Computational Discovery of Inhibitors Targeting Alphavirus nsP2 Proteases

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***Abstract*— The paper tackles the absence of approved drugs for debilitating alphavirus infections by targeting the nonstructural protein 2 protease, a key enzyme for viral replication. A pharmacophore-guided virtual screening workflow combined with molecular docking and all-atom molecular dynamics simulations was employed to investigate potential inhibitors. Within the scope of computational biology and biotechnological applications, this work reports new results from a pharmacophore-guided screening and docking of the NuBBE database and subsequent structural dynamics simulations. Molecular dynamics analysis demonstrated that the ligand nubbe417 exhibits a dual-inhibition potential against both Chikungunya and Mayaro viruses by structurally locking the catalytic loop in a rigid, closed state. The paper concludes that integrating *in silico* screening with dynamic structural analysis is a viable strategy to identify natural compounds, laying the groundwork for future *in vitro* validation of nubbe417 and expanded screening of additional compound databases to develop effective therapies against emerging arboviruses.**

Keywords- Chikungunya virus; Mayaro virus; pharmacophore modeling; molecular dynamics.

I. INTRODUCTION

Alphaviruses are enveloped, single-stranded positive-sense RNA viruses belonging to the Togaviridae family, primarily transmitted by mosquitoes and divided into two clinical groups: arthritogenic and encephalitic [1]. Among the arthritogenic viruses, Chikungunya (CHIKV) and Mayaro (MAYV) stand out as major public health threats, causing acute fever accompanied by debilitating polyarthralgia and, frequently, chronic pain that can persist for months or even years [2]. CHIKV caused global outbreaks, while MAYV remains South American but shows expansion potential [1], [2]. These infections significantly reduce quality of life and productivity, particularly in resource-limited endemic areas [2].

Despite their global impact, CHIKV and MAYV remain neglected tropical diseases, with no specific therapies or widely available vaccines [3]. Current treatment is limited to analgesics and anti-inflammatory drugs, and although efforts in antiviral development exist, none have yet been approved [3]. This therapeutic gap highlights the urgent need for new drug discovery strategies.

Alphavirus replication depends on the expression and processing of nonstructural polyproteins, mediated by the nsP2 protein, which contains a C-terminal cysteine protease domain, whose proteolytic activity is essential for releasing components of the replicase complex, being indispensable for viral replication [4], [5]. Structural studies reveal conserved pockets across different alphavirus species, suggesting potential for broad-spectrum antivirals [6].

The druggability of nsP2 has already been demonstrated: covalent inhibitors with electrophilic groups and computational approaches such as virtual screening, docking, and repurposing of approved drugs have identified promising compounds [4], [5], [7]. Previous computational studies [4], [5] have successfully utilized virtual screening and docking to identify potential inhibitors for alphavirus nsP2 proteases. However, a common weakness in existing literature is the primary focus on single-virus targets and the reliance on static binding models, which often fail to account for the dynamic flexibility of the interdomain loop that regulates active-site access.

This raises a critical research question: can a single natural compound achieve broad-spectrum inhibition by effectively modulating the conformational dynamics of this conserved loop across different alphaviruses? The purpose of this article is to address this gap by identifying a dual-inhibitor for both Chikungunya (CHIKV) and Mayaro (MAYV) viruses. Unlike standard virtual screening workflows, this study introduces a novelty by proposing a 'loop-locking' inhibition mechanism, where the ligand stabilizes the catalytic loop in a rigid, closed state to prevent substrate processing. Although this approach is currently limited by its purely *in silico* nature and the necessity for future *in vitro* validation, it provides a mechanistic framework for developing broad-spectrum therapies targeting the structural dynamics of emerging arboviruses.

Despite the global burden of CHIKV and MAYV, effective treatments remain elusive. To address this gap, we target the highly conserved nsP2 protease, a critical component for viral replication. By utilizing bioinformatics tools for rational drug design, this study aims to identify potent inhibitors against nsP2, establishing a foundation for their subsequent pharmaceutical application.

The rest of the paper is structured as follows. Section 2 presents the Materials and Methods, Section 3 the Results, Section 4 the Discussion, and Section 5 the Conclusion and Future Work.

II. MATERIALS AND METHODS

To identify potential inhibitors, this study utilized an integrated computational pipeline combining structure-based pharmacophore modeling with virtual

screening, molecular docking, and all-atom molecular dynamics simulations. The computational resources and software protocols utilized in this study are detailed below.

A. Infrastructure

All preprocessing, pharmacophore construction, virtual screening, and docking were performed on PowerEdge T550 workstation (Dell Technologies, United States), equipped with an Intel® Xeon® Silver 4316 processor (32 physical cores, 2.3 GHz). These tasks were executed primarily within the Molecular Operating Environment (MOE, v. 2024.0601, Chemical Computing Group, Canada).

Molecular Dynamics (MD) simulations were performed with GROMACS (v. 2023.2, GROMACS Development Team, Sweden/Germany) on the Santos Dumont Supercomputer (Laboratório Nacional de Computação Científica — LNCC, Brazil). Simulations were executed on Intel Xeon Gold 6154 ("Skylake") processors per node (36 physical cores total, 2.1 GHz).

B. Structure Preparation

The crystal structure of the nsP2 protease from Chikungunya virus (CHIKV) (PDB ID: 3TRK) was retrieved from the Protein Data Bank (Protein Data Bank, RCSB, United States). The protease from Mayaro virus (MAYV), for which no experimental structure exists, was modeled using ColabFold/AlphaFold2 (v 1.5.5, ColabFold Project) based on its sequence retrieved from National Center for Biotechnology Information (NCBI) (NCBI Reference Sequence: NP_740688.1). MAYV model quality was assessed via SAVES v.6.0 (Ramachandran plot). Both nsP2 structures were prepared in MOE using QuickPrep.

C. Pharmacophore Modeling, Virtual Screening and Docking

Binding pockets were identified using the Site Finder module, and pharmacophoric interaction points of the receptor were mapped using the Pharmacophore Query Editor. Electrostatic potential surfaces and interaction-energy grids were analyzed to refine essential pharmacophoric features.

Because no crystallized ligands or experimentally confirmed inhibitors exist for MAYV nsP2, pharmacophore validation was performed only for the CHIKV nsP2 using known inhibitors reported in the literature. Validation utilized property-matched decoys generated via DUDE-Z. Docking scores and pharmacophore metrics were analyzed to calculate ROC curves and enrichment factors using a specific MOE script. (ROC Calculator for MOE Docking, v. XX; R. Staub, GitHub repository) [8]. The validated pharmacophore model was subsequently employed to screen the NUBBE Database (Núcleo de Bioensaios, Biossíntese e Ecofisiologia de Produtos Naturais, UNESP, Brazil). It should be noted that pharmacophore validation was performed exclusively for CHIKV nsP2 due to the absence of experimentally confirmed MAYV inhibitors. The application of this model to MAYV assumes structural and functional conservation of the catalytic site, which is supported by sequence and structural similarity but remains a limitation of the present study. Database compounds were curated and protonation-normalized using OpenEye Applications Software (v. 24.05.15.0, OpenEye Scientific, United States), including pKa adjustment at physiological pH (7.4), tautomer enumeration, and 3D conformer generation.

Filtered ligands were docked into both nsP2 proteases. The validated pharmacophore was used as a 3D constraint during ligand placement, followed by scoring with London dG and refinement via induced-fit optimization, generating 30 poses per ligand. A consensus selection strategy was adopted, in which only ligands appearing within the top five ranked sets for both receptors were advanced to MD simulations.

D. Molecular Dynamics

In order to perform MD, the ligand topologies were generated using SwissParam (Swiss Institute of Bioinformatics, Switzerland). The system was solvated and neutralized by adding counterions, and the ionic strength was adjusted to 0.15 M NaCl to mimic physiological conditions. Energy minimization was performed using the steepest-descent algorithm. Equilibration consisted of a 100-ps NVT phase followed by a 100-ps NPT phase. Temperature was maintained at 300 K using the V-rescale thermostat. Pressure was controlled at 1 atm using the Parrinello–Rahman barostat with isotropic coupling. Production MD runs (200 ns) were carried out using the TIP3P water model and the CHARMM27 force field. Analyses included protein–ligand hydrogen bonding, protein RMSD, RMSF and comparison between the first and last MD frames.

III. Results

The outcomes of our integrated computational pipeline are detailed in this section, covering the complete trajectory from initial target characterization to ligand identification. These findings provide a comprehensive overview of the structural insights gained through both static modeling and dynamic simulations of the protease complexes.

E. Protein Molecular Modeling

The crystal structure of CHIKV nsP2 (PDB ID: 3TRK; 2.40 Å resolution) was used as the reference model, while the MAYV nsP2 structure was obtained through AlphaFold2. Structural validation of the MAYV model demonstrated good stereochemistry, with 92.6% of residues in favored regions of the Ramachandran plot, supporting its suitability for subsequent analyses. In both proteins, the identified active site corresponds to the same catalytic amino-acid ensemble described in the literature for CHIKV, including the residues that form the flexible interdomain loop controlling access to the catalytic pocket. Despite differences in residue numbering relative to the reference study, structural equivalence was confirmed.

This loop plays a critical functional role, as it contains residues essential for catalysis and substrate recognition. As reported previously [8], mutation of Asn547 (equivalent to Asn79 in the present numbering) results in a threefold

increase in Km, underscoring its importance for proper substrate positioning. Thus, alterations in the dynamics of this loop directly affect the catalytic competence of the protease.

F. *Pharmacophore Modeling, Virtual screening and Docking*

Pharmacophore analysis of the catalytic site revealed a robust set of interaction points, which were subsequently refined using electrostatic and energy-interaction maps. Validation with known CHIKV inhibitors yielded satisfactory performance (AUC = 0.88), reinforcing the discriminatory power of the model and supporting its use in the virtual screening of the NuBBE database. Docking allowed the selection of the top five ranked compounds for each protease; among them, only nubbe417 appeared at the top for both, motivating its selection for molecular dynamics simulations.

G. *Molecular Dynamics: Structural Stability from RMSD and Local Fluctuations (RMSF)*

The global structural stability and local residue flexibility of the nsP2 proteases were evaluated through Root Mean Square Deviation (RMSD) and Root Mean Square Fluctuation (RMSF) analyses. The resulting profiles, which reveal distinct behaviors between the apo and ligand-bound forms for both CHIKV and MAYV, are comprehensively presented in Figure 1.

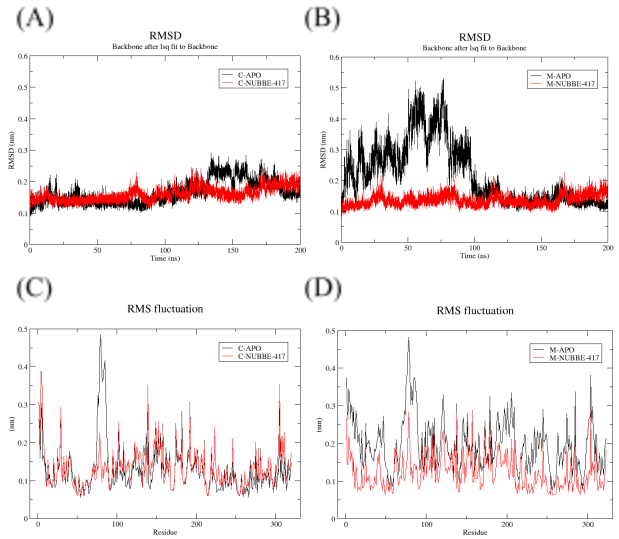

Figure 1. Structural stability and local fluctuations of CHIKV and MAYV nsP2 proteases. (A) RMSD profile for CHIKV nsP2 systems; (B) RMSD profile for MAYV nsP2 systems; (C) RMSF analysis of CHIKV nsP2 residues; (D) RMSF analysis of MAYV nsP2 residues. Black lines represent the apo systems, while red lines indicate the nubbe417-bound complexes.

The RMSD profiles obtained after the MD analysis revealed distinct behaviors between apo and complex forms. For CHIKV nsP2, both systems remained globally stable and showed similar fluctuations; 0.165 nm and 0.160 nm to apo and complex systems, respectively. In contrast, MAYV nsP2 displayed a pronounced difference: the apo form showed substantial initial instability, stabilizing only after approximately 100 ns with a mean of 0.219 nm, whereas the complex remained stable from the onset with a mean of 0.136 nm. Nubbe417 stabilized conformations, especially in the more flexible MAYV nsP2.

RMSF analysis further highlights the stabilizing effect of the ligand. In both proteases, the most mobile segment corresponds to the catalytic loop (residues 76–86 in the numbering used here). For CHIKV nsP2, the apo form displayed fluctuations of 0.3887 ± 0.0596 nm, while the complex reduced this mobility to 0.1456 ± 0.0349 nm. Similarly, in MAYV nsP2, the apo form fluctuated at 0.3798 ± 0.0554 nm, whereas the complex decreased mobility to 0.1649 ± 0.0547 nm. Reduced mobility shows the ligand pins the loop, promoting catalytic-site closure.

H. *Molecular Dynamics: Dynamic Stability and Conformational Changes*

The dynamic stability and conformational changes within the catalytic loop were investigated by comparing the first and last Molecular Dynamics (MD) frames (Figure 2). In this visualization, initial positions are represented by light colors—yellow for CHIKV, light blue for MAYV, and light gray for nubbe417—whereas the final states are depicted in dark colors—orange for CHIKV, dark blue for MAYV, and dark gray for the ligand. A distinct 'loop opening' effect was observed in both apo systems, leading to significant structural expansion of the catalytic pocket. Conversely, the presence of the inhibitor prevents this movement, inducing a prominent closure of the loop in CHIKV and promoting overall structural stabilization in MAYV. This 'loop-locking' mechanism is reinforced by persistent interactions with residues such as Asn79 and Leu202, which effectively maintain the protease in an inhibited, closed conformation .

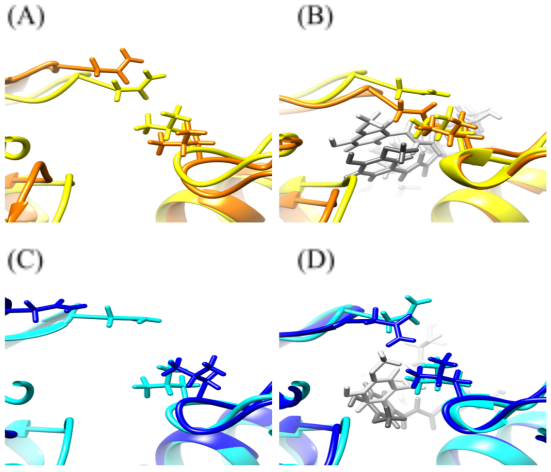

Figure 2. Conformational transitions of the nsP2 catalytic loop during MD simulations. Comparison between the initial (light colors) and final frames (dark colors) for the apo and complexed systems of both viruses. Protein is shown in ribbon representation, with catalytic residues and nubbe417 displayed in sticks.

Loop dynamics were quantified by comparing the distances between the α-carbons (Cα) of residues Asn79 and Leu202 across the initial and final conformations of the MD simulations1. As detailed in Table I, these distance measurements provide a numerical basis for assessing the ligand-induced closure of the catalytic pocket in both CHIKV and MAYV systems.

TABLE I. DISTANCE VARIATION BETWEEN Cα ATOMS OF ASN79 AND LEU202 IN APO AND LIGAND-BOUND NSP2 SYSTEMS.

System	Distance measurements (Cα - Asn79–Leu202)		
	First Frame (Å)	*Last Frame (Å)*	*Δ (Å)*
Apo CHIKV	7.96	10.83	+2.87
CHIKV-nubbe417	8.18	6.58	-1.60
Apo MAYV	9.83	14.19	+4.36
MAYV-nubbe417	9.39	8.74	-0.65

In the apo systems, the distance between Asn79 and Leu202 increased by 2.87 Å in CHIKV and expanded by a substantial 4.36 Å in MAYV relative to the initial structures. In sharp contrast, the inhibitor-bound complexes exhibited a narrowing of this distance. The CHIKV-nubbe417 complex tightened by 1.60 Å, while the MAYV-nubbe417 complex stabilized with a reduction of 0.65 Å. In these bound systems, the Asn79 side chain is specifically oriented toward the ligand and Leu202.

I. Molecular Dynamics: Hydrogen Bond Interactions

The stability of the protein-ligand complexes was further investigated by quantifying the number of hydrogen bonds formed throughout the 200-ns molecular dynamics simulations. As presented in Figure 3, the persistent nature of these interactions for both CHIKV and MAYV systems highlights the robust anchoring of nubbe417 within the catalytic pocket.

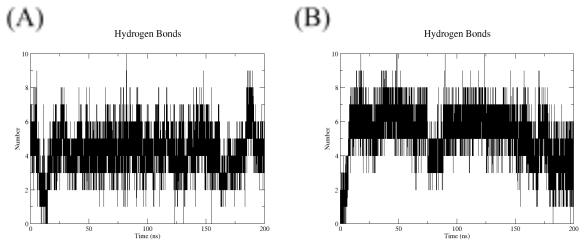

Figure 3. Time-dependent evolution of protein-ligand hydrogen bond interactions. The plots show the number of hydrogen bonds maintained between nubbe417 and the catalytic pocket for (A) CHIKV and (B) MAYV nsP2 systems throughout the 200-ns MD simulations.

The complexes maintained a consistent number of hydrogen bonds throughout the simulations, reinforcing the observed stability. In CHIKV–nubbe417, the average number of hydrogen bonds was 4.17 ± 1.24, while in MAYV–nubbe417 it was 5.21 ± 1.48. These interactions keep the ligand firmly anchored within the catalytic pocket, reduce local flexibility, and propagate stabilization to adjacent regions.

IV. DISCUSSION

The combined analyses of RMSD, RMSF, hydrogen-bond stability, and structural comparisons provide a coherent mechanistic interpretation of ligand-induced stabilization. The reduction in RMSF within the catalytic loop correlates strongly with the conformational transitions observed from initial to final frames: apo forms display a flexible, open loop, whereas ligand-bound complexes modulates the conformational state of the loop, stabilizing a closed architecture essential for protease inhibition. Persistent hydrogen bonds anchor nubbe417, dampening loop motion and enforcing inhibitory compactness. Taken together, these results are consistent with the regulatory 'gating' role described by Narwal et al. [8], where the loop modulates active site access. Nubbe417 exploits this by structurally locking the loop in a closed conformation, effectively barricading the catalytic dyad. Therefore, the stabilization induced by nubbe417 may potentially affect the protease functionality by reducing local flexibility, enforcing a closed active-site geometry, and maintaining interactions that reinforce this inhibited structural state.

The performance of nubbe417 in both proteases—structural stability, reduction of catalytic-loop mobility, and formation of persistent interactions—demonstrates that natural-product-derived molecules represent valuable sources of bioactive scaffolds with antiviral potential. Their ability to stabilize key functional regions suggests a mechanism of action capable of interfering with the proteolytic processing essential for viral replication. From a biomedical perspective, identifying compounds capable of modulating the catalytic loop is particularly significant, given its dual role in substrate access and positioning. Ligands that stabilize closed conformations may act as competitive or allosteric inhibitors. Additionally, the effective performance of nubbe417 against two distinct alphavirus proteases highlights its potential as a broad-spectrum antiviral lead.

These computational findings gain further relevance when contextualized with phytochemical and biological studies of *Chioccoca alba* (Rubiaceae). A previous work reported the isolation and structural characterization of iridoid and seco-iridoid glucosides from *C. alba*, including the compound identified in our study as nubbe417 [9]. The chemical framework of these metabolites confirmed the presence of functional groups capable of hydrogen bonding and stabilizing protein–ligand interactions, consistent with our simulation results. More recently, another study evaluated methanolic root extracts of *C. alba* against Chikungunya and Mayaro viruses, demonstrating inhibition levels above 70% at 60 µg/mL [10]. Notably, nubbe417 was originally isolated from *C. alba* roots , the exact plant organ exhibiting antiviral activity. Moreover, the methanolic extraction used in these biological assays is chemically suitable for solubilizing polar glycosides, supporting the physical presence of this iridoid in the active extract.

While Pires et al. [10] attributed the extract's potency primarily to flavonoids via docking, the use of crude extracts allows for the presence of other bioactive compounds. Thereby, our results suggest that iridoids like nubbe417—previously isolated from *C. alba* [9]—could be significant contributors to this antiviral activity, potentially acting synergistically or as potent specific inhibitors of nsP2. This hypothesis is further supported by the established bioactivity of the iridoid class, which has been previously documented to inhibit other cysteine proteases [11]. However, this hypothesis requires direct confirmation through *in vitro* testing of nubbe417 to establish its specific role as nsP2 protease inhibitor.

As with any structure-based virtual screening approach, potential limitations include docking score bias, force-field dependency, and the risk of false-positive predictions. Molecular dynamics simulations mitigate some of these limitations by incorporating protein flexibility and solvent effects; however, experimental validation remains essential to confirm binding affinity and inhibitory activity.

In summary, when compared with existing computational studies that primarily emphasize docking scores or covalent inhibition, the present work highlights a complementary mechanism based on dynamic stabilization of the catalytic loop. The dual-virus consistency observed for nubbe417, combined with its natural origin and previously reported antiviral activity at the extract level, distinguishes this study by proposing a mechanistically informed, cross-alphavirus inhibition strategy rather than a virus-specific solution.

V. Conclusion and Future Work

The present study demonstrates that an integrated computational workflow combining pharmacophore modeling, docking, and molecular dynamics simulations can identify natural product-derived compounds capable of modulating the structural dynamics of alphavirus nsP2 proteases. The ligand nubbe417 consistently stabilized the catalytic loop of both Chikungunya and Mayaro virus proteases, supporting a loop-locking inhibition mechanism with potential broad-spectrum relevance.

As future work, expanded virtual screening campaigns involving additional natural and synthetic compound libraries and *in vitro* protease inhibition assays, antiviral cell-based experiments are planned. These efforts will be essential to experimentally validate the proposed mechanism and advance nubbe417 or related scaffolds toward lead optimization.

Acknowledgment

The authors thank the Federal University of Uberlândia (UFU) and the Institute of Biotechnology (IBTEC/UFU) for institutional support. We also acknowledge the Santos Dumont supercomputing facility for providing the computational resources essential for this study. Financial support from FAPEMIG, CNPq, and CAPES is gratefully acknowledged.

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