In silico studies and *in vitro* evaluation of isatin-pyridine oximes hybrids as novel reactivators of acetylcholinesterase inhibited by an A-230 surrogate

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Abstract: Recent events involving A-series nerve agents, a once elusive class of chemical warfare agents, have provoked a great concern in the international

community. In this paper, continuing our research efforts in Medicinal Chemistry at IDQBRN, a OPCW Designated Laboratory for environmental samples, we explore ANMP, aA-230 surrogate, as an inhibitor, in the search for new treatment options for intoxication caused by these chemicals. We evaluated the potential of five isatin-pyridine oximes hybrids as reactivators using a modified Ellman's assay. The results suggest that isatin-oxime monocationic hybrids structures with 5 methylene units and its oxa-analog could be promising for the design of new AChE reactivator.

Keywords: nerve agents, isatin hybrids, acetylcholinesterase, antidotes, Chemical Weapons Convention.

Chemical warfare agents (CWAs) are toxic compounds designed to cause injury or incapacitation. They are more readily synthesized compared to biological and radiological/nuclear agents.[1–4] Among the highly toxic CWAs are the organophosphorus-based nerve agents (OPNA, Figure 1), which are potent inhibitors of acetylcholinesterase (AChE). AChE is a pivotal enzyme found in the central nervous system (CNS) and neuromuscular junctions, responsible for the hydrolysis of neurotransmitter acetylcholine (ACh). OPNA intoxication may lead to hyperexcitability (cholinergic syndrome)and, depending on the dose and route of exposure, can result in rapid death.[5,6]

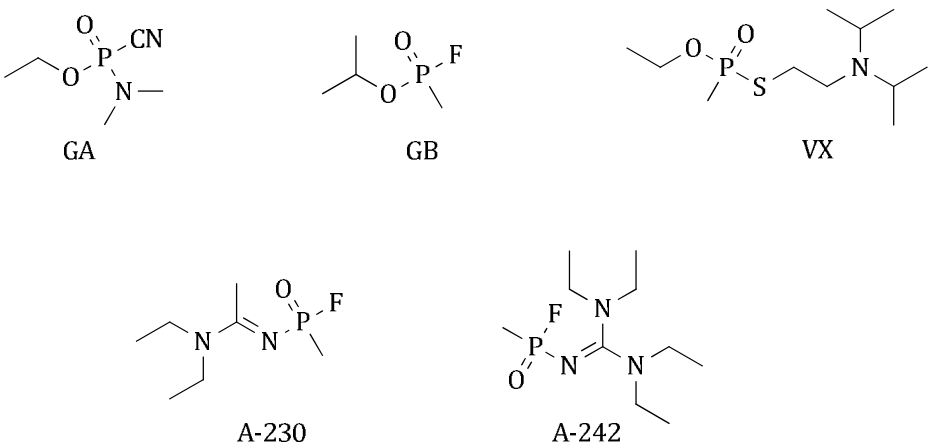

Figure 1. Structure of some nerve agents: tabun (GA), sarin (GB), VX, A-230 and A-242.

The most comprehensive and effective international agreement to control CWAs, the Chemical Weapons Convention (CWC), which entered into force in 1997, was not sufficient to avoid theuse of OPNA, as observed in Syria (2011), Malaysia (2017), UK (2018), and Russia (2020). The latter two cases drawn attention because they involvedattempts of intoxication using the once elusive A-series nerve agents, a class of OPNAfor which information on analysis and toxicology remains limited, albeit the increaseof research publications in recent years.[7–10] An amendment to the CWC expanded the list of controlled compounds to includeover 10,000 A-series scaffolds.[11,12] AsInstitute of Chemical, Biological Radiological and Nuclear Defense (IDQBRN) is the OPCW Designated Laboratory in the Group of Latin America and Caribbean (GRULAC) region, it is mandatoryto be ready to support the Organisation for the Prohibition of Chemical Weapons (OPCW), the international body overseeing the CWC compliance. This includesthe research on the peaceful uses of Chemistry, such as the development of medical countermeasures againsttoxic chemicals.

Currently, the therapeutic strategy for OPNA poisoning consists in up to three drugs. First, anticholinergicssuch as atropine are administered to antagonizethe effects of ACh accumulation in the synaptic cleft. Additionally, benzodiazepines (*e.g.*,diazepam or midazolam) are used to reduce seizures. Finally, an AChE reactivator is employed to restore the enzyme's biological activity.So far, oximes have beenthe most successful antidotes for OPNA poisoning. Nonetheless, all tested compounds still present limited efficacy, including commercial AChE reactivators (**Erro! Fonte de referência não encontrada.**).[13,14]

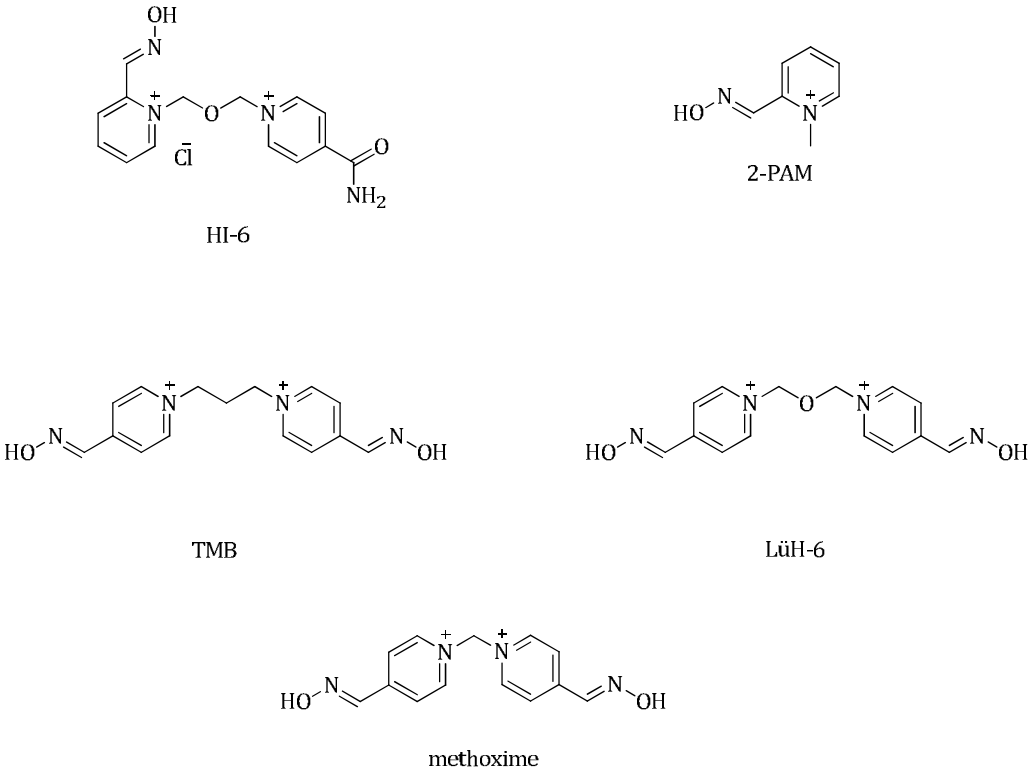

Figure 2. Commercial AChE reactivators oximes: Asoxime (HI-6), pralidoxime (2-PAM), trimedoxime (TMB),obidoxime (LüH-6) and methoxime.

In order to develop a broaderand more effective approach for treating A-series OPNA poisoning, we have tested the potential of isatin-oxime derivatives (1-5, Figure 3)as reactivators for the AChE inhibited by4-nitrophenyl (E)-*N*-(1-(diethylamino)ethylidene)-*P*-methylphosphonamidate (ANMP), a A-230surrogate (Figure 4).Literature suggests that ANMP is a valuable tool to study A-230 behavior as an AChE inhibitor and for assessing the profile of countermeasure candidates. Previously, we demonstrated that five isatin-oximes derivatives may be promising AChE reactivators against VX surrogate NEMP and paraoxon (Figure 4).[15–17]

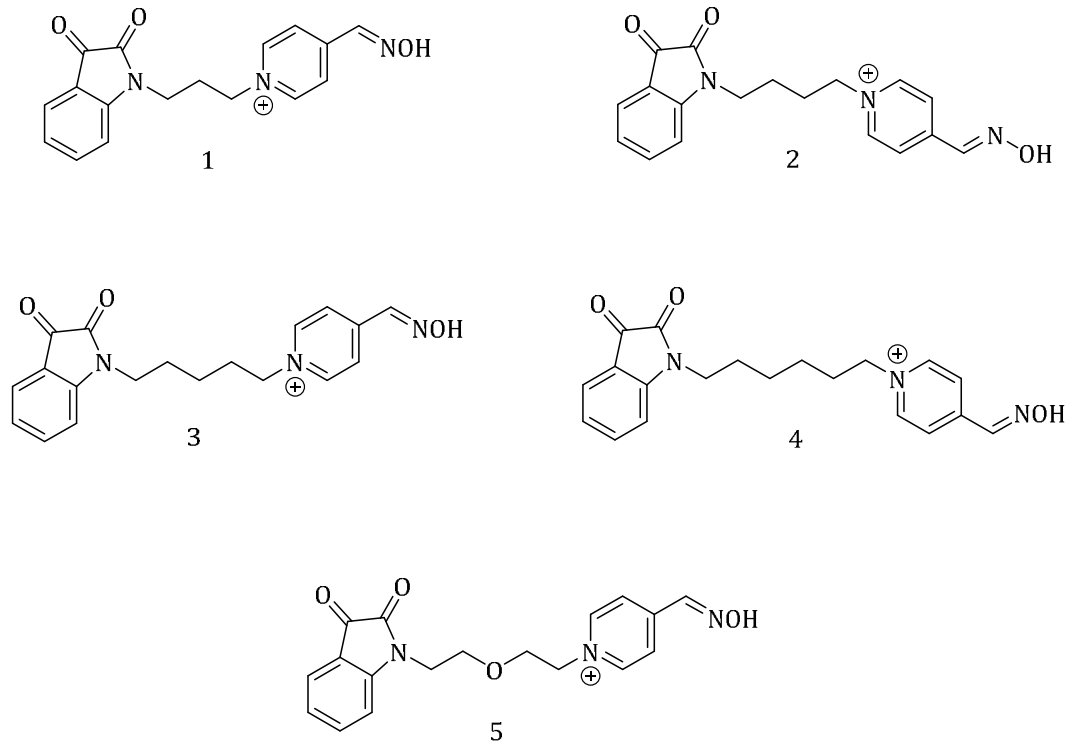

Figure 3. Structure of isatin-oxime derivatives studied in this work.

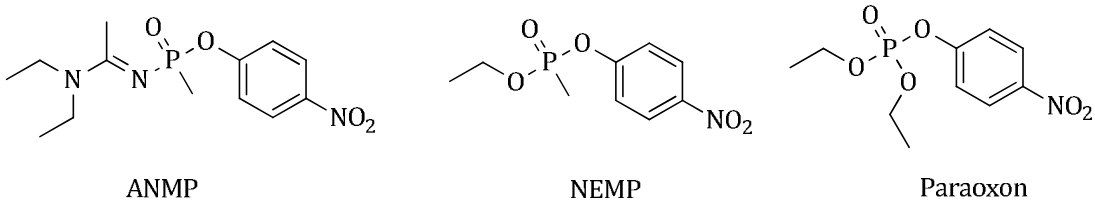

Figure 4. Structures of OP surrogates.

The evaluation of AChE inhibition by ANMP and reactivation by the five isatin-oxime hybrids were performed by modified Ellman's assay, following established protocols.[15,18]Only compounds 3 and 5 presented reactivation above 5% (Table 1), suggesting interactions with the AChE inhibited by ANMP.[19]These compounds also demonstrated the highest reactivation in this study, consistent with the results from similar studies with other OP.[16,17] Although 2-PAM, TMB, and LüH-6showed higher reactivation potential, compounds 3 and 5 outperformed HI-6 and methoxime.Reactivation potential of themolecules tested in this work for AChE inhibited by ANMP is lower compared to paraoxon- and NEMP-inhibited models,[15,16] suggesting a specific performance of isatin-oxime hybrids for each OP.

Table 1. Reactivation of AChE inhibited by ANMP in % (mean ± standard deviation).

Reactivator	1 µM	10 µM	100 µM
1	1±0	0±0	2±1
2	0±0	0±0	3±0
3	0±0	2±1	8±0
4	0±0	2±0	4±1
5	0±0	0±0	8±0
2-PAM*	2±0	7±0	27±0
TMB*	0 ± 0	2 ± 0	16 ± 1
LüH-6*	1 ± 1	1 ± 0	10 ± 3
HI-6*	0 ± 0	0 ± 0	1 ± 0
Methoxime*	0 ± 0	0 ± 0	1 ± 0

***source: Bernardo and co-workers[15]**

Aiming to assess the AChE reactivation mechanism, *in silico* studies were performed. Molecular docking simulations were performed with the human AChE (*Hss*AChE) inhibited by A-230 model along with the isatin-oxime derivatives structuresas established in the literature.[20]The near-attack conformation (NAC) approach was employed to analyze the poses,[21] and the angle-distance ratio $R_{\theta d}$ were calculated(Table S1-S10). It was observed that the non-deprotonated forms of compounds 3, 5, and 6 hadbetter scores than their deprotonated counterparts,indicating a better interaction with *Hss*AChE/A-230 model (Compound 4 showed the most negativetotal hydrogen bonding energy(E_H)calculated values among all ligands and formed more H-bonds with a greater number of residues in both deprotonated and non-deprotonated states (**Erro! Auto-referência de indicador não válida.** and Table 3). Although it showed lower reactivation in the Ellman's assay, these *in silico* results suggest the better interactions with the enzyme and greater stability forcompound 4, indicatinga potential for exploring this structure inother applications.[23,24]

Table 2). On the other hand, the $R_{\theta d}$ values werehigher for the deprotonated species of compounds 3 and 6, suggesting a better approach from these ligands to the phosphorus atom of Ser203 adduct (

Table 3). Comparing the theoretical data with the results from theEllman's assay, a greater similarity was observed in the calculations for deprotonated

species and the *in vitro* results, supporting theproposed mechanism in literature that oximate species provide the attack on phosphorus atom.[22]

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Table 3). Although it showed lower reactivation in the Ellman's assay, these *in silico* results suggest the better interactions with the enzyme and greater stability forcompound 4, indicatinga potential for exploring this structure inother applications.[23,24]

Table 2. Molecular docking data from the non-deprotonated species of the ligands.

Ligand	E_{inter} (kcal.mol^{-1})[a]	E_H (kcal.mol^{-1})[b]	H-bonding[c]	$R_{\theta d}$
1	−179.5	−1.8	Tyr124, Tyr337	21.2
2	−185.9	−4.3	Tyr124, Tyr337	24.2
3	−181.1	−6.0	Tyr124, Ser125	26.2
4	−171.2	−6.3	Tyr72, Tyr124, Ser125	27.6
5	−176.5	−3.5	Tyr124, Tyr337	22.8

[a] intermolecular interaction energy
[b] total hydrogen bonding energy
[c] amino acid residues

Table 3. Molecular docking data from the deprotonated species of the ligands.

Ligand	E_{inter} (kcal.mol^{-1})[a]	E_H (kcal.mol^{-1})[b]	H-bonding[c]	$R_{\theta d}$
1	−181.6	−4.6	Ser298	11.9
2	−183.1	−2.8	Tyr124, Tyr337	22.9
3	−168.8	−2.4	Tyr124, Tyr337	27.2
4	−179.4	−9.1	Tyr124 (x2), Ser125, Ser298 (x2)	25.4
5	−180.6	−6.2	Tyr124, Phe295, Arg296, Tyr337	25.2

[a] intermolecular interaction energy
[b] total hydrogen bonding energy
[c] amino acid residues

Ligand-protein 2D interaction maps (Figure 4-8) revealed Π-Π stacking interactions between the isatin moiety of all ligands and the Tyr124 and Trp286 residues, except for the deprotonated form of compound 4 deprotonated form. It was also observed Π-stacking interaction of the pyridinium ring with Tyr341 for the deprotonated species of compounds 1, 3, and 5, as well as for both non-deprotonated and deprotonated species of compound 2. These interactions meet the designing expectations forthe studied ligands, suggesting strong interactions with peripheral anionic site (PAS),[16]particularlyin the structures of compounds 3 and 5 .

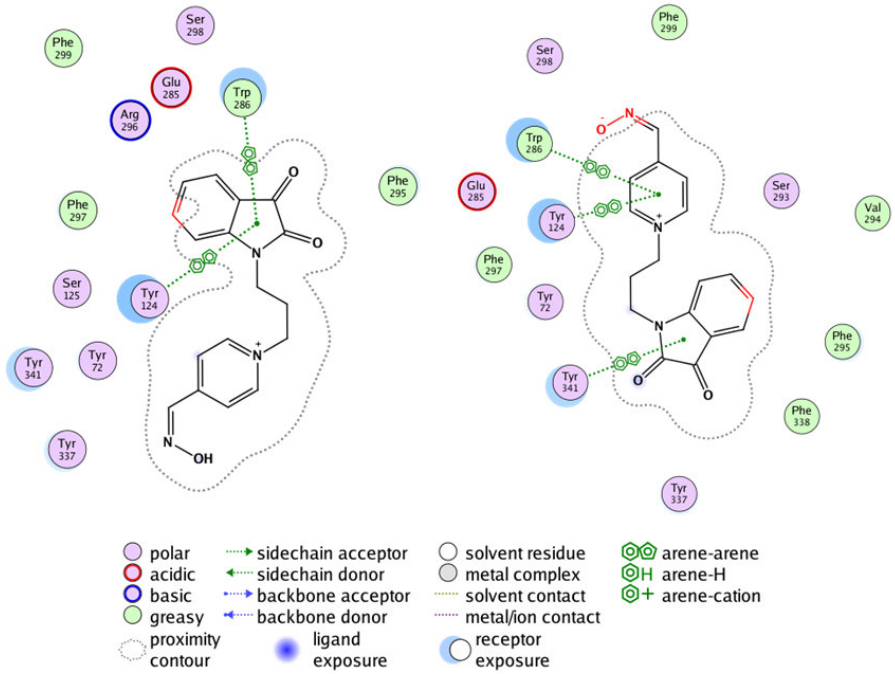

Figure 5. Ligand interaction maps (amino acids within range of 4 Å) for compound1 (left) and its deprotonated form (right).

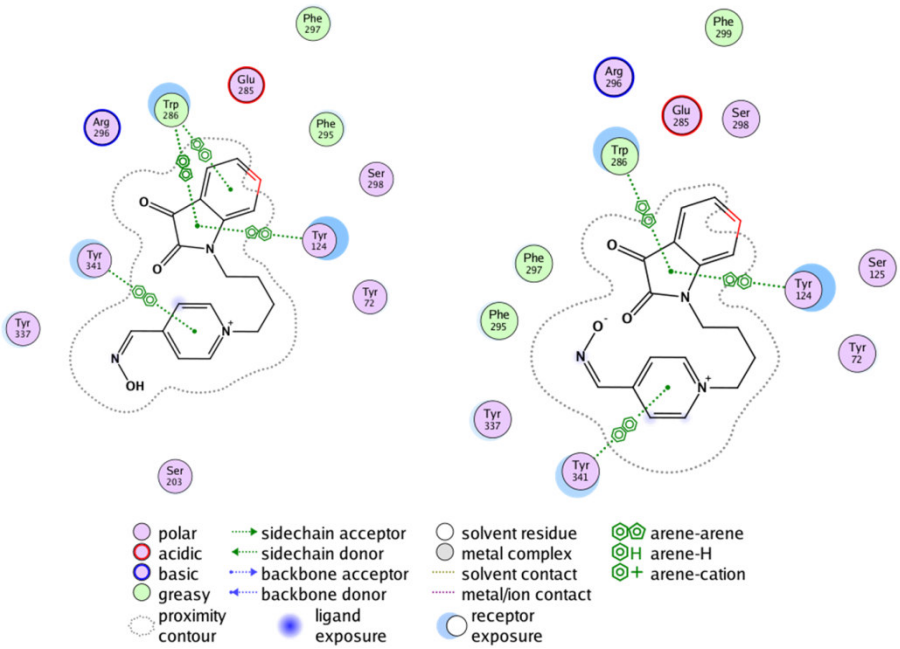

Figure 6. Ligand interaction maps (amino acids within range of 4 Å) for compound2 (left) and its deprotonated form (right).

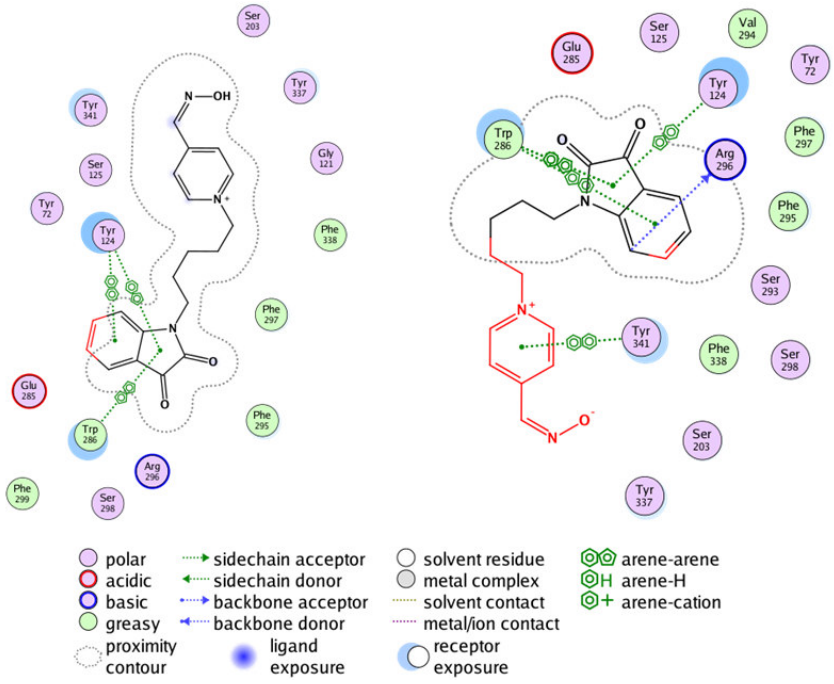

Figure 7. Ligand interaction maps (amino acids within range of 4 Å) for compound3 (left) and its deprotonated form (right).

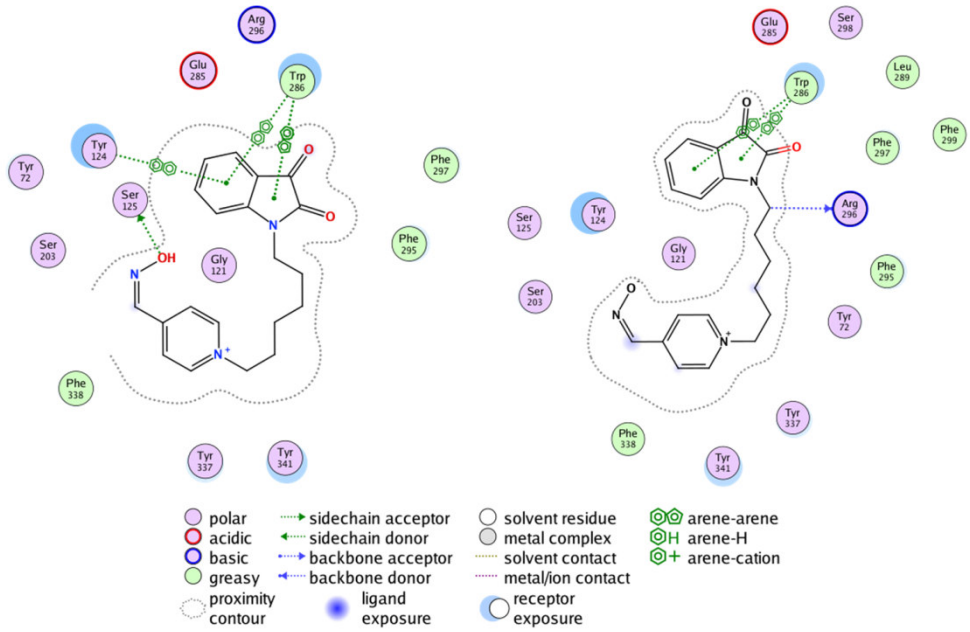

Figure 8. Ligand interaction maps (amino acids within range of 4 Å) for compound 4 (left) and its deprotonated form (right).

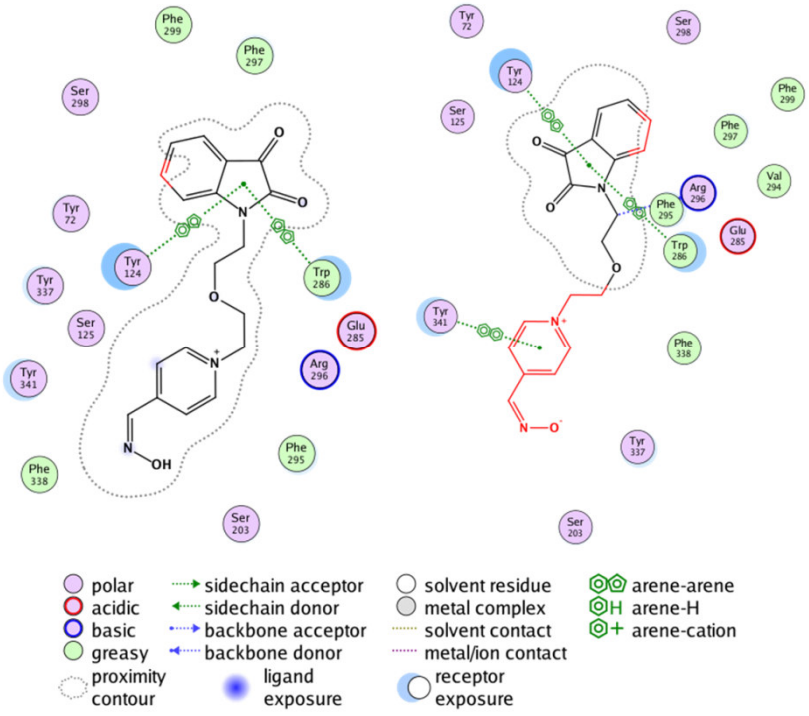

Figure 9. Ligand interaction maps (amino acids within range of 4 Å) for compound5 (left) and its deprotonated form (right).

This work presented information on the reliability of ANMP as an A-230 surrogate for toxicological assessments, confirming its importance as a toxicological tool. Additionally, we compared in *in silico* and *in vitro*AChE

reactivation data obtained for five isatin-oxime monocationic hybrids.Although these compounds did not perform betterthan all commercial oximes as reactivators for ANMP-inhibited AChE, we found that the compounds with 5 methylene units and theiroxa-analog showed the best results.This suggeststhat the distance of 5 atoms between isatin and hydroxyimino moiety provides optimalinteraction with the PAS and the stearic site, respectively. These findingsdemonstrated the potential for exploring isatin-oxime monocationic hybrids with 5 methylene units in the design of new AChE reactivators.

Disclaimer

Authors and Publisher should not be held responsible or accountable for accidents that may occur from incorrect handling of toxic organophosphorus compounds or misuse, in disagreement with the Chemical Weapons Convention. Readers must access the website of the Organisation for the Prohibition of Chemical Weapons (OPCW, www.opcw.org) to obtain further information about the legal framework for research in certain aspects of the organophosphorus chemistry.

Authors' contributions

L.B.B.: synthesis methodology, spectral data acquisition, data analysis, writing - review and editing. **L.A.V.**: molecular modeling methodology, data analysis, writing - review and editing. **C.V.N.B.**: spectral data acquisition, writing - review and editing. **P.A.G.B.**: synthesis methodology, writing - review and editing. **K.K.**: writing - review and editing. **T.C.C.F.**: Software, Modeling analysis, writing, revision. **S.F.A.C.**: synthesis methodology, data analysis, writing - review and editing. **R.B.S.**: writing - review and editing. **A.L.S.L.**: writing - review and editing. **D.A.S.K.**: conceptualization, *in vitro* assays methodology, spectrophotometric data acquisition, data analysis, writing - original draft, review andediting.

Funding

Authors would like to thank Brazilian Army for financial support.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The Authors are grateful to the Brazilian Army, Military Institute of Engineering (IME), Institute of Chemical, Biological Radiological and Nuclear Defense (IDQBRN) for infrastructure and University of Hradec Kralové (UHK) for technical cooperation.

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