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1 **Title: Inhibition by pesticides of the DJ-1/Park7 protein related to Parkinson** 2 **disease.**

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17 **Abbreviations:** AGE, advanced glycation end products; BSA, bovine serum albumin; DTT,
18 dithiothreitol; GMP, guanosine monophosphate; MGO, methylglyoxal; PD, Parkinson's
19 disease; pNPA, para-nitro-phenyl acetate.

20 **Abstract:**

21 Parkinson's disease is a severe neurodegenerative disease. Several environmental contaminants
22 such as pesticides have been suspected to favor the appearance of this pathology. The protein
23 DJ-1 (or Park7) protects against the development of Parkinson's disease. Thus, the possible
24 inhibitory effects of about a hundred pesticides on human DJ-1 have been studied. We identified
25 fifteen of them as strong inhibitors of DJ-1 with IC50 values between 0.02 and 30 μ M.
26 Thiocarbamates are particularly good inhibitors, as shown by thiram that acts as an irreversible
27 inhibitor of an esterase activity of DJ-1 with an IC50 value of 0.02 μ M. Thiram was also found
28 as a good inhibitor of the protective activity of DJ-1 against glycation. Such inhibitory effects
29 could be one of the various biological effects of these pesticides that may explain their
30 involvement in the development of Parkinson's disease.

31 **Keywords:** Neurodegenerative diseases, Pesticides, Park7, thiocarbamates.

32 **1. Introduction**

33 Parkinson's disease (PD) is the second most common neurodegenerative disease with
34 complex etiology and variable pathology (Kalia and Lang 2015). Most cases originate from a
35 combination of factors that are not fully characterized. Nevertheless, several molecular
36 mechanisms were historically identified as common markers of the disease, including α -
37 synuclein misfolding and aggregation, and such processes play a central role in the occurrence
38 of PD (Kazantsev and Kolchinsky 2008). Regarding its complex etiology, although
39 multifactorial, the causes of PD have mainly two components, a genetic component, and an
40 environmental component. Among various environmental factors, pesticides exposure has been
41 found as a major candidate in PD pathogenesis. Indeed, an increased risk of developing PD has
42 been observed in people exposed for a long period of time to pesticides (Baldi et al. 2021;
43 Brown et al. 2006). Some studies were able to investigate the risks of specific chemicals, such

44 as paraquat, maneb, organochlorines and rotenone (Vellingiri et al. 2022). A review by Tanner
1 45 et al. summarized the state of knowledge on this topic (Tanner et al. 2011). The chemicals for
2 46 which most data link them to an increased PD risk are paraquat and rotenone, with exposure
3 47 associated with a 2-3-fold higher PD risk over the general population (Yan et al. 2018). More
4 48 recently a study estimated the consequences of exposure for 157 pesticides in the Netherlands
5 49 population. Environmental exposure to pesticides was estimated using a spatio-temporal model,
6 50 based on agricultural crops around the residential address. Four of these 157 pesticides were
7 51 considered a priori relevant for PD: paraquat, maneb, lindane, and benomyl (Brouwer et al.
8 52 2017). Interestingly, two of them are carbamates or thiocarbamates (R-NH-CO-NH-R or R-
9 53 NH-CS-S-R), that are highly reactive towards biomolecules through modification of their thiol
10 54 residues.

14 55 Thus, the association between exposure to agricultural pesticides and PD has long been
15 56 a topic of study in the field of environmental health. However, the various molecular
16 57 mechanisms that are at the origin of the effects of pesticides on the development of PD are not
17 58 known in most cases.

19 59 A major factor contributing to cellular and organism aging is the accumulation of
20 60 advanced glycation end products (AGEs) on proteins, lipids and nucleic acids (Chaudhuri et al.
21 61 2018; Miranda et al. 2016; Singh et al. 2001). The AGEs comprise a large number of
22 62 heterogeneous chemical structures and a majority of them have a propensity to crosslink
23 63 proteins, which alters their structure and function. The glycation process (also called non-
24 64 enzymatic glycosylation) is initiated by a non-enzymatic reaction between sugar-derived
25 65 aldehydes and protein or nucleic acid nucleophiles such as amino or thiol groups. This reaction
26 66 was discovered by the French chemist Louis-Camille Maillard in 1912 and the major glycating
27 67 agents are reducing sugars (glucose, fructose, ribose, and their phosphorylated derivatives) and
28 68 glyoxals such as methylglyoxal (MGO, CH₃CO-CHO) (Maillard 1912). Glyoxals are formed
29 69 as by-products of glucose metabolism and are responsible for 65% of cellular glycation events.
30 70 The condensation reaction begins with the rapid formation of a hemithioacetal with thiols and
31 71 of aminocarbinols with NH₂ groups of biomolecules. Nucleic acids undergo permanent
32 72 glycation by MGO and the most susceptible nucleotides are guanosine and deoxyguanosine
33 73 (Thornalley 2008). Then, a series of dehydrations, oxidations and rearrangements result in a
34 74 myriad of products: Amadori's, AGEs and protein and/or DNA crosslinks. Several recent
35 75 studies implicate glycation as an important process in the pathogenesis of PD (Miranda et al.
36 76 2016; Sadowska-Bartosz and Bartosz 2016). Thus, it has been shown that α -synuclein itself, a
37 77 protein that plays a central role in PD, can be glycated in models of this pathology (Guerrero et
38 78 al. 2013). Recently, Outeiro and colleagues established that the glycation process enhances α -
39 79 synuclein toxicity *in vitro* and *in vivo*, in *Drosophila* and mice.

41 80 The protein DJ-1 (or Park7) has a protective role towards the development of PD since
42 81 the PARK7 gene is associated with recessive and sporadic forms of PD (Bonifati et al. 2003).
43 82 Intensive studies have been undertaken to figure out DJ-1 function(s) and its role in the etiology
44 83 of this neurodegenerative disease. Thus, DJ-1 has been proposed to take part in various
45 84 physiological pathways related to the promotion of cell survival (Oh and Mouradian 2017). It
46 85 also modulates oxidative and electrophilic stresses. DJ-1 activates the Nrf2-mediated
47 86 antioxidant response (Neves et al. 2022). It catalytically protects and/or repairs various
48 87 biomolecules against glycation by glyoxals (Matsuda et al. 2017; Richarme et al. 2017;
49 88 Richarme et al. 2015) or detoxifies reactive compounds produced during glycolysis (Heremans
50 89 et al. 2022; Lee et al. 2012). Although the nature of its physiological substrates is still a matter
51 90 of controversy (Andreeva et al. 2019; Galligan et al. 2018; Jun and Kool 2020; Matsuda et al.

91 2017; Mazza et al. 2022; Mulikova et al. 2021; Pfaff et al. 2017a, b; Richarme 2017; Richarme
1 92 and Dairou 2017; Richarme et al. 2017; Richarme et al. 2015) DJ-1 is involved in the protection
2 93 against the glycation processes.

3 94 This article reports the effects of about 100 pesticides belonging to the main chemical
4 95 classes, such as organophosphates, organochlorines, arylureas, carbamates, aryloxyalkanoic
5 96 acids, triazoles, neonicotinoids and pyrethroids, on human recombinant DJ-1. It shows that
6 97 some of them are irreversible inhibitors of DJ-1 at the sub-micro molar level, revealing a new
7 98 mode of action of these molecules. This strong inhibitory effect could be involved in the role
8 99 of those pesticides in the development of PD.

100 **2. Materials and methods**

101 **2.1. Materials.**

102 Most chemical and biochemical reactants were purchased from Sigma-Aldrich or Merck (St.
103 Louis, United States). Plasmids for human DJ-1 were obtained from Dr. Sun-Sin Cha (Lee et
104 al. 2003). Proteins were desalted with Micro Bio-Spin 6 columns (Biorad, Hercules, United
105 States). UV-Visible spectra were recorded on a Carry 300 or Biotek PowerWave XS
106 spectrometers. Gels were imaged on a LAS 4000 (GE Healthcare, Chicago, United States). The
107 Liquid Chromatography (LC) system was composed of Shimadzu apparatus (Kyoto, Japan)
108 equipped with a LC30AD pump, a SiL30AC auto-sampler coupled with a photodiode array
109 detector PDA20A and a triple quadrupole mass detector 8060.

110 **2.2. Human DJ-1 production and purification**

111 Human DJ-1 was expressed and purified as previously described (Richarme et al. 2015).
112 Briefly, the DJ-1 expression strain was grown in Luria-Bertani medium to an A600 = 0.6 and
113 then induced with 1 mM isopropyl β -D-1-thiogalactopyranoside for 4 h. Bacteria were lysed by
114 ultrasonic disruption, and the 100,000 g supernatant was used for DJ-1 purification. DJ-1 was
115 first purified on a DEAE-Sephacel (Pharmacia, NJ, United States) column equilibrated in 30
116 mM tris buffer, pH 8, containing 20 mM NaCl, 0.5 mM EDTA, and 1 mM dithiothreitol at 4°C.
117 Protein was eluted with a linear gradient of 20–400 mM NaCl in equilibration buffer. DJ-1 was
118 then purified on a hydroxyapatite column (Bio-Gel HTP from Bio-Rad) equilibrated in 20 mM
119 phosphate buffer, pH 8, containing 20 mM NaCl, 0.5 mM EDTA and 1 mM dithiothreitol at
120 4°C. Protein was eluted with a linear gradient of 20–250 mM sodium phosphate buffer, pH 8.
121 Then, DJ-1 was dialyzed overnight against 50 mM phosphate buffer (Na₂HPO₄), pH 8, 20 mM
122 NaCl, 0.5 mM EDTA and 1 mM dithiothreitol at 4 °C. Purified protein was quantitated with
123 Bradford reagent (Bradford 1976). Purity was assessed by SDS-PAGE and/or western blot with
124 anti-DJ-1 as antibody. Proteins were kept at -80°C until use.

125 **2.3. Enzyme Assay—Detection of DJ-1 esterase and deglycase activity**

126 Prior to any experiment, DJ-1 enzyme was reduced by 10 mM dithiothreitol (DTT) for 30 min
127 and then dialyzed overnight at 4°C against 50 mM phosphate buffer. Protein concentration was
128 determined using a Bradford assay with BSA (Bovine serum albumin) as standard (Bio-rad).
129 DJ-1 esterase activity was performed as described previously (Vazquez-Mayorga et al. 2016)
130 in a total volume of 200 μ l: ten μ L of DJ-1 solution (0.2 μ M final) were added to 190 μ L of a
131 2.8 mM solution of p-nitrophenylacetate (pNPA), prepared in PBS from a 200 mM stock
132 solution in dimethylsulfoxide. DJ-1 esterase activity was determined by monitoring the slope
133 of the absorbance at 405 nm vs time at 37°C.

134 The activity of DJ-1 concerning the protection against guanosine monophosphate glycation by
135 methylglyoxal was determined by LC as previously reported (Richarme et al. 2015). Briefly
136 guanosine monophosphate (GMP) 100 μ M was incubated at 37°C in N₂-gassed 50 mM

137 phosphate buffer pH 7.0, with 400 μ M MGO, in the absence or presence of 4 μ M DJ-1. The
138 samples were analyzed by LC on a C18 Reverse phase LC Kinetex® - Phenomenex column
139 equilibrated in water with formic acid 0.1% at 45°C, and eluted with a linear gradient with
140 acetonitrile with 0.1% formic acid. The kinetics of GMP protection by DJ-1 was followed by
141 measuring the area under the peak of the glycated GMP peak of the LC chromatogram
142 (absorbance at 254 nm and Mass Spectrometry detection).

143 ***2.4. Effects of pesticides on DJ-1 activity***

144 The inhibitory effects of pesticides were determined by running the enzymatic assay as
145 described above, in the presence of pesticide (5 μ M). This activity was compared to the activity
146 of a similar DJ-1 solution without pesticide (100%). pNPA hydrolysis by pesticides without
147 DJ-1 was also used as control and showed no significant esterase activity unless otherwise
148 stated. For pesticides that show DJ-1 inhibition greater than 25% under the previous conditions,
149 IC50 determinations were made.

150 To test whether the reaction of DJ-1 with pesticide was irreversible, purified enzyme (1 μ M
151 final) was first incubated in presence or absence of pesticide for 30 min at 37°C. The samples
152 were then dialyzed overnight at 4°C against phosphate buffer prior to enzyme assay. Controls
153 were carried out with non-dialyzed samples.

154 Kinetic data with thiram were obtained at 37°C as follows: to 1 mL of a 50 nM solution of DJ-
155 1 was added 1 μ L of a 1 mM solution of thiram (final concentrations: 1 μ M). The esterase
156 activity was recorded at various time intervals by monitoring of pNPA hydrolysis. The
157 bimolecular rate constant k_{inac} was determined by Kitz & Wilson methodology proposed in
158 1962, the time-course of an irreversible inhibition assay can be described by a rising exponential
159 curve by fitting (Kitz and Wilson 1962).

160 ***2.5. Effects of pesticides on the oxidation state of DJ-1***

161 Western blotting was performed as previously described (Bahmed et al. 2016). We used
162 antibodies against native and Cys106-oxidized DJ-1 (Sigma) and the blots were then developed
163 using an enhanced chemiluminescence western blotting kit according to the manufacturer's
164 instructions (Amersham Pharmacia Biotech, Piscataway, NJ). Images were quantitated using
165 ImageJ software.

166 ***2.6. Statistical Analyses***

167 Each experiment was performed independently at least three times. The results shown in figures
168 are, if not otherwise stated, mean values \pm S.E.M of 3–6 independent experiments. Statistical
169 analysis was performed using analysis of ANOVA test using Prism (GraphPad Software).

170 **3. Results and discussion**

171 About hundred pesticides belonging to the main pesticide chemical classes were tested as
172 possible inhibitors of DJ-1. First, they were tested as possible inhibitors of the esterase activity
173 of human recombinant DJ-1 using pNPA as substrate (Figure 1) (Maksimovic et al. 2021;
174 Vazquez-Mayorga et al. 2016). Table 1 shows the percentage of inhibition observed after 5 min
175 of incubation of 0.2 μ M DJ-1 in the presence of 2.8 mM pNPA and 5 μ M pesticide. Under these
176 conditions, only a little number of pesticides led to a significant inhibition of DJ-1. This was
177 the case of three carbamates (benomyl, formetanate and oxamyl), five organochlorines (captan,
178 DDE, dienochlore, folpet and lindane), one pyrethroid (resmethrin), two organophosphates,
179 (diazinon and pirimidiphos methyl) and two unclassified pesticides (paraquat and dithianon)
180 with various potencies (from 10 to 85% of inhibition). However, one family of pesticides
181 emerges as major inhibitors of DJ-1, the thiocarbamate series (from 70 to 100% of inhibition).

182 Interestingly, a thiocarbamate used as a drug, disulfiram, was recently reported as a good
183 inhibitor of the esterase activity of DJ-1 (Wu et al. 2022).

184 Table 2 indicates the IC₅₀ values found for those pesticides having led to a significant inhibition
185 of DJ-1 (higher than 25 %). Almost all the tested thiocarbamates and dithianon led to
186 remarkably low IC₅₀ values, equal or lower than 1 μM. Thiram was found to be the best inhibitor
187 under these conditions, with an IC₅₀ value of about 0.02 μM. It is important to compare these
188 IC₅₀ values with the concentrations found after human exposure to those pesticides. Thus, for
189 example, several recent studies have revealed that humans are exposed through their
190 consumption of fruit and vegetables and their drinking water to concentrations of maneb up to
191 450 μg/L (ie 1.7 μM) (Asghar et al. 2022; Carrasco Cabrera and Medina Pastor 2021; Onwona-
192 Kwakye et al. 2020; Ozhan and Alpertunga 2008). We found an IC₅₀ for maneb of 0.06 μM
193 that is much lower than the concentration reported upon maneb exposure.

194 Experiments based on preincubation of DJ-1 in the presence of the inhibitors were then
195 performed to determine the type of inhibition of those thiocarbamates. Figure 2a shows that the
196 inhibition level observed with 1 μM thiram clearly increased with the time of preincubation of
197 DJ-1 in the presence of this inhibitor. A complete inhibition of DJ-1 was observed after 5 min
198 preincubation of DJ-1 with 1 μM thiram, indicating that this pesticide acts as an irreversible
199 inhibitor of DJ-1. Determination of the k_{inact} led to a value of 3.5 ± 0.5 10⁴ M⁻¹.s⁻¹. Dialysis at
200 equilibrium experiments of the incubates of DJ-1 in the presence of thiram confirmed that DJ-
201 1 remained inactive under these conditions and that thiram acted as an irreversible inhibitor
202 (figure 2b).

203 Some of the pesticides having led to a strong inhibitory effect on the esterase activity of DJ-1
204 were tested on a previously described assay measuring the protecting activity of DJ-1 towards
205 glycated GMP formation upon reaction of GMP with MGO (Figure 3), resulting in lactate
206 formation, (Richarme et al. 2017). Figure 4A shows the LCMS chromatograms of glycated
207 GMP (m/z = 436.22), alone (a) or after incubation with DJ-1 (b), or after incubation with 4 μM
208 DJ-1 previously treated during 30 min at room temperature with 10 μM thiram (c). Figure 4B
209 shows the formation of lactate (m/z=89.00) under identical conditions. Those results show that
210 thiram is also an inhibitor of this protective activity of DJ-1 against glycated GMP formation.
211 Similar results were obtained with maneb and dithianon.

212 As a first attempt to determine the mechanism of the inhibition of DJ-1 by the pesticides
213 indicated in Table 2, experiments have been done to know whether some of them could lead to
214 an irreversible oxidation of cysteine 106, a crucial residue of the DJ-1 active site (Blackinton
215 et al. 2009; Canet-Aviles et al. 2004; Kinumi et al. 2004). For that purpose, western-blot
216 analyses of DJ-1 incubation mixtures were done by using a specific antibody against an
217 oxidized form of DJ-1, specifically at the level of Cys106. Figure 5 compares the analysis of
218 untreated DJ-1 with DJ-1 pretreated with 100 μM H₂O₂ and confirmed that the antibody
219 specifically recognizes S-oxidized DJ-1 (line 1 vs 2). Interestingly, analysis of DJ-1 pretreated
220 with maneb (line 5) showed the presence of S-oxidized DJ-1 whereas this was not the case for
221 DJ-1 pretreated with thiram or zineb. The oxidation of Cys106 observed upon treatment of DJ-
222 1 with maneb, that is a Mn(II) complex, could be due to the binding and reaction of dioxygen
223 (O₂) with its Mn(II) center with formation of superoxide. Accordingly, incubation of DJ-1 with
224 thiram or zineb that did not contain such a Mn(II) redox active center did not lead to Cys106
225 oxidation (Figure 5).

226 Similar experiments performed on DJ-1 pretreated with dithianon (figure 5) showed the
227 formation of S-oxidized DJ-1 whereas this was not the case for DJ-1 pretreated with folpet,
228 formetamate, lindane, resmethrine, oxamyl or triallate (data not shown). This oxidation of
229 Cys106 by dithianon could be due to its particular activated quinone structure able to receive
230 an electron from Cys106 with formation of a stable radical anion.

231 From those data, it seems that oxidation of Cys106 would only be involved in the inhibition of
232 DJ-1 by a few pesticides, such as maneb, mancozeb (data not shown) or dithianon. The
233 mechanisms of inhibition of DJ-1 by the other pesticides mentioned in Table 2 remain to be
234 determined.

235 4. Conclusion

236 Our study of the possible inhibitory effects of about hundred pesticides on human DJ-1 has
237 shown that some twenty of them are inhibitors of the esterase activity of DJ-1 (Table 1). Fifteen
238 of them are good inhibitors with IC₅₀ values between 0.02 and 30 μM (Table 2). The pesticides
239 of the thiocarbamate series are particularly good inhibitors. Thus, thiram was found as an
240 irreversible DJ-1 inhibitor with an IC₅₀ value of 0.02 μM. All those fifteen pesticides have been
241 linked to the development of PD (Brouwer et al. 2017; Brown et al. 2006; Tanner et al. 2011).
242 Interestingly, a recent study of the consequences of the exposure for 157 pesticides in the
243 Netherland population has shown that four of those pesticides, paraquat, maneb, lindane and
244 benomyl, were relevant for the development of PD (Brouwer et al. 2017). Interestingly, those
245 four pesticides were found to act as inhibitors of DJ-1 (Tables 1 and 2).

246 Several biological effects of some pesticides have been reported to contribute to the
247 development of PD. Thus, for instance, rotenone act as inhibitor of mitochondrial complex I
248 (Betarbet et al. 2006; Betarbet et al. 2000; Schiller and Zickermann 2022), Paraquat increases
249 the formation of free radicals and oxidative stress (Ranjbar et al. 2018) and benomyl and some
250 dithiocarbamates act as inhibitors of the aldehyde dehydrogenase that is responsible for the
251 detoxication of a dopamine metabolite, 3,4-dihydroxyphenyl acetaldehyde (Burke et al. 2014;
252 Fitzmaurice et al. 2014; Fitzmaurice et al. 2013). Our results show that some of the hundred
253 pesticides tested in our study are strong inhibitors of DJ-1. However, the esterase activity of
254 DJ-1 has so far no identified physiological role and further experiments using *in cellulo* and *in*
255 *vivo* models are necessary to show that this inhibition of DJ-1 could be one of the various
256 biological effects of those pesticides involved in the development of PD.

257 **5. Appendices: Tables and figures legends**

258 **5.1. Table 1. Inhibition of human DJ-1 by pesticides.**

259 % inhibition of the DJ1 esterase activity (using pNPA as substrate) upon incubation of DJ-1 in
 260 the presence of 2.8 mM pNPA and 5 μ M pesticide; mean values +/- S.E.M. from 6 to 9
 261 experiments.

Chemical Class	Compounds	% of Inhibition
Aryloxy alkanolic acids	Cyhalofop-butyl	<5%
	Diclofop-methyl	<5%
	Fenoxaprop-P	<5%
	Fluazifop-P-butyl	<5%
	MCPA	<5%
	MCPB	<5%
	Mecoprop-P	<5%
	Quizalofop-P-ethyl	<5%
Aryl ureas	Chlortoluron	<5%
	Foramsulfuron	<5%
	Linuron	<5%
	Metobromuron	<5%
	Metsulfuron-methyl	<5%
	Nicosulfuron	<5%
	Rimsulfuron	<5%
	Tribenuron-methyl	<5%
Carbamates	Tritosulfuron	<5%
	Asulam	<5%
	Benomyl	85 \pm 6%
	Carbaryl	<5%
	Carbendazim	<5%
	Chlorpropham	<5%
	Diethofencarb	<5%
	Formetanate	56 \pm 8%
	Iprovalicarb	<5%
	Methomyl	<5%
	Oxamyl	22 \pm 7%
	Phenmedipham	<5%
	Pirimicarb	<5%
Propamocarb	<5%	
Prosulfocarb	<5%	
Thiabendazole	<5%	
Neonicotinoids	Imidacloprid	<5%
	Thiacloprid	<5%
	Thiamethoxam	<5%
Organochlorines	Boscalid	<5%
	Bixafen	<5%

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	captan	57 ± 9%
	Chlordecon	<5%
	DDE	17 ± 4%
	DDT	<5%
	Dieldrin	<5%
	Dienochlore	35 ± 5%
	Endosulfan	<5%
	Folpet	25 ± 4%
	Lindane	12 ± 4%
	Metolachlore	<5%
	Acephate	<5%
	Diazinon	10 ± 4%
	Dichlorvos	<5%
	Dimethoate	<5%
	Ethion	<5%
	Fenitrothion	<5%
	Glyphosate	<5%
	Isocarbophos	<5%
	Malathion	<5%
	Methyl parathion	<5%
	Monocrotophos	<5%
	Parathion (ethyl)	<5%
	Phosmet	<5%
	pirimiphos methyl	14 ± 3%
	Profenofos	<5%
	Tolclofos-methyl	<5%
	Triazophos	<5%
	Allethrin	<5%
	Bifenthrin	<5%
	Cyfluthrin	<5%
	Cypermethrin	<5%
	Deltamethrin	<5%
	Esfenvalerate	<5%
	Fenvalerate	<5%
	Fluvalinate	<5%
	Permethrin	<5%
	Phenothrin	<5%
	Resmethrin	26 ± 4%
	Tetramethrin	<5%
	Tralomethrin	<5%
	Mancozeb	100 ± 5%
	Maneb	100 ± 6%
	Propineb	100 ± 3%
	Thiram	100 ± 1%
	Triallat	70 ± 12%
	Zineb	100 ± 7%
	Ziram	100 ± 6%

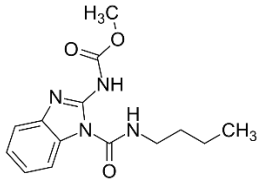
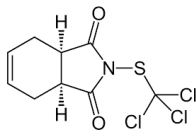
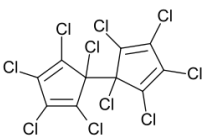
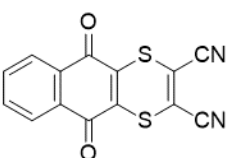
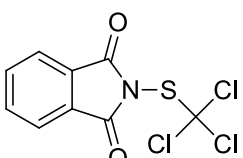
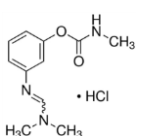
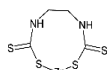
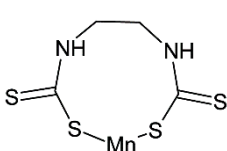
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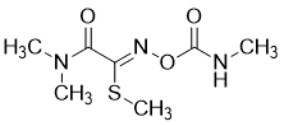
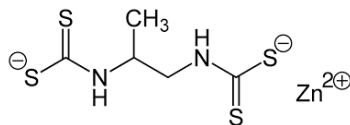
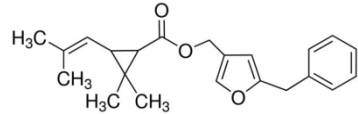
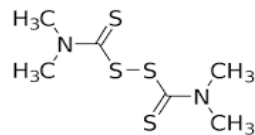
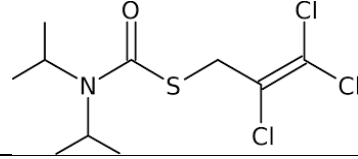
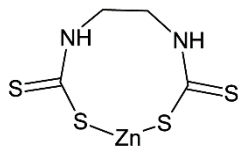
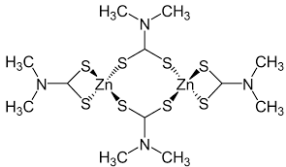
Triazoles	Difenoconazole	<5%
	Hexaconazole	<5%
	Metconazole	<5%
	Myclobutanil	<5%
	Penconazole	<5%
	Tebuconazole	<5%
	Triticonazole	<5%
Others	Atrazine	<5%
	Cyprodinil	<5%
	Dinitramin	<5%
	Dithianon	100 ± 4%
	Iprodione	<5%
	Mepanipyrim	<5%
	Mesotrione	<5%
	Paraquat	20 ± 4%
	Rotenone	<5%
	Sedaxane	<5%
Tembotrione	<5%	

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263 **5.2. Table 2. IC₅₀ values obtained for the inhibition of DJ1 (esterase activity) by**
 264 **pesticides.**

265 Mean values expressed in μM +/- S.E.M. from 3 to 6 experiments.

Compounds	STRUCTURE	IC ₅₀ (μM)
Benomyl		0.92 ± 0.1
Captan		6.1 ± 0.3
Dienochlore		19.2 ± 0.2
Dithianon		1.1 ± 0.2
Folpet		15.1 ± 2.3
Formetanate		3.2 ± 0.3
Mancozeb		0.09 ± 0.01
Maneb		0.06 ± 0.01

Oxamyl		24.8 ± 0.3
Propineb		0.17 ± 0.02
Resmethrine		18.1 ± 0.8
Thiram		0.018 ± 0.002
Triallat		0.99 ± 0.11
Zineb		0.17 ± 0.02
Ziram		0.15 ± 0.02

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5.3. Figure 1. Esterase activity of DJ-1 towards pNPA as substrate.

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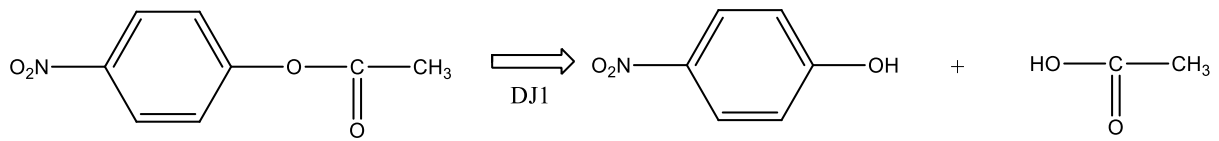
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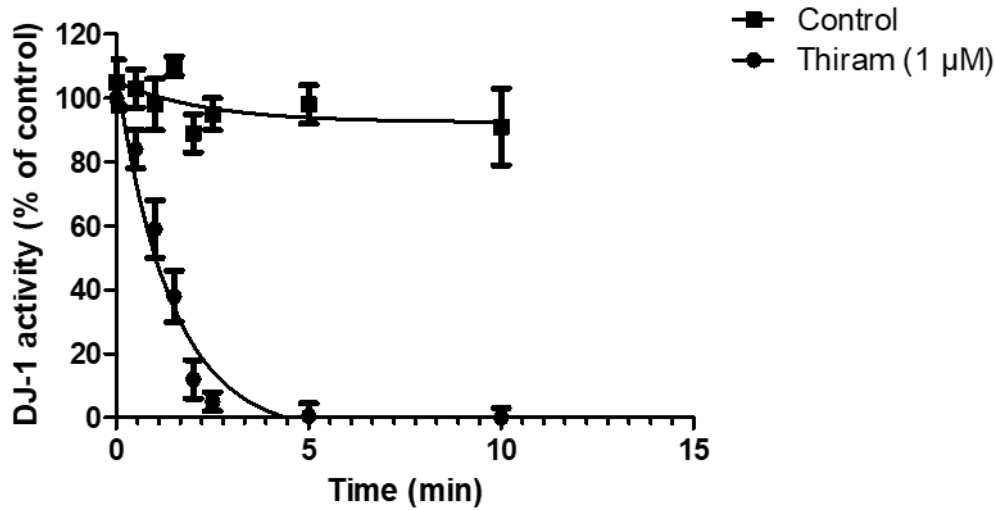
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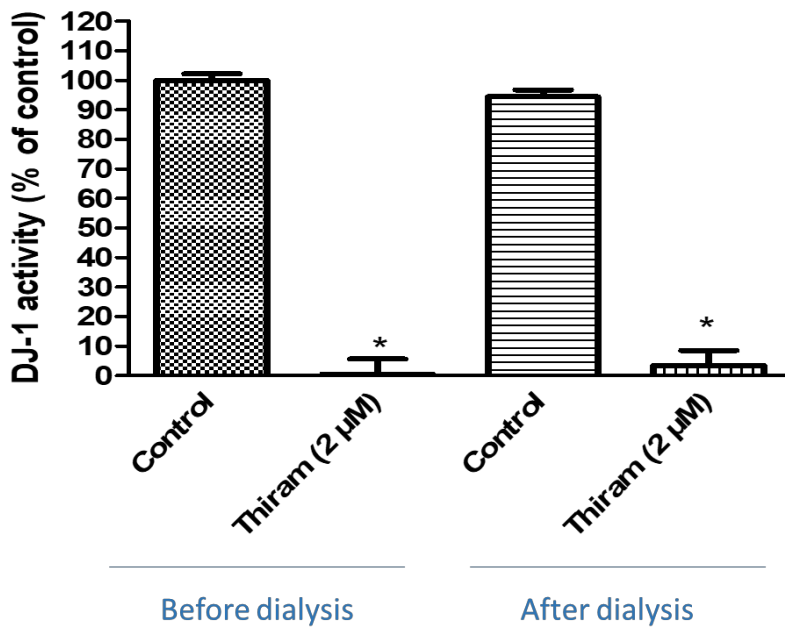


273 **5.4. Figure 2. Kinetic analysis of DJ-1 inhibition by thiram**

1 274 a: Inhibition of the DJ-1 esterase activity as a function of the time of preincubation of DJ-1 with
2 275 thiram. Mean values from three independent experiments.
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25 279 b: Inhibition of the DJ-1 esterase activity with thiram before and after dialysis. Mean values
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5.5. Figure 3. Protective activity of DJ1 against glycation of GMP by MGO.

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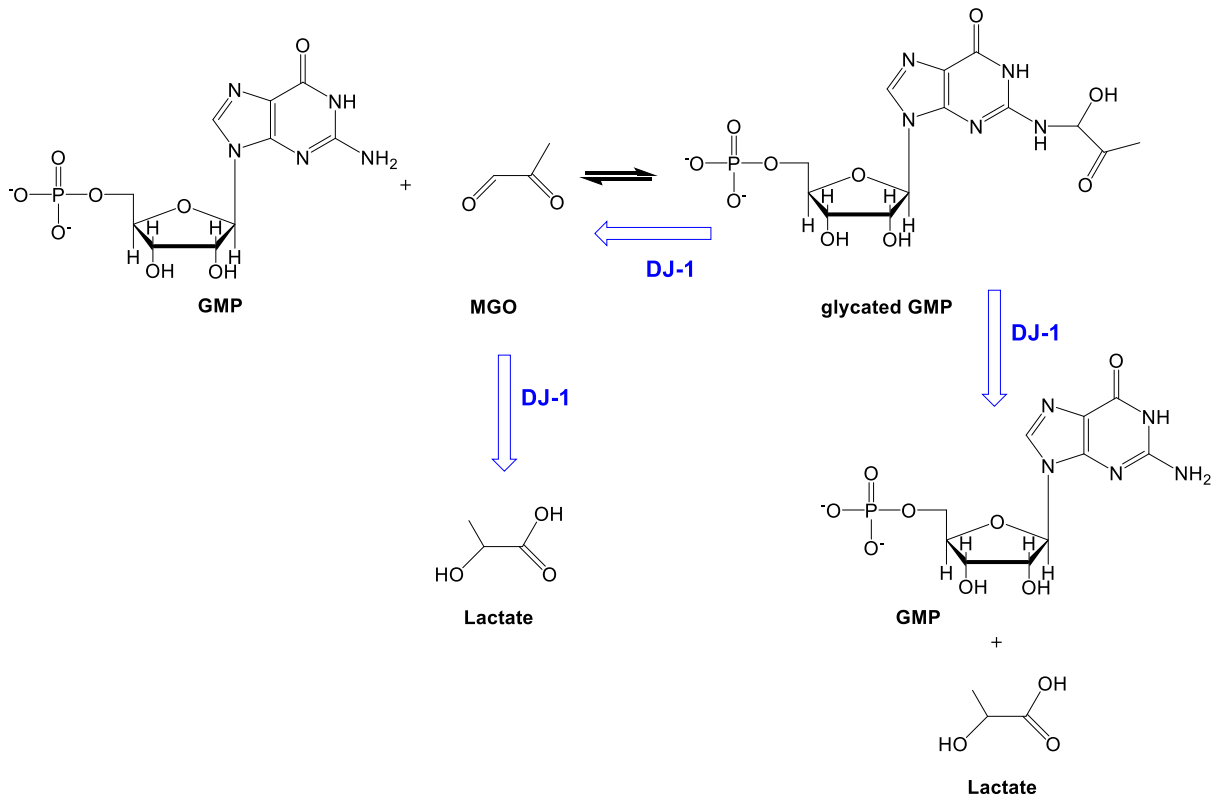
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5.6. Figure 4. Inhibition by thiram of the protective effect of DJ-1 against GMP glycation

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A. LCMS profiles of glycated GMP ($m/z = 436.22$) under the following conditions: (a) GMP (100 μM) incubated with MGO (400 μM), (b) GMP (100 μM) incubated with MGO (400 μM) and DJ-1 (4 μM), (c) GMP (100 μM) incubated with MGO (400 μM), DJ-1 (4 μM), and Thiram (10 μM).

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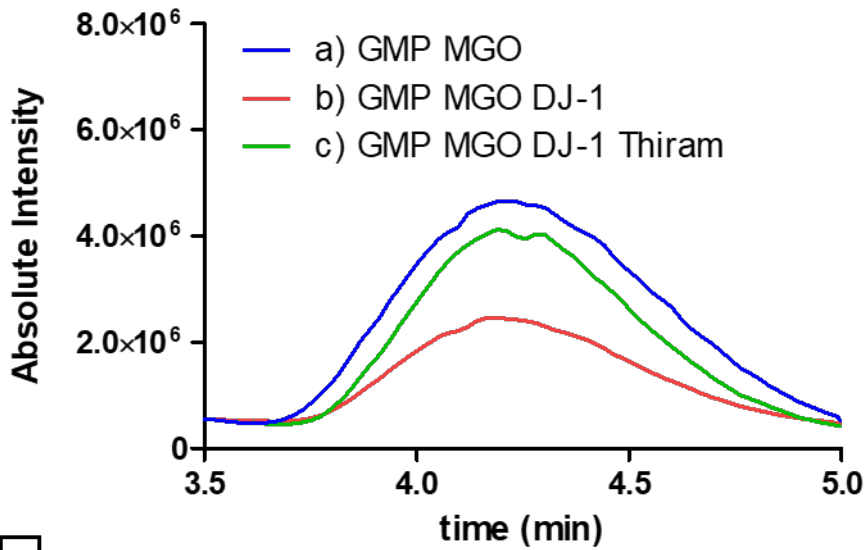
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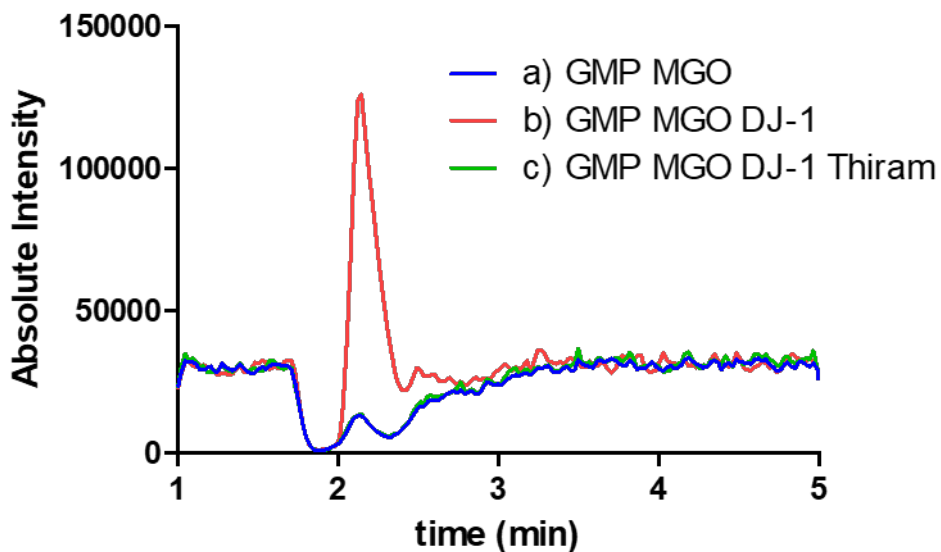
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B. LCMS profiles of lactate ($m/z = 89.00$) under the following conditions: (a) GMP (100 μM) incubated with MGO (400 μM), (b) GMP (100 μM) incubated with MGO (400 μM) and DJ-1 (4 μM), (c) GMP (100 μM) incubated with MGO (400 μM), DJ-1 (4 μM), and Thiram (10 μM).

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5.7. Figure 5. Effects of pesticides on the oxidation state of DJ-1

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Ponceau-stained full blot (above) and the immunostained DJ-1 band (below). Western blotting

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was performed with an antibody against Cys106-oxidized DJ-1 under the following conditions:

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1. DJ-1 (10 μ M), 2. DJ-1 (10 μ M) with 100 μ M H₂O₂, 3. DJ-1 (10 μ M) with 100 μ M dithianon,

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4. DJ-1 (10 μ M) with 100 μ M thiram, 5. DJ-1 (10 μ M) with 100 μ M maneb and 6. DJ-1 (10

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μ M) with 100 μ M zineb.

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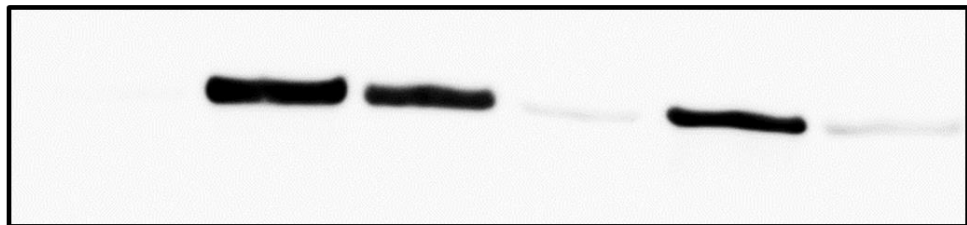
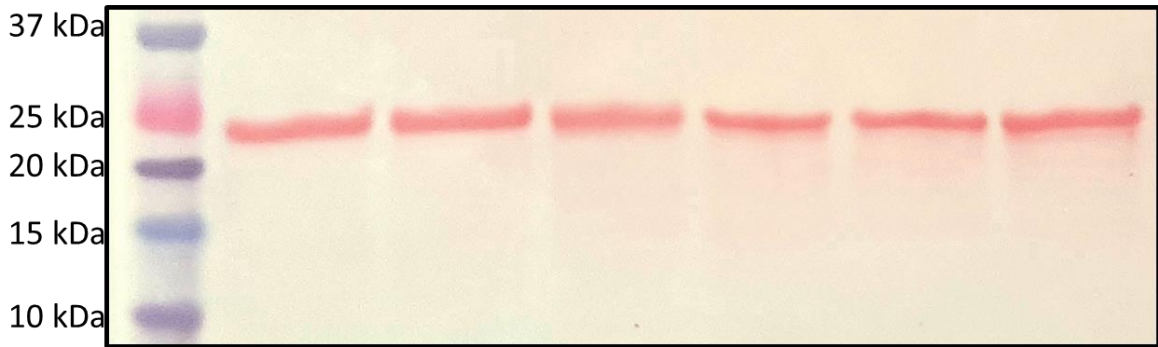
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1: Control 2: H₂O₂ 3: Dithianon 4: Thiram 5: Maneb 6: Zineb



1: Control 2: H₂O₂ 3: Dithianon 4: Thiram 5: Maneb 6: Zineb

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Title: Inhibition by pesticides of the DJ-1/Park7 protein related to Parkinson disease.

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Abbreviations: AGE, advanced glycation end products; BSA, bovine serum albumin; DTT, dithiothreitol; GMP, guanosine monophosphate; MGO, methylglyoxal; PD, Parkinson's disease; pNPA, para-nitro-phenyl acetate.

Abstract:

Parkinson's disease is a severe neurodegenerative disease. Several environmental contaminants such as pesticides have been suspected to favor the appearance of this pathology, The protein DJ-1 (or Park7), an esterase/déglycase protein, protects against the development of Parkinson's disease. Thus, the possible inhibitory effects of about a hundred pesticides on human DJ-1 have been studied. We identified fifteen of them as strong inhibitors of DJ-1 with IC50 values between 0.02 and 30 μ M. Thiocarbamates are particularly good inhibitors, as shown by thiram that acts as an irreversible inhibitor of an esterase activity of DJ-1 with an IC50 value of 0.02 μ M. Thiram was also found as a good inhibitor of a-déglycasethe protective activity of DJ-1 against glycation. Such inhibitory effects could be one of the various biological effects of these pesticides that may explain their involvement in the development of Parkinson's disease.

Keywords: Neurodegenerative diseases, Pesticides, Park7, thiocarbamates.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease with complex etiology and variable pathology (Kalia and Lang 2015). Most cases originate from a combination of factors that are not fully characterized. Nevertheless, several molecular mechanisms were historically identified as common markers of the disease, including α -synuclein misfolding and aggregation, and such processes play a central role in the occurrence of PD (Kazantsev and Kolchinsky 2008). Regarding its complex etiology, although multifactorial, the causes of PD have mainly two components, a genetic component, and an environmental component. Among various environmental factors, pesticides exposure has been enviectedfound as a major candidate in PD pathogenesis. Indeed, an increased risk of developing PD has been observed in people exposed for a long period of time to pesticides (Baldi et al. 2021; Brown et al. 2006). Some studies were able to investigate the risks of specific

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7 44 chemicals, such as paraquat, maneb, organochlorines and rotenone (Vellingiri et al. 2022). A
8 45 review by Tanner et al. summarized the state of knowledge on this topic (Tanner et al. 2011).
9 46 The chemicals for which most data link them to an increased PD risk are paraquat and rotenone,
10 47 with exposure associated with a 2-3-fold higher PD risk over the general population (Yan et al.
11 48 2018). More recently a study estimated the consequences of exposure for 157 pesticides in the
12 49 Netherlands population. Environmental exposure to pesticides was estimated using a spatio-
13 50 temporal model, based on agricultural crops around the residential address. Four of these 157
14 51 pesticides were considered a priori relevant for PD: paraquat, maneb, lindane, and benomyl
15 52 (Brouwer et al. 2017). Interestingly, two of them are carbamates or thiocarbamates (R-NH-CO-
16 53 NH-R or R-NH-CS-S-R), that are highly reactive towards biomolecules through modification
17 54 of their thiol residues.

18 55 Thus, the association between exposure to agricultural pesticides and PD has long been
19 56 a topic of study in the field of environmental health. However, the various molecular
20 57 mechanisms that are at the origin of the effects of pesticides on the development of PD are not
21 58 known in most cases.

22 59 A major factor contributing to cellular and organism aging is the accumulation of
23 60 advanced glycation end products (AGEs) on proteins, lipids and nucleic acids (Chaudhuri et al.
24 61 2018; Miranda et al. 2016; Singh et al. 2001). The AGEs comprise a large number of
25 62 heterogeneous chemical structures and a majority of them have a propensity to crosslink
26 63 proteins, which alters their structure and function. The glycation process (also called non-
27 64 enzymatic glycosylation) is initiated by a non-enzymatic reaction between sugar-derived
28 65 aldehydes and protein or nucleic acid nucleophiles such as amino or thiol groups. This reaction
29 66 was discovered by the French chemist Louis-Camille Maillard in 1912 and the major glycating
30 67 agents are reducing sugars (glucose, fructose, ribose, and their phosphorylated derivatives) and
31 68 glyoxals such as methylglyoxal (MGO, CH₃CO-CHO) (Maillard 1912). Glyoxals are formed
32 69 as by-products of glucose metabolism and are responsible for 65% of cellular glycation events.
33 70 The condensation reaction begins with the rapid formation of a hemithioacetal with thiols and
34 71 of aminocarbonyls with NH₂ groups of biomolecules. Nucleic acids undergo permanent
35 72 glycation by MGO and the most susceptible nucleotides are guanosine and deoxyguanosine
36 73 (Thornalley 2008). Then, a series of dehydrations, oxidations and rearrangements result in a
37 74 myriad of products: Amadori's, AGEs and protein and/or DNA crosslinks. Several recent
38 75 studies implicate glycation as an important process in the pathogenesis of PD (Miranda et al.
39 76 2016; Sadowska-Bartosz and Bartosz 2016). Thus, it has been shown that α -synuclein itself, a
40 77 protein that plays a central role in PD, can be glycated in models of this pathology (Guerrero et
41 78 al. 2013). Recently, Outeiro and colleagues established that the glycation process enhances α -
42 79 synuclein toxicity *in vitro* and *in vivo*, in *Drosophila* and mice.

43 80 The protein DJ-1 (or Park7) has a protective role towards the development of PD since
44 81 the PARK7 gene is associated with recessive and sporadic forms of PD (Bonifati et al. 2003).
45 82 Intensive studies have been undertaken to figure out DJ-1 function(s) and its role in the etiology
46 83 of this neurodegenerative disease. Thus, DJ-1 has been proposed to take part in various
47 84 physiological pathways related to the promotion of cell survival (Oh and Mouradian 2017). It
48 85 also modulates oxidative and electrophilic stresses. DJ-1 activates the Nrf2-mediated
49 86 antioxidant response (Neves et al. 2022). It catalytically protects and/or repairs various
50 87 biomolecules against glycation by glyoxals (Matsuda et al. 2017; Richarme et al. 2017;
51 88 Richarme et al. 2015) or detoxifies reactive compounds produced during glycolysis (Heremans
52 89 et al. 2022; Lee et al. 2012). ~~Although the nature of its physiological substrates is still a matter
53 90 of controversy (Jun and Koo 2020).~~ Although the nature of its physiological substrates is still

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7 91 [a matter of controversy \(Andreeva et al. 2019; Galligan et al. 2018; Jun and Kool 2020; Matsuda](#)
8 92 [et al. 2017; Mazza et al. 2022; Mulikova et al. 2021; Pfaff et al. 2017a, b; Richarme 2017;](#)
9 93 [Richarme and Dairou 2017; Richarme et al. 2017; Richarme et al. 2015\)](#) DJ-1 is involved in the
10 94 protection against the glycation processes.

11 95 This article reports the effects of about 100 pesticides belonging to the main chemical
12 96 classes, such as organophosphates, organochlorines, arylureas, carbamates, aryloxyalkanoic
13 97 acids, triazoles, neonicotinoids and pyrethroids, on human recombinant DJ-1. It shows that
14 98 some of them are irreversible inhibitors of DJ-1 at the sub-micro molar level, revealing a new
15 99 mode of action of these molecules. This strong inhibitory effect could be involved in the role
16 100 of those pesticides in the development of PD.

17 18 101 **2. Materials and methods**

19 102 **2.1. Materials.**

20 103 Most chemical and biochemical reactants were purchased from Sigma-Aldrich or Merck (St.
21 104 Louis, United States). Plasmids for human DJ-1 were obtained from Dr. Sun-Sin Cha (Lee et
22 105 al. 2003). Proteins were desalted with Micro Bio-Spin 6 columns (Biorad, Hercules, United
23 106 States). UV-Visible spectra were recorded on a Carry 300 or Biotek PowerWave XS
24 107 spectrometers. Gels were imaged on a LAS 4000 (GE Healthcare, Chicago, United States). The
25 108 Liquid Chromatography (LC) system was composed of Shimadzu apparatus (Kyoto, Japan)
26 109 equipped with a LC30AD pump, a SiL30AC auto-sampler coupled with a photodiode array
27 110 detector PDA20A and a triple quadrupole mass detector 8060.

28 111 **2.2. Human DJ-1 production and purification**

29 112 Human DJ-1 was expressed and purified as previously described (Richarme et al. 2015).
30 113 Briefly, the DJ-1 expression strain was grown in Luria-Bertani medium to an A600 = 0.6 and
31 114 then induced with 1 mM isopropyl β -D-1-thiogalactopyranoside for 4 h. Bacteria were lysed by
32 115 ultrasonic disruption, and the 100,000 g supernatant was used for DJ-1 purification. DJ-1 was
33 116 first purified on a DEAE-Sephacel (Pharmacia, NJ, United States) column equilibrated in 30
34 117 mM tris buffer, pH 8, containing 20 mM NaCl, 0.5 mM EDTA, and 1 mM dithiothreitol at 4°C.
35 118 Protein was eluted with a linear gradient of 20–400 mM NaCl in equilibration buffer. DJ-1 was
36 119 then purified on a hydroxyapatite column (Bio-Gel HTP from Bio-Rad) equilibrated in 20 mM
37 120 phosphate buffer, pH 8, containing 20 mM NaCl, 0.5 mM EDTA and 1 mM dithiothreitol at
38 121 4°C. Protein was eluted with a linear gradient of 20–250 mM sodium phosphate buffer, pH 8.
39 122 Then, DJ-1 was dialyzed overnight against 50 mM phosphate buffer (Na₂HPO₄), pH 8, 20 mM
40 123 NaCl, 0.5 mM EDTA and 1 mM dithiothreitol at 4 °C. Purified protein was quantitated with
41 124 Bradford reagent (Bradford 1976). Purity was assessed by SDS-PAGE and/or western blot with
42 125 anti-DJ-1 as antibody. Proteins were kept at -80°C until use.

43 126 **2.3. Enzyme Assay—Detection of DJ-1 esterase and deglycase activity**

44 127 Prior to any experiment, DJ-1 enzyme was reduced by 10 mM dithiothreitol (DTT) for 30 min
45 128 and then dialyzed overnight at 4°C against 50 mM phosphate buffer. Protein concentration was
46 129 determined using a Bradford assay with BSA (Bovine serum albumin) as standard (Bio-rad).
47 130 DJ-1 esterase activity was performed as described previously (Vazquez-Mayorga et al. 2016)
48 131 in a total volume of 200 μ l: ten μ L of DJ-1 solution (0.2 μ M final) were added to 190 μ L of a
49 132 2.8 mM solution of p-nitrophenylacetate (pNPA), prepared in PBS from a 200 mM stock
50 133 solution in dimethylsulfoxide. DJ-1 esterase activity was determined by monitoring the slope
51 134 of the absorbance at 405 nm vs time at 37°C.

52 135 The ~~deglycase~~ activity of DJ-1 concerning the protection against guanosine monophosphate
53 136 glycation by methylglyoxal was determined by LC as previously reported (Richarme et al.

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2015). Briefly guanosine monophosphate (GMP) 100 μM was incubated at 37°C in N₂-gassed 50 mM phosphate buffer pH 7.0, with 400 μM MGO, in the absence or presence of 4 μM DJ-1. The samples were analyzed by LC on a C18 Reverse phase LC Kinetex® - Phenomenex column equilibrated in water with formic acid 0.1% at 45°C, and eluted with a linear gradient with acetonitrile with 0.1% formic acid. The kinetics of GMP protection by DJ-1 was followed by measuring the ~~surface~~-area under the peak of the glycated GMP peak of the LC chromatogram (absorbance at 254 nm and Mass Spectrometry detection).

2.4. Effects of pesticides on DJ-1 activity

The inhibitory effects of pesticides were determined by running the enzymatic assay as described above, in the presence of pesticide (5 μM). This activity was compared to the activity of a similar DJ-1 solution without pesticide (100%). pNPA hydrolysis by pesticides without DJ-1 was also used as control and showed no significant esterase activity unless otherwise stated. For pesticides that show DJ-1 inhibition greater than 25% under the previous conditions, IC₅₀ determinations were made.

To test whether the reaction of DJ-1 with pesticide was irreversible, purified enzyme (1 μM final) was first incubated in presence or absence of pesticide for 30 min at 37°C. The samples were then dialyzed overnight at 4°C against phosphate buffer prior to enzyme assay. Controls were carried out with non-dialyzed samples.

Kinetic data with thiram were obtained at 37°C as follows: to 1 mL of a 50 nM solution of DJ-1 was added 1 μL of a 1 mM solution of thiram (final concentrations: 1 μM). The esterase activity was recorded at various time intervals by monitoring of pNPA hydrolysis. The bimolecular rate constant k_{inac} was determined by Kitz & Wilson methodology proposed in 1962, the time-course of an irreversible inhibition assay can be described by a rising exponential curve by fitting (Kitz and Wilson 1962).

2.5. Effects of pesticides on the oxidation state of DJ-1

Western blotting was performed as previously described (Bahmed et al. 2016). We used antibodies against native and Cys106-oxidized DJ-1 (Sigma) and the blots were then developed using an enhanced chemiluminescence western blotting kit according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ). Images were quantitated using ImageJ software.

2.6. Statistical Analyses

Each experiment was performed independently at least three times. The results shown in figures are, if not otherwise stated, mean values \pm S.E.M of 3–6 independent experiments. Statistical analysis was performed using analysis of ANOVA test using Prism (GraphPad Software).

3. Results and discussion

About hundred pesticides belonging to the main pesticide chemical classes were tested as possible inhibitors of DJ-1. First, they were tested as possible inhibitors of the esterase activity of human recombinant DJ-1 using pNPA as substrate (Figure 1) (Maksimovic et al. 2021; Vazquez-Mayorga et al. 2016). Table 1 shows the percentage of inhibition observed after 5 min of incubation of 0.2 μM DJ-1 in the presence of 2.8 mM pNPA and 5 μM pesticide. Under these conditions, only a little number of pesticides led to a significant inhibition of DJ-1. This was the case of three carbamates (benomyl, formetanate and oxamyl), five organochlorines (captan, DDE, dienochlore, folpet and lindane), one pyrethroid (resmethrin), two organophosphates, (diazinon and pirimidiphos methyl) and two unclassified pesticides (paraquat and dithianon) with various potencies (from 10 to 85% of inhibition). However, one family of pesticides

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7 182 emerges as major inhibitors of DJ-1, the thiocarbamate series (from 70 to 100% of inhibition).
8 183 Interestingly, a thiocarbamate used as a drug, disulfiram, was recently reported as a good
9 184 inhibitor of the esterase activity of DJ-1 (Wu et al. 2022).

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11 185 Table 2 indicates the IC₅₀ values found for those pesticides having led to a significant inhibition
12 186 of DJ-1 (higher than 25 %). Almost all the tested thiocarbamates and dithianon led to
13 187 remarkably low IC₅₀ values, equal or lower than 1 μM. Thiram was found to be the best inhibitor
14 188 under these conditions, with an IC₅₀ value of about 0.02 μM. It is important to compare these
15 189 IC50 values with the concentrations found after human exposure to those pesticides. Thus, for
16 190 example, several recent studies have revealed that humans are exposed through their
17 191 consumption of fruit and vegetables and their drinking water to concentrations of maneb up to
18 192 450 μg/L (ie 1.7 μM) (Asghar et al. 2022; Carrasco Cabrera and Medina Pastor 2021; Onwona-
19 193 Kwakye et al. 2020; Ozhan and Alpertunga 2008). We found an IC50 for maneb of 0.06 μM
20 194 that is much lower than the concentration reported upon maneb exposure.

21 195 Experiments based on preincubation of DJ-1 in the presence of the inhibitors were then
22 196 performed to determine the type of inhibition of those thiocarbamates. Figure 22a shows that
23 197 the inhibition level observed with 1 μM thiram clearly increased with the time of preincubation
24 198 of DJ-1 in the presence of this inhibitor. A complete inhibition of DJ-1 was observed after 5
25 199 min preincubation of DJ-1 with 1 μM thiram, indicating that this pesticide acts as an irreversible
26 200 inhibitor of DJ-1. Determination of the k_{inact} led to a value of 3.5 ± 0.5 10⁴ M⁻¹.s⁻¹. Dialysis at
27 201 equilibrium experiments of the incubates of DJ-1 in the presence of thiram confirmed that DJ-
28 202 1 remained inactive under these conditions (~~data not shown~~) and that thiram acted as an
29 203 irreversible inhibitor- (figure 2b).

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31 204 Some of the pesticides having led to a strong inhibitory effect on the esterase activity of DJ-1
32 205 were tested on a previously described assay measuring the protecting activity of DJ-1 towards
33 206 glycated GMP formation upon reaction of GMP with MGO (Figure 3), resulting in lactate
34 207 formation, (Richarme et al. 2017). Figure 4A shows the LCMS chromatograms of glycated
35 208 GMP (m/z = 436.22), alone (a) or after incubation with DJ-1 (b), or after incubation with 4 μM
36 209 DJ-1 previously treated during 30 min at room temperature with 10 μM thiram (c). Figure 4B
37 210 shows the formation of lactate (m/z=89.00) under identical conditions. Those results show that
38 211 thiram is also an inhibitor of this deglycaseprotective activity of DJ-1 against glycated GMP
39 212 formation. Similar results were obtained with maneb and dithianon (~~data not shown~~).

40 213 As a first attempt to determine the mechanism of the inhibition of DJ-1 by the pesticides
41 214 indicated in Table 2, experiments have been done to know whether some of them could lead to
42 215 an irreversible oxidation of cysteine 106, a crucial residue of the DJ-1 active site (Blackinton
43 216 et al. 2009; Canet-Aviles et al. 2004; Kinumi et al. 2004). For that purpose, western-blot
44 217 analyses of DJ-1 incubation mixtures were done by using a specific antibody against an
45 218 oxidized form of DJ-1, specifically at the level of Cys106. Figure 5 compares the analysis of
46 219 untreated DJ-1 with DJ-1 pretreated with 100 μM H₂O₂ and confirmed that the antibody
47 220 specifically recognizes S-oxidized DJ-1 (line 1 vs 2). Interestingly, analysis of DJ-1 pretreated
48 221 with maneb (line 5) showed the presence of S-oxidized DJ-1 whereas this was not the case for
49 222 DJ-1 pretreated with thiram or zineb. The oxidation of Cys106 observed upon treatment of DJ-
50 223 1 with maneb, that is a Mn(II) complex, could be due to the binding and reaction of dioxygen
51 224 (O₂) with its Mn(II) center with formation of superoxide. Accordingly, incubation of DJ-1 with

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thiram or zineb that did not contain such a Mn(II) redox active center did not lead to Cys106 oxidation (Figure 5).

Similar experiments performed on DJ-1 pretreated with dithianon (figure 5) showed the formation of S-oxidized DJ-1 whereas this was not the case for DJ-1 pretreated with folpet, formetamate, lindane, resmethrine, oxamyl or triallate (data not shown). This oxidation of Cys106 by dithianon could be due to its particular activated quinone structure able to receive an electron from Cys106 with formation of a stable radical anion.

From those data, it seems that oxidation of Cys106 would only be involved in the inhibition of DJ-1 by a few pesticides, such as maneb, mancozeb (data not shown) or dithianon. The mechanisms of inhibition of DJ-1 by the other pesticides mentioned in Table 2 remain to be determined.

4. Conclusion

Our study of the possible inhibitory effects of about hundred pesticides on human DJ-1 has shown that some twenty of them are inhibitors of the esterase activity of DJ-1 (Table 1). Fifteen of them are good inhibitors with IC₅₀ values between 0.02 and 30 μM (Table 2). The pesticides of the thiocarbamate series are particularly good inhibitors. Thus, thiram was found as an irreversible DJ-1 inhibitor with an IC₅₀ value of 0.02 μM. All those fifteen pesticides have been linked to the development of PD (Brouwer et al. 2017; Brown et al. 2006; Tanner et al. 2011). Interestingly, a recent study of the consequences of the exposure for 157 pesticides in the Netherland population has shown that four of those pesticides, paraquat, maneb, lindane and benomyl, were relevant for the development of PD (Brouwer et al. 2017). ~~Those Interestingly those~~ four pesticides were found to act as inhibitors of DJ-1 (Tables 1 and 2), ~~suggesting that inhibition of DJ-1 would participate in their role in the development of PD.~~

Several biological effects of some pesticides have been reported to contribute to the development of PD. Thus, for instance, rotenone ~~and paraquat~~ act as ~~inhibitors~~inhibitor of mitochondrial complex1 (Betarbet et al. 2006; Betarbet et al. 2000; Schiller and Zickermann 2022), ~~Paraquat increases the formation of free radicals and oxidative stress (Ranjbar et al. 2018)~~ and benomyl and some dithiocarbamates act as inhibitors of the aldehyde dehydrogenase that is responsible for the detoxication of a dopamine metabolite, 3,4-dihydroxyphenyl acetaldehyde (Burke et al. 2014; Fitzmaurice et al. 2014; Fitzmaurice et al. 2013). Our results show that some of the hundred pesticides tested in our study are strong inhibitors of DJ-1. ~~This inhibition of DJ-1~~ However, the esterase activity of DJ-1 has so far no identified physiological role and further experiments using *in cellulo* and *in vivo* models are necessary to show that this ~~inhibition of DJ-1~~ could be one of the various biological effects of those pesticides involved in the development of PD. ~~These results could also be interesting in the regulatory framework for the implementation of AOPs (for Adverse Outcome Pathways, (Barouki et al. 2021)) linking DJ-1 to the etiology of PD and the weight of evidence of key event relationships. AOPs are increasingly used in regulatory toxicology to define pathways from a molecular initiating event (here, DJ-1 inhibition) to an adverse outcome (here Parkinson's disease), through several key events. The built up of such an AOP linking DJ-1 inhibition to Parkinson's disease would be interesting in the future to identify potential neurodegenerative molecules before their marketing.~~

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268 **5. Appendices: Tables and figures legends**

8
269 **5.1. Table 1. Inhibition of human DJ-1 by pesticides.**

9
270 % inhibition of the DJ1 esterase activity (using pNPA as substrate) upon incubation of DJ-1 in
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271 the presence of 2.8 mM pNPA and 5 μM pesticide; mean values +/- S.E.M. from 6 to 9
11
272 experiments.

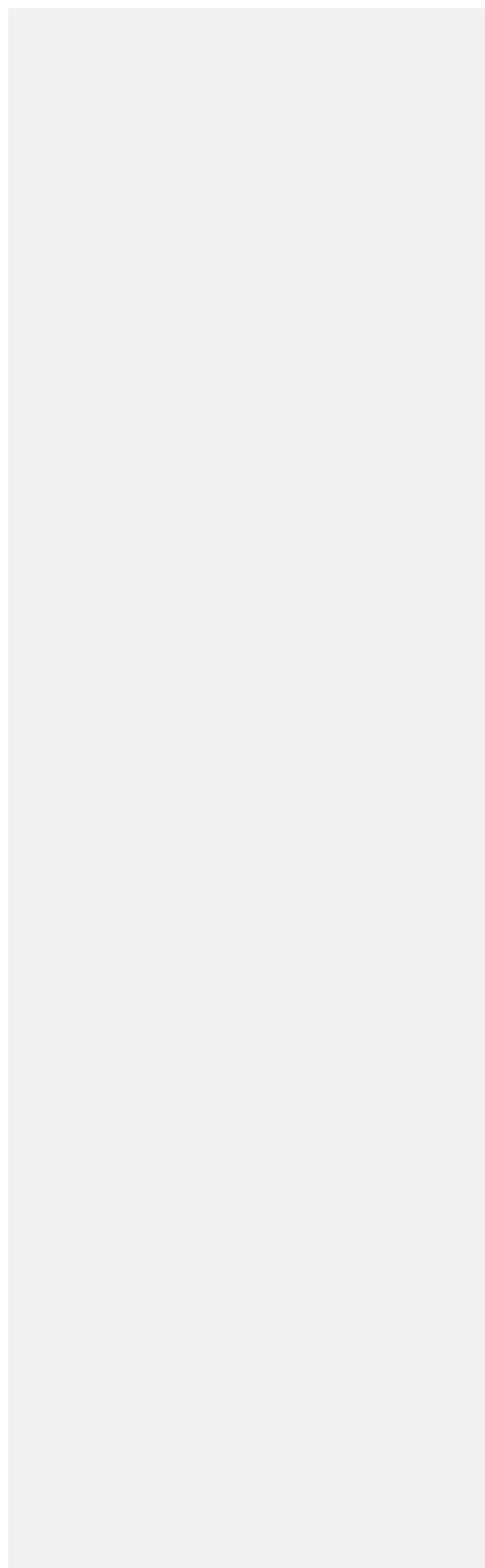
Chemical Class	Compounds	% of Inhibition
Aryloxy alcanoic acids	Cyhalofop-butyl	<5%
	Diclofop-methyl	<5%
	Fenoxaprop-P	<5%
	Fluazifop-P-butyl	<5%
	MCPA	<5%
	MCPB	<5%
	Mecoprop-P	<5%
	Quizalofop-P-ethyl	<5%
Aryl ureas	Chlortoluron	<5%
	Foramsulfuron	<5%
	Linuron	<5%
	Metobromuron	<5%
	Metsulfuron-methyl	<5%
	Nicosulfuron	<5%
	Rimsulfuron	<5%
	Tribenuron-methyl	<5%
	Tritosulfuron	<5%
	Asulam	<5%
Carbamates	Benomyl	85 ± 6%
	Carbaryl	<5%
	Carbendazim	<5%
	Chlorpropham	<5%
	Diethofencarb	<5%
	Formetanate	56 ± 8%
	Iprovalicarb	<5%
	Methomyl	<5%
	Oxamyl	22 ± 7%
	Phenmedipham	<5%
	Pirimicarb	<5%
	Propamocarb	<5%
	Prosulfocarb	<5%
	Thiabendazole	<5%
	Neonicotinoids	Imidacloprid
Thiacloprid		<5%
Thiamethoxam		<5%
Organochlorines	Boscalid	<5%
	Bixafen	<5%

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Organophosphates	captan	57 ± 9%
	Chlordecon	<5%
	DDE	17 ± 4%
	DDT	<5%
	Dieldrin	<5%
	Dienochlore	35 ± 5%
	Endosulfan	<5%
	Folpet	25 ± 4%
	Lindane	12 ± 4%
	Metolachlore	<5%
	Acephate	<5%
	Diazinon	10 ± 4%
	Dichlorvos	<5%
	Dimethoate	<5%
Ethion	<5%	
Fenitrothion	<5%	
Glyphosate	<5%	
Isocarbophos	<5%	
Malathion	<5%	
Methyl parathion	<5%	
Monocrotophos	<5%	
Parathion (ethyl)	<5%	
Phosmet	<5%	
pirimiphos methyl	14 ± 3%	
Profenofos	<5%	
Tolclofos-methyl	<5%	
Triazophos	<5%	
Pyrethroids	Allethrin	<5%
	Bifenthrin	<5%
	Cyfluthrin	<5%
	Cypermethrin	<5%
	Deltamethrin	<5%
	Esfenvalerate	<5%
	Fenvalerate	<5%
	Fluvalinate	<5%
	Permethrin	<5%
	Phenothrin	<5%
	Resmethrin	26 ± 4%
	Tetramethrin	<5%
	Tralomethrin	<5%
	Thiocarbamates	Mancozeb
Maneb		100 ± 6%
Propineb		100 ± 3%
Thiram		100 ± 1%
Triallat		70 ± 12%
Zineb		100 ± 7%
Ziram	100 ± 6%	

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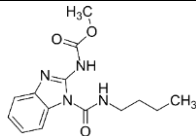
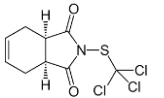
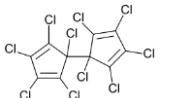
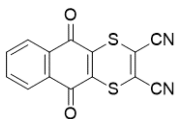
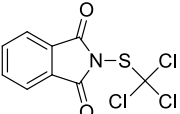
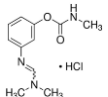
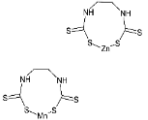
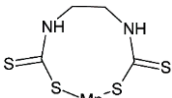
Triazoles	Difenoconazole	<5%
	Hexaconazole	<5%
	Metconazole	<5%
	Myclobutanil	<5%
	Penconazole	<5%
	Tebuconazole	<5%
	Triticonazole	<5%
Others	Atrazine	<5%
	Cyprodinil	<5%
	Dinitramin	<5%
	Dithianon	100 ± 4%
	Iprodione	<5%
	Mepanipyrim	<5%
	Mesotrione	<5%
	Paraquat	20 ± 4%
	Rotenone	<5%
	Sedaxane	<5%
Tembotrione	<5%	



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5.2. Table 2. IC₅₀ values obtained for the inhibition of DJ1 (esterase activity) by pesticides.

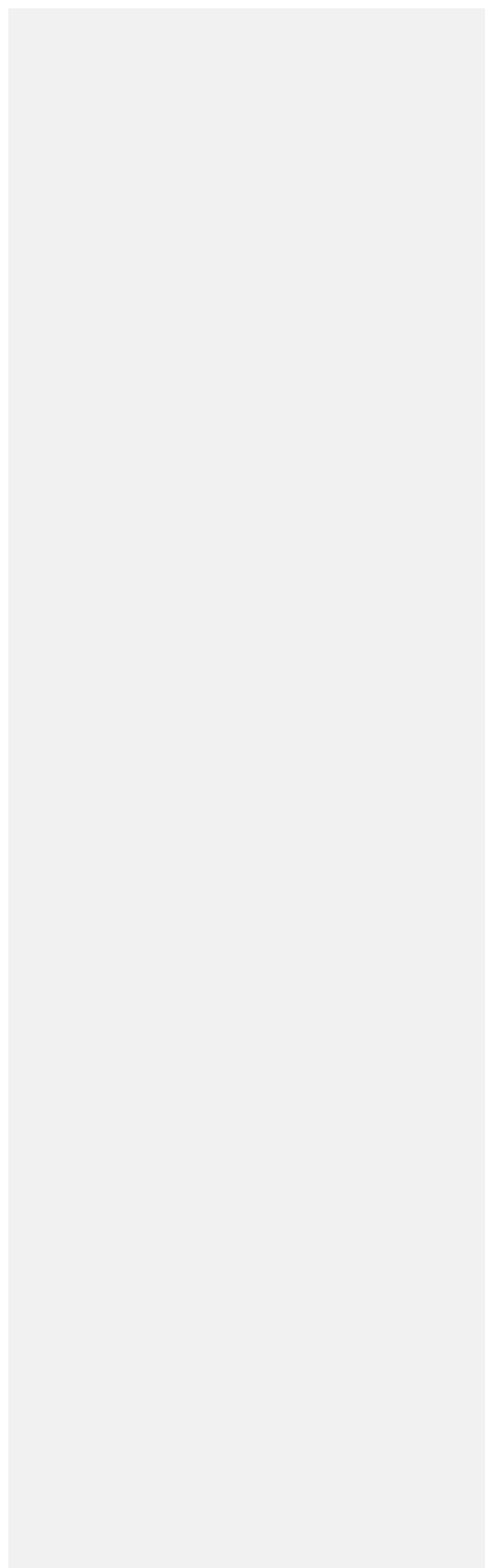
Mean values expressed in μM +/- S.E.M. from 3 to 6 experiments.

Compounds	STRUCTURE	IC ₅₀ (μM)
Benomyl		0.92 ± 0.1
Captan		6.1 ± 0.3
Dienochlore		19.2 ± 0.2
Dithianon		1.1 ± 0.2
Folpet		15.1 ± 2.3
Formetanate		3.2 ± 0.3
Mancozeb		0.09 ± 0.01
Maneb		0.06 ± 0.01

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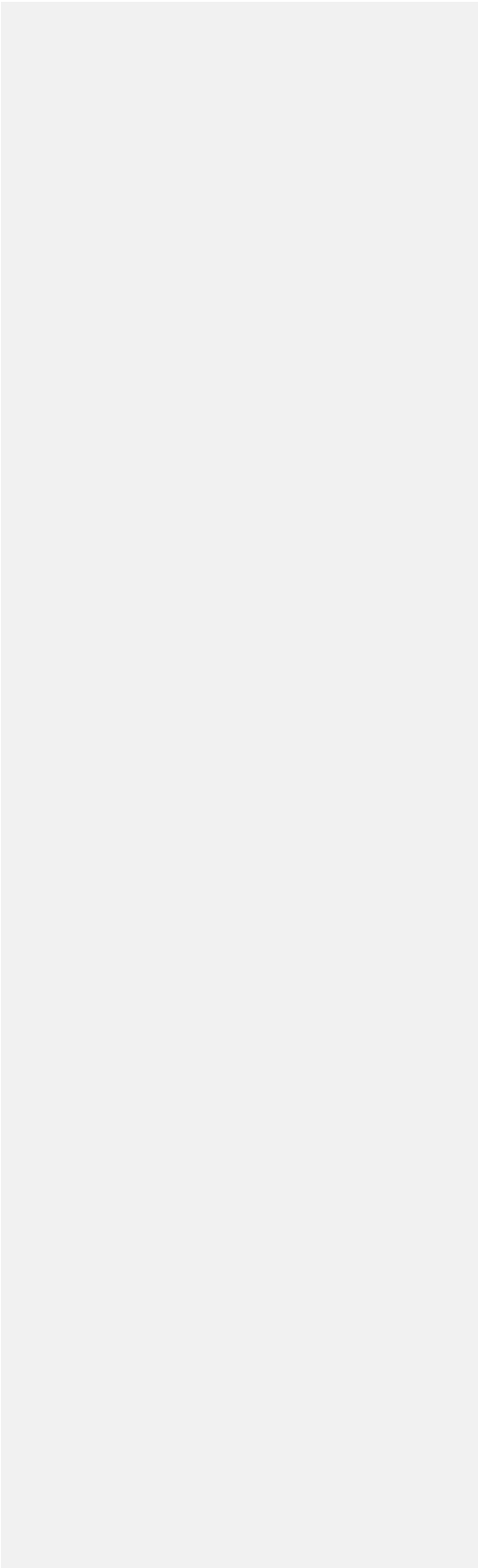
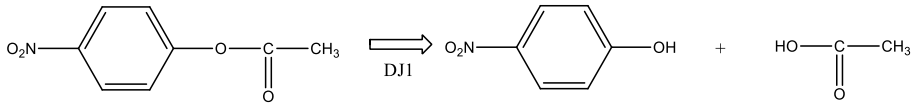
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Oxamyl		24.8 ± 0.3
Propineb		0.17 ± 0.02
Resmethrine		18.1 ± 0.8
Thiram		0.018 ± 0.002
Triallat		0.99 ± 0.11
Zineb		0.17 ± 0.02
Ziram		0.15 ± 0.02



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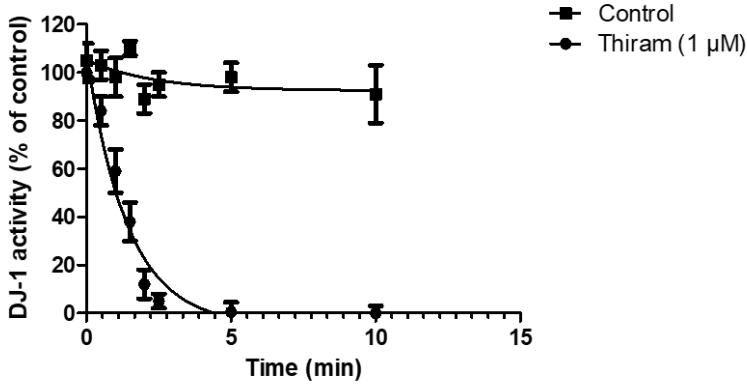
5.3. Figure 1. Esterase activity of DJ-1 towards pNPA as substrate.



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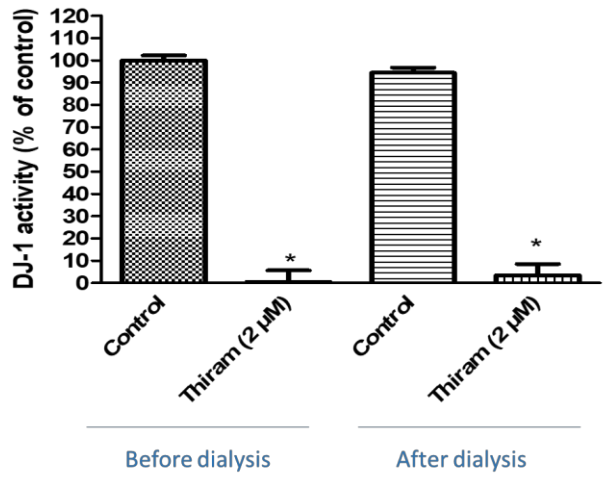
5.4. Figure 2. Kinetic analysis of DJ-1 inhibition by thiram

a: Inhibition of the DJ-1 esterase activity as a function of the time of preincubation of DJ-1 with thiram. Mean values from three independent experiments.



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b: Inhibition of the DJ-1 esterase activity with thiram before and after dialysis. Mean values from three independent experiments.

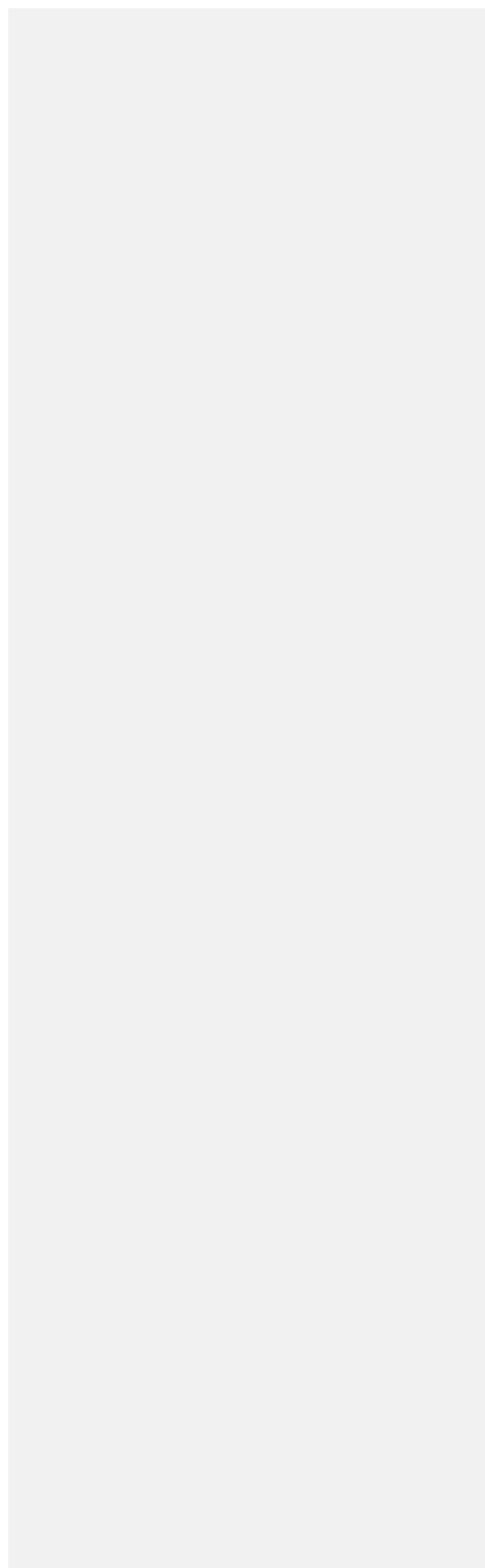
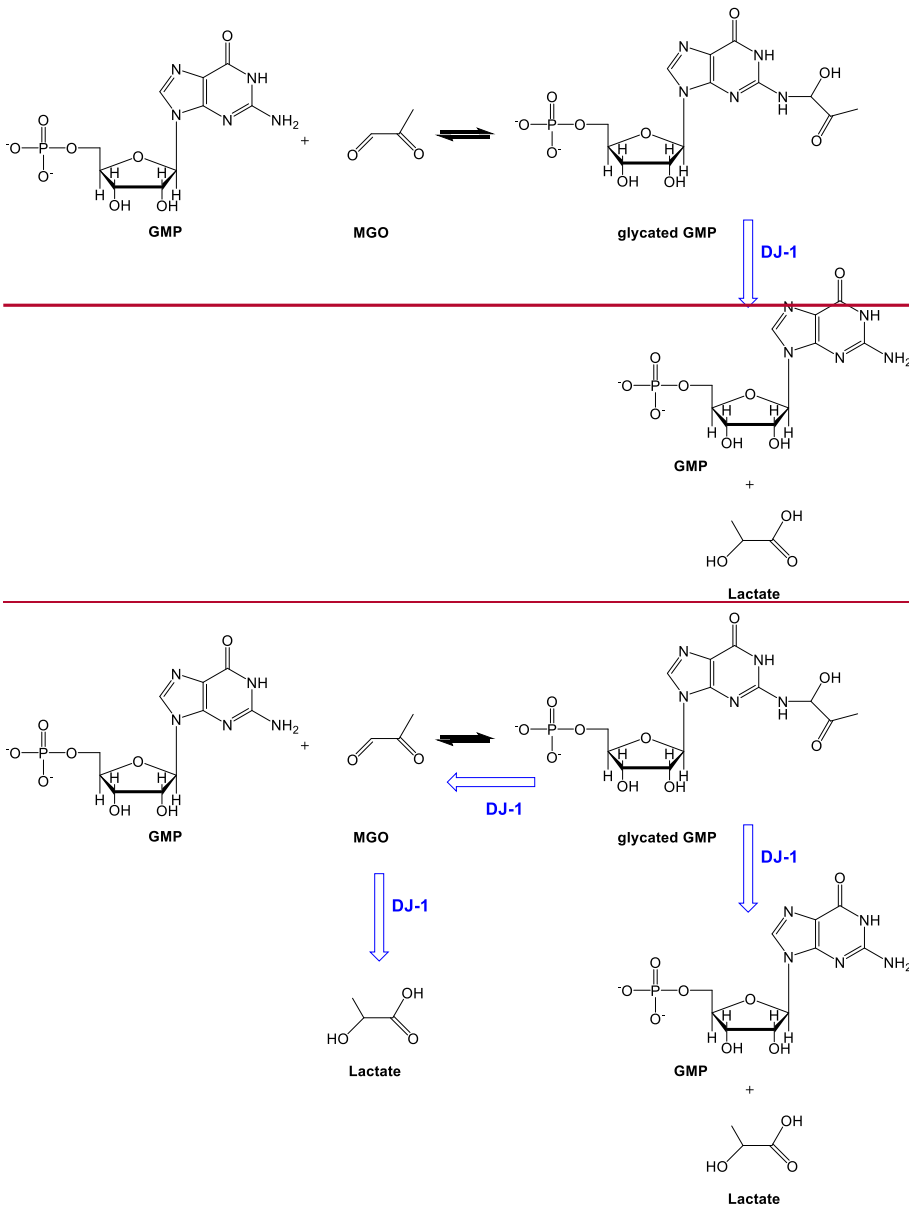


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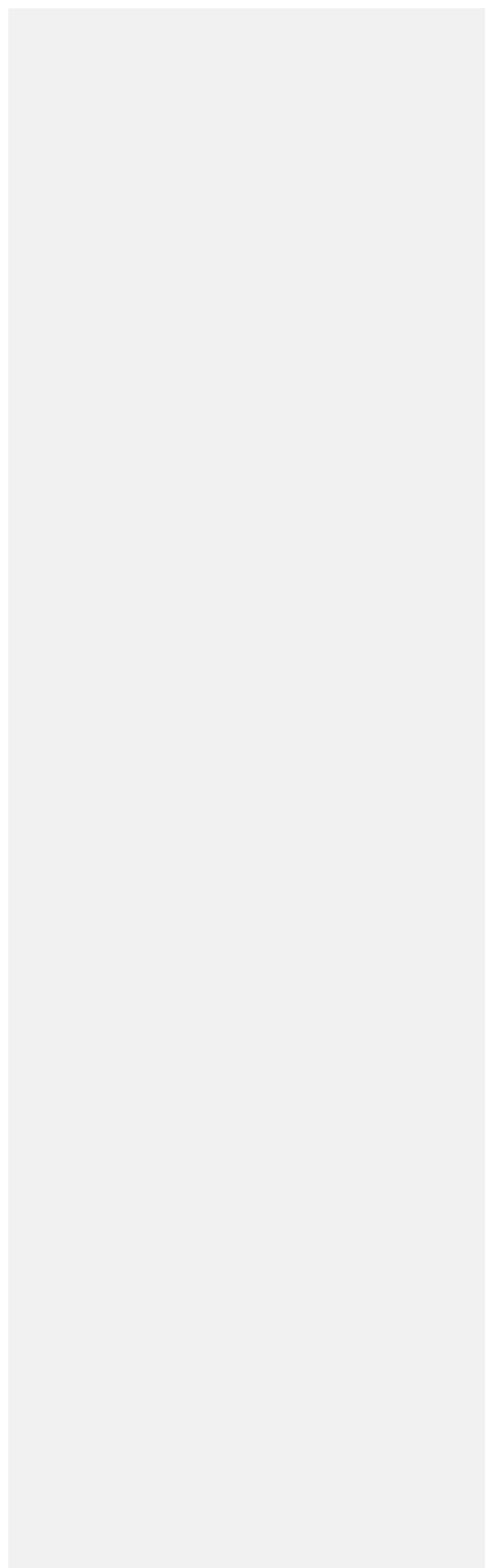
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295 **5.5. Figure 3. DeglycaseProtective activity of DJ1 with glycated against glycation of GMP**
296 **as substrate MGO.**
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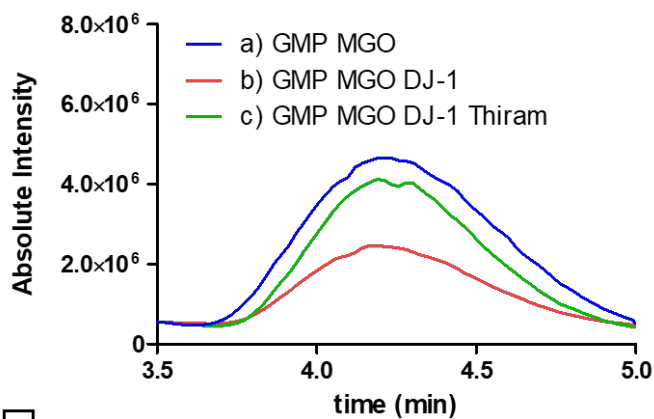
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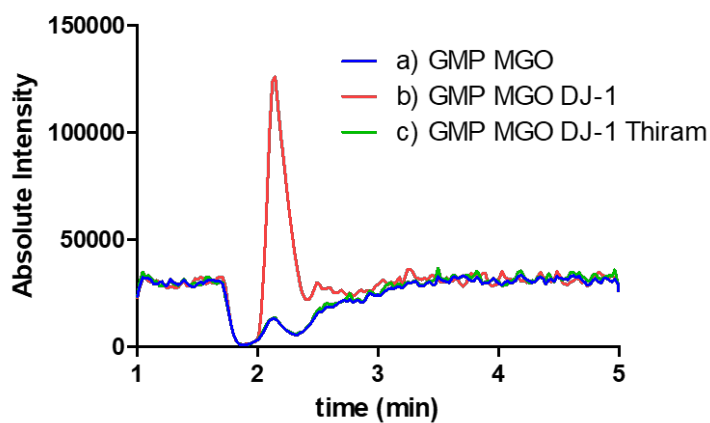
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306 **5.6. Figure 4. Inhibition by thiram of the protective effect of DJ-1 against GMP glycation**

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- A. LCMS profiles of glycated GMP ($m/z = 436.22$) under the following conditions: (a) GMP (100 μM) incubated with MGO (400 μM), (b) GMP (100 μM) incubated with MGO (400 μM) and DJ-1 (4 μM), (c) GMP (100 μM) incubated with MGO (400 μM), DJ-1 (4 μM), and Thiram (10 μM).
- B. LCMS profiles of lactate ($m/z = 89.00$) under the following conditions: (a) GMP (100 μM) incubated with MGO (400 μM), (b) GMP (100 μM) incubated with MGO (400 μM) and DJ-1 (4 μM), (c) GMP (100 μM) incubated with MGO (400 μM), DJ-1 (4 μM), and Thiram (10 μM).

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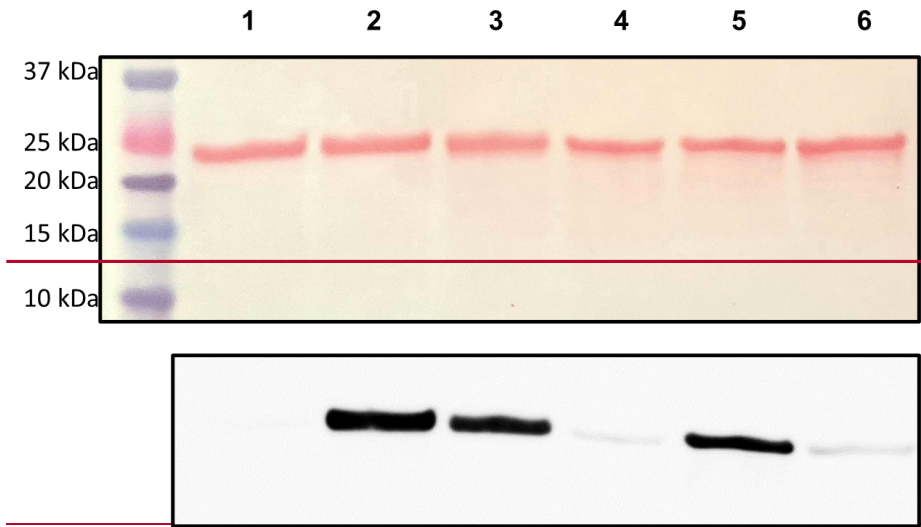
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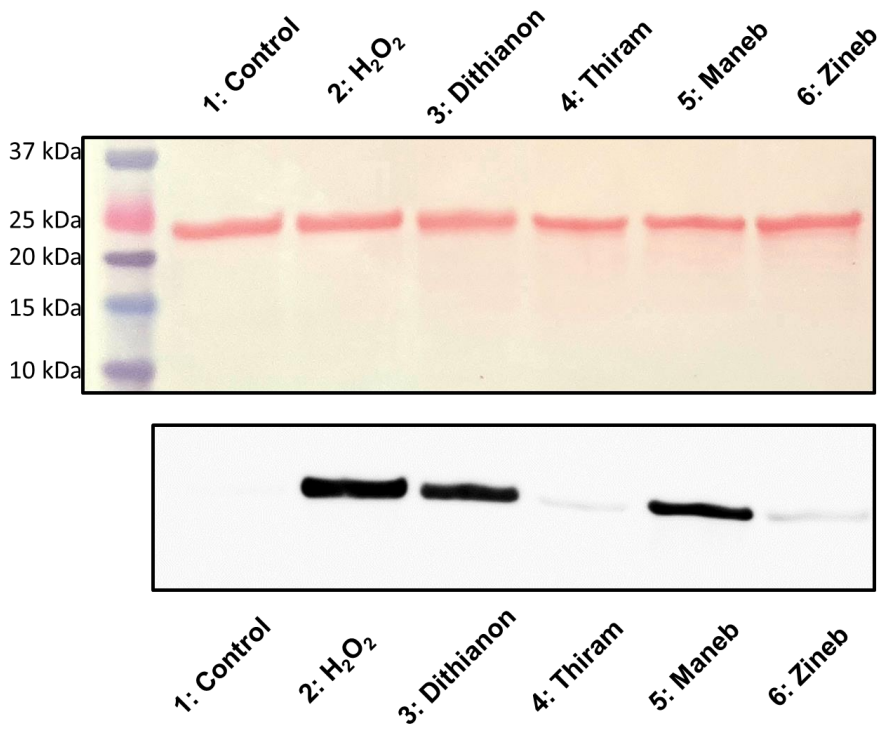
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5.7. Figure 5. Effects of pesticides on the oxidation state of DJ-1

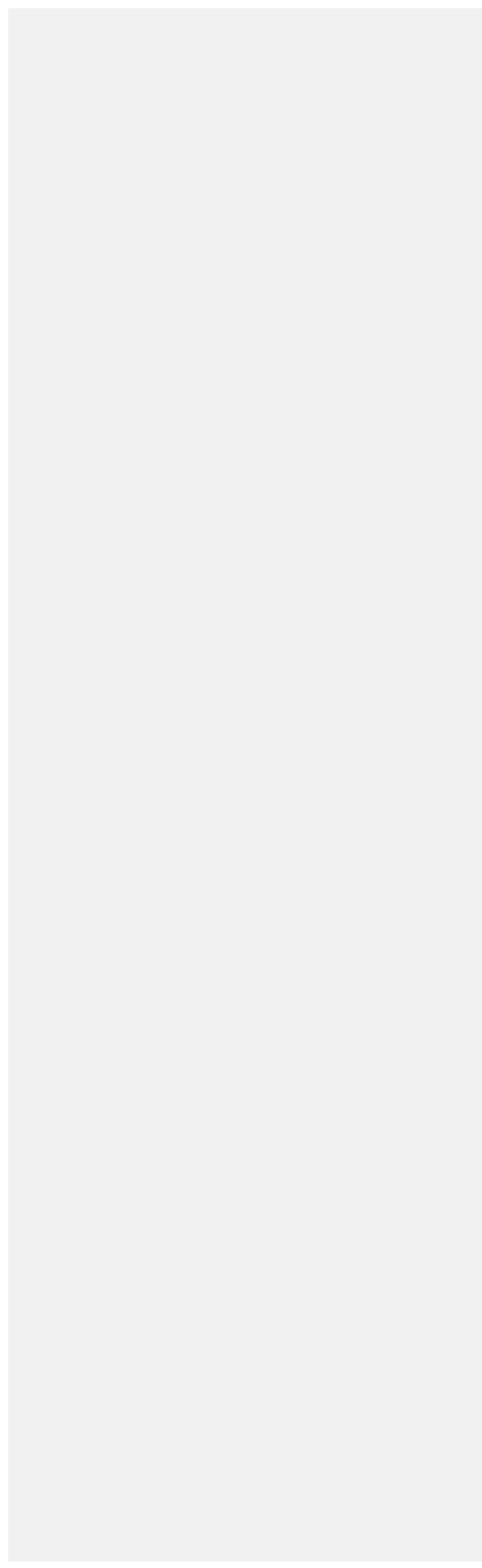
Ponceau-stained full blot (above) and the immunostained DJ-1 band (below). Western blotting was performed with an antibody against Cys106-oxidized DJ-1 under the following conditions: 1. DJ-1 (10 μ M), 2. DJ-1 (10 μ M) with 100 μ M H₂O₂, 3. DJ-1 (10 μ M) with 100 μ M dithianon, 4. DJ-1 (10 μ M) with 100 μ M thiram, 5. DJ-1 (10 μ M) with 100 μ M maneb and 6. DJ-1 (10 μ M) with 100 μ M zineb.



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