The structural requirements of organophosphorus insecticides (OPI) for reducing chicken embryo NAD⁺ content in OPI-induced teratogenesis in chickens

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Abstract

The objective of this study was to determine the structural requirements of organophosphorus insecticides (OPI) for reducing chicken embryo nicotinamide adenine dinucleotide (NAD⁺) content in OPI-induced teratogenesis and compare them with those needed for OPI inhibition of yolk sac membrane kynurenine formamidase (KFase), the proposed primary target for OPI teratogens in chicken embryos. The comparative molecular field analysis (COMFA) of three-dimensional quantitative structure-activity relationship (3D QSAR) revealed the electrostatic and steric fields as good predictors of OPI structural requirements to reduce NAD⁺ content in chicken embryos. The dominant electrostatic interactions were localized at nitrogen-1, nitrogen-3, nitrogen of 2-amino substituent of the pyrimidinyl of pyrimidinyl phosphorothioates, and at the oxygen of crotonamide carbonyl in crotonamide phosphates. Bulkiness of the substituents at carbon-6 of the pyrimidinyls and/or N-substituents of crotonamides was the steric structural component that contributed to superiority of those OPI for reducing embryonic NAD⁺ levels. Both electrostatic and steric requirements are similar to those defined in our previous study for OPI inhibition of chicken embryo yolk sac membrane KFase. The findings of this study provide another piece of evidence for the cause-and-effect relationship between yolk sac membrane KFase inhibition and reduced embryo NAD⁺ content in NAD-associated OPI-induced teratogenesis in chickens.

Key words: chicken embryo yolk sac membranes
kynurenine formamidase inhibition
nicotinamide adenine dinucleotide
organophosphorus insecticides
teratogenesis in chickens
three-dimensional quantitative structure-activity relationship

1. Introduction

Organophosphorus insecticides (OPI) have been the largest class of insecticides used and investigated in the past 70 years. Their primary toxicity is due to their effect on the neural cholinergic system in both target and nontarget organisms. Because of OPI reactivity and structural diversity, some OPI may also produce toxic effects that do not originate from their interaction with the neural cholinergic system.

NAD-related teratogenicity induced by some OPI in chickens (reviewed in [1]) is an example of OPI toxicity not related to the disruption of the cholinergic system. Micromelia and abnormal feathering are the malformations formed in chicks following OPI administration into yolk of the fertile chicken eggs. Severity of those malformations correlates with a lowered embryo nicotinamide adenine dinucleotide (NAD⁺) content [2, 3]. The pyrimidinyl phosphorothioate and crotonamide phosphate insecticides are the most potent OPI in inducing NAD-related teratogenesis.

N-formyl-L-kynurenine formamidase (KFase) [EC. 3.5.1.9] of chicken embryo yolk sac membranes is the proposed primary target for OPI teratogens in NAD-associated teratogenesis [6]. OPI inhibition of KFase, the second enzyme in the L-tryptophan to NAD⁺ biosynthetic pathway, would disrupt a flow of the metabolites through the L-tryptophan to NAD⁺ pathway and result in a reduced formation of NAD⁺. KFase inhibition correlates with both OPI teratogenicity and the reduction in embryonic NAD⁺ content. The structural requirements for chicken embryo yolk sac membrane KFase inhibition by OPI were defined recently [7]. The comparative molecular field analysis (COMFA) of three-dimensional quantitative structure-activity relationship (3D QSAR) revealed the electrostatic and steric fields as good predictors of OPI inhibitory potency. It also identified structural elements that made pyrimidinyl phosphorothioate and crotonamide phosphate teratogens most potent KFase inhibitors.

In this project, we searched for more evidence that would further support our proposal [6] about the cause-and-effect relationship between OPI inhibition of chicken embryo yolk sac membrane KFase and the reduced embryo NAD⁺ content. By applying the COMFA of 3D QSAR on the data obtained in our previous studies [2, 3, 6], we examined structural features that make pyrimidinyl phosphorothioates and crotonamide phosphates most effective compounds in lowering embryonic NAD⁺ levels. The conclusions of the COMFA were then compared to those obtained for OPI inhibition of yolk sac membrane KFase [7].

Abbreviations: COMFA - comparative molecular field analysis; 3D QSAR - three-dimensional quantitative structure-activity relationship; ED₅₀ - a dose of an OPI injected into the yolk of a fertile chicken egg causing a 50% reduction of the embryo NAD⁺ content; KFase - N-formyl-L-kynurenine formamidase; NAD⁺ – nicotinamide adenine dinucleotide; OPI - organophosphorus insecticide(s).

2. Materials and Methods

2.1. COMFA of 3D QSAR for OPI and their ability to lower chicken embryo NAD⁺ content

OPI potency to reduce chicken embryo NAD⁺ content was expressed as a negative logarithm (plnED₅₀) of the ED₅₀'s (a dose of an OPI injected into the yolk of a fertile chicken egg causing a 50% reduction of the embryo NAD⁺ content). ED₅₀'s of 18 OPI (the structures listed in Table 1) were calculated from the data obtained in [2, 3, 6] by using a formula $ED_{50} = [\%NAD^+ / (100 - \%NAD^+)] \times I$ (where $\underline{NAD}^+$ is the embryo NAD⁺ content after egg treatment with a dose $\underline{I}$ of an OPI) [8].

The QSAR module in Sybyl 7.3 molecular modeling program package (Tripos Associates, St. Louis, MO, USA) was used for COMFA and other associated computations as reported in our previous study [8]. OPI conformers at their lowest minimal global energy were aligned manually on a common OPI substructure >P(=O or =S)-O- and superimposed on diazinon as a template molecule. The COMFA used the electrostatic and steric fields with the spacing grid 0.2 nm and sp³ hybridized carbon atom with a charge +1 as a probe atom. The data matrix was analyzed by the partial least squares method. The cross-validation correlation coefficient q² was computed by the leave-one-out method. A noncross-validated coefficient r² was calculated by using all compounds of the set.

2.2. OPI isopotential contours and the changes in plnED₅₀'s due to manipulation of the electric charges of the elements in the critical molecular regions

OPI isopotential contours were constructed for the electrical field -8 kcal/mol. The atoms selected for charge modifications were those located in the regions where COMFA predicted that a more electronegative field would likely result in an increased reduction of the embryo NAD⁺ content. Electric charges of the selected atoms were computer-altered to zero and +1/-1 charges, respectively. The plnED₅₀'s of the pyrimidinyl phosphorothioates and crotonamide phosphates with altered electric charges on the selected atoms were predicted by using the Sybyl program and exploring the model created by the COMFA.

2.3. Contouring correlation for OPI structures and the inhibition of yolk sac membrane KFase

The COMFA electrostatic and steric contours of the chicken embryo yolk sac membrane KFase inhibition by OPI were constructed by exploring results from our previous study [7].

3. Results

The electrostatic and steric fields contributed equally to COMFA-based model. The cross-validated coefficient q^2 of the relationship between the explored fields and OPI plnED_{50} was 0.441. The non-crossvalidated coefficient r^2 was 0.999 with a standard error 0.087. The plnED_{50} 's measured and those predicted by the COMFA are listed in Table 1.

The COMFA electrostatic maps contoured at 20 % (polyhedrals I) and 80% (polyhedrals II) of the overall range of the COMFA field are shown in Fig. 1. The electrostatic maps obtained at the -0.020 and +0.009 kcal/mol levels (S.D. x field coefficient) (Fig. 1 inset) show regions where the electrostatic fields would likely have the most significant effects on OPI ability to reduce embryo NAD^+ content. Region I, where an increased electronegativity would likely result in the lowest embryonic NAD^+ levels, is located in the space of OPI leaving groups X. It envelopes pyrimidinyl nitrogen-1 of pyrimidinyl phosphorothioates (Figs. 1 and 2). In crotonamide phosphates, region I surrounds a carbonyl of the crotonamide moiety (Fig. 2). Region II, where an increase in positive charge would likely result in lower embryonic NAD^+ levels, is adjacent to $>\text{P}=\text{O}$ ($>\text{P}=\text{S}$) fragment (Fig. 1).

The isopotential contours of several OPI recorded at -8 kcal/mol together with COMFA electrostatic map contoured at -0.025 and +0.020 kcal/mol levels are shown in Figs. 3-5. Those of diazinon and dicrotophos overlap with the region where an increase in electronegativity would likely be supportive of the reduction in embryonic NAD^+ levels (Figs. 3C, 4C). A similar relationship was seen for other pyrimidinyl phosphorothioates as well as the crotonamide phosphate monocrotophos. The negative isopotential contours of other OPI, such as e.g. parathion, methamidophos and leptophos (Fig. 5), have minimal or no common interface with the region where the increased electronegativity is expected to enhance reduction in embryonic NAD^+ levels.

The changes in OPI isopotential contours due to the altered charges on the selected atoms together with COMFA electrostatic maps are shown in Figs. 3 and 4. The resulting changes in OPI plnED_{50} 's are summarized in Table 2. The increased negative charges on pyrimidinyl nitrogens and/or the nitrogen of carbon-2 substituent resulted in an increased OPI potency to lower embryonic NAD^+ levels. Imposing a no charge and/or a positive charge on those atoms impaired OPI ability to reduce the embryo NAD^+ content. In crotonamide phosphates, the increased negative charge on oxygen of the crotonamide carbonyl resulted in a greater plnED_{50} . A zero charge and/or an increased positive charge on the oxygen impaired OPI ability to lower the embryo NAD^+ content. Minor or no changes in plnED_{50} occurred due to the altered charges on the nitrogen of the crotonamide moiety. The COMFA steric contour maps shown for -0.003 and +0.007 kcal/mol levels identified a space around a substituent on carbon-6 of the pyrimidinyl ring of pyrimidinyl phosphorothioates as a region where bulkiness would most significantly enhance OPI potency to lower embryonic NAD^+ levels (Fig. 6). The methyl substituents of the amide group of crotonamide phosphates conform to these regions too. None other OPI fully satisfied those steric requirements (Fig. 7).

Table 1 list also plnED_{50} 's predicted for diazinon rotamer and/or stereoisomers of dicrotophos and leptophos. Diazinon with the torsion angle of parathion was less potent in reducing embryonic NAD^+ levels than the parental compound. E-isomer of dicrotophos was better KFase inhibitor and more potent in reducing the embryo NAD^+ content than the corresponding Z-isomer. S-enantiomer of leptophos was more active in that sense than its R-chiral image.

4. Discussion

Validity of the COMFA-based model was demonstrated by the value of the cross-validated coefficient and by its high predictive power (Table 1). Our search for the structural features that make pyrimidinyl phosphorothioates and crotonamide phosphates most potent OPI in lowering embryonic NAD^+ levels considered both OPI electrostatic and steric fields since those fields contributed equally to COMFA-based model, similarly to the relationship between OPI structures and chicken embryo yolk sac membrane KFase inhibition.

Regions where an increase in electronegativity (region I) and/or electropositivity (region II) are predicted to support reduction in embryonic NAD^+ levels are located in the spaces similar to those that define OPI potency to inhibit chicken embryo yolk sac membrane KFase (Fig.1). We focused on region I since region II, where an increased electropositivity would likely favor lowering the embryonic NAD^+ levels, is located around the $>\text{P}(\text{O} \text{ or } \text{S})-$ fragment, the structural feature common to all investigated OPI, and thus does not seem to be a factor that would alone determine selectivity of pyrimidinyl phosphorothioates and crotonamide phosphates to lower the embryonic NAD^+ levels. Similarly to OPI structural requirements for yolk sac membrane KFase inhibition, nitrogen-1, nitrogen-3 and the nitrogen of 2-amino substituent of the pyrimidinyl of pyrimidinyl phosphorothioates, and the oxygen of crotonamide carbonyl in crotonamide phosphates are the structural elements that most likely make those OPI distinguishably potent in lowering embryonic NAD^+ levels. The evidence that supports this conclusion is essentially the same as that revealed for KFase inhibition by OPI (Seifert, 2013): First, only the pyrimidinyl and crotonamide moieties of OPI leaving groups are located in the space, where an increased electronegativity is predicted to enhance OPI inhibitory potency. Second, the negative isopotential contours of pyrimidinyl phosphorothioates and crotonamide phosphates are the only ones that overlap with region I (Figs. 3 and 4) in contrast to those of less effective OPI with leaving groups such as 4-nitrophenyl of methyl parathion and parathion, 4-bromo-2, 5-dichlorophenyl of leptophos, amino-group of methamidophos (Fig. 5), diethoxycarboxy- of malathion, dichlorovinyl of dichlorvos and chromen of coumaphos. And third, changing electric charges of atoms that are the structural elements of the negative electrostatic potential formed around the pyrimidinyl and crotonamide moieties altered OPI plnED_{50} 's in the sense predicted by the COMFA (Table 2). More negative charges imposed on those elements resulted in a greater reduction in the embryonic NAD^+ levels. With more positive charges imposed, the negative isopotential contours ceased to exist in region I space (Figs. 3 A, B and 4 A, B) and OPI ability to lower the embryo NAD^+ content was markedly reduced (Table 2).

The OPI steric requirements for reducing chicken embryo NAD^+ content are as well similar to those that support inhibition of yolk sac membrane KFase (Fig. 6). The location of 6-substituents on a pyrimidinyl moiety of pyrimidinyl phosphorothioates in the space, where the COMFA predicted a positive correlation between OPI structural bulkiness and their ability to lower embryonic NAD^+ levels, is another structural feature that contributes to supreme potency of those OPI to reduce embryonic NAD^+ levels. N-substituents of the amide group enhance those qualities in crotonamide phosphates. None of less potent OPI has such structural features, either due to a lack of bulkiness of small molecules such as methamidophos or due to the location of large size moieties such as 4-bromo-2,5-dichlorophenyl of leptophos, 4-nitrophenyl of parathion or 3,5,6-trichloro-2-pyridinyl of methyl chlorpyrifos in the region where the COMFA predicts negative effects of bulkiness on OPI ability to lower the embryo NAD^+ content (Fig. 7). Pattern of the changes in pInED_{50} 's of diazinon rotamer and/or the stereoisomers of dicrotophos and leptophos followed that of KFase inhibition and is another feature of similarity between OPI structural requirements for a reduction of the embryo NAD^+ content and yolk sac membrane KFase inhibition.

4.1. Conclusion

NAD^+ is the terminal product of L-tryptophan metabolism with KFase critical for catalyzing hydrolysis of N-formyl-L-kynurenine, the oxidative product of L-tryptophan at the beginning of the L-tryptophan to NAD^+ metabolic pathway. The similarity between OPI structural requirements for lowering chicken embryo NAD^+ content and yolk sac membrane KFase inhibition is another piece of evidence for the cause-and-effect relationship between yolk sac membrane KFase inhibition and the reduced embryonic NAD^+ levels in OPI-induced teratogenesis in chickens.

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Figure legends

Fig. 1. The COMFA electrostatic contour plots for reducing embryo NAD^+ content and inhibition of yolk sac membrane KFase by OPI. The contour plots with inserted diazinon for reducing embryo NAD^+ content (A) and KFase inhibition (B) are shown at 20 % (I) and 80% (II) of the overall COMFA field range. Polyhedrals I represent a region where an increased electronegativity would likely promote OPI reducing/inhibitory potency. Polyhedrals II represent a region where an increased electropositivity would likely promote OPI reducing/inhibitory potency. Inset are the electrostatic contour plots for a general OPI structure showing regions where the electrostatic fields would have the greatest effects. The contour plots were obtained at -0.020 (I) and 0.009 (II) kcal/mol levels for reducing embryo NAD^+ content (A) and at -0.009 (I) and +0.006 (II) kcal/mol for KFase inhibition (B). R_1 , R_2 , X (a leaving group) in OPI formula are defined in Table 1.

Fig. 2. The COMFA electronegative contour plots for reducing embryo NAD^+ content and inhibition of yolk sac membrane KFase by OPI. The contour plots with inserted diazinon and dicotophos obtained at -0.02/0.04 and -0.01/0.01 kcal/mol levels for reducing embryo NAD^+ content (A) and KFase inhibition (B), respectively, indicate regions where the electronegative field would likely have the greatest impact on OPI potency to lower the embryo NAD^+ content and/or inhibit KFase.

Fig. 3. The effects of altering charges of pyrimidinyl nitrogens on the isopotential contours of diazinon. The isopotential contours (wire frames) are shown for -8 kcal/mol potential. The COMFA electrostatic plot contours (filled areas) obtained at -0.025 and +0.020 kcal/mol levels indicate the region where electronegativity would likely have the greatest impact on OPI potency to lower the embryo NAD^+ content. A – a positive charge +1 on pyrimidinyl N-1; B – zero charge on pyrimidinyl N-1, N-3; C – actual charges. Other pyrimidinyl phosphorothioates showed a similar pattern of isopotential contours like diazinon.

Fig. 4. The effects of altering charges of the oxygen of crotonamide carbonyl on the isopotential contours of dicotophos. The isopotential contours (wire frames) are shown for -8 kcal/mol potential. The COMFA electrostatic plot contours (filled areas) obtained at -0.025 and +0.020 kcal/mol levels indicate the region where electronegativity would likely have the greatest impact on OPI potency to lower the embryo NAD^+ content. A – a positive charge +1 on the carbonyl oxygen, B – a zero charge on the carbonyl oxygen; C – actual charges. The crotonamide phosphate monocotophos showed a similar pattern of isopotential contours like dicotophos.

Fig. 5. The COMFA electronegative contour plots and the isopotential contours of OPI less potent in reducing embryonic NAD^+ levels. The isopotential contours (wire frames) are shown for -8 kcal/mol potential. The COMFA electrostatic contour plots (filled areas) obtained at -0.025 and +0.020 kcal/mol level indicate the region where electronegativity would likely have the greatest impact on OPI potency to lower the embryo NAD^+ content.

Fig. 6. The COMFA steric contour plots with inserted diazinon and dicotophos indicating regions where bulkiness would likely enhance OPI potency to lower embryonic NAD⁺ levels. The contour plots were obtained at -0.003/0.007 and -0.007/0.005 kcal/mol, respectively, for reducing embryo NAD⁺ content (A) and KFase inhibition (B).

Fig. 7. The COMFA steric contour plots with inserted OPI less potent in lowering embryonic NAD⁺ levels. Area I represent a region where bulkiness would likely enhance OPI potency to lower embryonic NAD⁺ levels. Areas II show regions where bulkiness would likely reduce OPI potency to lower embryonic NAD⁺ levels. The contour plots were obtained at -0.003 and 0.007 kcal/mol.

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Table 1. The measured and COMFA-predicted OPI plnED₅₀'s for reducing embryo NAD⁺ content

OPI	R ₁ R ₂ P(=O or =S)-O(or S)-X			plnED ₅₀	
	P=O (P=S)	R ₁ /R ₂	-O-X or S-X	Measured	Predicted ¹
coumaphos	P=S	C ₂ H ₅ O/C ₂ H ₅ O	O-3-chloro-4-methyl-2-oxo-2H-chromen-7-yl	11.42	11.42
diazinon	P=S	C ₂ H ₅ O/C ₂ H ₅ O	O-2-isopropyl-6-methyl-4-pyrimidinyl	13.95	14.04 10.93 ²
dichlorvos	P=O	CH ₃ O/CH ₃ O	O-2,2-dichlorovinyl	8.83	8.93
dicrotophos	P=O	CH ₃ O/CH ₃ O	O-1[E]-methyl-3-(dimethylamino)-3-oxo-1-propenyl	14.11	14.05 10.67 ³
dimethoate	P=S	CH ₃ O/CH ₃ O	S-(2-ethylamino)-2-oxoethyl	10.44	10.41
ethylpirimiphos	P=S	C ₂ H ₅ O/C ₂ H ₅ O	O-2-(diethylamino)-6-methyl-4-pyrimidinyl	13.76	13.64
etrimfos	P=S	CH ₃ O/CH ₃ O	O-2-ethyl-6-ethoxy-4-pyrimidinyl	13.85	13.86
leptophos	P=S	phenyl/ CH ₃ O	O-4-bromo-2,5-dichlorophenyl	8.33	8.34 10.44 ⁴
malathion	P=S	CH ₃ O/CH ₃ O	S-[1,2-bis(ethoxy-carbonyl) ethyl	10.81	10.79
methamidophos	P=O	CH ₃ O/CH ₃ S	Amino	11.74	11.69
methylchlorpyriphos	P=S	CH ₃ O/CH ₃ O	O-3,5,6-trichloro-2-pyridinyl	8.09	8.00
methylparathion	P=S	CH ₃ O/CH ₃ O	O-4-nitrophenyl	11.27	11.36
methylpirimiphos	P=S	CH ₃ O/CH ₃ O	O-2-(diethylamino)-6-methyl-4-pyrimidinyl	13.48	13.44
mevinphos	P=O	CH ₃ O/CH ₃ O	O-1[E]-methyl-3-methoxy-3-oxo-1-propenyl	10.33	10.36
monocrotophos	P=O	CH ₃ O/CH ₃ O	O-1[E]-methyl-3-(methylamino)-3-oxo-1-propenyl	13.70	13.85
parathion	P=S	C ₂ H ₅ O/C ₂ H ₅ O	O-4-nitrophenyl	11.69	11.61
phorate	P=S	C ₂ H ₅ O/C ₂ H ₅ O	S-ethylthiomethyl	11.08	11.14
phosphamidon	P=O	C ₂ H ₅ O/C ₂ H ₅ O	O-1[Z]-methyl-2-chloro-3-(diethylamino)-3-oxo-1-propenyl	12.82	12.76

¹Correlation coefficient r² 0.999; X - a leaving group; ² the torsion angle of a pyrimidinyl/P-O- fragments in diazinon modified to that of 4-nitrophenyl/P-O- fragment in parathion; ³ Z-isomer (for KFase inhibition, plnED₅₀ for E-isomer was 13.68 and for Z-isomer 11.68); ⁴ S-enantiomer (for KFase inhibition, plnED₅₀ for R-enantiomer was 11.56 and 12.23 for S-enantiomer).

Table 2. Effects of altering charges on the selected atoms of pyrimidinyl phosphorothioate and crotonamide phosphates on their plnED₅₀'s

OPI selected atom	Actual charges ¹	0 charge	plnED ₅₀ +1 charge	-1 charge
Diazinon	14.04			
N1		12.22	11.60	15.03
N3		12.86	11.70	14.76
Pirimiphos-methyl	13.44			
N1		11.84	11.11	14.38
N3		11.93	10.56	14.36
N(C2)		10.91	9.12	13.93
Pirimiphos-ethyl	13.64			
N1		11.65	10.88	14.75
N3		12.18	10.92	14.50
N(C2)		11.12	9.20	14.13
Etrimfos	13.86			
N1		11.94	11.06	15.04
N3		12.18	10.49	14.80
O(C6)		13.78	13.88	14.00
Dicrotophos	14.05			
O(carbonyl)		10.59	8.16	15.74
N(amide)		13.83	12.93	14.09
Monocrotophos	13.85			
O(carbonyl)		10.59	8.32	15.47
N(amide)		13.70	12.77	13.85

¹Actual charges: N1, N3 both -0.62; N(C2) -0.84; O(C6) -0.36; carbonyl O -0.57; N-amide of crotonamide phosphates -0.73.

**STRUCTURAL REQUIREMENTS FOR ORGANOPHOSPHORUS
INSECTICIDES TO LOWER CHICKEN EMBRYO NAD^+ CONTENT
AND INHIBIT N-FORMYL-L-KYNURENINE FORMAMIDASE**

pyrimidinyl phosphorothioates

crotonamide phosphates

lowered NAD^+ content kynurenine formamidase inhibition

- * 3D QSAR: I - dominant electronegative interactions II - bulkiness favored
- * similarity of the structural requirements supports the cause-and-effect relationship between kynurenine formamidase inhibition and lowered embryo NAD^+ content in NAD-associated OPI teratogenesis