 values, we used the mean of the ranks of the tied chemicals to calculate Spearman's correlation coefficient. In the case of dioxacarb, where two values of the oral acute LD50 were reported for rats, a mean value was used. We obtained LD50s from the Registry of Toxic Effects of Chemical Substances (RTECS) database. For the AChE activity assay, we performed an ANOVA among the concentrations of a particular chemical, with Tukey's analysis to determine which concentration groups differed at a significance level of 0.05 (SAS Inc.; Cary, NC, USA).

Results

We exposed *C. elegans* to 11 carbamates, a class of environmental contaminants with a well-characterized mode of toxicity in mammals, i.e. reversible AChE inhibition. Following exposure, we generated movement–concentration plots and an EC50 was determined for each pesticide in order to test whether the order of potency corresponded to the ranking order of oral acute LD50s for the rat and mouse. We chose LD50s as a basis for comparison because they are uniformly generated and, unlike a comparable behavioral endpoint, available for all of these chemicals in rats and mice. As the cause of death for the rat and mouse lethality studies is understood to be inhibition of AChE, lethality is an endpoint along a continuum of neurotoxic effects. We last measured the AChE activity to cor-

roborate that the cause of toxicity is the same in *C. elegans* as it is in rats and mice.

Movement

We began by recording movement values for all concentration groups at 30, 45, 60, 75, and 90 min post-exposure to determine reversibility of the compounds as measured by movement. We observed no significant change between 45 and 90 min. Therefore, we standardized the post-exposure movement analysis to 1 h post-exposure for every subsequent replicate. In this way, we were able to construct 11 movement–concentration plots using the chosen carbamates (Fig. 1). Most movement responses (100–0% of control movement) of each carbamate occurred across two orders of magnitude of concentration, while two compounds (ethiofencarb and formetanate) responded over a concentration range of one order of magnitude or less. Overall, the EC50 values fall within approximately 2.5 orders of magnitude of concentration. The ranking of the carbamates using *C. elegans*' movement was significant to $\alpha = 0.05$ when compared to both rat and mouse oral acute LD50 values using Spearman's correlation coefficient (Table 1). The combined values of the control replicates for all 11 carbamates tested were normally distributed with a mean value of 3.52 μm/s (95% confidence interval 3.41–3.62).

Acetylcholinesterase activity

Using ANOVA, concentration was a significant predictor of AChE activity for all carbamates ($p < 0.01$). Following exposure to the NOEC-movement concentrations, AChE activity was significantly less than control in 8 of the 11 carbamates. This increased to 9 of 11 for the EC50 concentrations and 10 of 11 for the EC80 concentration groups. The only carbamate not to show a statistically significant reduction between any concentration group and its control was ethiofencarb, which caused both comparatively less and more variable AChE inhibition than other carbamates. AChE activities among controls were uni-

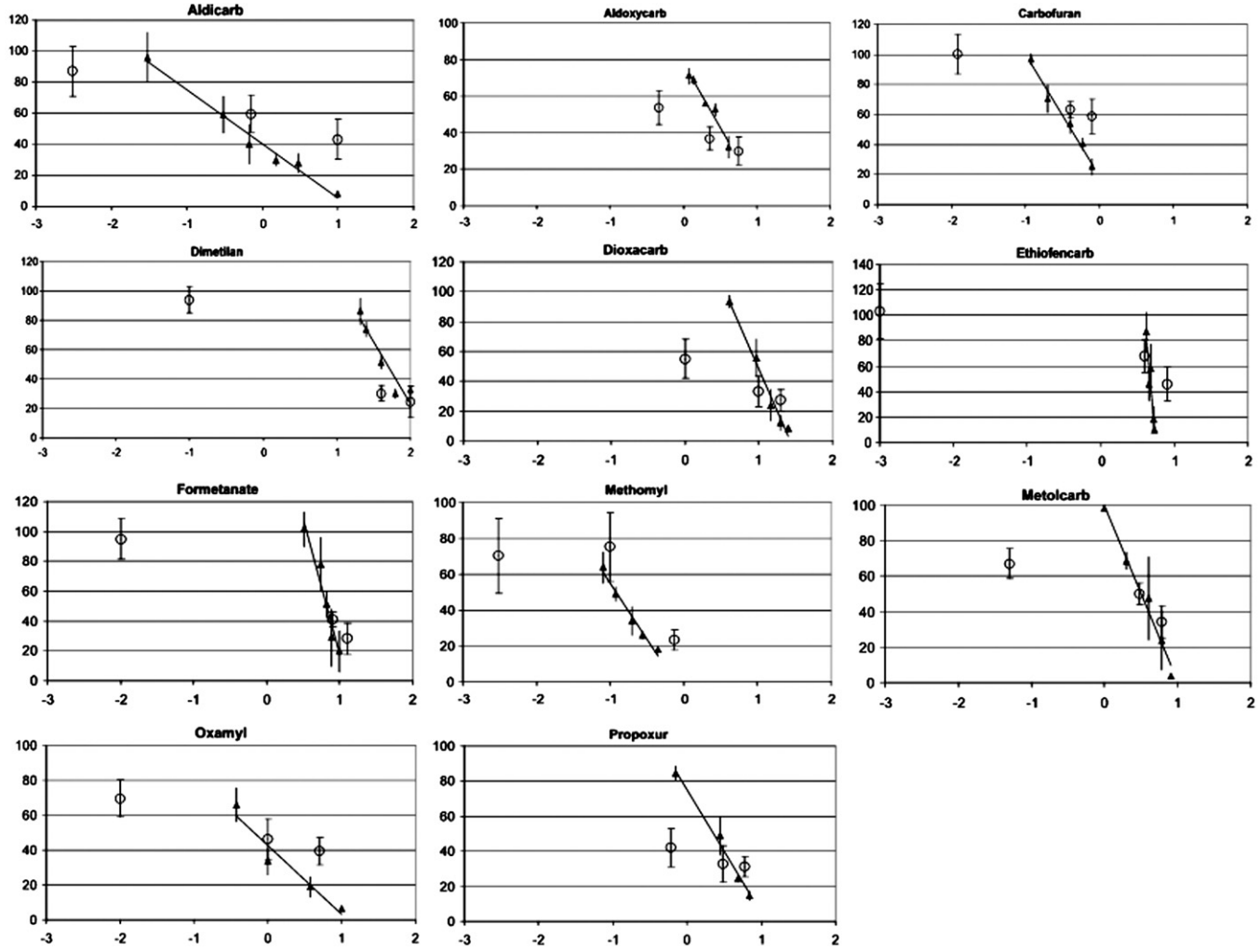

Fig. 1. In alphabetical order, the individual plots of movement (▲) and AChE activity (○) shown as percent control against a Log(mM) scale for all 11 carbamates tested are displayed with their corresponding standard error bars. Solid black line denotes least-squares regression line for movement.

Table 1
Comparison of *Caenorhabditis elegans*' EC50s with rat and mouse oral acute LD50s using Spearman correlation coefficient

Chemical	EC50 (mM)	95% Confidence interval (mM)	Rat LD50 (mg/kg)	Mouse LD50 (mg/kg)
Methomyl	0.1039	(0.08390, 0.1285)	14.7	10
Carbofuran	0.4044	(0.3504, 0.4668)	5	2
Aldicarb	0.5263	(0.3348, 0.8274)	0.46	0.3
Oxamyl	0.6745	(0.4006, 1.135)	2.5	2.3
Propoxur	2.350	(1.997, 2.764)	41	23.5
Aldoxycarb	2.541	(1.961, 3.292)	20	NA[a]
Metolcarb	3.161	(2.404, 4.158)	268	109
Ethiofencarb	4.529	(3.781, 5.425)	200	71
Formetanate	6.683	(5.461, 8.176)	20	18
Dioxacarb	9.635	(8.612, 10.78)	40	48
Dimetilan	40.18	(34.56, 46.70)	25	60
		Number of comparisons[b]	11	10
		Rs[c]	0.6059[d]	0.6848[d]

[a] LD50 data for Aldoxycarb in mice not available in RTECS.
[b] Rat ranking data contained 11 comparisons, while mouse data compared 10.
[c] Spearman correlation coefficient (Rs).
[d] Both comparisons are significant to $\alpha = 0.05$.

formly distributed with a mean value of 11.0 nmol/min mg protein (95% confidence interval 9.2, 12.8).

Discussion

We standardized the time interval between the end of the exposure and when either movement was tracked or when the worms were snap-frozen for subsequent AChE activity analysis at 1 h. By doing so, we found changes in movement of populations of *C. elegans* to be a valid predictor of mammalian neurotoxicity. Additionally, our findings suggest that carbamate toxicity appears to occur via AChE inhibition in *C. elegans* as in rats and mice.

Movement

The steep slopes of the movement–concentration plots constructed after exposure to carbamates are comparable to the movement–concentration plots generated by Cole et al. [7], using organophosphates. In addition, the toxicity ranges covered by both classes are similar to mammalian data. The rank correlation performed by Cole et al. [7] found EC50 values for *C. elegans*' movement across 4.5 orders of magnitude of organophosphate concentration, which roughly corresponded to the organophosphate toxicity ranges of 3.5 orders of magnitude for rats and 4 orders of magnitude for mice. In the present experiment, the rank correlation found EC50 values for *C. elegans*' movement that spanned approximately 2.5 orders of magnitude of carbamate concentration; this toxicity range also corresponds to the toxicity range of the chosen carbamates in rats and mice, or approximately 2.5 orders of magnitude for both species.

Exposure to *C. elegans* is via the oral route, as the cuticle layer of *C. elegans* is thought to be relatively impermeable. This is supported by a recent experiment utilizing synchrotron X-ray techniques to study metals distributions in *C. elegans* that showed spatial distribution in several internal compartments but none in the cuticle layer following aqueous exposure [22]. Because the worms were exposed by ingesting the carbamates and because we desired to maintain an aqueous exposure vehicle, this experiment was subject to the limitations of the water solubilities of the carbamate class. Out of the 25 carbamates that we originally identified for inclusion into the study based upon commercial availability, we prepared the maximum concentrations obtainable in water for the 16 that possessed the best toxicity-to-aqueous-solubility ratios. The highest obtainable concentrations of five compounds (Asulam, Carbaryl, Propham, Promecarb, Pirimicarb) in K-medium failed to reduce movement by $\geqslant 50\%$, and were therefore not evaluated further. Therefore, we performed the movement rank comparison over a relatively small toxicity range of carbamates. Despite this, we found a significant correlation between relative toxicities caused by carbamates.

Acetylcholinesterase activity

We measured AChE activity as the 'total AChE activity' normalized to each replicate's control value at concentrations preceding and corresponding to the constructed movement curves. All 11 carbamates show concentration to be a significant predictor of AChE activity ($p < 0.01$). Only ethiofencarb failed to show a significant decline in AChE activity versus control at any exposure group. Although a review of the structure and metabolism of ethiofencarb offers no clues as to why this is observed ethiofencarb possesses an unusually steep movement curve ($\sim$1/2 order of magnitude), which required us to test ethiofencarb concentrations that differed minimally. Perhaps more importantly, the ethiofencarb movement–concentration curve revealed nematode hyperactivity over a wider concentration range than was seen among other carbamates. This required us to test a NOEC that was relatively lower

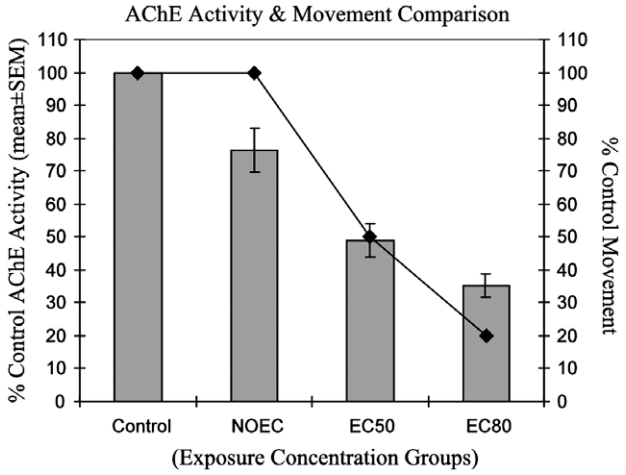

Fig. 2. Mean values of 'AChE activity as a percent of control' for all carbamates tested at each of the three concentrations (NOEC, EC50, and EC80) and control are displayed as bar graphs with standard error bars. The black diamonds connected by a solid line represent the 'movement as a percent of control' at each exposure level: NOEC, 'no difference in movement vs. controls'; EC50, 50% reduction in movement vs. controls; EC80, 80% reduction in movement vs. controls.

than corresponding NOECs of other carbamates tested. Nonetheless, in view of all 11 carbamates showing inhibition of AChE activity directly preceding a decrease in movement, we believe the data suggests that the mechanism of toxicity of carbamates in *C. elegans* is AChE inhibition, as it is in mammals.

Movement vs. cholinesterase activity

A comparison of inhibition of AChE activity in *C. elegans* to its movement decrease provides an additional basis for comparison to vertebrates. From the pooled carbamate data (Fig. 2), we see an average of 24% inhibition of AChE before an effect on movement is seen (NOEC-movement). This is a similar result to Sheets et al. [23], who found that all treatment-related neurobehavioral findings occurred only at dietary levels that produced more than 20% inhibition of plasma, RBC, and brain cholinesterase activity using Fischer 344 rats.

Summary

Rank order correlation coefficients for carbamate potency were significant when compared to either rat or mouse oral acute LD50s. The toxicity range of the carbamates tested was comparable between *C. elegans* and both rat and mouse. A concentration-dependent decrease in AChE activity correlating to a decrease in movement was observed for each carbamate. AChE activity declined before phenotypic effects were observed, as is seen in mammals. In view of the correlation between the actions of neurotoxicants in *C. elegans* and mammals, and because large numbers of *C. elegans* can be raised quickly, inexpensively and without many of the restrictions of animal welfare issues, we believe *C. elegans* has potential as a sensitive, reproducible, and quantitative screen that is, by its nature, rapid and inexpensive.

Acknowledgments

We thank Dr. David Dusenberry for the use of his tracking program; the Interdisciplinary Toxicology Program at the University of Georgia for partial funding of this project; and the *Caenorhabditis* Genetics Center (CGC), which is funded by the NIH National Center for Research Resources (NCRR), for supplying the nematode strain used in this research.

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