



ISABELA APARECIDA MILITANI

**INVESTIGAÇÃO *IN SILICO* DO SISTEMA COLINÉRGICO:
DESENVOLVIMENTO DE ANTÍDOTOS CONTRA ARMAS
QUÍMICAS E PROSPECÇÃO DE FÁRMACOS PARA A DOENÇA
DE ALZHEIMER**

**LAVRAS – MG
2026**

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Tese apresentada à Universidade Federal de
Lavras, como parte das exigências do Programa
de Pós-Graduação em Agroquímica, área de
concentração em Química/Bioquímica, para a
obtenção do título de Doutora.

Prof. Dr. Teodorico de Castro Ramalho
Orientador

Prof. Dr. Alexandre Alves de Castro e Prof.^a Dra. Daiana Teixeira Mancini
Coorientadores

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ALZHEIMER**

***IN SILICO* INVESTIGATION OF THE CHOLINERGIC SYSTEM: DEVELOPMENT
OF ANTIDOTES AGAINST CHEMICAL WEAPONS AND DRUG PROSPECTING FOR
ALZHEIMER'S DISEASE**

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*A Deus, minha proteção divina, pois cada passo desta jornada
foi guiado por Sua vontade; aos meus pais, por tudo o que
puderam oferecer; ao meu esposo, presença fiel em
todos os momentos; e ao meu filho, razão da minha força.
Sem eles, esta conquista não poderia ser possível.*

Dedico

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*“Nada na vida deve ser temido, apenas compreendido.
Agora é hora de compreender mais, para temer menos.”*

Marie Curie

RESUMO

O sistema colinérgico desempenha papel fundamental tanto na neurotoxicidade causada por compostos organofosforados (OPs, do inglês organophosphorus) quanto na fisiopatologia da Doença de Alzheimer (AD, do inglês Alzheimer's disease). As colinesterases (ChEs, do inglês cholinesterases) surgem como importantes alvos moleculares para o desenvolvimento de antídotos contra agentes químicos neurotóxicos e de novas estratégias terapêuticas para a AD. Este trabalho teve como objetivo investigar, por meio de abordagens *in silico*, mecanismos de modulação de colinesterases humanas (*HssChEs*) associados à reativação e à inibição enzimática. Para isso, foram empregadas técnicas computacionais como o *docking* molecular, cálculos híbridos de mecânica quântica/mecânica molecular (QM/MM) e dinâmica molecular (MD, do inglês Molecular Dynamics) visando à avaliação estrutural, energética e mecanística de diferentes compostos bioativos. Os resultados permitiram identificar oximas com potencial para a reativação de enzimas inibidas por OPs, bem como candidatos promissores para a inibição da acetilcolinesterase (AChE, do inglês acetylcholinesterase) no contexto terapêutico da AD. As análises teóricas revelaram interações favoráveis com os alvos biológicos, estabilidade conformacional e perfis energéticos compatíveis com a atividade biológica esperada. Dessa forma, os resultados reforçam a importância de aplicar métodos computacionais no planejamento racional de compostos direcionados ao sistema colinérgico, contribuindo para o desenvolvimento de estratégias mais eficazes e seguras.

Palavras-chave: Sistema colinérgico. Colinesterases. Organofosforados. Oximas. Doença de alzheimer. Química computacional.

ABSTRACT

The cholinergic system plays a central role in both the neurotoxicity induced by organophosphorus compounds (OPs) and the pathophysiology of Alzheimer's disease (AD). Cholinesterases (ChEs) have emerged as important molecular targets for the development of antidotes against neurotoxic chemical agents as well as novel therapeutic strategies for AD. This study aimed to investigate, through *in silico* approaches, the mechanisms underlying the modulation of human cholinesterases (*HssChEs*) associated with enzyme reactivation and inhibition. To this end, computational techniques including molecular *docking*, hybrid quantum mechanics/molecular mechanics (QM/MM) calculations, and molecular dynamics (MD) simulations were employed to evaluate the structural, energetic, and mechanistic properties of different bioactive compounds. The results identified oximes with potential to reactivate OP-inhibited enzymes, as well as promising candidates for acetylcholinesterase (AChE) inhibition in the therapeutic context of AD. Theoretical analyses revealed favorable interactions with the biological targets, conformational stability, and energetic profiles consistent with the expected biological activity. Overall, these findings highlight the value of computational approaches in the rational design of compounds targeting the cholinergic system, contributing to the development of more effective and safer therapeutic and protective strategies.

Keywords: Cholinergic system. Cholinesterases. Organophosphorus. Oximes. Alzheimer's disease. Computational chemistry.

INDICADORES DE IMPACTO

Os resultados obtidos nesta tese apresentam impactos diretos nas esferas científica e tecnológica, com potenciais desdobramentos nas áreas de saúde, toxicologia, defesa química e desenvolvimento de novos candidatos a fármacos. A pesquisa contribui para o avanço do conhecimento sobre o sistema colinérgico por meio da aplicação de métodos de química computacional na investigação dos mecanismos de interação entre compostos bioativos e enzimas colinesterases (ChEs).

Impacto científico e tecnológico: A pesquisa contribuiu para o avanço do conhecimento científico na área de química computacional, com a investigação de estratégias moleculares voltadas à reativação de ChEs inibidas por agentes organofosforados (OPs) e à prospecção de novos compostos com potencial aplicação terapêutica na doença de Alzheimer (AD).

Desenvolvimento de candidatos a antídotos contra agentes OPS: O estudo de oximas como potenciais reativadores de ChEs contribui para a compreensão dos mecanismos moleculares envolvidos na reativação enzimática após a inibição por agentes neurotóxicos OPs. Os resultados obtidos fornecem informações relevantes para o direcionamento racional de novos candidatos a antídotos, com potencial contribuição para as áreas de toxicologia e defesa química.

Prospecção de novos candidatos a fármacos para a AD: a investigação de compostos OPs como potenciais inibidores da acetilcolinesterase (AChE) contribui para a identificação e caracterização de candidatos promissores para o desenvolvimento de estratégias terapêuticas relacionadas à AD. A utilização de abordagens computacionais permitiu avaliar aspectos estruturais, energéticos, farmacológicos e toxicológicos dos compostos investigados, contribuindo para a seleção racional de moléculas com características potencialmente favoráveis ao desenvolvimento de novos agentes terapêuticos.

Impacto na saúde: os resultados apresentam impacto potencial na área da saúde ao contribuir para a compreensão de mecanismos moleculares relacionados à intoxicação por agentes OPs e à modulação do sistema colinérgico no contexto da AD. Embora os resultados obtidos sejam de natureza predominantemente computacional e ainda demandem validação experimental, as informações geradas podem subsidiar estudos futuros voltados ao desenvolvimento de novos antídotos e candidatos a fármacos.

Contribuição para a inovação científica: A aplicação de técnicas computacionais como o *docking* molecular, cálculos de QM/MM, simulações de dinâmica molecular e avaliações *in silico* de propriedades farmacológicas e toxicológicas demonstra o potencial da química

computacional como ferramenta para a investigação e o direcionamento racional de moléculas bioativas. Essa abordagem contribui para a redução do número de candidatos a serem avaliados experimentalmente e para a priorização de compostos com características moleculares potencialmente mais adequadas às aplicações investigadas.

Alinhamento com os Objetivos de Desenvolvimento Sustentável (ODS) da ONU: Os resultados desta pesquisa apresentam alinhamento com os seguintes objetivos:

- ODS 3 (Saúde e Bem-Estar): contribui para a investigação de estratégias relacionadas ao tratamento da AD e para o desenvolvimento de potenciais antídotos contra intoxicações por agentes neurotóxicos;
- ODS 9 (Indústria, Inovação e Infraestrutura): contribui para o desenvolvimento e a aplicação de estratégias computacionais voltadas à descoberta e ao desenvolvimento racional de novas moléculas de interesse farmacêutico e toxicológico.

Áreas temáticas da política nacional de extensão: os impactos potenciais da pesquisa relacionam-se principalmente às áreas de saúde, tecnologia e produção, considerando sua contribuição para a investigação de doenças neurodegenerativas, para a compreensão dos mecanismos de intoxicação por agentes OPS e para o desenvolvimento racional de novos candidatos a antídotos e fármacos. No âmbito científico e tecnológico, os resultados também contribuem para o fortalecimento da pesquisa em química computacional e para a formação de recursos humanos qualificados na pós-graduação *Stricto sensu* da UFLA.

IMPACT INDICATORS

The results obtained in this thesis have direct impacts in the scientific and technological spheres, with potential implications for healthcare, toxicology, chemical defense, and the development of new drug candidates. This research contributes to advancing knowledge of the cholinergic system through the application of computational chemistry methods to investigate the interaction mechanisms between bioactive compounds and cholinesterase enzymes (ChEs).

Scientific and technological impact: The research contributed to advancing scientific knowledge in the field of computational chemistry by investigating molecular strategies aimed at the reactivation of ChEs inhibited by organophosphorus (OP) agents and at the screening of new compounds with potential therapeutic applications in Alzheimer's disease (AD).

Development of antidote candidates against OP agents: The study of oximes as potential ChE reactivators contributes to understanding the molecular mechanisms involved in enzymatic reactivation following inhibition by neurotoxic OP agents. The results obtained provide relevant information to support the rational design of new antidote candidates, with potential contributions to the fields of toxicology and chemical defense.

Screening of new drug candidates for AD: The investigation of OP compounds as potential acetylcholinesterase (AChE) inhibitors contributes to the identification and characterization of promising candidates for the development of therapeutic strategies related to AD. The use of computational approaches enabled the evaluation of structural, energetic, pharmacological, and toxicological aspects of the investigated compounds, contributing to the rational selection of molecules with potentially favorable characteristics for the development of new therapeutic agents.

Impact on health: The results have potential impacts in the healthcare field by contributing to the understanding of molecular mechanisms related to OP agent poisoning and the modulation of the cholinergic system in the context of AD. Although the results obtained are predominantly computational in nature and still require experimental validation, the information generated may support future studies aimed at developing new antidotes and drug candidates.

Contribution to scientific innovation: The application of computational techniques, such as molecular *docking*, QM/MM calculations, molecular dynamics simulations, and *in silico* assessments of pharmacological and toxicological properties, demonstrates the potential of computational chemistry as a tool for the investigation and rational prioritization of bioactive molecules. This approach contributes to reducing the number of candidates to be experimentally

evaluated and to prioritizing compounds with molecular characteristics that may be more suitable for the investigated applications.

Alignment with the United Nations Sustainable Development Goals (SDGs): The results of this research are aligned with the following goals:

- SDG 3 (Good Health and Well-Being): contributes to the investigation of strategies related to the treatment of AD and to the development of potential antidotes against poisoning by neurotoxic agents;
- SDG 9 (Industry, Innovation and Infrastructure): contributes to the development and application of computational strategies aimed at the discovery and rational development of new molecules of pharmaceutical and toxicological interest.

Thematic areas of the National Extension Policy: The potential impacts of this research are primarily related to the areas of health, technology, and production, considering its contribution to the investigation of neurodegenerative diseases, the understanding of the mechanisms underlying poisoning by OP agents, and the rational development of new antidote and drug candidates. Within the scientific and technological spheres, the results also contribute to strengthening research in computational chemistry and to the training of highly qualified human resources in *stricto sensu* graduate education at UFLA.

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LISTA DE SIGLAS

PRIMEIRA PARTE

ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ADME	Absorption, Distribution, Metabolism, and Excretion
BCh	Butyrylcholine
BChE	Butyrylcholinesterase
Ch	Choline
ChAT	Choline Acetyltransferase
ChEs	Cholinesterases
DFT	Density Functional Theory
<i>HssAChE</i>	Human Acetylcholinesterase
<i>HssBChE</i>	Human Butyrylcholinesterase
<i>HssCHEs</i>	Human Cholinesterases
LD ₅₀	Lethal Dose, 50%
MD	Molecular Dynamics
MM	Molecular Mechanics
OP	Organophosphorus compound
OPs	Organophosphorus compounds
QM	Quantum Mechanics
RMN	Ressonância Magnética Nuclear
RMSD	Root Mean Square Deviation
S _N 2	Substituição Nucleofílica Bimolecular
SNC	Sistema Nervoso Central
SNP	Sistema Nervoso Periférico
VAcHT	Vesicular Acetylcholine Transporter

SEGUNDA PARTE

ARTIGO 1

ACh	Acetylcholine
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ADME	Absorption, Distribution, Metabolism, and Excretion
ADMET	Absorption, Distribution, Metabolism, Excretion, and Toxicity
Ch	Choline
ChEs	Cholinesterases
CWAs	Chemical Warfare Agents
DFT	Density Functional Theory
<i>HssAChE</i>	Human Acetylcholinesterase
<i>HssBChE</i>	Human Butyrylcholinesterase
LD ₅₀	Lethal Dose, 50%
MM	Molecular Mechanics
MVD	Molegro Virtual Docker
MW	Molecular Weight
OP	Organophosphate
OPs	Organophosphates
PDB	Protein Data Bank
PLP	Piecewise Linear Potential
QM	Quantum Mechanics
RMSD	Root Mean Square Deviation
S _N 2	Bimolecular Nucleophilic Substitution

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ARTIGO 2

AD	Alzheimer's disease
ACh	Acetylcholine
AIM	Atoms In Molecules
APP	Amyloid Precursor Protein
BBB	Blood- Brain Barrier
CMO	Canonical Molecular Orbitals
DFT	Density Functional Theory
GEM	Generic Evolutionary Method
<i>HssAChE</i>	Human Acetylcholinesterase
LD ₅₀	Lethal Dose, 50%
MD	Molecular Dynamics

MK	Merz-Kollman
MM	Molecular Mechanics
MLR	Multiple Linear Regression
MVD	Molegro Virtual Docker
NBO	Natural Bond Orbital
NBOMO	NBO Molecular Orbital
OP	Organophosphorus
PBSA	Poisson- Boltzmann Surface Area
PCA	Principal Component Analysis
PDB	Protein Data Bank
PLP	Piecewise Linear Potential
QM	Quantum Mechanics
RMSD	Root Mean Square Deviation
SASA	Solvent- Accessible Surface Area

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PRIMEIRA PARTE

Introdução e Referencial Teórico

1 INTRODUÇÃO

O sistema colinérgico é um dos principais sistemas de neurotransmissão do organismo, exercendo papel fundamental na comunicação neuronal e na regulação de funções fisiológicas no sistema nervoso central (SNC) e no sistema nervoso periférico (SNP) (ANANTH *et al.*, 2023; BERTRAND; WALLACE, 2020; FERREIRA-VIEIRA *et al.*, 2016; GEULA *et al.*, 2021; WOOLF, 1991). A neurotransmissão colinérgica é mediada pela acetilcolina (ACh, do inglês *acetylcholine*), sintetizada no terminal pré-sináptico pela colina acetiltransferase (ChAT, do inglês *choline acetyltransferase*) (BERTRAND; WALLACE, 2020; FERREIRA-VIEIRA *et al.*, 2016; GEULA *et al.*, 2021; ŚWIT *et al.*, 2023; WOOLF, 1991). Sua ação é rapidamente interrompida pela hidrólise catalisada pelas colinesterases (ChEs, do inglês *cholinesterases*), representadas pela enzima acetilcolinesterase (AChE, do inglês *acetylcholinesterase*) e, em menor grau, pela butirilcolinesterase (BChE, do inglês *butyrylcholinesterase*). Estas enzimas são essenciais para a manutenção da homeostase colinérgica e do equilíbrio sináptico (BERTRAND; WALLACE, 2020; DIRAK; CHAN; KOLEMEN, 2024; FERREIRA-VIEIRA *et al.*, 2016).

A acetilcolinesterase humana (*HssAChE*) apresenta elevada eficiência catalítica e estrutura altamente organizada, com sítio ativo responsável pelo reconhecimento e hidrólise da ACh (RADIĆ, 2024; SOREQ *et al.*, 1990; SOREQ; SEIDMAN, 2001; DVIR *et al.*, 2010). Essa eficiência, embora essencial para a fisiologia neuronal, também a torna um alvo sensível a compostos capazes de interferir em sua atividade enzimática (DVIR *et al.*, 2010; HÖRNBERG; TUNEMALM; EKSTRÖM, 2007; RADIĆ, 2024). A butirilcolinesterase humana (*HssBChE*), por sua vez, apresenta maior plasticidade estrutural e ampla especificidade de substratos, desempenhando papel complementar na regulação colinérgica e em processos farmacológicos e toxicológicos (CHATONNET; LOCKRIDGE, 1989; DIRAK; CHAN; KOLEMEN, 2024; GIACOPPO *et al.*, 2014; MACK; ROBITZKI, 2000; PATOCKA *et al.*, 2005; SVOBODOVA; SOUKUP; KORABECNY, 2026).

Os compostos organofosforados (OPs, do inglês *organophosphorus*) constituem uma classe química amplamente utilizada na agricultura, pecuária e no controle de pragas devido à sua elevada eficácia biológica (CHU *et al.*, 2026; COSTA, 2018; GUPTA 2020; MALI *et al.*, 2023). Além dos OPs serem empregados como pesticidas, eles também são utilizados como agentes neurotóxicos de guerra, incluindo o sarin, o ciclosarin e o VX, compostos capazes de provocar intoxicações graves devido à sua elevada toxicidade e potente ação anticolinesterásica (MERCEY *et al.*, 2012; ROBB; REGINA; BAKER, 2023). Entretanto, sua toxicidade está

diretamente relacionada à capacidade de inibir ChEs por fosforilação do sítio ativo enzimático, levando à disfunção da neurotransmissão colinérgica (COSTA, 2018; IYER; IKEN; LEON, 2015; ROBB; REGINA; BAKER, 2023). Essa inibição resulta em acúmulo de ACh nas sinapses, hiperestimulação de receptores colinérgicos e manifestações clínicas da síndrome colinérgica, podendo evoluir para quadros graves e potencialmente fatais (COSTA, 2018; ROBB; REGINA; BAKER, 2023). Em alguns casos, o complexo enzima–organofosforado sofre o processo de envelhecimento, tornando a inibição irreversível e dificultando a reativação da enzima por agentes terapêuticos convencionais. Sendo assim, a intervenção terapêutica antes da ocorrência do envelhecimento é fundamental para aumentar as possibilidades de reativação das ChEs (KUCA *et al.*, 2004; MASSON; NACHON, 2017; MERCEY *et al.*, 2012; MILLARD *et al.*, 1999; WOREK *et al.*, 2016).

No tratamento das intoxicações por OPs, uma das principais estratégias terapêuticas consiste no uso de oximas, que são compostos nucleofílicos capazes de promover a reativação das ChEs por meio da desfosforilação do sítio ativo enzimático (CHAMBERS *et al.*, 2020; EDDLESTON *et al.*, 2008; KUCA *et al.*, 2004; MERCEY *et al.*, 2012). A eficácia desses agentes depende fortemente de fatores como a estrutura química da oxima, a natureza do OP envolvido, o grau de envelhecimento do complexo enzima–inibidor e a afinidade pelo alvo molecular (CHAMBERS *et al.*, 2020; FIGUEROA-VILLAR *et al.*, 2021; KOVARIK *et al.*, 2019; MASSON; NACHON, 2017; MERCEY *et al.*, 2012; MILLARD *et al.*, 1999; RAMALHO *et al.*, 2023).

A Doença de Alzheimer (AD, do inglês Alzheimer's disease) é uma enfermidade neurodegenerativa progressiva e irreversível, caracterizada pelo declínio cognitivo e pela perda neuronal em regiões como hipocampo e córtex cerebral. A doença é associada à presença de placas β -amiloide e emaranhados neurofibrilares formados pela proteína tau hiperfosforilada (BRUNEAU; AKAABOUNE, 2006; KNOPMAN *et al.*, 2021; LONG; HOLTZMAN, 2019; VENTURA *et al.*, 2010). Evidências indicam que a disfunção do sistema colinérgico desempenha papel central nos sintomas da doença, fundamentando a hipótese colinérgica. Segundo esta hipótese, a redução da neurotransmissão mediada por ACh contribui significativamente para os déficits cognitivos observados nos pacientes (ANANTH *et al.*, 2023; FERREIRA-VIEIRA *et al.*, 2016).

Os fármacos anticolinesterásicos constituem a principal abordagem terapêutica sintomática da AD. A função destes fármacos é promover o aumento da disponibilidade de ACh na fenda sináptica por meio da inibição da AChE. Embora não interrompam a progressão da doença, esses medicamentos podem promover melhora temporária das funções cognitivas e da

capacidade funcional dos pacientes (COLOVIĆ *et al.*, 2013; FERREIRA-VIEIRA *et al.*, 2016; KANDY *et al.*, 2026; KNOPMAN *et al.*, 2021; PEREIRA; RODRIGUES, 2015; TAN *et al.*, 2014; TAYLOR, 2018).

Além dos seus efeitos neurotóxicos, os OPs apresentam grande importância farmacológica, pois contribuem para o entendimento dos mecanismos de inibição das ChEs e do papel da AChE na neurotransmissão colinérgica (IYER; IKEN; LEON, 2015). O conhecimento obtido a partir dessas investigações impulsionou o desenvolvimento racional de novos inibidores da AChE, com maior seletividade e menor toxicidade, além de favorecer o desenvolvimento de candidatos terapêuticos capazes de modular a neurotransmissão colinérgica, particularmente em doenças neurodegenerativas associadas ao déficit de ACh, como a AD (COLOVIĆ *et al.*, 2013; SINGH *et al.*, 2013; SORIN *et al.*, 2021). A investigação dos mecanismos moleculares envolvidos tanto na inibição quanto na reativação das ChEs é fundamental para compreender os efeitos dos compostos OPs e orientar o desenvolvimento de estratégias terapêuticas mais eficazes. A abordagem computacional, por permitir a análise das interações moleculares e dos mecanismos envolvidos nesses processos, constitui uma ferramenta relevante (BRAUN *et al.*, 2019; CARVALHO, 2003; GONÇALVES *et al.*, 2014).

No primeiro estudo desenvolvido nesta tese, foram empregadas técnicas computacionais para investigar, em nível molecular, a interação de OPs, com ênfase nos agentes neurotóxicos sarin, ciclosarin e VX, com as *HssChEs* (BRAUN *et al.*, 2019; CARVALHO, 2003; GONÇALVES *et al.*, 2014). Foram avaliadas diferentes oximas quanto à sua capacidade de promover a reativação da *HssAChE* e da *HssBChE* previamente inibidas por OPs, utilizando métodos de *docking* molecular para elucidar os modos de interação, bem como cálculos híbridos QM/MM (em inglês, Quantum Mechanics/Molecular Mechanics) para investigar os mecanismos moleculares envolvidos no processo de desfosforilação enzimática (YU *et al.*, 2026).

No segundo estudo desenvolvido nesta tese, foram empregadas técnicas computacionais para investigar o potencial de diferentes compostos OPs como inibidores da *HssAChE*, visando sua aplicação terapêutica no tratamento da AD (KANDY *et al.*, 2026). Inicialmente, foram avaliadas as características estruturais, eletrônicas e toxicológicas dos compostos, utilizando abordagens estatísticas e quimiométricas para auxiliar na seleção dos candidatos. Posteriormente, foram investigadas as interações dos compostos selecionados com a *HssAChE* por meio de *docking* molecular, cálculos híbridos QM/MM e simulações de Dinâmica Molecular (MD, do inglês Molecular Dynamics), além da avaliação de suas propriedades

farmacológicas e toxicológicas, com o objetivo de identificar candidatos mais eficientes e de menor toxicidade (YU *et al.*, 2026).

Em conjunto, os estudos desenvolvidos nesta tese contribuíram para ampliar a compreensão das interações entre compostos OPs e as ChEs, abordando diferentes perspectivas de interesse científico e farmacológico. A realização desses estudos justifica-se pela relevância de compreender, em nível molecular, os mecanismos de interação entre OPs e ChEs, considerando tanto os efeitos associados à exposição a agentes neurotóxicos quanto o potencial de determinados compostos dessa classe para aplicações terapêuticas. O emprego de abordagens computacionais possibilita a investigação detalhada das interações moleculares e dos mecanismos envolvidos nesses processos, contribuindo para o direcionamento racional da busca por estratégias de reativação enzimática e por candidatos com potencial terapêutico e perfil de toxicidade mais favorável. Diante desse contexto, foram estabelecidos os objetivos desta tese, apresentados a seguir, com o propósito de investigar, por meio de abordagens computacionais, os mecanismos moleculares envolvidos na modulação das ChEs por compostos OPs, contemplando tanto a reativação enzimática quanto a prospecção de potenciais agentes terapêuticos.

2 OBJETIVOS

2.1 Objetivos gerais:

Investigar, por meio de abordagens computacionais, os mecanismos moleculares de modulação das *HssChEs* envolvidos nos processos de reativação e inibição enzimática, visando à identificação e avaliação de compostos bioativos com potencial aplicação no tratamento da intoxicação causada por OPs, bem como no tratamento da AD.

2.2 Objetivos específicos:

- 1) Analisar as interações intermoleculares entre oximas e os sítios ativos das *HssChEs* por meio de técnicas de *docking* molecular;
- 2) Investigar os mecanismos reacionais envolvidos na reativação das *HssChEs* por oximas, utilizando cálculos híbridos QM/MM para a estimativa das barreiras energéticas associadas ao processo de desfosforilação;

- 3) Estimar parâmetros teóricos de toxicidade das oximas, incluindo valores de LD₅₀ com o objetivo de avaliar sua segurança potencial;
- 4) Avaliar propriedades farmacocinéticas e toxicológicas das oximas por meio de análises de ADME, visando à avaliação de seu perfil farmacológico;
- 5) Identificar e selecionar oximas com potencial de ação universal para a reativação de *HssChEs* inibidas por diferentes agentes OPs;
- 6) Investigar a relação entre estrutura química e atividade inibitória dos compostos OPs por meio de cálculos quânticos e tratamentos estatísticos, visando identificar características estruturais associadas à maior eficácia como inibidores anticolinesterásicos;
- 7) Analisar as interações intermoleculares entre agentes anticolinesterásicos OPs e a enzima *HssAChE* por meio de técnicas de *docking* molecular;
- 8) Avaliar o potencial de inibição da *HssAChE* por diferentes compostos OPs, com base em parâmetros de afinidade de ligação e reconhecimento molecular por meio de técnicas de MD;
- 9) Comparar a eficácia e toxicidade de novos compostos OPs com o composto de referência triclorfon (metrifonato), quanto à afinidade e padrão de interação com a *HssAChE*;
- 10) Comparar os padrões farmacológicos dos compostos OPs selecionados com o fármaco de referência rivastigmina;
- 11) Selecionar compostos OPs com maior potencial de inibição seletiva da *HssAChE* e menor toxicidade relativa, visando sua aplicação como candidatos promissores no tratamento da AD.

3 REFERENCIAL TEÓRICO

3.1 Sistema colinérgico

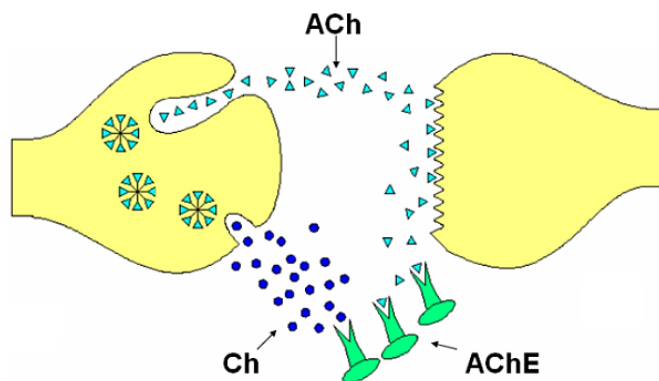
O sistema colinérgico constitui um dos principais sistemas de neurotransmissão do organismo e desempenha papel fundamental na comunicação entre neurônios e células efectoras. Esse sistema é composto pela ACh, seus receptores e o aparato enzimático responsável por sua síntese e degradação (BERTRAND; WALLACE, 2020; GEULA *et al.*, 2021; VENTURA *et al.*, 2010). Sua atuação estende-se por todo o SNC e SNP, participando da regulação de diversas funções fisiológicas essenciais. No SNC, a neurotransmissão colinérgica está envolvida em

processos cognitivos como aprendizagem, memória, atenção e processamento de informações. No SNP, desempenha papel central na transmissão neuromuscular e na regulação das funções autonômicas, incluindo o controle da atividade cardiovascular, respiratória, gastrointestinal e glandular. Assim, a integridade do sistema colinérgico é indispensável para a manutenção da homeostase e do adequado funcionamento do organismo (ANANTH *et al.*, 2023; BERTRAND; WALLACE, 2020; FERREIRA-VIEIRA *et al.*, 2016; GEULA *et al.*, 2021; WOOLF, 1991).

A ACh é o principal neurotransmissor do sistema colinérgico e desempenha papel fundamental na transmissão de sinais nervosos. Sua síntese ocorre no terminal pré-sináptico por ação da ChAT, que catalisa a reação entre colina (Ch, do inglês choline) e acetil-coenzima A. Após sua formação, a ACh é transportada para o interior das vesículas sinápticas pelo VACHT, onde permanece armazenada até a chegada de um potencial de ação. A despolarização da membrana pré-sináptica promove a liberação da ACh por exocitose na fenda sináptica, permitindo sua interação com receptores colinérgicos localizados nas membranas pré- e pós-sinápticas. A ativação desses receptores desencadeia respostas celulares responsáveis pela propagação do sinal nervoso e pela regulação de diversas funções fisiológicas (BERTRAND; WALLACE, 2020; FERREIRA-VIEIRA *et al.*, 2016; GEULA *et al.*, 2021; ŚWIT *et al.*, 2023; WOOLF, 1991).

A neurotransmissão colinérgica depende não apenas da liberação da ACh, mas também da rápida interrupção de sua ação na fenda sináptica (FIGURA 1). Esse processo ocorre por meio da hidrólise da ACh em Ch e acetato, catalisada principalmente pela AChE (PETRONILHO *et al.*, 2011). Além da AChE, outra enzima pertencente a essa família é a BChE, amplamente distribuída nos tecidos e capaz de hidrolisar diferentes ésteres de Ch. A elevada eficiência catalítica dessas enzimas garante o término adequado do estímulo colinérgico e a manutenção da homeostase do sistema nervoso. Em razão de seu papel central na regulação da neurotransmissão, as ChEs constituem importantes alvos moleculares tanto para o desenvolvimento de antídotos contra intoxicações por compostos OPs quanto para estratégias terapêuticas voltadas ao tratamento de doenças neurodegenerativas, como a AD (COLOVIĆ *et al.*, 2013; SOREQ; SEIDMAN, 2001; SUN *et al.*, 2024).

Figura 1- Neurotransmissão colinérgica mediada pela AChE.



Fonte: Petronilho *et al.* (2011).

A modulação da atividade das ChEs pode produzir efeitos fisiológicos distintos, dependendo do grau e da natureza da interação com essas enzimas. A inibição quase irreversível causada por compostos OPs promove o acúmulo excessivo de ACh nas sinapses e junções neuromusculares, resultando na hiperestimulação dos receptores colinérgicos e em graves quadros de neurotoxicidade (CHEN *et al.*, 2024; EDDLESTON *et al.*, 2008; IYER; IKEN; LEON, 2015). Por outro lado, a inibição controlada da AChE tem sido empregada como estratégia terapêutica para aumentar a disponibilidade de ACh em doenças caracterizadas por déficit colinérgico, como a AD (BARTUS *et al.*, 1982; COLOVIĆ *et al.*, 2013; HAMPEL *et al.*, 2018). Dessa forma, tanto a reativação quanto a inibição das ChEs representam abordagens de grande interesse científico e farmacológico para o desenvolvimento de antídotos contra intoxicações por compostos OPs e de novos agentes terapêuticos (CHAMBERS *et al.* 2020; COLOVIĆ *et al.*, 2013; WOREK *et al.*, 2016).

3. 2 Colinesterases humanas

As *HssChEs* constituem uma família de enzimas fundamentais para o funcionamento do sistema colinérgico, atuando na regulação da neurotransmissão por meio da hidrólise da ACh e de outros ésteres de Ch. Essas enzimas são amplamente distribuídas no organismo e desempenham papel essencial na manutenção da homeostase colinérgica. A função dessas enzimas é promover o término adequado da transmissão sináptica, evitando assim a estimulação excessiva dos receptores colinérgicos. Em humanos, as ChEs são classificadas em duas subclasses principais: a acetilcolinesterase (AChE, EC 3.1.1.7) e a butirilcolinesterase (BChE, EC 3.1.1.8). A AChE é a principal enzima responsável pela hidrólise da ACh nas sinapses colinérgicas, estando presente em elevadas concentrações nas junções neuromusculares, nos

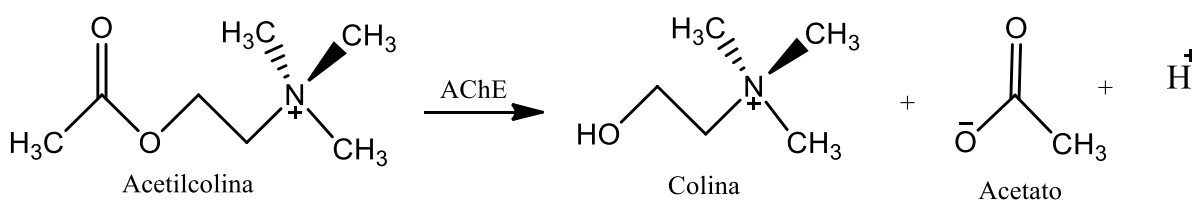
eritrócitos e em diversas regiões do SNC e SNP (CHATONNET e LOCKDRIDE, 1989; COLOVIĆ *et al.*, 2013; KUTTY, 1980; LUSHCHEKINA; MASSON, 2018; SUN *et al.*, 2024).

Diferentemente da AChE, a BChE apresenta maior versatilidade quanto aos substratos que pode hidrolisar, atuando não apenas sobre a ACh, mas também sobre outros ésteres de Ch, como a butirilcolina (BCh) (COLOVIĆ *et al.*, 2013; DIRAK; CHAN; KOLEMEN, 2024; LOCKRIDGE, 2015). Em função de sua ampla distribuição tecidual e de seu perfil catalítico, a BChE participa de diversos processos fisiológicos relacionados à modulação da neurotransmissão colinérgica (COLOVIĆ *et al.*, 2013; DARVESH; HOPKINS; GEULA, 2003; LOCKRIDGE, 2015; MESULAM *et al.*, 2002). Dessa forma, a AChE e a BChE constituem importantes alvos farmacológicos e toxicológicos, despertando crescente interesse no desenvolvimento de novos agentes terapêuticos e reativadores enzimáticos (COLOVIĆ *et al.*, 2013; LOCKRIDGE, 2015; MERCEY *et al.*, 2012; SVOBODOVÁ; SOUKUP; KORABECNÝ, 2026). Entre essas enzimas, a AChE destaca-se por seu papel central na neurotransmissão colinérgica e por sua relevância em processos patológicos e terapêuticos (COLOVIĆ *et al.*, 2013; FERREIRA-VIEIRA *et al.*, 2016; YU; LANE; LIN, 2021).

3. 2. 1 Acetilcolinesterase

A *HssAChE* é uma hidrolase de serina cujo papel biológico principal é interromper rapidamente a transmissão do impulso nervoso nas sinapses colinérgicas, através da hidrólise da ACh em Ch e acetato (FIGURA 2) (COLOVIĆ *et al.*, 2013; DOS SANTOS *et al.*, 2007; QUINN, 1987; SILMAN; SUSSMAN, 2005; TAYLOR; BROWN, 1994).

Figura 2- Hidrólise da ACh pela AChE.



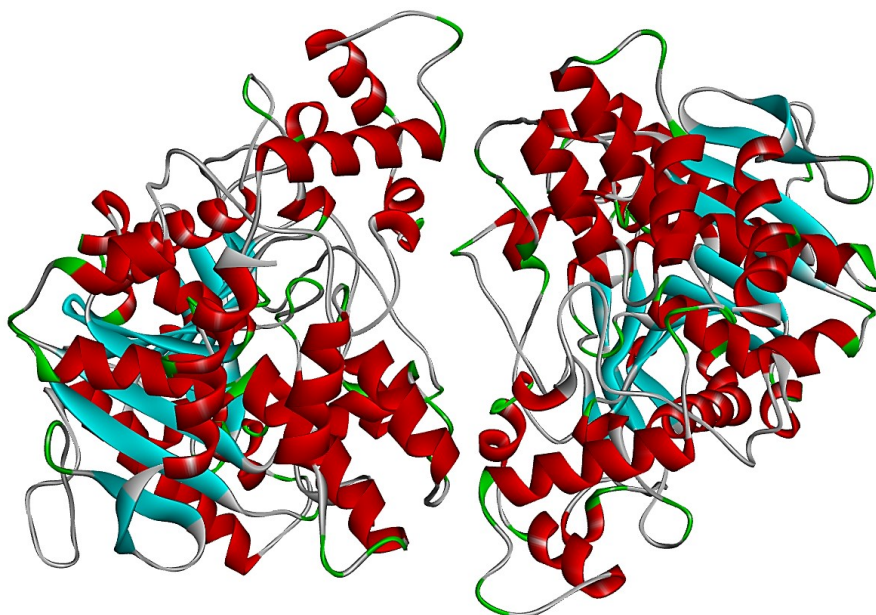
Fonte: Adaptado de Dos Santos *et al.* (2007).

Além de sua função clássica na neurotransmissão, evidências indicam que a AChE também desempenha funções não colinérgicas, participando de processos biológicos importantes, como adesão celular, diferenciação celular, proliferação celular e desenvolvimento tecidual. Essas funções adicionais sugerem que a enzima exerce papéis fisiológicos mais

amplios do que aqueles tradicionalmente associados à neurotransmissão colinérgica (LUO *et al.*, 2021; SILMAN, 2021; SOREQ; SEIDMAN, 2001; TSIM; SOREQ, 2013).

A AChE possui uma estrutura de formato elipsoidal (FIGURA 3), com dimensões aproximadas de $45 \text{ \AA} \times 60 \text{ \AA} \times 65 \text{ \AA}$. Seu monômero pertence ao tipo α/β , apresentando uma folha β central mista composta por 12 fitas β , que é circundada por 14 hélices α . Um aspecto estrutural bastante característico é a presença de um “*gorge*” profundo e estreito, com cerca de $20 \text{ \AA}$ de extensão, que se projeta para o interior da enzima até cerca de metade de sua profundidade e se amplia na região mais basal (COLOVIĆ *et al.*, 2013; HUNG *et al.*, 2025; MANAVALAN; TAYLOR; JOHNSON Jr., 1985). O sítio catalítico localiza-se aproximadamente a $4 \text{ \AA}$ do fundo dessa cavidade e é organizado em dois subsítios principais: o subsítio aniônico e o subsítio esterásico (COLOVIĆ *et al.*, 2013; HUNG *et al.*, 2025; NACHMANSOHN; WILSON, 1951). O primeiro é responsável pelo reconhecimento de substratos catiônicos, por meio de múltiplas interações com um conjunto de 14 resíduos aromáticos que revestem o *gorge*, todos altamente conservados evolutivamente; entre eles, o triptofano 84 (Trp84) se destaca por sua importância funcional, já que sua mutação para alanina pode provocar uma queda de cerca de 3000 vezes na atividade enzimática (COLOVIĆ *et al.*, 2013; HUNG *et al.*, 2025; ORDENTLICH *et al.*, 1995; TOUGU, 2001). O subsítio esterásico, por sua vez, é onde ocorre a hidrólise da ACh, e contém a tríade catalítica formada pelos resíduos serina (Ser203), histidina (His447) e glutamato (Glu334). Essa organização estrutural é responsável por sua notável eficiência catalítica na hidrólise da ACh, bem como por sua elevada suscetibilidade à ação de inibidores e agentes neurotóxicos (COLOVIĆ *et al.*, 2013; HUNG *et al.*, 2025).

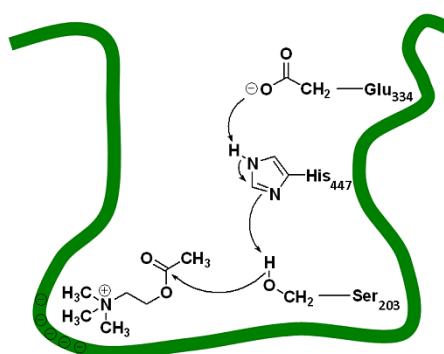
Figura 3- Estrutura tridimensional da *HssAChE*.



Fonte: Do autor (2026).

Durante o processo catalítico, a Ser203 atua como nucleófilo, promovendo o ataque ao grupo éster do substrato, enquanto a His447 e a Glu334 participam de uma rede de transferência de prótons que estabiliza e favorece a reação. A ação coordenada desses resíduos confere à enzima sua elevada eficiência catalítica, permitindo a rápida degradação da ACh e o término da neurotransmissão colinérgica (FIGURA 4) (HUNG *et al.*, 2025; PETRONILHO *et al.*, 2011; SILMAN; SUSSMAN, 2005).

Figura 4- Mecanismo de hidrólise da ACh no sítio ativo da *HssAChE*.



Fonte: Petronilho *et al.* (2011).

Embora a AChE seja a principal enzima responsável pela hidrólise da ACh nas sinapses colinérgicas, outra ChE de relevância fisiológica e farmacológica é a BChE. Apesar de compartilhar características estruturais e mecânicas com a AChE, a BChE apresenta

diferenças quanto à distribuição tecidual, especificidade por substratos e funções biológicas (HUNG *et al.*, 2025; LOCKRIDGE, 2015; SILMAN, 2021).

3. 2. 2 Butirilcolinesterase

A *HssBChE* está amplamente distribuída nos tecidos humanos, mas suas funções fisiológicas ainda não estão completamente elucidadas. A enzima contribui para a transmissão colinérgica e participa do metabolismo de lipídios e da grelina. Ela apresenta grande importância farmacológica e toxicológica devido à sua capacidade de hidrolisar diversos ésteres e neutralizar compostos tóxicos. Evidências indicam que a *HssBChE* pode atuar como enzima acessória no metabolismo de colinésteres, apresentando contribuição variável dependendo do tecido e do contexto fisiológico, o que reforça seu potencial envolvimento em diferentes processos biológicos (DARVESH *et al.*, 2003; HAJIMOHAMMADI; LOCKRIDGE; MASSON, 2025; LOCKRIDGE, 2016; SILMAN, 2021).

A BChE apresenta atividade hidrolítica sobre a ACh e outros ésteres de Ch, contribuindo para a modulação da sinalização colinérgica em diferentes contextos fisiológicos. Embora a AChE seja a principal enzima responsável pela degradação da ACh nas sinapses colinérgicas, a BChE pode atuar como uma enzima acessória, apresentando participação variável na regulação dos níveis de ACh de acordo com o tecido e o estado fisiológico. Essa capacidade de atuar sobre diferentes substratos colinérgicos confere à BChE relevância funcional em processos neuroquímicos e periféricos, ainda que seu papel fisiológico exato permaneça menos definido quando comparado ao da AChE (FREITAS; PAZ; CASTILHO, 2009; GOULART *et al.*, 2021; HAJIMOHAMMADI; LOCKRIDGE; MASSON, 2025; SILMAN, 2021).

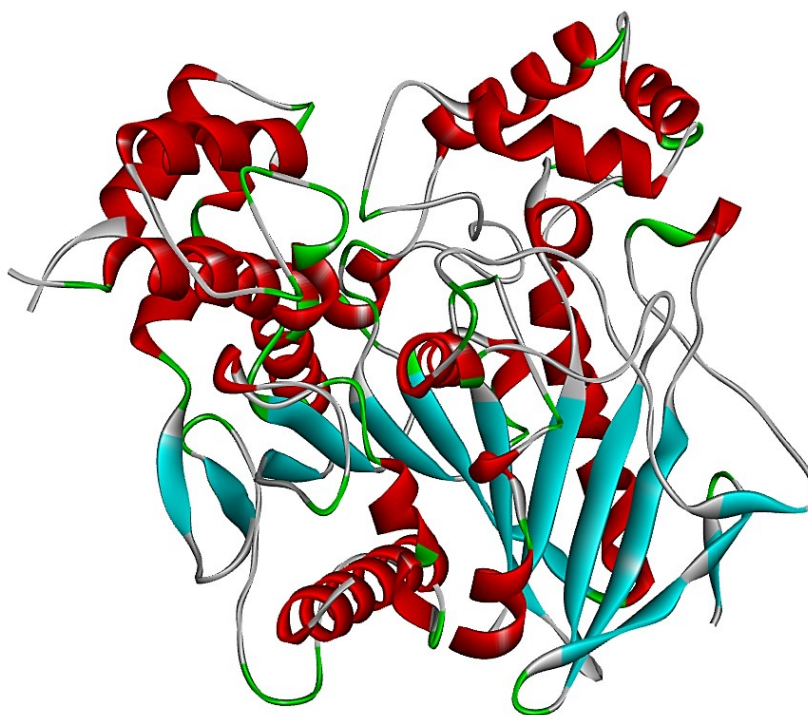
Uma característica relevante das *HssChEs* é a presença de diferenças estruturais entre a AChE e a BChE, especialmente em relação à composição dos resíduos de aminoácidos e ao volume da cavidade catalítica. Essas diferenças determinam a especificidade por substratos, a afinidade por inibidores e a cinética enzimática, além de desempenharem papel fundamental no planejamento racional de compostos bioativos em estudos de modelagem molecular (NICOLET *et al.*, 2003; SUN *et al.*, 2024; SUSSMAN *et al.*, 1991; ROSENBERRY *et al.*, 2017).

A *HssBChE*, também denominada ChE sérica ou pseudocolinesterase, é uma esterase sintetizada principalmente no fígado e liberada na corrente sanguínea. Apresenta meia-vida plasmática relativamente longa, variando entre 12 e 14 dias, o que contribui para sua estabilidade na circulação. No organismo humano, essa enzima possui ampla distribuição tecidual, sendo detectada em diferentes órgãos e tecidos, incluindo pele, pâncreas, substância

branca cerebral, coração, músculos lisos e tecido adiposo (FREITAS; PAZ; CASTILHO, 2009; GOULART *et al.*, 2021; LOCKRIDGE, 2015; SUN *et al.*, 2024).

A BChE é uma enzima tetramérica com massa molecular aproximada de 340 kDa, sendo constituída por quatro subunidades idênticas de cerca de 85 kDa cada. Estruturalmente, apresenta uma conformação tridimensional muito semelhante à da AChE, com um enovelamento do tipo α/β -hidrolase e um sítio ativo localizado no fundo de um *gorge* profundo e estreito. No entanto, diferenças na composição dos aminoácidos que revestem o *gorge* conferem à BChE uma cavidade catalítica mais ampla, permitindo a acomodação e a hidrólise de substratos de maior volume em comparação com a AChE. O sítio catalítico é organizado em diferentes subsítios funcionais. O centro catalítico é composto pela tríade catalítica formada pelos resíduos Ser198, His438 e Glu325, responsável pela hidrólise de ésteres de Ch. Na região de ligação do substrato, destaca-se o sítio aniônico, onde ocorre a interação com o grupo quaternário de amônio da Ch por meio de interações do tipo cátion- π . Além disso, o sítio de acilação contribui para a formação do intermediário acil-enzima durante o processo catalítico, enquanto o sítio acil, predominantemente hidrofóbico, modula o acesso do substrato ao sítio ativo e influencia a especificidade enzimática (FIGURA 5) (GIACOBINI, 2003; NICOLET *et al.*, 2003; ROSENBERRY *et al.*, 2017; SUN *et al.*, 2024).

Figura 5- Estrutura tridimensional da *HssBChE*.



Fonte: Do autor (2026).

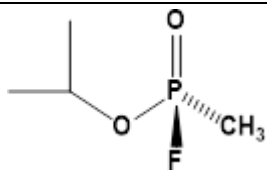
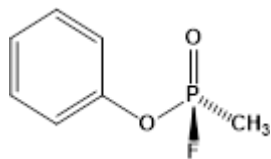
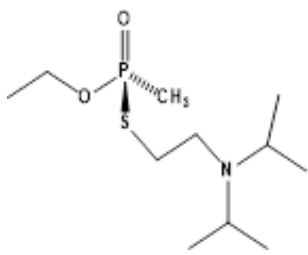
Em razão de suas características estruturais e catalíticas, a BChE também constitui um importante alvo dos agentes OPs. A inibição dessa enzima, juntamente com a da AChE, contribui para alterações na neurotransmissão colinérgica. A interação dos OPs com as ChEs apresenta relevância tanto para a compreensão dos mecanismos envolvidos na toxicidade desses compostos quanto para o desenvolvimento de estratégias destinadas à prevenção e ao tratamento da intoxicação. Assim, a compreensão das características dos OPs e de seus mecanismos de ação é fundamental para elucidar os efeitos decorrentes da exposição a esses agentes (DARVESH *et al.*, 2003; GIACOBINI, 2003; MASSON; NACHON, 2017; ROSENBERRY *et al.*, 2017; SUN *et al.*, 2024; WANG *et al.*, 2026).

3. 3 Organofosforados como agentes tóxicos

Os compostos OPs constituem uma classe de substâncias reconhecida por sua elevada toxicidade ao sistema nervoso. Estes compostos são amplamente utilizados na agricultura, na indústria e em outros setores, como na fabricação de plastificantes, componentes de medicamentos e materiais poliméricos. Na agricultura, destacam-se pelo controle eficiente de pragas, contribuindo para o aumento da produtividade e da qualidade dos produtos. Entretanto, seu uso extensivo está associado a riscos significativos à saúde humana e ao meio ambiente (BIGLEY; RAUSHEL, 2019; CHU *et al.*, 2026; EDDLESTON *et al.*; 2008; RAJAK *et al.*, 2021). Além das aplicações civis, alguns OPs foram desenvolvidos e utilizados como agentes de guerra química devido ao seu elevado potencial neurotóxico (MARRS; MAYNARD; SIDELL, 2007; NOGA; MICHALSKA; JUROWSKI, 2023; WOREK *et al.*, 2020). Em razão de sua ampla utilização, o risco de intoxicação é elevado, tornando essencial compreender seus efeitos tóxicos e sua participação no desenvolvimento e agravamento de condições patológicas (BELINSKAIA *et al.*, 2019; CHU *et al.*, 2026; LIMA, 2008; RAJAK *et al.*, 2021; VINHAL; SOARES, 2018).

Entre os principais agentes de guerra química, destacam-se o sarin, o ciclosarin e o VX (TABELA 1), que representam importantes ameaças à saúde pública e à segurança devido à sua elevada toxicidade. A compreensão detalhada dos mecanismos de ação desses agentes é essencial para o desenvolvimento de terapias eficazes e estratégias que possam minimizar seus efeitos tóxicos. Um dos principais alvos dessas substâncias são as ChEs, enzimas essenciais para a transmissão nervosa (MARRS; MAYNARD; SIDELL, 2007; NOGA; MICHALSKA; JUROWSKI, 2023; SILVA; JR; FIGUEROA-VILLAR, 2012; WOREK *et al.*, 2020).

Tabela 1. Estruturas químicas dos agentes de guerra química: sarin, ciclosarin e VX.

Agentes	Estruturas 2D
Sarin	
Ciclosarin	
VX	

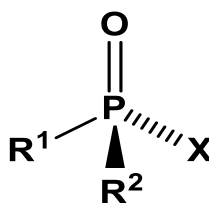
Fonte: Do autor (2026).

A toxicidade dos OPs decorre principalmente da inibição quase irreversível das ChEs, especialmente da AChE. Esses agentes ligam-se covalentemente ao resíduo de serina presente no sítio ativo da enzima, promovendo sua fosforilação e impedindo a hidrólise da ACh. Como a reativação espontânea da AChE é extremamente lenta, ocorre acúmulo de ACh na fenda sináptica, resultando em hiperestimulação dos receptores colinérgicos. Esse processo desencadeia manifestações clínicas como agitação, fraqueza muscular, fasciculações, miose, hipersalivação e sudorese. Nos casos mais graves, podem ocorrer insuficiência respiratória, confusão mental, convulsões, perda da consciência e evolução para óbito (ALDRIDGE; REINER, 1972; BELINSKAIA *et al.*, 2019; BOUBLIK *et al.*, 2002; COLOVIĆ *et al.*, 2013; EDDLESTON *et al.*; 2008; WOREK *et al.*, 2020).

Os compostos OPs são ésteres ou tioésteres derivados dos ácidos fosfórico, fosfônico, fosfinico ou fosforamídico. Em sua estrutura geral, o átomo de fósforo encontra-se ligado a dois substituintes orgânicos, denominados R1 e R2, frequentemente representados por grupos alquila ou arila, como metil ou etil (FIGURA 6). Além disso, apresenta um grupo de saída (X), que pode corresponder a diferentes substituintes químicos, incluindo halogênios, grupos

alifáticos ou aromáticos, o qual pode ser liberado durante reações de hidrólise. O fósforo também está ligado a um átomo de oxigênio por meio de uma ligação dupla (P=O), característica estrutural que contribui de forma significativa para a elevada reatividade química dessa classe de compostos (BAJGAR, 2004; BELINSKAIA *et al.*, 2019; COLOVIĆ *et al.*, 2013; KANEKAR *et al.*, 2004; SOGORB; VILANOVA, 2002; SMULDERS *et al.*, 2004; STODDART, 1979; WOREK *et al.*, 2020).

Figura 6- Representação estrutural geral dos compostos OPs.



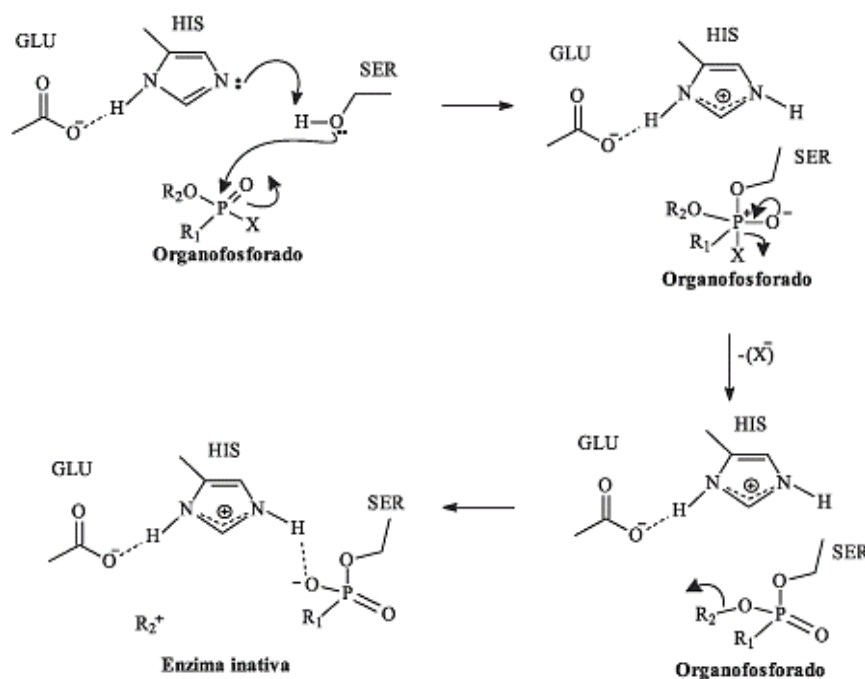
Fonte: Adaptado de Stoddart (1979).

3. 3. 1 Mecanismo de inibição das colinesterases por organofosforados

Como descrito anteriormente, os compostos OPs exercem sua principal ação tóxica por meio da inibição das enzimas ChEs. Este processo está associado à elevada reatividade do átomo de fósforo presente em sua estrutura. Essa interação com enzimas-chave do sistema colinérgico resulta em alterações significativas na neurotransmissão mediada pela ACh, comprometendo a comunicação sináptica. Como consequência, esses compostos estão diretamente relacionados ao desenvolvimento de quadros de neurotoxicidade, devido ao desequilíbrio funcional do sistema colinérgico (ABOU-DONIA, 2003; BELINSKAIA *et al.*, 2019; COLOVIĆ *et al.*, 2013; LIMA, 2008; RAJAK *et al.*, 2021; WOREK *et al.*, 2020).

O mecanismo de inibição (FIGURA 7) inicia-se com o ataque nucleofílico do oxigênio do grupo hidroxila do resíduo de serina catalítica (Ser203), que se liga ao átomo de fósforo eletrofílico do OP. Essa interação resulta na formação de um complexo fosforilado covalente entre o OP e a enzima, estabelecendo a ligação OP e a AChE ou BChE e promovendo o bloqueio do sítio ativo (COLOVIĆ *et al.*, 2013; GUPTA *et al.* 2023; WOREK *et al.*, 2020). Como consequência imediata desse processo, ocorre a perda da atividade catalítica das ChEs, impedindo a hidrólise da ACh. Esse bloqueio enzimático leva ao comprometimento da terminação da sinalização colinérgica, caracterizando o efeito tóxico inicial dos OPs sobre o sistema nervoso (ARONIADOU-ANDERJASKA *et al.*, 2023; CAVALCANTI *et al.*, 2016; COLOVIĆ *et al.*, 2013; EDDLESTON *et al.*, 2008).

Figura 7- Mecanismo geral de inibição da *HssAChE* por compostos OPs.



Fonte: Adaptado de Colović *et al.* (2013).

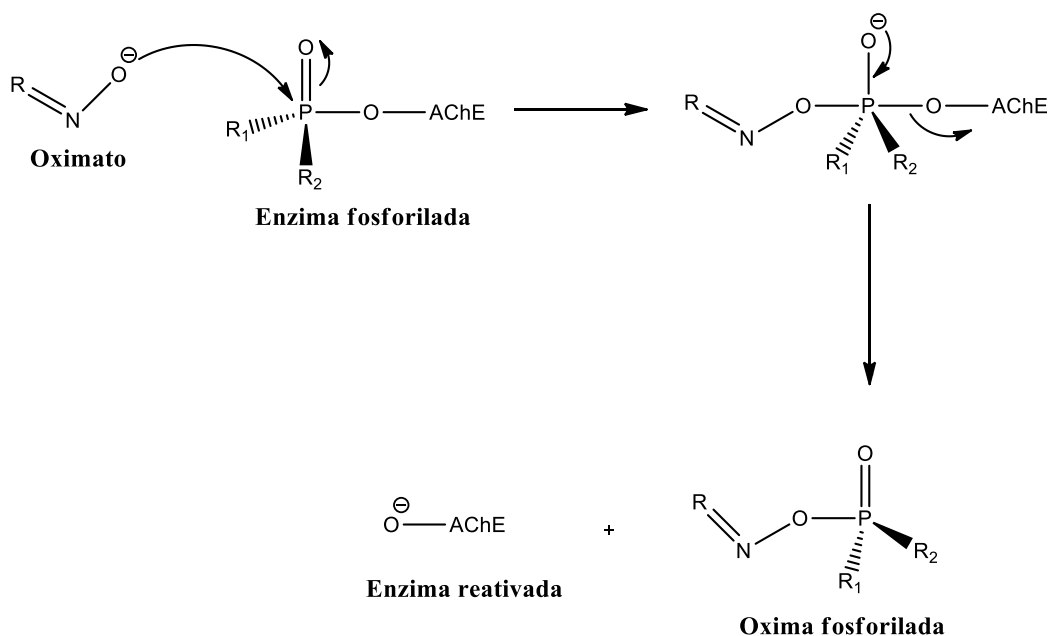
3. 3. 2 Processo de envelhecimento

O processo de envelhecimento (em inglês, *aging*) corresponde a uma modificação química progressiva do complexo enzima–OP após a inibição das ChEs. Nesse processo, ocorre a desalquilação de um dos grupos alquila ligados ao átomo de fósforo, resultando em uma reorganização estrutural que aumenta significativamente a estabilidade da ligação covalente entre o fósforo do OP e o oxigênio do resíduo de serina presente no sítio catalítico (MASSON; NACHON, 2017; MILLARD *et al.*, 1999; WOREK *et al.*, 2016). Como consequência, a reativação da enzima por oximas torna-se inviável, tornando a inibição irreversível e fazendo com que a recuperação da atividade enzimática dependa da síntese de novas moléculas de ChE (QUINN, 1987; SAXENA *et al.*, 2009; WOREK *et al.*, 2016). Dessa forma, o processo de envelhecimento representa um fator determinante na perda da capacidade de reativação das ChEs, constituindo uma etapa crítica na toxicidade dos compostos OPs. Como a reativação é possível apenas antes da ocorrência desse processo, o conhecimento dos mecanismos envolvidos na reativação das ChEs é essencial para o desenvolvimento de estratégias terapêuticas eficazes (EDDLESTON *et al.*, 2008; MASSON; NACHON, 2017; MILLARD *et al.*, 1999; WOREK *et al.*, 2016).

3.3.3 Reativação das colinesterases por oximas

As oximas são agentes nucleofílicos capazes de reativar as ChEs inibidas por compostos OPs, desde que o processo de envelhecimento do complexo enzima–OP ainda não tenha ocorrido. Nessa condição, o complexo permanece suscetível ao ataque nucleofílico da oxima, que promove a clivagem da ligação entre o grupo fosforilado e o resíduo de serina presente no sítio ativo da enzima, restaurando sua atividade catalítica (CHAMBERS; MEEK, 2021; MASSON; NACHON, 2017; MATOS, 2012; MILLARD *et al.*, 1999; WOREK *et al.*, 2016). Esse mecanismo permite a recuperação da atividade das ChEs e, conseqüentemente, contribui para o restabelecimento da neurotransmissão colinérgica. Entretanto, a eficácia das oximas depende da estrutura química do OP responsável pela inibição, uma vez que determinados reativadores apresentam elevada atividade frente a alguns agentes, mas baixa ou nenhuma eficácia contra outros. Essa especificidade limita sua aplicação clínica e reforça a necessidade do desenvolvimento de oximas de amplo espectro, capazes de reativar as ChEs inibidas por diferentes compostos OPs (CHAMBERS; DAIL; MEEK, 2020; MATOS, 2012; WOREK; THIERMANN; WILLE, 2020).

Figura 8- Mecanismo de reativação da AChE.



Fonte: Adaptado de Delfino; Figueroa-Villar (2009).

Na Figura 8, ilustra-se o mecanismo geral de reativação da AChE mediado por oximas. Inicialmente, a enzima encontra-se fosforilada em decorrência da reação entre um composto

OP e o grupo hidroxila da serina presente no sítio catalítico, formando uma ligação covalente estável do tipo fósforo–oxigênio. Essa modificação impede que a serina participe da hidrólise da ACh, levando ao acúmulo do neurotransmissor na fenda sináptica e à hiperestimulação dos receptores colinérgicos (CHAMBERS; DAIL; MEEK, 2020; DELFINO; FIGUEROA-VILLAR, 2009; WONG *et al.*, 2000; WOREK *et al.*, 2007).

Como já mencionado, as oximas atuam como agentes nucleofílicos devido à presença do grupo funcional oxima (CHAMBERS; MEEK, 2021; MATOS, 2012). Em pH fisiológico, uma fração dessas moléculas encontra-se na forma desprotonada, espécie que apresenta elevada densidade eletrônica sobre o átomo de oxigênio e, consequentemente, maior nucleofilicidade. Após o reconhecimento do complexo enzima–OP, a oxima posiciona-se no interior do sítio ativo da AChE, aproximando o oxigênio nucleofílico do centro eletrofílico representado pelo átomo de fósforo do grupo fosforila (CHAMBERS; DAIL; MEEK, 2020; DELFINO; FIGUEROA-VILLAR, 2009; WONG *et al.*, 2000; WOREK *et al.*, 2007).

O mecanismo de reativação ocorre por meio de uma reação de substituição nucleofílica bimolecular (S_N2). O ânion oximato realiza um ataque ao átomo de fósforo, formando um intermediário pentacoordenado, característico das reações de substituição em compostos OPs. Esse intermediário é instável e sofre colapso com a clivagem da ligação fósforo–oxigênio estabelecida entre o OP e o resíduo de serina da enzima. Como consequência, a serina recupera seu grupo hidroxila livre, restaurando o sítio catalítico da AChE, enquanto o grupo fosforila permanece ligado à oxima, originando um complexo fosforil-oxima, que posteriormente pode sofrer hidrólise ou ser eliminado do organismo. A eficiência desse processo depende de diversos fatores estruturais e cinéticos, incluindo a nucleofilicidade da oxima, sua capacidade de alcançar o sítio ativo da enzima, a orientação espacial adequada para o ataque nucleofílico e a natureza química do OP responsável pela inibição (CHAMBERS; DAIL; MEEK, 2020; DELFINO; FIGUEROA-VILLAR, 2009; WONG *et al.*, 2000; WOREK *et al.*, 2007).

Diversas oximas têm sido desenvolvidas e avaliadas como reativadores das ChEs inibidas por compostos OPs, como o sarin, ciclosarin e VX, apresentando diferenças estruturais que influenciam sua afinidade pela enzima, capacidade de reativação e espectro de ação frente a diferentes agentes anticolinesterásicos (WOREK *et al.*, 2020). Na tabela 2, estão as estruturas químicas das oximas que serviram como base para os estudos de interação molecular e reativação das ChEs.

Tabela 2. Estruturas químicas das oximas.

Oximas	Estruturas 2D	Fórmula Molecular
1		$C_{15}H_{18}Cl_2N_4O_2$
2		$C_{15}H_{18}Cl_2N_4O_2$
3		$C_{15}H_{18}Cl_2N_4O_2$
4		$C_{15}H_{16}Cl_4N_4O_2$
5		$C_{15}H_{18}F_2N_4O_2$
6		$C_{15}H_{18}Br_2N_4O_2$
7		$C_{15}H_2ON_4O_2$
8		$C_{15}H_2ON_4O_2$
9		$C_{15}H_2ON_4O_2$

Fonte: Do autor (2026).

3.4 Organofosforados como agentes terapêuticos

Embora os compostos OPs sejam amplamente reconhecidos por sua toxicidade e frequentemente associados a pesticidas e agentes neurotóxicos, diversos representantes dessa classe química também apresentam potencial terapêutico (BIGLEY; RAUSHEL, 2019; CHANG *et al.*, 2017; DEMKOWICZ *et al.*, 2016; RAJAK *et al.*, 2021). Estudos recentes destacam que alguns OPs têm sido utilizados no tratamento de diferentes patologias e que, quando racionalmente projetados, administrados e avaliados, podem constituir importantes ferramentas farmacológicas. Na AD, a hipótese colinérgica sugere que o aumento dos níveis de ACh no SNC pode melhorar a função cognitiva (ATTA *et al.*, 2025; CONTESTABILE, 2011; FRANCIS *et al.*, 1999; SAHA *et al.*, 2024; SINGH; PRASAD; KUMAR, 2024). Os compostos OPs capazes de promover uma inibição parcial da AChE vêm sendo investigados como potenciais agentes terapêuticos, uma vez que a redução da atividade dessa enzima aumenta a disponibilidade de ACh nas sinapses, favorecendo a neurotransmissão colinérgica e podendo contribuir para a melhora dos sintomas cognitivos. Apesar da conhecida toxicidade associada a essa classe de compostos, evidências demonstram que os compostos OPs também podem desempenhar papel relevante na química medicinal, desde que seu desenvolvimento e aplicação sejam cuidadosamente controlados (AHLMANN; HEMPEL, 2016; DELFINO; FIGUEROA-VILLAR, 2009; EMADI; JONES; BRODSKY, 2009; GARDINER *et al.*, 2019; SCHMIDT *et al.*, 2010).

3.4.1 Doença de Alzheimer

A AD é uma enfermidade neurodegenerativa progressiva e irreversível de instalação insidiosa. Esta é a principal causa de demência, predominante na população idosa (SAMPAIO *et al.*, 2025, SMITH, 1999). Caracteriza-se por um declínio gradual das funções cognitivas, incluindo memória, reconhecimento de pessoas e espaços, além de prejuízos na realização de atividades diárias, afetando de forma significativa a autonomia e a qualidade de vida do paciente (HUO *et al.*, 2022; SAMPAIO *et al.*, 2025).

Do ponto de vista neuropatológico, a doença é marcada por alterações estruturais específicas no SNC. Entre as principais características estão a deposição extracelular de placas senis compostas por peptídeo β -amiloide ($A\beta$) e a formação intracelular de emaranhados neurofibrilares constituídos por proteína tau hiperfosforilada. Essas alterações estão associadas à disfunção sináptica, estresse oxidativo, neuroinflamação e perda progressiva de neurônios,

especialmente em regiões como o hipocampo e o córtex cerebral, áreas fundamentais para os processos de memória e aprendizagem (ABYADEH *et al.*, 2024; KINNEY *et al.*, 2018; MACHADO; CARVALHO; SOBRINHO, 2020).

Embora sua etiologia não seja totalmente compreendida, a AD é uma patologia multifatorial e de causa idiopática, e fatores genéticos e ambientais podem influenciar na sua progressão (BREIJYEH; KARAMAN, 2020; CÂMARA, 2019; LANE; HARDY; SCHOTT, 2018). Entre as principais hipóteses fisiopatológicas, destaca-se a hipótese colinérgica, que relaciona parte dos sintomas cognitivos à redução da neurotransmissão colinérgica no cérebro, especialmente devido à morte ou comprometimento da função dos neurônios liberadores e produtores da ACh. Esse neurotransmissor possui papel fundamental nos processos de memória, concentração, aprendizagem e demais funções cognitivas (DALMAGRO; CAZARIN; ZENAIDE, 2020; FALCO *et al.*, 2016; FERREIRA-VIEIRA *et al.*, 2016).

Atualmente, os tratamentos disponíveis não são capazes de reverter a doença. Os mesmos são voltados principalmente para o controle sintomático e tentativa de retardar a progressão da doença (LANE; HARDY; SCHOTT, 2018). O entendimento dos mecanismos neuropatológicos da AD é fundamental para o desenvolvimento de novas abordagens terapêuticas, especialmente aquelas voltadas à modulação do sistema colinérgico (COELHO FILHO; BARBOSA, 2023; KANDY *et al.*, 2026; SREE *et al.*, 2025; YU; LANE; LIN, 2021).

3.4.2 Sistema colinérgico e doença de Alzheimer

O sistema de neurotransmissão colinérgica é constituído pela ACh, seus receptores e o aparato enzimático responsável por sua síntese e degradação. A ACh é um mediador químico de sinapses no SNC, bem como no SNP e junção neuromuscular (BRUNEAU; AKAABOUNE, 2006; VENTURA *et al.*, 2010). Esse sistema é constituído por neurônios colinérgicos amplamente distribuídos no cérebro humano e desempenham papel fundamental nos processos cognitivos. A transmissão dos sinais colinérgicos envolvidos na memória e na aprendizagem depende da ação da ACh. Por meio da ativação de receptores nicotínicos e muscarínicos, esse neurotransmissor participa da modulação da atividade neuronal e da comunicação entre diferentes circuitos cerebrais (FUJII; TAKADA-TAKATORI; KAWASHIMA, 2008; FUKUNAGA; YABUKI, 2018; LI *et al.*, 2020; MING; WANG; GENTILE, 2024).

Na AD, alterações no sistema colinérgico estão entre os principais mecanismos associados ao comprometimento cognitivo. A perda progressiva de neurônios colinérgicos do prosencéfalo basal, especialmente daqueles que compõem o núcleo basal de Meynert, leva à

redução da neurotransmissão colinérgica em regiões cerebrais envolvidas com memória e atenção. Como consequência, ocorre diminuição da disponibilidade de ACh, contribuindo para os déficits cognitivos característicos da doença. Embora a AD seja marcada pelo acúmulo de placas amiloides e emaranhados neurofibrilares, evidências indicam que a perda sináptica e a disfunção colinérgica apresentam forte associação com a progressão do declínio cognitivo observado nos pacientes (BARTUS *et al.*, 1982; FERREIRA-VIEIRA *et al.*, 2016).

A disfunção do sistema colinérgico desempenha papel central na fisiopatologia da AD, justificando o emprego de estratégias farmacológicas voltadas ao aumento da neurotransmissão colinérgica. Entre essas, destacam-se os inibidores da AChE, que elevam os níveis cerebrais de ACh e promovem melhora temporária dos sintomas cognitivos, sem modificar a evolução da doença (KANDY *et al.*, 2026; VENTURA *et al.*, 2010).

3.4.3 Fármacos anticolinesterásicos no tratamento da doença de Alzheimer

Os anticolinesterásicos ou inibidores das ChEs são fármacos que constituem a principal classe terapêutica utilizada no tratamento sintomático da AD em estágios leves a moderados. Esses medicamentos estimulam a transmissão colinérgica indiretamente inibindo a AChE, que hidrolisa e inativa a ACh nas fendas sinápticas do SNC. Assim, os agentes dessa classe prolongam a permanência da ACh na fenda sináptica, aumentando sua disponibilidade para interação com os receptores pós-sinápticos e, consequentemente, melhorando a neurotransmissão colinérgica (COLOVIĆ *et al.*, 2013; KANDY *et al.*, 2026; TAYLOR, 2018).

Os anticolinesterásicos podem ser classificados em inibidores reversíveis e irreversíveis das ChEs, de acordo com o mecanismo de interação com a enzima. Os inibidores reversíveis incluem compostos que se ligam ao sítio ativo da AChE por meio de interações não covalentes, como a donepezila e a galantamina, bem como carbamatos, como a fisostigmina, a neostigmina e a rivastigmina, que promovem a carbamilação reversível da enzima. Nesses casos, a atividade enzimática é restabelecida após a dissociação do complexo enzima–inibidor ou pela hidrólise espontânea do grupo carbamilado. Em contrapartida, os inibidores irreversíveis, representados principalmente pelos compostos OPs, promovem a fosforilação do resíduo de serina presente no sítio catalítico da AChE, formando um complexo covalente altamente estável. Essa ligação pode tornar-se progressivamente mais resistente à hidrólise em decorrência do processo de envelhecimento, resultando em inibição prolongada ou permanente da atividade enzimática (COLOVIĆ *et al.*, 2013; MASSON; NACHON, 2017; MILLARD *et al.*, 1999; TAYLOR, 2018; WOREK *et al.*, 2016).

Os principais fármacos anticolinesterásicos utilizados clinicamente incluem a donepezila, a rivastigmina e a galantamina (BOND *et al.*, 2012; COLOVIĆ *et al.*, 2013; HANSEN *et al.*, 2008). A donepezila atua como um inibidor da AChE de ação central, rápida e reversível. A rivastigmina, por sua vez, inibe tanto a AChE quanto a BChE, atuando como um inibidor não competitivo e apresentando ação pseudoirreversível (de reversão lenta). Já a galantamina é um inibidor seletivo, competitivo e reversível da AChE, atuando também como potencializador alostérico dos receptores nicotínicos de ACh (KANDIAH *et al.*, 2017; RATHI, 2021; SELTZER, 2005).

Os anticolinesterásicos atuam na disfunção neuronal secundária à patologia e ajudam a estabilizar ou a retardar o declínio cognitivo, funcional e comportamental. Não existem estratégias capazes de reverter o declínio cognitivo ou impedir a progressão da doença; os recursos atualmente disponíveis são medicamentos sintomáticos que visam retardar sua evolução (PEREIRA; RODRIGUES, 2015; TAN *et al.*, 2014). As evidências sobre a eficácia dos anticolinesterásicos a longo prazo são escassas, e os resultados ainda não são suficientes para esclarecer, de forma definitiva, o uso ou não desses medicamentos em fases avançadas da doença (OLAZARÁN *et al.*, 2013; PEREIRA; RODRIGUES, 2015). Dessa forma, os anticolinesterásicos permanecem como uma terapia consolidada, porém de eficácia limitada, o que justifica a busca por novas opções terapêuticas com maior seletividade, melhor perfil de segurança e potencial para modificar a progressão da doença (PEREIRA; RODRIGUES, 2015; TAN *et al.*, 2014).

Baseado na hipótese colinérgica, alguns compostos OPs de baixa toxicidade são investigados como potenciais agentes terapêuticos devido à sua capacidade de inibir a AChE de forma prolongada. Estudos mostram que o metrifonato destaca-se por apresentar resultados promissores em estudos clínicos, com melhora modesta da função cognitiva em pacientes com AD. Entretanto, preocupações relacionadas à segurança e aos efeitos colinérgicos adversos impedem sua utilização clínica. Apesar dessas limitações, esses estudos contribuem para ampliar a compreensão da relação entre estrutura química, inibição enzimática e atividade farmacológica, fornecendo subsídios para o desenvolvimento de novos inibidores da AChE com maior seletividade e melhor perfil de segurança (COLOVIĆ *et al.*, 2013; GIOVANNINI *et al.*, 1998; LOPEZ-ARRIETA; SCHNEIDER, 2006; SINGH *et al.*, 2013). Sendo assim, a aplicação de métodos computacionais tem se mostrado uma estratégia relevante para investigar, em nível molecular, as interações entre compostos bioativos e seus alvos enzimáticos, permitindo avaliar propriedades estruturais, eletrônicas, energéticas e farmacológicas e, assim, auxiliar na

identificação e no direcionamento racional de potenciais candidatos a fármacos (KANDY *et al.*, 2026).

3.5 Métodos computacionais

A disponibilidade dos programas computacionais de química e os bancos de dados em rede são, atualmente, ferramentas fundamentais para a descoberta e o *design* de novos fármacos. Essas ferramentas permitem avaliar, de forma rápida e econômica, propriedades estruturais e físico-químicas de uma série de moléculas de interesse, contribuindo para a compreensão de suas interações com alvos terapêuticos específicos. Os modelos moleculares gerados pelo computador são resultantes de equações matemáticas que estimam as posições e propriedades dos elétrons e núcleos. Os cálculos realizados exploram as características de uma estrutura, fornecendo uma molécula sob nova perspectiva. Entre as diversas ferramentas computacionais disponíveis, destacam-se o *docking* molecular, os cálculos QM/MM e as simulações de MD, que permitem avaliar os modos de interação entre ligantes e biomoléculas, investigar mecanismos de reação e analisar a estabilidade estrutural e dinâmica dos complexos formados. Essas abordagens são fundamentadas em diferentes níveis de descrição molecular, sendo a Mecânica Molecular (MM, do inglês Molecular Mechanics) particularmente importante para a investigação de sistemas biomoleculares, uma vez que permite descrever a estrutura e as interações moleculares por meio de modelos baseados em campos de força (CARVALHO *et al.*, 2003; MANCINI, 2011; SLIWOSKI *et al.*, 2014; YU *et al.*, 2026).

3.5.1 Mecânica Molecular

A modelagem molecular consiste em um conjunto de métodos computacionais destinados a representar e prever o comportamento de moléculas e sistemas moleculares. Com o avanço dos recursos computacionais, essas técnicas passaram a desempenhar um papel fundamental na química, permitindo a investigação de estruturas, propriedades e interações moleculares. Entre essas abordagens, destaca-se a MM, que utiliza modelos baseados na mecânica clássica para descrever sistemas moleculares por meio de funções matemáticas capazes de representar as interações entre os átomos (CRAMER 2013; JENSEN 2017; LEACH, 2001).

Devido ao menor custo computacional em comparação aos métodos baseados na mecânica quântica, a MM permite o estudo de sistemas contendo um grande número de átomos,

sendo amplamente empregada em química computacional, bioquímica e ciência dos materiais. Entre suas principais aplicações estão a otimização geométrica de moléculas, estudos conformacionais, simulações de MD e investigação de interações intermoleculares. Entretanto, por utilizar uma descrição clássica dos átomos, essa metodologia apresenta limitações em processos que envolvem alterações na estrutura eletrônica, como formação e quebra de ligações químicas, sendo frequentemente combinada com métodos quânticos em abordagens híbridas, como QM/MM. No contexto da investigação das interações intermoleculares, os métodos baseados em MM também fundamentam abordagens de *docking* molecular, utilizadas para prever os modos de ligação e avaliar a afinidade de ligantes em sítios específicos de proteínas, constituindo uma importante ferramenta na investigação computacional de sistemas biomoleculares (CRAMER 2013; JENSEN 2017; LEACH, 2001).

3.5.1.1 *Docking* molecular

A MM é uma das principais abordagens dos métodos clássicos teóricos (COELHO *et al.*, 1999). Esse método permite modificar os ângulos e comprimentos das ligações entre os átomos de uma molécula, gerando novas conformações e calculando as energias associadas a essas alterações. No contexto do *docking* molecular, a MM é utilizada para investigar as interações entre um ligante e um receptor, identificando conformações energeticamente favoráveis e as principais interações intermoleculares envolvidas. O *docking* molecular explora diferentes orientações e conformações do ligante no sítio de ligação, buscando arranjos estruturais mais estáveis e com menor energia de interação. O objetivo do *docking* molecular, associado aos métodos de MM, é prever os modos de interação entre o ligante e o alvo molecular, fornecendo informações sobre a orientação do ligante no sítio ativo da enzima, as interações intermoleculares envolvidas e a estabilidade relativa dos complexos formados (CARVALHO, 2003; PATRICK; FLANAGAN, 2001).

O ancoramento molecular (em inglês, *molecular docking*) é um processo que indica a conformação do ligante e a sua orientação dentro do sítio ativo da enzima. Para compreender os mecanismos de inibição ou reativação enzimática, é necessário conhecer a posição tridimensional do ligante durante a interação molecular com a enzima, bem como sua geometria e as principais interações envolvidas no reconhecimento molecular (IWATA *et al.*, 2000; MANCINI, 2011; MIZUTANI; NIWA; TANAKA, 1994).

A simulação computacional por *docking* molecular é uma técnica muito utilizada para investigar as interações entre ligantes e enzimas a partir de suas estruturas tridimensionais. Por

meio dessa abordagem, diferentes conformações e orientações do ligante são geradas e avaliadas, permitindo identificar os modos de interação mais favoráveis e os principais resíduos envolvidos no reconhecimento molecular. As estruturas experimentais utilizadas nesses estudos são geralmente obtidas por técnicas como difração de raios x ou espectroscopia de ressonância magnética nuclear (RMN). Devido às dificuldades experimentais associadas à determinação estrutural de todos os complexos de interesse, o *docking* molecular constitui uma ferramenta valiosa para a investigação preliminar das interações entre ligantes e proteínas (GUIMARÃES, 2010; MANCINI, 2011; SILVEIRA, 2003).

Os valores da função utilizada no *docking* (*Docking Scoring Function*), E_{score} , são definidos pela equação 1:

$$E_{score} = E_{inter} + E_{intra} \quad (1)$$

O termo E_{inter} , é a energia de interação ligante-enzima e é definido de acordo com a equação 2:

$$E_{inter} = \sum_{i \in \text{ligante}} \sum_{j \in \text{proteína}} \left[E_{PLP}(r_{ij}) + 332.0 \frac{q_i q_j}{4r_{ij}^2} \right] \quad (2)$$

Na equação 2, o primeiro termo E_{PLP} é um potencial linear por partes que utiliza dois conjuntos diferentes de parâmetros, um para a aproximação do termo estérico (Van de Waals) entre átomos e o outro para o potencial para a ligação de hidrogênio. Já o segundo termo descreve as interações eletrostáticas entre átomos carregados, ou seja, é um potencial de Coulomb com uma constante dielétrica dependente da distância ($D(r) = 4r$). O valor numérico de 332,0 fixa as unidades de energia eletrostática para kcal mol⁻¹ (MANCINI, 2011; RAMALHO *et al.*, 2009; THOMSEN; CHRISTENSEN, 2006). O termo E_{intra} é a energia interna do ligante e é definido de acordo com a equação 3:

$$E_{intra} = \sum_{i \in \text{ligante}} \sum_{j \in \text{ligante}} E_{PLP}(r_{ij}) + \sum_{\text{ligações flexíveis}} A[1 - \cos(m \cdot \theta - \theta_0)] + E_{penalizada} \quad (3)$$

As duas primeiras somas referem-se a todos os pares de átomos do ligante, exceto os pares de átomos conectados por duas ligações. Já o segundo termo refere-se a energia de torção, onde o θ é o ângulo de torção da ligação. Para determinar diversas torções, é utilizada a média da contribuição de torção da ligação da energia. O último termo, $E_{penalizada}$ (termo de correção), atribui uma penalidade de 1.000 se a distância entre dois átomos pesados (mais de duas ligações distantes) for menor que 2,0 Å, punindo conformações inexistentes do ligante. Todas essas

funções são usadas para ancorar automaticamente uma molécula flexível em uma molécula molde (enzima) (MANCINI, 2011; THOMSEN; CHRISTENSEN, 2006).

A técnica de *docking* molecular gera diferentes poses para o ligante no sítio de ligação da enzima e as classifica por meio de funções de pontuação, permitindo identificar as conformações energeticamente mais favoráveis e os principais resíduos envolvidos na interação entre o ligante e a enzima. Após a análise dos diferentes modos de interação, considerando a flexibilidade conformacional do ligante a ser introduzido no sítio de ligação da enzima, são identificadas as conformações mais favoráveis (MANCINI, 2011; MIZUTANI *et al.*, 1994). No entanto, por representar, em geral, uma abordagem de natureza essencialmente estática, o *docking* molecular não permite avaliar de forma abrangente a estabilidade e a evolução temporal do complexo ligante-enzima. As simulações de MD constituem uma abordagem complementar, possibilitando investigar o comportamento dinâmico do sistema e a estabilidade das interações ao longo do tempo, considerando a flexibilidade conformacional dos componentes do complexo (LEACH, 2001; MARTÍNEZ; BORIN; SKAF, 2007; SLIWOSKI *et al.*, 2014).

3.5.1.2 Dinâmica Molecular

A MD é uma técnica computacional baseada nos princípios da mecânica clássica que permite investigar o comportamento e a evolução de sistemas moleculares por meio da simulação do movimento dos átomos ao longo do tempo. Essa abordagem possibilita a análise detalhada das interações intermoleculares, das mudanças conformacionais e da estabilidade estrutural de biomoléculas em condições próximas às fisiológicas. A técnica pode ser empregada no estudo de sistemas com diferentes níveis de complexidade, desde pequenas moléculas até grandes sistemas macromoleculares. Devido à sua capacidade de descrever fenômenos dependentes do tempo em escala atômica, a MD tornou-se uma das ferramentas mais amplamente utilizadas para compreender e prever propriedades estruturais, dinâmicas e funcionais de macromoléculas biológicas em função do tempo (BRAUN *et al.*, 2019; DE VIVO *et al.*, 2016).

Nas simulações de MD clássica, os átomos são representados como partículas pontuais interagindo por meio de potenciais empíricos descritos pelos campos de força. Nesse formalismo, as ligações químicas são geralmente modeladas como oscilações harmônicas em torno de posições de equilíbrio, permitindo a descrição eficiente da dinâmica conformacional de sistemas moleculares. Como os elétrons não são tratados explicitamente, fenômenos

eletrônicos, tais como formação e ruptura de ligações químicas, transferência de carga, polarização eletrônica e interações entre orbitais moleculares, não podem ser adequadamente representados por essa abordagem (BRAUN *et al.*, 2019; DE VIVO *et al.*, 2016).

Os programas de MD utilizam algoritmos de integração numérica para resolver as equações de movimento dos átomos ao longo do tempo, gerando uma trajetória que descreve a evolução temporal do sistema em estudo por meio de suas coordenadas e momentos. A análise dessas trajetórias permite a obtenção de diversas propriedades estruturais, dinâmicas e energéticas, fornecendo informações detalhadas sobre o comportamento molecular do sistema. Entre os parâmetros mais frequentemente avaliados estão a energia do sistema, o número médio de ligações de hidrogênio formadas durante a simulação, o desvio quadrático médio da raiz (RMSD), a flutuação quadrática média da raiz (RMSF), as distâncias intermoleculares e o raio de giro, entre outros (VAN DER SPOEL *et al.*, 2005).

Em simulações de sistemas biomoleculares, como proteínas, o sistema é geralmente inserido em uma caixa de solvatação contendo moléculas de água explícitas (*water box*). Essa estratégia minimiza os efeitos de borda e reproduz um ambiente semelhante ao encontrado em sistemas macroscópicos. Nesse modelo, réplicas da caixa de simulação são distribuídas ao redor da célula principal, formando um sistema periódico que busca representar o limite termodinâmico. Nesse limite, o número de moléculas (N) e o volume (V) tendem ao infinito, enquanto a densidade numérica (N/V) permanece constante. Como consequência, o movimento das partículas não é artificialmente restringido pelas paredes da caixa de simulação, uma vez que as réplicas periódicas garantem a continuidade do sistema em todas as direções (MARK *et al.*, 2000; MARTÍNEZ, 2007).

A descrição clássica da energia de um sistema molecular é realizada por meio do Hamiltoniano (H), definido como a soma das contribuições cinética (K) e potencial (V) de todas as partículas do sistema. Esse formalismo é expresso em função das coordenadas generalizadas q_l e dos momentos generalizados p_l dos N átomos presentes no sistema (TUCKERMAN; MARTYNA, 2000):

$$H(\{q_l, p_l\}) = K(\{p_l\}) + V(\{q_l\}) \quad (\text{Eq. 4})$$

onde:

$$q_l = q_1, q_2, q_3, \dots, q_N \quad \text{e} \quad p_l = p_1, p_2, p_3, \dots, p_N$$

O termo de energia potencial $V(\{\vec{q}^i\})$, representa as interações intra e intermoleculares de curto e longo alcance presentes no sistema. Já a energia cinética é expressa pela Equação 5, na qual m_i corresponde à massa do átomo i :

$$K(\{\vec{p}^i\}) = \sum_{i=1 \rightarrow N} \vec{p}^i{}^2 / 2m_i \quad (\text{Eq. 5})$$

A dinâmica das partículas que compõem o sistema pode ser descrita por equações de movimento derivadas do formalismo Hamiltoniano, cujas derivadas conduzem às equações de movimento de Newton:

$$\dot{q}_i = \partial H / \partial p_i \text{ e } \dot{p}_i = -\partial H / \partial q_i \quad (\text{Eq. 6})$$

$$\dot{r}_i = p_i / m_i = v_i \quad (\text{Eq. 7})$$

$$\dot{p}_i = m_i \ddot{r}_i = -\partial V(\{\vec{r}^i\}) / \partial r_i = F_i \quad (\text{Eq. 8})$$

O termo $\ddot{r}_i$ representa a aceleração do átomo i , enquanto F_i corresponde à força resultante que atua sobre esse átomo (NAMBA *et al.*, 2008).

A técnica de MD baseia-se na resolução numérica das Equações 7 e 8, seguida de sua integração sucessiva ao longo do tempo por meio de algoritmos computacionais eficientes. Esse procedimento possibilita acompanhar a evolução temporal das posições e velocidades das partículas que compõem o sistema, gerando trajetórias que descrevem seu comportamento dinâmico em nível atômico. A partir dessas trajetórias, podem ser obtidas diversas propriedades estruturais, dinâmicas e termodinâmicas, permitindo a análise do comportamento macroscópico do sistema com base nos princípios da MM clássica (NAMBA *et al.*, 2008).

Embora o *docking* molecular e a MD sejam ferramentas poderosas para a análise de interações proteína-ligante, as mesmas não descrevem adequadamente processos químicos envolvendo formação e ruptura de ligações covalentes. Dessa forma, a elucidação de mecanismos de reação em sistemas enzimáticos requer métodos complementares, como os cálculos híbridos QM/MM. Dessa forma, a investigação detalhada de mecanismos de reação em sistemas enzimáticos requer métodos capazes de descrever explicitamente a estrutura eletrônica das espécies envolvidas. A Mecânica Quântica (QM, do inglês Quantum Mechanics) permite uma descrição mais detalhada das propriedades eletrônicas e dos processos químicos,

sendo frequentemente empregada em combinação com métodos de MM por meio de abordagens híbridas QM/MM (GONÇALVES *et al.*, 2014).

3.5.2 Mecânica Quântica

Em 1926, o físico austríaco Erwin Schrödinger, inspirado pela tese de De Broglie e pela teoria de Hamilton-Jacobi (uma formulação da mecânica clássica), desenvolveu a equação de onda da mecânica ondulatória. Essa equação foi aplicada ao modelo atômico de Bohr, permitindo demonstrar que os valores das energias quantizadas obtidos estavam em concordância com os resultados experimentais e eram exatamente equivalentes aos determinados por um método mais abstrato proposto, um ano antes, por Werner Heisenberg. Atualmente conhecida como equação de Schrödinger, essa formulação constitui a base dos cálculos de energia de átomos e moléculas, sendo um dos pilares fundamentais da QM (ALCÁCER, 2007; CRAMER, 2013; JENSEN, 2017).

A equação de Schrödinger independente do tempo pode ser generalizada para as três dimensões do espaço:

$$\left[-\frac{\hbar^2}{2m} \left(\frac{\partial^2}{\partial x^2} + \frac{\partial^2}{\partial y^2} + \frac{\partial^2}{\partial z^2} \right) + V(x, y, z) \right] \Psi(x, y, z) = E \Psi(x, y, z) \quad (\text{Eq. 9})$$

Sabendo que esse termo representa o operador laplaciano, normalmente denotado por ∇^2 ou Δ , a equação de Schrödinger pode ser expressa da seguinte maneira:

$$\left[-\frac{\hbar^2}{2m} \nabla^2 + V(x, y, z) \right] \Psi(x, y, z) = E \Psi(x, y, z) \quad (\text{Eq. 10})$$

Na realidade, todo o termo entre colchetes corresponde a um operador matemático denominado Hamiltoniano, geralmente representado pela letra H . Dessa forma, a equação de Schrödinger pode ser escrita de maneira mais simplificada, conforme apresentado na Equação 11, em que $\mathbf{r}$ representa o conjunto das três coordenadas espaciais, $\mathbf{r} = (\mathbf{x}, \mathbf{y}, \mathbf{z})$. Na maioria dos problemas da química, o objetivo é calcular os possíveis valores de energia do sistema, representados por E (ALCÁCER, 2007; CRAMER, 2013; JENSEN, 2017).

$$\hat{H}\psi(r) = E\psi(r) \quad (\text{Eq. 11})$$

Essa equação descreve as funções de onda (ou estados quânticos) das partículas quando suas energias estão bem definidas, ou seja, quando descreve estados estacionários. Por meio dela, é

possível obter a maioria dos resultados da QM não relativística, como o espectro de energia do átomo de hidrogênio. A equação de Schrödinger possui uma importância fundamental nos cálculos de química quântica, abrangendo diversos métodos computacionais. Nesse contexto, destaca-se a Teoria do Funcional da Densidade (DFT, do inglês *Density Functional Theory*), uma técnica amplamente utilizada (ALCÁCER, 2007; CRAMER, 2013; JENSEN, 2017). A aplicação de métodos quânticos a sistemas biomoleculares de grande dimensão pode apresentar elevado custo computacional. Nesse contexto, os métodos híbridos QM/MM combinam a descrição quântica da região de interesse químico com a descrição clássica do restante do sistema, permitindo investigar processos químicos complexos em sistemas biomoleculares com maior eficiência computacional (SENN; THIEL, 2009; GONÇALVES *et al.*, 2014).

3.5.3 Métodos Híbridos QM/MM

As técnicas híbridas QM/MM são amplamente utilizadas na modelagem computacional para grandes sistemas moleculares, que combinam a descrição eletrônica da QM com a eficiência computacional da MM (GONÇALVES *et al.*, 2014). A integração dessas abordagens amplia significativamente a aplicação de métodos computacionais a sistemas químicos e biológicos complexos (BORMAN, 2004). Nessa metodologia, o sistema é dividido em dois subsistemas: uma região tratada por QM, onde são considerados explicitamente os elétrons e os núcleos atômicos, e uma região tratada por MM, descrita por potenciais clássicos. Em geral, a região QM engloba os átomos diretamente envolvidos no processo químico de interesse, enquanto a região MM representa o ambiente molecular circundante. Os cálculos quânticos são normalmente baseados na aproximação de Born–Oppenheimer, que permite tratar separadamente os movimentos dos núcleos e dos elétrons, simplificando a determinação da energia e das propriedades do sistema molecular (WARSHEL; LEVITT, 1976; SZABO; OSTLUND, 1996).

A QM permite descrever rearranjos eletrônicos associados à quebra e à formação de ligações químicas. Em mecanismos de reação enzimática, a região QM consiste no sítio ativo da enzima, incluindo os átomos diretamente envolvidos no processo químico, como resíduos catalíticos, substratos e cofatores. A parte restante do sistema, que não participa diretamente da reação, é descrita por MM, utilizando um campo de força previamente parametrizado. Uma característica importante dos métodos híbridos QM/MM é a existência de diferentes formas de acoplamento entre as regiões quântica e clássica. A forma mais simples de acoplamento faz uso de cargas pontuais na região QM que interagem com o restante da região MM (LONSDALE;

RANAGHAN; MULHOLLAND, 2010). No desempenho desse cálculo, a energia QM/MM do sistema total é calculado como mostrado na equação 12:

$$E_{TOTAL}^{QM/MM} = E_{TOTAL}^{MM} + E_{QM\,região}^{QM} - E_{QM\,região}^{MM} \quad (\text{Eq. 12})$$

Onde E_{TOTAL}^{MM} é a energia MM de todo o sistema, $E_{QM\,região}^{QM}$ é a energia QM da região QM e $E_{QM\,região}^{MM}$ é a energia MM da região QM isolada. Diferentes métodos QM/MM incluem também o efeito de polarização na região QM pelo ambiente MM, tal efeito é importante no contexto de várias enzimas (SENTHILKUMAR *et al.*, 2008). Esses métodos estão relacionados com as interações eletrostáticas entre as regiões QM e MM no cálculo (LONSDALE; RANAGHAN; MULHOILLAND, 2010; MULHOLLAND, 2007; VAN DER KAMP; MULHOLLAND, 2013).

A energia total de um sistema tratado por métodos QM/MM não pode ser descrita apenas pela soma das energias calculadas separadamente para as regiões QM e MM, uma vez que as interações entre esses subsistemas também devem ser consideradas (SENN; THIEL, 2009). Dessa forma, as condições de acoplamento na interface QM/MM desempenham papel fundamental na descrição adequada do sistema. Dependendo da estratégia de acoplamento adotada, pode ser necessária a definição de uma região de fronteira entre as partes quântica e clássica, incluindo átomos de ligação ou outros procedimentos destinados a representar corretamente as ligações químicas que atravessam a interface (HEYDEN; LIN; TRUHLAR, 2007; SENN; THIEL, 2009).

Os métodos de QM de alto nível são amplamente utilizados no estudo de reações enzimáticas, e permitem o cálculo das barreiras energéticas para o processo da reação. Além disso, os efeitos exercidos por grandes sistemas moleculares sobre a região reativa, podem ser considerados por meio da utilização de métodos híbridos QM/MM (BRAUN-SAND *et al.*, 2005; MULHOLLAND, 2007). Dessa forma, a abordagem QM/MM tem sido constantemente aplicada na investigação de mecanismos de catálise enzimática, possibilitando a análise de intermediários reacionais, estados de transição e barreiras energéticas envolvidas nos processos químicos (LONSDALE; RANAGHAN; MULHOILLAND, 2010).

A abordagem QM/MM é considerada uma ferramenta de grande relevância, pois além de ser utilizada na modelagem de sistemas biomoleculares, também encontra aplicações no estudo de sistemas inorgânicos e organometálicos, materiais de estado sólido e processos em solvente explícito (SENN; THIEL, 2009). Em sistemas biológicos, essa metodologia tem se

mostrado particularmente útil para a investigação de mecanismos de reação, permitindo descrever, em nível molecular, etapas fundamentais como formação e ruptura de ligações químicas, estados de transição e barreiras energéticas. Assim, os métodos híbridos QM/MM constituem uma abordagem robusta para o estudo de processos enzimáticos complexos, complementando as informações obtidas por técnicas como o *docking* molecular e contribuindo para a compreensão detalhada dos mecanismos de reação em sistemas biológicos (GONÇALVES *et al.*, 2014). Embora a compreensão dos mecanismos moleculares e da interação com os alvos biológicos seja fundamental para a prospecção de potenciais agentes terapêuticos, a avaliação de sua segurança também constitui uma etapa essencial no processo de desenvolvimento de candidatos a fármacos. A investigação da toxicidade dos compostos permite estimar seus possíveis efeitos adversos e contribui para a seleção de moléculas com maior potencial terapêutico e perfil de segurança mais favorável (FOWLER *et al.*, 2022; LUO *et al.*, 2021).

3.5.4 Testes de Toxicidade e ADME

3.5.4.1 Teste de Toxicidade

Os testes de toxicidade *in silico* representam uma etapa importante no processo de descoberta e desenvolvimento de novos compostos bioativos. Essas abordagens permitem a avaliação preliminar dos possíveis efeitos tóxicos de uma molécula por meio de modelos computacionais, contribuindo para a seleção de candidatos mais seguros e reduzindo custos associados às etapas experimentais. Entre os parâmetros mais utilizados destaca-se a dose letal média (LD₅₀), definida como a quantidade de uma substância capaz de causar a morte de 50% de uma população experimental, sendo amplamente empregada como indicador de toxicidade aguda. Dessa forma, a predição computacional da toxicidade constitui uma ferramenta valiosa para a avaliação inicial da segurança de potenciais candidatos terapêuticos (LUO *et al.*, 2021; ZWICKL *et al.*, 2022).

3.5.4.2 Teste de Absorção, Distribuição, Metabolismo e Excreção (ADME)

A análise das propriedades de absorção, distribuição, metabolismo e excreção (ADME) desempenha papel fundamental no desenvolvimento de novos fármacos, uma vez que a atividade biológica de um composto depende não apenas de sua interação com o alvo molecular,

mas também de seu comportamento farmacocinético no organismo. Essas propriedades influenciam diretamente parâmetros como biodisponibilidade, distribuição tecidual, biotransformação e eliminação do composto. Dessa forma, a avaliação *in silico* de parâmetros ADME permite identificar precocemente limitações farmacocinéticas e selecionar moléculas com maior potencial para desenvolvimento terapêutico. Em conjunto com as demais abordagens computacionais, a avaliação de propriedades ADME contribui para uma caracterização mais abrangente dos compostos candidatos, permitindo integrar aspectos estruturais, energéticos, farmacológicos e toxicológicos no processo de seleção racional de moléculas com potencial terapêutico. A aplicação integrada dessas estratégias constitui uma ferramenta relevante para a investigação e o direcionamento de novos candidatos a fármacos, especialmente diante da necessidade de identificar compostos que apresentem não apenas atividade biológica, mas também propriedades farmacológicas e perfil de segurança adequados (FOWLER *et al.*, 2022; LUO *et al.*, 2021).

REFERÊNCIAS

- ABOU-DONIA, M. B. Organophosphorus ester-induced chronic neurotoxicity. **Archives of Environmental Health**, v. 58, p. 484–497, 2003.
- ABYADEH, M. *et al.* Amyloid-beta and tau protein beyond Alzheimer's disease. **Neural Regeneration Research**, v. 19, 2024.
- AHLMANN, M.; HEMPEL, G. The effect of cyclophosphamide on the immune system: implications for clinical cancer therapy. **Cancer Chemotherapy and Pharmacology**, v. 78, p. 661–671, 2016.
- ALCÁCER, L. **Introdução à química quântica computacional**. Lisboa: Instituto Superior Técnico, 2007.
- ALDRIDGE, W. N.; REINER, E. Acylated amino acids in inhibited B-esterases. In: NEUBERGER, A.; TATUM, E. L. (ed.). **Enzyme inhibitors as substrates**. Amsterdam: North-Holland Publishing Company, p. 170–175, 1972.
- ANANTH, M. R. *et al.* Basal forebrain cholinergic signalling: development, connectivity and roles in cognition. **Nature Reviews Neuroscience**, v. 24, n. 4, p. 233–251, 2023.
- ARONIADOU-ANDERJASKA, V.; FIGUEIREDO, T. H.; DE ARAUJO FURTADO, M.; PIDOPLICHKO, V. I.; BRAGA, M. F. M. Mechanisms of organophosphate toxicity and the role of acetylcholinesterase inhibition. **Toxics**, v. 11, n. 10, p. 866, 2023.
- ATTA, A. M.; ELKALLA, W. S.; OMRAN, S. A.; ELGAWISH, M. S. Innovations in acetylcholinesterase inhibitors: emerging strategies for Alzheimer's disease therapy (2019–2024). **Egyptian Pharmaceutical Journal**, v. 24, n. 2, p. 152–170, 2025. DOI: 10.21608/epj.2025.407574.
- BAJGAR, J. Organophosphates/nerve agent poisoning: mechanism of action, diagnosis, prophylaxis, and treatment. **Advances in Clinical Chemistry**, v. 38, p. 151–216, 2004.
- BARTUS, R. T.; DEAN III, R. L.; BEER, B.; LIPPA, A. S. The cholinergic hypothesis of geriatric memory dysfunction. **Science**, v. 217, n. 4558, p. 408–414, 1982.
- BELINSKAIA, D. A. *et al.* Rational *in silico* design of aptamers for organophosphates based on the example of paraoxon. **Computational Biology and Chemistry**, v. 80, p. 452–462, 2019.
- BERTRAND, D.; WALLACE, T. L. A review of the cholinergic system and therapeutic approaches to treat brain disorders. In: SHOAI, M.; WALLACE, T. L. (ed.). **Current Topics in Behavioral Neurosciences**, v. 45. Cham: Springer, p. 1–28, 2020.
- BIGLEY, A. N.; RAUSHEL, F. M. The evolution of phosphotriesterase for decontamination and detoxification of organophosphorus chemical warfare agents. **Chemico-Biological Interactions**, v. 308, p. 80–88, 2019.

BOND, M. *et al.* The effectiveness and cost-effectiveness of donepezil, galantamine, rivastigmine and memantine for the treatment of Alzheimer's disease (review of Technology Appraisal No. 111): a systematic review and economic model. **Health Technology Assessment**, v. 16, n. 21, p. 1–470, 2012.

BORMAN, S. A. Much to do about enzyme mechanism. **Chemical & Engineering News**, v. 82, n. 8, p. 35–39, 2004.

BOUBLIK, Y.; SAINT-AGUET, P.; LOUGARRE, A.; ARNAUD, M.; VILLATTE, F.; ESTRADA-MONDACA, S.; FOURNIER, D. Acetylcholinesterase engineering for detection of insecticide residues. **Protein Engineering, Design & Selection**, v. 15, p. 43–50, 2002.

BRAUN, E.; GILMER, J.; MAYES, H. B.; MOBLEY, D. L.; MONROE, J. I.; PRATAPANENI, S.; ZUCKERMAN, D. M. Best practices for foundations in molecular simulations. **Living Journal of Computational Molecular Science**, v. 1, n. 1, p. 5957, 2019.

BRAUN-SAND, S.; OLSSON, M. H. M.; WARSHEL, A. Computer modeling of enzyme catalysis and its relationship to concepts in physical organic chemistry. In: RICHARD, J. P. **Advances in Physical Organic Chemistry**. Amsterdam: Elsevier, v. 40, p. 201–245, 2005.

BREIJYEH, Z.; KARAMAN, R. Comprehensive review on Alzheimer's disease: causes and treatment. **Molecules**, v. 25, n. 24, 2020.

BRUNEAU, E. G.; AKAABOUNE, M. Running to stand still: ionotropic receptor dynamics at central and peripheral synapses. **Molecular Neurobiology**, v. 34, n. 2, p. 137–151, 2006.

CÂMARA, A. B. Receptores neurais e a doença de Alzheimer: uma revisão sistemática da literatura sobre as famílias de receptores mais associadas à doença, suas funções e áreas de expressão. **Jornal Brasileiro de Psiquiatria**, Rio de Janeiro, v. 68, n. 3, p. 161–176, 2019.

CARVALHO, I. *et al.* Introdução à modelagem molecular de fármacos no curso experimental de química farmacêutica. **Química Nova**, São Paulo, v. 26, n. 3, p. 428–438, 2003.

CAVALCANTI, L. P. A. N.; AGUIAR, A. P.; LIMA, J. A.; LIMA, A. L. S. Intoxicação por organofosforados: tratamento e metodologias analíticas empregadas na avaliação da reativação e inibição da acetilcolinesterase. **Revista Virtual de Química**, v. 8, n. 3, p. 739–766, 2016.

CHAMBERS, J. E.; DAIL, M. B.; MEEK, E. C. Oxime-mediated reactivation of organophosphate-inhibited acetylcholinesterase with emphasis on centrally-active oximes. **Neuropharmacology**, v. 181, p. 108201, 2020.

CHAMBERS, J. E.; MEEK, E. C. Central neuroprotection demonstrated by novel oxime countermeasures to nerve agent surrogates. **Annals of the New York Academy of Sciences**, New York, v. 1503, n. 1, p. 137–149, 2021.

CHANG, M. M. F.; GINJOM, I. R.; NG, S. M. Single-shot "turn-off" optical probe for rapid detection of paraoxon-ethyl pesticide on vegetable utilising fluorescence carbon dots. **Sensors and Actuators B: Chemical**, v. 242, p. 1050–1056, 2017.

CHATONNET, A.; LOCKRIDGE, O. Comparison of butyrylcholinesterase and acetylcholinesterase. **Biochemical Journal**, v. 260, p. 625, 1989.

CHEN, Y.; YANG, Z.; NIAN, B. *et al.* Mechanisms of neurotoxicity of organophosphate pesticides and their relation to neurological disorders. **Neuropsychiatric Disease and Treatment**, v. 20, p. 2237–2254, 2024.

CHU, Y. *et al.* Organophosphorus Pesticides in the Natural Environment: Detection Methods, Distribution, and Toxic Effects. **Water Environment Research**, v. 98, n. 4, e70375, 2026.

COELHO FILHO, L. N.; BARBOSA, M. F. D. Doença de Alzheimer: novas abordagens terapêuticas. **Brazilian Journal of Implantology and Health Sciences**, v. 5, n. 4, p. 2270–2278, 2023.

COELHO, L. W. *et al.* Aplicação de mecânica molecular em química inorgânica. **Química Nova**, v. 22, n. 3, 1999.

COLOVIĆ, M. B. *et al.* Acetylcholinesterase inhibitors: pharmacology and toxicology. **Current Neuropharmacology**, v. 11, n. 3, p. 315–335, 2013.

CONTESTABILE, A. The history of the cholinergic hypothesis. **Behavioural Brain Research**, v. 221, p. 334–340, 2011.

COSTA, L. G. Organophosphorus compounds at 80: some old and new issues. **Toxicological Sciences**, v. 162, n. 1, p. 24–35, 2018.

CRAMER, C. J. **Essentials of Computational Chemistry: Theories and Models**. 2. ed. Chichester: John Wiley & Sons, 2013.

DALMAGRO, A. P.; CAZARIN, C. A.; ZENAIDE, F. S. Atualização no estudo das bases bioquímicas e moleculares da doença de Alzheimer. **Brazilian Applied Science Review**, v. 4, n. 1, p. 118–130, 2020.

DARVESH, S.; HOPKINS, D. A.; GEULA, C. Neurobiology of butyrylcholinesterase. **Nature Reviews Neuroscience**, v. 4, n. 2, p. 131–138, 2003.

DELFINO, R. T.; RIBEIRO, T. S.; FIGUEROA-VILLAR, J. D. Organophosphorus compounds as chemical warfare agents: a review. **Journal of the Brazilian Chemical Society**, v. 20, n. 3, p. 407–428, 2009.

DEMKOWICZ, S.; RACHON, J.; DAŠKO, M.; KOZAK, W. Selected organophosphorus compounds with biological activity. Applications in medicine. **RSC Advances**, Cambridge, v. 6, n. 9, p. 7101–7112, 2016.

DE VIVO, M.; MASETTI, M.; BOTTEGONI, G.; CAVALLI, A. Role of Molecular Dynamics and Related Methods in Drug Discovery. **Journal of Medicinal Chemistry, Washington**, v. 59, n. 9, p. 4035–4061, 2016.

DIRAK, M.; CHAN, J.; KOLEMEN, S. Optical imaging probes for selective detection of butyrylcholinesterase. **Journal of Materials Chemistry B**, v. 12, n. 5, p. 1149–1167, 2024.

DOS SANTOS, V. M. R.; DONNICI, C. L. Compostos organofosforados pentavalentes: histórico, métodos sintéticos de preparação e aplicações como inseticidas e agentes antitumorais. **Química Nova**, v. 30, n. 1, p. 159–170, 2007.

DVIR, H. *et al.* Acetylcholinesterase: from 3D structure to function. **Chemico-Biological Interactions**, v. 187, n. 1–3, p. 10–22, 2010.

EMADI, A.; JONES, R. J.; BRODSKY, R. A. Cyclophosphamide and cancer: golden anniversary. **Nature Reviews Clinical Oncology**, v. 6, p. 638–647, 2009.

EDDLESTON, M.; BUCKLEY, N. A.; EYER, P.; DAWSON, A. H. Management of acute organophosphorus pesticide poisoning. **The Lancet**, v. 371, n. 9612, p. 597–607, 2008.

FALCO, A. *et al.* Doença de Alzheimer: hipóteses etiológicas e perspectivas de tratamento. **Química Nova**, v. 39, p. 63–80, 2016.

FERREIRA-VIEIRA, T. H.; GUIMARÃES, I. M.; SILVA, F. R.; RIBEIRO, F. M. Alzheimer's disease: targeting the cholinergic system. **Current Neuropharmacology**, v. 14, n. 1, p. 101–115, 2016.

FIGUEROA-VILLAR, J. D.; PETRONILHO, E. C.; KUCA, K.; FRANCA, T. C. C. Review about structure and evaluation of reactivators of acetylcholinesterase inhibited with neurotoxic organophosphorus compounds. **Current Medicinal Chemistry**, v. 28, n. 7, p. 1422–1442, 2021.

FOWLER, S. *et al.* Addressing today's absorption, distribution, metabolism, and excretion (ADME) challenges in the translation of in vitro ADME characteristics to humans: a case study of the SMN2 mRNA splicing modifier risdiplam. **Drug Metabolism and Disposition**, v. 50, n. 1, p. 65–75, 2022.

FRANCIS, P. T.; PALMER, A. M.; SNAPE, M.; WILCOCK, G. K. The cholinergic hypothesis of Alzheimer's disease: a review of progress. **Journal of Neurology, Neurosurgery & Psychiatry**, v. 66, p. 137–147, 1999.

FREITAS, H. F.; PAZ, O. S.; CASTILHO, M. S. Estudos de QSAR 3D para um conjunto de inibidores de butirilcolinesterase humana. **Química Nova**, v. 32, n. 8, p. 2114–2121, 2009.

FUJII, T.; TAKADA-TAKATORI, Y.; KAWASHIMA, K. Basic and clinical aspects of non-neuronal acetylcholine: expression of an independent, non-neuronal cholinergic system in lymphocytes and its clinical significance in immunotherapy. **Journal of Pharmacological Sciences**, v. 106, n. 2, p. 186–192, 2008.

FUKUNAGA, K.; YABUKI, Y. SAK3-induced neuroprotection is mediated by nicotinic acetylcholine receptors. In: AKAIKE, A.; SHIMOHAMA, S.; MISU, Y. (ed.). **Nicotinic acetylcholine receptor signaling in neuroprotection**. Singapore: Springer, p. 159–171, 2018.

GARDINER, B. J.; STEWARDSON, A. J.; ABBOTT, I. J.; PELEG, A. Y. Nitrofurantoin and fosfomycin for resistant urinary tract infections: old drugs for emerging problems. **Australian Prescriber**, v. 42, p. 14–19, 2019.

GEULA, C. *et al.* Basal forebrain cholinergic system in the dementias: vulnerability, resilience, and resistance. **Journal of Neurochemistry**, v. 158, p. 1394–1411, 2021.

GIACOBINI, E. **Butyrylcholinesterase: Its Function and Inhibition**. 1. ed. United Kingdom: Martin Dunitz Ltd., p. 192, 2003.

GIOVANNINI, M. G.; SCALI, C.; BARTOLINI, L.; SCHMIDT, B.; PEPEU, G. Effect of subchronic treatment with metrifonate and tacrine on brain cholinergic function in aged F344 rats. **European Journal of Pharmacology**, v. 354, n. 1, p. 17–24, 1998.

GONÇALVES, A. da S. *et al.* Reactivation steps by 2-PAM of tabun-inhibited human acetylcholinesterase: reducing the computational cost in hybrid QM/MM methods. **Journal of Biomolecular Structure and Dynamics**, v. 32, n. 2, p. 301–307, 2014.

GOULART, P. N. *et al.* Butirilcolinesterase (BuChE): um potencial alvo para o desenvolvimento de fármacos para o tratamento da doença de Alzheimer. **Revista Virtual de Química**, v. 13, n. 1, 2021.

GUIMARÃES, A. P. **Estudos por ancoramento e dinâmica molecular de potenciais inibidores da nucleosídeo hidrolase de Bacillus anthracis**. Dissertação (Mestrado em Agroquímica) — Universidade Federal de Lavras, Lavras, p. 170, 2010.

GUPTA, R. C. **Handbook of toxicology of chemical warfare agents**. 3. ed. Academic Press, 2020.

GUPTA, S.; BAWEJA, G. S.; GUPTA, G. D.; ASATI, V. Identification of potential N-substituted 5-benzylidenethiazolidine-2,4-dione derivatives as α -amylase inhibitors: computational cum synthetic studies. **Journal of Molecular Structure**, v. 1287, p. 135596, 2023.

HAJIMOHAMMADI, S.; LOCKRIDGE, O.; MASSON, P. New views on physiological functions and regulation of butyrylcholinesterase and potential therapeutic interventions. **Frontiers in Molecular Biosciences**, v. 12, art. 1625318, 2025.

HAMPEL, H.; MESULAM, M.-M.; CUELLO, A. C.; FARLOW, M. R.; GIACOBINI, E.; SNYDER, P. J.; KHACHATURIAN, Z. S. The cholinergic system in the pathophysiology and treatment of Alzheimer's disease. **Brain**, v. 141, n. 7, p. 1917–1933, 2018.

HANSEN, R. A.; GARTLEHNER, G.; WEBB, A. P.; MORGAN, L. C.; MOORE, C. G.; JONAS, D. E. Efficacy and safety of donepezil, galantamine, and rivastigmine for the treatment of Alzheimer's disease: a systematic review and meta-analysis. **Clinical Interventions in Aging**, v. 3, n. 2, p. 211–225, 2008.

HEYDEN, A.; LIN, H.; TRUHLAR, D. G. Adaptive partitioning in combined quantum mechanical and molecular mechanical calculations of potential energy functions for

multiscale simulations. **The Journal of Physical Chemistry B**, v. 111, n. 9, p. 2231–2241, 2007.

HÖRNBERG, A.; TUNEMALM, A.; EKSTRÖM, F. Crystal structures of acetylcholinesterase in complex with organophosphorus compounds suggest that the acyl pocket modulates the aging reaction by precluding the formation of the trigonal bipyramidal transition state. **Biochemistry**, v. 46, p. 4815–4825, 2007.

HUNG, L. W.; SANBONMATSU, K. Y.; WILLIAMS, R. F.; CHEN, J. C. H. Acetylcholinesterase: structure, dynamics, and interactions with organophosphorus compounds. **Protein Science**, v. 34, n. 10, e70297, 2025.

HUO, S. *et al.* Effects of eszopiclone on sleep quality and cognitive function in elderly patients with Alzheimer's disease and sleep disorder: a randomized controlled trial. **Brain and Behavior**, v. 12, n. 2, e2488, 2022.

IWATA, Y. *et al.* Conformational analysis and *docking* study of potent factor XIIIa inhibitors having a cyclopropanone ring. **Journal of Molecular Graphics and Modelling**, v. 18, n. 6, p. 591–599, 2000.

IYER, R.; IKEN, B.; LEON, A. Developments in alternative treatments for organophosphate poisoning. **Toxicology Letters**, v. 233, n. 2, p. 200–206, 2015.

JENSEN, F. **Introduction to Computational Chemistry**. 3. ed. Chichester: John Wiley & Sons, 2017.

KANEKAR, P. P. *et al.* Biodegradation of organophosphorus pesticides. **Proceedings of the Indian National Science Academy**, v. 70, n. 1, p. 57–70, 2004.

KANDIAH, N.; CHAN, Y. F.; CHEN, C. *et al.* Rivastigmine: the advantages of dual inhibition of acetylcholinesterase and butyrylcholinesterase in Alzheimer's disease and Parkinson's disease dementia. **Neurology and Therapy**, v. 6, suppl. 2, p. 57–70, 2017.

KANDY, A. T.; KUMAR, T. D. A.; SELVARAJ, D.; JUPUDI, S. Repurposing FDA-approved drugs as acetylcholinesterase inhibitors for Alzheimer's disease: an integrated *in silico* approach. **ChemistrySelect**, v. 11, n. 24, e06836, 2026.

KINNEY, J. W. *et al.* Inflammation as a central mechanism in Alzheimer's disease. **Alzheimer's & Dementia: Translational Research & Clinical Interventions**, v. 4, p. 575–590, 2018.

KNOPMAN, D. S. *et al.* Alzheimer disease. **Nature Reviews Disease Primers**, v. 7, art. 33, 2021.

KOVARIK, Z.; MAČEK HRVAT, N.; KALISIAK, J.; KATALINIĆ, M.; SIT, R. K.; ZORBAZ, T.; RADIĆ, Z.; FOKIN, V. V.; SHARPLESS, K. B.; TAYLOR, P. Counteracting tabun inhibition by reactivation by pyridinium aldoximes that interact with active center gorge mutants of acetylcholinesterase. **Toxicology and Applied Pharmacology**, v. 372, p. 40–46, 2019.

KUCA, K.; PATOCKA, J.; CABAL, J.; JUN, D. Reactivation of organophosphate-inhibited acetylcholinesterase by quaternary pyridinium aldoximes. **Neurotoxicity Research**, v. 6, n. 7–8, p. 565–570, 2004.

KUTTY, K. M. Biological function of cholinesterase. **Clinical Biochemistry**, v. 13, p. 265–275, 1980.

LANE, S. A.; HARDY, J.; SCHOTT, J. M. Alzheimer's disease. **European Journal of Neurology**, v. 25, n. 1, p. 59–70, 2018.

LEACH, A. R. **Molecular modelling: principles and applications**. 2. ed. Harlow: Prentice Hall, 2001.

LIMA, P. J. P. **Possíveis doenças físicas e mentais relacionadas ao manuseio de agrotóxicos em atividades rurais, na região de Atibaia, SP/Brasil**. Dissertação (Mestrado em Saúde Pública) — Faculdade de Saúde Pública, Universidade de São Paulo, São Paulo, 2008.

LI, Y.; FAN, H.; SUN, J.; NI, M.; ZHANG, L.; CHEN, C.; HONG, X.; FANG, F.; ZHANG, W.; MA, P. Circular RNA expression profile in Alzheimer's disease and its clinical significance as biomarkers for the disease risk and progression. **International Journal of Biochemistry and Cell Biology**, v. 123, p. 105747, 2020.

LOCKRIDGE, O.; NORNGREN, R. B.; JOHNSON, R. C.; BLAKE, T. A. Naturally occurring genetic variants of human acetylcholinesterase and butyrylcholinesterase and their potential impact on the risk of toxicity from cholinesterase inhibitors. **Chemical Research in Toxicology**, v. 29, n. 9, p. 1381–1392, 2016.

LOCKRIDGE, O. Review of human butyrylcholinesterase structure, function, genetic variants, history of use in the clinic, and potential therapeutic uses. **Pharmacology & Therapeutics**, v. 148, p. 34–46, 2015.

LONG, J. M.; HOLTZMAN, D. M. Alzheimer disease: an update on pathobiology and treatment strategies. **Cell**, v. 179, n. 2, p. 312–339, 2019.

LONSDALE, R.; RANAGHAN, K. E.; MULHOLLAND, A. J. Computational enzymology. **Chemical Communications**, v. 46, n. 14, p. 2354–2372, 2010.

LOPEZ-ARRIETA, J. M.; SCHNEIDER, L. Metrifonate for Alzheimer's disease. **Cochrane Database of Systematic Reviews**, n. 1, CD003155, 2006.

LUO, X.; LAUWERS, M.; LAYER, P. G.; WEN, C. Non-neuronal role of acetylcholinesterase in bone development and degeneration. **Frontiers in Cell and Developmental Biology**, v. 8, art. 620543, 2021.

LUSHCHEKINA, S.; MASSON, P. Catalytic bioscavengers against organophosphorus agents: mechanistic issues of self-reactivating cholinesterases. **Toxicology**, v. 409, p. 91–102, 2018.

MACHADO, A. P. R.; CARVALHO, I. O.; SOBRINHO, H. M. R. Neuroinflamação na doença de Alzheimer. **Revista Brasileira Militar de Ciências**, v. 6, n. 14, p. 30–38, 2020.

MACK, A.; ROBITZKI, A. The key role of butyrylcholinesterase during neurogenesis and neural disorders: an antisense-5'-butyrylcholinesterase-DNA study. **Progress in Neurobiology**, v. 60, p. 607–628, 2000.

MALI, H.; SHAH, C.; RAGHUNANDAN, B. H.; PRAJAPATI, A. S.; PATEL, D. H.; TRIVEDI, U.; SUBRAMANIAN, R. B. Organophosphate pesticides: an emerging environmental contaminant: pollution, toxicity, bioremediation progress, and remaining challenges. **Journal of Environmental Sciences**, v. 127, p. 234–250, 2023.

MANAVALAN, P.; TAYLOR, P.; JOHNSON JR., W. C. Circular dichroism studies of acetylcholinesterase conformation: comparison of the 11 S and 5.6 S species and the differences induced by inhibitory ligands. **Biochimica et Biophysica Acta – Protein Structure and Molecular Enzymology**, v. 829, n. 3, p. 365–370, 1985.

MANCINI, D. T. **Estudos computacionais de potenciais inibidores da enzima nucleosídeo hidrolase de Brucella suis**. Dissertação (Mestrado) — Universidade Federal de Lavras, Lavras, 2011.

MARK, E. T.; GLENN, J. M. Understanding modern molecular dynamics: techniques and applications. **Journal of Physical Chemistry B**, v. 104, p. 159–178, 2000.

MARRS, T. C.; MAYNARD, R. L.; SIDELL, F. R. **Chemical warfare agents: toxicology and treatment**. 2. ed. Chichester: John Wiley & Sons, p. 738, 2007.

MARTÍNEZ, L.; BORIN, I. A.; SKAF, M. S. Fundamentos de Simulação por Dinâmica Molecular. In: MORGON, N. H.; COUTINHO, K. (eds.). **Métodos de Química Teórica e Modelagem Molecular**. São Paulo: Editora Livraria da Física, p. 413–452, 2007.

MASSON, P.; NACHON, F. Cholinesterase reactivators and bioscavengers for pre- and post-exposure treatments of organophosphorus poisoning. **Journal of Neurochemistry**, v. 142, supl. 2, p. 26–40, 2017.

MATOS, K. S. **Aspectos moleculares da reativação da acetilcolinesterase inibida por ciclosarin e carbofurano**. Dissertação (Mestrado) — Universidade Federal de Lavras, Lavras, p. 148, 2012.

MERCEY, G.; VERDELET, T.; RENOU, J.; KLIACHYNA, M.; BAATI, R.; NACHON, F.; JEAN, L.; RENARD, P. Y. Reactivators of acetylcholinesterase inhibited by organophosphorus nerve agents. **Accounts of Chemical Research**, v. 45, n. 5, p. 756–766, 2012.

MESULAM, M. M.; GUILLOZET, A.; SHAW, P.; LEVEY, A.; DUYSSEN, E. G.; LOCKRIDGE, O. Acetylcholinesterase knockouts establish central cholinergic pathways and can use butyrylcholinesterase to hydrolyze acetylcholine. **Neuroscience**, v. 110, n. 4, p. 627–639, 2002.

MILLARD, C. B.; LOCKRIDGE, O.; BROOMFIELD, C. A. Crystal structures of aged phosphonylated acetylcholinesterase: nerve agent reaction products at the atomic level. **Biochemistry**, v. 38, n. 22, p. 7032–7039, 1999.

MING, C. L. C.; WANG, X.; GENTILE, C. Protective role of acetylcholine and the cholinergic system in the injured heart. **iScience**, v. 27, p. 110726, 2024.

MIZUTANI, M. Y.; TOMIOKA, N.; ITAI, A. Rational automatic search method for stable *docking* models of protein and ligand. **Journal of Molecular Biology**, v. 243, n. 2, p. 310–326, 1994.

MULHOLLAND, A. J. Chemical accuracy in QM/MM calculations on enzyme-catalysed reactions. **Chemistry Central Journal**, v. 1, art. 19, p. 1–5, 2007.

NACHMANSOHN, D.; WILSON, I. B. The enzymic hydrolysis and synthesis of acetylcholine. **Advances in Enzymology**, v. 12, p. 259–339, 1951.

NAMBA, A. M.; DA SILVA, V. B.; DA SILVA, C. H. T. P. Dinâmica molecular: Teoria e aplicações em planejamento de fármacos. **Ecletica Quimica**, v. 33, n. 4, p. 13–24, 2008.

NICOLET, Y.; LOCKRIDGE, O.; MASSON, P.; FONTECILLA-CAMPS, J. C.; NACHON, F. Crystal structure of human butyrylcholinesterase and of its complexes with substrate and products. **Journal of Biological Chemistry**, v. 278, n. 42, p. 41141–41147, 2003.

NOGA, M.; MICHALSKA, A.; JUROWSKI, K. Review of possible therapies in treatment of Novichoks poisoning and HAZMAT/CBRNE approaches: state of the art. **Journal of Clinical Medicine**, v. 12, p. 2221, 2023.

OLAZARÁN, J.; NAVARRO, E.; ROJO, J. M. Persistence of cholinesterase inhibitor treatment in dementia: insights from a naturalistic study. **Dementia and Geriatric Cognitive Disorders Extra**, v. 3, p. 48–59, 2013.

ORDENTLICH, A.; BARAK, D.; KRONMAN, C.; ARIEL, N.; SEGALL, Y.; VELAN, B.; SHAFFERMAN, A. Contribution of aromatic moieties of tyrosine 133 and of the anionic subsite tryptophan 86 to catalytic efficiency and allosteric modulation of acetylcholinesterase. **Journal of Biological Chemistry**, v. 270, n. 5, p. 2082–2091, 1995.

PATOČKA, J. *et al.* Oxime reactivation of acetylcholinesterase inhibited by toxic phosphorus esters: in vitro kinetics and thermodynamics. **Journal of Applied Biomedicine**, v. 3, p. 91–99, 2005.

PATRICK, G. L. **An introduction to medicinal chemistry**. 2. ed. Oxford: Oxford University Press, p. 432, 2001.

PEREIRA, K. K.; RODRIGUES, V. M. Uso de anticolinesterásicos na doença de Alzheimer. **Debates em Psiquiatria**, v. 5, n. 3, p. 18–23, 2015.

PETRONILHO, E. C.; PINTO, A. C.; VILLAR, J. D. F. Acetilcolinesterase: Alzheimer e guerra química. **Revista Militar de Ciência e Tecnologia**, 2011.

QUINN, D. M. Acetylcholinesterase: enzyme structure, reaction dynamics, and virtual transition states. **Chemical Reviews**, v. 87, p. 955–979, 1987.

RADIĆ, Z. Connectivity between surface and interior in catalytic subunits of acetylcholinesterases inferred from their X-ray structures. **Journal of Neurochemistry**, v. 168, p. 386–396, 2024.

RAJAK, P. *et al.* Immunotoxic role of organophosphates: an unseen risk escalating SARS-CoV-2 pathogenicity. **Food and Chemical Toxicology**, v. 149, art. 112007, 2021.

RAMALHO, T. C.; CASTRO, A. A.; LEAL, D. H. S.; TEIXEIRA, J. P.; CUNHA, E. F. F.; KUČA, K. Assessing the therapeutic and toxicological profile of novel acetylcholinesterase reactivators: value of *in silico* and in vitro data. **Current Medicinal Chemistry**, v. 30, n. 36, p. 4149–4166, 2023.

RAMALHO, T. C. *et al.* Construction and assessment of reaction models of class I EPSP synthase: molecular *docking* and density functional theoretical calculations. **Journal of Biomolecular Structure and Dynamics**, v. 27, n. 2, p. 195–208, 2009.

RATHI, V. Galantamine. In: BELWAL, T.; NABAVI, S. M.; NABAVI, S. F.; DEHPUR, A. R.; SHIROOIE, S. (ed.). **Naturally Occurring Chemicals Against Alzheimer's Disease**. Cambridge: Academic Press, p. 83–92, 2021.

ROBB, E. L.; REGINA, A. C.; BAKER, M. B. Organophosphate toxicity. Atualizado em: 12 nov. 2023. In: **StatPearls**. Treasure Island (FL): StatPearls Publishing, 2026.

ROSENBERRY, T. L.; BRAZZOLOTTO, X.; MACDONALD, I. R.; WANDHAMMER, M.; TROVASLET-LEROY, M.; DARVESH, S.; NACHON, F. Comparison of the binding of reversible inhibitors to human butyrylcholinesterase and acetylcholinesterase: a crystallographic, kinetic and calorimetric study. **Molecules**, v. 22, n. 12, p. 2098, 2017.

SAHA, B.; DAS, A.; JANGID, K.; KUMAR, A.; KUMAR, V.; JAITAK, V. Identification of coumarin derivatives targeting acetylcholinesterase for Alzheimer's disease by field-based 3D-QSAR, pharmacophore model-based virtual screening, molecular *docking*, MM/GBSA, ADME and MD simulation study. **Current Research in Structural Biology**, v. 7, p. 100124, 2024.

SAMPAIO, C. A.; SILVA, B. M.; LIMA, L. C. *et al.* Perspectivas futuras do tratamento farmacológico para doença de Alzheimer: uma revisão integrativa. **Revista JRG de Estudos Acadêmicos**, v. 8, n. 18, 2025.

SAXENA, A.; SUN, W.; LUO, C.; MYERS, T. M.; DOCTOR, B. P. Human serum butyrylcholinesterase: structure, function and detoxification of organophosphates. **Mini-Reviews in Medicinal Chemistry**, v. 9, n. 2, p. 166–176, 2009.

SCHMIDT, K. G.; HOROWITZ, Y.; BUCKMAN, G.; SEGEV, E.; LEVINGER, E.; GEYER, O. Lowering of IOP by echothiophate iodide in pseudophakic eyes with glaucoma. **Current Eye Research**, v. 35, p. 698–702, 2010.

SELTZER, B. Donepezil: a review. **Expert Opinion on Drug Metabolism & Toxicology**, v.

1, n. 3, p. 527–536, 2005.

SENN, H. M.; THIEL, W. QM/MM methods for biomolecular systems. **Angewandte Chemie International Edition**, v. 48, n. 7, p. 1198–1229, 2009.

SENTHILKUMAR, K. *et al.* Analysis of polarization in QM/MM modelling of biologically relevant hydrogen bonds. **Journal of the Royal Society Interface**, v. 5, n. 3, p. 207–216, 2008.

SINGH, Y. P.; PRASAD, S.; KUMAR, H. A comprehensive analysis on galantamine-based hybrids for the management of Alzheimer's disease. **Chemical Biology & Drug Design**, v. 104, p. e70004, 2024.

SILMAN, I.; SUSSMAN, J. L. Acetylcholinesterase: 'classical' and 'non-classical' functions and pharmacology. **Current Opinion in Pharmacology**, v. 5, n. 3, p. 293–302, 2005.

SILMAN, I. The multiple biological roles of the cholinesterases. **Progress in Biophysics and Molecular Biology**, v. 162, p. 41–56, 2021.

SILVA, G. R.; JÚNIOR, I. B.; FIGUEROA-VILLAR, J. D. Defesa química: histórico, classificação dos agentes de guerra e ação dos neurotóxicos. **Química Nova**, v. 35, n. 10, p. 2083–2091, 2012.

SILVEIRA, R. L. V. de A. Fitotoxicidade de glifosato em Eucalyptus. **Addubare**, Piracicaba, v. 2, n. 9, p. 4–7, 2003.

SINGH, M.; KAUR, M.; KUKREJA, H.; CHUGH, R.; SILAKARI, O.; SINGH, D. Acetylcholinesterase inhibitors as Alzheimer therapy: from nerve toxins to neuroprotection. **European Journal of Medicinal Chemistry**, v. 70, p. 165–188, 2013.

SLIWOSKI, G.; KOTHIWALE, S.; MEILER, J.; LOWE JR., E. W. Computational methods in drug discovery. **Pharmacological Reviews**, Baltimore, v. 66, n. 1, p. 334–395, 2014.

SMITH, M. A. C. Doença de Alzheimer. **Revista Brasileira de Psiquiatria**, São Paulo, v. 21, supl. 2, p. 3–7, 1999.

SMULDERS, C. J.; BUETERS, T. J.; VAILATI, S.; VAN KLEEF, R. G.; VIJVERBERG, H. P. Block of neuronal nicotinic acetylcholine receptors by organophosphate insecticides. **Toxicological Sciences**, v. 82, p. 545–554, 2004.

SOGORB, M. A.; VILANOVA, E. Enzymes involved in the detoxification of organophosphorus, carbamate and pyrethroid insecticides through hydrolysis. **Toxicology Letters**, v. 128, p. 215–228, 2002.

SORIN *et al.* A comprehensive review of cholinesterase modeling and simulation. **Biomolecules**, 2021.

SOREQ, H. *et al.* Molecular cloning and construction of the coding region for human acetylcholinesterase reveals a G+C-rich attenuating structure. **Proceedings of the National Academy of Sciences of the United States of America**, v. 87, p. 9688–9692, 1990.

SOREQ, H.; SEIDMAN, S. Acetylcholinesterase: new roles for an old actor. **Nature Reviews Neuroscience**, v. 2, p. 294–302, 2001.

SREE, R. R.; KALYAN, M.; ANAND, N.; MANI, S.; GORANTLA, V. R.; SAKHAR-KAR, M. K.; SONG, B.-J.; CHIDAMBARAM, S. B. Newer therapeutic approaches in treating Alzheimer's disease: a comprehensive review. **ACS Omega**, v. 10, 2025.

STODDART, J. F. **Comprehensive organic chemistry: the synthesis and reaction of organic compounds**. Oxford: Pergamon Press, 1979. v. 6, p. 1228.

SVOBODOVA, B.; SOUKUP, O.; KORABECNY, J. Revisiting butyrylcholinesterase in Alzheimer's disease: a hub linking cholinergic, metabolic and affective pathways. **Ageing Research Reviews**, v. 119, art. 103187, 2026.

SUN, T.; ZHEN, T.; HARAKANDI, C. H.; WANG, L.; GUO, H.; CHEN, Y.; SUN, H. New insights into butyrylcholinesterase: pharmaceutical applications, selective inhibitors and multitarget-directed ligands. **European Journal of Medicinal Chemistry**, v. 275, p. 116569, 2024.

SUSSMAN, J. L. *et al.* Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototypic acetylcholine-binding protein. **Science**, v. 253, p. 872–879, 1991.

ŚWIT, P.; POLLAP, A.; ORZEŁ, J. Spectroscopic determination of acetylcholine (ACh): a representative review. **Topics in Current Chemistry**, v. 381, n. 4, p. 16, 2023.

SZABO, A.; OSTLUND, N. S. **Modern quantum chemistry**. United Kingdom: Constable and Company, 1996. n. 3, p. 162–164.

TAN, C. C. *et al.* Efficacy and safety of donepezil, galantamine, rivastigmine, and memantine for the treatment of Alzheimer's disease: a systematic review and meta-analysis. **Journal of Alzheimer's Disease**, v. 41, p. 615–631, 2014.

TAYLOR, P.; BROWN, J. H. Nicotinic receptors. In: SIEGEL, G. J.; AGRANOFF, B. W.; ALBERS, R. W. (ed.). **Basic neurochemistry: molecular, cellular, and medical aspects**. Philadelphia: Raven Press, 1994. p. 248–254.

TAYLOR, P. Cholinesterase inhibitors. In: BRUNTON, L. L.; HILAL-DANDAN, R.; KNOLLMANN, B. C. (ed.). **Goodman & Gilman's: The pharmacological basis of therapeutics**. 13. ed. New York: McGraw-Hill Education, p. 161–176, 2018.

THOMSEN, R.; CHRISTENSEN, M. H. MolDock: a new technique for high-accuracy molecular *docking*. **Journal of Medicinal Chemistry**, Washington, v. 49, n. 11, p. 3315–3332, 2006.

TOUGU, V. Acetylcholinesterase: mechanism of catalysis and inhibition. **Current Medicinal Chemistry – CNS Agents**, v. 1, p. 155–170, 2001.

TSIM, K. W. K.; SOREQ, H. Acetylcholinesterase: old questions and new developments. **Frontiers in Molecular Neuroscience**, v. 5, art. 101, 2013.

TUCKERMAN, M. E.; MARTYNA, G. J. Understanding Modern Molecular Dynamics: Techniques and Applications. **Journal of Physical Chemistry B**, v. 104, p. 159–178, 2000.

VAN DER KAMP, M. W.; MULHOLLAND, A. J. Combined quantum mechanics/molecular mechanics (QM/MM) methods in computational enzymology. **Biochemistry, Washington**, v. 52, n. 16, p. 2708–2728, 2013.

VAN DER SPOEL, D.; LINDAHL, E.; HESS, B.; GROENHOF, G.; MARK, A. E.; BERENDSEN, H. J. C. GROMACS: Fast, flexible, and free. **Journal of Computational Chemistry**, v. 26, p. 1701–1718, 2005.

VENTURA, A. L. M. *et al.* Sistema colinérgico: revisitando receptores, regulação e a relação com a doença de Alzheimer, esquizofrenia, epilepsia e tabagismo. **Revista de Psiquiatria Clínica**, v. 37, n. 2, p. 66–72, 2010.

VINHAL, D. C.; SOARES, V. H. C. Intoxicação por organofosforados: uma revisão da literatura. **Revista Científica FacMais**, v. 14, n. 3, p. 2238–8427, 2018.

WANG, C. *et al.* PEGylated organophosphorus hydrolase combined with butyrylcholinesterase: dual-enzyme synergistic biocatalysis for acute organophosphate poisoning. **Bioorganic Chemistry**, v. 175, 109836, 2026.

WARSHEL, A.; LEVITT, M. Theoretical studies of enzymic reactions: dielectric, electrostatic and steric stabilization of carbonium-ion in reaction of lysozyme. **Journal of Molecular Biology**, v. 103, p. 227–249, 1976.

WONG, L. *et al.* Mechanism of oxime reactivation of acetylcholinesterase analyzed by chirality and mutagenesis. **Biochemistry, Washington**, v. 39, n. 19, p. 5750–5757, 2000.

WOOLF, N. J. Cholinergic systems in mammalian brain and spinal cord. **Progress in Neurobiology**, v. 37, p. 475–524, 1991.

WOREK, F. *et al.* Reappraisal of indications and limitations of oxime therapy in organophosphate poisoning. **Human & Experimental Toxicology**, v. 26, n. 3, p. 215–219, 2007.

WOREK, F.; THIERMANN, H.; WILLE, T. Organophosphorus compounds and oximes: a critical review. **Archives of Toxicology**, v. 94, n. 7, p. 2275–2292, 2020.

WOREK, F.; THIERMANN, H.; WILLE, T. Catalytic bioscavengers in nerve agent poisoning: a promising approach?. **Toxicology Letters**, Amsterdam, v. 244, p. 143–148, 2016.

YU, J.; FU, Y.; FAN, F.; CAO, Z. Degradation of Novichok nerve agent A234 by phosphotriesterase: insight into the pH-regulated mechanism from QM/MM and MM MD simulations. **Physical Chemistry Chemical Physics**, v. 28, n. 4, p. 3079–3088, 2026.

YU, T. W.; LANE, H. Y.; LIN, C. H. Novel therapeutic approaches for Alzheimer's disease: an updated review. **International Journal of Molecular Sciences**, v. 22, n. 15, p. 8208, 2021.

ZWICKL, C. M.; GRAHAM, J.; JOLLY, R.; BASSAN, A. Principles and procedures for assessment of acute toxicity incorporating *in silico* methods. **Computational Toxicology**, v. 24, p. 100237, 2022.

SEGUNDA PARTE

Artigo 1

Reactivation of cholinesterases to combat sarin, cyclosarin and VX poisoning: an *in silico* study

Artigo aceito pela revista Combinatorial Chemistry & High Throughput Screening

ABSTRACT

Introduction: Neurotoxic agents are potent inhibitors of cholinesterase enzymes. Inhibited cholinesterases can be reactivated using oximes. In this line, computational techniques were used to study the reactivation of the human acetylcholinesterase (*HssAChE*) and butyrylcholinesterase (*HssBChE*) enzymes.

Methods: The 3D structures of the oximes were built in the GaussView® program and optimized in Gaussian 09W®, at the Density Functional Theory level, with the 6-31g (d, p) basis set and B3LYP density functional. In the molecular *docking* software, the oximes were docked in the active site of the *HssAChE* and *HssBChE* enzymes, regarding the residues within a 12 Å radius around the active ligand as flexible. The most stable conformation of the oximes was selected for the quantum mechanics / molecular mechanics (QM/MM) calculations, wherein QM calculations were conducted using density functional theory (DFT) with the Gaussian 09 software package.

Results: Our theoretical results obtained from molecular *docking* showed that oximes 2, 4, and 5 exhibit the lowest intermolecular interaction energies. In the reaction mechanism simulation, the most effective compounds for reactivating cholinesterases inhibited by VX (the most toxic chemical agent) are oximes 3 and 6. The activation energy analyses align with the experimental findings of Kr for the reference oxime K868. The LD50 value of oxime 3 (2.936) exceeds that of oxime K868 (2.836), indicating lower toxicity, while oxime 6 (2.833) exhibits similar toxicity to K868 (2.836).

Conclusion: Through this theoretical study, it was possible to investigate new oximes with the potential to effectively reactivate cholinesterases, aiming to identify oximes suitable for universal applications.

Keywords: Theoretical study, Neurotoxic agents, Oximes, Cholinesterases, Molecular *docking*, QM/MM

1. INTRODUCTION

Chemical warfare agents (CWAs) were developed to be used by governments during times of war, causing mass casualties and, consequently, severe damage to the environment. These agents are considered toxic when they cause serious injuries or death, and can even be incapacitating when they produce only temporary effects and do not lead to death, depending on the dose used [1,2]. CWAs are classified as neurotoxic due to their high toxicity to the nervous system. These agents are widely used by both military forces and terrorist groups due to their low production cost [3,4]. Among neurotoxic agents, organophosphorus compounds (OPs) are among the most commonly used chemical weapons, designed to cause mass destruction. This means these weapons are frequently used in terrorist attacks, peripheral conflicts, and political assassinations [1,5]. Due to the widespread use of OPs, many people are poisoned by them, as their primary mechanism of action is the inhibition of human cholinesterase enzymes (*HssChEs*) [6-8].

Among the OPs, the agents sarin, cyclosarin, and VX have been extensively investigated due to their wide use and high toxicity. Sarin (methyl-isopropoxyfluorophosphine oxide), cyclosarin (fluoromethylphosphoryloxycyclohexane), and VX (ethyl-S-2-diisopropylaminoethylmethylphosphonothiolate) are synthetic compounds used as chemical weapons. These compounds bind very strongly and stably to the esteratic site of cholinesterases (ChEs), thereby inhibiting their activity [1,9].

The ChEs, human acetylcholinesterase (*HssAChE*) and human butyrylcholinesterase (*HssBChE*), are enzymes responsible for hydrolyzing acetylcholine (ACh). These enzymes are found in the nervous system, where they work together to catalyze the hydrolysis of ACh, reducing its levels and interrupting communication between nerve cells [10]. ACh is a neurotransmitter located in the axon terminal, the region at the end of neurons. It is stored in synaptic vesicles, but when a nerve impulse reaches the terminal axon, ACh is released into the synaptic cleft, where it binds to cholinergic receptors on the next neuron. This interaction regenerates the nerve impulse in the next neuron, continuing the transmission of the signal [11-14]. After transmission, the interaction of ACh with its receptors must be interrupted to prevent excess nerve stimulation, which could disrupt normal bodily functions [11]. *HssAChE* facilitates the breakdown of ACh into choline (Ch) and acetate. However, in the case of poisoning, the inhibition of *HssAChE* can lead to serious health problems, including cholinergic syndrome and even death [9,15,16].

HssAChE is located in both the central and peripheral nervous systems and is renowned for its exceptional hydrolytic efficiency [9, 17, 18]. Each monomer of the *HssAChE* enzyme contains a catalytic center, which includes the catalytic subsite, the catalytic triad, and the anionic subsite, where the positively charged nitrogen of ACh binds [9, 16]. The enzyme's active site, located in the "throat" region, can be divided into two distinct and significant areas. The first is the esterase site, which contains the catalytic triad. The second is the anionic site, where ACh interacts with the Ch component. The human catalytic triad consists of serine, glutamate, and histidine residues (Ser203, Glu334, and His447), which are situated at the posterior region of the active site's "throat" [17, 19, 20]. Inhibition of *HssAChE* by OPs occurs via the S_N2 (bimolecular nucleophilic substitution) reaction. This inhibitory process occurs when the oxygen atom of the Ser203 residue (nucleophile) attacks the phosphorus center of the OP [21].

When comparing *HssAChE* with *HssBChE*, it is important to note that *HssBChE* has a lower interaction with ACh, but it becomes essential when ACh levels are high [22]. The physiological role of *HssBChE* is not fully understood. *HssBChE* can degrade a wide range of ester-containing compounds, performing important pharmacological and toxicological functions, such as in the metabolism of lipoproteins and drugs [23, 24].

In cases of poisoning by OPs, oximes are chemical agents used to reactivate ChEs. For example, the oxime K868 (Figure 1), proposed by Zorbaz (2018), is a potent dichlorinated analogue of oxime K027 (Figure 1). Oxime K868 has demonstrated significantly improved efficiency in reactivating both *HssAChE* and *HssBChE*, thanks to its enhanced interaction and molecular recognition [25,26].

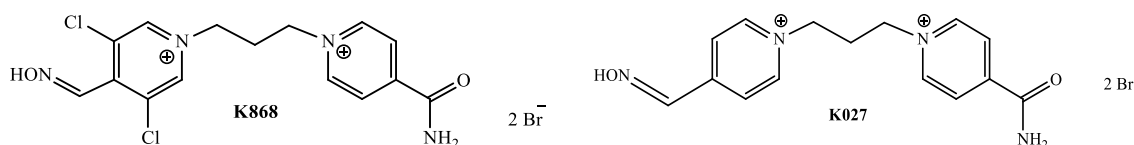


Figure 1. Chemical structures of the oximes K868 and K027.

In this study, oxime K868 and its derivatives (oximes 1–6; Table 1), as well as oxime K027 (oxime 7; Table 1) and its derivatives (oximes 8 and 9; Table 1), were used to assess their ability to reactivate the *HssAChE* and *HssBChE* enzymes, which had been inhibited by OPs. Computational methods, such as molecular *docking*, were employed to gain insights into the interaction modes of these oximes within the active site of both enzymes. Additionally, the

QM/MM approach was used to investigate the reaction mechanisms involved in reactivating the inhibited *HssAChE* and *HssBChE* enzymes using different oximes.

2. METHODS

2.1. Molecular *docking*

To determine the initial conformation of the oximes, their three-dimensional structures were generated using GaussView[®] software and optimized in Gaussian 09W[®] at the DFT level, with the 6-31G(d,p) basis set and the B3LYP density functional [27]. The atomic partial charges were calculated using the same level of theory [28-30].

After constructing the 3D structures of the oximes, the molecules were imported into the Molegro Virtual Docker (MVD) program [31, 32], where the oximes were docked in the crystallographic structure of the *HssAChE* enzyme (PDB code 6U37; resolution = 2.25 Å; active ligand = RS194B) [33, 34]. In addition, the oximes were also docked in the *HssBChE* active site (PDB code 1P0I; resolution = 2.00 Å) [33, 34], following the same procedures used in prior studies [29, 30, 35-37]. The structures of *HssAChE* and *HssBChE* taken from the Protein Data Bank (PDB) were modeled, being inhibited by sarin, cyclosarin and VX. In the *docking* procedure, the identification of the ligand interaction modes is an iterative process that involves evaluating different ligand conformations and orientations, as well as estimating their interaction energies with the protein. The most favorable solutions are selected for further analysis [38].

To achieve optimal results, residues within a 12 Å radius around the active ligand were treated as flexible. The most favorable conformation of the oximes was chosen based on its highest degree of spatial similarity to the active ligand. Due to the nature of *docking* methods, the calculations were carried out generating 30 poses (conformation and orientation) for each ligand. For *docking* validation processes, the re-*docking* calculations were used. Re-*docking* is a crucial step in validating the *docking* protocol and assessing the reliability of the *docking* results. It involves *docking* a ligand back into the binding site of its target protein, typically using its experimentally known binding pose (e.g., from X-ray crystallography or NMR). The re-*docking* results are then compared to the experimentally observed structure to evaluate the accuracy of the *docking* algorithm and parameters [39].

The MolDock scoring function employed in the MVD program comes from the piecewise linear potential (PLP), a simplified potential whose parameters are fitted to protein-

ligand structures, binding data scoring functions and further extended in Generic Evolutionary Method for molecular *docking* with a new hydrogen bonding term and new charge schemes [40]. The *docking* scoring function values, E_{score} , are defined by Eq. 1:

$$E_{\text{score}} = E_{\text{inter}} + E_{\text{intra}} \quad (1)$$

Wherein:

$$E_{\text{inter}} = \sum_{i \in \text{ligand}} \sum_{j \in \text{protein}} \left[E_{\text{PLP}}(r_{ij}) + 332.0 \frac{q_i q_j}{4r_{ij}^2} \right] \quad (2)$$

E_{PLP} represents “piecewise linear potential”, which consists of the use of two different parameter sets, as described forward: one for approximation of the steric term (Van der Waals) among atoms, and the other potential for the hydrogen bonding. The second term is related to the electrostatic interactions among overloaded atoms. It is a Coulomb potential with a dielectric constant dependent on the distance ($D(r) = 4r$). The numerical value of 332.0 is responsible for the electrostatic energy unit to be given in kilocalories per molecule [34]. E_{intra} is the internal energy of the ligand:

$$E_{\text{intra}} = \sum_{i \in \text{ligand}} \sum_{j \in \text{ligand}} E_{\text{PLP}}(r_{ij}) + \sum_{\text{flexiblebonds}} A[1 - \cos(m \cdot \theta - \theta_0)] + E_{\text{clash}} \quad (3)$$

The first part of the equation (double summation) is among all pairs of atoms in the ligand, taking off those which are connected by two bonds. The second one characterizes the torsional energy, wherein θ is the torsional angle of the bond. If several torsions could be determined, each torsional energy is considered and there is the use of an average among them. The last term, E_{clash} , assigns a penalty of 1,000 if the distance between two heavy atoms (more than two bonds apart) is smaller than 2.0 Å, not taking into account infeasible ligand conformations [34]. The *docking* search algorithm that is applied in the MVD program considers an evolutionary algorithm, interactive optimization techniques which are inspired by Darwinian evolution theory, and a new hybrid search algorithm called guided differential evolution. This hybrid combines the differential evolution optimization technique with a cavity prediction algorithm during the search process, allowing that way a fast and accurate identification of potential binding modes (poses) [34, 38, 41].

The most stable conformation of the oximes in the active sites of *HssAChE* and *HssBChE* was determined and chosen for the QM/MM calculations. Prior to this, the toxicities

of the proposed oximes were investigated. In our study, we selected molecular *docking* and QM/MM as the primary computational methods due to their ability to provide both a detailed and efficient analysis of the interactions between our compounds and their targets [42]. Molecular *docking* is an established and widely used tool for predicting the binding modes and interactions of ligands to proteins, making it ideal for the initial stages of our investigation. This method allows for high-throughput screening of potential compounds in a computationally feasible manner [43].

For more precise modeling of key interactions, particularly in cases where electronic effects are important, we employed QM/MM. This hybrid approach combines the computational efficiency of molecular mechanics for the bulk of the system with the accuracy of quantum mechanics for the critical active site, allowing us to capture detailed information that is crucial for understanding the binding mechanism at the atomic level [44]. More details about this method are displayed ahead. These methods have been successfully applied in similar studies [45, 46], and their combination allows for a more comprehensive and accurate assessment of molecular interactions and reaction mechanisms. Therefore, *docking* and QM/MM were chosen as the most appropriate tools for achieving both computational efficiency and the required level of detail for this study.

2.2. Toxicity Prediction

2.2.1. LD50

The website (<https://biosig.lab.uq.edu.au/pkcsim/prediction>) allows for the safe measurement of compound toxicity based on computational methods. In this case, the pkCSM server was used to determine the toxic effects of the 10 reactivators with the best interaction energy and best overlap selected in the molecular *docking* [36]. The server in question measures the mean lethal dose (LD₅₀) [40, 47].

2.2.2. Absorption, Distribution, Metabolism and Excretion Test (ADME)

Using the Swiss-ADME server (www.swissadme.ch/index.php), the solubility and pharmacokinetics of the 10 reactivators suggested as pharmaceuticals for the treatment of OP's poisoning were analyzed [48]. ADME analysis is an important test performed on molecules with the potential to become therapeutic agents [40]. Diverse compounds with the potential to

become pharmaceuticals do not come close to testing due to the lack of their pharmacokinetic properties [48].

2.3. QM/MM approach

Highly correlated QM methods are not suitable for treating large systems like proteins due to their significant computational requirements. In this study, a QM/MM approach was employed, integrating quantum mechanics (QM) with molecular mechanics (MM) methods to investigate the reactivation reactions of *HssAChE* and *HssBChE* enzymes by oximes. To achieve more precise results and assess electronic effects, QM calculations were carried out using density functional theory (DFT) with the Gaussian 09 software package (DFT) level with the Gaussian 09 package [29, 30, 49]. DFT methods are widely used and demonstrate satisfactory performance when applied to large systems, including biomolecules [29, 30, 49, 50].

Calculations were carried out based on the generalized gradient approximation [36, 51]. Final geometries were obtained using DFT, Becke's three-parameter method, and Lee, Yang, and Paar's corrected gradient functional (B3LYP) [28], with a 6-31g (d, p) basis set. The QM system for reactivation of the *HssAChE* enzyme by oximes included Ser203, along with the organophosphorus compound, oximes and amino acid residues within a radius of 5 Å, while the QM system for reactivation of the *HssBChE* consisted of Ser198 of the catalytic triad, the organophosphorus compound, oximes and amino acid residues, also within a radius of 5 Å. This radius covers a region capable of simulating the mechanism pathway with the essential species that directly or indirectly participate in the reaction.

3. RESULTS AND DISCUSSION

3.1. Molecular *Docking*

3.1.1. *Docking* study between oximes and the *HssAChE* enzyme inhibited by sarin

The molecular *docking* technique is crucial in toxicity assessment because it provides an ethical alternative to animal testing in research, allowing for the computational prediction of chemical interactions with biological targets. By simulating how a molecule binds to a receptor or enzyme, *docking* predicts intermolecular interactions that may indicate toxic effects, such as

the inhibition of essential biological processes. This reduces the need for *in vivo* experiments, promoting a more efficient and ethical study [52-54].

Furthermore, molecular *docking*-based toxicity evaluation can identify potential toxins before their actual exposure to the organism, allowing for the assessment of substances at different stages of development or industrial use. This becomes a valuable tool in chemical safety, helping toxicologists predict and assess the risks of new substances or those in contact with the environment. Thus, the combination of *docking* and toxicity analysis offers a faster, cheaper, and more ethical way to study the impacts of chemicals on human and environmental health [53-55].

The use of ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) evaluation is fundamental in this context because it allows for a more comprehensive analysis of the feasibility of compounds for therapeutic use. ADMET provides essential information about how compounds may behave within the human body, including their ability to be absorbed, effectively distributed, metabolized safely, and excreted without causing toxic accumulation [56, 57].

The binding site of the *HssAChE* enzyme inhibited by sarin was identified using Molegro Virtual Docker (MVD) [34]. A cavity prediction algorithm was employed, utilizing a three-dimensional box with a cavity volume of 89.6 Å³. To validate this method, the Root Mean Square Deviation (RMSD) of the *re-docking* results was computed. After superimposing the structures of two molecules, their atomic coordinates were compared. This is the most common and effective method for evaluating structural and positional differences in *in silico* studies [58].

In the *re-docking* calculation, the co-crystallized ligand from the enzyme (PDB: 6U37) was subjected to molecular *docking*. After extracting the best pose with the lowest interaction energy from the 30 poses generated, it was superimposed onto the initial structure of the co-crystallized ligand. The RMSD was then calculated based on the differences in atomic positions [59]. *Re-docking* serves as an essential quality control step in molecular *docking* studies, ensuring that the computational approach is reliable and appropriately parameterized for the system under study. By verifying the protocol with experimentally validated complexes, researchers can confidently extend their predictions to novel ligands, improving the reliability of the *docking* outcomes [39].

When the structure of the RS194B reactivator (active ligand) was overlaid onto the *HssAChE* active site, the RMSD for the alignment was 1.26 Å. Literature indicates that RMSD values below 2.00 Å are considered satisfactory [60-62]. This result validates the theoretical method used for the molecular *docking* calculations. The intermolecular interaction energy

obtained from re-*docking* was $-101.46 \text{ kcal mol}^{-1}$. The active ligand formed a hydrogen bond with the Tyr72 residue, with the interaction occurring between the oxygen atom of the amino acid and the hydroxyl group of the active ligand.

Once the molecular *docking* method was validated, the oximes listed in Table 1 were docked into the active site of the sarin-inhibited *HssAChE* enzyme. To understand the differences in interaction modes among the oximes within the enzyme's active site and identify the factors influencing each compound's activity, intermolecular interactions, including ligand-protein hydrogen bonds, were assessed (see Table 2). The interaction of the ligand with the receptor was measured by predicting the preferred orientation and minimum binding energy [63]. The *docking* process can predict various types of intermolecular interactions, such as hydrogen bonds, Van der Waals forces, hydrophobic interactions, and electrostatic forces [64].

Table 1. Chemical structures of the K868 oxime analogues studied in this work.

Oximes	2D Structures	Molecular Formula
1		$C_{15}H_{18}Cl_2N_4O_2$ 2 Br ⁻
2		$C_{15}H_{18}Cl_2N_4O_2$ 2 Br ⁻
3		$C_{15}H_{18}Cl_2N_4O_2$ 2 Br ⁻
4		$C_{15}H_{16}Cl_4N_4O_2$ 2 Br ⁻
5		$C_{15}H_{18}F_2N_4O_2$ 2 Br ⁻
6		$C_{15}H_{18}Br_2N_4O_2$ 2 Br ⁻
7		$C_{15}H_2ON_4O_2$ 2 Br ⁻
8		$C_{15}H_2ON_4O_2$ 2 Br ⁻
9		$C_{15}H_2ON_4O_2$ 2 Br ⁻

Table 2. Values of intermolecular interaction energy (kcal mol⁻¹), hydrogen bond energy (kcal mol⁻¹) and the amino acid residues of *HssAChE* inhibited by sarin, obtained by molecular *docking*.

Interactions between sarin-inhibited <i>HssAChE</i> and oximes			
Oximes	Intermolecular interaction energy (kcal mol ⁻¹)	Hydrogen bond energy (kcal mol ⁻¹)	Amino acid residues
2	-160.42	-7.57	Glu202, Gly120, Phe295, Tyr124, Tyr133
K868	-157.51	-10.15	Glu202, Gly120, Phe295, Tyr124, Tyr133, Arg296
6	-152.60	-6.53	Gly120, Tyr124
3	-150.58	-12.36	Glu202, Gly120, Phe295, Tyr124, Tyr337
4	-150.18	-10.64	Gly120, Tyr124
5	-149.82	-10.41	Glu202, Gly120, Tyr124, Tyr337
1	-147.20	-8.29	Glu202, Gly120, Phe295, Tyr124
8	-138.89	-10.80	Phe295, Tyr124, Ser125
9	-135.48	-9.05	Glu202, Phe295, Tyr124, Arg296, Ser203, His447
7 (K027)	-134.32	-8.55	Glu202, Gly120, Tyr337

*Values of intermolecular interaction energy (kcal mol⁻¹) refer to the forces that bind the molecule to the enzyme's active site, influencing the stability of the interactions. Hydrogen bond energy (kcal mol⁻¹) measures the strength of interactions between hydrogen atoms and electronegative atoms in the enzyme's amino acid residues and the oximes. The amino acid residues of *HssAChE*, inhibited by sarin, interact with the oximes as obtained through molecular *docking*. These parameters enable the evaluation of interaction stability, contributing to the understanding of the enzyme's reactivation potential by the studied oximes.

As shown in Table 2, oxime 2 exhibited the highest stability in terms of intermolecular interaction energy, with a value of -160.42 kcal mol⁻¹, indicating that it has the most stabilizing interaction within the *HssAChE* active site. Additionally, the molecular *docking* calculations revealed the hydrogen bonds formed between each oxime and the enzyme, facilitating the

identification of which amino acid residues interact most strongly with the ligands considered in this study. Regarding the intermolecular interactions, oxime 2 displayed a hydrogen bond energy value of $-7.57 \text{ kcal mol}^{-1}$ and interacted through hydrogen bonding in the active site with the glutamate, glycine, phenylalanine, and tyrosine residues (Figure 2). The interaction with tyrosine is particularly relevant, as this amino acid is often associated with the stabilization of ligands in the active site of enzymes due to its aromatic properties, which facilitate the formation of π - π interactions and other non-covalent interactions. The presence of phenylalanine may be important for modulating enzyme specificity, as the aromatic chain of this residue can help orient the oxime in an optimized manner for enzyme reactivation.

The second compound with the highest interaction with the active site is oxime K868, which serves as the reference compound in these studies, with an interaction energy value of $-157.51 \text{ kcal mol}^{-1}$. The energy difference between oxime K868 and oxime 2 is $2.91 \text{ kcal mol}^{-1}$. Oxime K868 showed a hydrogen bond energy of $-10.15 \text{ kcal mol}^{-1}$ and interacted with the glutamate, glycine, phenylalanine, tyrosine, and arginine residues (Figure 3). The interaction with arginine is intriguing, as the side chain of the guanidine group can form strong ionic bonds, potentially providing additional stability to the interaction in the active site. The third compound with the most stabilizing intermolecular interaction energy is oxime 6, with an energy of $-152.60 \text{ kcal mol}^{-1}$. The energy difference between oxime 6 and oxime K868 is $4.91 \text{ kcal mol}^{-1}$. Oxime 6 displayed a hydrogen bond energy of $-6.53 \text{ kcal mol}^{-1}$ and interacted with glycine and tyrosine. Although oxime 6 exhibits a less stable interaction compared to the previous ones, the interaction with tyrosine remains relevant for the stability of the complex. The fourth compound with the most stabilizing energy is oxime 3, which showed an interaction energy of $-150.58 \text{ kcal mol}^{-1}$. The energy difference between oxime 3 and oxime K868 is $6.93 \text{ kcal mol}^{-1}$. Oxime 3 exhibited a hydrogen bond energy of $-12.36 \text{ kcal mol}^{-1}$ and interacted with glutamate, glycine, phenylalanine, and tyrosine.

Moreover, the energy differences between the oximes and K868 were as follows: $7.33 \text{ kcal mol}^{-1}$ for oxime 4, $7.69 \text{ kcal mol}^{-1}$ for oxime 5, $10.31 \text{ kcal mol}^{-1}$ for oxime 1, $18.62 \text{ kcal mol}^{-1}$ for oxime 8, $22.03 \text{ kcal mol}^{-1}$ for oxime 9, and $23.19 \text{ kcal mol}^{-1}$ for oxime 7 (K027). These values highlight the varying degrees of stability and interaction strength of each oxime compared to the reference compound K868. The greatest energy difference occurred between oximes 7 (K027) and K868, with a value of $23.19 \text{ kcal mol}^{-1}$. Among the compounds investigated, oxime 7 (K027) exhibited the lowest stability in the *HssAChE* active site, with an energetic value of $-134.32 \text{ kcal mol}^{-1}$. It also displayed a hydrogen bond energy of $-8.55 \text{ kcal mol}^{-1}$, interacting with the residues glutamate, glycine, and tyrosine.

An important aspect to mention is that all oximes, including K868, interacted with the tyrosine residue. Additionally, oxime 2, when compared to oxime K868, commonly interacted with the residues glutamate, glycine, phenylalanine, and tyrosine. These interactions reveal not only the importance of tyrosine as a key residue but also the relevance of other interactions, such as glutamate and phenylalanine, for the stability of the complex.

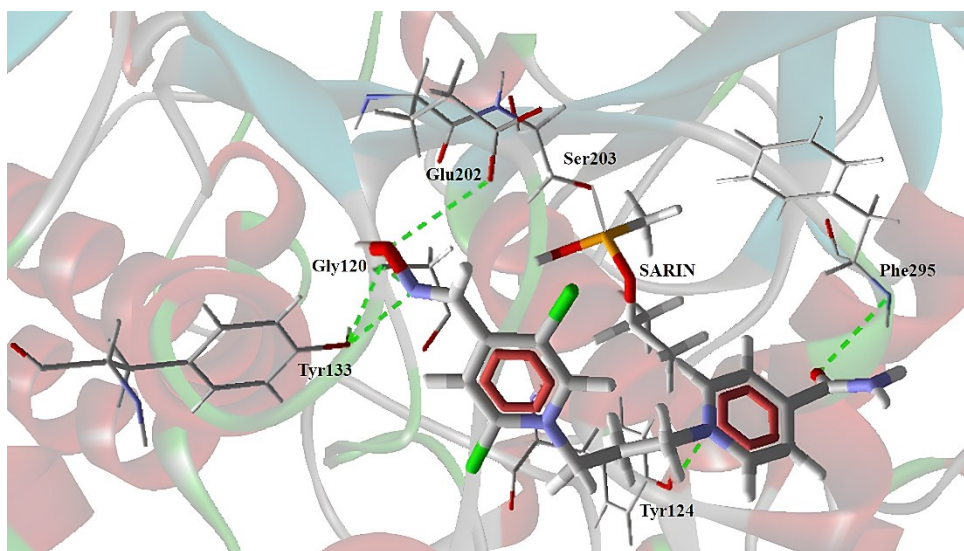


Figure 2. Hydrogen bonds (green dashed lines) between oxime 2 and the residues of the active site of *HssAChE* inhibited by sarin (PDB code 6U37; resolution = 2.25 Å; active ligand = RS194B) are shown. These interactions demonstrate the conformational stability of the oxime in the active site of *HssAChE*, highlighting the specific residues involved in the hydrogen bonds: Glutamic Acid 202, Glycine 120, Phenylalanine 295, and Tyrosines 124 and 133 [36, 37].

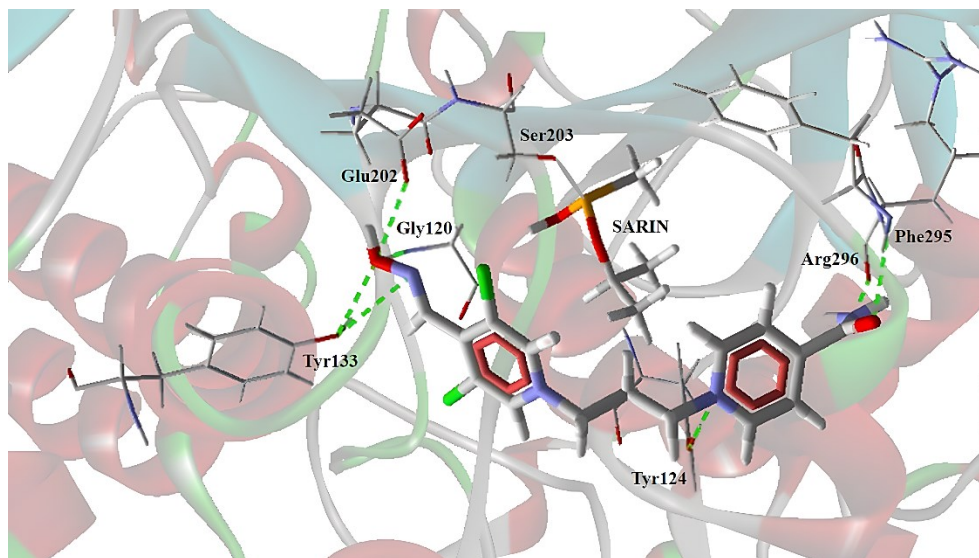


Figure 3. Hydrogen bonds (green dashed lines) between oxime K868 and the residues of the active site of *HssAChE* inhibited by sarin (PDB code 6U37; resolution = 2.25 Å; active ligand = RS194B) are shown. These interactions demonstrate the conformational stability of the oxime in the active site of *HssAChE*, highlighting the specific residues involved in the hydrogen bonds: Arginine 296, Glutamic Acid 202, Glycine 120, Phenylalanine 295, and Tyrosines 124 and 133 [36, 37].

Through molecular *docking* calculations, the conformation assumed by each of the reactivators was observed. Among the oximes investigated, oxime 2 demonstrated the highest interaction within the *HssAChE* active site, suggesting that this compound likely adopts a more favorable conformation for reactivating the *HssAChE* enzyme inhibited by sarin.

3.1.2. *Docking* study between oximes and the *HssAChE* enzyme inhibited by cyclosarin

The oximes listed in Table 3 were docked into the active site of the cyclosarin-inhibited *HssAChE* enzyme. Intermolecular interactions, including ligand-protein hydrogen bonds, were analyzed to assess the differences in interaction modes among the oximes within the enzyme's active site and to identify the factors contributing to the activity of each compound.

Table 3. Values of intermolecular interaction energy (kcal mol⁻¹), hydrogen bond energy (kcal mol⁻¹) and amino acid residues of *HssAChE* inhibited by cyclosarin, obtained by molecular *docking*.

Interactions between cyclosarin-inhibited <i>HssAChE</i> and oximes			
Oximes	Intermolecular interaction energy (kcal mol ⁻¹)	Hydrogen bond energy (kcal mol ⁻¹)	Amino acid residues
4	-165.99	-10.01	Glu202, Gly120, Tyr124, Tyr133
3	-159.67	-12.76	Glu202, Gly120, Phe295, Tyr124, Tyr337
2	-159.37	-11.22	Glu202, Gly120, Tyr124, Tyr337
5	-152.19	-8.34	Phe295, Tyr124, Arg296
9	-150.63	-8.32	Glu202, Gly120, Tyr124, Asp74
7 (K027)	-145.46	-11.36	Phe295, Tyr124, Trp86, Arg296
8	-145.05	-9.08	Glu202, Gly120, Phe295, Tyr124
K868	-143.15	-6.29	Phe295, Tyr133, Tyr337
6	-140.10	-8.20	Phe295, Tyr124, Arg296
1	-130.82	-7.13	Gly120, Phe295, Arg296

*Values of intermolecular interaction energy (kcal mol⁻¹) refer to the forces that bind the molecule to the enzyme's active site, influencing the stability of the interactions. Hydrogen bond energy (kcal mol⁻¹) measures the strength of interactions between hydrogen atoms and electronegative atoms in the enzyme's amino acid residues and the oximes. The amino acid residues of *HssAChE*, inhibited by cyclosarin, interact with the oximes as obtained through molecular *docking*. These parameters enable the evaluation of interaction stability, contributing to the understanding of the enzyme's reactivation potential by the studied oximes.

As shown in Table 3, from most stable to least stable, oxime 4 exhibited the most stabilizing intermolecular interaction energy (-165.99 kcal mol⁻¹). The energy difference between oximes 4 and K868 was 22.84 kcal mol⁻¹. Regarding hydrogen bonds, oxime 4 had a hydrogen bond interaction energy of -10.01 kcal mol⁻¹ and interacted through hydrogen bonding

with the amino acid residues glutamate, glycine, and tyrosine (Figure 4). Tyrosine helps to stabilize ligands in the active site of enzymes through non-covalent interactions, such as π - π bonds, which are crucial for the stabilization of the complex.

The compound with the second most favorable interaction energy is oxime 3, with a value of $-159.67 \text{ kcal mol}^{-1}$. This represents a $16.52 \text{ kcal mol}^{-1}$ energy difference compared to the reference compound, oxime K868, indicating greater stabilization. Oxime 3 exhibited a hydrogen bond energy of $-12.76 \text{ kcal mol}^{-1}$ and interacted with the amino acid residues glutamate, glycine, phenylalanine, and tyrosine. The presence of phenylalanine, an aromatic residue, contributes to the stabilization of the interaction through π - π interactions with the ligand. The third most favorable compound is oxime 2, with an interaction energy of $-159.37 \text{ kcal mol}^{-1}$, $16.22 \text{ kcal mol}^{-1}$ lower than that of oxime K868. Oxime 2 showed a hydrogen bond energy of $-11.22 \text{ kcal mol}^{-1}$ and formed interactions with glutamate, glycine, and tyrosine residues. Oxime 2, like oxime 3, interacted with tyrosine, a residue important for the formation of stable interactions in the active site of *HssAChE*. Overall, the majority of the oximes investigated demonstrated more stabilizing interaction energies compared to the reference compound, oxime K868.

The energy differences relative to oxime K868 were as follows: $9.04 \text{ kcal mol}^{-1}$ for oxime 5, $7.48 \text{ kcal mol}^{-1}$ for oxime 9, $2.31 \text{ kcal mol}^{-1}$ for oxime 7 (K027), $1.90 \text{ kcal mol}^{-1}$ for oxime 8, $3.05 \text{ kcal mol}^{-1}$ for oxime 6, and $12.33 \text{ kcal mol}^{-1}$ for oxime 1. The largest energy difference was observed for oxime 4, with a value of $22.84 \text{ kcal mol}^{-1}$.

The reference compound, oxime K868, exhibited an interaction energy of $-159.37 \text{ kcal mol}^{-1}$ and a hydrogen bond energy of $-6.29 \text{ kcal mol}^{-1}$, forming interactions with phenylalanine and tyrosine (Figure 5). In contrast, oxime 1 was the least stable compound in the group, with an interaction energy of $-130.82 \text{ kcal mol}^{-1}$ and a hydrogen bond energy of $-6.29 \text{ kcal mol}^{-1}$, also interacting with phenylalanine and tyrosine.

Oximes 2, 3, and 4 demonstrated greater stability within the *HssAChE* active site compared to the reference oxime, K868. These oximes consistently interacted with the tyrosine residue via hydrogen bonding. Notably, oxime 3 also interacted with phenylalanine, similar to the reference oxime. Among all the proposed oximes, those that shared a common interaction with tyrosine were oximes 2, 3, 4, 5, 6, 7, 8, and 9. In contrast, the reactivators that interacted with phenylalanine were oximes 1, 3, 5, 6, 7, and 8. These interactions provide valuable insights into the mechanisms of complex stabilization, where tyrosine stands out as a crucial residue for the formation of stable interactions.

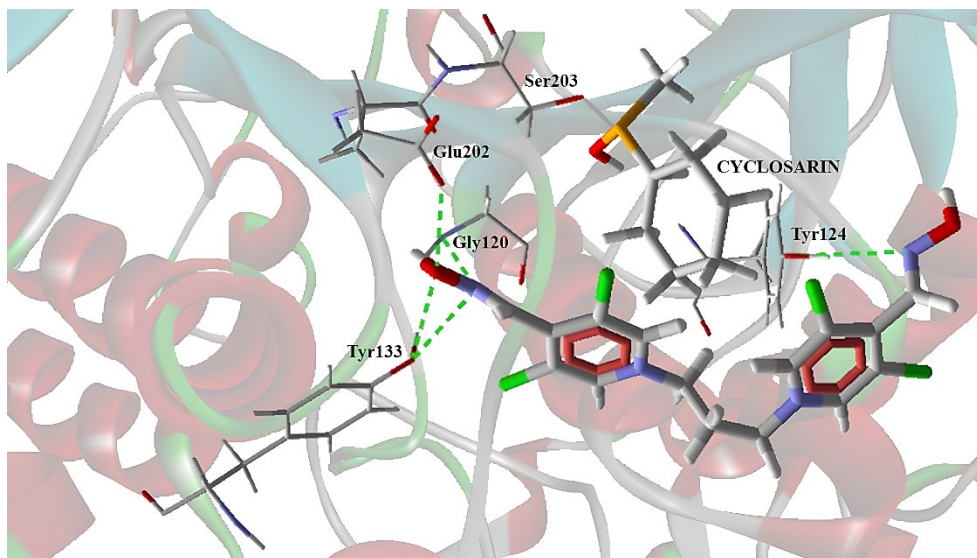


Figure 4. Hydrogen bonds (green dashed lines) between oxime 4 and the residues of the active site of *HssAChE* inhibited by cyclosarin (PDB code 6U37; resolution = 2.25 Å; active ligand = RS194B) are shown. These interactions demonstrate the conformational stability of the oxime in the active site of *HssAChE*, highlighting the specific residues involved in the hydrogen bonds: Glutamic Acid 202, Glycine 120, and Tyrosines 124 and 133 [36, 37].

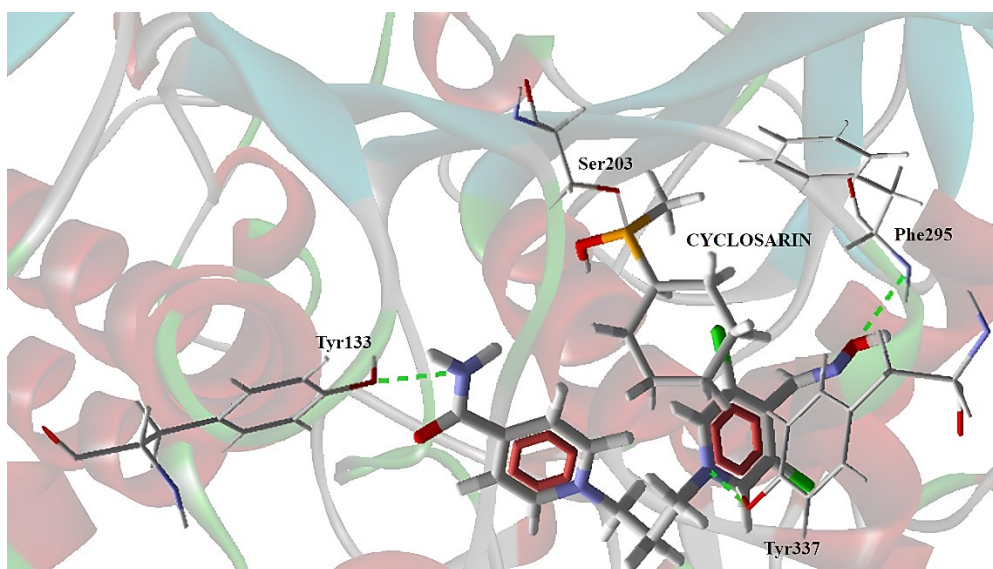


Figure 5. Hydrogen bonds (green dashed lines) between oxime K868 and the residues of the active site of *HssAChE* inhibited by cyclosarin (PDB code 6U37; resolution = 2.25 Å; active ligand = RS194B) are shown. These interactions demonstrate the conformational stability of the oxime in the active site of *HssAChE*, highlighting the specific residues involved in the hydrogen bonds: Phenylalanine 295, and Tyrosines 133 and 337 [36, 37].

Molecular *docking* calculations revealed that, among the reactivators investigated, oxime 4 exhibited the greatest stability within the *HssAChE* active site. These findings suggest that oxime 4 provides a more favorable conformation for reactivating the inhibited *HssAChE* enzyme.

3.1.3. Docking study between oximes and the *HssAChE* enzyme inhibited by VX

The oximes listed in Table 4 were docked into the active site of VX-inhibited *HssAChE*. The table presents the intermolecular interaction energies, hydrogen bond energies, and the corresponding interacting amino acid residues.

Table 4. Values of intermolecular interaction energy (kcal mol^{-1}), hydrogen bond energy (kcal mol^{-1}) and amino acid residues of *HssAChE* inhibited by VX, obtained by molecular *docking*.

Interactions between VX-inhibited <i>HssAChE</i> and oximes			
Oximes	Intermolecular interaction energy (kcal mol^{-1})	Hydrogen bond energy (kcal mol^{-1})	Amino acid residues
4	-159.94	-9.28	Glu202, Gly120, Phe295, Tyr124, Tyr133, Tyr337
3	-152.87	-8.96	Glu202, Gly120, Tyr337
K868	-152.30	-12.32	Arg296, Phe295, Ser293, Tyr124, Tyr133
5	-150.67	-9.22	Arg296, Phe295
1	-148.41	-5.89	Phe295, Tyr124
8	-147.50	-11.36	Arg296, Phe295, Trp86, Tyr124
2	-146.95	-14.37	Arg296, Phe295, Trp86, Tyr124
7 (K027)	-140.25	-11.67	Arg296, Phe295, Ser125, Trp86, Tyr124
6	-139.73	-5.75	Phe295, Tyr337
9	-138.30	-7.92	Phe295, Ser125, Trp86

*Values of intermolecular interaction energy (kcal mol^{-1}) refer to the forces that bind the molecule to the enzyme's active site, influencing the stability of the interactions. Hydrogen bond energy (kcal mol^{-1}) measures the strength of interactions between hydrogen atoms and electronegative atoms in the enzyme's amino acid residues and the oximes. The amino acid residues of *HssAChE*, inhibited by VX, interact with the oximes as obtained through molecular *docking*. These parameters enable the evaluation of interaction stability, contributing to the understanding of the enzyme's reactivation potential by the studied oximes.

As shown in Table 4, oxime 4 was the most stable reactivator in terms of intermolecular interaction energy, with a value of $-159.94 \text{ kcal mol}^{-1}$. The energy difference between oxime 4 and the reference oxime, K868, was $7.64 \text{ kcal mol}^{-1}$. Regarding hydrogen bonds, oxime 4 exhibited an energy value of $-9.28 \text{ kcal mol}^{-1}$ and interacted with key amino acid residues in the active site, including glutamate, glycine, phenylalanine, and tyrosine (Figure 6). The presence of phenylalanine and tyrosine, both aromatic residues, is crucial for the stability of the interaction through π - π bonds, suggesting that oxime 4 has the potential to form strong non-covalent interactions.

The second most favorable reactivator in terms of interaction energy is oxime 3, with a value of $-152.87 \text{ kcal mol}^{-1}$. The energy difference between oxime 3 and the reference oxime, K868, was $0.57 \text{ kcal mol}^{-1}$. Oxime 3 exhibited a hydrogen bond energy of $-8.96 \text{ kcal mol}^{-1}$ and interacted with the amino acid residues glutamate, glycine, and tyrosine. Although oxime 3 shows a slightly weaker interaction than oxime 4, the interaction with tyrosine remains an important factor for its stability in the active site, as tyrosine plays a key role in stabilizing enzyme complexes. The third most stable compound is the reference oxime, K868, with an interaction energy of $-152.30 \text{ kcal mol}^{-1}$. Oxime K868 had a hydrogen bond energy of $-12.32 \text{ kcal mol}^{-1}$ and interacted with the amino acid residues arginine, phenylalanine, serine, and tyrosine (Figure 7). The interaction with arginine, a residue with a charged side chain, may promote the formation of additional ionic interactions, which is relevant for the stabilization of the enzyme complex and could contribute to the reactivation efficacy of the oxime. The fourth reactivator with the most stabilizing interaction energy is oxime 5, with a value of $-150.67 \text{ kcal mol}^{-1}$. The energy difference between oxime 5 and oxime K868 was $1.63 \text{ kcal mol}^{-1}$. Oxime 5 exhibited a hydrogen bond energy of $-9.22 \text{ kcal mol}^{-1}$ and interacted with the residues arginine and phenylalanine. The interaction with arginine is particularly relevant, as this residue can form electrostatic interactions that stabilize the oxime in the active site of *HssAChE*.

The energy differences between oximes and the reference oxime, K868, were as follows: $3.89 \text{ kcal mol}^{-1}$ for oxime 1, $4.80 \text{ kcal mol}^{-1}$ for oxime 8, $5.35 \text{ kcal mol}^{-1}$ for oxime 2, $12.05 \text{ kcal mol}^{-1}$ for oxime 7 (K027), $12.57 \text{ kcal mol}^{-1}$ for oxime 6, and $14.00 \text{ kcal mol}^{-1}$ for oxime 9. The largest energy difference occurred between oxime 9 and K868, with a difference of $14.00 \text{ kcal mol}^{-1}$. Furthermore, oxime 9 was the least stable compound in the *HssAChE* active site, with an interaction energy of $-138.30 \text{ kcal mol}^{-1}$. It exhibited a hydrogen bond energy of $-7.92 \text{ kcal mol}^{-1}$ and interacted with the amino acid residues phenylalanine, serine, and tryptophan. Although the interaction with phenylalanine and serine is advantageous for the stabilization of the oxime, the lack of a strong interaction with tyrosine may explain its lower stability.

Oxime 4, compared to oxime K868, shared hydrogen bond interactions with phenylalanine and tyrosine. Among the proposed oximes, oximes 2, 5, 7, and 8 commonly interacted with arginine, while oximes 1, 2, 4, 5, 6, 7, 8, and 9 commonly interacted with phenylalanine. Additionally, oximes 1, 2, 3, 4, 6, 7, and 8 commonly interacted with tyrosine. Tyrosine, in particular, appears as a critical residue for the formation of stabilizing hydrogen bonds, while arginine and phenylalanine may contribute to electrostatic and π - π stabilization.

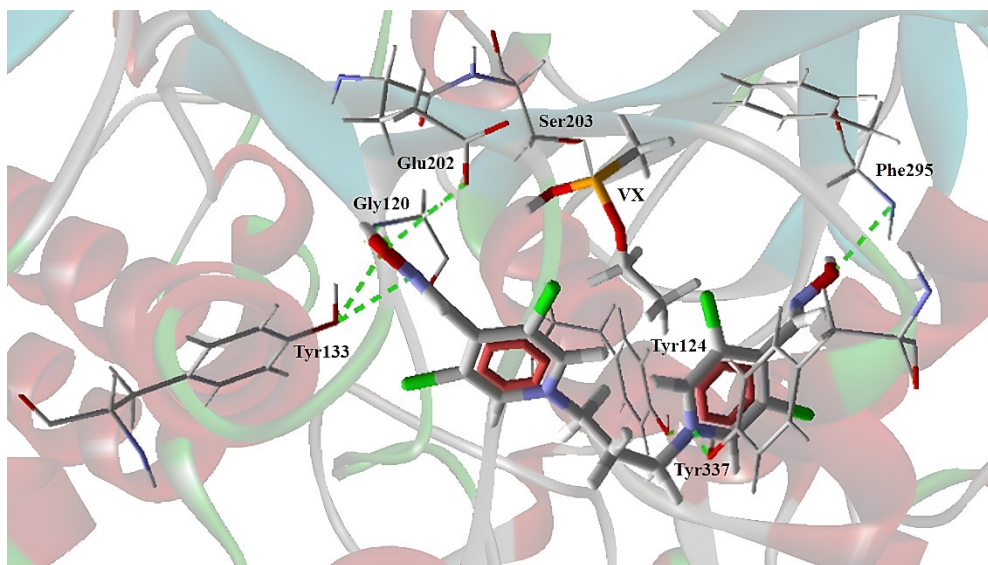


Figure 6. Hydrogen bonds (dashed in green) between oxime 4 and the residues of the active site of *HssAChE* inhibited by VX (PDB code 6U37; resolution = 2.25 Å; active ligand = RS194B) are shown. These interactions demonstrate the conformational stability of the oxime in the active site of *HssAChE*, highlighting the specific residues involved in the hydrogen bonds: Glutamic Acid 202, Glycine 120, Phenylalanine 295, and Tyrosines 124, 133 and 337 [36, 37].

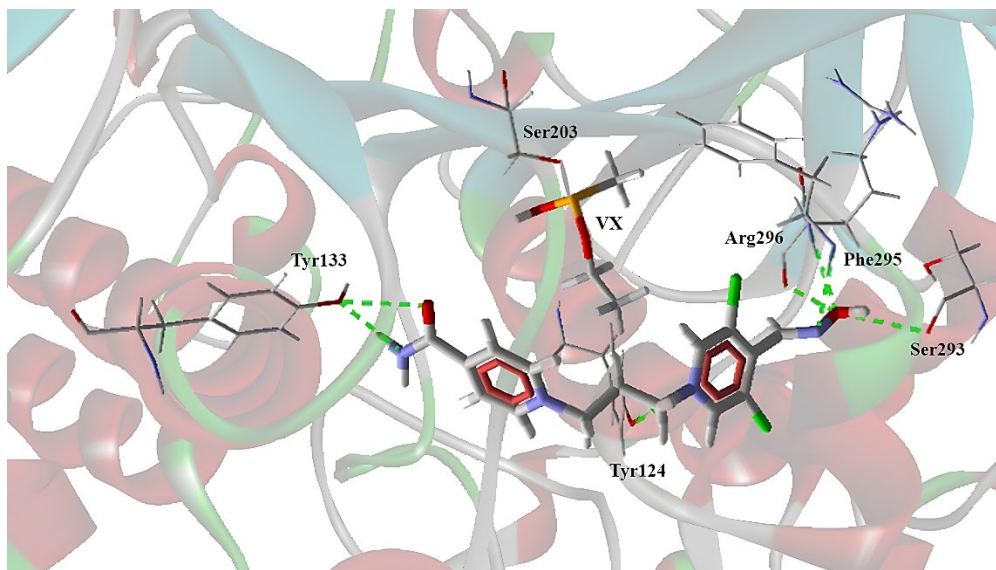


Figure 7. Hydrogen bonds (green dashed lines) between oxime K868 and the residues of the active site of *HssAChE* inhibited by VX (PDB code 6U37; resolution = 2.25 Å; active ligand = RS194B) are shown. These interactions demonstrate the conformational stability of the oxime in the active site of *HssAChE*, highlighting the specific residues involved in the hydrogen bonds: Arginine 296, Phenylalanine 295, Serine 293, and Tyrosines 124 and 133 [36, 37].

These studies revealed that, among the oximes examined, oxime 4 exhibited the highest stability within the *HssAChE* active site, offering the most favorable conformation for reactivating the inhibited *HssAChE* enzyme.

3.1.4 Docking study between oximes and the *HssBChE* enzyme inhibited by sarin

The binding site of the sarin-inhibited *HssBChE* enzyme was modeled using the Molegro Virtual Docker (MVD) program [34], which employs a three-dimensional cavity prediction algorithm. This approach identified a cavity with a volume of 62.464 Å³. The oximes were then docked into the active site of the sarin-inhibited *HssBChE* enzyme. To investigate the differences in interaction modes of the compounds within the enzyme's active site, intermolecular interactions, including ligand-protein hydrogen bonds, were analyzed. This analysis aimed to identify the factors influencing the compounds' activity.

Table 5. Values of intermolecular interaction energy (kcal mol^{-1}), hydrogen bond energy (kcal mol^{-1}) and the amino acid residues of *HssBChE* inhibited by sarin, obtained by molecular *docking*.

Interactions between sarin-inhibited <i>HssBChE</i> and oximes			
Oximes	Intermolecular interaction energy (kcal mol^{-1})	Hydrogen bond energy (kcal mol^{-1})	Amino acid residues
4	-143.15	-14.48	Ala229, Asp395, Pro230, Tyr396, Val 233
6	-120.54	-4.08	Asp395, Tyr396
1	-118.08	-4.75	Cys400, Pro230, Thr523, Val233
2	-114.16	-2.50	Tyr396
5	-112.22	-5.01	Cys400, Thr523, Val233
3	-109.68	-6.68	Cys400, Pro401, Thr523, Val233
K868	-108.68	-3.00	Tyr396, Val233
8	-106.78	0.00	-
9	-105.35	-2.50	Asp304
7 (K027)	-104.63	-2.93	Pro401, Thr523

*Values of intermolecular interaction energy (kcal mol^{-1}) refer to the forces that bind the molecule to the enzyme's active site, influencing the stability of the interactions. Hydrogen bond energy (kcal mol^{-1}) measures the strength of interactions between hydrogen atoms and electronegative atoms in the enzyme's amino acid residues and the oximes. The amino acid residues of *HssBChE*, inhibited by sarin, interact with the oximes as obtained through molecular *docking*. These parameters enable the evaluation of interaction stability, contributing to the understanding of the enzyme's reactivation potential by the studied oximes.

As shown in Table 5, oxime 4 exhibited the highest stability, with an intermolecular interaction energy of $-143.15 \text{ kcal mol}^{-1}$, indicating the most substantial interaction within the *HssBChE* active site. The energy difference between oxime 4 and the reference oxime K868 was $34.47 \text{ kcal mol}^{-1}$. Regarding hydrogen bonds, oxime 4 demonstrated a stable hydrogen bond energy of $-14.48 \text{ kcal mol}^{-1}$ and interacted with the amino acid residues alanine, aspartate, proline, tyrosine, and valine (Figure 8). Tyrosine, being an aromatic amino acid, has a phenolic

hydroxyl group, which allows hydrogen bonding with carbonyl or amine groups, enhancing the stability of oxime 4 in the enzyme's active site. Additionally, valine, a nonpolar amino acid, contributes to hydrophobic stabilization.

The second most effective reactivator in terms of interaction energy is oxime 6, with a value of $-120.54 \text{ kcal mol}^{-1}$. The energy difference between oxime 6 and oxime K868 is $11.86 \text{ kcal mol}^{-1}$. Oxime 6 exhibited a hydrogen bond energy of $-4.08 \text{ kcal mol}^{-1}$ and interacted with aspartate and tyrosine residues. The interaction with tyrosine is relevant, as this interaction may be important for stabilizing oxime 6 in the active site, as observed with oxime 4. The third most stable oxime is oxime 1, with an interaction energy of $-118.08 \text{ kcal mol}^{-1}$. The energy difference between oxime 1 and oxime K868 is $9.40 \text{ kcal mol}^{-1}$. Oxime 1 showed a hydrogen bond energy of $-4.75 \text{ kcal mol}^{-1}$ and interacted with cysteine, proline, threonine, and valine residues. Cysteine is an essential amino acid for enzymatic function due to its reactive thiol group, which can form hydrogen bonds and interact with specific groups in the enzyme's active site. The interaction with valine, which is nonpolar, also suggests that oxime 1 may bind favorably to the hydrophobic region of the active site, which could be relevant for enhancing the stability of the complex.

The energy differences between the oximes and the reference oxime K868 were as follows: $5.48 \text{ kcal mol}^{-1}$ for oxime 2, $3.54 \text{ kcal mol}^{-1}$ for oxime 5, $1.00 \text{ kcal mol}^{-1}$ for oxime 3, $1.90 \text{ kcal mol}^{-1}$ for oxime 8, $3.33 \text{ kcal mol}^{-1}$ for oxime 9, and $4.05 \text{ kcal mol}^{-1}$ for oxime 7 (K027). The largest energy difference occurred between oxime 4 and oxime K868, with a difference of $34.47 \text{ kcal mol}^{-1}$. On the other hand, oxime 7 (K027) showed the lowest stability in the *HssBChE* active site, with an energy value of $-104.63 \text{ kcal mol}^{-1}$. Oxime 7 (K027) exhibited a hydrogen bond energy of $-2.93 \text{ kcal mol}^{-1}$ and interacted with proline and threonine. These interactions are also relevant, as both proline and threonine play important roles in stabilizing protein structures. The reference oxime K868 had an energy value of $-108.68 \text{ kcal mol}^{-1}$ and a hydrogen bond energy of $-3.00 \text{ kcal mol}^{-1}$, interacting with tyrosine and valine (Figure 9). An interesting observation is that, compared to K868, oxime 4 commonly interacted with valine, and oximes 1, 3, and 5 also interacted with this amino acid residue. This comparison suggests that the presence of tyrosine in the interactions contributes to the stability of the complex. The interactions of the oximes with residues such as tyrosine, valine, and cysteine play an important role in reactivation efficacy. Tyrosine, due to its aromatic hydroxyl group, facilitates the formation of hydrogen bonds, while hydrophobic residues like valine enhance the stability of the oximes in the active site of *HssBChE*.

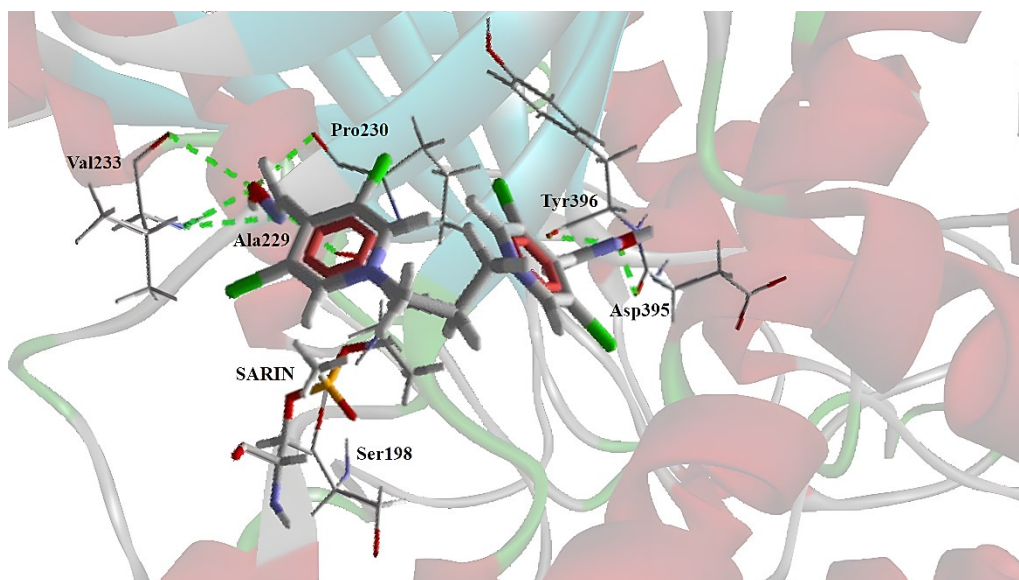


Figure 8. Hydrogen bonds (green dashed lines) between oxime 4 and the residues of the active site of *HssBChE* inhibited by sarin (PDB code 1P0I; resolution = 2.00 Å) are shown. These interactions demonstrate the conformational stability of the oxime in the active site of *HssBChE*, highlighting the specific residues involved in the hydrogen bonds: Alanine 229, Aspartic Acid 395, Proline 230, Tyrosine 396, and Valine 233 [36, 37].

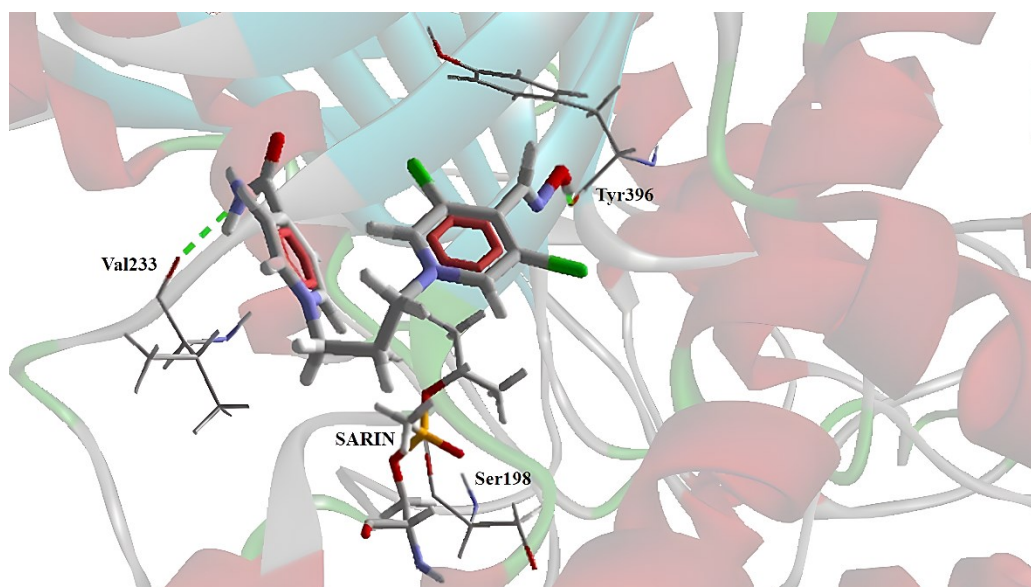


Figure 9. Hydrogen bonds (green dashed lines) between oxime K868 and the residues of the active site of *HssBChE* inhibited by sarin (PDB code 1P0I; resolution = 2.00 Å) are shown. These interactions demonstrate the conformational stability of the oxime in the active site of *HssBChE*, highlighting the specific residues involved in the hydrogen bonds: Tyrosine 396 and Valine 233 [36, 37].

Among the oximes studied, oxime 4 exhibited the highest stability within the *HssBChE* active site, suggesting that it likely adopts a more favorable conformation for reactivating the inhibited *HssBChE* enzyme.

3.1.5. Docking study between oximes and the *HssBChE* enzyme inhibited by cyclosarin

The binding site of the *HssBChE* enzyme inhibited by cyclosarin was identified using the MVD program, which revealed a cavity volume of 452.608 Å³. Oximes were subsequently docked into the active site of the cyclosarin-inhibited *HssBChE*, with the results detailed in the corresponding table.

Table 6. Values of intermolecular interaction energy (kcal mol⁻¹), hydrogen bond energy (kcal mol⁻¹) and the amino acid residues of *HssBChE* inhibited by cyclosarin, obtained by molecular *docking*.

Interactions between cyclosarin-inhibited <i>HssBChE</i> and oximes			
Oximes	Intermolecular interaction energy (kcal mol ⁻¹)	Hydrogen bond energy (kcal mol ⁻¹)	Amino acid residues
5	-130.74	-15.78	Ala199, Asn68, Gln119, Glu197, Glu276, Gly116, Gly117
4	-129.84	-6.81	Ala328, Gln67, Gly121, Trp82
8	-129.68	-12.19	Asn68, Glu276, Gly116, Gly117
2	-128.29	-1.52	Asp70, Phe118
K868	-126.38	-5.00	Tyr128
6	-126.10	-8.55	Ile69, Tyr128
1	-124.63	-7.49	Glu197, Gly116, Gly117
7 (K027)	-124.43	-5.87	Ala328, Thr122, Tyr128
9	-119.75	-2.68	Asp70, Trp82
3	-119.22	-11.70	Gln71, Gly78, Ile69, Trp82, Trp430, Tyr440

*Values of intermolecular interaction energy (kcal mol⁻¹) refer to the forces that bind the molecule to the enzyme's active site, influencing the stability of the interactions. Hydrogen bond energy (kcal mol⁻¹) measures the strength of interactions between hydrogen atoms and electronegative atoms in the enzyme's amino acid residues and the oximes. The amino acid residues of *HssBChE*, inhibited by cyclosarin, interact with the oximes as obtained through molecular *docking*. These parameters enable the evaluation

of interaction stability, contributing to the understanding of the enzyme's reactivation potential by the studied oximes.

As shown in Table 6, oxime 5 exhibited a more stable intermolecular interaction energy ($-130.74 \text{ kcal mol}^{-1}$). The energy difference between oxime 5 and the reference oxime K868 was $4.36 \text{ kcal mol}^{-1}$. Regarding the hydrogen bonds, oxime 5 displayed a hydrogen bond energy of $-15.78 \text{ kcal mol}^{-1}$ and interacted with the amino acid residues alanine, asparagine, glutamine, glutamate, and glycine (Figure 10). The interaction with alanine and glycine, which are small and hydrophobic amino acids, may promote the stabilization of the oximes in the hydrophobic region of the active site. On the other hand, interactions with asparagine, glutamine, and glutamate, which have polar groups, can facilitate the formation of hydrogen bonds, increasing oxime 5's affinity for the active site.

Additionally, the second oxime with the highest interaction energy is oxime 4, with an energy value of $-129.84 \text{ kcal mol}^{-1}$. The energy difference between oxime 4 and the reference oxime K868 is $3.46 \text{ kcal mol}^{-1}$. Oxime 4 exhibited a hydrogen bond energy of $-6.81 \text{ kcal mol}^{-1}$, interacting with the amino acid residues alanine, glutamine, glycine, and tryptophan. Tryptophan, with its aromatic structure and indole group, can form hydrophobic and π - π interactions with the oxime, contributing to stability in the active site. Additionally, the interaction with glutamine, a polar amino acid, also favors the formation of hydrogen bonds, which can help stabilize the complex. The third most stabilized oxime is oxime 8, which has an energy value of $-129.68 \text{ kcal mol}^{-1}$. The energy difference between oxime 8 and oxime K868 is $3.30 \text{ kcal mol}^{-1}$. Oxime 8 showed a hydrogen bond energy of $-12.19 \text{ kcal mol}^{-1}$ and interacted with asparagine, glutamate, and glycine. Asparagine, being a polar amino acid, can form hydrogen bonds, further stabilizing the oxime in the active site. Glutamate, with its carboxyl group, can also aid in stabilization due to its ability to form ionic interactions or hydrogen bonds with the oxime.

The energy differences between the oximes and K868 were as follows: $1.91 \text{ kcal mol}^{-1}$ for oxime 2, $0.28 \text{ kcal mol}^{-1}$ for oxime 6, $1.75 \text{ kcal mol}^{-1}$ for oxime 1, $1.95 \text{ kcal mol}^{-1}$ for oxime 7 (K027), $6.63 \text{ kcal mol}^{-1}$ for oxime 9, and $7.16 \text{ kcal mol}^{-1}$ for oxime 3. The greatest energy difference was observed between oxime 3 and K868, with a value of $7.16 \text{ kcal mol}^{-1}$.

Among the proposed oximes, oxime 3 demonstrated the least stability in the *HssBChE* active site, with an energy value of $-119.22 \text{ kcal mol}^{-1}$. Oxime 3 exhibited a hydrogen bond energy of $-11.70 \text{ kcal mol}^{-1}$ and interacted with the amino acid residues glutamine, glycine, isoleucine, tryptophan, and tyrosine. Although oxime 3 showed a relatively weaker interaction

compared to the other oximes, the presence of tyrosine, an aromatic amino acid with a hydroxyl group, suggests an important interaction for stabilizing the oxime in the active site. The reference oxime K868 showed an energy value of $-126.38 \text{ kcal mol}^{-1}$, with a hydrogen bond energy of $-5.00 \text{ kcal mol}^{-1}$. Oxime K868 interacted only with the tyrosine residue (Figure 11). Comparing all oximes with the reference compound, it is noteworthy that oximes 3, 6, and 7 consistently formed hydrogen bonds with tyrosine. Interactions with amino acids such as tyrosine, glutamine, glycine, and tryptophan are crucial for the stability of the oximes in the active site of *HssBChE*. Tyrosine plays a central role, promoting the formation of hydrogen bonds and contributing to the enhanced stability of the complex.

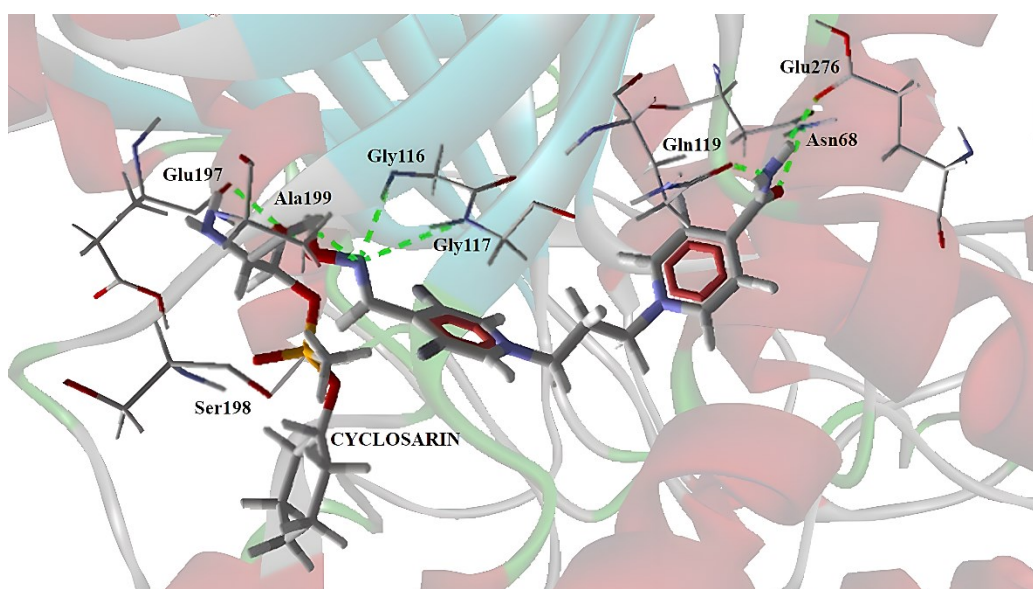


Figure 10. Hydrogen bonds (green dashed lines) between oxime 5 and the residues of the active site of *HssBChE* inhibited by cyclosarin (PDB code 1P0I; resolution = $2.00 \text{ \AA}$) are shown. These interactions demonstrate the conformational stability of the oxime in the active site of *HssBChE*, highlighting the specific residues involved in the hydrogen bonds: Alanine 199, Asparagine 68, Glutamine 119, Glutamates 197 and 276, and Glycines 116 and 117 [36, 37].

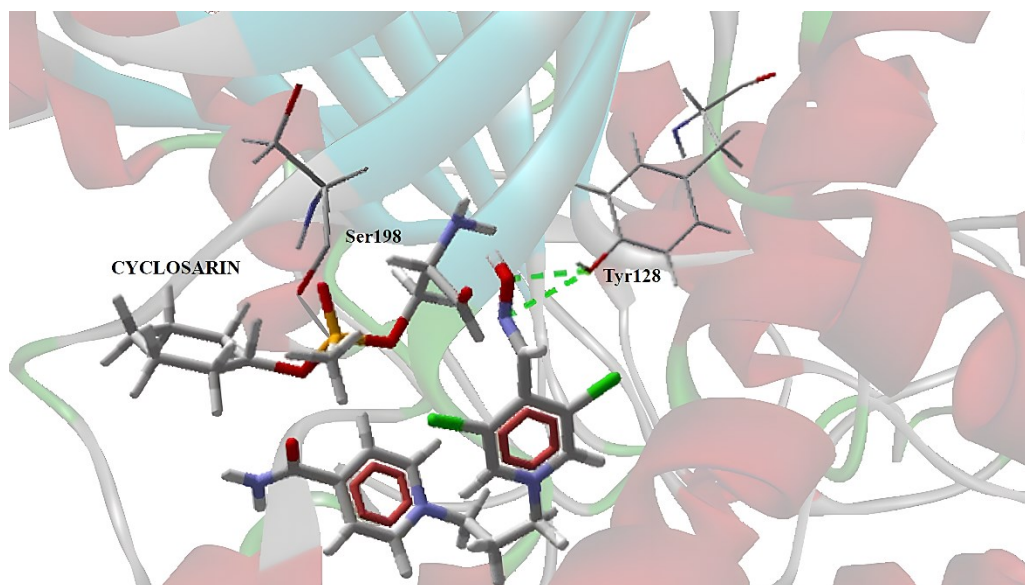


Figure 11. Hydrogen bonds (green dashed lines) between oxime K868 and the residues of the active site of *HssBChE* inhibited by cyclosarin (PDB code 1P0I; resolution = 2.00 Å) are shown. These interactions demonstrate the conformational stability of the oxime in the active site of *HssBChE*, highlighting the specific residues involved in the hydrogen bonds: Tyrosine 128 [36, 37].

Molecular *docking* calculations revealed that, among the oximes studied, oxime 5 exhibited the highest stability within the *HssBChE* active site. This suggests that oxime 5 likely adopts a more favorable conformation for reactivating the inhibited *HssBChE* enzyme.

3.1.6. *Docking* study between oximes and the *HssBChE* enzyme inhibited by VX

The binding site of the VX-inhibited *HssBChE* enzyme was identified using the MVD program, revealing a cavity volume of 451.072 Å³. The oximes were docked into the active site of the VX-inhibited *HssBChE*. The molecular *docking* results are summarized in Table 7.

Table 7. Values of intermolecular interaction energy (kcal mol⁻¹), hydrogen bond energy (kcal mol⁻¹) and the amino acid residues of *HssBChE* inhibited by VX, obtained by molecular *docking*.

Interactions between VX-inhibited <i>HssBChE</i> and oximes			
Oximes	Intermolecular interaction energy (kcal mol ⁻¹)	Hydrogen bond energy (kcal mol ⁻¹)	Amino acid residues
5	-141.64	-5.94	Gly116, Gly121, His438, Leu286, Thr120
4	-140.27	-4.82	Tyr128
8	-138.92	-10.45	His438, Thr122, Trp82, Tyr128
1	-135.05	-7.82	Thr122, Tyr128
9	-134.89	-12.40	Glu197, Gly116, Gly117, Tyr128
3	-132.44	-14.76	Ala201, Asn68, Asn289, Glu197, Glu276, Gly116, Gly117
6	-132.07	-11.14	Gly115, His438, Thr122, Trp82, Tyr128, Tyr440
K868	-128.17	-7.92	Ala199, Glu197, Gly116, Gly117
2	-121.74	-4.45	Tyr128
7 (K027)	-117.78	-2.33	Asn68, Asp70, Gln67, Gly116

*Values of intermolecular interaction energy (kcal mol⁻¹) refer to the forces that bind the molecule to the enzyme's active site, influencing the stability of the interactions. Hydrogen bond energy (kcal mol⁻¹) measures the strength of interactions between hydrogen atoms and electronegative atoms in the enzyme's amino acid residues and the oximes. The amino acid residues of *HssBChE*, inhibited by VX, interact with the oximes as obtained through molecular *docking*. These parameters enable the evaluation of interaction stability, contributing to the understanding of the enzyme's reactivation potential by the studied oximes.

As shown in Table 7, oxime 5 exhibited the most stable intermolecular interaction energy value of -141.64 kcal mol⁻¹, indicating the strongest interaction within the *HssBChE*

active site. The energy difference between oxime 5 and oxime K868 was 13.47 kcal mol⁻¹. Regarding hydrogen bonds, oxime 5 displayed a hydrogen bond energy of -5.94 kcal mol⁻¹ and interacted with the amino acid residues glycine, histidine, leucine, and threonine in the active site (Figure 12). The interaction with glycine and threonine, which are small amino acids, may promote the stability of the complex. Additionally, histidine, a basic amino acid, plays a crucial role in the stability of the complex.

The second oxime with the most favorable interaction energy is oxime 4, with an energy value of -140.27 kcal mol⁻¹. The energy difference between oxime 4 and the reference oxime K868 is 12.10 kcal mol⁻¹. Oxime 4 exhibited a hydrogen bond energy of -4.82 kcal mol⁻¹ and interacted only with the tyrosine amino acid residue. The third most stabilized oxime is oxime 8, which has an energy value of -138.92 kcal mol⁻¹. The energy difference between oxime 8 and oxime K868 is 10.75 kcal mol⁻¹. Oxime 8 displayed a hydrogen bond energy of -10.45 kcal mol⁻¹ and interacted with the amino acid residues histidine, threonine, tryptophan, and tyrosine. The interaction with tryptophan and tyrosine, two aromatic amino acids, may indicate the formation of hydrophobic and π - π interactions, enhancing the stability of the oxime in the active site.

The energy differences between the oximes and K868 were as follows: 6.88 kcal mol⁻¹ for oxime 1, 6.72 kcal mol⁻¹ for oxime 9, 4.27 kcal mol⁻¹ for oxime 3, 3.90 kcal mol⁻¹ for oxime 6, 6.43 kcal mol⁻¹ for oxime 2, and 10.39 kcal mol⁻¹ for oxime 7 (K027). The largest energy difference was observed between oxime 5 and K868, with a value of 13.47 kcal mol⁻¹.

Oxime 7 (K027) exhibited the lowest stability in the *HssBChE* active site, with an interaction energy value of -117.78 kcal mol⁻¹. It displayed a hydrogen bond energy of -3.99 kcal mol⁻¹ and interacted with the amino acid residues asparagine, aspartate, glutamine, and glycine. Despite the interaction with polar amino acids such as glutamine and asparagine, the overall stability was lower than that of the other oximes, indicating that these interactions may not be sufficient to ensure a strong binding in the active site. The reference oxime K868 had an interaction energy value of -128.17 kcal mol⁻¹ and a hydrogen bond energy of -7.92 kcal mol⁻¹, interacting with alanine, glutamate, and glycine residues (Figure 13).

Compared to oxime K868, oxime 5 predominantly interacted through hydrogen bonding with the glycine residue. Oximes 3, 6, and 7 frequently interacted with glycine, while oximes 3 and 9 commonly interacted with the glutamine residue. These results reinforce that residues such as tyrosine, glycine, and histidine play critical roles in the stability of the oximes in the active site of *HssBChE*. Tyrosine, with its aromatic interactions, appears to be particularly

important for the stability of the complex. Smaller residues, such as glycine and alanine, also contribute to the stability of the complex.

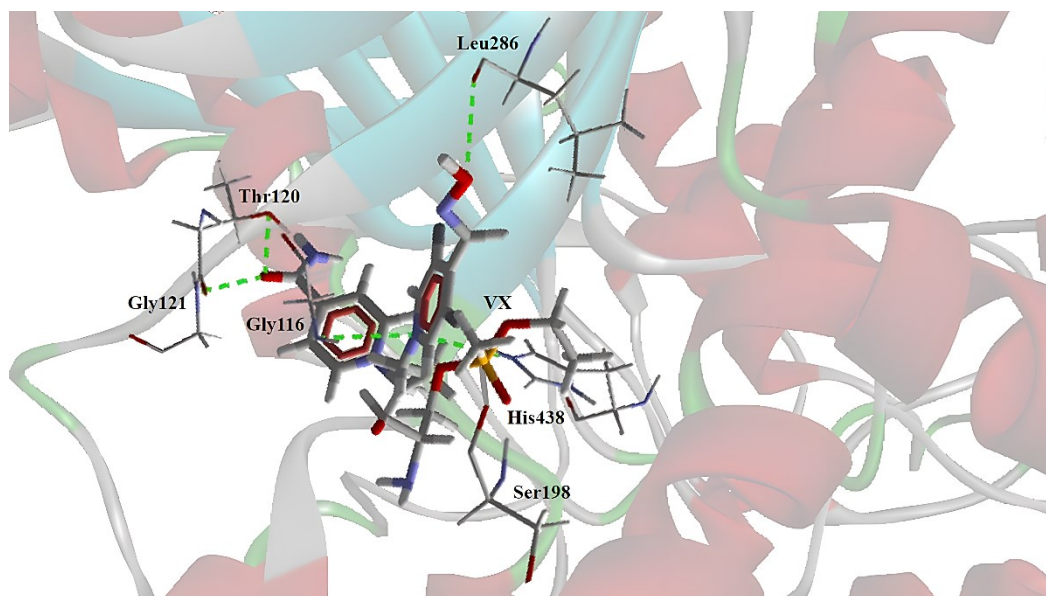


Figure 12. Hydrogen bonds (green dashed lines) between oxime 5 and the residues of the active site of *HssBChE* inhibited by VX (PDB code 1P0I; resolution = 2.00 Å) are shown. These interactions demonstrate the conformational stability of the oxime in the active site of *HssBChE*, highlighting the specific residues involved in the hydrogen bonds: Glycines 116 and 121, Histidine 438, Leucine 286, and Threonine 120 [36, 37].

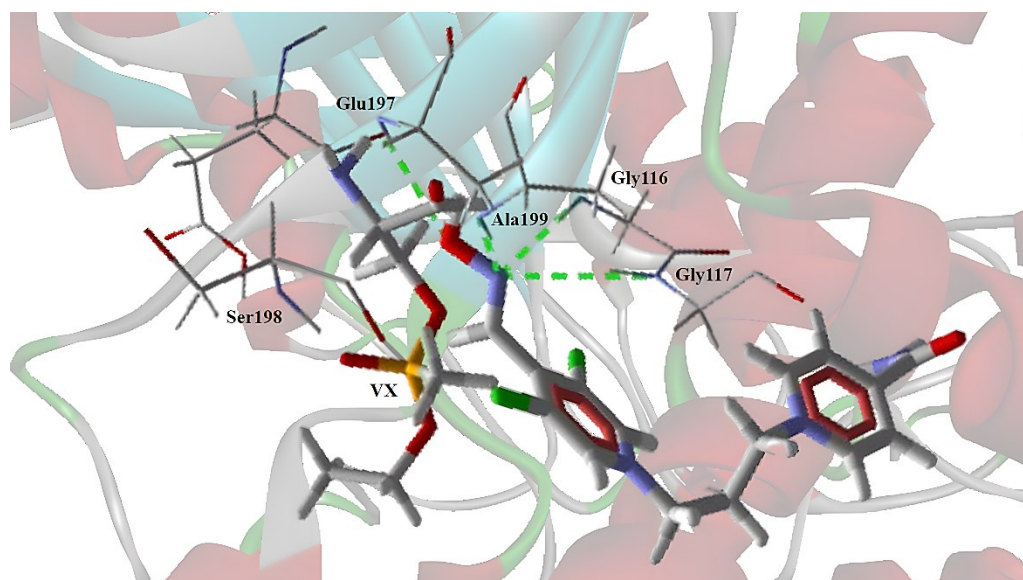


Figure 13. Hydrogen bonds (green dashed lines) between oxime K868 and the residues of the active site of *HssBChE* inhibited by VX (PDB code 1P0I; resolution = 2.00 Å) are shown. These interactions demonstrate the conformational stability of the oxime in the active site of *HssBChE*, highlighting the specific residues involved in the hydrogen bonds: Alanine 199, Glutamate 197, and Glycines 116 and 117 [36, 37].

Molecular *docking* studies revealed that, among the oximes analyzed, oxime 5 demonstrated the highest stability within the *HssBChE* active site. These findings suggest that oxime 5 likely adopts a more favorable conformation for reactivating the inhibited *HssBChE* enzyme. Additionally, reaction mechanism simulations were performed. Initially, the toxicity of the oximes, along with other relevant parameters, was assessed. The evaluation of these parameters was a fundamental prerequisite for advancing the study.

3.2. Toxicity Prediction

A chemical compound must undergo a toxicity prediction before it can progress to further studies. This prediction is crucial for ensuring that a promising drug with the potential to treat a disease can ultimately be developed into a safe and effective medicine [50, 65]. The prediction of LD₅₀ (lethal dose for 50% of the population) is a common subject in scientific literature, particularly in the fields of pharmacology, toxicology, and environmental health. Researchers and toxicologists often predict or estimate the LD₅₀ to evaluate the potential toxicity of chemicals, drugs, or environmental agents. These methods are interesting because they use various machine learning techniques, molecular descriptors, and data from existing toxicological studies to estimate toxicity [66].

3.2.1. LD₅₀

Table 8 presents the theoretical LD₅₀ results obtained using pkCSM (<https://biosig.lab.uq.edu.au/pkcsm/prediction>), which are indicative of the toxicity levels of the oximes reported in this study.

Table 8. LD₅₀ values obtained from pkCSM.

Oximes	Tox. (LD ₅₀)
HI-6	2.298
7 (K027)	2.169
8	2.197
5	2.239
9	2.311
6	2.833
K868	2.836
2	2.853
1	2.862
3	2.936
4	2.937

As shown, many proposed derivatives exhibited LD₅₀ values higher than that of the reference oxime K868, suggesting lower toxicity. To validate the theoretical LD₅₀ results, the known oximes HI-6 and K027 were also analyzed. Oxime HI-6 has an experimental LD₅₀ value of 671.3 mg/kg, while oxime K027 has an experimental value of 577.0 mg/kg [67]. These experimental LD₅₀ results align with the theoretical LD₅₀ values for HI-6 (2.298 mol/kg) and K027 (2.169 mol/kg). The theoretical results indicate that oxime HI-6 has lower toxicity than oxime K027, a trend that is consistent with the experimental data.

3.2.2. Absorption, Distribution, Metabolism and Excretion Test (ADME)

Swiss-ADME is a platform that provides key parameters such as lipophilicity (WLOGP, TPSA), water solubility (ESOL Log S), drug similarity rules, and medicinal chemistry properties [68]. The ADME prediction study offers physicochemical properties of potential oral drug candidates based on the combined criteria of Lipinski, Veber, Ghose, Egan, and Muegge [69-70].

Table 9. ADME properties of the molecules, including molecular weight (MW), number of rotatable bonds, hydrogen bond acceptors, hydrogen bond donors, solubility prediction (ESOL LogS), topological polar surface area (TPSA), and lipophilicity (WLOGP).

Molecule	MW (g/mol)	Rotable Bonds	H-bonds acceptors	H-bonds donors	ESOL LogS	TPSA (Å²)	WLOGP
K868	357.24	6	3	2	-2.55	82.16	1.20
1	357.24	6	3	2	-3.10	82.16	1.20
2	357.24	6	3	2	-2.86	82.16	1.20
3	357.24	6	4	2	-3.08	71.66	2.18
4	426.13	6	4	2	-3.94	71.66	3.31
5	324.33	6	5	2	-1.68	82.16	1.51
6	446.14	6	3	2	-3.18	82.16	1.52
7 (K027)	288.34	6	3	2	-1.69	82.16	0.07
8	288.34	6	3	2	-1.69	82.16	0.07
9	288.34	6	3	2	-1.99	82.16	0.07

The ADME results (Table 9) show that, in terms of molecular mass, all ligands fall within the range necessary to be considered potential drugs, as per Muegge's Rule, which states that molecular weight (MW) values should be between 200 and 600 g/mol. Regarding rotational bonds, all oximes adhere to the guidelines proposed by Scholars, who recommend that for pharmacological potential, the number of rotational bonds should not exceed 15. Additionally, the number of hydrogen bond acceptors should not be greater than 10, and all oximes meet this criterion. The number of hydrogen bond donors should not exceed 5, which all oximes also comply with.

For solubility, the higher the log S value, the more soluble the compound. In this regard, oximes 5, 7, and 8 are the most soluble. The TPSA value should be less than or equal to 140 Å², and all ligands meet this requirement. The WlogP value should be less than or equal to 5.88, and all oximes also satisfy this condition. To be a good medicine, a drug candidate should possess both water and lipid solubility. In this study, the most hydrophobic ligands are oximes K868, 1, 2, 3, 4, and 6, while the most hydrophilic ligands, with greater solubility in water, are oximes 5, 7, 8, and 9 [68, 71]. Overall, the ADME results indicate that all ligands comply with the proposed rules for drug-like molecules.

The Swiss-ADME platform was also used to evaluate other relevant parameters of oximes, such as GI absorption (gastrointestinal tract absorption), BBB permeant (blood-brain barrier permeability), Lipinski #violations (violations of Lipinski's rule), Ghose #violations (violations of Ghose's rule), Veber #violations (violations of Veber's rule) and PAINS #alerts (PAINS alerts) [72].

Table 10. ADME properties of the molecules, including gastrointestinal absorption (GI absorption), blood-brain barrier permeability (BBB permeant), the number of violations of Lipinski, Ghose, and Veber rules, as well as PAINS.

Molecule	GI absorption	BBB permeant	Lipinski #violations	Ghose #violations	Veber #violations	PAINS #alerts
K868	High	No	0	0	0	0
1	High	No	0	0	0	0
2	High	No	0	0	0	0
3	High	Yes	0	0	0	0
4	High	Yes	0	0	0	0
5	High	No	0	0	0	0
6	High	No	0	0	0	0
7 (K027)	High	No	0	0	0	0
8	High	No	0	0	0	0
9	High	No	0	0	0	0

According to Table 10, all oximes exhibited high gastrointestinal absorption (GI absorption), highlighting favorable characteristics for oral absorption. This result underscores the potential of these compounds as viable candidates for orally administered drugs. Regarding blood-brain barrier permeability (BBB permeant), only oximes 3 and 4 demonstrated the ability to cross this barrier, indicating their potential usefulness in the development of drugs targeting the central nervous system.

Concerning the Lipinski, Ghose, and Veber criteria, all oximes showed results without violations, suggesting a high likelihood of good oral bioavailability. For PAINS (Pan-Assay Interference Compounds) parameters, the oximes yielded zero alerts, being considered structurally clean and with a low risk of producing false positives in biochemical assays.

Incorporating a detailed discussion on ADME and LD₅₀ values, along with their implications for clinical use, can transform a purely scientific study into one that provides holistic insights into the drug's real-world potential. This discussion bridges the gap between laboratory data and clinical practice, allowing researchers to make more informed decisions regarding a drug's feasibility, safety, and effectiveness in diverse patient populations. Ultimately, by emphasizing the practical implications of these key pharmacological metrics, the study can guide drug development toward more personalized treatments, safer therapeutic options, and more equitable access to innovative medications [73, 74].

3.3. QM/MM

3.3.1. QM/MM study between oximes and the *Hss*AChE inhibited by OP's

To develop new and more selective reactivators, it is essential to understand how oximes facilitate reactivation. Hybrid quantum mechanics/molecular mechanics (QM/MM) simulations are especially effective for this purpose, as they combine the accuracy of quantum mechanics (QM) with the efficiency of molecular mechanics (MM). This approach allows for a detailed study of chemical processes within solutions and proteins [75]. In the quantum framework of these simulations, the computations focus on the electronic structure, energy, and related derivatives.

The reaction mechanism is evaluated by examining the activation energy, a critical factor determining whether a reaction will proceed. Activation energy represents the minimum energy required for reactants to initiate a chemical reaction [76]. Table 11 presents the relative activation energy values ($\Delta\Delta E^\ddagger$) for the reactivation of *Hss*AChE inhibited by sarin, cyclosarin, and VX.

Table 11. Relative activation energy values ($\Delta\Delta E^\ddagger$) for reactivation of the *HssAChE* inhibited by OP's.

	Sarin	Cyclosarin	VX
Oximes	$\Delta\Delta E^\ddagger$ (kcal mol ⁻¹)	$\Delta\Delta E^\ddagger$ (kcal mol ⁻¹)	$\Delta\Delta E^\ddagger$ (kcal mol ⁻¹)
K868	0.43	0.00	11.19
1	0.03	1.61	2.65
2	0.05	3.12	1.37
3	0.02	1.60	1.00
4	2.99	1.57	1.59
5	0.01	1.56	1.27
6	0.00	1.59	0.00
7 (K027)	4.21	1.60	1.22
8	0.06	1.62	1.39
9	0.01	1.51	3.60

The reaction mechanism results for the reactivation of *HssAChE* inhibited by sarin (Table 11) show that oxime 6 exhibited the lowest relative activation energy. Oximes 5 and 9 displayed activation barriers very close to that of oxime 6, with a minimal energy difference of just 0.01 kcal mol⁻¹, followed by oximes 3 and 1, and so on. Oxime 7 (K027) presented the highest activation barrier (4.21 kcal mol⁻¹) compared to the more stabilized oxime 6. This trend suggests that oxime 7 (K027) is less stable within the *HssAChE* active site, which limits its ability to effectively reactivate the inhibited enzyme.

According to Zorbaz (2018), oxime K868 is considered a potent dichlorinated analogue of oxime K027, which has shown satisfactory results in reactivating the *HssAChE* enzyme [25,26]. As observed, oximes 1, 2, 3, 5, 6, 8, and 9 exhibited lower activation energies compared to the reference oxime K868. These results suggest that these oximes are capable of effectively reactivating the inhibited *HssAChE* enzyme. Among the reactivators studied, oxime 6 demonstrated the highest potential, as it exhibited the lowest relative activation energy. Therefore, oxime 6 can be considered a potent analogue of oxime K868, with strong efficacy in reactivating *HssAChE* inhibited by sarin.

Table 11 also presents the relative activation energy values ($\Delta\Delta E^\ddagger$) for the reactivation of *HssAChE* inhibited by cyclosarin. The results from the reaction mechanism for reactivating *HssAChE* inhibited by cyclosarin (Table 11) show that the reference oxime K868 exhibited the

lowest relative activation energy. This finding aligns with the work of Zorbaz (2018), thereby validating the calculation method employed in this study.

Oxime 9 exhibited a higher relative activation energy compared to oxime K868, with an energy difference of $1.51 \text{ kcal mol}^{-1}$. As a result, oxime 9 was the second most stabilizing compound in terms of energy barrier and can be considered a potent analogue of oxime K868, with strong potential to reactivate the inhibited *HssAChE* enzyme.

Oxime 5 exhibited a higher relative activation energy, with an energy difference of $1.56 \text{ kcal mol}^{-1}$ compared to oxime K868, while oxime 4 also showed a slightly higher relative activation energy ($1.57 \text{ kcal mol}^{-1}$), followed by oxime 6 ($1.59 \text{ kcal mol}^{-1}$), and so on. Oxime 2 had the highest relative activation energy ($3.12 \text{ kcal mol}^{-1}$) compared to oxime K868. This result suggests that oxime 2 is less stable within the *HssAChE* active site, which reduces its effectiveness in reactivating the inhibited *HssAChE* enzyme.

Among the reactivators investigated, the reference oxime K868 demonstrated the greatest potential to reactivate the inhibited *HssAChE*, as it exhibited the lowest relative activation energy. According to Zorbaz (2018), oxime K868 is a potent dichlorinated analogue of oxime K027 and has shown satisfactory results in reactivating the inhibited *HssAChE* enzyme [25,26].

Additionally, Table 11 presents the relative activation energy values ($\Delta\Delta E^\ddagger$) for the reactivation of *HssAChE* inhibited by VX. The results indicate that oxime 6 exhibited the lowest relative activation energy, making it the most stabilized compound in this context. Oxime 3 showed a slightly higher energy barrier compared to oxime 6, with an energy difference of $1.00 \text{ kcal mol}^{-1}$, followed by oxime 7 (K027), with a difference of $1.22 \text{ kcal mol}^{-1}$. Similarly, oxime 5 displayed a higher relative activation energy ($1.27 \text{ kcal mol}^{-1}$) compared to oxime 6, and so forth. Notably, the reference oxime K868 exhibited the highest relative activation energy ($11.19 \text{ kcal mol}^{-1}$) compared to the most stabilized oxime 6. This result suggests that oxime K868 is less stable within the *HssAChE* active site, which reduces its effectiveness in reactivating the inhibited enzyme.

The results show that all oximes exhibited lower relative activation energy compared to oxime K868 [25,26]. This suggests that all the tested oximes have the potential to reactivate the *HssAChE* inhibited by VX. Among the analyzed compounds, oxime 6 demonstrated the lowest relative activation energy, positioning it as a highly potent analogue of oxime K868.

3.3.2. QM/MM study between oximes and the *HssBChE* inhibited by OP's

The reactivation mechanism of *HssBChE* inhibited by sarin, cyclosarin, and VX was evaluated. Table 12 presents the computed relative activation energy values ($\Delta\Delta E^\ddagger$) obtained from these simulations.

Table 12. Relative activation energy values ($\Delta\Delta E^\ddagger$) for reactivation of the *HssBChE* inhibited by OP's.

	Sarin	Cyclosarin	VX
Oximes	$\Delta\Delta E^\ddagger$ (kcal mol ⁻¹)	$\Delta\Delta E^\ddagger$ (kcal mol ⁻¹)	$\Delta\Delta E^\ddagger$ (kcal mol ⁻¹)
K868	1.12	6.97	9.80
1	0.08	0.00	0.61
2	0.62	37.79	0.56
3	3.54	9.76	0.00
4	0.02	10.16	1.14
5	2.94	16.57	0.36
6	0.04	14.43	0.60
7 (K027)	0.00	6.48	0.43
8	0.04	1.35	0.93
9	0.02	18.59	0.36

The results of the reaction mechanism for reactivating *HssBChE* inhibited by sarin (Table 12) reveal that oxime 7 (K027) exhibited the lowest relative activation energy. Oximes 4 and 9 demonstrated very similar activation energies to oxime 7 (K027), with an energy difference of only 0.02 kcal mol⁻¹. In contrast, oxime 3 showed the highest relative activation energy (3.54 kcal mol⁻¹) compared to oxime 7 (K027). This indicates that oxime 3 is less stabilized within the *HssBChE* active site, reducing its effectiveness in reactivating the inhibited enzyme.

According to Zorbaz (2018), oxime K868 has also demonstrated satisfactory results in reactivating the inhibited *HssBChE* enzyme. Our findings indicate that oximes 1, 2, 4, 6, 7, 8, and 9 exhibit significant potential for reactivating *HssBChE*, as they achieved lower relative activation energy values compared to the reference oxime K868. Among the compounds analyzed, oxime 7 (K027) stands out as the most promising candidate for reactivating *HssBChE* inhibited by sarin [25,26].

Table 12 also presents the relative activation energy values ($\Delta\Delta E^\ddagger$) for the reactivation of *HssBChE* inhibited by cyclosarin. The results indicate that oxime 1 exhibited the lowest energy barrier, making it the most promising candidate for reactivating *HssBChE* inhibited by cyclosarin. Oxime 8 displayed a slightly higher relative activation energy compared to oxime 1, with a difference of 1.35 kcal mol⁻¹. Oxime 7 (K027) had a higher relative activation energy than oxime 1, with a difference of 6.48 kcal mol⁻¹. Conversely, oxime 2 presented the highest relative activation energy (37.79 kcal mol⁻¹) among the oximes, indicating that it is the least stabilized within the *HssBChE* active site, which significantly impairs its ability to facilitate reactivation.

According to these results, oximes 1 and 8 exhibit strong potential for reactivating *HssBChE* inhibited by cyclosarin, as they demonstrated lower relative activation energy values compared to the reference oxime K868 [25,26].

In addition, Table 12 presents the relative activation energy values ($\Delta\Delta E^\ddagger$) for the reactivation of *HssBChE* inhibited by VX. The results related to the reaction mechanism for reactivating VX-inhibited *HssBChE* (Table 12) reveal that oxime 3 exhibited the lowest relative activation energy. Oximes 5 and 9 showed slightly higher relative activation energies compared to oxime 3, with an energy difference of 0.36 kcal mol⁻¹. On the other hand, the reference oxime K868 displayed the highest relative activation energy (9.80 kcal mol⁻¹) among the compounds investigated. This suggests that oxime K868 is less stabilized within the *HssBChE* active site than the other reactivators, potentially limiting its effectiveness.

In comparison to oxime 7 (K027), oximes 1, 2, 3, 4, 5, 6, 8, and 9 demonstrate a greater potential for reactivating the inhibited *HssBChE* enzyme, as indicated by their lower relative activation energy values when compared to the reference oxime K868. Among these, oxime 3 stands out as the most effective reactivator for *HssBChE* inhibited by VX, showcasing the greatest efficacy [25,26].

3.4. Experimental values of oxime K868

Table 13 presents the experimental values of K_r for oxime K868 in the reactivation of *HssAChE* and *HssBChE* inhibited by cyclosarin, sarin, and VX.

Table 13. Values of K_r for oxime K868 in the *HssAChE* and *HssBChE* active sites.

	<i>HssAChE</i>	<i>HssBChE</i>
	Experimental result of K_r ($M^{-1} \text{ min}^{-1}$)	Experimental result of K_r ($M^{-1} \text{ min}^{-1}$)
Cyclosarin	25900 ± 6400 [26]	240 ± 70 [25]
Sarin	12000 ± 2500 [26]	360 ± 20 [25]
VX	7660 ± 1280 [26]	150 ± 40 [25]

From these data, it is possible to observe that the trends obtained from the theoretical results are consistent with those observed in the experimental data. According to Table 11, the reference oxime K868 presented the best mechanistic result for the reactivation of *HssAChE* inhibited by cyclosarin, with the lowest reaction barrier compared to the other oximes. This trend aligns with the best experimental result for the reactivation of *HssAChE* inhibited by cyclosarin (Table 13). Additionally, for the enzyme inhibited by sarin, which still shows a good experimental K_r value, the reference oxime remained more promising than most of the oximes investigated (Table 11). For the reactivation of *HssAChE* inhibited by VX, the experimental result for K868 showed the least promising trend. This experimental trend for VX also corroborates the theoretical results, where the reference oxime K868 shows the highest reaction barrier compared to the other oximes analyzed, indicating decreased efficiency. On the other hand, Table 13 also presents the experimental values of K_r for oxime K868 in the reactivation of *HssBChE* inhibited by sarin, cyclosarin, and VX.

For the reactivation process of the *HssBChE* enzyme, we can observe that the trends obtained from the theoretical results also align with those observed in the experimental data. Regarding the reference oxime K868, the best result is observed for sarin (Tables 12, 13), followed by cyclosarin (Tables 12, 13). The least promising trend is found for the enzyme inhibited by VX (Tables 12, 13), likely due to the high toxicity of this warfare nerve agent. In this case, the reference oxime K868 showed the highest reaction barrier compared to the other oximes investigated. Zorbaz *et al.* (2019) demonstrated in their work that the reference oxime K868 (Table 13) is more efficient in the reactivation of sarin ($K_r = 360 \pm 20 \text{ M}^{-1} \text{ min}^{-1}$), followed by cyclosarin ($K_r = 240 \pm 70 \text{ M}^{-1} \text{ min}^{-1}$) and VX ($K_r = 150 \pm 40 \text{ M}^{-1} \text{ min}^{-1}$) [25].

According to Kovarik *et al.* (2010), *HssBChE* exhibited lower affinities for nearly all tested oximes compared to *HssAChE*, with the exception of TMB-4, where both enzymes

displayed similar interaction levels. In experiments with the *HssAChE* enzyme, oxime 9, described in the literature as ICD-585, showed a slightly better affinity ($K_i = 0.028 \pm 0.002$ mmol dm⁻³) than oxime K027 ($K_i = 0.073 \pm 0.01$ mmol dm⁻³) and oxime HI-6 ($K_i = 0.031$ mmol dm⁻³). Note that ICD-585 is an analogue of oxime HI-6. These experimental results were very close, indicating the good affinity of these oximes for the *HssAChE* active site, which corroborates the stabilizing interaction energies obtained from molecular *docking* techniques. Additionally, in experiments with the *HssBChE* enzyme, oxime HI-6 ($K_i = 0.23 \pm 0.02$ mmol dm⁻³) showed greater affinity than K027 ($K_i = 0.66 \pm 0.11$ mmol dm⁻³) in the *HssBChE* active site. The experimental results also show that oximes HI-6 and K027 exhibit better affinity for *HssAChE* compared to *HssBChE*. This trend aligns well with the theoretical results from molecular *docking* [77].

In their study, Zorbaz *et al.* (2018) demonstrated that oxime HI-6 exhibits greater efficacy in reactivating *HssAChE* and *HssBChE* inhibited by cyclosarin, compared to those inhibited by sarin and VX. This finding was detailed in their paper entitled 'Potent 3-hydroxy-2-pyridine aldoxime reactivators of organophosphate-inhibited cholinesterases with predicted blood-brain barrier penetration' [78]. Additionally, another study by Zorbaz *et al.* (2018) provided experimental evidence indicating that oxime K027 displays higher affinity towards *HssAChE* compared to *HssBChE*. This work also showed that oxime K027 achieves a maximal reactivation percentage of 100% for *HssAChE* inhibited by cyclosarin, sarin, and VX [26].

4. CONCLUSIONS

The molecular *docking* results indicated that oxime 2 exhibited greater stability when *HssAChE* was inhibited by sarin, while oxime 4 showed more stable intermolecular interactions with *HssAChE* inhibited by cyclosarin and VX. Similarly, oxime 4 had lower intermolecular interaction energy when *HssBChE* was inhibited by sarin, whereas oxime 5 demonstrated greater stability when *HssBChE* was inhibited by cyclosarin and VX. In the reaction mechanism simulation, oximes 6, 9, and 6 presented the lowest activation energies for reactions with *HssAChE* inhibited by sarin, cyclosarin, and VX, respectively. Likewise, oximes 7, 1, and 3 demonstrated a more favorable conformation in the active site of *HssBChE* inhibited by sarin, cyclosarin, and VX, respectively. We considered the results related to cholinesterases inhibited by VX, as it is regarded as the most toxic chemical agent among those studied. In this context, we observed that, among the studied oximes, oximes 3 and 6 demonstrated the highest reactivation potential due to specific structural features, such as the presence of two oximates

in oxime 3 and bromine in oxime 6. The LD₅₀ values indicated that oxime 3 has lower toxicity compared to the reference oxime K868, while oxime 6 exhibits toxicity similar to K868. This study revealed the potential of novel oximes in reactivating cholinesterases, providing valuable insights for the development of more efficient universal compounds against neurotoxic agents.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

- [1] Petronilho, E.C.; Figueroa-Villar, J.D. Agentes para defesa contra guerra química: reativadores da acetilcolinesterase inibida com organofosforados neurotóxicos. *Rev. Virtual de Quim.*, **2014**, 6(3), 671-686.
- [2] Silva, G.R.; Jr, I.B.; Figueroa-Villar, J.D. Defesa química: histórico, classificação dos agentes de guerra e ação dos neurotóxicos. *Quim. Nova*, **2012**, 35(10), 2083-2091.
- [3] França, T.C.C.; Silva, G.R.; De Castro, A.T. Defesa química: uma nova disciplina no ensino de química. *Rev. Virtual de Quim.*, **2010**, 2(2), 84-104.
- [4] Torres, F.O.; Colasso, C. Armas químicas - Agentes neurotóxicos: toxicologia e desenvolvimento de novos tratamentos. *Rev. Bras. Estudos Defesa*, **2018**, 5(2), 37-59.
- [5] Figueroa-Villar, J.D. *et al.* Review about structure and evaluation of reactivators of acetylcholinesterase inhibited with neurotoxic organophosphorus compounds. *Curr. Med. Chem.*, **2021**, 28(7), 1422-1442.
- [6] Belinskaia, D.A. *et al.* Rational *in silico* design of aptamers for organophosphates based on the example of paraoxon. *Comput. Biol. and Chem.*, **2019**, 80, 452-462.
- [7] De Lima, P.; Junior, P. Possíveis doenças físicas e mentais relacionadas ao manuseio de agrotóxicos em atividades rurais, na região de Atibaia, SP/Brasil. Tese de Doutorado, Universidade de São Paulo, **2008**.
- [8] Vinhal, D.C.; Soares, V.H.C. Intoxicação por organofosforados: uma revisão da literatura organophosphates intoxication: a literature review. *Rev. Científica FacMais*, **2018**, 14(3), 2238-8427.
- [9] Giacoppo, J.O.S. *et al.* Guerra química: perspectivas no estudo de reativadores da enzima acetilcolinesterase inibida por organofosforados. *Rev. Virtual de Quim.*, **2014**, 6(3), 653-670.
- [10] Goulart, P.N.; Caruso, L.; Nadur, N.F.; Franco, D.P.; Kümmerle, A.E.; Lacerda, R.B. Butirilcolinesterase – BuChE: um potencial alvo para o desenvolvimento de fármacos para o tratamento da Doença de Alzheimer. *Rev. Virtual de Quim.*, **2021**, 13(1).
- [11] Patrick, G.L. An Introduction to Medicinal Chemistry. 2th ed. *Ed Oxford*, **2001**, 432.
- [12] Petronilho, E.C.; Pinto, A.C.; VILLAR, J.D.F. Acetilcolinesterase: Alzheimer e guerra química. *Rev. Militar Ciência Tecnol.*, **2011**, 28,1.
- [13] Rang, H.P.; Dale, M.M.; Ritter, J.M. Drogas que inibem a colinesterase. 4. ed. Rio de Janeiro: Guanabara Koogan, **2001**.
- [14] Ventura, A.L.M. Sistema colinérgico: revisitando receptores, regulação e a relação com a doença de Alzheimer, esquizofrenia, epilepsia e tabagismo. *Rev. Psiq. Clin.*, **2010**; 37(2), 66-72.
- [15] Albuquerque, E.X. *et al.* Effective countermeasure against poisoning by organophosphorus insecticides and nerve agents. *Proc. Natl. Acad. Sci. U.S.A.*, **2006**, 103,13220.
- [16] Patocka, J. *et al.* Oxime reactivation of acetylcholinesterase inhibited by toxic phosphorus esters: in vitro kinetics and thermodynamics. *J. Appl. Biomed.*, **2005**, 3, 91.
- [17] Hörnberg, A.; Tunemalm, A.; Ekström, F. Crystal structures of acetylcholinesterase in complex with organophosphorus compounds suggest that the acyl pocket modulates the aging reaction by precluding the formation of the trigonal bipyramidal transition state. *Biochem.*, **2007**, 46, 4815.

- [18] Soreq, H. *et al.* Molecular cloning and construction of the coding region for human acetylcholinesterase reveals a G + C-rich attenuating structure. *Proc. Natl. Acad. Sci. U.S.A.*, **1990**, 87, 9688.
- [19] Dvir, H. *et al.* Acetylcholinesterase: from 3D structure to function. *Chem. Biol. Interact.* **2010**, 187(1-3), 10-22.
- [20] Soreq, H.; Seidman, S. Acetylcholinesterase – new roles for an old actor. *Nature*, **2001**, 2, 294-302.
- [21] Dos Santos, V.M.R.; Donnici, C.L.; Da Costa, J.B.N.; Caixeiro, J.M.R. Compostos organofosforados pentavalentes: histórico, métodos sintéticos de preparação e aplicações como inseticidas e agentes antitumorais. *Quim. Nova*, **2007**, 30(1), 159-170.
- [22] Zilbeyaz, K.; Stellenboom, N.; Guney, M.; Oztekin, A.; Senturk, M. Effects of aryl methanesulfonate derivatives on acetylcholinesterase and butyrylcholinesterase. *J. Biochem. Mol. Toxicol.*, **2018**, 32, e22210.
- [23] Chatonnet, A.; Lockridge, O. Comparison of butyrylcholinesterase and acetylcholinesterase. *J. Biochem.*, **1989**, 260, 625.
- [24] Mack, A.; Robitzki, A. The key role of butyrylcholinesterase during neurogenesis and neural disorders: an antisense- 5'butyrylcholinesterase-DNA study. *Prog. Neurobiol.*, **2000**, 60,607.
- [25] Zorbaz, T.; Malinakb, D.; Kuca, K.; Musilekb, K.; Kovarika, Z. Butyrylcholinesterase inhibited by nerve agents is efficiently reactivated with chlorinated pyridinium oximes. *Chem. Biol. Interac.*, **2019**, 307, 16–20.
- [26] Zorbaz, T.; Malinak, D.; Marakovic, N.; Hrvat, N.M.; Zandona, A.; Novotny, M.; Skarka, A.; Andrys, R.; Benkova, M.; Soukup, O.; Katalinic, M.; Kuca, K.; Kovarik, Z.; Musilek, K. Pyridinium Oximes with Ortho-Positioned Chlorine Moiety Exhibit Improved Physicochemical Properties and Efficient Reactivation of Human Acetylcholinesterase Inhibited by Several Nerve Agents. *J. Med. Chem.*, **2018**, 61, 10753–10766.
- [27] Frisch, M.J.; Trucks, G.W.; Schlegel, H.B.; Scuseria, G.E.; Robb, M.A.; Cheeseman, J.R.; Zakrzewski, V.G.; Montgomery, J.A.; Stratmann, R.E.; Burant, J.C.; Dapprich, S.; Millam, J.M.; Daniels, A.D.; Kudin, K.N.; Strain, M.C.; Farkas, O.; Tomasi, J.; Barone, V.; Cossi, M.; Cammi, R.; Mennucci, B.; Pomelli, C.; Adamo, C.; Clifford, S.; Ochterski, J.; Petersson, G.A.; Ayala, P.Y.; Cui, Q.; Morokuma, K.; Salvador, P.; Dannenberg, J.J.; Malick, D.K.; Rabuck, A.D.; Raghavachari, K.; Foresman, J.B.; Cioslowski, J.; Ortiz, J.V.; Baboul, A.G.; Stefanov, B.B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Gomperts, R.; Martin, R.L.; Fox, D.J.; Keith, T.; Al-Laham, M.A.; Peng, C.Y.; Nanayakkara, A.; Challacombe, M.; Gill, P.M.W.; Johnson, B.; Chen, W.; Wong, M.W.; Andres, J.L.; Gonzalez, C.; Head-Gordon, M.; Replogle, E.S.; Pople, J.A. Gaussian, Inc., Pittsburgh PA, **1998**.
- [28] Ramalho, T.C.; Rocha, M.V.; Da Cunha, E.F.F.; Oliveira, L.C.A.; Carvalho, K.T. Understanding the Molecular Behavior of Organotin compounds to Design their Effective use as Agrochemicals: Exploration via Quantum Chemistry and Experiments. *J. Biomol. Struct. Dyn.*, **2010**, 28, 227-238.
- [29] França, T.C.C.; Pascutti, P.; Ramalho, T.C.; Figueroa-Villar, J.D. A three-dimensional structure of Plasmodium falciparum serine hydroxymethyltransferase in comple with Glycine and 5-Formyl- Tetrahydrofolate. Homology Modeling and Molecular Dynamics. *Biophys. Chem.*, **2005**, 115, 1-10.

- [30] Ramalho, T.C.; Buhl, M. Probing NMR Parameters, Structure and Dynamics of 5-Nitroimidazole Derivatives. Density Functional Study of Prototypical Radiosensitizers. *Magn. Reson. Chem.*, **2005**, *43*, 139-146.
- [31] Da Cunha, E.F.F.; Ramalho, T.C.; Reynolds, R.C. Binding mode analysis of 2,4-diamino-5-methyl-5-deaza-6-substituted pteridines with Mycobacterium tuberculosis and human dihydrofolate reductases. *J. Biomol. Struct. Dyn.*, **2008**, *25*(4), 377–385.
- [32] De Castro, A.A.; Soares, F.V.; Pereira, A.F.; Silva, T.C.; Silva, D.R.; Mancini, D.T.; Caetano, M.S.; Da Cunha, E.F.F.; Ramalho, T.C. Asymmetric biodegradation of the nerve agents Sarin and VX by human dUTPase: Chemometrics, Molecular *Docking* and Hybrid QM/MM calculations. *J. Biomol. Struct. Dyn.*, **2019**, *37*(8), 2154-2164.
- [33] Militani, I.A.; Mancini, D.T.; De Castro, A.A.; Ramalho, T.C. Oxidative Biotransformation of Organophosphotioate Pesticides and Acetylcholinesterase Enzymatic Inhibition. *Lett. in Drug Des. Discov.*, **2023**, *20*. DOI: 10.2174/1570180820666230503095544.
- [34] Thomsen, R.; Christensen, M.H. *MolDock*: a new technique for high-accuracy molecular docking. Washington: *J. Med. Chem.*, **2006**, *49*(11), 3315-3332.
- [35] Bitencourt-Ferreira, G.; De Azevedo, W.F. Molegro Virtual Docker for *Docking*. In: de Azevedo Jr., W. (eds) *Docking Screens for Drug Discovery. Methods Mol. Biol.*, **2019**, (2053).
- [36] Westbrook, J.D.; Stephen, S.K. How Structural Biologists and the Protein Data Bank Contributed to Recent FDA New Drug Approvals. *Structure*, **2019**, *27*(2), 211-217.
- [37] Berman, H.M. *et al.* The protein data bank. *Nucleic Acids Res.*, **2000**, *28*(1), 235-242.
- [38] Da Cunha, E.F.F.; Barbosa, E.F.; Oliveira, A.A.; Ramalho, T.C. Molecular Modeling of Mycobacterium Tuberculosis DNA Gyrase and its Molecular *Docking* Study with Gatifloxacin Inhibitors. *J. Biomol. Struct. Dyn.*, **2010**, *27*(5), 619–625.
- [39] Hevener, K.E.; Zhao, W.; Ball, D.M.; Babaoglu, K.; Qi, J.; White, S.W.; Lee, R.E. Validation of Molecular *Docking* Programs for Virtual Screening against Dihydropteroate Synthase. *J. Chem. Inf. Model.*, **2009**, *49*, 444–460.
- [40] Ramalho, T.C. *et al.* Construction and assessment of reaction models of class I EPSP synthase: molecular docking and density functional theoretical calculations. New York: *J. Biomol. Struct. Dyn.*, **2009**, *27*(2), 195-208.
- [41] Souza, T.C.S.; Josa, D.; Ramalho, T.C.; Caetano, M.S.; da Cunha, E.F.F. Molecular Modelling of Mycobacterium Tuberculosis Acetolactate Synthase Catalytic Subunit and Its Molecular *Docking* Study with Inhibitors. *Mol. Simul.*, **2008**, *34*, 707–713.
- [42] Manathunga, M.; Götz, A.W.; Merz, K.M. Computer-Aided Drug Design, Quantum-Mechanical Methods for Biological Problems. *Curr. Opin. Struct. Biol.*, **2022**, *75*, 102417.
- [43] Agu, P.C.; Afiukwa, C.A.; Orji, O.U.; Ezech, E.M.; Ofoke, I.H.; Ogbu, C.O.; Ugwuja, E.I.; Aja, P.M. Molecular *Docking* as a Tool for the Discovery of Molecular Targets of Nutraceuticals in Diseases Management. *Sci. Rep.*, **2023**, *13*, 13398.
- [44] Senn, H.M.; Thiel, W. QM/MM Methods for Biomolecular Systems. *Angew. Chem. Int. Ed. Engl.*, **2009**, *48*, 1198–1229.
- [45] de Castro, A.A.; Polisel, D.A.; Pereira, B.T.L.; da Cunha, E.F.F.; Kuca, K.; Nepovimova, E.; Ramalho, T.C. Understanding the Interaction Modes and Reactivity of Trimedoxime toward MmAChE Inhibited by Nerve Agents: Theoretical and Experimental Aspects. *International Journal of Molecular Sciences*, **2020**, *21*, 6510.

- [46] de Castro, A.A.; Caetano, M.S.; Silva, T.C.; Mancini, D.T.; Rocha, E.P.; da Cunha, E.F.F.; Ramalho, T.C. Molecular *Docking*, Metal Substitution and Hydrolysis Reaction of Chiral Substrates of Phosphotriesterase. *Comb. Chem. High Throughput Screen.*, **2016**, *19*, 334–344.
- [47] Da Cunha, E.F.F.; Mancini, D.T.; Ramalho, T.C. Molecular modeling of the Toxoplasma gondii adenosine kinase inhibitors. *Med. Chem. Res.*, **2012**, *21*, 590-600.
- [48] Mancini, D.T. Estudos Computacionais de Potenciais Inibidores da Enzima Nucleosídeo Hidrolase de Brucella Suis. Dissertação (mestrado), Universidade Federal de Lavras, **2011**.
- [49] Arulanandam, C.D.; Hwang, J.S.; Rathinam, A.J.; Dahms, H.U. Evaluating different web applications to assess the toxicity of plasticizers. *Nature*, **1968**, *12*, 1-8.
- [50] Luo, L.; Ai, Z.; Qu, W.; Zheng, T. Structure Based Pharmacophore Modeling, Virtual Screening, Molecular *Docking*, ADMET and Molecular Dynamics (MD) Simulation for the discovery of Potential Inhibitors of PD-L1 From Library of Marine Natural Products. *Mar. Drugs*, **2021**, *20*(1), 29, 1-17.
- [51] Bakchi, B.; Krishna, A.D.; Sreecharan, E.; Ganes, V.B.J.; Niharika, M.; Maharshi, S.; Puttagunta, S.B.; Sigalapalli, D.K.; Bhandare, R.R.; Afzal, B.; Shaik, A.B. An overview on applications of SwissADME web tool in the design and development of anticancer, antitubercular and antimicrobial agents: A medicinal chemist's perspective. *J. Mol. Struct.*, **2022**, *1259*, 1-32.
- [52] Gupta, M.; Sharma, R.; Kumar, A. *Docking* Techniques in Pharmacology: How Much Promising? *Comput. Biol. Chem.*, **2018**, *76*, 210–217.
- [53] Gupta, M.; Sharma, R.; Kumar, A. *Docking* Techniques in Toxicology: An Overview. *Current Bioinformatics*, **2020**, *15*.
- [54] Dey, D.; Kumar, A. Structural-based Study to Identify the Repurposed Candidates Against Bacterial Infections. *Current Medicinal Chemistry*, **2024**, *31*.
- [55] Trisciuzzi, D.; Alberga, D.; Leonetti, F.; Novellino, E.; Nicolotti, O.; Mangiatordi, G.F. Molecular *Docking* for Predictive Toxicology. *Methods Mol. Biol.*, **2018**, *1800*, 181–197.
- [56] Navyashree, V., Kant, K., & Kumar, A. Natural chemical entities from *Arisaema* genus might be a promising break-through against Japanese encephalitis virus infection: a molecular *docking* and dynamics approach. *Journal of Biomolecular Structure and Dynamics*, **2020**, *39*(4), 1404–1416.
- [57] Ferreira, L.L.G.; Andricopulo, A.D. ADMET Modeling Approaches in Drug Discovery. *Drug Discov. Today*, **2019**, *24*, 1157–1165.
- [58] Sargsyan, K.; Grauffel, C.; Lim, C. How Molecular Size Impacts RMSD Applications in Molecular Dynamics Simulations. *J. Chem. Theory and Comput.*, **2020**, *13*(4), 1518-1524.
- [59] Vankayala, S.L.; Warrensford, L.C.; Pittman, A.R.; Pollard, B.C.; Kearns, F.C.; Larkin, J.D.; Woodcock, H.L. CifDock: A novel CHARMM-based flexible receptor–flexible ligand *docking* protocol. *J. Comput. Chem.*, **2021**, *43*(2), 84-95.
- [60] Guimarães, A.P. Estudos por ancoramento e dinâmica molecular de potenciais inibidores da nucleosídeo hidrolase de bacillus anthracis. Dissertação (Mestrado em Agroquímica), Universidade Federal de Lavras **2010**, *170*.
- [61] Banerjee, P.; Ulker, O.C. Combinative *ex vivo* studies and *in silico* models ProTox-II for investigating the toxicity of chemicals used mainly in cosmetic products. *Toxicol. Mech. Methods*, **2022**, *32*(7), 542-548.

- [62] Warren, D.K.; Guth, R.M.; Coopersmith, C.M.; Merz, L.R.; Zack, J.E.; Fraser, V.J. Epidemiology of methicillin- resistant *Sthaphylococcus aureus* colonization in a surgical intensive care unit. *Infect. Control. Hosp. Epidemiol.*, **2006**, *27*(10), 1137-1139.
- [63] Fowler, S.; Brink, A.; Cleary, Y.; Günther, A.; Heinig, K.; Husser, C.; Kletz, H.; Kratochwil, N.; Mueller, L.; Savage, M.; Stillhart, C.; Tuerck, D.; Ullah, M.; Umehara, K.; Poirier, A. Addressing Today's Absorption, Distribution, Metabolism, and Excretion (ADME) Challenges in the Translation of In Vitro ADME Characteristics to Humans: A Case Study of the SMN2 mRNA Splicing Modifier Risdiplam. *Drug Metab. and Dispos.*, **2022**, *50*(1), 65-75.
- [64] Zubair, M.S.; Maulana, S.; Widodo, A.; Mukaddas, A.; Pitopang R. Docking Study on Anti-HIV-1 Activity of Secondary Metabolites from Zingiberaceae Plants. *J. Pharm. Bioallied Sci.*, **2020**, *12*(6), 763-767.
- [65] Salha, D.; Andaç, M.; Denizli, A. Molecular *docking* of metal ion immobilized ligands to proteins in affinity chromatography. *J. Mol. Recognit.*, **2020**, *34*(2), 1-11.
- [66] Cavasotto, C.N.; Scardino, V. Machine Learning Toxicity Prediction: Latest Advances by Toxicity End Point. *ACS Omega*, **2022**, *7*, 47536–47546.
- [67] Kassa, J.; Kuca, K.; Bartosova, L.; Kunesova, G. The development of new structural analogues of oximes for the antidotal treatment of poisoning by nerve agents and the comparison of their reactivating and therapeutic efficacy with currently available oximes. *Curr. Org. Chem.*, **2007**, *11*, 267-283.
- [68] Bragana, M.E.; Daina, A.; Pérez, M.A.S.; Michielin, O.; Zoete, V. The SwissSimilarity 2021 Web Tool: Novel Chemical Libraries and Additional Methods for an Enhanced Ligand-Based Virtual Screening Experience. *Int. J. Mol. Sci.*, **2021**, *23*(2), 1-23.
- [69] Lipinski, C.A.; Lomabardo, F.; Dominy, B.W.; Feeney, P.J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev.*, **2001**, *64*, 4–17.
- [70] Muegge, I.; Bergner, A.; Kriegl, J.M. Computer-aided drug design at Boehringer Ingelheim. *J. Comput. Aided. Mol. Des.*, **2017**, *31*, 275-285.
- [71] Protti, I.C.; Rodrigues, D.R.; Fonseca, S.K.; Alves, R.J.; Oliveira, R.B.; Maltarollo, V.G. Do Drug-likeness Rules Apply to Oral Prodrugs? *ChemMedChem*, **2021**, *16*, 1446-1456.
- [72] Daina, A.; Michielin, O.; Zoete, V. SwissADME: A Free Web Tool to Evaluate Pharmacokinetics, Drug-Likeness and Medicinal Chemistry Friendliness of Small Molecules. *Sci. Rep.*, **2017**, *7*, 42717.
- [73] Su, J.; Yang, L.; Sun, Z.; Zhan, X. Personalized Drug Therapy: Innovative Concept Guided With Proteoformics. *Mol. Cell. Proteomics*, **2024**, *23*, 100737.
- [74] Tibbitts, J.; Canter, D.; Graff, R.; Smith, A.; Khawli, L.A. Key Factors Influencing ADME Properties of Therapeutic Proteins: A Need for ADME Characterization in Drug Discovery and Development. *MAbs*, **2016**, *8*, 229–245.
- [75] Borman, S.A. Much to do about enzyme mechanism. *Chem. Eng.*, **2004**, *8*, 35-39.
- [76] Lima, L.S. Energia de ativação. *Rev. Ciência Elem.*, **2015**, *3*(2), 035.
- [77] Kovarik, Z.; Katalinic, M.; Bosak, A.; Sinko, G. Cholinesterase Interactions with Oximes. *Curr. Bioact. Compd.*, **2010**, *6*, 9–15.

- [78] Zorbaz, T.; Braïki, A. Maraković N. *et al.* Potent 3-Hydroxy-2-Pyridine Aldoxime Reactivators of Organophosphate-Inhibited Cholinesterases with Predicted Blood–Brain Barrier Penetration. *Chem - A Eur. J.*, **2018**, *24*, 675–9691.

TERCEIRA PARTE

Artigo 2

***In silico* studies into the therapeutic potential of organophosphorus compounds against
Alzheimer's disease**

Artigo aceito pela revista Letters in Drug Design & Discovery

ABSTRACT

Background: Alzheimer's disease (AD) is the most prevalent form of dementia in the elderly and is characterized by a progressive decline in cognitive function. Therapeutic strategies for AD have largely been guided by the cholinergic hypothesis, which has driven the investigation of acetylcholinesterase inhibitors, including trichlorfon, as potential therapeutic agents. **Objectives:** This study aims to propose novel organophosphorus (OP) compounds as *HssAChE* inhibitors, based on the cholinergic hypothesis. **Methods:** A diverse array of OP compounds was systematically evaluated using advanced computational approaches, along with rigorous statistical analyses. The most promising candidates were further investigated through drug-likeness assessments, binding interaction analyses, and mechanistic simulations. **Results:** In particular, inhibitors **12**, **13**, **20**, and **28** emerged as promising candidates for the treatment of AD. However, following a detailed safety analysis, inhibitor **12** was identified as the least toxic inhibitor, exhibiting favorable interactions with the molecular target. Molecular dynamics simulations indicated that inhibitor **12** is more energetically stabilized in the *HssAChE* active site than the reference compound trichlorfon. QM/MM calculations further revealed a lower energy barrier for the *HssAChE* inhibition mechanism. **Conclusions:** These findings may facilitate the development of more effective and safer therapeutic options for the treatment of AD derived from this class of compounds.

Keywords: Alzheimer's disease, acetylcholinesterase, cholinergic hypothesis, trichlorfon, pharmacology, organophosphorus compounds.

1.0 INTRODUCTION

Alzheimer's disease (AD) is widely regarded as a prevalent disorder, affecting over 50 million individuals globally [1]. Although there are currently no disease-modifying therapies for AD, significant advances in understanding its molecular pathogenesis have identified two primary therapeutic targets. The first of these is amyloid- β ($A\beta$), a peptide that aggregates to form insoluble neuritic plaques. A variety of compounds aimed at modifying the formation, aggregation, or clearance of $A\beta$ are currently under investigation [1–3]. The formation of $A\beta$ deposits appears to play a central role in the pathogenesis of AD [3,4], making it a key target for disease-modifying therapies. $A\beta$ is generated through sequential cleavages of its precursor, amyloid precursor protein (APP), by β -secretase (also known as BACE-1) and γ -secretase [5,6]. Another promising therapeutic target in AD is neurofibrillary tangles, which result from the aggregation and hyperphosphorylation of tau protein. One approach to targeting this pathology involves developing mechanisms that can reduce tau phosphorylation by inhibiting specific kinases [1,3,7].

The few drugs currently approved for clinical use, such as donepezil and rivastigmine, offer only symptomatic relief and provide short-term benefits [8–10]. There is an urgent need to identify novel and effective interventions capable of preventing or delaying the onset of AD, slowing its progression and, equally important, improving the symptoms associated with its pathological framework [11,12]. Academic research laboratories, along with biotechnology and pharmaceutical companies, are actively exploring new therapeutic targets, identifying and optimizing potential compounds, and advancing clinical trials to discover more promising treatments [13–15].

With the emerging understanding of acetylcholine (ACh) in learning and memory, the cholinergic hypothesis of AD was proposed [16,17]. This hypothesis suggests that several factors significantly contribute to the deterioration and decline in cognitive function observed in AD patients. Notably, the degeneration of cholinergic neurons in the basal forebrain, coupled with the loss of cholinergic neurotransmission in the cerebral cortex and related regions, plays a central role in this cognitive decline [16,18]. A key prediction of the cholinergic hypothesis is that drugs designed to enhance central cholinergic function could improve cognition and potentially alleviate behavioral issues associated with AD [17–21]. In this context, compounds that can partially inhibit human acetylcholinesterase (*Hs*sAChE) warrant careful investigation, as *Hs*sAChE hydrolyzes ACh. OP compounds are frequently associated with toxic side effects. However, several members of this chemical class have been used as therapeutic agents for

different types of pathologies [22–26]. In this regard, despite the sensitivity surrounding this group, this chemical class can be therapeutically relevant when appropriately designed, administered and tested. The mechanism of AChE inhibition by OP inhibitors is shown in Figure 1. Partial inhibition of this enzyme could increase ACh levels at synapses, potentially providing beneficial effects for AD patients by improving cholinergic neurotransmission [19,20,27,28].

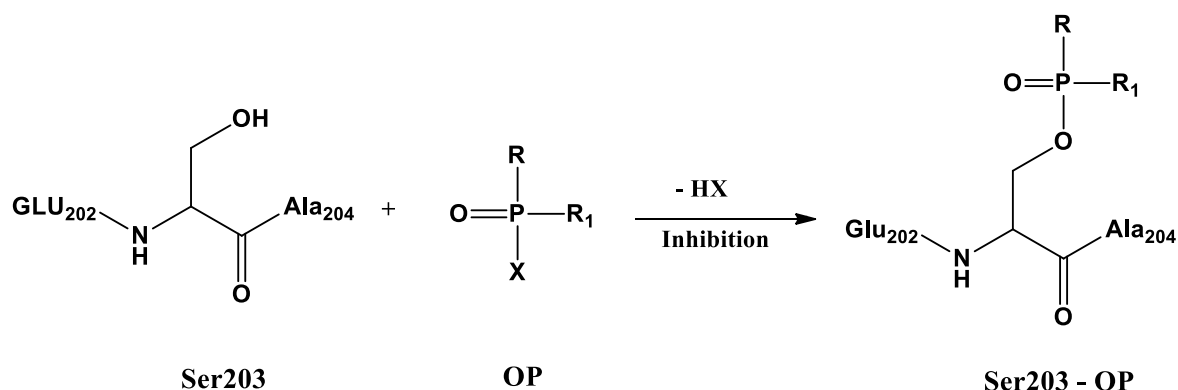


Figure 1. Mechanism of AChE inhibition by OP inhibitors.

HssAChE adopts a typical α/β hydrolase globular fold (Figure 2). Each monomer contains a single catalytic center, which consists of two distinct compartments: the catalytic site and the anionic site, the latter of which accommodates the positively charged quaternary nitrogen group of ACh [29]. Further details regarding the crystallographic structure of *HssAChE* can be found in the literature [30]. For example, it has been demonstrated that the catalytic triad (Ser203–Glu334–His447) is located at the bottom of the active site *gorge*, surrounded by key structural elements essential for catalytic activity: the acyl pocket (comprising residues Phe295, Phe297, and Phe338), the oxyanion hole (formed by Gly121, Gly122, and Ala204), and the choline-binding site (containing Trp86 and Tyr337) [29,31].

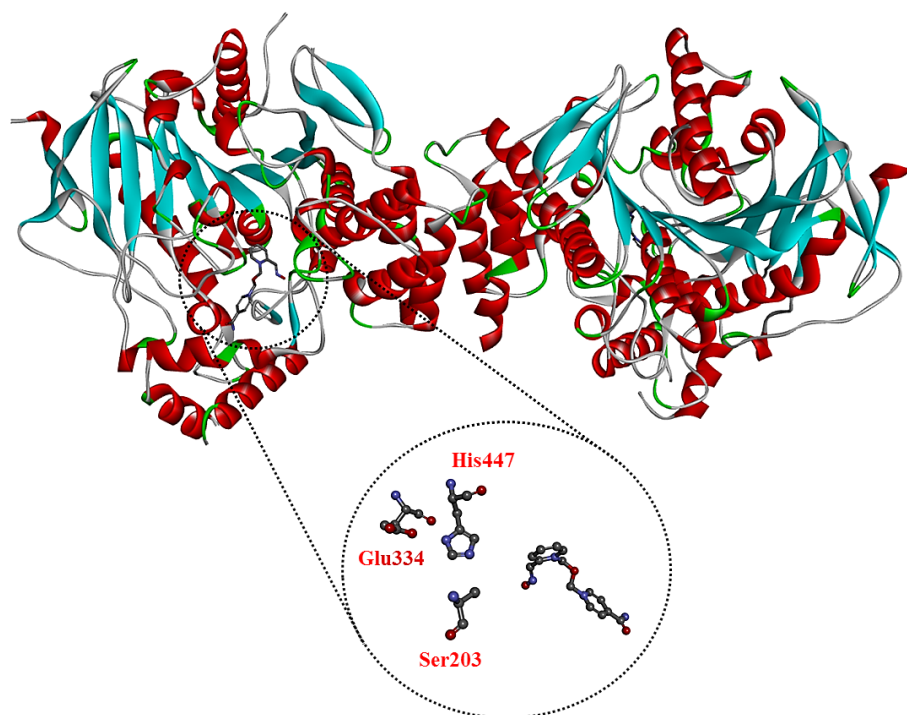


Figure 2. Representation of the three-dimensional structure of *HssAChE* (PDB code = 5HF9) [32] and its catalytic triad.

Given that AD is a global health concern, the search for novel therapies that can significantly improve the underlying pathology is of paramount importance. Therefore, this theoretical investigation aims to explore the therapeutic potential of a wide range of OP compounds, identifying those that are more efficient and less toxic than trichlorfon. The chemical structure of trichlorfon is shown next.

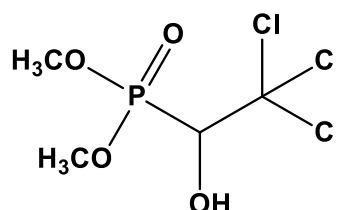


Figure 3. Chemical structure of trichlorfon (metrifonate).

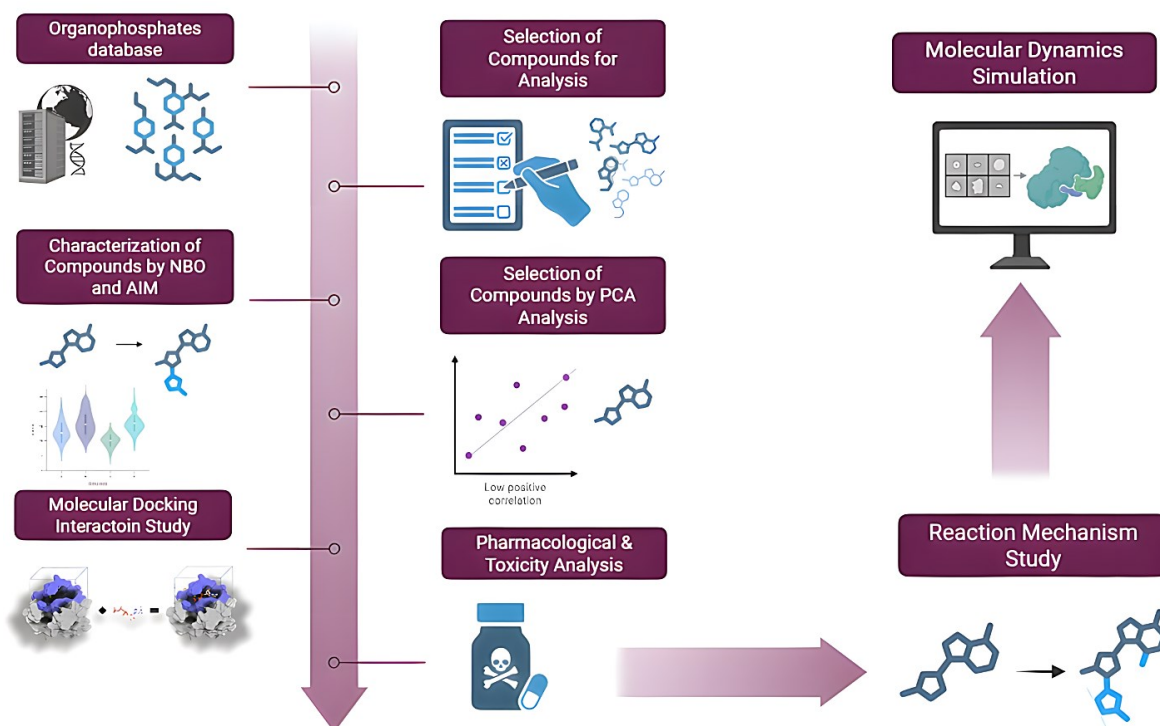
Trichlorfon, also known as metrifonate, inhibits both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activity in rodents and has also been studied for its effects on enzymes in the human brain cortex [33,34]. In addition to playing a key role in its pharmacological effects through cholinesterase inhibition, this agent also inhibits other serine enzymes, such as esterases and phosphatases. However, its use became unfeasible due to side effects reported in several trials [35]. To date, despite the uncommon use of OP compounds for

the treatment of AD, the literature reports potential benefits of administering compounds in this class [36,37]. In this study, various computational tools were used to analyze a wide range of OP compounds in an effort to identify inhibitors that are more efficient than trichlorfon and less toxic for clinical use.

Thus, this study investigates OP compounds as potential inhibitors of cholinesterase enzymes for the treatment of AD, based on the cholinergic hypothesis [9–11]. By developing safer OP agents that effectively inhibit *HssAChE*, these compounds aim to enhance ACh availability in the brain, potentially improving cognitive function in AD [10]. Additionally, these compounds are expected to be more efficient than older agents like trichlorfon, while also offering a reduced toxicity profile, making them safer candidates for clinical use. This methodology could lead to the discovery of new drug candidates or even help understand the underlying biological interactions that could be targeted for therapy. This approach highlights the potential of OP compounds beyond their conventional uses, opening new avenues for research into their neuropharmacological properties.

2.0 THEORETICAL METHODOLOGY

2.1 Computational Workflow for the Analysis of *HssAChE* inhibitors



2.2 Construction of the inhibitors

The OP compounds listed in Table S1 were selected from a database available at <https://www.inchem.org/documents/pims/chemical/pimg001.htm#SectionTitle:1.1%20%20Substance>. This database provides a comprehensive collection of OP compounds, which are chemicals commonly used in agricultural applications. These compounds were chosen to explore their potential as therapeutic agents for the treatment of AD. Rather than applying a stringent selection criterion, the compounds were selected without initial filtering. This approach was intentionally adopted to include a diverse range of OP inhibitors in order to ensure a broader scope for investigation. By choosing a wide variety of compounds, the study aims to explore novel and under-investigated molecules that could demonstrate unexpected or unique effects on AD pathology.

The chemical structures of the OP inhibitors (with the subscript index corresponding to the phosphorus atom as the stereocenter, when applicable) used in this computational study were constructed using PC Spartan Pro[®] software. These structures were then subjected to a pre-optimization process at the semi-empirical level (PM6), followed by conformational analysis calculations. Conformational analysis was important for identifying the lowest-energy conformer, as many of these compounds exhibit significant rotational flexibility due to rotatable σ -bonds. Both *Rp* and *Sp* enantiomers were considered for compounds with stereocenters.

After this step, the lowest-energy conformer of each OP inhibitor was fully optimized to the energy minimum using Density Functional Theory (DFT) with the B3LYP functional and the 6-311+G(2df,2pd) basis set, and SCF = (direct, maxcycle=300, conver=6). Solvent effects were incorporated using an implicit solvent model, along with empirical dispersion methods. The optimization calculations were performed with Gaussian09 software [38]. The compounds included in this theoretical study are listed in Table S1.

2.3 Atoms in Molecules (AIM)

AIM (Atoms in Molecules) calculations, based on Bader's Theory [39] are important for gaining a deeper understanding of the nature of chemical bonds, such as whether the bond exhibits covalent, partially covalent, or non-covalent character [40]. In this study, AIM calculations were performed for all the compounds listed in Table S1, taking into account the stereochemistry of those compounds containing stereocenters. These calculations were carried out at the DFT level using the B3LYP functional and the 6-311+G(2df,2pd) basis set to generate the AIM input files. Solvent effects and empirical dispersion were also incorporated at this

stage. The calculations were then carried out and analyzed using the AIM program [41].

2.4 Natural Bond Orbital (NBO)

The Natural Bond Orbital (NBO) technique uses a set of mathematical algorithms designed to analyze electronic wavefunctions in terms of localized, Lewis-like chemical bonds. The NBO method enables in-depth analysis of electron densities using advanced wavefunction technologies [42]. The NBO calculations were performed at the same level of theory as in the previous calculations (B3LYP/6-311+G(2df,2pd)), incorporating solvent effects and empirical dispersion, using Gaussian09 software [38]. In this calculation, the localized analysis of Canonical Molecular Orbitals (CMO) was considered. CMO analysis offers an effective alternative to the NBO molecular orbital (NBOMO) method by categorizing the primary NBO contributions, such as bonding, nonbonding, or antibonding, and providing a concise NBO report for each CMO [43].

2.5 Multivariate analysis of principal components (PCA)

In this statistical treatment, all compounds were included in the chemometric analysis. The parameters analyzed consisted of those derived from AIM calculations (atomic charge, $\nabla^2\rho(r)$, $G+V$, $-G/V$) and those from NBO calculations (NBO charge, occupancy, energy). A data matrix was constructed with the data from the AIM and NBO calculations as preprocessing. Principal component analysis (PCA) was then applied using MATLAB software, version R2012b [44]. The self-scaling technique was employed during the data preprocessing step.

2.6 Docking Procedure

For the *docking* calculations, the inhibitors selected from PCA were used. The chemical structures of these compounds were previously constructed and individually optimized using the calculation method described earlier. The partial charges of the atoms were determined using the CHELPG method [45]. The OP inhibitors were individually docked into the crystallographic structure of *HssAChE* (PDB code: 5HF9, resolution: 2.20 Å) [32], using the Molegro Virtual Docker program (MVD[®]) [46]. For the *docking* calculations, a radius of 5 Å centered on the active site was defined, and the residues within the binding site were treated as flexible. A total of 50 poses, each representing a distinct conformation and orientation, were generated for each ligand. For the *docking* configuration, the constraint parameters were set to $X = -11.08$, $Y = -44.40$, and $Z = 31.82$, and Radius = 5 Å. The scoring function applied was

the MolDock Score [GRID], and the optimization algorithm used was the MolDock Optimizer. Water molecules were removed for calculations. In addition, the enzyme was protonated at physiological pH (7.4). For *docking* replicates, the MolDock SE algorithm was additionally evaluated. The size of the constraint region was also varied (Radius = 6 Å) to assess the reproducibility and consistency of the results.

The MolDock scoring function, used in the MVD program, is based on the piecewise linear potential (PLP). This simplified potential was parameterized using protein-ligand structures and binding data. The scoring function is further enhanced in the Generic Evolutionary Method (GEM) for molecular *docking*, incorporating a new hydrogen bonding term and updated charge schemes [47]. The *docking* scoring function values, E_{score} , are defined by Eq. 1:

$$E_{\text{score}} = E_{\text{inter}} + E_{\text{intra}} \quad (1)$$

Wherein:

$$E_{\text{inter}} = \sum_{i \in \text{ligand}} \sum_{j \in \text{protein}} \left[E_{\text{PLP}}(r_{ij}) + 332.0 \frac{q_i q_j}{4r_{ij}^2} \right] \quad (2)$$

E_{PLP} , or "Piecewise Linear Potential", employs two distinct parameter sets. One set approximates the steric (Van der Waals) interactions among atoms, while the second set models hydrogen bonding. The hydrogen bonding term is complemented by a Coulomb potential that accounts for electrostatic interactions among charged atoms. The electrostatic term uses a distance-dependent dielectric constant, represented as $D(r) = 4r$, where the numerical value of 332.0 ensures that the electrostatic energy is expressed in kcal mol⁻¹ [46]. E_{intra} is the internal energy of the ligand:

$$E_{\text{intra}} = \sum_{i \in \text{ligand}} \sum_{j \in \text{ligand}} E_{\text{PLP}}(r_{ij}) + \sum_{\text{flexiblebonds}} A[1 - \cos(m \cdot \theta - \theta_0)] + E_{\text{clash}} \quad (3)$$

The first part of the equation (the double summation) accounts for all pairs of atoms within the ligand, excluding those that are directly connected by two bonds. The second term represents the torsional energy, where θ is the torsional angle of a bond. If multiple torsions are possible

within the molecule, the torsional energies for each are calculated and averaged. The final term, E_{clash} , imposes a penalty of 1,000 if the distance between two heavy atoms (more than two bonds apart) is less than 2.0 Å, effectively rejecting infeasible ligand conformations that result in steric clashes [46]. The *docking* search algorithm employed in the MVD program integrates an evolutionary algorithm and interactive optimization techniques inspired by Darwinian evolutionary theory, along with a novel hybrid search method known as guided differential evolution. This hybrid approach combines the differential evolution optimization technique with a cavity prediction algorithm during the search process, enabling rapid and precise identification of potential binding modes [46]. The *docking* protocol validation was performed through *redocking* of co-crystallized ligand.

2.7 Toxicological Profiling and Drug-Likeness Prediction

The toxicological profiles were predicted using the ProTox 3.0 platform (<https://tox.charite.de/protox3/>), an *in-silico* toxicity prediction framework that integrates molecular similarity analysis, structural alert detection, and machine learning based classification models. The methodology relies on curated experimental data from publicly available toxicological databases and employs Random Forest and Deep Neural Network algorithms trained on multiple molecular representations, including MACCS keys and Morgan circular fingerprints. ProTox 3.0 evaluates toxicity across seven hierarchical levels, encompassing acute oral toxicity (LD₅₀), organ toxicities, toxicological endpoints, toxicity-related biological pathways, molecular initiating events, metabolism-related targets, and toxicity targets. Model performance was optimized using advanced data-balancing strategies and validated through cross-validation and independent external test sets, ensuring balanced sensitivity and specificity. For each endpoint, the platform outputs binary or multiclass predictions accompanied by confidence scores, enabling a comprehensive, mechanism informed assessment of chemical toxicity while supporting early-stage risk evaluation and reduction of animal testing [48]. The ProTox 3.0 platform was also used to predict the blood-brain barrier (BBB) permeability and hepatotoxicity of the compounds investigated. To assess the drug-likeness of inhibitors based on Lipinski, PAINS, Brenk, and Veber, the Swiss-ADME platform (<https://www.swissadme.ch/>) was employed [49].

2.8 QM/MM approach

The integration of mixed quantum and molecular mechanics (QM/MM) methods enables the modeling of larger systems, such as enzymes, by combining quantum chemical methods with classical molecular mechanics. This hybrid approach enhances computational efficiency while reducing the overall computational cost [29,50–52]. In this study, QM/MM methods, combined with molecular *docking* simulations, were used to assess the most favorable interaction mode of each inhibitor within the active site of *HssAChE*. This approach is essential for simulating the inhibition reaction pathway, a critical step in identifying compounds with great therapeutic potential for the treatment of AD. To obtain more precise results and better capture electronic effects, QM calculations were performed at the DFT level using the Gaussian09 software package [38].

In this study, a sequential QM/MM (S-QM/MM) approach was used [53], in which the QM and MM subsystems were treated using separate computational programs. This method is particularly advantageous because it prevents issues related to the boundary region between the two subsystems. The whole enzymatic system was treated by MM calculations through molecular *docking* [54–60]. The software does not use a traditional molecular mechanics force field [46]. It uses a custom empirical scoring function built into its *docking* engine called MolDock Score, which estimates the interaction energy between a ligand and a protein using simplified potential terms [46]. On the other hand, the QM calculations were performed on the enzyme active site to achieve a more accurate description of electronic effects [54–60].

In this method, the active site was cut off, and all atoms within a 5 Å radius were treated at the DFT level using the Gaussian09 package [38]. The calculations were performed using B3LYP functional and 6-31G (d, p) basis set, and SCF = (direct, maxcycle=300, conver=2). The QM system included the neighboring peptide bonds, link atoms, the catalytic triad (Ser203–Glu334–His447), the inhibitor, and other amino acid residues within a 5 Å radius. The transition states involved were calculated and characterized by identifying imaginary frequencies [54–60].

2.9 RESP charges fitting and molecular dynamics simulations

The structures of inhibitor 12 and trichlorfon (reference) were optimized at the DFT level (B3LYP/6-31G*) considering a charge of +1 and multiplicity 2 using Gaussian09. Using the same software and charge/multiplicity scheme, the optimized structures were subjected to

electrostatic potential (ESP) calculations at the Hartree-Fock level (HF/6-31G*). The Merz-Kollman (MK) algorithm was employed for grid generation, configured with a high density of concentric layers (IOps 6/33=2 and 6/42=6) and strict SCF convergence (SCF=Tight), following the standard AMBER force field parameterization philosophy [61]. The calculated ESP was then used in the AmberTools25 [62] suite to derive partial charges via RESP fitting (fitting statistics and dipole comparisons are available in Table S2 of the Supplementary Material). Remaining ligand parameters were assigned according to the GAFF2 library, and the results were converted using ACPYPE [63] to the standard GROMACS format, which was the engine used for all molecular dynamics (MD) simulations.

The *HssAChE* structure was parameterized in GROMACS (v. 2023) [64] using the AMBER14sb force field, maintaining the original protonation state from the *docking* stage. Two protein-ligand complexes were built with inhibitor 12 and trichlorfon. A cubic simulation box was defined with edges extending 1.5 nm from the complex boundaries. The system was solvated with 38,765 TIP3P water molecules and neutralized by the addition of 8 sodium ions.

Minimization was performed in two steps using the Steepest Descent algorithm (with and without position restraints), configured for a maximum of 20,000 steps or until the maximum force was below 1000 kJ/(mol nm). The systems were equilibrated using NVT (310 K) and NPT (1 bar) ensembles, respectively, employing the leap-frog integrator, a 2 fs time step, H-bond constraints, and a total duration of 500 ps per ensemble (convergence plots for temperature, density, and potential energy are shown in Figure S1 of the Supplementary Material). An additional 500 ps simulation was performed post-equilibration for solvent relaxation. Finally, each system was subjected to a 200 ns MD production run with a time step of 2 fs, temperature maintained at 310 K, pressure at 1 bar, using the leap-frog integrator. All analyses were performed over the entire trajectory using GROMACS, and XMGRACE was used for plotting.

2.9.1 MM-PBSA calculations

End-state Gibbs free energy calculations were performed using the Molecular Mechanics Poisson-Boltzmann Surface Area (MM-PBSA) method with `gmx_MMPBSA` software [65] (v. 1.6.4). The calculations used the final 50 ns of the production trajectories for all complexes, totaling 1,250 frames with a sampling interval of 2 frames. The MM portion employed the AMBER14sb force field for the protein and GAFF2 for the ligand. The solute and solvent dielectric constants were set to 2 and 80, respectively. The linear Poisson-

Boltzmann equation was used for the polar solvation model, while the non-polar solvation contribution was estimated based on the solvent-accessible surface area (SASA). The interaction entropy model was adopted for entropy estimation, and all calculations were conducted at 310 K. Convergence was assessed using the Cumulative Averages [66] of the binding free energy ($\Delta G_{gas+solv}$).

3.0 RESULTS AND DISCUSSION

3.1 Statistical treatment and Principal Components Analysis (PCA)

The first stage of the statistical analysis involved linear regression, with multiple linear regression (MLR) used to perform this evaluation. As a predictive tool, MLR is employed to explain the relationship between a continuous dependent variable and two or more independent variables, which may be either continuous or categorical. This approach assumes that the regression residuals are normally distributed, that a linear relationship exists between the dependent and independent variables, and that multicollinearity is absent, meaning the independent variables are not highly correlated [67–69].

Based on the model's performance evaluation, the correlation coefficient is high ($R^2 = 0.8585$), indicating a strong correlation among the samples (OP inhibitors). Additionally, the low root mean square error (RMSE = 0.04057) suggests minimal error, which aligns with the strong linear correlation observed. An external validation (test set) was performed, which is important because it demonstrates how well the model performs on new data, provides a more realistic assessment of its predictive ability, and helps confirm that the results are reliable rather than merely fitted to the original dataset. The MLR graph, which illustrates these findings, is presented in Figure 4.

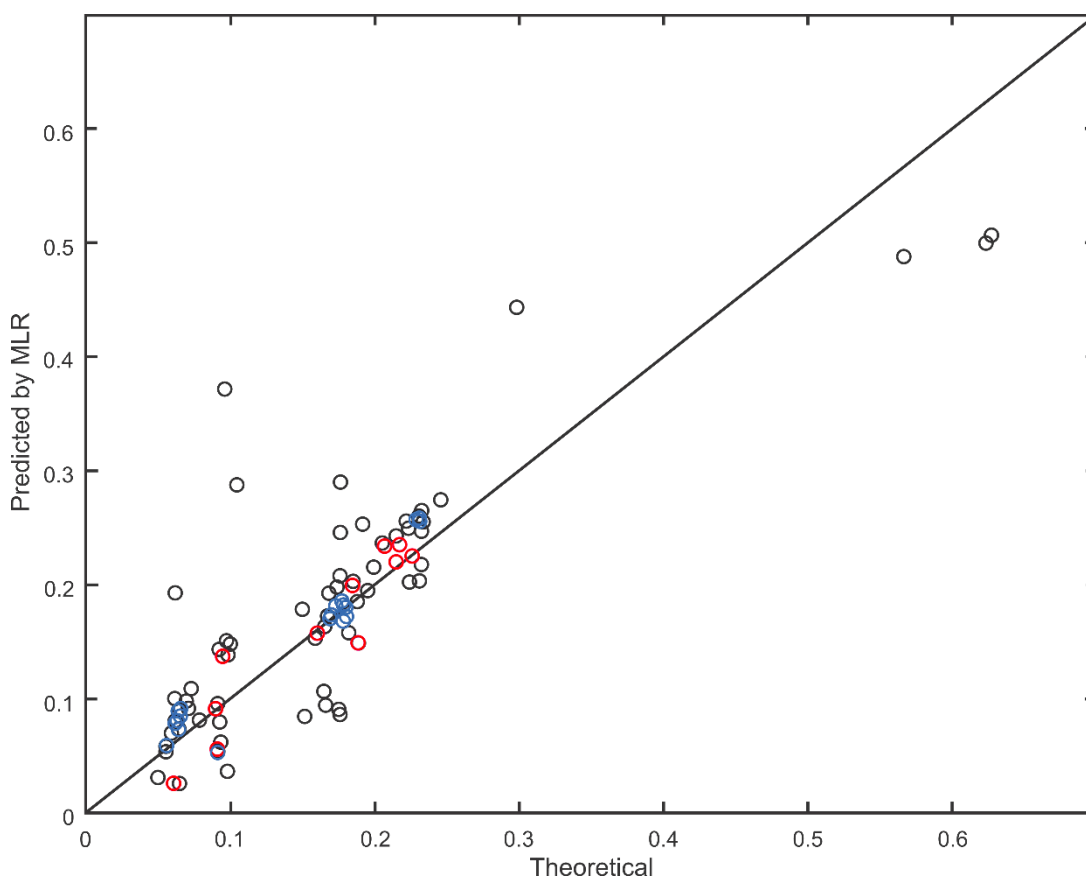


Figure 4. Representation of the MLR graph without outliers (blue = independent variables; red = external validation).

It is important to note that the first seven compounds, listed in Table S1, were identified as outliers due to their high toxicity levels. These toxic compounds were intentionally included in the analysis to validate the methodology, and as anticipated, they showed a significant discrepancy compared to the other compounds. As a result, they were considered outliers and subsequently removed from the dataset. According to the multiple linear regression (MLR) analysis, a reasonable degree of linearity is observed among the external validation samples.

The foundation of most methods for analyzing multivariate data is Principal Component Analysis (PCA), which was used in this study. PCA involves transforming the data matrix to reduce its dimensionality by exploiting correlations among variables. When these variables exhibit strong similarities, they are considered collinear. High collinearity suggests that it is possible to identify a new set of linearly independent variables that can effectively describe the data, expressed as linear combinations of the original variables [70]. In the context of selecting OP compounds most similar to the reference compound trichlorfon, PCA serves as an important tool by analyzing various physicochemical and electronic properties of the compounds, which are essential for predicting behavior, toxicity, and effectiveness [56].

By applying PCA, one can efficiently evaluate and compare compounds across multiple parameters, including Q (atomic charge), $\nabla^2\rho(r)$ (Laplacian of electron density), V (volume), G (Gibbs free energy), $G+V$ (combined Gibbs free energy and volume), $-G/V$ (negative change in Gibbs free energy per unit volume), NBO (natural bond orbital), MO Occup (molecular orbital occupancy), and E (energy). Each of these parameters provides valuable insight into the molecular characteristics of the compounds, such as their stability, reactivity, and interaction with biological systems [70]. This allows researchers to identify the most significant features that differentiate compounds, facilitating the identification of those that exhibit structural and electronic similarities to the reference compound. By projecting the data onto the principal components, compounds that exhibit chemical and physical characteristics similar to the reference compound can be identified with greater precision [56,70].

These new variables are extracted in descending order of the amount of statistical information they capture. Specifically, the first principal component (PC1) represents the direction of most significant data variation; the second principal component (PC2), which is orthogonal to the first one, describes the largest remaining variation, and so on. Each principal component thus accounts for a specific percentage of the total variance in the data. Based on the PCA analysis, the inhibitors most similar to trichlorfon were compounds **8** (*Rp*), **8** (*Sp*), **12**, **13**, **20**, **28**, **29**, **64** (*Sp*), and **69** (Figure 5). Consequently, these inhibitors were selected for further analysis. This selection is important for subsequent studies on interaction modes, reactivity, drug-likeness and toxicity levels.

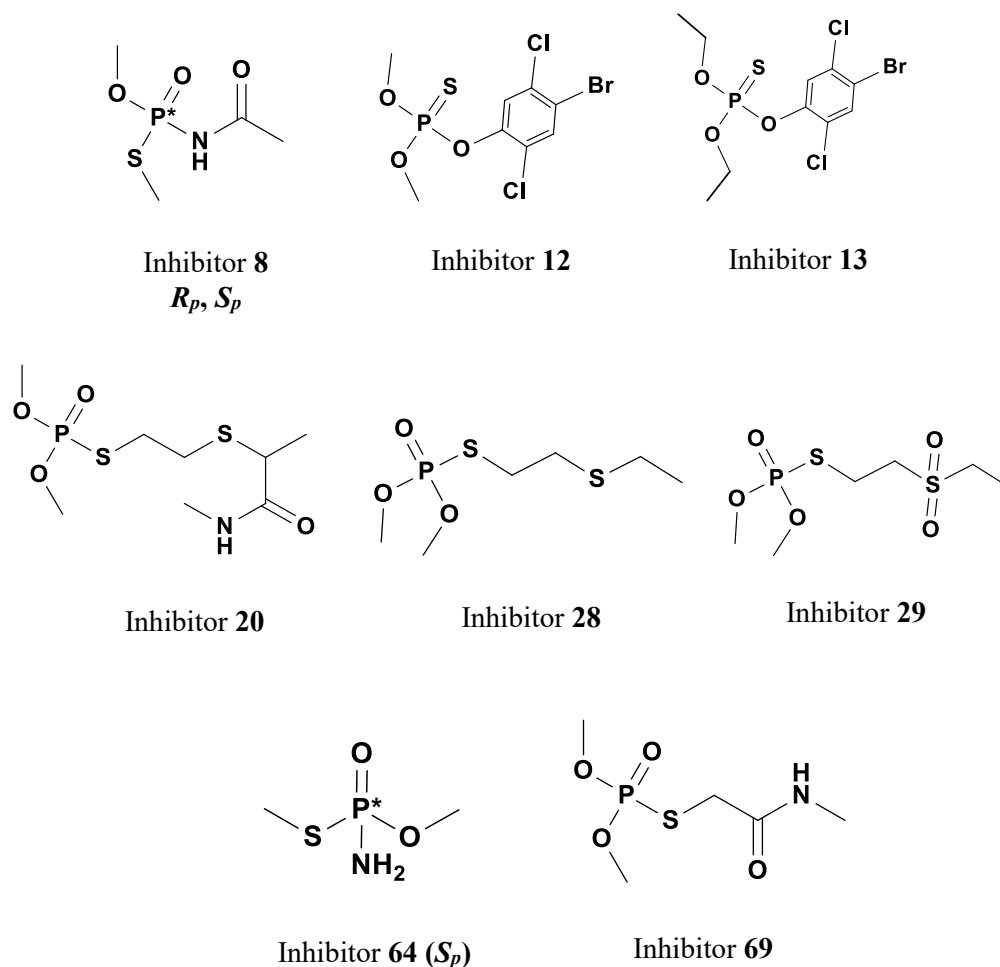


Figure 5. Chemical structures of selected inhibitors from the PCA.

As mentioned previously, in PCA, it is well-established that the percentage of variance explained by PC1 is always greater than that explained by PC2, and so on. Therefore, the projections that best represent the dataset are typically PC1 vs. PC2 or PC1 vs. PC3, since $PCN > PCN+1$. The PC1 vs. PC2 plot provides a more comprehensive representation of the original dataset; therefore, it was selected for this study [70].

The selected compounds differ from trichlorfon in both steric and electronic properties. Trichlorfon presents a relatively small structure containing a trichloromethyl group, while the investigated compounds contain longer thioether chains, halogenate aromatic rings, and amide groups. In terms of steric properties, compounds containing larger groups, such as the thioether chains and halogenate aromatic rings tend to increase steric complexity and conformational flexibility for interaction with the enzyme when compared to trichlorfon. On the other hand, regarding the electronic properties, in trichlorfon, the trichloromethyl group exerts a strong electron-withdrawing inductive effect, due to the presence of three highly electronegative chlorine atoms bonded to a sp^3 carbon, generating a strongly polarized electronic environment.

In contrast, some of the investigated compounds contain halogenated aromatic rings, in which halogen atoms exhibit both inductive electron-withdrawing effects and resonance interactions with the aromatic π -system, resulting in a more delocalized electronic distribution. Furthermore, compounds with thioether chains contain the sulfur atom, which is less electronegative but highly polarizable and contains lone electron pairs, producing a weaker inductive effect and increased electronic polarizability.

3.2 AIM and NBO analyses

The data computed using the AIM technique are presented in Table 1. It is important to note that for ionic character, the following conditions hold: $\nabla^2\rho(r) > 0$, $-G/V > 1$, and $G + V > 0$. For covalent character, the conditions are: $\nabla^2\rho(r) < 0$, $-G/V < 1$, and $G + V < 0$ [71]. The aim of this study was to investigate the chemical bonds of potential inhibitors and gain a deeper understanding of the pharmacophoric groups that contribute most to the covalent or ionic character of the phosphorus-leaving group binding. These analyses were conducted prior to *docking* the inhibitors into the *HssAChE* active site. It is important to note that these bond characteristics, along with the reactivity parameters obtained from NBO calculations, are essential for selecting inhibitors with more stable transition states and lower energetic barriers for inhibiting *HssAChE*.

Table 1. The AIM B3LYP/6-311+g (2df, 2pd) parameters, Laplacian ($\nabla^2\rho(r)$), kinetic electron energy density ($G(r)$), potential electron energy density ($V(r)$) for bond critical point (BCP) of the phosphorus-leaving group*, and reactivity parameters from NBO.

Inhibitor	$\nabla^2\rho(r)$	G+V	-G/V	LUMO Energy	Occupancy
8(<i>R_p</i>)	0.13066	-0.18142	0.54129	0.09602	0.17494
8(<i>S_p</i>)	0.14046	-0.18226	0.54394	0.09623	0.16712
12	-0.27508	-0.13291	0.32554	0.17514	0.23347
13	-0.27480	-0.13214	0.32433	0.17595	0.23464
20	-0.22657	-0.08973	0.26936	0.10001	0.15679
28	-0.21927	-0.08709	0.27042	0.09450	0.15994
29	-0.22736	-0.08939	0.26693	0.09277	0.15753
64(<i>S_p</i>)	-0.21686	-0.08751	0.27558	0.09817	0.19069
69	-0.23355	-0.09153	0.26588	0.09787	0.15554
T	-0.45142	-0.15227	0.20564	0.17655	0.15893

*Computed results were performed by using the AIM and Gaussian09 programs. T stands for trichlorfon.

After fully optimizing the OP inhibitors, AIM calculations were performed to investigate the chemical bond character between the phosphorus atom and the leaving group of the selected compounds from the PCA. It is important to emphasize that the greater the ionic character of the bond, the more readily the leaving group can depart from the OP inhibitor during the inhibition process. From the previous table, it is clear that inhibitor **8 (*R_p* and *S_p*)** exhibits the highest ionic character in the chemical bond, suggesting that the leaving group dissociates more readily, lowering the energy barrier at the transition state. This trend is particularly evident in the higher values of $\nabla^2\rho(r)$ and $-G/V$ compared to the other compounds. The toxicity (LD₅₀) of these inhibitors is discussed later in this work, providing further insight into these trends. Thus, the results suggest that inhibitor **8** could exhibit higher inhibitory activity than trichlorfon. Additionally, the low LUMO energy is relevant in this analysis as it suggests greater reactivity. It should be noted that these results are preliminary, as this analysis was conducted outside the

enzyme environment. Once the compounds are docked into the *HssAChE* active site, the emergence of intermolecular interactions could modify these initial trends; however, this provides a solid foundation for further theoretical studies.

According to the data presented in Table 1, the OP inhibitors most similar to trichlorfon were inhibitors **12** and **13**. This conclusion is primarily supported by the computed values of $G + V$ and LUMO energies. It is known that the LUMO energy corresponds to the virtual orbital of the phosphorus-leaving group, and lower LUMO energy values are generally indicative of higher reactivity, considering the *HssAChE* inhibition mechanism [72]. The PCA analysis further supports this trend, showing that inhibitors **12** and **13** exhibited the least variation from trichlorfon. Additionally, both inhibitors share similar functional groups, which may provide favorable *docking* and stabilization within the *HssAChE* active site.

The literature reports that trichlorfon exhibited side effects in clinical trials, with toxicity levels deemed intolerable [35,73]. In this context, the search for compounds with both efficacy and lower toxicity levels is of significant interest.

3.3 Docking results

Molecular *docking* calculations were performed to position the OP inhibitors within the *HssAChE* active site, yielding stable conformations and assessing their interactions with the biomacromolecule. A cavity prediction algorithm based on a 3D box was used to identify the binding sites, with the calculated volume of the active site cavity being 169.472 Å³. Table 2 presents the intermolecular interaction energies and the hydrogen bonds formed between the ligands and the amino acid residues within the active cavity. The *docking* protocol validation was performed through *redocking* of co-crystallized ligand, with a value of 1.7141. Generally, a RMSD value lower than 2 Å indicates a successful binding mode prediction. To improve the reliability and accuracy of the *docking* results, replicates were performed using a different algorithm and constraint size. The correlation between the results was very high ($R^2 = 0.9882$), demonstrating the strong performance of the method. The correlation graph is shown in Figure S2.

Table 2. Values of the parameters obtained from molecular *docking* calculations for the inhibitors within the *HssAChE* active site.

Inhibitor	Intermolecular Interaction Energy (kcal mol ⁻¹)	H-bond Strength (kcal mol ⁻¹)	H-bond Length (Å)	Residues
8 (<i>R_p</i>)	-60.57	-0.226	3.189	His447
		-2.50	2.970	Ser203
8 (<i>S_p</i>)	-61.55	-2.50	2.890	His447
		-2.50	2.774	Ser203
		-2.20	2.799	Gly121
		-1.98	2.693	Gly122
12	-79.71	-0.86	3.145	Gly121
		-2.50	2.925	Ser203
		-1.59	3.281	Ser203
13	-90.97	-0.307	3.017	Gly121
		-2.50	2.849	Ser203
20	-89.96	-1.11	3.340	Asp74
		-0.090	2.982	Ser125
		-0.456	3.136	Gly121
		-1.44	3.311	Ser203
28	-79.87	-1.84	2.703	Gly121
		-1.78	2.854	Gly122
		-2.50	3.004	Ser203
		-2.30	2.831	Ser203
29	-81.96	-0.994	2.850	Gly121
		-0.453	3.062	Gly122
		-2.50	2.612	Tyr337
		-2.07	3.186	Ser203
64 (<i>S_p</i>)	-44.89	-0.396	3.337	Ser203
		-1.74	3.031	Gly121
		-1.57	3.053	Gly122

		-2.50	3.016	Ser203
69	-61.98	-2.50	2.764	Ser203
		-2.03	2.765	Gly121
		-1.55	2.559	Gly122
		-2.50	2.705	Tyr124
		-2.50	3.070	Ser203
		-1.69	3.261	Ser203
Trichlorfon	-67.19	-1.13	2.774	Gly121
		-1.13	3.167	Gly122
		-2.50	3.087	Ser203
		-0.305	3.239	Ser203

According to the data presented in Table 2, the inhibitors showed significant variations in their intermolecular interaction energies. Trichlorfon, the reference compound in this study, exhibited an interaction energy of $-67.19 \text{ kcal mol}^{-1}$. Inhibitor **8** (*Rp* and *Sp*) displayed close values of $-60.57 \text{ kcal mol}^{-1}$ and $-61.55 \text{ kcal mol}^{-1}$, respectively, which are slightly more stabilizing than that of trichlorfon, indicating less interaction. Notably, inhibitor **69** also showed a close interaction energy of $-61.98 \text{ kcal mol}^{-1}$. Inhibitor **8** (*Rp* and *Sp*) formed a stable hydrogen bond with the catalytic residue Ser203, as detailed in Table 2 and Figure 6, and also interacted with the catalytic residue His447.

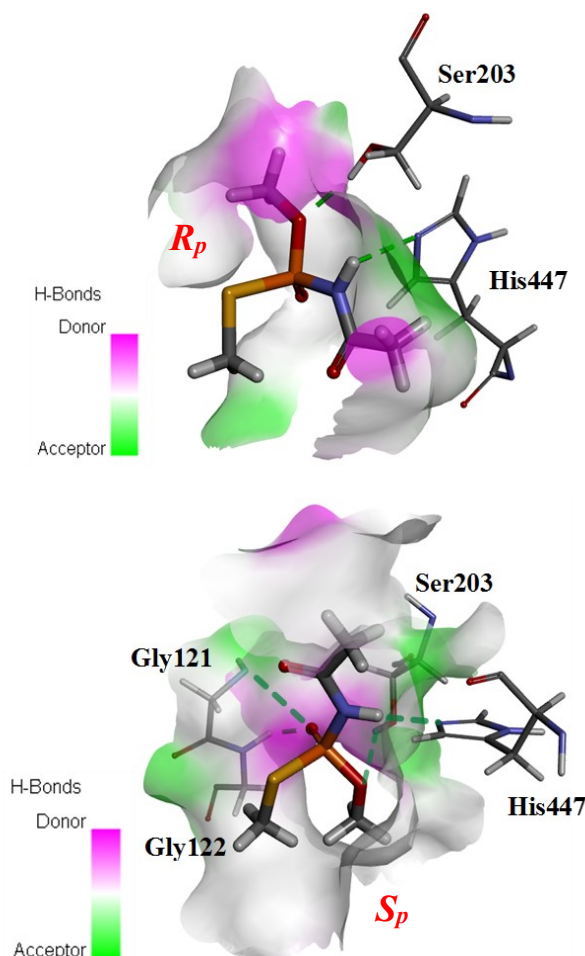


Figure 6. Hydrogen bonds formed by inhibitor 8 (*R_p* and *S_p*) in the *HssAChE* active site.

According to Figure 6, the *S_p* enantiomer of inhibitor **8** formed more hydrogen bonds than the *R_p* enantiomer, interacting with the residues Gly121, Gly122, Ser203, and His447. This suggests that the stereochemistry significantly altered the interaction modes of the ligand. Additionally, inhibitor **69** formed hydrogen bonds with Gly121, Gly122, and Tyr124, along with a stable interaction with Ser203. The formation of stable intermolecular interactions with Ser203 is crucial for the *docking* and stabilization of these inhibitors in the active site, as well as for the subsequent inhibition reaction mechanism.

From the *docking* calculations, a group of OP inhibitors exhibited very similar intermolecular interaction energies. These compounds include inhibitors **12** ($-79.71 \text{ kcal mol}^{-1}$), **28** ($-79.87 \text{ kcal mol}^{-1}$), and **29** ($-81.96 \text{ kcal mol}^{-1}$). These compounds shared common interactions with Gly121 and Ser203. Additionally, inhibitors **28** and **29** formed extra hydrogen bonds with Gly122, and inhibitor **29** also interacted with Tyr337, as detailed in Table 2. Inhibitor

29 appears to be better stabilized in the active site, likely due to the greater variety of residues with which it forms hydrogen bonds.

According to Table 2, one OP inhibitor stands out due to its distinct interaction energy. Inhibitor **64** (*Sp*) exhibited the least stabilizing interaction energy, with a computed value of $-44.89 \text{ kcal mol}^{-1}$. Although it formed hydrogen bonds with amino acid residues such as Gly121 and Gly122, and a stable interaction with Ser203, these interactions were insufficient to stabilize the inhibitor in the active site, resulting in a less stabilizing interaction energy. This suggests that more effective *docking* requires a diverse range of intermolecular interactions, including long-range, hydrophobic, and electrostatic interactions, which collectively influence the binding modes of the ligands. These types of interactions are illustrated for trichlorfon in Figure 7. The hydrophobic interaction surface of trichlorfon (Figure 7A) reveals its interaction with both hydrophobic regions (shown in red and lilac) and hydrophilic regions (shown in blue) within the active site.

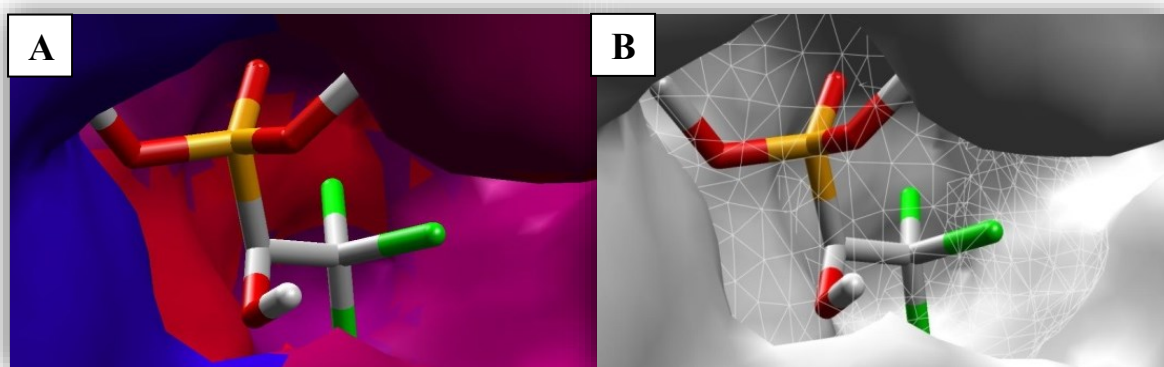


Figure 7. Hydrophobic interactions (A) and electrostatic interactions (B) for trichlorfon in the *HssAChE* active site.

The OP inhibitors that exhibited the most stable interaction energies (i.e., the lowest energies) were inhibitors **13**, **20**, **29**, **28**, and **12**, with computed values of $-90.97 \text{ kcal mol}^{-1}$, $-89.96 \text{ kcal mol}^{-1}$, $-81.96 \text{ kcal mol}^{-1}$, $-79.87 \text{ kcal mol}^{-1}$, and $-79.71 \text{ kcal mol}^{-1}$, respectively. Regarding intermolecular interactions, inhibitor **13** formed two hydrogen bonds with Gly121 and Ser203, demonstrating the best interaction energy within the enzyme. Inhibitor **12** also formed hydrogen bonds with these same residues. Inhibitor **20** showed an interaction energy very close to that of inhibitor **13** and formed a larger number of hydrogen bonds, with residues Asp74, Ser125, Gly121, and Ser203. As expected, all these inhibitors formed stable interactions with the catalytic Ser203 residue. The size of the inhibitor also contributed to the overall

intermolecular interaction energy. For example, inhibitor **13**, which has ethyl groups attached to the central phosphorus, displayed a more stabilizing interaction energy than inhibitor **12**, which contains methyl groups (Figure 5, Table 2). Their intermolecular interactions within the enzyme are shown in Figure S3. Additionally, a pharmacological analysis of the selected inhibitors was conducted, as shown in Table 3.

Table 3. Pharmacological properties predicted for the best-ranked inhibitors.

Inhibitor	Commercial name	BBB Permeability (Probability)	Hepatotoxicity	LD ₅₀ (mg kg ⁻¹)
8 (<i>R_p</i>)	Acephate	0.91	Inactive	233
8 (<i>S_p</i>)	Acephate	0.91	Inactive	233
12	Bromophos	0.87	Inactive	1600
13	Bromophos-ethyl	0.85	Inactive	52
20	Vamidotion	0.85	Inactive	40
28	Demeton-S-methyl	0.89	Inactive	10
29	Demeton-S-Methylsulphon	0.86	Inactive	30
64 (<i>S_p</i>)	Methamidophos	0.92	Inactive	8
69	Omethoate	0.87	Inactive	19
Trichlorfon	-	0.93	Inactive	210
Rivastigmine	-	0.93	Inactive	1000

Based on ProTox 3.0 predictions, Table 3 was constructed showing some of the ADMET profiles for each inhibitor studied in this work, such as blood-brain barrier (BBB) permeability, hepatotoxicity and LD₅₀. All inhibitors showed high probability of BBB permeation when compared to the reference compound trichlorfon, suggesting favorable central nervous system accessibility. Moreover, none of the compounds exhibited hepatotoxicity profile, but a variety of LD₅₀ values was observed, ranging from 8 to 1600 mg kg⁻¹, indicating substantial variability in safety profiles among the inhibitors. Despite these promising results, further experimental studies, including *in vivo* testing, are necessary to confirm their safety profile and therapeutic potential in clinical applications.

The promising inhibitors **12**, **13**, **20**, and **28** were further evaluated by comparing their experimental acute oral toxicity (LD₅₀) values with the theoretical LD₅₀ predictions obtained

from the ProTox 3.0 platform, aiming to gain deeper insights into their safety profiles and potential administration risks. The theoretical LD₅₀ values estimates acute oral toxicity in rodents through a consensus approach combining two-dimensional chemical similarity analysis and toxic fragment identification. Initially, the input compound is compared against a curated database of approximately 38,000 compounds with experimentally determined rodent LD₅₀ values, using molecular fingerprints and Tanimoto similarity metrics. The LD₅₀ prediction is primarily derived from the median LD₅₀ values of the most structurally similar compounds. In parallel, the presence of toxic fragments overrepresented in highly toxic compounds is assessed through a propensity score analysis. The final LD₅₀ estimate integrates both similarity-based predictions and fragment-based toxicity alerts, resulting in a conservative, mechanism-informed approximation of acute oral toxicity [74–76]

Inhibitor **12** (Bromophos) exhibited an experimental LD₅₀ of 1600 mg/kg in rats [77], which was in excellent agreement with the theoretical LD₅₀ value of 1600 mg/kg predicted. Inhibitor **13** (Bromophos-ethyl) showed experimental LD₅₀ values ranging from 52 to 170 mg/kg in rats [78], while its predicted LD₅₀ was 52 mg/kg, corresponding to the lower bound of the experimental range. For inhibitor **20** (Vamidothion), the experimental LD₅₀ was reported as 64 mg/kg [79], compared with a predicted value of 40 mg/kg. Similarly, inhibitor **28** (Demeton-S-methyl) presented an experimental LD₅₀ of 30 mg/kg [80], whereas the theoretical prediction was 10 mg/kg. From a toxicological standpoint, among the evaluated compounds, inhibitor **12** displays the least acute toxicity profile, even when compared to the toxicity reference compound Rivastigmine, which exhibited a LD₅₀ of 1000 mg kg⁻¹. The experimental and predicted LD₅₀ values showed good proximity in both magnitude and trend. The overall trends align, indicating that the predictions were largely accurate in terms of relative toxicity. In addition, drug-likeness analyses were performed, and the results are presented in Table 4.

Table 4. Drug-likeness assessment of inhibitors based on Lipinski, PAINS, Brenk and Veber.

Inhibitor	Lipinski #violations	PAINS #alerts	Brenk #alerts	Veber #violations
8 (<i>R_p</i>)	0	0	1	0
8 (<i>S_p</i>)	-	-	-	-
12	0	0	2	0
13	0	0	2	0
20	0	0	1	0
28	0	0	1	0
29	0	0	1	0
64 (<i>S_p</i>)	0	0	1	0
69	0	0	1	0
Trichlorfon	0	0	2	0
Rivastigmine	0	0	0	0

According to Lipinski *et al.*, on the guideline named ‘rule of 5’, it suggests that molecules whose properties fell inside of these boundaries would be more likely to be orally absorbed. In other words, these rules are: molecular mass ≤ 500 ; calculated octanol/water partition coefficient (ClogP) ≤ 5 ; Number of hydrogen bond donors ≤ 5 ; Number of hydrogen bond acceptors ≤ 10 . As shown in Table 4, the selected inhibitors, and the reference compound trichlorfon as well, performed no violations, which may indicate drug-likeness [81].

Table 4 summarizes other drug-likeness criteria, such as PAINS, Brenk and Veber. According to PAINS, none of the evaluated inhibitors or the reference compound trichlorfon triggered any alerts, indicating a low probability of interference and nonspecific bioactivity. Regarding the Brenk filter, most OP inhibitors presented one or two alerts, reflecting the presence of structural motifs commonly associated with toxicity or reactivity, such as thiophosphate, phosphate or halogenated ring. On the other hand, rivastigmine, a molecule lacking these motifs, exhibited no alerts. Remarkably, like Lipinski and PAINS, none of the compounds violated Veber rules, suggesting that these molecules retain acceptable drug-like characteristics while warranting careful toxicological consideration.

Besides that, toxicity profiling also revealed that inhibitor **20**, inhibitor **29**, inhibitor **69**, and trichlorfon were predicted as AMES positive, indicating a potential mutagenic risk. In contrast, inhibitors **8 (*R_p* and *S_p*)**, **12**, **13**, **28**, and **64**, as well as Rivastigmine, were predicted as AMES negative, suggesting no indication of mutagenic potential for these compounds.

Furthermore, no compounds were predicted to inhibit hERG channels, suggesting a low risk of cardiotoxicity associated with QT interval prolongation. Together, these results highlight a differentiated safety profile in which mutagenicity emerges as a more relevant concern than cardiotoxicity for the evaluated OP compounds.

Pre-filtering strategies based on drug-likeness rules and toxicity prediction help prioritize compounds with more favorable pharmacokinetic and safety profiles, aiding in identifying viable drug candidates. Furthermore, applying the filters before QM analysis can significantly reduce the number of compounds subjected to computationally requested calculations, thereby improving the overall efficiency of the workflow.

3.4 Mechanistic studies: QM/MM

A comprehensive understanding of the mechanism of action of these inhibitors is essential for the effective design of novel and potent pharmacophoric agents for therapeutic use. In this context, it is crucial to evaluate and characterize the action of these inhibitors against AD, which act by inhibiting the *HssAChE* enzyme, in line with the cholinergic hypothesis. The Quantum Mechanics/Molecular Mechanics (QM/MM) approach offers a powerful tool to investigate disease-related reactional systems, providing valuable data to support this theoretical study [82,83]. The energy profile graphs for the reaction mechanisms of inhibitor 12 and trichlorfon are presented in Figure 8.

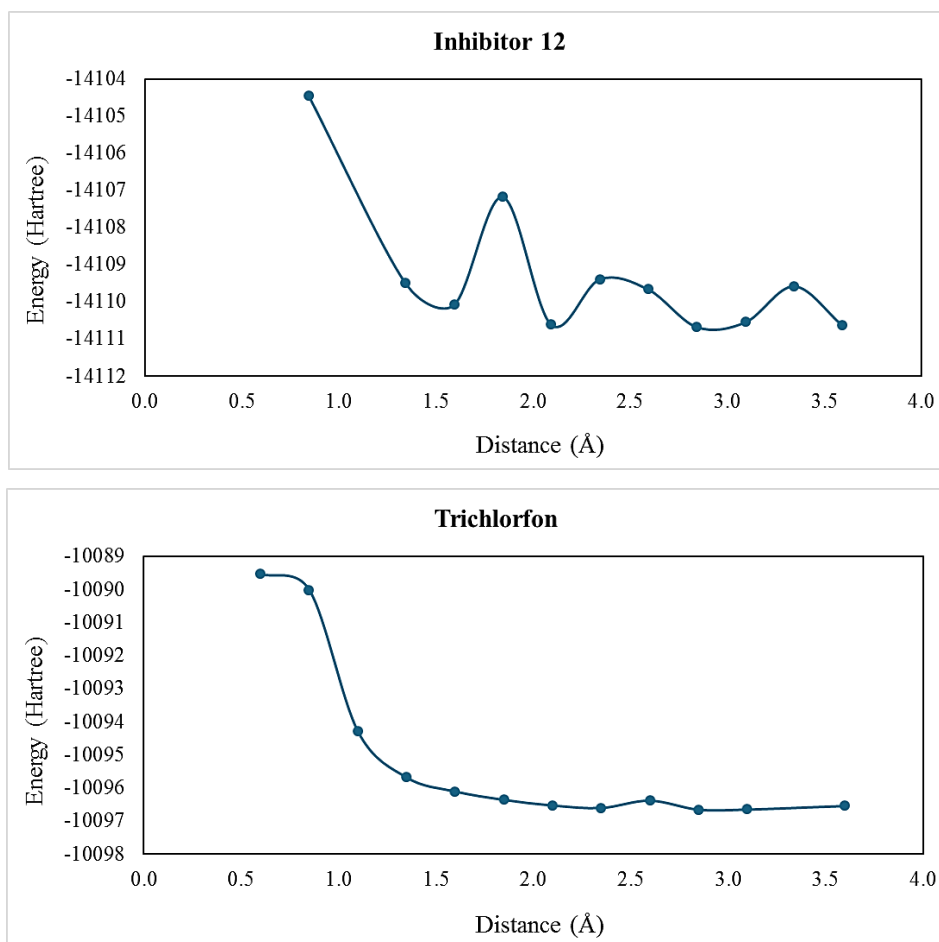


Figure 8. Energy profiles calculated for inhibitor **12** and trichlorfon in the inhibition reaction mechanism of *HssAChE*.

Based on the pharmacological parameters presented in Table 3, inhibitor **12** was selected for the reaction mechanism simulation due to its lowest toxicity, with LD₅₀ value of 1600 mg kg⁻¹, making it a promising candidate. This compound, commonly known as bromophos, exhibits lower toxicity than trichlorfon and has a lower energetic barrier, with a relative value of 44 kcal mol⁻¹ smaller than that of trichlorfon. This finding suggests that inhibitor **12** is more active toward the *HssAChE* enzyme. Given this trend, inhibitor **12** warrants further investigation to better assess its potential therapeutic effects in the treatment of neurodegenerative diseases.

According to Figure 8, the calculated energy profiles along the reaction coordinate reveal distinct mechanistic features for inhibitor **12** and trichlorfon. For inhibitor **12**, the energy profile exhibits a non-monotonic behavior, characterized by multiple fluctuations along the reaction coordinate. After an initial decrease in energy, a well-defined local maximum is observed, followed by additional variations before reaching the final stabilized region. These larger oscillations in energy are also due to the bulkier group attached to the central phosphorus.

In contrast, the trichlorfon energy profile displays a smoother and more monotonic trend after the initial region. A pronounced decrease in energy is observed, followed by gradual stabilization without significant oscillations. However, inhibitor 12 presented a lower energy barrier than trichlorfon, probably due to its stronger interaction within the active site. This study could serve as a starting point for the development of new therapies to treat AD. Experimental assays are essential to further evaluate the pharmacological and kinetic properties of these drug candidates.

Lower energetic barriers for inhibitor binding and stabilization of the enzyme-inhibitor complex indicate that the compound can more readily and persistently occupy the catalytic site of the *HssAChE*, reducing the energy required to form a nonproductive state of the enzyme. This energetic favorability enhances inhibitory efficiency by limiting catalytic turnover and slowing ACh hydrolysis. As a consequence, synaptic ACh levels increase, improving cholinergic neurotransmission. As discussed previously, restoration of cholinergic signaling mitigates cognitive symptoms by compensating for the neurotransmitter deficit characteristic of the disorder. Therefore, inhibitors associated with lower energetic barriers and more stable binding profiles are predicted to be more effective in modulating AChE activity and supporting symptomatic treatment of AD.

3.5 Molecular dynamics simulations and MM-PBSA calculations

The molecular dynamics results confirmed the conformational stability of inhibitor **12** within the *HssAChE* active site. Figure 9A presents the RMSD results for the ligands complexed with the protein. Trichlorfon exhibited stability at an RMSD of 0.125 nm predominantly throughout the entire trajectory, displaying a single peak in the graph containing the normalized histograms. Inhibitor **12** stabilized at an RMSD of 0.075 nm from approximately 30 ns until the end of the trajectory, exhibiting high conformational stability with average RMSD values lower than the reference compound. This generated a single peak in the normalized histogram, indicating the high frequency of low RMSD conformations.

Figure 9B shows the RMSD variation of the *HssAChE* protein complexed with the ligands throughout the simulations. The RMSD profiles are similar across the systems, showing low fluctuations and an absence of significant structural change, which indicates that the binding of the different compounds does not induce relevant global conformational alterations in the protein structure. This behavior suggests that the observed differences in complex stability are mainly associated with the local dynamics of the ligands within the active site,

rather than structural rearrangements of the enzyme as a whole. Overall, from 100 ns onwards, the protein stabilized within a narrow RMSD range, encompassing values from 0.125 to 0.175 nm.

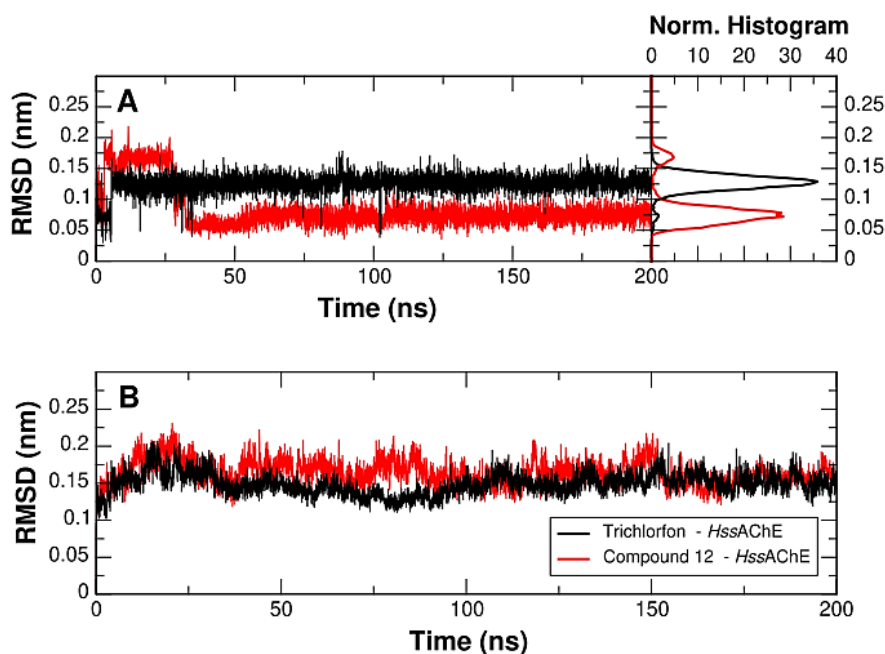


Figure 9. Structural stability analysis of the *HssAChE*-ligand complexes along 200 ns molecular dynamics simulations. (A) Time evolution of the ligand RMSD in complex with *HssAChE*: trichlorfon (black, reference ligand), inhibitor 12 (red). On the right, normalized RMSD histograms are shown for each ligand, highlighting their conformational distributions along the trajectory. (B) RMSD variation of the *HssAChE* protein complexed with the two ligands, indicating the overall structural stability of the enzyme during the simulations.

MM-PBSA calculations were performed to assess the energetic stability of the ligands. The convergence results are displayed in Figure 10, presenting cumulative average data of energy [84]. The values shown in the graph correspond to the free energy calculated considering only the enthalpic, Poisson-Boltzmann solvation and SASA contributions, without entropy correction, using frames from the last 50 ns of the molecular dynamics simulation. It was observed that the ΔG values converged for all systems from approximately 170 ns onwards, resulting in $-22.06 \text{ kcal mol}^{-1}$ for trichlorfon and $-27.48 \text{ kcal mol}^{-1}$ for inhibitor **12**.

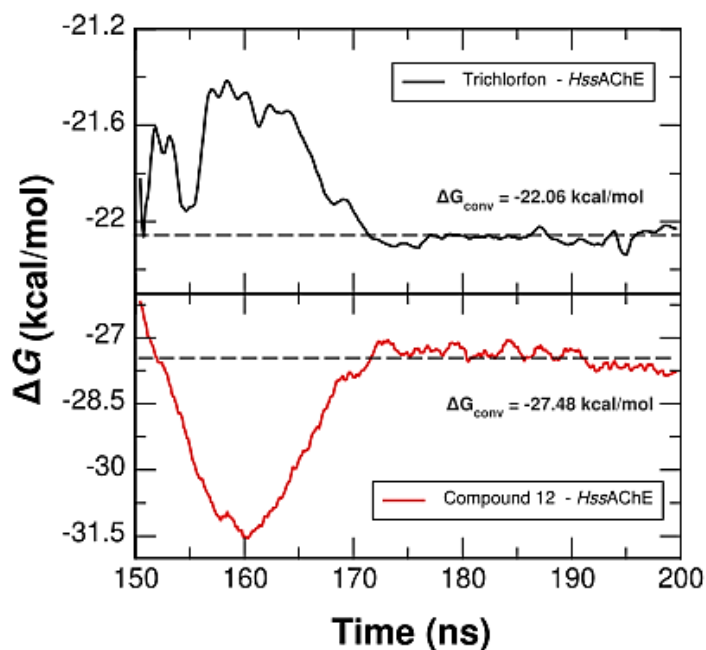


Figure 10. Convergence of the binding free energy ($\Delta G_{\text{gas+solv}}$) calculated by MM-PBSA without entropic correction for the *HssAChE*-trichlorfon (black) and *HssAChE*-inhibitor 12 (red) complexes. Dashed lines indicate the converged mean values (ΔG_{conv}) at the end of the trajectory, demonstrating stabilization of the cumulative averages and the statistical reliability of the energy estimates.

Table 5 presents the decomposition of the binding energies calculated by MM-PBSA for the complexes between *HssAChE* and the two ligands analyzed (trichlorfon – reference and inhibitor **12**), including the van der Waals terms (ΔE_{vdW}), electrostatic (ΔE_{el}), free energy in vacuum added to polar/nonpolar solvation ($\Delta G_{\text{gas+solv}}$), entropic contribution ($-T\Delta S$) and final binding free energy (ΔG_{bind}).

Table 5. MM-PBSA binding energy decomposition for the *HssAChE*-ligand complexes: van der Waals contribution (ΔE_{vdW}), electrostatic contribution (ΔE_{el}), combined gas-phase and solvation free energy ($\Delta G_{\text{gas+solv}}$), entropic term ($-T\Delta S$), and final binding free energy (ΔG_{bind}) for trichlorfon and inhibitor **12**.

Compound	MM-PBSA energies (kcal mol ⁻¹)				
	ΔE_{vdW}	ΔE_{el}	$\Delta G_{\text{gas+solv}}$	$-T\Delta S$	ΔG_{bind}
Trichlorfon	-22.53 ± 0.39	-283.64 ± 1.09	-22.06 ± 1.62	11.01 ± 0.33	-11.31 ± 3.18
12	-34.44 ± 0.44	-284.16 ± 1.28	-27.48 ± 2.35	9.55 ± 0.41	-17.93 ± 2.94

It is observed that, for all systems, the electrostatic term (ΔE_{el}) exhibits a significantly larger magnitude (more negative) than the van der Waals term (ΔE_{vdW}). This behavior may be directly related to the chemical nature of the ligands. All compounds possess strongly polar and

charged groups, specifically the positively charged phosphorus center (P^+), in addition to electronegative heteroatoms (O, S, N) capable of establishing intense electrostatic interactions, such as hydrogen bonds, ion-dipole, and dipole-dipole interactions with charged and polar residues of the active site. In particular, trichlorfon presents a phosphoryl group associated with a highly polarized center, whereas inhibitor **12** contain multiple heteroatoms and sulfur-phosphorus containing functional groups, favoring long-range electrostatic interactions [85]. This explains why ΔE_{el} energetically dominates over ΔE_{vdW} , which represents short-range interactions, essentially hydrophobic and steric packing forces.

However, this strong electrostatic stabilization in the bound state is partially offset by a high solvation penalty, reflected in the less favorable $\Delta G_{gas+solv}$ values and, consequently, in the final binding free energy. This effect stems from the fact that highly polar and charged ligands are strongly stabilized by the solvent (water) in the free state through extensive solvation networks and electrostatic interactions with the medium [86,87]. Upon complexation with the protein, part of this stabilization is lost due to the desolvation of the ligand and, to a lesser extent, the active site, generating a high energetic cost of solvation. Thus, although protein-ligand electrostatic interactions are energetically very favorable in a vacuum (highly negative ΔE_{el}), the PBSA model reintroduces this cost via the polar solvation term, resulting in significant compensation.

From a comparative standpoint, inhibitor **12** presents the most favorable ΔG_{bind} value ($-17.93 \text{ kcal mol}^{-1}$), the result of a more efficient balance between intense electrostatic interactions, relevant van der Waals contributions, and a solvation penalty that is relatively compensated by the energetic gain in the complex, while trichlorfon exhibits the lowest affinity value ($-11.31 \text{ kcal mol}^{-1}$), indicating lower overall binding efficiency. The entropic contribution ($-T\Delta S$), which is relatively similar among the two systems, suggests that the affinity differences are predominantly governed by enthalpic factors, especially the competition between protein-ligand electrostatic stabilization and the desolvation penalty. Taken together, these results indicate that the binding of the compounds to *HssAChE* is entropically similar yet enthalpically distinct, being controlled by a delicate balance between strong electrostatic interactions and high solvation costs.

In an integrated manner, the molecular dynamics and MM-PBSA results converge to a coherent picture of structural stability and energetic differentiation among the *HssAChE*-ligand complexes. The RMSD analyses demonstrate that all systems are conformationally stable throughout the simulations, with no global perturbations in the enzyme structure, indicating that affinity differences do not stem from conformational rearrangements of the protein, but rather

from local dynamics and the mode of accommodation of the ligands within the active site. Consistent with this structural behavior, the MM-PBSA calculations reveal a binding profile governed predominantly by enthalpic factors, marked by intense electrostatic interactions partially offset by high desolvation costs, and by similar entropic contributions across the systems. In this context, inhibitor **12** stands out for presenting the most favorable balance between intermolecular stabilization and solvation penalty, reflecting the highest calculated affinity and a more efficient interaction with *HssAChE*. Collectively, these findings indicate that inhibitor **12** is the most promising candidate, providing a consistent molecular basis for its prioritization in experimental studies and in the rational design of new *HssAChE* inhibitors.

CONCLUSIONS

As time progresses, AD has become an increasingly alarming global health concern. According to the cholinergic hypothesis, partial inhibition of the *HssAChE* enzyme can elevate neurotransmitters levels in synapses, thereby providing symptomatic relief and improving cognitive function. In this context, trichlorfon emerged as a potential compound, despite its well documented adverse effects. Consequently, a diverse set of OP compounds was evaluated in this study to identify candidates capable of achieving the efficiency of trichlorfon while exhibiting lower toxicity for clinical application. Among the evaluated molecules, inhibitors **12**, **13**, **20**, and **28** emerged as promising candidates for AD treatment, based on their favorable interaction profiles with *HssAChE* and their predicted safety parameters. Notably, inhibitor **12** displayed an energy barrier significantly lower than that of trichlorfon and, according to the toxicity analysis, exhibited a more favorable LD₅₀ profile compared with the reference drug Rivastigmine, highlighting its potential as a lead compound for AD therapy. Furthermore, the extent of toxicity of OP compounds is strongly dependent on the molecular structure, dose, and pharmacokinetics properties. For instance, the pharmacological effect of irreversible acetylcholinesterase inhibitors is limited by the natural turnover and resynthesis of the enzyme, which allows controlled levels of inhibition over time, avoiding cumulative toxicity [88,89]. Overall, this theoretical investigation provides a solid foundation for subsequent experimental studies aimed at further elucidating the pharmacological and kinetic properties of these inhibitors.

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AUTHORS' CONTRIBUTIONS

AAC designed the study. AAC, HFS, IAM and APLM contributed equally to this work, as well as performed calculations and data analyses. AAC, HFS, IAM and APLM contributed to manuscript preparation. TCR, EFFC and PHM conceived the project, supervised students and contributed to the revision of the whole manuscript. All authors discussed the results and contributed to the final manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study did not involve human participants or animals. Therefore, ethics approval and informed consent were not required.

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REFERENCES

- [1] Frisoni, G.B.; Aho, E.; Brayne, C.; Ciccarelli, O.; Dubois, B.; Fox, N.C.; Frederiksen, K.S.; Gabay, C.; Garibotto, V.; Hofmarcher, T.; Jack Jr, C.R.; Kivipelto, M.; Petersen, R.C.; Ribaldi, F.; Rowe, C.C.; Walsh, S.; Zetterberg, H.; Hansson, O. Alzheimer's Disease Outlook: Controversies and Future Directions. *The Lancet*, **2025**, *406*, 1424–1442.
- [2] Bradshaw, A.C.; Georges, J. Anti-Amyloid Therapies for Alzheimer's Disease: An Alzheimer Europe Position Paper and Call to Action. *J. Prev. Alzheimers Dis.*, **2024**, *11*, 265–273.
- [3] Abyadeh, M.; Gupta, V.; Paulo, J.A.; Mahmoudabad, A.G.; Shadfar, S.; Mirshahvaladi, S.; Gupta, V.; Nguyen, C.T.O.; Finkelstein, D.I.; You, Y.; Haynes, P.A.; Salekdeh, G.H.; Graham, S.L.; Mirzaei, M. Amyloid-Beta and Tau Protein beyond Alzheimer's Disease. *Neural Regen. Res.*, **2024**, *19*.
- [4] Walsh, D.M.; Selkoe, D.J. A Beta Oligomers - a Decade of Discovery. *J. Neurochem.*, **2007**, *101*, 1172–1184.
- [5] Grill, J.D.; Cummings, J.L. Current Therapeutic Targets for the Treatment of Alzheimer's Disease. *Expert Rev. Neurother.*, **2010**, *10*, 711–728.
- [6] De Strooper, B.; Vassar, R.; Golde, T. The Secretases: Enzymes with Therapeutic Potential in Alzheimer Disease. *Nat. Rev. Neurol.*, **2010**, *6*, 99.
- [7] Kim, T.-W. Drug Repositioning Approaches for the Discovery of New Therapeutics for Alzheimer's Disease. *Neurotherapeutics*, **2015**, *12*, 132–142.
- [8] Klafki, H.-W.; Staufenbiel, M.; Kornhuber, J.; Wiltfang, J. Therapeutic Approaches to Alzheimer's Disease. *Brain*, **2006**, *129*, 2840–2855.
- [9] de Castro, A.A.; Silva, H.F.; Garcia, M.V.F.P.; Homem-de-Mello, P.; Ramalho, T.C. Molecular Modeling Insights on the Pharmaceuticals and Hypotheses of Alzheimer's Disease. *Curr. Med. Chem.*, **2024**.
- [10] de Castro, A.A.; Soares, F. V; Pereira, A.F.; Polisel, D.A.; Caetano, M.S.; Leal, D.H.S.; da Cunha, E.F.F.; Nepovimova, E.; Kuca, K.; Ramalho, T.C. Non-Conventional Compounds with Potential Therapeutic Effects against Alzheimer's Disease. *Expert Rev. Neurother.*, **2019**, *19*, 375–395.
- [11] de Castro, A.A.; da Cunha, E.F.F.; Pereira, A.F.; Soares, F. V; Leal, D.H.S.; Kuca, K.; Ramalho, T.C. Insights into the Drug Repositioning Applied to the Alzheimer's Disease Treatment and Future Perspectives. *Curr. Alzheimer Res.*, **2018**, *15*, 1161–1178.
- [12] Sales, T.A.; Prandi, I.G.; Castro, A.A. de; Leal, D.H.S.; Cunha, E.F.F. da; Kuca, K.; Ramalho, T.C. Recent Developments in Metal-Based Drugs and Chelating Agents for Neurodegenerative Diseases Treatments. *Int. J. Mol. Sci.*, **2019**, *20*, 1829.
- [13] Prajapati, S.K.; Pathak, A.; Samaiya, P.K. Alzheimer's Disease: From Early Pathogenesis to Novel Therapeutic Approaches. *Metab. Brain Dis.*, **2024**, *39*, 1231–1254.
- [14] Asati, V.; Thakur, S.; Upmanyu, N.; Bharti, S. Virtual Screening, Molecular Docking, and DFT Studies of Some Thiazolidine-2,4-diones as Potential PIM-1 Kinase Inhibitors. *ChemistrySelect*, **2018**, *3*, 127–135.
- [15] Ritu; Sharma, P.; Gupta, G.D.; Asati, V. Design, Synthesis and Antidiabetic Study of Triazole Clubbed Indole Derivatives as α -Glucosidase Inhibitors. *Bioorg. Chem.*, **2023**, *139*, 106750.

- [16] Sharma, A.; Rudrawar, S.; Bharate, S.B.; Jadhav, H.R. Recent Advancements in the Therapeutic Approaches for Alzheimer's Disease Treatment : Current and Future Perspective †. **2025**, 652–693.
- [17] Singh, Y.P.; Prasad, S.; Kumar, H. A Comprehensive Analysis on Galantamine Based Hybrids for the Management of Alzheimer's Disease. *Chem. Biol. Drug Des.*, **2024**, *104*, e70004.
- [18] Francis, P.T.; Palmer, A.M.; Snape, M.; Wilcock, G.K. The Cholinergic Hypothesis of Alzheimer's Disease: A Review of Progress. *Journal of Neurology, Neurosurgery & Psychiatry*, **1999**, *66*, 137 LP – 147.
- [19] Contestabile, A. The History of the Cholinergic Hypothesis. *Behavioural brain research*, **2011**, *221*, 334–340.
- [20] Atta, A.M.; Elkalla, W.S.; Omran, S.A.; Elgawish, M.S. Innovations in Acetylcholinesterase Inhibitors : Emerging Strategies for Alzheimer's Disease Therapy (2019 – 2024). **2024**, 152–170.
- [21] Saha, B.; Das, A.; Jangid, K.; Kumar, A.; Kumar, V.; Jaitak, V. Identification of Coumarin Derivatives Targeting Acetylcholinesterase for Alzheimer's Disease by Field-Based 3D-QSAR, Pharmacophore Model-Based Virtual Screening, Molecular Docking, MM/GBSA, ADME and MD Simulation Study. *Curr. Res. Struct. Biol.*, **2024**, *7*, 100124.
- [22] Ahlmann, M.; Hempel, G. The Effect of Cyclophosphamide on the Immune System: Implications for Clinical Cancer Therapy. *Cancer Chemotherapy and Pharmacology*, **2016**, *78*, 661–671.
- [23] Emadi, A.; Jones, R.J.; Brodsky, R.A. Cyclophosphamide and Cancer: Golden Anniversary. *Nature Reviews Clinical Oncology*, **2009**, *6*, 638–647.
- [24] Schmidt, K.G.; Horowitz, Y.; Buckman, G.; Segev, E.; Levinger, E.; Geyer, O. Lowering of IOP by Echthiophate Iodide in Pseudophakic Eyes with Glaucoma. *Curr. Eye Res.*, **2010**, *35*, 698–702.
- [25] Díez-Aguilar, M. *New Microbiological Aspects of Fosfomycin*.
- [26] Gardiner, B.J.; Stewardson, A.J.; Abbott, I.J.; Peleg, A.Y. Nitrofurantoin and Fosfomycin for Resistant Urinary Tract Infections: Old Drugs for Emerging Problems. *Aust. Prescr.*, **2019**, *42*, 14–19.
- [27] Lleo, A.; Greenberg, S.M.; Growdon, J.H. Current Pharmacotherapy for Alzheimer's Disease. *Annu. Rev. Med.*, **2006**, *57*, 513–533.
- [28] Kandy, A.T.; Venkatesan, R.; Mohan, D.; Chadapullykolumbu Ismail, S.; Jupudi, S.; Selvaraj, D. Acetylcholinesterase Inhibitors from Carbamate and Benzo-Fused Heterocyclic Scaffolds: Promising Therapeutics for Alzheimer's Disease. *Medicinal Chemistry Research*, **2025**, *34*, 1200–1211.
- [29] Ramalho, T.C.; de Castro, A.A.; Silva, D.R.; Silva, M.C.; Franca, T.C.C.; Bennion, B.J.; Kuca, K. Computational Enzymology and Organophosphorus Degrading Enzymes: Promising Approaches Toward Remediation Technologies of Warfare Agents and Pesticides. *Curr. Med. Chem.*, **2016**, *23*, 1041–1061.
- [30] Bagrowska, W.; Karasewicz, A.; Góra, A. Comprehensive Analysis of Acetylcholinesterase Inhibitor and Reactivator Complexes: Implications for Drug Design and Antidote Development. *Drug Discov. Today*, **2024**, *29*, 104217.
- [31] Hörnberg, A.; Tunemalm, A.K.; Ekström, F. Crystal Structures of Acetylcholinesterase in Complex with Organophosphorus Compounds Suggest That the Acyl Pocket Modulates the

Aging Reaction by Precluding the Formation of the Trigonal Bipyramidal Transition State. *Biochemistry*, **2007**, *46*, 4815–4825.

- [32] Franklin, M.C.; Rudolph, M.J.; Ginter, C.; Cassidy, M.S.; Cheung, J. Structures of Paraoxon-Inhibited Human Acetylcholinesterase Reveal Perturbations of the Acyl Loop and the Dimer Interface. *Proteins*, **2016**, *84*, 1246–1256.
- [33] Giovannini, M.G.; Scali, C.; Bartolini, L.; Schmidt, B.; Pepeu, G. Effect of Subchronic Treatment with Metrifonate and Tacrine on Brain Cholinergic Function in Aged F344 Rats. *Eur. J. Pharmacol.*, **1998**, *354*, 17–24.
- [34] Lopez-Arrieta, J.M.; Schneider, L. Metrifonate for Alzheimer's Disease. *Cochrane Database Syst. Rev.*, **2006**, CD003155.
- [35] Mori, F.; Lai, C.C.; Fusi, F.; Giacobini, E. Cholinesterase Inhibitors Increase Secretion of APPs in Rat Brain Cortex. *Neuroreport*, **1995**, *6*, 633–636.
- [36] Blass, J.P.; Cyrus, P.A.; Bieber, F.; Gulanski, B. Randomized, Double-Blind, Placebo-Controlled, Multicenter Study to Evaluate the Safety and Tolerability of Metrifonate in Patients with Probable Alzheimer Disease. The Metrifonate Study Group. *Alzheimer Dis. Assoc. Disord.*, **2000**, *14*, 39–45.
- [37] Heinig, R.; Sachse, R. The Effect of Food and Time of Administration on the Pharmacokinetic and Pharmacodynamic Profile of Metrifonate. *Int. J. Clin. Pharmacol. Ther.*, **1999**, *37*, 456–464.
- [38] J. Frisch, M.; W. Trucks, G.; B Schlegel, H.; E Scuseria, G.; Robb, M.; Cheeseman, J.; Scalmani, G.; Barone, V.; Mennucci, B.; A. H. Petersson, G.; Nakatsuji, H.; Caricato, M.; Li, X.; P Hratchian, H.; F Izmaylov, A.; Bloino, J.; Zheng, G.; L Sonnenberg, J.; Hada, M.; Fox, D. *Gaussian 09 (Revision A02)*; **2009**.
- [39] KUMAR, P.S.V.; RAGHAVENDRA, V.; SUBRAMANIAN, V. Bader's Theory of Atoms in Molecules (AIM) and Its Applications to Chemical Bonding. *Journal of Chemical Sciences*, **2016**, *128*, 1527–1536.
- [40] Silva, T.C.; de Almeida, K.J.; dos Santos Pires, M.; de Castro, A.A.; Gonçalves, M.A.; da Cunha, E.F.F.; Ramalho, T.C. Theoretical Structural and Electronic Analyses with Emphasis on the Reactivity of Iron Oxide Prototypes in Methane C–H Bond Activation. *Reaction Kinetics, Mechanisms and Catalysis*, **2017**, *120*, 195–208.
- [41] KUMAR, P.S.V.; RAGHAVENDRA, V.; SUBRAMANIAN, V. Bader's Theory of Atoms in Molecules (AIM) and Its Applications to Chemical Bonding. *Journal of Chemical Sciences*, **2016**, *128*, 1527–1536.
- [42] D., G.E.; R., L.C.; Frank, W. Natural Bond Orbital Methods. *Wiley Interdiscip. Rev. Comput. Mol. Sci.*, **2011**, *2*, 1–42.
- [43] Weinhold, F.; Landis, C.R. *Discovering Chemistry With Natural Bond Orbitals*; Wiley: Hoboken, NJ, **2012**.
- [44] Hanselman, D.; C. Littlefield, B. *Mastering MATLAB 5: A Comprehensive Tutorial and Reference*; **2001**.
- [45] Oliveira, B.G.; Vasconcellos, M.L.A.A. Hydrogen Bonds in Alcohols:Water Complexes: A Theoretical Study about New Intramolecular Interactions via CHELPG and AIM Calculations. *Journal of Molecular Structure: THEOCHEM*, **2006**, *774*, 83–88.
- [46] Thomsen, R.; Christensen, M.H. MolDock: A New Technique for High-Accuracy Molecular Docking. *J. Med. Chem.*, **2006**, *49*, 3315–3321.

- [47] Ramalho, T.C.; Caetano, M.S.; da Cunha, E.F.F.; Souza, T.C.S.; Rocha, M.V.J. Construction and Assessment of Reaction Models of Class I EPSP Synthase: Molecular *Docking* and Density Functional Theoretical Calculations. *J. Biomol. Struct. Dyn.*, **2009**, *27*, 195–207.
- [48] Banerjee, P.; Kemmler, E.; Dunkel, M.; Preissner, R. ProTox 3.0: A Webserver for the Prediction of Toxicity of Chemicals. *Nucleic Acids Res.*, **2024**, *52*, W513–W520.
- [49] Anant, A.; Ali, A.; Ali, A.; Gupta, G.D.; Asati, V. A Computational Approach to Discover Potential Quinazoline Derivatives against CDK4/6 Kinase. *J. Mol. Struct.*, **2021**, *1245*, 131079.
- [50] de Castro, A.A.; Assis, L.C.; Silva, D.R.; Corrêa, S.; Assis, T.M.; Gajo, G.C.; Soares, F. V; Ramalho, T.C. Computational Enzymology for Degradation of Chemical Warfare Agents: Promising Technologies for Remediation Processes. *catalysis*, **2017**, *12*, 13.
- [51] 3D-QSAR and Virtual Screening Studies of Thiazolidine-2,4-Dione Analogs.
- [52] Gupta, S.; Baweja, G.S.; Gupta, G.D.; Asati, V. Identification of Potential N-Substituted 5-Benzylidenethiazolidine-2,4-dione Derivatives as α -Amylase Inhibitors: Computational Cum Synthetic Studies. *J. Mol. Struct.*, **2023**, *1287*, 135596.
- [53] Coutinho, K.; Rivelino, R.; Georg, H.C.; Canuto, S. A New Technique for High-Accuracy Molecular *Docking*. In: Canuto, S., Ed.; Springer Netherlands: Dordrecht, **2008**; pp. 159–189.
- [54] Sartorelli, J.; de Castro, A.A.; Ramalho, T.C.; Giacoppo, J.O.S.; Mancini, D.T.; Caetano, M.S.; da Cunha, E.F.F. Asymmetric Biocatalysis of the Nerve Agent VX by Human Serum Paraoxonase 1: Molecular *Docking* and Reaction Mechanism Calculations. *Medicinal Chemistry Research*, **2016**, *25*, 2521–2533.
- [55] de Castro, A.A.; Caetano, M.S.; Silva, T.C.; Mancini, D.T.; Rocha, E.P.; da Cunha, E.F.F.; Ramalho, T.C. Molecular *Docking*, Metal Substitution and Hydrolysis Reaction of Chiral Substrates of Phosphotriesterase. *Comb. Chem. High Throughput Screen.*, **2016**, *19*, 334–344.
- [56] Soares, F. V; de Castro, A.A.; Pereira, A.F.; Leal, D.H.S.; Mancini, D.T.; Krejcar, O.; Ramalho, T.C.; da Cunha, E.F.F.; Kuca, K. Theoretical Studies Applied to the Evaluation of the DFPase Bioremediation Potential against Chemical Warfare Agents Intoxication. *Int. J. Mol. Sci.*, **2018**, *19*.
- [57] Polisel, D.A.; de Castro, A.A.; Mancini, D.T.; da Cunha, E.F.F.; França, T.C.C.; Ramalho, T.C.; Kuca, K. Slight Difference in the Isomeric Oximes K206 and K203 Makes Huge Difference for the Reactivation of Organophosphorus-Inhibited AChE: Theoretical and Experimental Aspects. *Chem. Biol. Interact.*, **2019**, *309*, 108671.
- [58] Pereira, A.F.; de Castro, A.A.; Soares, F.V.; Soares Leal, D.H.; da Cunha, E.F.F.; Mancini, D.T.; Ramalho, T.C. Development of Technologies Applied to the Biodegradation of Warfare Nerve Agents: Theoretical Evidence for Asymmetric Homogeneous Catalysis. *Chem. Biol. Interact.*, **2019**, *308*, 323–331.
- [59] de Castro, A.A.; Soares, F.V.; Pereira, A.F.; Silva, T.C.; Silva, D.R.; Mancini, D.T.; Caetano, M.S.; da Cunha, E.F.F.; Ramalho, T.C. Asymmetric Biodegradation of the Nerve Agents Sarin and VX by Human DUTPase: Chemometrics, Molecular *Docking* and Hybrid QM/MM Calculations. *J. Biomol. Struct. Dyn.*, **2019**, *37*, 2154–2164.
- [60] de Castro, A.A.; Polisel, D.A.; Pereira, B.T.L.; da Cunha, E.F.F.; Kuca, K.; Nepovimova, E.; Ramalho, T.C. Understanding the Interaction Modes and Reactivity of Trimedoxime toward MmAChE Inhibited by Nerve Agents: Theoretical and Experimental Aspects. *International Journal of Molecular Sciences*, **2020**, *21*.

- [61] Wang, J.; Wolf, R.M.; Caldwell, J.W.; Kollman, P.A.; Case, D.A. Development and Testing of a General Amber Force Field. *J. Comput. Chem.*, **2004**, *25*, 1157–1174.
- [62] Case, D.A.; Aktulga, H.M.; Belfon, K.; Cerutti, D.S.; Cisneros, G.A.; Cruzeiro, V.W.D.; Forouzeshe, N.; Giese, T.J.; Götz, A.W.; Gohlke, H.; Izadi, S.; Kasavajhala, K.; Kaymak, M.C.; King, E.; Kurtzman, T.; Lee, T.-S.; Li, P.; Liu, J.; Luchko, T.; Luo, R.; Manathunga, M.; Machado, M.R.; Nguyen, H.M.; O’Hearn, K.A.; Onufriev, A. V; Pan, F.; Pantano, S.; Qi, R.; Rahnamoun, A.; Risheh, A.; Schott-Verdugo, S.; Shajan, A.; Swails, J.; Wang, J.; Wei, H.; Wu, X.; Wu, Y.; Zhang, S.; Zhao, S.; Zhu, Q.; Cheatham, T.E.I.I.I.; Roe, D.R.; Roitberg, A.; Simmerling, C.; York, D.M.; Nagan, M.C.; Merz, K.M.Jr. AmberTools. *J. Chem. Inf. Model.*, **2023**, *63*, 6183–6191.
- [63] Sousa da Silva, A.W.; Vranken, W.F. ACPYPE - AnteChamber PYthon Parser InterfacE. *BMC Res. Notes*, **2012**, *5*, 367.
- [64] Abraham, M.J.; Murtola, T.; Schulz, R.; Páll, S.; Smith, J.C.; Hess, B.; Lindahl, E. GROMACS: High Performance Molecular Simulations through Multi-Level Parallelism from Laptops to Supercomputers. *SoftwareX*, **2015**, *1–2*, 19–25.
- [65] Valdés-Tresanco, M.S.; Valdés-Tresanco, M.E.; Valiente, P.A.; Moreno, E. Gmx_MMPBSA: A New Tool to Perform End-State Free Energy Calculations with GROMACS. *J. Chem. Theory Comput.*, **2021**, *17*, 6281–6291.
- [66] Fereidounpour, P.; Steinmann, C.; Larsen, K.L. Prediction of the Free Energy of Binding for Cyclodextrin-Steroid Complexes: Phase Solubility and Molecular Dynamics Studies. *J. Incl. Phenom. Macrocycl. Chem.*, **2024**, *104*, 535–546.
- [67] Jobson, J.D. Multiple Linear Regression BT - Applied Multivariate Data Analysis: Regression and Experimental Design. In: Jobson, J.D., Ed.; Springer New York: New York, NY, **1991**; pp. 219–398.
- [68] Asati, V.; Bharti, S.; Rathore, A.; Kar Mahapatra, D. SWFB and GA Strategies for Variable Selection in QSAR Studies for the Validation of Thiazolidine- 2,4-Dione Derivatives as Promising Antitumor Candidates. *Indian Journal of Pharmaceutical Education and Research*, **2017**, *51*, 436–451.
- [69] Rathore, A.; Asati, V.; Mishra, M.; Das, R.; Kashaw, V.; Kashaw, S.K. Computational Approaches for the Design of Novel Dopamine D2 and Serotonin 5-HT2A Receptor Dual Antagonist towards Schizophrenia. *In Silico Pharmacol.*, **2022**, *10*, 7.
- [70] Lima, W.E.A. de; Pereira, A.F.; Castro, A.A. de; Ramalho, E.F.F. da C. and T.C. Flexibility in the Molecular Design of Acetylcholinesterase Reactivators: Probing Representative Conformations by Chemometric Techniques and Docking/QM Calculations. *Letters in Drug Design & Discovery*, **2016**, *13*, 360–371.
- [71] Silva, T.C.; de Almeida, K.J.; dos Santos Pires, M.; de Castro, A.A.; Gonçalves, M.A.; da Cunha, E.F.F.; Ramalho, T.C. Theoretical Structural and Electronic Analyses with Emphasis on the Reactivity of Iron Oxide Prototypes in Methane C–H Bond Activation. *Reaction Kinetics, Mechanisms and Catalysis*, **2017**, *120*, 195–208.
- [72] Fukui, K. Role of Frontier Orbitals in Chemical Reactions. *Science*, **1982**, *218*, 747–754.
- [73] Lamb, H.M.; Faulds, D. Metrifonate. *Drugs Aging*, **1997**, *11*, 490–496.
- [74] Lai, K.-C.; Hsieh, H.-H.; Lu, Y.-L.; Liu, S.-Y.; Ha, H.-A.; Hour, M.-J. Design and Synthesis of Holu-7, a Quinazolinone Derivative With an Active Stereo Geometry and Polar 2,4-Dihydroxyphenyl Group, as a Selective Anticancer Agent. *Chem. Biol. Drug Des.*, **2025**, *106*, e70209.

- [75] Jurowski, K.; Niżnik, Ł.; Frydrych, A.; Kobylarz, D.; Noga, M.; Krośniak, A.; Fijałkowska, O.; Świdniak, A.; Ahuja, V. Toxicological Profile of Acovenoside A as an Active Pharmaceutical Ingredient – Prediction of Missing Key Toxicological Endpoints Using *in Silico* Toxicology Methodology. *Chem. Biol. Interact.*, **2025**, *408*, 111404.
- [76] Banerjee, P.; Ulker, O.; Ozkan, I.; Ulker, O.C. The Investigation of the Toxicity of Organophosphorus Flame Retardants (OPFRs) by Using *in Silico* Toxicity Prediction Platform ProTox- 3.0. *Toxicol. Mech. Methods*, **2025**, *35*, 32–42.
- [77] Hazardous Substances Data Bank (HSDB) : 6573 - PubChem.
<https://pubchem.ncbi.nlm.nih.gov/source/hsdb/6573> (Accessed Feb 18, **2026**).
- [78] Hazardous Substances Data Bank (HSDB) : 6574 - PubChem.
<https://pubchem.ncbi.nlm.nih.gov/source/hsdb/6574> (Accessed Feb 18, **2026**).
- [79] Hazardous Substances Data Bank (HSDB) : 6746 - PubChem.
<https://pubchem.ncbi.nlm.nih.gov/source/hsdb/6746#section=Non-Human-Toxicity-Excerpts-Complete> (Accessed Feb 18, **2026**).
- [80] Hazardous Substances Data Bank (HSDB) : 6410 - PubChem.
<https://pubchem.ncbi.nlm.nih.gov/source/hsdb/6410> (Accessed Feb 18, **2026**).
- [81] Walters, W.P. Going Further than Lipinski’s Rule in Drug Design. *Expert Opin. Drug Discov.*, **2012**, *7*, 99–107.
- [82] Senn, H.M.; Thiel, W. QM/MM Methods for Biomolecular Systems. *Angew. Chem. Int. Ed Engl.*, **2009**, *48*, 1198–1229.
- [83] Militani, I.A.; Mancini, D.T.; Castro, A.A. de; Ramalho, T.C. Oxidative Biotransformation of Organophosphotioate Pesticides and Acetylcholinesterase Enzymatic Inhibition. *Letters in Drug Design & Discovery*, **2024**, *21*, 1847–1857.
- [84] Xu, X.; Zhou, F.; Zheng, L.; Wang, S.; Peng, X.; Li, D. Sampling Challenges of MM/PBSA Binding Energy Calculations. *J. Phys. Chem. B*, **2025**, *129*, 11666–11678.
- [85] Simonson, T.; Archontis, G.; Karplus, M. Continuum Treatment of Long-Range Interactions in Free Energy Calculations. Application to Protein–Ligand Binding. *J. Phys. Chem. B*, **1997**, *101*, 8349–8362.
- [86] Balamurugan, K.; Pisabarro, M.T. Stabilizing Role of Water Solvation on Anion– π Interactions in Proteins. *ACS Omega*, **2021**, *6*, 25350–25360.
- [87] Szalai, T.V.; Bajusz, D.; Börzsei, R.; Zsidó, B.Z.; Ilaš, J.; Ferenczy, G.G.; Hetényi, C.; Keserű, G.M. Effect of Water Networks On Ligand Binding: Computational Predictions vs Experiments. *J. Chem. Inf. Model.*, **2024**, *64*, 8980–8998.
- [88] Moss, D.E. Improving Anti-Neurodegenerative Benefits of Acetylcholinesterase Inhibitors in Alzheimer’s Disease: Are Irreversible Inhibitors the Future? *International Journal of Molecular Sciences*, **2020**, *21*.
- [89] Moss, D.E.; Perez, R.G.; Kobayashi, H.; Ochoa, J.F. Cholinesterase Inhibitor Therapy in Alzheimer’s Disease: The Limits and Tolerability of Irreversible CNS-Selective Acetylcholinesterase Inhibition in Primates. *Journal of Alzheimer’s Disease*, **2017**, *55*, 1285–1294.

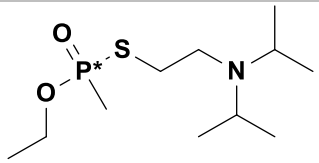
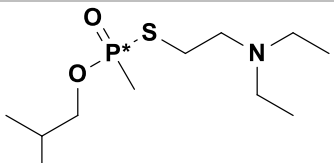
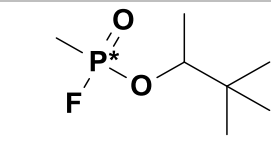
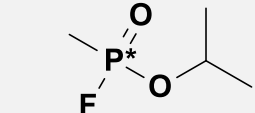
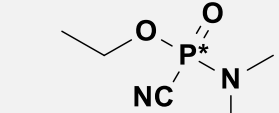
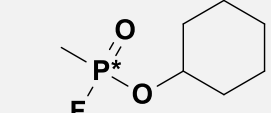
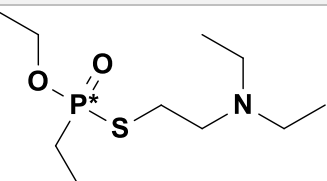
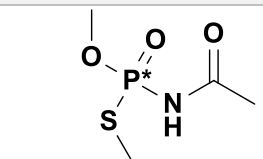
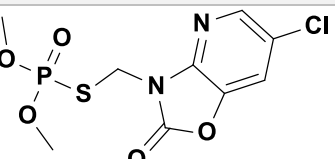
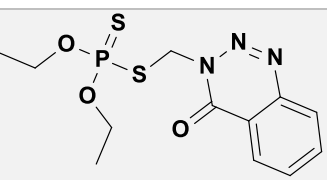
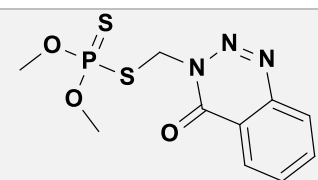
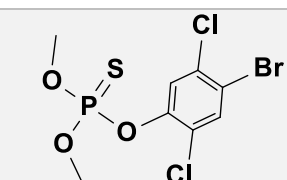
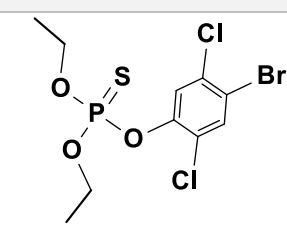
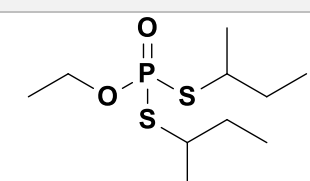
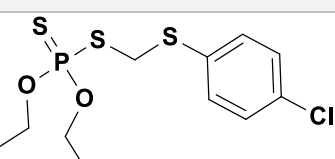
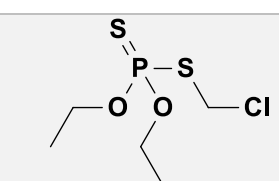
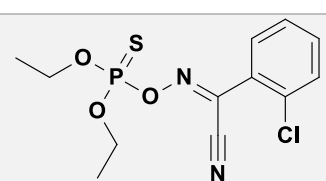
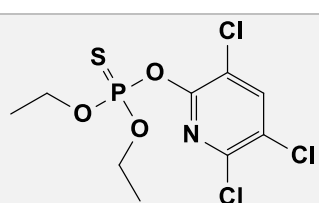
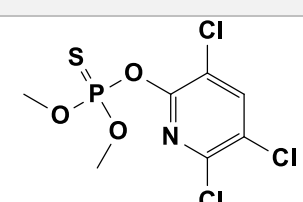
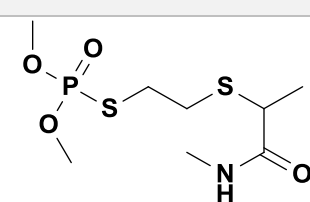
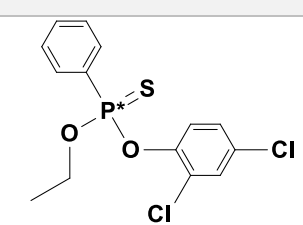
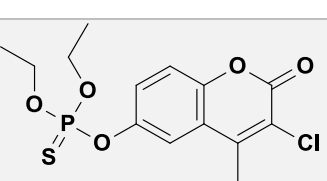
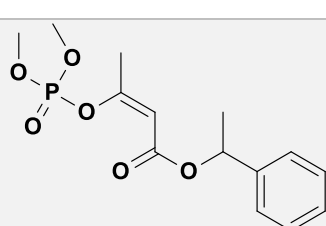
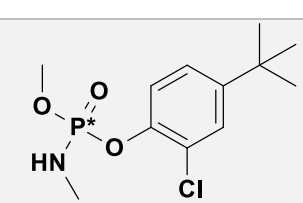
CONCLUSÕES GERAIS

O sistema colinérgico representa um importante alvo molecular tanto para o desenvolvimento de antídotos contra agentes neurotóxicos quanto para a prospecção de novas estratégias terapêuticas para o tratamento da AD. Este trabalho demonstrou a aplicabilidade de abordagens computacionais no estudo de mecanismos de modulação de *HssChEs*, envolvendo processos de reativação e inibição enzimática. Os resultados obtidos a partir das análises de *docking* molecular e cálculos QM/MM permitiram identificar oximas com elevado potencial para a reativação das enzimas AChE e BChE inibidas por compostos OPs, especialmente frente ao agente VX, considerado um dos neurotóxicos mais letais. As análises indicaram que características estruturais específicas influenciam diretamente a estabilidade intermolecular, a barreira energética das reações e o potencial de reativação das enzimas, mostrando a relevância do planejamento racional de moléculas para o desenvolvimento de antídotos mais eficientes e seguros. Paralelamente, a investigação de OPs como inibidores da AChE revelou candidatos promissores para aplicação terapêutica na AD, apresentando interações favoráveis com o alvo biológico, estabilidade conformacional e perfis toxicológicos compatíveis com a atividade farmacológica desejada. Os resultados reforçam a importância da relação entre estrutura molecular, propriedades farmacocinéticas e toxicidade no desenvolvimento de novos compostos bioativos. De maneira geral, os resultados demonstram que ferramentas *in silico* constituem estratégias robustas e eficientes para a investigação de mecanismos moleculares, avaliação toxicológica e planejamento racional de compostos direcionados ao sistema colinérgico. Além disso, os resultados fornecem bases teóricas relevantes para futuros estudos experimentais voltados ao desenvolvimento de moléculas com potencial aplicação em diversas áreas.

MATERIAL SUPPLEMENTAR**Artigo 2**

***In silico* studies into the therapeutic potential of organophosphorus compounds against
Alzheimer's disease**

Table S1. Chemical structures of the OP inhibitors investigated.

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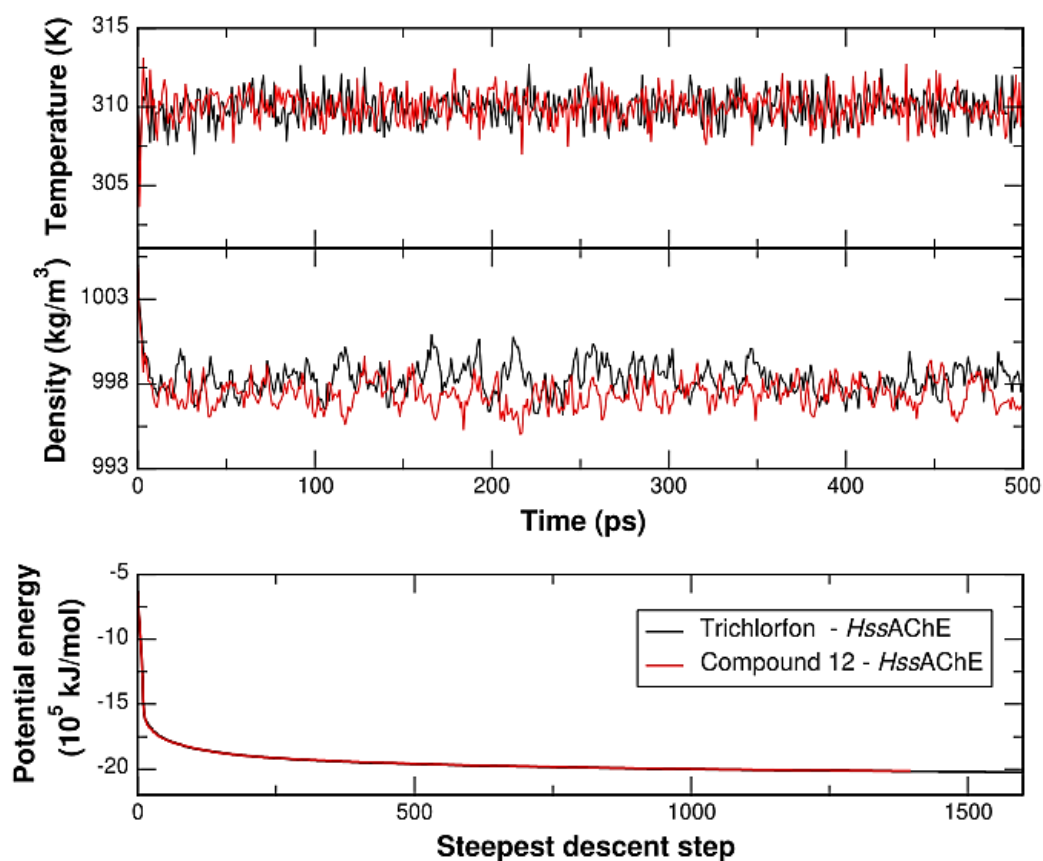


Figure S1. Temperature (NVT), pressure and density (NPT) equilibration graphs and potential energy minimization.

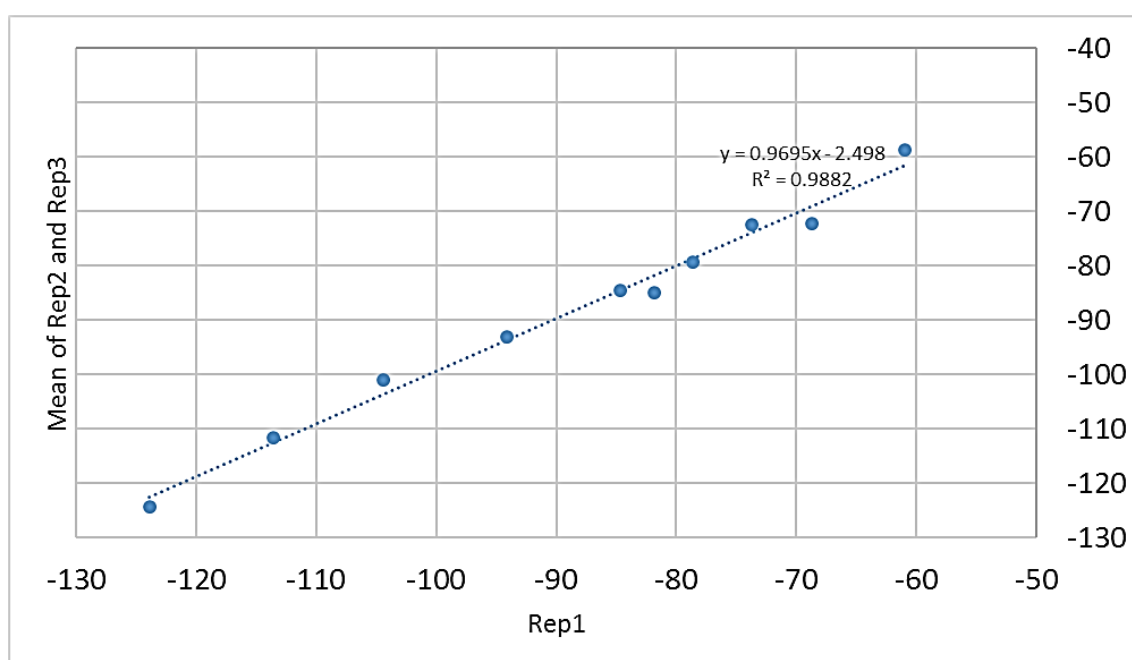


Figure S2. Correlation between *docking* replicates.

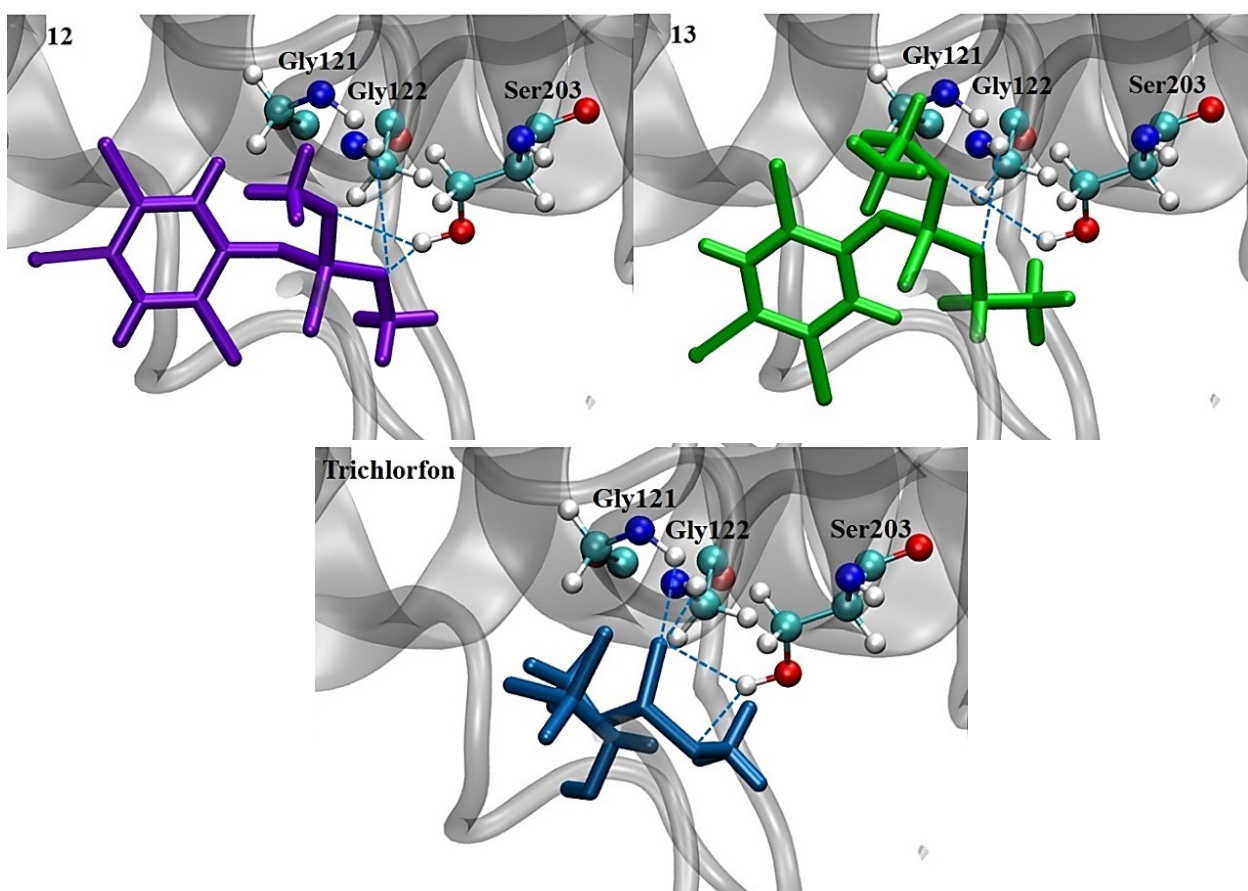


Figure S3. Hydrogen bonds formed by trichlorfon and inhibitors **12** and **13** in the *HssAChE* active site.

Table S2. Validation metrics for RESP charge parametrization.

Compound	ESP relative RMS (a. u)	STD error estimate (a. u.)	QM dipole (Debye)	MM dipole (Debye)	Dipole error
12	0.03377	0.00335	10.2671	10.01383	0.02467
Trichlorfon	0.02505	0.00277	3.87620	4.18816	0.08048

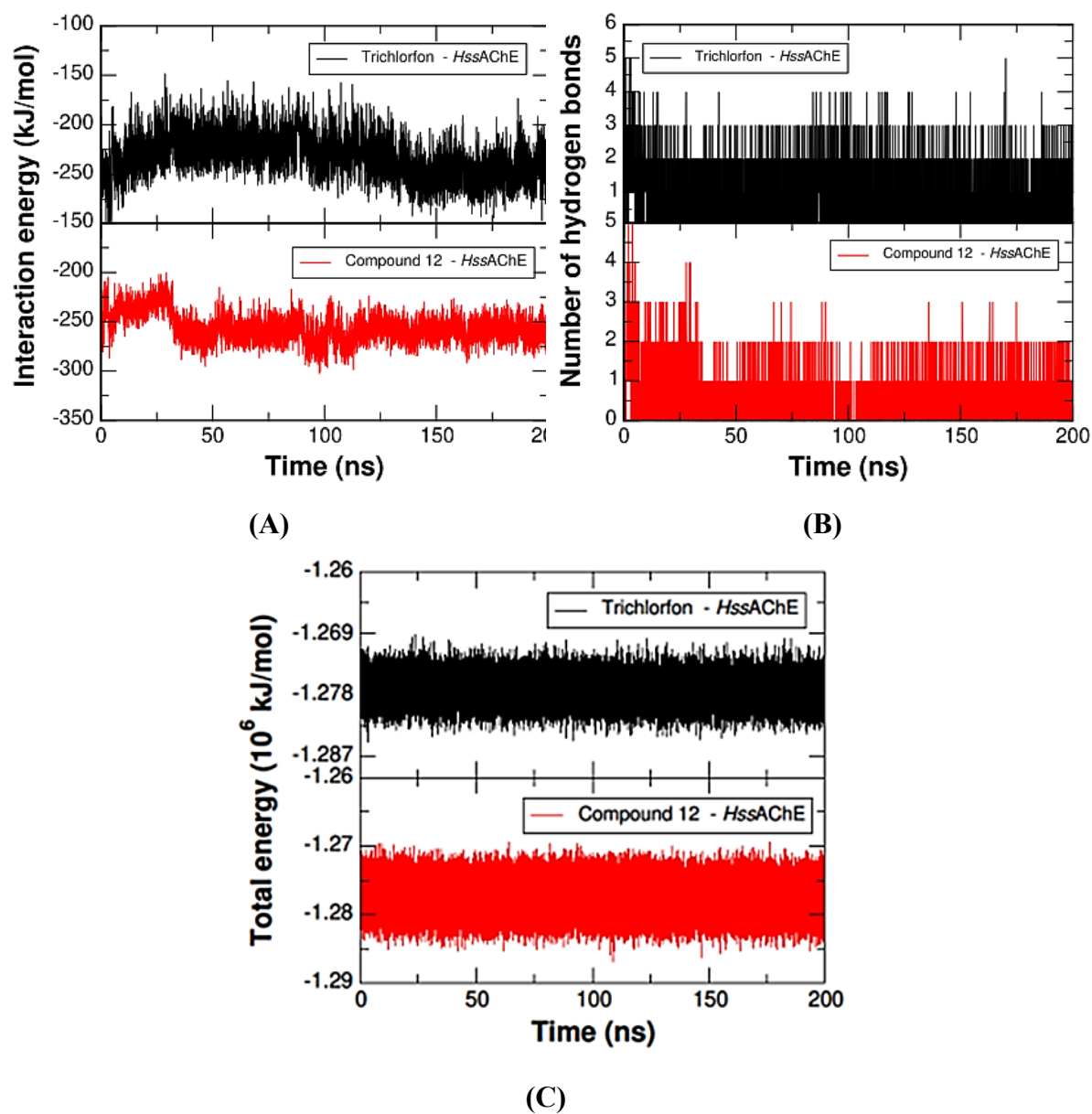


Figure S4. Graphs of interaction energy by molecular mechanics (Coulomb energy + Lennard-Jones short range) (A), number of hydrogen bonds (B) and simulation's total energy (C).