Chemical Research in Toxicology

Article

pubs.acs.org/crt

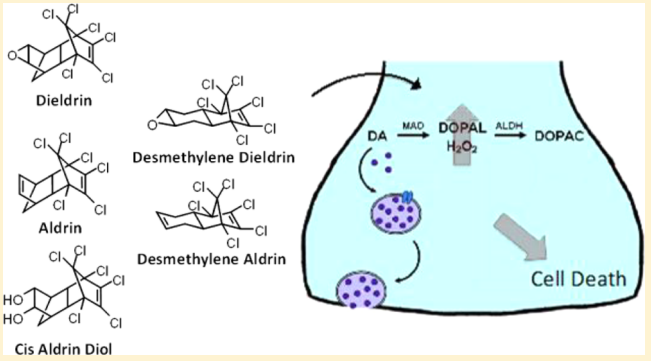
Cellular Localization of Dieldrin and Structure−Activity Relationship of Dieldrin Analogues in Dopaminergic Cells

Erin M. G. Allen,[†] Virginia R. Florang, Laurie L. Davenport, Yunden Jinsmaa,[‡] and Jonathan A. Doorn*

Division of Medicinal and Natural Products Chemistry, College of Pharmacy, The University of Iowa, Iowa City, Iowa 52242, United States

Supporting Information

ABSTRACT: The incidence of Parkinson's disease (PD) correlates with environmental exposure to pesticides, such as the organochlorine insecticide, dieldrin. Previous studies found an increased concentration of the pesticide in the striatal region of the brains of PD patients and also that dieldrin adversely affects cellular processes associated with PD. These processes include mitochondrial function and reactive oxygen species production. However, the mechanism and specific cellular targets responsible for dieldrin-mediated cellular dysfunction and the structural components of dieldrin contributing to its toxicity (toxicophore) have not been fully defined. In order to identify the toxicophore of dieldrin, a structure−activity approach was used, with the toxicity profiles of numerous analogues of dieldrin (including aldrin, endrin, and *cis*-aldrin diol) assessed in PC6-3 cells. The MTT and lactate dehydrogenase (LDH) assays were used to monitor cell viability and membrane permeability after treatment with each compound. Cellular assays monitoring ROS production and extracellular dopamine metabolite levels were also used. Structure and stereochemistry for dieldrin were found to be very important for toxicity and other end points measured. Small changes in structure for dieldrin (e.g., comparison to the stereoisomer endrin) yielded significant differences in toxicity. Interestingly, the *cis*-diol metabolite of dieldrin was found to be significantly more toxic than the parent compound. Disruption of dopamine catabolism yielded elevated levels of the neurotoxin, 3,4-dihydroxyphenylacetaldehyde, for many organochlorines. Comparisons of the toxicity profiles for each dieldrin analogue indicated a structure-specific effect important for elucidating the mechanisms of dieldrin neurotoxicity.



INTRODUCTION

While the exact mechanism of neuronal loss in Parkinson's disease (PD) is unknown,[1] a majority of PD cases are thought to be due to exposure to environmental toxicants, such as insecticides, herbicides, and heavy metals.[2−6] The correlation between pesticide exposure and PD has been corroborated by an increased concentration of pesticides found in the brains of PD patients.[2,4−6] One pesticide of interest is dieldrin, as its level was elevated in PD patients, a result not found in the brains of patients with other neurodegenerative diseases, such as Alzheimer's disease, or in the brains of healthy, control patients.[4] In addition, previous studies have also shown dieldrin to affect a number of cellular processes associated with PD. The consequences of these effects include increased oxidative stress, disruptions in the metabolism and trafficking of dopamine (DA), apoptosis susceptibility, and mitochondrial dysfunction.[1,7−12] Many of the reported adverse effects caused by exposure to dieldrin, such as reactive oxygen species (ROS) production and apoptosis, lead to selective dopaminergic cell dysfunction and/or death.[7,9,12,13] A question that remains is, what is the mechanism via which dieldrin mediates such adverse cellular effects (e.g., ROS formation and disruption of DA trafficking)? Where does dieldrin localize in a cell, and what are the targets? How does dieldrin interact with proteins, covalently or noncovalently, given that it has an epoxide?

Previous studies have assessed how structural changes to dieldrin affect its pesticidal activity in various species of insects.[14−16] Significant alterations to insect toxicity were found even with small changes to dieldrin's structure, such as removal of chlorine atoms or a carbon bridge.[14−16] Such data suggest that specific structural elements are critical for its insecticidal properties. Of question then is how these same structural features relate to dieldrin's dopaminergic toxicity in humans. Do the compounds with the most potent insecticidal activity also demonstrate the most detrimental effects in a model of dopaminergic cells?

To determine dieldrin's structure−activity relationship, three aspects of its structure were assessed (Figure 1: 1). The relative 3-dimensional orientation and presence of the methano bridge (Groups 1 and 2). The identity of the polar moiety (olefin, *cis*-diol, or epoxide) located opposite the chlorine atoms (Groups

Received: November 14, 2012

   dx.doi.org/10.1021/tx300458b | *Chem. Res. Toxicol.* XXXX, XXX, XXX−XXX

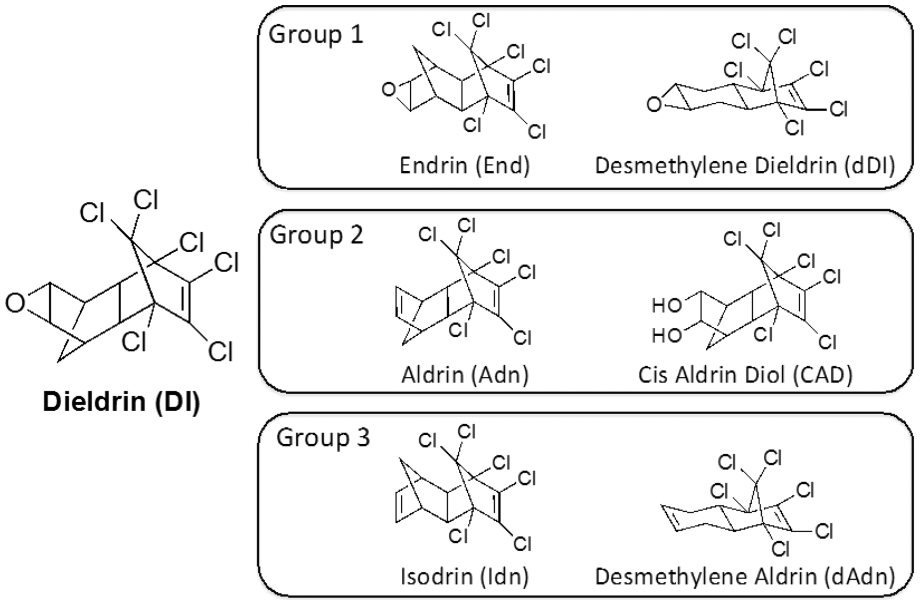


Figure 1. Structures and groupings of dieldrin analogues. Compared to dieldrin, each of the three groups have varied structure. Group 1: methano bridge, opposite configuration (endrin) or absent (desmethylene dieldrin). Group 2: epoxide, hydrolyzed to diol (*cis*-aldrin diol) or absent (aldrin). Group 3: no epoxide and opposite configuration of the methano bridge (isodrin) or no epoxide and methano bridge (desmethylene aldrin).

2 and 3). The combined effect of the 3-dimensional orientation and polar group (Group 3). The effects of these compounds on cell viability, DA metabolism, and ROS production were investigated.

Comparison of these features and their activity will provide valuable information about the cellular targets of these compounds and will be used to gain valuable insight into the mechanistic link between dieldrin exposure and the development of PD. Ultimately, how does dieldrin exposure predispose individuals to PD? The selectivity of dieldrin toward dopaminergic neuronal death *in vivo* is predicted to facilitate the development of PD. Therefore, it is of high importance that the mechanism of dopaminergic toxicity from dieldrin exposure is understood.

EXPERIMENTAL PROCEDURES

Caution: ***The following compounds are hazardous and should be handled carefully: dieldrin, aldrin, endrin, isodrin, cis-aldrin diol, desmethylene dieldrin, and desmethylene aldrin. All are highly lipophilic and contact with skin, eyes, and clothing should be avoided.*** **Chemicals.** Dieldrin, aldrin, endrin, and isodrin were purchased from Chem Service (West Chester, PA). All other reagents, unless otherwise noted, were purchased from Sigma Aldrich (St. Louis, MO) without further purification. Organochlorine analogues were named based on the von Baeyer/IUPAC system for polycyclic compounds and as discussed previously.[17]

***cis*-Aldrin Diol (1,8,9,10,11,11-Hexachloro-4,5-(exo)*cis*-dihydroxy-2,3-7,6-endo-2,1-7,8-exotetracyclo[6.2.1.1^{3,6}.0^{2,7}]dodec-9-ene).** *cis*-Aldrin diol was synthesized using a method adapted from the literature.[18,19] Briefly, aldrin (86 mg, 0.24 mmol) was combined with dry ether (6.4 mL), pyridine (0.15 mL), and osmium tetroxide (50 mg, 0.20 mmol). The reaction was stirred for 2 h at room temperature and then allowed to stand overnight in the dark. The ether was evaporated with nitrogen, giving a brown residue that was then dissolved in dichloromethane (10 mL) and stirred vigorously overnight with a solution of water (6.5 mL), potassium hydroxide (71 mg, 1.3 mmol), and D-mannitol (630 mg, 3.46 mmol). The dichloromethane layer was removed, washed with water, and dried with sodium sulfate, giving the product in 90% yield. ^{1}H NMR (DMSO-d_6): δ 1.12 (d, 1H, CH_2), 1.60 (d, 1H, CH_2), 2.06 (m, 2H, CH), 2.60 (m, 2H, CH), 3.57 (m, 2H, CH), 4.93 (m, 2H, OH); ^{13}C NMR (DMSO-d6): δ 28.3, 43.0, 51.7, 73.6, 81.0, 104.7, 130.8; MS (EI) m/z calcd for $C_{12}H_{10}Cl_6O_2$ 395.9; found m/z 396.1.

Desmethylene Aldrin (1,8,9,10,11,11-Hexachloro-2,3-7,6-endotricyclo[6.2.1.0^{2,7}]undeca-4,9-diene). Using methods adapted from literature methods,[14,19−21] we placed hexachlorocyclopentadiene (1.0 g, 0.004 mol, 0.6 mL) and 1,4-cyclohexadiene (1.3 g, 0.016 mol, 1.51 mL) under argon and stirred them at 110 °C for 44 h. The product was purified by column chromatography on silica gel with hexanes as mobile phase, resulting in a 25% yield. ^{1}H NMR ($CDCl_3$): δ 1.90−1.99 and 2.35−2.44 (m, 4H, CH_2), 2.98−3.09 (m, 2H, CH), 5.86 (m, 2H, HC═CH). ^{13}C NMR ($CDCl_3$): δ 21.9, 45.8, 82.5, 103.2, 126.7, 131.6; MS (EI) m/z calculated for $C_{11}H_8Cl_6$ 349.9; found m/z 349.9.

Desmethylene Dieldrin (1,8,9,10,11,11-Hexachloro-4,5-epoxy-2,3-7,6-endotricyclo[6.2.1.0^{2,7}]undec-9-ene). Using methods adapted from literature methods,[14,19,21,22] we added 3-chloroperbenzoic acid (*m*CPBA) (0.08 g, 0.5 mmol) in 2.0 mL of dichloromethane dropwise to desmethylene aldrin (0.1 g, 0.3 mmol) in 2.0 mL of dichloromethane and stirred at room temperature for 2 h. The reaction mixture was extracted twice with water. The organic layer was then dried with magnesium sulfate and the product purified by column chromatography on silica gel with a mobile phase of 4:1 hexanes to acetone, resulting in a 50% yield. ^{1}H NMR ($CDCl_3$): δ 1.59−1.67 and 2.40−2.48 (m, 4H, CH_2), 2.91−2.99 (m, 2H, CH), 3.20−3.24 (m, 2H, CH−O). ^{13}C NMR ($CDCl_3$): δ 22.2, 42.2, 49.1, 82.3, 102.8, 132.0; MS (EI) m/z calculated for $C_{11}H_8Cl_6O$ 365.9; found m/z 365.9.

Thiol and Amine Reactivity. Thiol reactivity of dieldrin was determined using Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)) and a procedure adapted from the literature.[23,24] *N*-Acetyl Cys (100 μM) was incubated with dieldrin (0−2.5 mM) overnight at 37 °C in DMSO. DTNB (0.5 mM) in DMSO was added along with 50 mM sodium phosphate buffer, pH 7.4 (25% v/v), and absorbance monitored at 412 nm using a Molecular Devices SpectraMax plate reader. The free thiol concentration in each well was determined by comparing the absorbance to a standard curve of DTNB reacted with varying concentrations of *N*-acetyl Cys.

The reactivity of dieldrin with primary amines was assessed using *N*-acetyl Lys. Dieldrin (0−100 μM in DMSO) was incubated with *N*-acetyl Lys (100 μM in DMSO) at 37 °C for 24 h. The concentration of dieldrin was monitored via an HPLC method adapted from Mowafy et al.[25] Briefly, the samples were analyzed on an Agilent 1100 Series

dx.doi.org/10.1021/tx300458b | *Chem. Res. Toxicol.* XXXX, XXX, XXX−XXX

Capillary HPLC with a Phenomenex C18 Jupiter column (5 μm, 300 Å, 150 × 1.0 mm) using isocratic conditions of water (25%) and methanol (75%), a flow rate of 50 μL/min, and the absorbance measured via photodiode array at 214 and 234 nm. Differences in peak area between dieldrin incubated with and without *N*-acetyl Lys are predicted to indicate reactivity between these two compounds, i.e., dieldrin and the dieldrin-Lys conjugate.

Cell Culture. PC6-3 cells, a generous gift from Stefan Strack (University of Iowa), were cultured in RPMI1640 medium (GIBCO, Invitrogen, Carlsbad, CA) supplemented with heat-inactivated horse serum (10%) (HyClone, Thermo Scientific, Rockford, IL), fetal bovine serum (5%) (GIBCO), penicillin (10 IU/mL), and streptomycin (10 mg/mL) and were grown in 100 mm^2 tissue culture dishes at 37 °C with 5% CO_2. Cells (3×10^4 cells/well or 6×10^4 cells/well for DA-supplemented metabolism experiments) were seeded into six-well plates and allowed to grow for four days at 37 °C with 5% CO_2. The cells were then differentiated with 2.5S nerve growth factor (NGF) (BD Biosciences, Bedford, MA) at 50 ng/mL for 4 days. The cells used in these experiments are a subline of PC12 cells, an established cellular model for dopaminergic neurons.[26−29] Previous work has demonstrated that PC6-3 cells express enzymatically active tyrosine hydroxylase and metabolize dopamine, thereby demonstrating their utility as a cellular model for dopaminergic neurons.[30−32]

Treatment of Cells with Dieldrin and Analogues. Media were removed from cells and replaced with HEPES-buffered saline containing 115 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.5 mM glucose, 1.0 mM NaH_2PO_4, and 15 mM HEPES (pH 7.4). The cells were treated with the compounds (dieldrin, aldrin, endrin, isodrin, *cis*-aldrin diol, desmethylene aldrin, or desmethylene dieldrin) at 0−900 μM in dimethylsulfoxide (DMSO, 0.6%) for 4 h. The concentrations used in this study (10−900 μM) are higher than the concentrations detected in the brains of Parkinson's patients (2.4 μM, based on reported values of μg of dieldrin per g of lipid in the striatum)[2,4−6] but were chosen to represent the full range of cell death in this model system (20−100% loss of cell viability). This range was required to calculate the IC_{50} values, which were used for accurate comparisons between compounds and were also comparable to the concentrations used in similar studies.[7,11,13,33−36]

Cell Fractionation and GC/MS Analysis. Upon 4 h of treatment of the PC6-3 cells with dieldrin (0−300 μM), the HEPES-buffered saline used for treatment was collected (extracellular fraction) and then lysis buffer (10 mM K_2HPO_4 with 0.1% Triton X-100 (v/v)) was added to each well. The cell lysate was collected and sonicated for 5 min using a sonicator bath. The membrane/lipid fraction was separated from the cytosol and lysed organelles (intracellular fraction) using centrifugation at 1000*g* for 10 min at 4 °C (Abcam Technical Bulletin, Plasma Membrane Protein Extraction Kit). The pellet (membrane/lipid fraction) was resuspended in phosphate-buffered saline, and all samples were stored at −70 °C pending analysis.

Prior to extraction, each sample was spiked with an internal standard (endrin, 1 μg/mL). The organochlorines were extracted three times using hexanes with a volume equal to the sample volume. If an emulsion formed, one part ethanol was added to the sample. The solvent was removed from the organic layer using nitrogen gas and then the sample dissolved in ethyl acetate. Dieldrin and endrin were separated using a Thermo Voyager gas chromatograph coupled with a single quadrapole mass spectrometer. Flow of the gas carrier was 20 mL/min, and the temperature was 50 °C for 2 min, increased by 10°/min to 280 °C and then held for 10 min. The mass spectrometer monitored ionization at 79 *m*/*z* and 263 *m*/*z*. Peak area quantification was completed using Xcaliber 2.0. Dieldrin peak area at 263 *m*/*z*, elution time 20.28 min, was first normalized to the internal standard (endrin at 263 *m*/*z*, elution time 19.88 min) and then compared to a standard curve. The amount of dieldrin (μg) in each of the fractions was compared to the amount of dieldrin extracted from treatment with 10 μM of dieldrin and are given as fold change of dieldrin. Values are reported as the mean ± SD, $n = 3$.

Cell Viability. The effect of each compound on cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay, in which the yellow, MTT tetrazolium is converted to a purple formazan salt by cellular reductases (e.g., succinate dehydrogenase). After a 4 h treatment of the cells with each compound, the cells were incubated for 1.25 h at 37 °C with MTT (0.5 mg/mL). The HEPES-buffered saline was then removed from each well and centrifuged at 10,000*g* for 5 min. This pellet and the formazan salt remaining in each well were dissolved in DMSO and combined. Absorbances were then measured at 570 and 650 nm using a Molecular Devices Spectra-Max plate reader. Results are presented as % of control.

IC_{50} Value Calculations. In order to calculate the IC_{50} values for each compound based on their cell viability, the dose−response data was normalized between 0 and 100%, and graphed against the log[inhibitor]. A nonlinear curve fitting was applied based on log[inhibitor] vs normalized response with variable slope. The concentration at which 50% normalized activity was observed is reported as the IC_{50} ± SEM.

Cytotoxicity Assay. The cytotoxicity of each compound was determined using Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche Applied Science, Mannheim, Germany), according to the manufacturer's specifications. Briefly, a high control for each set consisted of cells incubated with 0.6% DMSO and then for the last 15 min of the experiment, lysis buffer, as provided by the manufacturer. The low control in each set was a well incubated with 0.6% DMSO and no lysis buffer. At 4 h, an aliquot of media was removed from each well and incubated with the reaction mixture provided for 20 min in the dark at RT. The reaction was then stopped and the absorbance at 492 and 690 nm measured using a Molecular Devices Spectra-Max plate reader. In order to determine the percent cytotoxicity, the value obtained for the low control was subtracted from each treated well, then divided by the difference between the high control and the low control. Dieldrin and the other analogues did not interfere with the absorbance readings for this assay.

The ability of each compound to inhibit LDH was evaluated by incubating rabbit L-LDH (0.5 U/mL) with various concentrations of each compound (0−900 μM, 0.6% DMSO) for 30 min in HEPES-buffered saline. At this point, the reaction mixture containing the necessary enzyme substrates and cofactors was added and incubated in the dark at room temperature for 5 h. Absorbances at 492 and 690 nm were then measured and the normalized absorbance determined by subtracting the 690 nm absorbance from the absorbance at 492 nm to assess enzyme activity.

Dopamine Metabolism. The effect of each compound on the extracellular concentration of DA metabolites was monitored. Cells were treated with 0, 10, 25, 50, 100, or 300 μM of each compound. An aliquot of the extracellular HEPES-buffered saline was removed at 0, 1, and 4 h and then mixed with perchloric acid (5% v/v) in order to precipitate the proteins and terminate any reaction. The samples were stored at −70 °C, thawed, and centrifuged at 10,000*g* for 5 min prior to HPLC analysis. Samples were analyzed by an Agilent 1200 Series Capillary HPLC with a Phenomenex C18 Luna column (1 × 150 mm). The dopamine metabolites 3,4-dihydroxyphenylacetaldehyde (DOPAL), 3,4-dihydroxyphenylacetic acid (DOPAC), and 3,4-dihydroxyphenylethanol (DOPET) were separated using isocratic conditions of 0.1% trifluoroacetic acid in water with 6% ACN (v/v), a flow rate of 50 μL/min, and detection with a photodiode array detector (absorbance at 202 and 280 nm). The peak area was then converted to concentration by comparison to a standard curve calculated from metabolite standards as previously described.[30,37−39] For the DA-supplemented metabolism experiments, cells were supplemented with 100 μM DA 15 min prior to dieldrin treatment to initiate DA metabolism.

Flow Cytometry. Differentiated PC6-3 cells were pretreated with dihydroethidium (DHE, 10 μM, 0.2% DMSO) and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, 50 μM, 0.2% DMSO) for 20 min at 37 °C in HEPES-buffered saline. Cells were then washed and treated with each dieldrin analogue at a nontoxic dose (<20% cell death according to MTT assay) for 1 h. The doses of each organochlorine used were dieldrin (25 μM), aldrin (25 μM), *cis*-aldrin diol (25 μM), desmethylene dieldrin (10 μM), isodrin (100 μM), and desmethylene aldrin (10 μM). Cells were then removed

dx.doi.org/10.1021/tx300458b | *Chem. Res. Toxicol.* XXXX, XXX, XXX−XXX

from the wells, washed with phosphate-buffered saline, and resuspended in HEPES-buffered saline prior to filtration (70 μm). Hoechst 33258 (4 μg/mL) was added and the absorbance within the cells measured using a Becton Dickinson LSR II flow cytometer with UV and 440/40 band-pass (BP) (Hoechst 33258), 530/30 BP (H_2DCFDA), and 610/20 BP (DHE). Data were processed using CellQuest (BD Bioscience), and results were reported as the geometric mean ± SEM.

Statistics. All statistics and curve fittings were calculated using GraphPad Prism, version 5.01c (GraphPad Software, San Diego, CA). Rules of error propagation were used to determine the standard deviation of the fold change of dieldrin concentration for the cellular localization experiments. Significance from control for the thiol and amine reactivity was determined using a two-tailed *t* test ($p < 0.05$). All other statistical analyses were calculated using one-way ANOVA with Newman-Keuls post-test, unless otherwise noted.

RESULTS

Reactivity of Dieldrin (Epoxide) with Cys and Lys. To determine the reactivity of dieldrin, specifically the epoxide, toward the protein nucleophiles Cys and Lys, dieldrin was incubated with *N*-acetyl Lys and Cys and the reaction monitored. The thiol reactivity of dieldrin was assessed using *N*-acetyl Cys, and the concentration of free thiols was monitored via DTNB. No decrease in thiol concentration was observed.

In addition, dieldrin was incubated with *N*-acetyl Lys and the concentration of dieldrin remaining over time measured using HPLC. No significant decrease in dieldrin concentration was observed upon addition of *N*-acetyl Lys, indicating no reaction under these conditions. These results indicate that dieldrin does not covalently modify target proteins under the conditions used and that any observed protein interactions are most likely noncovalent, despite the presence of an epoxide on the organochlorine.

Localization of Dieldrin in Dopaminergic PC6-3 Cells. To determine the cellular localization of dieldrin following the exposure of PC6-3 cells to the organochlorine, cells were fractionated and dieldrin measured using GC/MS. Upon treatment with dieldrin (0–300 μM), the PC6-3 cells were fractionated giving extracellular, membrane/lipid, and intracellular fractions. Representative chromatograms of the extract of a cellular fraction are shown in Supporting Information, Figure 1. As depicted in Figure 2A, the amount of dieldrin in the membrane/lipid fraction increased with the dose of dieldrin. Cells treated with 3.8 μg (10 μM), 9.5 μg (25 μM), 19 μg (50 μM), 38 μg (100 μM), and 114 μg (300 μM) dieldrin had measured lipid/membrane organochlorine amounts of 0.022 μg, 0.16 μg, 0.89 μg, 3.1 μg, and 17 μg, respectively. When dieldrin in the intracellular fraction was quantified (Figure 2B), it was found that the amount of dieldrin increased with dosage through 50 μM treatment but not to the same extent as that observed for the membrane/lipid fraction. Cells treated with 3.8 μg (10 μM), 9.5 μg (25 μM), 19 μg (50 μM), 38 μg (100 μM), and 114 μg (300 μM) dieldrin had measured intracellular organochlorine amounts of 0.34 μg, 0.83 μg, 1.3 μg, 0.77 μg, and 2.3 μg, respectively. Such results indicate the limited capacity of the intracellular fraction for dieldrin and that the majority of the organochlorine remains in the membrane/lipid component.

Cell Viability. To assess the structure–activity relationship of dieldrin toxicity, the viability of PC6-3 cells was monitored after 4 h of treatment with various concentrations of each dieldrin analogue via the MTT assay (Figure 4). Results are

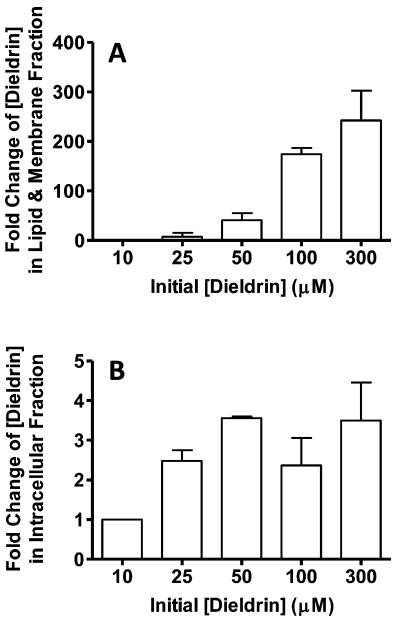


Figure 2. Amount of dieldrin, normalized to internal standard and standard curve, following hexane extraction of cellular fractions from cells treated with 0–300 μM dieldrin. Each bar represents the fold change in dieldrin concentration compared to the amount extracted after treatment of cells with 10 μM dieldrin (±SD, $n = 3$). (A) membrane/lipid fractions; (B) intracellular fractions.

depicted for dieldrin in Figure 3A, Group 1 analogues in Figure 3B and C, Group 2 in Figure 3D and E, and Group 3 shown in Figure 3F and G. All of the compounds, except for endrin, displayed a dose-dependent decrease in cell viability in the PC6-3 cells after 4 h. On the basis of these results, IC_{50} values were calculated in order to easily compare the potency of each compound (Table 1).

The parent compound dieldrin showed a dose-dependent increase in toxicity as determined via the decrease in cell viability, with an IC_{50} value of 293 μM. Endrin did not exhibit a dose-dependent increase in toxicity (Figure 3B); therefore, no IC_{50} value could be calculated. When the methano bridge was removed, yielding a more flexible structure (desmethylene dieldrin), the compound was substantially more toxic than dieldrin (Figure 3C) with an IC_{50} value of 22 μM.

When dieldrin was compared to Group 2, compounds with changes to the polar moiety, it was found that the substitution of the epoxide for an olefin (aldrin, Figure 3D) or a *cis*-diol (*cis*-aldrin diol, Figure 3E) both resulted in decreased cell viability (IC_{50} values of 46 and 36 μM, respectively), as compared to that for dieldrin. Such a result implies that a moiety capable of being a hydrogen bond donor (i.e., cis diol) enhances the toxicity, as does the presence of an olefin.

The last group of compounds takes into account the presence of the olefin, already shown to enhance toxicity, as well as the 3-dimensional orientation of the compound. The isomer of aldrin (isodrin, Figure 3F) showed very little toxicity (IC_{50} value was 578 μM). When the desmethylene analogue of aldrin was assessed (desmethylene aldrin, Figure 3G), it resulted in the most substantial decrease of cell viability of all of the analogues with an IC_{50} value of 16 μM. These results imply that the presence of the olefin augments the toxicity of dieldrin, and when this modification is coupled with the removal of the methano bridge, the toxicity is further enhanced.

dx.doi.org/10.1021/tx300458b | *Chem. Res. Toxicol.* XXXX, XXX, XXX–XXX

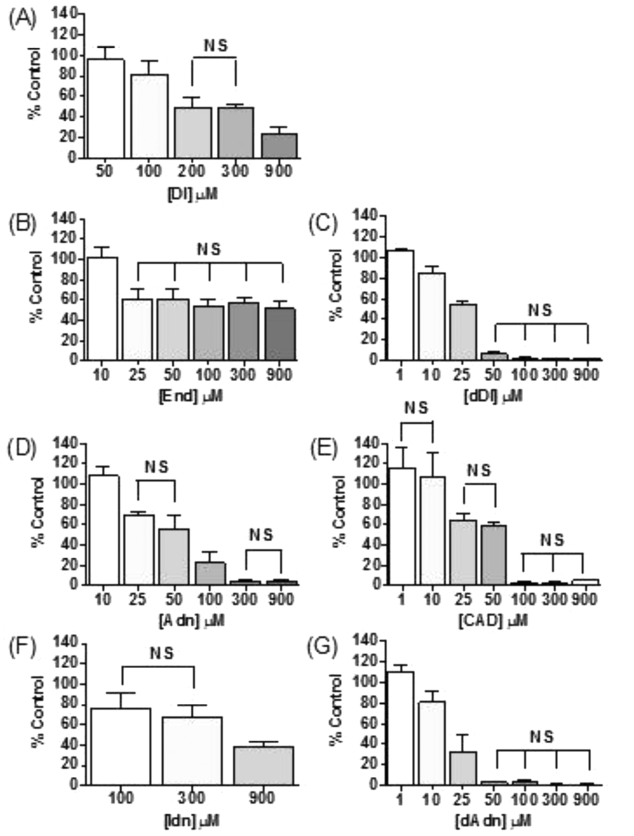


Figure 3. Cell viability of PC6-3 cells treated with dieldrin, Group 1, Group 2, or Group 3 compounds (% control ± SD, $n = 4–6$) obtained from MTT assay after 4 h of incubation with (A) dieldrin, (B) endrin, (C) desmethylene dieldrin, (D) aldrin, (E) *cis*-aldrin diol, (F) isodrin, and (G) desmethylene aldrin. The difference between each pair of treated groups is significant ($p < 0.05$) unless otherwise noted (NS).

Table 1. IC_{50} Values (μM ± SEM) for Each Compound Based on the Concentration of Each Inhibitor at 50% of the Normalized Cell Viability

compd	IC_{50} (μM)[a]
dieldrin	293 ± 1.08
endrin	ND[b]
desmethylene dieldrin	22.3 ± 1.05
aldrin	46.4 ± 1.08
cis-aldrin diol	36.9 ± 1.11
isodrin	578 ± 1.22
desmethylene aldrin	15.8 ± 1.05

[a]Values represent the mean ± SEM. [b]Not determined. The measured toxicity was not dose dependent.

Release of Lactate Dehydrogenase. To further assess the structure–activity relationship of dieldrin toxicity, the viability of PC6-3 cells was monitored after 4 h of treatment with various concentrations of each dieldrin analogue via the LDH assay. For each of the compounds, a dose-dependent decrease in cell viability with respect to that of the control was observed, except for endrin, which showed no cytotoxicity, even at high concentrations (900 μM) (Figure 4). The results from the LDH experiment correlate well to those observed using MTT to monitor cell viability (Figure 3) and data from a previous study.[7] The relative order of toxicity between each analogue was also comparable, with the desmethylene

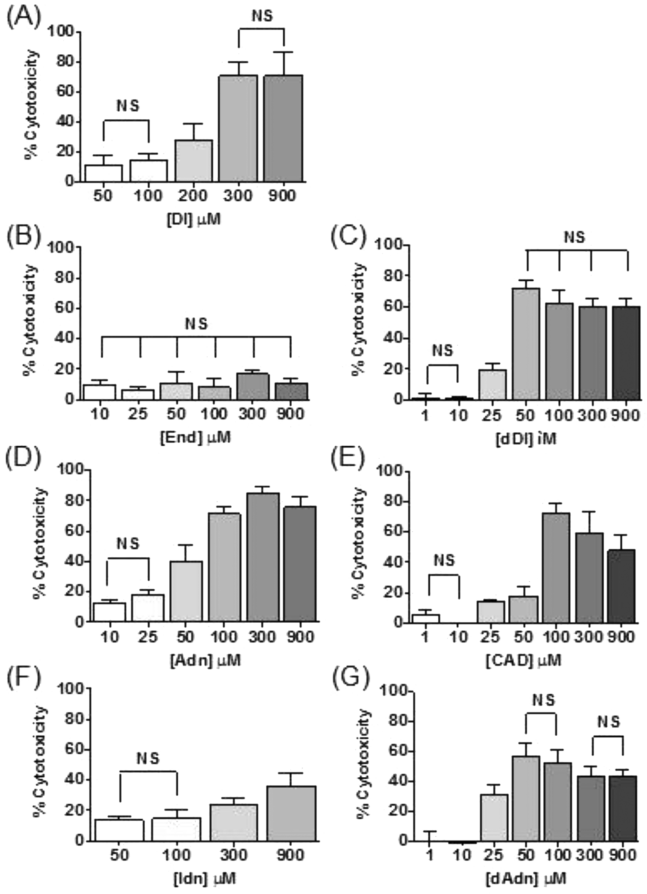


Figure 4. Percent cytotoxicity by lactate dehydrogenase release from PC6-3 cells after 4 h of incubation with dieldrin, Group 1, Group 2, or Group 3 compounds (% cytotoxicity ± SD, $n = 4–6$). (A) Dieldrin, (B) endrin, (C) desmethylene dieldrin, (D) aldrin, (E) *cis*-aldrin diol, (F) isodrin, and (G) desmethylene aldrin. The difference between each pair of treated groups is significant ($p < 0.05$) unless otherwise noted (NS).

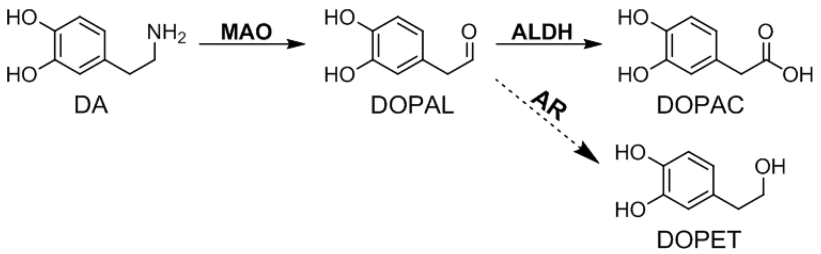
analogues being the most potent, followed by both compounds in Group 2 (aldrin and *cis*-aldrin), then dieldrin. Isodrin and endrin were the least toxic.

In the LDH results, a deviation from the dose-dependent trend was observed at higher concentrations of organochlorine, particularly with the more toxic derivatives. Each compound showed increased release of LDH until the concentration at which roughly 65% cytotoxicity was achieved. At organochlorine concentrations that would be expected to yield greater than 65% cytotoxicity, the values representing LDH release and activity remained constant (desmethylene dieldrin, Figure 4C) or decreased (*cis*-aldrin diol, Figure 4E). This deviation from the expected dose–response was not seen with the MTT results. It was determined that the compounds did not absorb at the wavelengths monitored or react with the reagents used in this experiment.

Since this experiment was based on enzyme activity, the compounds were then tested for inhibition of the enzyme, LDH. When each compound was incubated with rabbit L-LDH at higher concentrations, a decrease in enzyme activity was observed as shown in Supporting Information, Figure 2. It can be inferred that when the cells were treated with higher concentrations of the dieldrin analogues, the outer membrane of the cells was disrupted, causing a release of LDH. However,

dx.doi.org/10.1021/tx300458b | *Chem. Res. Toxicol.* XXXX, XXX, XXX–XXX

Scheme 1. DA Metabolism[a]



[a]DA is metabolized by monoamine oxidase (MAO) to the aldehyde, DOPAL. This intermediate is oxidized by aldehyde dehydrogenase (ALDH) to the acid metabolite (DOPAC) or reduced by aldehyde reductase (AR) to an alcohol, DOPET.

Table 2. Concentration of Dopamine Metabolites (DOPAL, DOPAC, or DOPET)[a]

compd	group[b]	[DOPAL] (μM)	[DOPAC] (μM)	[DOPET] (μM)
control		0.76 ± 0.21	2.94 ± 0.87	1.67 ± 0.32
dieldrin		1.53 ± 0.25^{NS}	2.87 ± 1.36^{NS}	1.58 ± 0.49^{NS}
endrin	1	0.60 ± 0.09^{NS}	3.51 ± 0.19^{NS}	1.78 ± 0.14^{NS}
des dieldrin	1	7.79 ± 2.49***	0.54 ± 0.15***	1.16 ± 0.27^{NS}
aldrin	2	7.75 ± 1.61***	1.15 ± 0.20**	1.24 ± 0.11^{NS}
cis-aldrin diol	2	10.7 ± 5.48***	0.53 ± 0.11***	0.58 ± 0.06***
isodrin	3	1.00 ± 0.19^{NS}	2.76 ± 0.95^{NS}	2.12 ± 0.44^{NS}
des aldrin	3	7.02 ± 2.19***	0.23 ± 0.20***	0.85 ± 0.06**

[a]Concentrations of dopamine metabolites (±SD, n = 3, except for controls where n = 21) after 4 h of treatment with 300 μM of each compound or vehicle control (0.6% DMSO). Difference from vehicle control was significant with $p < 0.001$ (**) or $p < 0.0001$ (***), or not significant with $p > 0.05$ (^{NS}). Statistical analysis was performed via ANOVA with Newman-Keuls post-test. The elevation of DOPAL following dieldrin treatment was significantly different from that of the control when analyzed by a two-tailed t test ($p < 0.0001$). [b]See Figure 1 for group assignments and structures.

due to the substantial amount of the dieldrin analogue still remaining in the extracellular media or that was released from the cell during apoptosis, the LDH was inhibited by the compound. This resulted in the decreased enzyme activity corresponding to the observed deviation from the dose-dependent increase in cytotoxicity for each analogue at higher concentrations (Figure 4).

Dopamine Catabolism. To determine the effect of dieldrin and analogues on DA catabolism, which is a process capable of generating reactive and toxic intermediates, PC6-3 cells were incubated with the organochlorines and extracellular DA metabolites measured, including DOPAL and DOPAC. Extracellular DA metabolites (Scheme 1) were quantified by HPLC, with the results presented in Table 2 and Figures 5 and 6. The concentration of extracellular DOPAL after 4 h of treatment is shown for the control (vehicle-treated) and all organochlorine compounds in Figure 5. When the cells were treated with dieldrin, a time- and concentration-dependent increase in DOPAL was observed. Small changes in the levels for the other DA metabolites, DOPAC and DOPET, were also observed (Figure 6 and Table 2). Such findings were also noted following treatment of the cells with the various isomers of dieldrin.

When endrin, the isomer of dieldrin, was analyzed, a slight decrease in DOPAL and an increase in DOPAC were observed. For desmethylene dieldrin (300 μM), the analogue of dieldrin lacking the methano bridge, the extracellular DOPAL concentration increased 10-fold. The concentration of DOPAC was significantly reduced from the control following treatment with this analogue (Figure 6A).

Group 2, with changes to the polar group opposite of the chlorine atoms, also showed a profound effect on the extracellular concentration of DOPAL in comparison to dieldrin, with both aldrin and *cis*-aldrin diol resulting in

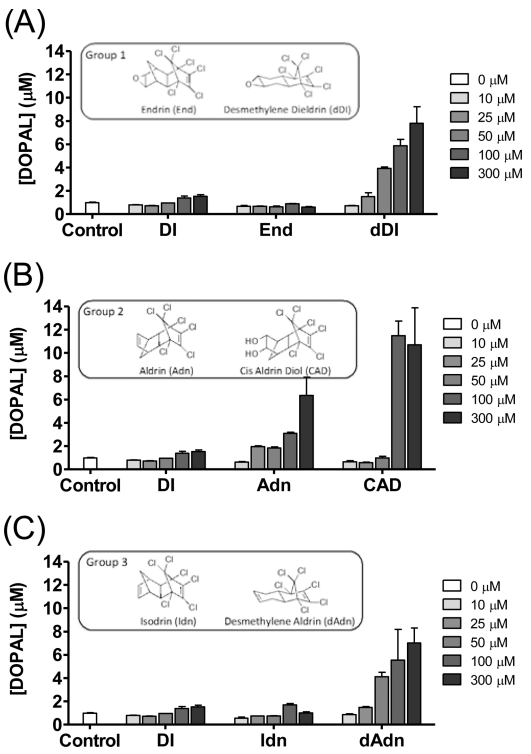


Figure 5. Quantification of the extracellular concentration of DOPAL from PC6-3 cells incubated for 4 h with vehicle control (0.6% DMSO), dieldrin, and Group 1 compounds (endrin and desmethylene dieldrin, A), Group 2 compounds (aldrin and *cis*-aldrin diol, B), or Group 3 compounds (isodrin and desmethylene aldrin, C). The values shown represent the mean ± SD (n = 3, except for controls where n = 21), where peak area was converted to concentration using a standard curve.

dx.doi.org/10.1021/tx300458b | *Chem. Res. Toxicol.* XXXX, XXX, XXX–XXX

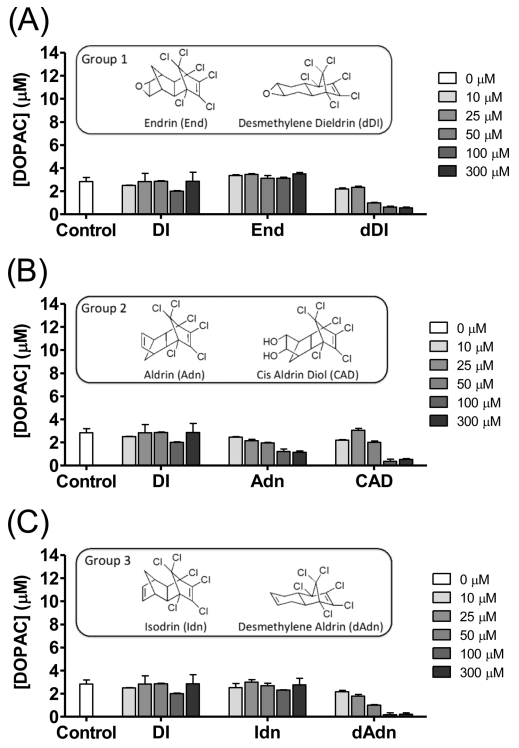


Figure 6. Quantification of the extracellular concentration of DOPAC from PC6-3 cells incubated for 4 h with vehicle control (0.6% DMSO), dieldrin, and Group 1 compounds (endrin and desmethylene dieldrin, A), Group 2 compounds (aldrin and *cis*-aldrin diol, B), or Group 3 compounds (isodrin and desmethylene aldrin, C). The values shown represent the mean ± SD (n = 3, except for controls where n = 21), where peak area was converted to concentration using a standard curve.

significant and substantial increases in DOPAL levels, as compared to the control (Figure 5B). This observation was accompanied by a marked decrease in the production of DOPAC (Figure 6B).

For the third group of compounds, isodrin and desmethylene aldrin, results similar to those of endrin and desmethylene dieldrin were observed, respectively. Isodrin showed only a small change from the control for both DOPAL (slight increase) and DOPAC (slight decrease), whereas desmethylene aldrin showed a marked increase in DOPAL concentration and a corresponding decrease in DOPAC (Figures 5C and 6C).

Because the PC6-3 cells released low levels of DA metabolites, the cells were spiked with 100 μM DA 15 min prior to dieldrin exposure in order to more accurately determine the influence of dieldrin on DA metabolism (Figure 7). DOPAL levels were measured as previously described and were expressed as % control [DOPAL] for treatment with 10, 30, 100, or 300 μM dieldrin. A dose-dependent trend was observed, with concentrations as low as 10 μM dieldrin generating a nearly 200% increase in extracellular DOPAL levels.

Reactive Oxygen Species. To determine the effect of nontoxic concentrations (<20% cell death) of each dieldrin analogue on the production of ROS, cells were incubated with the organochlorines, and ROS was measured via flow cytometry. General ROS production was determined using H_2DCFDA, and a significant increase in oxidative stress was observed upon treatment with each of the compounds studied (endrin was not included in this experiment) (Figure 8A).

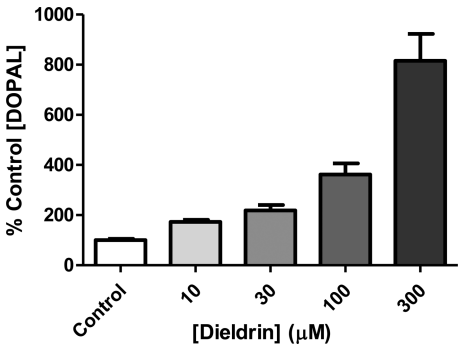


Figure 7. Percent control of the concentration of DOPAL released from PC6-3 cells pretreated with 100 μM dopamine prior to 4 h of exposure to 10, 30, 100, or 300 μM dieldrin. The values shown represent the mean ± SEM (n = 3 or 4), normalized to the percent control of DOPAL release. The percent control of DOPAL release after treatment with 100 and 300 μM dieldrin was significantly different from that of the control (p < 0.05).

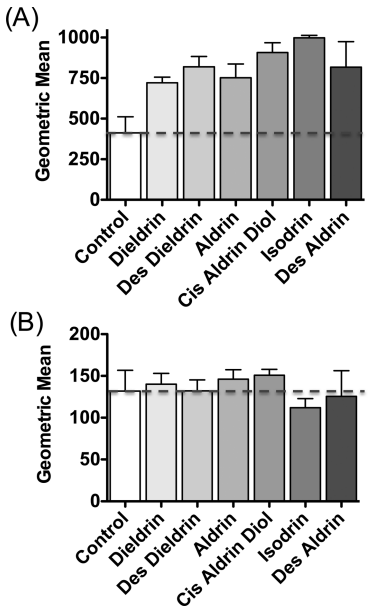


Figure 8. Production of ROS monitored via flow cytometry after 1 h of treatment with nontoxic concentrations of each compound (10 μM for desmethylene aldrin and desmethylene dieldrin, 25 μM for dieldrin, aldrin, and *cis*-aldrin diol, and 100 μM for isodrin). Results depicted as geometric mean (±SD, n = 3) for (A) H_2DCFDA (general ROS) or (B) DHE ($O_2^{\bullet-}$). Change in geometric mean upon treatment with each compound in A for H_2DCFDA fluorescence was significantly different from that of the control (p < 0.0001), except for desmethylene aldrin; whereas in B, for DHE fluorescence, no significant change was observed with any of the compounds (p > 0.05).

When levels of superoxide anion were monitored using DHE, no significant change from the control was observed (Figure 8B), indicating that the accumulation of ROS from dieldrin analogue exposure is most likely from H_2O_2 in the PC6-3 cells.

DISCUSSION

The goal of this study was to elucidate the interaction of the dieldrin with components of dopaminergic cells and determine structure−activity relationships in order to better understand the mechanism of toxicity for this organochlorine pesticide. To complete this goal, a series of experiments were conducted to

dx.doi.org/10.1021/tx300458b | *Chem. Res. Toxicol.* XXXX, XXX, XXX−XXX

determine the protein reactivity, localization, effect on DA metabolism, and structure−activity relationship of dieldrin for toxicity using dopaminergic cells. Such work is significant given that the structure−activity relationship of dieldrin is not well-defined in mammals.[40]

Dieldrin contains an epoxide, a moiety that is typically electrophilic and highly reactive toward nucleophiles.[41] It is conceivable that the nucleophilic sites on proteins (i.e., Cys or Lys residues) would be able to react with the epoxide, causing the opening of this three-membered ring and protein modification.[41] Previous studies have shown that dieldrin "binds" to proteins, such as those found in spinal cord homogenate from rats and cockroaches and, to a lesser extent, albumin.[42,43] While isolated amino acids cannot completely model the unique environment of these amino acids in each possible protein target, they can provide a valuable reactivity profile. In the current study, it was determined that dieldrin was not capable of covalently modifying Lys or Cys residues, supporting the previous assumptions of noncovalent interactions. The dieldrin epoxide could be stabilized due to the rigid polycyclic structure, thereby limiting the likelihood of nucleophilic attack by amino acids such as Lys and Cys.

The cellular localization of dieldrin in PC6-3 cells was investigated using cell fractionation and GC/MS. On the basis of the results (Figure 2), dieldrin was found to accumulate in the cell, particularly in the membrane and lipid fraction (Figure 2A). Saturation of the membrane fraction was not observed with organochlorine concentrations used, and dieldrin levels increased as treatment levels increased. The amount of dieldrin in the intracellular fraction increased as organochlorine treatment concentration (up to 50 μM) increased. At higher treatment concentrations (>50 μM), the amount of dieldrin in the intracellular fraction began to level out, indicating decreased diffusion or saturation of binding sites. From the results in Figure 2, it may be concluded that dieldrin readily enters PC6-3 cells and accumulates in the membrane and lipid-containing portion; however, binding to intracellular components can be saturated at low micromolar levels. This trend is most likely due to the lipophilicity of the organochlorine.

The adverse cellular effects of dieldrin have been previously investigated, particularly with respect to the effect of dieldrin on various aspects of mitochondrial activity.[7,11,44,45] Bergen et al.[44] demonstrated dieldrin's ability to disrupt respiration in rat liver mitochondria and inhibit electron transport. In a dopaminergic system similar to that of the PC6-3 cells (i.e., PC12 cells), dieldrin has been shown to cause a 50% decrease in cell viability (MTT) at 143 μM at 1 h in PC12 cells.[7] Even with variations in cell line, cell preparation, and time frame, this value is comparable with the IC_{50} value of 293 μM obtained using the MTT assay in the current study.

In an effort to assess the structure−activity relationship of dieldrin, as it relates to its toxicity in dopaminergic cells, six analogues were used (Figure 1). These compounds were chosen based on their chemical structure, allowing for the assessment of three structural characteristics: (1) the importance of the 3-dimensional orientation and presence of the methano bridge (Group 1, endrin and desmethylene dieldrin), (2) the effect of the polar group opposite of the chlorine atoms (Group 2, aldrin and *cis*-aldrin diol), and (3) the interplay between both the polar moiety and the 3-dimensional orientation (Group 3, isodrin and desmethylene aldrin).

Dieldrin contains an epoxide opposite of the chlorine atoms, whereas the compounds in Group 2 contain either an olefin (aldrin) or a *cis*-diol (*cis*-aldrin diol). Aldrin was widely used as a pesticide around the same time as dieldrin but is slightly less toxic in various species of insects.[14,46] Aldrin has a shorter half-life than dieldrin, mostly due to increased volatility and rate of metabolism in the environment and in mammals.[47] Despite these characteristics limiting the adverse effects of aldrin exposure, it is still listed as #25 on the CERCLA priority list of hazardous substances[48] and has been detected in the post-mortem brains of Parkinson's disease patients.[2,4−6] *cis*-Aldrin diol is a metabolite of dieldrin that can then be epimerized to *trans*-diol in mammals.[49] This compound is less toxic to insects, as compared to dieldrin. [50]

The third group aimed to assess the combined effects of altering the polar moiety (epoxide to olefin) along with the 3-dimensional structure. The two compounds in this group are based on aldrin, with isodrin being its isomer, and desmethylene aldrin lacking the methano bridge found in aldrin and dieldrin. Isodrin was never used commercially as a pesticide, but in *Musca domestica*, it is only slightly less toxic than endrin.[14] The toxicity profile of desmethylene aldrin has not yet been investigated. These organochlorines (Groups 1, 2, and 3) were chosen based on their environmental and metabolic relevancy and also to assess the role of dieldrin's epoxide.

The order of potency for each group of compounds on cell viability was assessed by LDH and MTT assays, both showing similar results. Group 2, aldrin and *cis*-aldrin diol, showed moderate toxicity, the dieldrin isomers (endrin and isodrin) evinced little to no toxicity, and the desmethylene compounds were the most toxic in the PC6-3 cells. Dieldrin, while substantially more toxic than endrin and isodrin, was markedly less toxic than the Group 2 analogues. Orientation of the bridge did greatly influence toxicity (the structural difference between dieldrin and endrin and between aldrin and isodrin), but its absence also increased toxicity as the desmethylene compounds were the most toxic. These results demonstrate the importance of the three-dimensional structure in the cytotoxic potency of the cyclodiene pesticides in a dopaminergic model. This structure−activity relationship differs significantly from the structural features found to be important for insect toxicity. The toxicities of many organochlorines have been assessed in various species of insects, including *Musca domestica* (house fly), *Anopheles stephensi* (mosquito), *Glossina austeni* (tsetse fly), and *Periplaneta americana* (American cockroach).[14−16,46,50] When considering only the compounds investigated in the current study, previous work has shown dieldrin to be the most toxic in insects, usually followed by aldrin, endrin, and then isodrin. *cis*-Aldrin diol, desmethylene aldrin, and desmethylene dieldrin were all found to have minimal toxicity in the insect species investigated. There were many other compounds also investigated in these studies (>51 compounds in total), and some species differences, in terms of the relative potency of each compound, were observed.[14−16]

The general characteristics of the structural features important for toxicity in both insects and dopaminergic cells are similar.[14−16] These characteristics include the importance of the 3-dimensional structure and the identity of electronegative/polar moiety opposite of the chlorine atoms (i.e., epoxide or *cis*-diol). For example, while dieldrin and endrin are stereoisomers of each other differing only the position/orientation of the epoxide and methylene bridge (Figure 1), the measured toxicity is significantly different. Similar results were found for the stereoisomers aldrin and isodrin. The mechanistic basis for such

differences in toxicity, e.g., dieldrin versus endrin, are unknown at this point but might include diversity in regards to cellular targets, metabolism, and disposition.[51,52] In regards to the latter, it is known that unlike dieldrin, endrin is rapidly excreted in humans and does not bioaccumulate.[51,52]

In addition, it seems the minor structural changes that make a molecule less toxic toward insects increase toxicity toward dopaminergic cells (i.e., orientation and presence of the methano bridge). There are significant differences in the model systems used between these studies, whole insect versus immortalized cell line, that affect the metabolism, localization, and absorption of these compounds. However, based on findings for the organochlorine isomers used in the current study (e.g., dieldrin and endrin), it may still be concluded that there are significant differences in the cellular targets that warrant further investigation. It is these differences that will be useful in developing new, potent insecticides that do not adversely affect the dopaminergic system of humans.

The *cis*-diol metabolite of dieldrin resulting from cleavage of the epoxide was found to be significantly more toxic than the parent compound dieldrin (Table 1).[49] Such a finding raises the question of whether dieldrin or its metabolites are the toxic insults relevant to neurodegenerative disease development.

Dieldrin has been associated with the disruption of many key processes in dopaminergic neurons, including the metabolism of the neurotransmitter, DA; mitochondrial function; and the balance of oxidative stress. Dysregulation of these processes and exposure to dieldrin have been correlated to PD.[4−6,53−57]

Previous studies have shown that exposure to dieldrin causes depletion of DA in both ring doves and mallards.[58,59] This effect has also been shown to be specific to DA, with no corresponding inhibition of GABA.[60] When this effect was further investigated in mice dosed with dieldrin, a decrease in DOPAC, the carboxylic acid metabolite of DA, was detected, along with a corresponding increase in cysteinyl-DA, cysteinyl-DOPAC, and elevated levels of protein carbonyls. This indicates a disruption in DA metabolism and trafficking, but instead of seeing elevated levels of the DA metabolites, increased protein modification was observed.[9]

Dieldrin has also been found to cause the release of striatal DA and DOPAC in mice.[12] Developmental exposure to dieldrin has also been shown to have long-term, adverse effects on murine DA metabolism. An increase in DOPAC was observed in the striatal region of these mice after they were aged to 12 weeks, and they were much more sensitive to MPTP toxicity.[10] In dopaminergic PC12 cells, treatment with dieldrin resulted in decreased intracellular DA and a corresponding increase in extracellular DA and DOPAC.[7]

Higher extracellular DA and DOPAC levels suggest release of vesicular DA and elevated DA turnover/metabolism to DOPAC. The metabolism of DA to DOPAC proceeds via the activity of monoamine oxidase and involves production of an aldehyde intermediate, DOPAL.[61] DOPAL is an endogenous neurotoxin, capable of covalently modifying proteins.[30,38,39,62,63] Aberrant levels of this reactive compound have been associated with an increased risk for Parkinson's disease.[62−67] In the current study, the compounds that exhibited the greatest toxicity caused the largest increase in extracellular levels of DOPAL (desmethylene aldrin, desmethylene dieldrin, and *cis*-aldrin diol). The least toxic compounds (endrin and isodrin) showed the least effect on DOPAL concentration, and minimal effect on the concentrations of the other DA metabolites quantified (DOPAC and DOPET). Additionally, pretreatment of the cells with DA demonstrated an even greater increase in DOPAL production following dieldrin treatment as compared to the control (Figure 7). While there appears to be a correlation between toxicity and extracellular levels of DOPAL, it is likely that the generation of DOPAL is a secondary effect of cell death in the current study given the full range of organochlorine compounds used with some concentrations higher than the measured LC_{50}. Further experiments should investigate the correlation between organochlorine treatment and generation of DOPAL, especially for chronic low-level dieldrin exposure.

Of question is how treatment of dopaminergic cells with dieldrin or other organochlorines at concentrations lower than needed for toxicity yield elevated DOPAL. As noted above, exposure of PC12 cells to dieldrin resulted in increased extracellular DA and DOPAC, indicative of organochlorine-mediated release of vesicular DA.[7] One other mechanism that could account for elevated DOPAL would be the impairment of aldehyde metabolism, and previous works have demonstrated aldehyde dehydrogenases and reductases to be inhibited by low levels of products of oxidative stress.[30,38] Further work is needed to determine whether either or both pathways (i.e., release of vesicular DA or oxidative stress products) are responsible for elevated DOPAL.

In addition to DA metabolism, the current study also investigated the effect of dieldrin and its analogues on the production of ROS. It was found that treatment of PC6-3 cells with nontoxic doses of each of the organochlorines resulted in a significant increase in H_2O_2 levels. This increased production of ROS was not necessarily indicative of toxicity, such as in the case of isodrin which caused the largest increase in ROS but showed the least toxicity. A similar trend has been observed for the organophosphates, another class of synthetic pesticides that includes compounds such as chlorpyrifos and malathion.[68,69] It was initially thought that all of the organophosphates had the same mechanism of action: irreversible inhibition of acetylcholinesterase. While there were some observed differences in relative potency, these could be easily attributed to structural changes.[68] It was later determined that there were exceptions to this original hypothesis and observed that organophosphates could produce similar levels of oxidative stress, but the cellular compensations and/or reactions to this insult were different depending on the toxicant.[34,36,70−76] Therefore, the same degree of initial insult can result in a varied degree of toxicity,[36] as observed with the dieldrin analogues investigated here.

At this point, it is not clear why elevated hydrogen peroxide but not superoxide anion was observed following organochlorine treatment. However, exposure of the cells to dieldrin and other analogues resulted in increased DOPAL, indicative of higher DA metabolism via monoamine oxidase, which would produce hydrogen peroxide as a byproduct.[61]

The information obtained regarding the reactivity and localization of dieldrin is important to better understand the interaction of this pesticide with proteins in dopaminergic cells. The major findings of this study are the following: (1) dieldrin readily diffuses into cells and accumulates in the membrane fraction of PC6-3 cells, while the intracellular solution demonstrated saturation. (2) The epoxide of dieldrin is not reactive toward Cys or Lys nucleophiles under conditions used. Therefore, dieldrin most likely associates with proteins noncovalently. (3) Minor structural changes can greatly alter toxicity for dieldrin. The most toxic analogues of dieldrin were the desmethyl isomers and the *cis*-diol metabolite. Interestingly,

dx.doi.org/10.1021/tx300458b | *Chem. Res. Toxicol.* XXXX, XXX, XXX−XXX

the *cis*-diol analogue is a metabolite of dieldrin, which raises a question as to whether dieldrin or a metabolite is the insulting species. (4) Treatment of PC6-3 cells with dieldrin and organochlorine analogues yielded elevated levels of the toxic DA metabolite DOPAL. (5) Exposure of PC6-3 cells to dieldrin and analogues resulted in elevated hydrogen peroxide but not superoxide anion. Given that minor structural changes to dieldrin resulted in significant alterations in toxicity and dopamine catabolism implies specificity in regards to molecular targets. Work is in progress to better understand the interaction of the pesticide dieldrin with dopaminergic cells, focusing on mechanisms for disruption of cell processes and identification of novel intracellular targets.

■ ASSOCIATED CONTENT

Supporting Information

Chromatographs from GC separation of extracted dieldrin and endrin and inhibition of rabbit LDH by organochlorines. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +1-319-335-8834. Fax: +1-319-335-8766. E-mail: jonathan-doorn@uiowa.edu.

Present Addresses

[†]E.M.G.A: Department of Pharmacology, Case Western Reserve University, Cleveland, Ohio.

[‡]Y.J.: National Institute for Neurological Disease and Stroke, National Institutes of Health, Bethesda, Maryland.

Funding

This work was supported by grants NIH R01 ES15507 (to J.A.D.), NIH T32 GM067795 (to E.M.G.A. and L.L.D.), and NIH T32 DK007319 (to E.M.G.A.), fellowships through the American Foundation for Pharmaceutical Education (to E.M.G.A. and L.L.D.), a scholarship from the Marshall H. and Nellie Alworth Memorial Fund (to L.L.D.), and University of Iowa College of Pharmacy Dissertation Fellowships (to E.M.G.A. and L.L.D.).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The GC/MS data were acquired by Dr. Lynn M. Teesch in the High Resolution Mass Spectrometry Facility at The University of Iowa.

■ ABBREVIATIONS

DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DOPAL, 3,4-dihydroxyphenylacetaldehyde; DOPAC, 3,4-dihydroxyphenylacetic acid; DOPET, 3,4-dihydroxyphenylethanol; *m*CPBA, 3-chloroperbenzoic acid; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; ALDH, aldehyde dehydrogenase; ALR, aldehyde reductase; Adn, aldrin; CAD, *cis*-aldrin diol; dAdn, desmethylene aldrin; dDI, desmethylene dieldrin; DI, dieldrin; DHE, dihydroethidium; DMSO, dimethylsufloxide; DA, dopamine; End, endrin; GC/MS, gas chromatography tandem mass spectrometer; Idn, isodrin; LDH, lactate dehydrogenase; NGF, nerve growth factor; PD, Parkinson's disease; ROS, reactive oxygen species; SOD, superoxide dismutase

■ REFERENCES

(1) Kanthasamy, A. G., Kitazawa, M., Kanthasamy, A., and Anantharam, V. (2005) Dieldrin-induced neurotoxicity: relevance to Parkinson's disease pathogenesis. *Neurotoxicology 26*, 701−719.

(2) Weisskopf, M. G., Knekt, P., O'Reilly, E. J., Lyytinen, J., Reunanen, A., Laden, F., Altshul, L., and Ascherio, A. (2010) Persistent organochlorine pesticides in serum and risk of Parkinson disease. *Neurology 74*, 1055−1061.

(3) Tanner, C. M., Ottman, R., Goldman, S. M., Ellenberg, J., Chan, P., Mayeux, R., and Langston, J. W. (1999) Parkinson disease in twins: an etiologic study. *J. Am. Med. Assoc. 281*, 341−346.

(4) Corrigan, F. M., Wienburg, C. L., Shore, R. F., Daniel, S. E., and Mann, D. (2000) Organochlorine insecticides in substantia nigra in Parkinson's disease. *J. Toxicol. Environ. Health, Part A 59*, 229−234.

(5) Corrigan, F. M., Murray, L., Wyatt, C. L., and Shore, R. F. (1998) Diorthosubstituted polychlorinated biphenyls in caudate nucleus in Parkinson's disease. *Exp. Neurol. 150*, 339−342.

(6) Corrigan, F. M., French, M., and Murray, L. (1996) Organochlorine compounds in human brain. *Hum. Exp. Toxicol. 15*, 262−264.

(7) Kitazawa, M., Anantharam, V., and Kanthasamy, A. G. (2001) Dieldrin-induced oxidative stress and neurochemical changes contribute to apoptopic cell death in dopaminergic cells. *Free Radical Biol. Med. 31*, 1473−1485.

(8) Mao, H., Fang, X., Floyd, K. M., Polcz, J. E., Zhang, P., and Liu, B. (2007) Induction of microglial reactive oxygen species production by the organochlorinated pesticide dieldrin. *Brain Res. 1186*, 267−274.

(9) Hatcher, J. M., Richardson, J. R., Guillot, T. S., McCormack, A. L., Di Monte, D. A., Jones, D. P., Pennell, K. D., and Miller, G. W. (2007) Dieldrin exposure induces oxidative damage in the mouse nigrostriatal dopamine system. *Exp. Neurol. 204*, 619−630.

(10) Richardson, J. R., Caudle, W. M., Wang, M., Dean, E. D., Pennell, K. D., and Miller, G. W. (2006) Developmental exposure to the pesticide dieldrin alters the dopamine system and increases neurotoxicity in an animal model of Parkinson's disease. *FASEB J. 20*, 1695−1697.

(11) Kitazawa, M., Anantharam, V., and Kanthasamy, A. G. (2003) Dieldrin induces apoptosis by promoting caspase-3-dependent proteolytic cleavage of protein kinase Cdelta in dopaminergic cells: relevance to oxidative stress and dopaminergic degeneration. *Neuroscience 119*, 945−964.

(12) Sava, V., Velasquez, A., Song, S., and Sanchez-Ramos, J. (2007) Dieldrin elicits a widespread DNA repair and antioxidative response in mouse brain. *J. Biochem. Mol. Toxicol. 21*, 125−135.

(13) Sanchez-Ramos, J., Facca, A., Basit, A., and Song, S. (1998) Toxicity of dieldrin for dopaminergic neurons in mesencephalic cultures. *Exp. Neurol. 150*, 263−271.

(14) Brooks, G. T., and Harrison, A. (1964) The effect of pyrethrin synergists, especially sesamex, on the insecticidal potency of hexachlorocyclopentadiene derivatives ("cyclodiene" insecticides) in the adult housefly, Musca domestica L. *Biochem. Pharmacol. 13*, 827−840.

(15) Brooks, G. T., Barlow, F., Hadaway, A. B., and Harris, E. G. (1981) The toxicities of some analogs of dieldrin, endosulfan and isobenzan to bloodsucking diptera, especially tsetse flies. *Pestic. Sci. 12*, 475−484.

(16) Brooks, G. T., and Mace, D. W. (1987) Toxicity and mode of action of reductively dechlorinated cyclodiene insecticide analogues on houseflies (Musca domestica L.) and other Diptera. *Pestic. Sci. 21*, 129−142.

(17) Bedford, C. T. (1974) Von Baeyer/IUPAC names and abbreviated chemical names of metabolites and artifacts of aldrin (HHDN), dieldrin (HEOD) and endrin. *Pestic. Sci. 5*, 473−489.

(18) Mckinney, J. D., Keith, L. H., Alford, A., and Fletcher, C. E. (1971) Proton magnetic resonance spectra of some chlorinated polycyclodiene pesticide metabolites - rapid assessment of stereochemistry. *Can. J. Chem. 49*, 1993−2003.

(19) Walker, M. P., McKinney, J. D., and Mudgal, V. G. (1976) Synthesis of 14C-desmethylene aldrin, desmethylene dieldrin and

desmethylene aldrin C is and trans-dihydrodiols. *J. Labelled Compd. Radiopharm. 12*, 23−30.
(20) Grimme, W., and Krauthauser, S. (1997) Degenerate cope rearrangement of tetracyclo[7.3.1.0(2,8).0(4,12)]trideca-5,10-diene. *Tetrahedron 53*, 9903−9912.
(21) Cox, R. H., and Mckinney, J. D. (1978) C-13 Nmr-spectra of some chlorinated polycyclodiene pesticides. *Org. Magn. Reson 11*, 541−546.
(22) Costa, V. E. U., Alifantes, J., and Martins, J. E. D. (1998) Evaluation of lipase from Candida rugosa in the resolution of endo-(±)-1,8,9,10,11,11-hexachloropentacyclo-[6.2.1.13,6.02,7.05,9]-dodecan-4-alcohol. *Tetrahedron: Asymmetry 9*, 2579−2585.
(23) Ellman, G. L. (1959) Tissue sulfhydryl groups. *Arch. Biochem. Biophys. 82*, 70−77.
(24) Allen, E. M., Anderson, D. G., Florang, V. R., Khanna, M., Hurley, T. D., and Doorn, J. A. (2010) Relative inhibitory potency of molinate and metabolites with aldehyde dehydrogenase 2: implications for the mechanism of enzyme inhibition. *Chem. Res. Toxicol. 23*, 1843−1850.
(25) Mowafy, L. I., Marzouk, M. A., el-Ahraf, A., and Willis, W. V. (1987) Determination of dieldrin concentrations in recycled cattle feed and manure by liquid chromatography. *Bull. Environ. Contam. Toxicol. 38*, 396−403.
(26) Pittman, R. N., Wang, S., DiBenedetto, A. J., and Mills, J. C. (1993) A system for characterizing cellular and molecular events in programmed neuronal cell death. *J. Neurosci. 13*, 3669−3680.
(27) Shafer, T. J., and Atchison, W. D. (1991) Transmitter, ion channel and receptor properties of pheochromocytoma (PC12) cells: a model for neurotoxicological studies. *Neurotoxicology 12*, 473−492.
(28) Seegal, R. F., Brosch, K., Bush, B., Ritz, M., and Shain, W. (1989) Effects of Aroclor 1254 on dopamine and norepinephrine concentrations in pheochromocytoma (PC-12) cells. *Neurotoxicology 10*, 757−764.
(29) Hirata, Y., Adachi, K., and Kiuchi, K. (1998) Activation of JNK pathway and induction of apoptosis by manganese in PC12 cells. *J. Neurochem. 71*, 1607−1615.
(30) Jinsmaa, Y., Florang, V. R., Rees, J. N., Anderson, D. G., Strack, S., and Doorn, J. A. (2009) Products of oxidative stress inhibit aldehyde oxidation and reduction pathways in dopamine catabolism yielding elevated levels of a reactive intermediate. *Chem. Res. Toxicol. 22*, 835−841.
(31) Jinsmaa, Y., Florang, V. R., Rees, J. N., Mexas, L. M., Eckert, L. L., Allen, E. M., Anderson, D. G., and Doorn, J. A. (2011) Dopamine-derived biological reactive intermediates and protein modifications: Implications for Parkinson's disease. *Chem.-Biol. Interact. 192*, 118−121.
(32) Higgins, C. A., Vermeer, L. M., Doorn, J. A., and Roman, D. L. (2012) Expression and purification of recombinant human tyrosine hydroxylase as a fusion protein in Escherichia coli. *Protein Expression Purif. 84*, 219−223.
(33) Sharma, H., Zhang, P., Barber, D. S., and Liu, B. (2010) Organochlorine pesticides dieldrin and lindane induce cooperative toxicity in dopaminergic neurons: role of oxidative stress. *Neurotoxicology 31*, 215−222.
(34) Slotkin, T. A., MacKillop, E. A., Ryde, I. T., Tate, C. A., and Seidler, F. J. (2007) Screening for developmental neurotoxicity using PC12 cells: comparisons of organophosphates with a carbamate, an organochlorine, and divalent nickel. *Environ. Health Perspect. 115*, 93−101.
(35) Slotkin, T. A., and Seidler, F. J. (2009) Protein kinase C is a target for diverse developmental neurotoxicants: transcriptional responses to chlorpyrifos, diazinon, dieldrin and divalent nickel in PC12 cells. *Brain Res. 1263*, 23−32.
(36) Slotkin, T. A., and Seidler, F. J. (2009) Oxidative and excitatory mechanisms of developmental neurotoxicity: transcriptional profiles for chlorpyrifos, diazinon, dieldrin, and divalent nickel in PC12 cells. *Environ. Health Perspect. 117*, 587−596.
(37) Florang, V. R., Rees, J. N., Brogden, N. K., Anderson, D. G., Hurley, T. D., and Doorn, J. A. (2007) Inhibition of the oxidative metabolism of 3,4-dihydroxyphenylacetaldehyde, a reactive intermediate of dopamine metabolism, by 4-hydroxy-2-nonenal. *Neurotoxicology 28*, 76−82.
(38) Rees, J. N., Florang, V. R., Anderson, D. G., and Doorn, J. A. (2007) Lipid peroxidation products inhibit dopamine catabolism yielding aberrant levels of a reactive intermediate. *Chem. Res. Toxicol. 20*, 1536−1542.
(39) Rees, J. N., Florang, V. R., Eckert, L. L., and Doorn, J. A. (2009) Protein reactivity of 3,4-dihydroxyphenylacetaldehyde, a toxic dopamine metabolite, is dependent on both the aldehyde and the catechol. *Chem. Res. Toxicol. 22*, 1256−1263.
(40) Coats, J. R. (1990) Mechanisms of toxic action and structure-activity relationships for organochlorine and synthetic pyrethroid insecticides. *Environ. Health Perspect. 87*, 255−262.
(41) Lopachin, R. M., and Decaprio, A. P. (2005) Protein adduct formation as a molecular mechanism in neurotoxicity. *Toxicol. Sci. 86*, 214−225.
(42) Haunerland, N. H., and Bowers, W. S. (1986) Binding of insecticides to lipophorin and arylphorin, 2 hemolymph-proteins of Heliothis-Zea. *Arch. Insect Biochem. Physiol. 3*, 87−96.
(43) Jakubowski, T., and Crowder, L. A. (1973) Binding of 36C1-dieldrin to suspected target and non-target proteins in vitro. *Bull. Environ. Contam. Toxicol. 10*, 217−224.
(44) Bergen, W. G. (1971) The in vitro effect of dieldrin on respiration of rat liver mitochondria. *Proc. Soc. Exp. Biol. Med. 136*, 732−735.
(45) Pardini, R. S., Heidker, J. C., and Payne, B. (1971) The effect of some cyclodiene pesticides, benzenehexachloride and toxaphene on mitochondrial electron transport. *Bull. Environ. Contam. Toxicol. 6*, 436−444.
(46) Hamilton, E. W. (1971) Relative toxicity of aldrin and dieldrin. *J. Agric. Food. Chem. 19*, 863−864.
(47) Kutz, F. W., Wood, P. H., and Bottimore, D. P. (1991) Organochlorine pesticides and polychlorinated biphenyls in human adipose tissue. *Rev. Environ. Contam. Toxicol. 120*, 1−82.
(48) Pietruszko, R., MacKerell, A. D., Jr., and Ferencz-Biro, K. (1985) Adducts of propiolaldehyde and 3,4-dihydroxyphenylacetaldehyde with human aldehyde dehydrogenase. *Prog. Clin. Biol. Res. 183*, 67−74.
(49) Matthews, H. B., and McKinney, J. D. (1974) Dieldrin metabolism to cis-dihydroaldrindiol and epimerization of cis- to trans-dihydroaldrindiol by rat liver microsomes. *Drug Metab. Dispos. 2*, 333−340.
(50) Kadous, A. A., and Matsumura, F. (1982) Toxicity of metabolites of dieldrin, photodieldrin, and endrin in the cockroach. *Arch. Environ. Contam. Toxicol. 11*, 635−643−643.
(51) Patterson, D. G., Jr., Wong, L. Y., Turner, W. E., Caudill, S. P., Dipietro, E. S., McClure, P. C., Cash, T. P., Osterloh, J. D., Pirkle, J. L., Sampson, E. J., and Needham, L. L. (2009) Levels in the U.S. population of those persistent organic pollutants (2003−2004) included in the Stockholm Convention or in other long range transboundary air pollution agreements. *Environ. Sci. Technol. 43*, 1211−1218.
(52) Sullivan, J. B., and Krieger, G. R. (2001) *Clinical Environmental Health and Toxic Exposures*, 2nd ed., Lippincott Williams & Wilkins, Philadelphia, PA.
(53) Uhl, G. R. (1998) Hypothesis: the role of dopaminergic transporters in selective vulnerability of cells in Parkinson's disease. *Ann. Neurol. 43*, 555−560.
(54) Jenner, P. (2003) Oxidative stress in Parkinson's disease. *Ann. Neurol. 53* (Suppl 3), S26−S36 discussion S36−S28..
(55) Fumagalli, F., Gainetdinov, R. R., Wang, Y. M., Valenzano, K. J., Miller, G. W., and Caron, M. G. (1999) Increased methamphetamine neurotoxicity in heterozygous vesicular monoamine transporter 2 knock-out mice. *J. Neurosci. 19*, 2424−2431.
(56) Gainetdinov, R. R., Fumagalli, F., Wang, Y. M., Jones, S. R., Levey, A. I., Miller, G. W., and Caron, M. G. (1998) Increased MPTP neurotoxicity in vesicular monoamine transporter 2 heterozygote knockout mice. *J. Neurochem. 70*, 1973−1978.

(57) Miller, G. W., Gainetdinov, R. R., Levey, A. I., and Caron, M. G. (1999) Dopamine transporters and neuronal injury. *Trends Pharmacol. Sci. 20*, 424−429.
(58) Sharma, R. P., Winn, D. S., and Low, J. B. (1976) Toxic, neurochemical and behavioral effects of dieldrin exposure in mallard ducks. *Arch. Environ. Contam. Toxicol. 5*, 43−53.
(59) Heinz, G. H., Hill, E. F., and Contrera, J. F. (1980) Dopamine and norepinephrine depletion in ring doves fed DDE, dieldrin, and Aroclor 1254. *Toxicol. Appl. Pharmacol. 53*, 75−82.
(60) Kirby, M. L., Barlow, R. L., and Bloomquist, J. R. (2002) Selective effects of cyclodiene insecticides on dopamine release in mammalian synaptosomes. *Toxicol. Appl. Pharmacol. 181*, 89−92.
(61) Eisenhofer, G., Kopin, I. J., and Goldstein, D. S. (2004) Catecholamine metabolism: a contemporary view with implications for physiology and medicine. *Pharmacol. Rev. 56*, 331−349.
(62) Mexas, L. M., Florang, V. R., and Doorn, J. A. (2011) Inhibition and covalent modification of tyrosine hydroxylase by 3,4-dihydroxyphenylacetaldehyde, a toxic dopamine metabolite. *Neurotoxicology 32*, 471−477.
(63) Wey, M. C., Fernandez, E., Martinez, P. A., Sullivan, P., Goldstein, D. S., and Strong, R. (2012) Neurodegeneration and motor dysfunction in mice lacking cytosolic and mitochondrial aldehyde dehydrogenases: implications for Parkinson's disease. *PLoS One 7*, e31522.
(64) Goldstein, D. S., Sullivan, P., Holmes, C., Kopin, I. J., Basile, M. J., and Mash, D. C. (2011) Catechols in post-mortem brain of patients with Parkinson disease. *Eur. J. Neurol. 18*, 703−710.
(65) Burke, W. J., Li, S. W., Chung, H. D., Ruggiero, D. A., Kristal, B. S., Johnson, E. M., Lampe, P., Kumar, V. B., Franko, M., Williams, E. A., and Zahm, D. S. (2004) Neurotoxicity of MAO metabolites of catecholamine neurotransmitters: role in neurodegenerative diseases. *Neurotoxicology 25*, 101−115.
(66) Burke, W. J., Li, S. W., Williams, E. A., Nonneman, R., and Zahm, D. S. (2003) 3,4-Dihydroxyphenylacetaldehyde is the toxic dopamine metabolite in vivo: implications for Parkinson's disease pathogenesis. *Brain Res. 989*, 205−213.
(67) Panneton, W. M., Kumar, V. B., Gan, Q., Burke, W. J., and Galvin, J. E. (2010) The neurotoxicity of DOPAL: behavioral and stereological evidence for its role in Parkinson disease pathogenesis. *PLoS One 5*, e15251.
(68) Krieger, R. I. (2001) *Handbook of Pesticide Toxicology*, 2nd ed., Academic Press, San Diego, CA.
(69) Klaassen, C. D., Watkins, J. B., and Casarett, L. J. (2010) *Casarett & Doull's Essentials of Toxicology*, 2nd ed., McGraw-Hill Medical, New York.
(70) Pope, C. N. (1999) Organophosphorus pesticides: do they all have the same mechanism of toxicity? *J. Toxicol. Environ. Health, Part B 2*, 161−181.
(71) Slotkin, T. A., Levin, E. D., and Seidler, F. J. (2006) Comparative developmental neurotoxicity of organophosphate insecticides: effects on brain development are separable from systemic toxicity. *Environ. Health Perspect. 114*, 746−751.
(72) Slotkin, T. A., and Seidler, F. J. (2007) Comparative developmental neurotoxicity of organophosphates in vivo: transcriptional responses of pathways for brain cell development, cell signaling, cytotoxicity and neurotransmitter systems. *Brain Res. Bull. 72*, 232−274.
(73) Slotkin, T. A., Seidler, F. J., and Fumagalli, F. (2007) Exposure to organophosphates reduces the expression of neurotrophic factors in neonatal rat brain regions: similarities and differences in the effects of chlorpyrifos and diazinon on the fibroblast growth factor superfamily. *Environ. Health Perspect. 115*, 909−916.
(74) Slotkin, T. A., Tate, C. A., Ryde, I. T., Levin, E. D., and Seidler, F. J. (2006) Organophosphate insecticides target the serotonergic system in developing rat brain regions: disparate effects of diazinon and parathion at doses spanning the threshold for cholinesterase inhibition. *Environ. Health Perspect. 114*, 1542−1546.
(75) Timofeeva, O. A., Roegge, C. S., Seidler, F. J., Slotkin, T. A., and Levin, E. D. (2008) Persistent cognitive alterations in rats after early postnatal exposure to low doses of the organophosphate pesticide, diazinon. *Neurotoxicol. Teratol. 30*, 38−45.
(76) Timofeeva, O. A., Sanders, D., Seemann, K., Yang, L., Hermanson, D., Regenbogen, S., Agoos, S., Kallepalli, A., Rastogi, A., Braddy, D., Wells, C., Perraut, C., Seidler, F. J., Slotkin, T. A., and Levin, E. D. (2008) Persistent behavioral alterations in rats neonatally exposed to low doses of the organophosphate pesticide, parathion. *Brain Res. Bull. 77*, 404−411.