Journal of Chromatography B, 878 (2010) 1312–1319

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Analytical approaches to investigate protein–pesticide adducts☆,☆☆

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ARTICLE INFO

Article history:
Received 22 July 2009
Accepted 14 October 2009
Available online 22 October 2009

Keywords:
Organophosphorus pesticide
Neurotoxicity
Immunotoxicity
Serine hydrolase
Diisopropylfluorophosphate
Protein adduct
Biomarker
Low-dose exposure
Proteomics
Albumin
Carboxylesterase 1

ABSTRACT

Organophosphorus pesticides primarily elicit toxicity via their common covalent adduction of acetylcholinesterase (AChE), but pesticide binding to additional sensitive secondary targets may also compromise health. We have utilised tritiated-diisopropylfluorophosphate (^{3}H-DFP) binding to quantify the levels of active immune and brain tissue serine hydrolases, and visualise them using autoradiography after protein separation by one-dimensional and two-dimensional techniques. Preincubation of protein extracts with pesticide *in vitro* or dosing of rats with pesticide *in vivo* was followed by ^{3}H-DFP radiolabelling. Pesticide targets were identified by a reduction in ^{3}H-DFP radiolabelling relative to controls, and characterised by their tissue presence, molecular weight, and isoelectric point. Conventional column chromatography was employed to enrich pesticide targets to enable their further characterisation, and/or identification by mass spectrometry. The major *in vivo* pesticide targets characterised were 66 kDa, serum albumin, and 60 kDa, likely carboxylesterase 1, both of which displayed differential pesticide binding character under conditions producing approximately 30% tissue AChE inhibition. The characterisation and identification of sensitive pesticide secondary targets will enable an evaluation of their potential contribution to the ill health that may arise from chronic low-dose pesticide exposures. Additionally, secondary targets may provide useful biomonitors and/or bioscavengers of pesticide exposures.

1. Introduction

Organophosphorus (OP) compounds have been utilised as commercial pesticides for over 50 years. OP pesticides are distinguished by a central phosphorus atom double bonded to either sulphur (thion) or oxygen (oxon), an R1 group, R2 group, and a leaving group. Pesticides in common commercial use possess R1 and R2 groups that are ester linked to dimethyl or diethyl groups (see Fig. 1). Pesticides covalently bind protein targets directly as oxons, or require liver bioactivation from phosphorothionates (thions) to their corresponding oxons.

OPs form covalent adducts with protein nucleophilic sites, such as the carbonyl group of the hydroxy amino acids serine or tyrosine, thereby producing a phosphorylated derivative. Hydrolysis and removal of this phosphate moiety may be relatively slow, or essentially irreversible if adduct side chain dealkylation (ageing) occurs. The serine hydrolase family of enzymes possess an active site serine that is susceptible to OP adduction, and this has been exploited in the use of pesticides to covalently bind and inactivate acetylcholinesterase (AChE) within synapses. AChE hydrolyses the neurotransmitter ACh resulting in a termination of nerve signal conduction, hence its inactivation by OP adduction results in ACh signal persistence and cholinergic toxicity.

Additionally, inadvertent OP protein adduction of other serine hydrolases may arise, whose enzymatic inactivation may also contribute to ill health [1–3]. Although OP pesticides constitute a generic compound class by virtue of their binding and inactivation of AChE, disparate binding and structure–activity relationships may well exist for secondary protein targets. Moreover, OP secondary targets may impact upon health status at OP exposures at or below the 30% inhibition of brain AChE that can trigger signs of cholinergic toxicity [2,4–9]. Additionally, secondary (blood) protein targets may provide useful adjuncts to measurements of cholinesterase inhibitions or analysis of urinary metabolites for pesticide exposure biomonitoring [10], and also provide bioscavengers capable of depleting toxic OPs [11].

Toxicologically relevant pesticide secondary targets can be evaluated systematically by analysis of pesticide structure–activity profiles, but this can only be accomplished if the tissue activity of a given serine hydrolase is known. An alternative approach has been to interrogate tissues directly for active serine hydrolases using compounds retaining an OP chemical signature, for example, affinity ligands that possess a fluorophosphonate (FP) probe [12–15]. As an alternative, we and others have utilised the OP compound diisopropylfluorophosphate (DFP) for which a tritiated version is commercially available, to provide a means to track active serine hydrolases by radiolabelling and autoradiography [16–20].

☆ This paper is part of the special issue 'Bioanalysis of Organophosphorus Toxicants and Corresponding Antidotes', Harald John and Horst Thiermann (Guest Editors).
☆☆ Presented at the 12th Medical Chemical Defence Conference, 22–23 April 2009, Munich, Germany.

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doi:10.1016/j.jchromb.2009.10.018

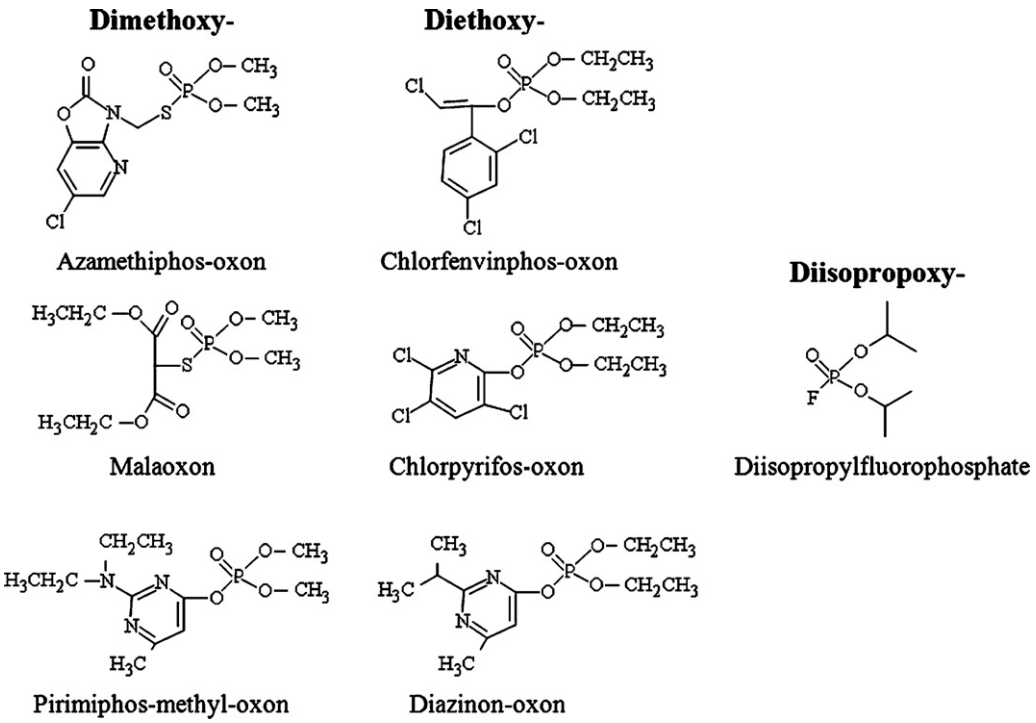

Fig. 1. Structures of the pesticides examined, drawn as their active oxons, and divided into dimethoxy-, diethoxy-, and diisopropoxy-forms.

Our studies have focussed upon brain tissue targets of pesticides (neurotoxicity), and tissues of the immune system and blood (immunotoxicity), since accumulating evidence suggests that the immune and neuro-immune system also represent important targets of OP toxicity [21,22]. In this paper we describe the use of ^{3}H-DFP to quantify active serine hydrolases within immune and brain tissues, and a strategy for detecting which of these active hydrolases is also a target of pesticide binding. This strategy also provided a means of purification, characterisation, and ultimately identification of pesticide targets.

2. Experimental

2.1. Materials

The organophosphorus pesticides: azamethiphos-oxon (*S*-6-chloro-2,3-dihydro-2-oxo-1,3-oxazolo[4,5-*b*]pyridin-3-ylmethyl *O*,*O*-dimethyl phosphorothioate), chlorfenvinphos-oxon (2-chloro-1-(2,4-dichlorophenyl)vinyl diethyl phosphate), and malathion (diethyl (dimethoxyphosphinothioylthio)succinate) and its corresponding oxon (malaoxon) were purchased from QMX Laboratories Ltd., Thaxted, UK, and were at 95–99.5% purity. Chlorpyrifos (*O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphorothioate) and diazinon (*O*,*O*-diethyl *O*-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate) as their corresponding oxons, and pirimiphos-methyl (*O*-2-diethylamino-6-methylpyrimidin-4-yl *O*,*O*-dimethyl phosphorothioate) were purchased from Greyhound Laboratories, Birkenhead, UK, at 97.2–99.4% purity. For *in vitro* assays, pesticides were prepared as 100 mM stock solutions in ethanol (Sigma, HPLC grade, <0.10% water), except azamethiphos-oxon which was at 50 mM, and were stored at 4 °C for up to 2 weeks. Pesticides were diluted in phosphate buffered saline (PBS) to required concentrations just prior to use. Tritiated-diisopropylfluorophosphate (^{3}H-DFP) at a specific activity of 150 GBq/mmol was purchased from Perkin Elmer, Boston, USA. NuPAGE Novex pre-cast gels (4–12% Bis–Tris gels for 1D SDS–PAGE and 4–12% Bis–Tris Zoom gels for 2D-PAGE), MOPS–SDS running buffer, transfer buffer, SeeBlue Plus2 prestained gel standards, and Safe stain were all obtained from the Invitrogen Corporation. Precision plus prestained protein standards, and IPG strips (pH 3–10, 7 cm length) were purchased from BioRad, with all isoelectric focussing performed using a BioRad Protean isoelectric focussing cell. Dithiothreitol (DTT), wide range molecular weight markers, ρ-nitrophenyl acetate, gel filtration molecular weight standards, and recombinant human AChE (C1682) were all purchased from Sigma.

2.2. Blood and tissue preparations

Male F344 strain rats weighing between 200 and 230 g were used for experiments. Rats were maintained in cages (four/cage) under controlled temperature (21 ± 1 °C) and light (16 h light/8 h dark cycle) with *ad libitum* access to food intake and water. All animal procedures were approved by the University of Nottingham Local Ethical Review Committee and were carried out in accordance with the Animals Scientific Procedures Act (UK) 1986. Rats were dosed orally by gavage with OPs in arachis oil. Rat blood, and whole tissue and cytosolic extract were prepared according to previous publications [19,20]. Control human blood (taken with University of Nottingham Ethical Review Committee approval) from one of the authors (male, 41 years of age) was collected into heparin and erythrocytes and plasma prepared as described previously [20]. Protein concentrations in homogenates were measured using the DC Protein assay (Biorad) using bovine serum albumin as a protein standard.

2.3. Acetylcholinesterase measurements

Blood or tissue AChE activity measurements were based upon the spectrophotometric method described by Ellman et al. [23]. Spectrophotometric measurements were performed at 412 nm in a Perkin Elmer Lambda 2S spectrophotometer operated using UV KinLab software as described previously [20].