

Research Article

Computational study of natural and FDA-approved compounds binding to wild-type and P135Q mutant HBcAg

Somayeh Farahmand^{1*} , Mobina Ashtari² , Reza Haji Hosseini³ , Gholamreza Bakhshi Khaniki⁴ 

1. Assistant Professor in Department of Biology, Payame Noor University, Tehran, Iran.
2. M.Sc. in Department of Biology, Payame Noor University, Tehran, Iran.
3. Professor in Department of Biology, Payame Noor University, Tehran, Iran.
4. Professor in Department of Biology, Payame Noor University, Tehran, Iran.

✉ **Correspondence:**
Somayeh Farahmand
Email: s.farahmand@pnu.ac.ir

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ABSTRACT

Hepatitis B virus (HBV) infection remains a global health challenge, and mutations in the core antigen (HBcAg) can compromise the efficacy of current antiviral therapies. This study aimed to identify potential natural and FDA-approved compounds targeting wild-type (WPr) and P135Q mutant (MPR) HBcAg using a comprehensive in silico approach. Natural compounds were filtered using Lipinski's Rule of Five and ADMET profiling. Molecular docking was performed using AutoDock Vina (version 1.2.x, 2021), and the top-ranked complexes were subjected to 500 ns molecular dynamics simulations using GROMACS 2023 with the AMBER99SB force field. Structural and energetic analyses included RMSD, RMSF, radius of gyration (Rg), solvent-accessible surface area (SASA), hydrogen bonding, principal component analysis (PCA), and MM/PBSA binding free energy calculations. Among 5,000 screened phytochemicals, Carnosic acid (Csa) and Nordentatin (Nor) demonstrated favorable binding affinities ($\Delta G = -8.85$ and -9.84 kcal/mol, respectively) with acceptable ADMET profiles. MD simulations revealed distinct binding mechanisms: Csa stabilized WPr through electrostatic interactions, while Nor stabilized MPR through persistent hydrogen bonds and van der Waals contacts involving Trp102, Ser106, and Tyr118. Binding free energy analysis suggested that Csa may have stronger affinity for the mutant form, whereas Nor appeared to favor the wild-type. The P135Q mutation significantly influenced ligand binding dynamics and interaction patterns, highlighting the structural differences between wild-type and mutant HBcAg. Our computational findings suggest that Csa and Nor may serve as potential candidates for HBcAg binding. However, these predictions are purely computational and require experimental validation to confirm their actual biological activity.

KEYWORDS

Anti-HBV, Carnosic acid, HBcAg, Molecular dynamics, Nordentatin, P135Q mutation..

1. INTRODUCTION

Hepatitis B virus (HBV) is a small DNA virus belonging to the *Hepadnaviridae* family and *Orthohepadnavirus* genus, and remains a major global health challenge with over 250 million chronic carriers worldwide [1]. The virus itself is not directly cytopathic; liver damage mainly results from immune-mediated attacks on infected hepatocytes [2]. An insufficient immune response—particularly against viral antigens such as HBV core antigen (HBcAg)—may lead to chronic infection [3].

HBcAg, as a core structural component of the viral nucleocapsid, plays a pivotal role in capsid assembly, genome replication, and modulation of host immune responses [4]. Its genome, approximately 3.2 kb in length, contains four open reading frames (ORFs): the S gene (encoding HBsAg), C gene (encoding HBcAg and HBeAg), P gene (encoding polymerase), and X gene [5]. A hallmark of HBV infection is the persistence of covalently closed circular DNA (cccDNA) in the nuclei of infected hepatocytes, which serves as a transcriptional template for viral RNA synthesis and remains stable even after suppression of viral replication by antiviral drugs [6]. This long-term persistence explains the inability of current treatments to achieve complete viral eradication and the frequent relapse following therapy withdrawal. Consequently, cccDNA has been identified as a major barrier to HBV cure, making indirect targeting of key viral proteins a potential strategy. The high mutation rate in the HBV genome, owing to the lack of proofreading activity in reverse transcriptase, results in multiple variants—some capable of immune escape or drug resistance. For instance, the P135Q mutation in the C gene, substituting proline with glutamine at position 135 of HBcAg, alters capsid assembly and reduces HBeAg expression.

These changes are associated with suppressed T-cell responses, increased viral persistence, higher viral loads, and disease progression [7, 8]. Understanding how such mutations affect the structural dynamics of HBcAg is critical for the rational design of compounds that remain effective against drug-resistant strains [9].

Despite the availability of effective vaccines, HBV treatment remains a major public health challenge. Vaccination shows limited efficacy in high-risk groups, such as immunocompromised patients and those undergoing dialysis [2], and does not confer lifelong immunity, necessitating booster doses. Existing therapeutic agents, including interferon- α (IFN- α) and nucleos(t)ide analogs such as lamivudine and adefovir, face several limitations—such as drug resistance, side effects, and failure to achieve sustained virological response [10]. Importantly, none of these therapies can eliminate cccDNA, making complete viral eradication unattainable [3, 6].

Recent advances in antiviral drug discovery have highlighted the potential of capsid assembly modulators (CAMs) as a novel class of anti-HBV agents. CAMs inhibit HBV replication by binding to a hydrophobic pocket (the HAP pocket) between HBcAg dimer-dimer interfaces [11, 12]. However, the emergence of CAM-resistant variants necessitates the continuous search for novel compounds with diverse chemical scaffolds, particularly from natural sources [13].

In this context, in silico drug discovery methods—including molecular docking, virtual screening, and molecular dynamics (MD) simulations—have been successfully applied to identify bioactive compounds with strong binding energies against HBV targets [9, 14]. The integration of computational approaches with natural product research offers a cost-effective and time-efficient strategy for identifying new

anti-HBV agents [15]. Previous computational studies have identified several natural compounds, such as those from *Phyllanthus niruri*, with favorable binding affinities for HBV core protein, highlighting the potential of phytochemicals as a source of novel antivirals [16]. However, most of these studies have focused on the wild-type protein and none have systematically evaluated ligand binding to the clinically relevant P135Q mutant using extended simulation times (Table S1, Supplementary File). Encouraged by these findings, the present study employed a comparative computational approach to investigate the interactions of selected natural compounds (Carnosic acid and Nordentatin) with wild-type and P135Q mutant HBcAg. Our objective was to evaluate their potential as HBcAg-binding compounds and to elucidate the molecular mechanisms by which the P135Q mutation affects ligand binding and protein dynamics. This computational study provides a foundation for the future development of HBcAg-targeting therapies, while acknowledging that experimental validation remains essential to confirm these *in silico* predictions.

2. MATERIALS AND METHODS

2.1. Structural crystallography preparation

The crystallography structures of the HBcAg protein were not available with sufficient resolution (mostly >2.5 Å) or contained sequence mutations not relevant to this study. Therefore, three-dimensional modeling was performed using the Swiss-Model server based on template 6htx.1.A, which had 96.72% sequence identity with the target (UniProt ID: P03147). The generated homology model was assessed for structural quality using the Ramachandran plot and QMEAN scoring parameters, and no missing

loops or steric clashes were detected in the putative binding site.

The P135Q mutation (proline to glutamine at position 135) was introduced into the HBcAg model using Visual Molecular Dynamics (VMD) software, and both wild-type (WPr) and mutant (MPr) structures were subjected to energy minimization prior to subsequent computational analyses.

2.2 Putative Binding Site Determination

The putative binding site of HBcAg was identified using PyMOL, UCSF Chimera, BIOVIA Discovery Studio Visualizer, and PDBsum. Based on previous structural studies, a set of key residues including Leu19, Phe23, Phe24, Pro25, Trp102, Phe103, Ser106, Val115, Tyr118, Leu119, Phe122, and Gly123 were selected as critical for ligand interactions [16-19]. These residues were subsequently used to define the docking grid.

2.3. Virtual database construction and ligand preparation

A library of 5,000 natural compounds was compiled from the PubChem database, focusing on compounds with previously reported antiviral, anti-inflammatory, or hepatoprotective activities. For each compound, the PubChem ID was retrieved, and preliminary drug-likeness properties—including molecular weight, number of hydrogen bond donors and acceptors, number of rotatable bonds, and XLogP3—were evaluated according to Lipinski's Rule of Five. Compounds violating more than one of Lipinski's criteria were excluded.

To further assess pharmacokinetic suitability, ADMET profiles (absorption, distribution, metabolism, excretion, and toxicity) were analyzed using PreADMET (<https://preadmet.webservice.bmdrc.org>), while

pharmacodynamic parameters were predicted using SwissADME (<http://www.swissadme.ch>). The analysis included metrics such as gastrointestinal absorption, plasma protein binding, blood–brain barrier permeability, P-glycoprotein interaction, bioavailability score, and hepatic metabolism potential.

After applying these screening criteria, 18 compounds exhibiting favorable pharmacokinetic properties and acceptable toxicity profiles were selected for molecular docking. To ensure chemical diversity, the selected compounds belonged to different chemical classes, including phenolic acids, terpenoids, alkaloids, and coumarins. The Canonical SMILES of each ligand were retrieved from the PubChem database and converted into PDB format using an online SMILES converter. These optimized ligand structures were subsequently used for molecular docking against both WPr and MPr.

2.4. Receptor grid generation and molecular docking

AutoDock Vina (version 1.2.x, 2021) facilitated the prediction of optimal ligand binding conformations within the putative binding site of HBcAg. The docking protocol employed an Iterated Local Search (ILS) algorithm with an exhaustiveness of 8, which controls the number of independent runs, within a defined grid box (Box Size: X = 60, Y = 60, Z = 60; Grid box center coordinates: X = 244.366, Y = 262.547, Z = 406.879) encompassing the binding pocket. The energy range was set to 3 kcal/mol, and a maximum of 9 binding modes were generated for each ligand. These parameters are the default settings of AutoDock Vina, except for the grid box dimensions and center coordinates, which were defined based on the putative binding site. The best docking pose for each protein–ligand complex was selected based on the smallest

cluster size and the lowest binding free energy (ΔG , kcal·mol⁻¹). Interaction analyses focused on binding energies (ΔG) and molecular interactions, including hydrogen bonds, hydrophobic contacts, and π – π stacking. PyMOL, Chimera, and Discovery Studio enabled visualization and validation of the molecular interactions. Based on these analyses, the best binding poses for the 18 pre-selected ligands were identified and selected for subsequent molecular dynamics simulations.

2.5. MD simulations

All simulations were performed using GROMACS 2022.6 with the Amber99SB force field [20]. The initial protein structures were obtained from the best docking poses. Ligand topologies and parameters were generated using the ACPYPE interface, with partial atomic charges derived using the AM1-BCC method. The TIP3P water model was selected due to its compatibility with the AMBER99SB force field. To mimic physiological conditions and neutralize the system charge, 64 Na⁺ and 52 Cl⁻ ions were added, yielding a final ionic concentration of 0.15 M. Protonation states of ionizable residues were assigned at physiological pH 7.4 using default AMBER99SB parameters. Under these conditions, Asp and Glu residues were modeled in their deprotonated (negatively charged) form, Lys and Arg in their protonated (positively charged) form, and His residues in their neutral form (HID/HIE) based on their local environment. No explicit pKa calculations were performed using external tools such as PROPKA, as the binding site residues do not contain titratable groups with anomalous pKa values at pH 7.4. Energy minimization was carried out using the steepest descent algorithm, followed by two equilibration phases: a 1-ns NVT ensemble at 310 K, and a 1-ns NPT ensemble at 1 bar, using the Nosé–Hoover thermostat and Parrinello–

Rahman barostat [21]. Equilibration convergence was verified by monitoring temperature (310 ± 2 K) and density (1009 ± 6 kg/m³) stability during the final 500 ps of each phase (Fig. S1, Supplementary File). Long-range electrostatic interactions were computed using the Particle Mesh Ewald (PME) method, van der Waals interactions were modeled via the Lennard-Jones potential, and bond constraints were applied using the LINCS algorithm [22, 23]. Production simulations were run for 500 ns under NPT conditions (Parrinello-Rahman barostat, 1 bar; velocity-rescaling thermostat, 310 K) with a 2 fs time step, and atomic coordinates were saved every 10 ps. Due to computational resource constraints, only a single trajectory was generated per system, and the absence of replica simulations is acknowledged as a limitation of this study.

2.6. MD trajectory analysis

The MD trajectories were analyzed to evaluate key structural and dynamic parameters of WPr, MPr, and their complexes with ligands. Analyses included root mean square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration (Rg), number of hydrogen bonds, solvent-accessible surface area (SASA), principal component analysis (PCA), and binding free energy estimations using the molecular mechanics Poisson-Boltzmann surface area (MM-PBSA) method. All analyses were performed using standard GROMACS modules.

3. RESULTS

3.1. Virtual screening and binding affinity analysis

Following virtual screening of 5,000 natural compounds—initially filtered according to Lipinski's Rule of Five—18 top candidates were selected based on their favorable drug-likeness

properties and desirable ADMET profiles (Tables S2–S4, Supplementary File). These compounds were subsequently subjected to molecular docking analyses against both WPr and MPr forms of HBcAg.

The predicted ΔG values among the tested ligands ranged widely (Table 1). Although some compounds exhibited more negative ΔG values, they were excluded from further evaluation due to unfavorable ADMET characteristics. Ultimately, Csa and Nor were selected for further analysis based on a multi-criteria scoring system (Table S5, Supplementary File). The selection criteria included: (1) binding affinity (ΔG), (2) ADMET profile, and (3) chemical diversity. Although some compounds such as Gingerol exhibited slightly better ΔG values (e.g., -9.89 kcal/mol for MPr), they were excluded due to unfavorable ADMET properties, including BBB permeability and P-glycoprotein inhibition. Csa demonstrated a favorable ADMET profile with no major liabilities, while Nor was selected despite its positive Ames test and BBB permeability, due to its superior binding affinity and chemical diversity as an alkaloid scaffold.

Additionally, as a comparative benchmark, docking results (Table 1), Lipinski's rule assessments, and pharmacokinetic and pharmacodynamic analyses of three clinically approved anti-HBV drugs—Entecavir (Ent), Lamivudine (Lam), and Adefovir (Ade)—were evaluated (Tables S2–S4, Supplementary File). Results indicated that all three reference drugs satisfied Lipinski's criteria, although variations were observed in absorption, permeability, and toxicity indices. Molecular docking results also suggested that these antiviral agents could bind to both WPr and MPr, with apparent differences in ΔG and predicted binding constant (K_i) values between the two forms.

Table 1. Binding affinities (ΔG , kcal/mol) and predicted binding constants (K_i , μM) of phytochemical ligands docked to WPr and MPr (P135Q) HBcAg, compared with FDA-approved drugs (Entecavir, Lamivudine, Adefovir).

No	ligand	WPr		MPr (P135Q)	
		ΔG	K_i	ΔG	K_i
1	Gingerol	-9.74	72.00	-9.89	56.30
2	Nordentatin	-9.7	61.23	-9.84	67.40
3	Populene D	-9.24	167.44	-9.23	172.79
4	Carnosic acid	-8.77	328.34	-8.85	371.06
5	Sophocarpine	-8.55	543.3	-8.44	646.49
6	dihydrocurcumenone	-5.03	206.29	-8.43	659.39
7	Nerolidol	-8.35	735.05	-8.41	678.80
8	Levomenol	-8.46	628.88	-8.26	888.96
9	Alpha-Bisabolol	-8.26	879.10	-8.25	893.52
10	Osthole	-8.16	1.04	-8.15	1.05
11	Matrine	-8.14	1.07	-8.12	1.11
12	sophoridine	-8.14	1.07	-8.12	1.11
13	beta-Thujaplicinol	-7.49	3.22	-7.38	3.91
14	Carvacrol	-6.59	14.68	-6.63	13.71
15	NEROL	-6.36	21.73	-6.37	21.47
16	Linalool	-6.37	21.41	-6.36	21.71
17	Geraniol	-6.38	21	-6.35	22.10
18	Menthol	-6.16	30.39	-6.17	29.78
1	Entecavir	-8.77	370.59	-8.73	402.14
2	Lamivudine	-7.59	2.74	-7.30	2.74
3	Adefovir	-7.28	4.58	-7.42	3.63

3.2. Molecular Docking Interaction Results within the Putative Binding Site

Analysis of the molecular docking results using VMD and LigPlot revealed that the selected ligands, Csa and Nor, were capable of forming stable hydrogen-bond interactions within the putative binding site of HBcAg in both WPr and MPr (P135Q) forms (Fig. 1). Specifically, Nor interacted with Thr109 in WPr, whereas in MPr it established an additional stable hydrogen bond

with Trp102. Moreover, the more negative ΔG observed for the mutant complex suggested a slightly enhanced binding affinity of Nor in the presence of the P135Q mutation.

For Csa, comparable hydrogen-bonding patterns were observed in both protein forms, primarily involving Ser106, suggesting a key role for this residue in ligand recognition and stabilization of the Csa–HBcAg complex.

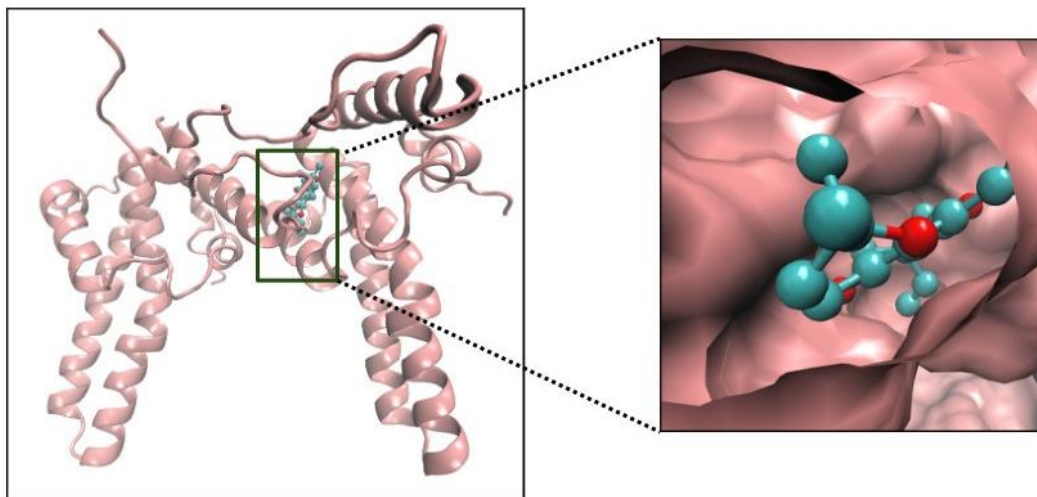
Other ligands displayed distinct interaction patterns. Ade formed multiple interactions with Trp102, Ser106, Tyr118, Tyr132, and Leu140 in MPr, and a similar set of contacts in WPr. Ent exhibited stable hydrogen bonds with Ser106 and

Tyr132 in both forms, whereas Lam primarily interacted with Tyr132, Leu140, and Ser141, forming an additional contact with Tyr118 in MPr (Table 2). These findings were generally consistent with the corresponding ΔG data (Table 1).

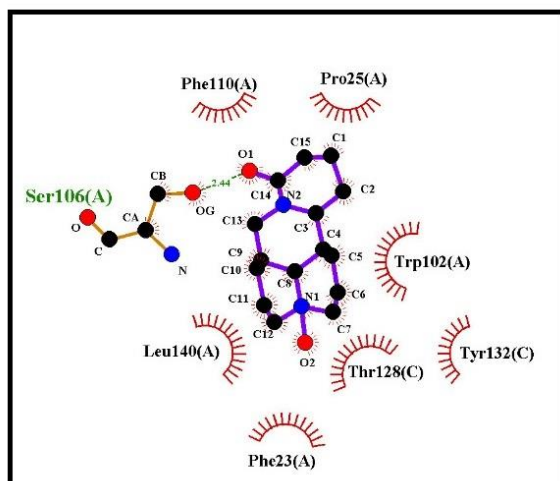
A

Pose view

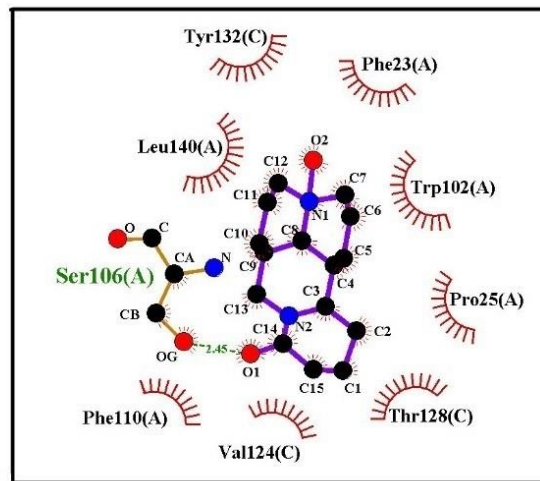
Surface view



B

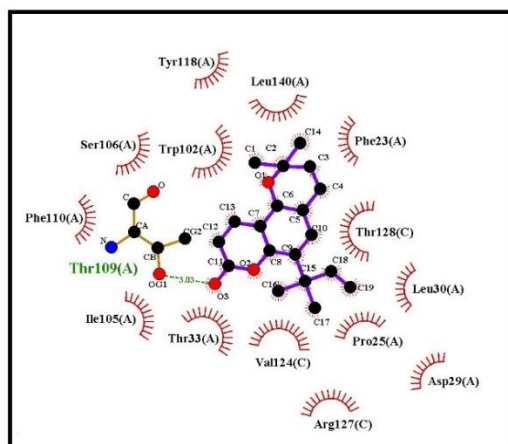


WPr-Csa

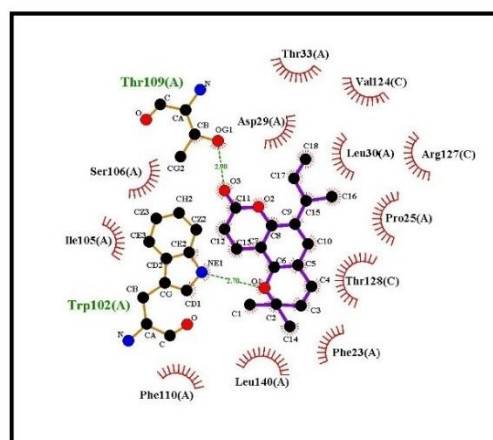


MPr-Csa

C

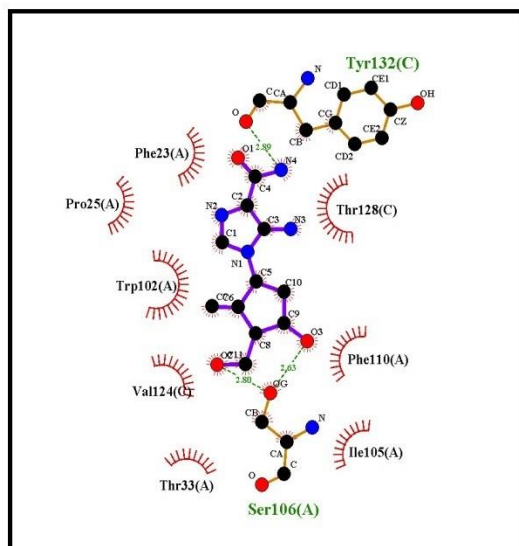


WPr-Nor

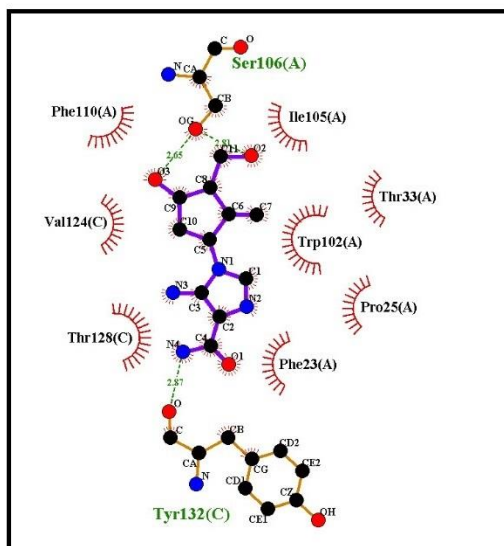


MPr-Nor

D

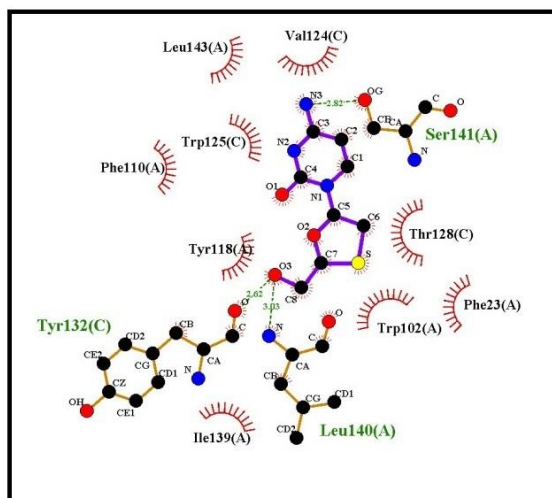


WPr-Ent

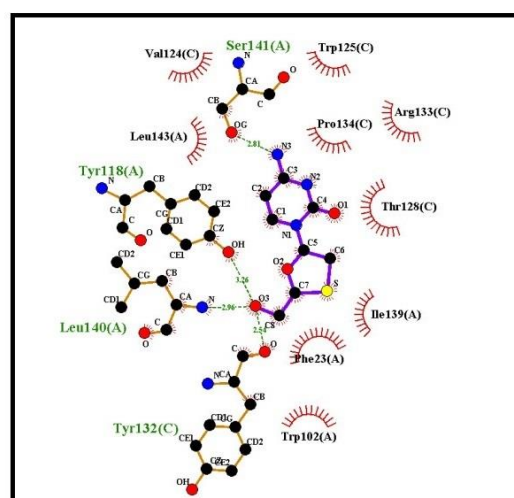


MPr-Ent

E



WPr-Lam



MPr-Lam

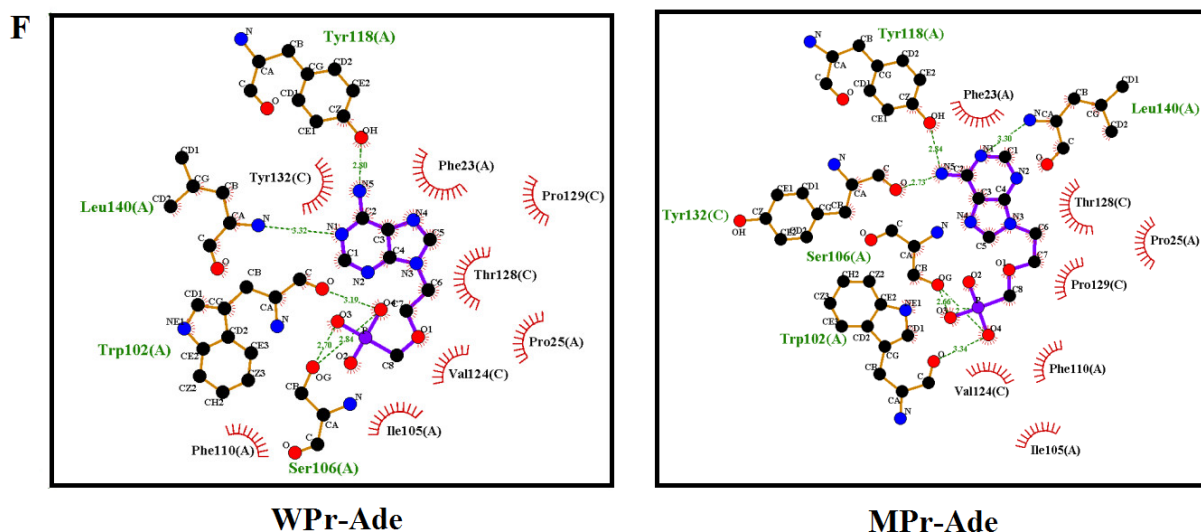


Fig. 1. Representative visualization of the interactions between WPr and MPr (P135Q) HBcAg with five ligands (Csa, Nor, Ent, Lam, and Ade) obtained from AutoDock docking analysis. (A) Pose and surface views are provided as representative examples to highlight the binding orientation and pocket accessibility of a selected ligand within the binding pocket. (B–F) LigPlot diagrams illustrate hydrogen bond interactions and hydrophobic contacts for each ligand in both WPr and MPr.

Table 2. Results of hydrogen-bond interaction analysis using VMD and LigPlot.

Name	Residue-WPr	Residue-MPr
Nor	Thr109	Trp102, Thr109
Csa	Ser106	Ser106
Ade	Trp102, Ser106, Tyr118, Lue140	Trp102, Ser106, Tyr118, Tyr132, Lue140
Ent	Ser106, Tyr132	Ser106, Tyr132
Lam	Tyr132, Lue140, Ser141	Tyr118, Tyr132, Lue140, Ser141

3.3. Molecular dynamics simulation of natural ligands (Csa and Nor)

To evaluate the structural stability of WPr and MPr (P135Q) HBcAg in complex with Csa and Nor, MD simulations were performed. Four structural parameters were analyzed: RMSD, RMSE, Rg, and SASA.

The average RMSD of MPr was slightly higher than that of WPr (Fig. 2A). Nor was associated with lower RMSD in MPr compared to WPr, whereas Csa showed lower RMSD in WPr relative to MPr (Fig. 2A).

Ligand binding was associated with generally reduced residue fluctuations in WPr. In MPr, Csa

binding corresponded to decreased flexibility, while Nor binding was associated with a slight increase in residue mobility (Fig. 2B).

The P135Q mutation was associated with a more compact protein structure. In both protein forms, Csa binding corresponded to lower Rg values, whereas Nor binding was associated with increased Rg values (Fig. 2C).

Csa binding was associated with reduced solvent-accessible surface area in both WPr and MPr. Nor binding to MPr corresponded to increased SASA, consistent with the increased Rg values. In WPr, Nor binding was associated with decreased SASA (Fig. 2D).

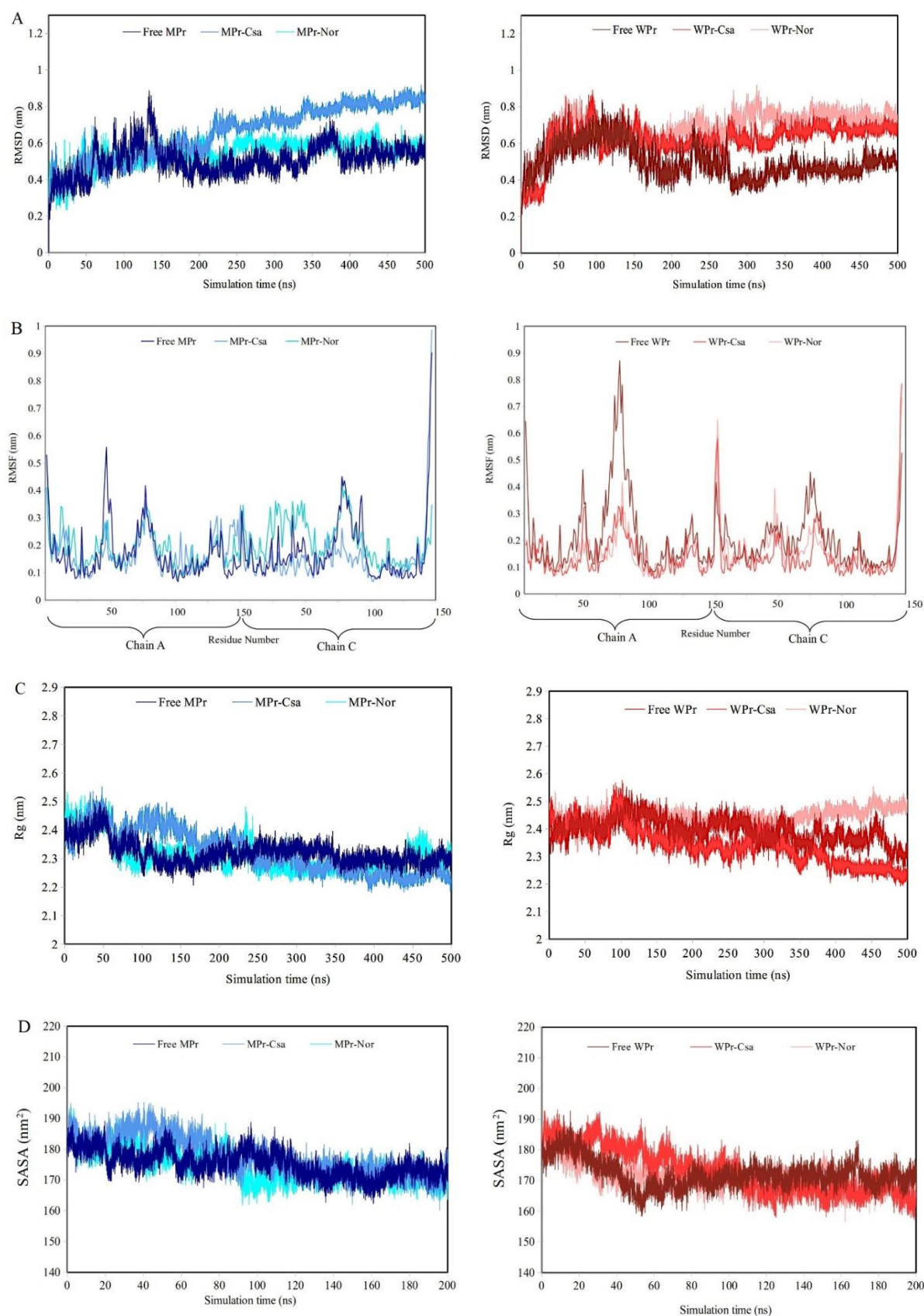


Fig. 2. Structural dynamics of WPr and MPr in the free form and upon binding with Csa and Nor during 500 ns MD simulations, as monitored by (A) RMSD, (B) RMSF, (C) Rg, and (D) SASA.

3.4. Principal Component Analysis (PCA)

PCA was performed to assess dominant motions and large-scale structural variations of HBcAg in the presence of Csa and Nor. Eigenvalue distribution plots for the first twenty eigenvectors showed that the first three principal components accounted for most motions across all systems.

For WPr systems, the first three eigenvectors of WPr, WPr-Csa, and WPr-Nor accounted for 71.44%, 51.91%, and 59.76% of total variance, respectively. The cumulative variance contributed by the first three eigenvectors decreased in the presence of ligands compared to free WPr.

For MPr systems, the first three eigenvectors of MPr, MPr-Csa, and MPr-Nor accounted for

51.86%, 61.67%, and 68.58% of total variance, respectively. In contrast to WPr, ligand binding was associated with increased cumulative variance in the mutant form (Fig. 3).

The projections of the first two principal components showed that Csa and Nor had different effects on the conformational space sampled by WPr and MPr. In WPr, both ligands were associated with reduced conformational space compared to the free protein. In MPr, Csa was associated with a more confined conformational ensemble, whereas Nor was associated with a broader sampled region (Fig. 3).

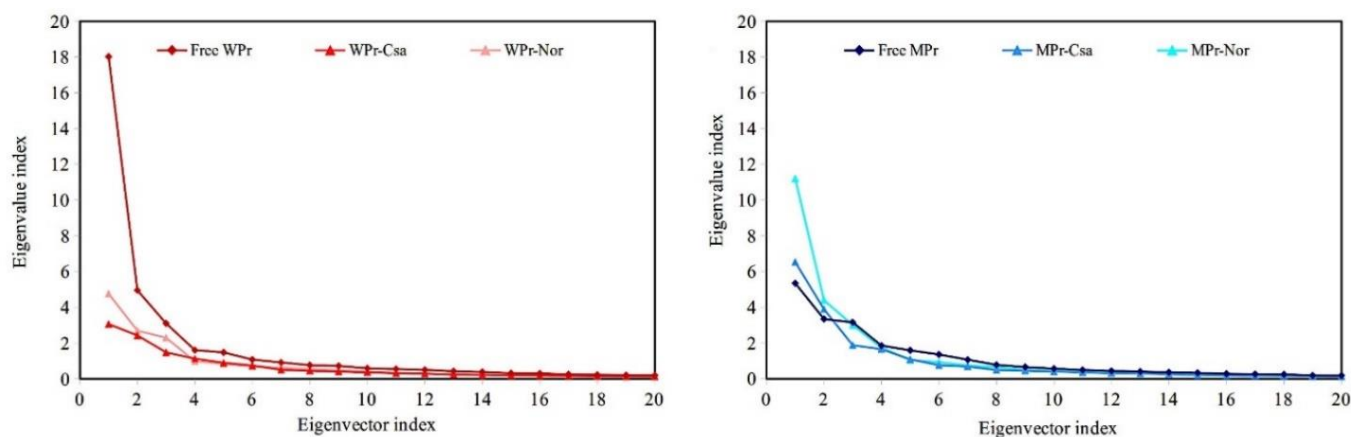


Fig. 3. Eigenvalue index plot derived from the PCA of molecular dynamics trajectories for WPr and MPr (P135Q) HBcAg in complex with Csa and Nor. The plot illustrates the magnitude of the first principal eigenvalues, showing the contribution of dominant collective motions within each system.

3.5. Hydrogen Bonding Patterns

Hydrogen bond formation and stability between WPr and MPr (P135Q) HBcAg with Csa and Nor were analyzed using a donor-acceptor distance cutoff of 0.35 nm and an angular cutoff of 30°, consistent with standard geometric criteria.

As shown in Fig. 4, the maximum number of hydrogen bonds formed was three for WPr-Csa and WPr-Nor, two for MPr-Csa, and four for

MPr-Nor. Hydrogen bonds between Csa and both protein forms were intermittently stable, whereas those involving Nor remained consistently stable throughout the trajectories.

Analysis of intramolecular (protein-protein) and protein-solvent hydrogen bonds during the final 50 ns revealed distinct patterns. In WPr-Nor and MPr-Csa, intramolecular hydrogen bonds increased while protein-solvent hydrogen bonds

decreased. In MPr–Nor and WPr–Csa, intramolecular hydrogen bonds decreased and protein–solvent hydrogen bonds increased. In

WPr–Csa, inconsistencies between hydrogen bond changes and SASA values were observed.

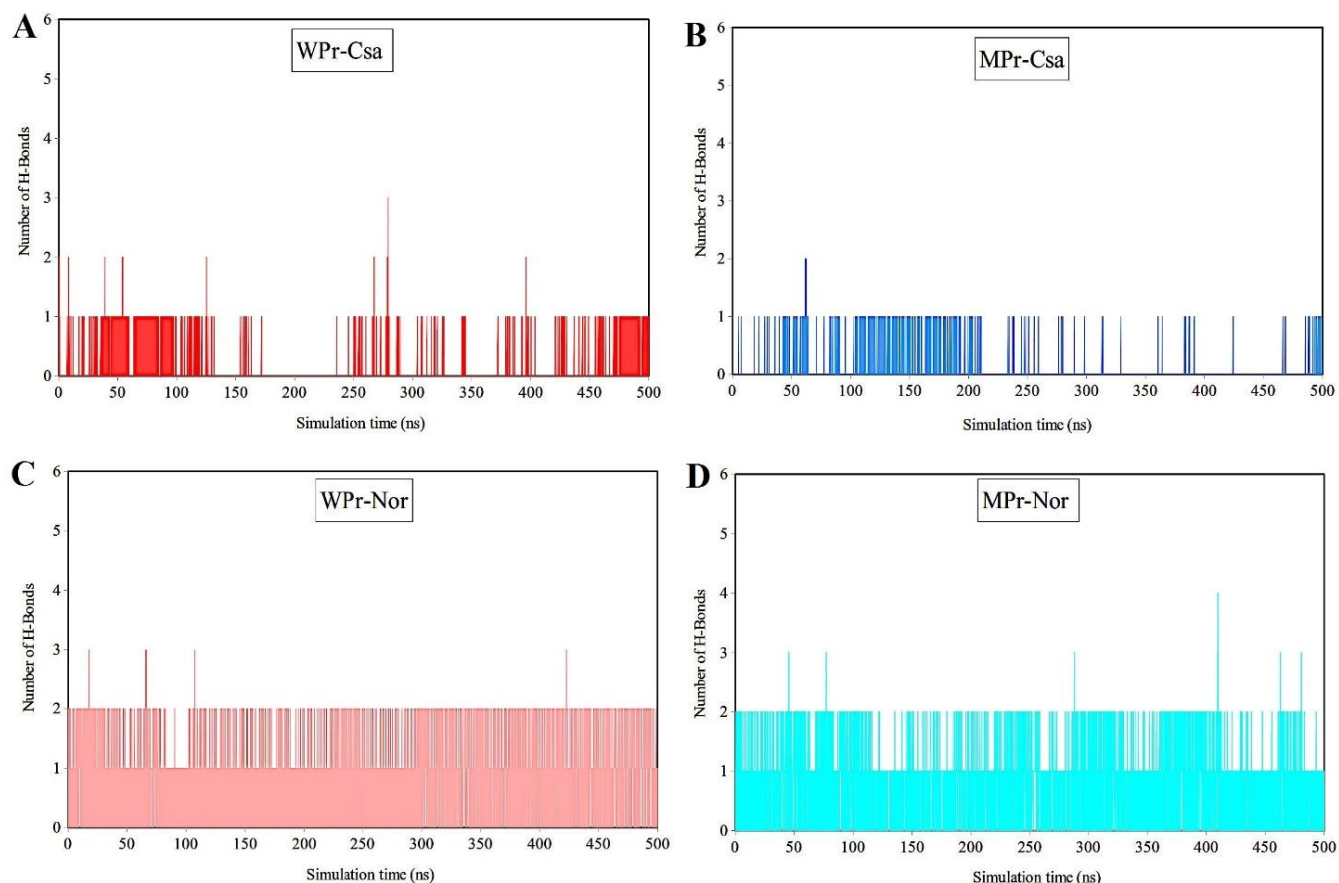


Fig. 4. Time-dependent profiles of hydrogen bonds observed between HBcAg proteins (WPr and MPr (P135Q) forms) and ligands (Csa, Nor) during the molecular dynamics simulations.

3.6. Ligand–Protein Interaction Patterns During MD Simulations

The interaction patterns between Nor and Csa and both WPr and MPr (P135Q) forms of HBcAg were analyzed at the end of the 500 ns MD simulations. After simulation, both ligands exhibited interaction patterns distinct from their initial docking poses.

For Csa, the WPr–Csa complex exhibited a hydrogen bond with Arg150 and alkyl contacts with Arg151. In contrast, in the MPr–Csa complex, only alkyl interactions with Phe110

were observed, and hydrogen bonding was diminished (Fig. 5A).

In the WPr–Nor complex, the ligand formed hydrogen bonds with Trp102 and Arg127, along with alkyl contacts with Val124, Tyr118, Leu30, Pro25, Phe23, Tyr132, and Leu140. In the MPr–Nor complex, the hydrogen bond with Trp102 was maintained throughout the simulation, accompanied by additional alkyl interactions involving Tyr118, Leu140, Pro129, Val124, Arg127, Leu30, and Pro25 (Fig. 5B).

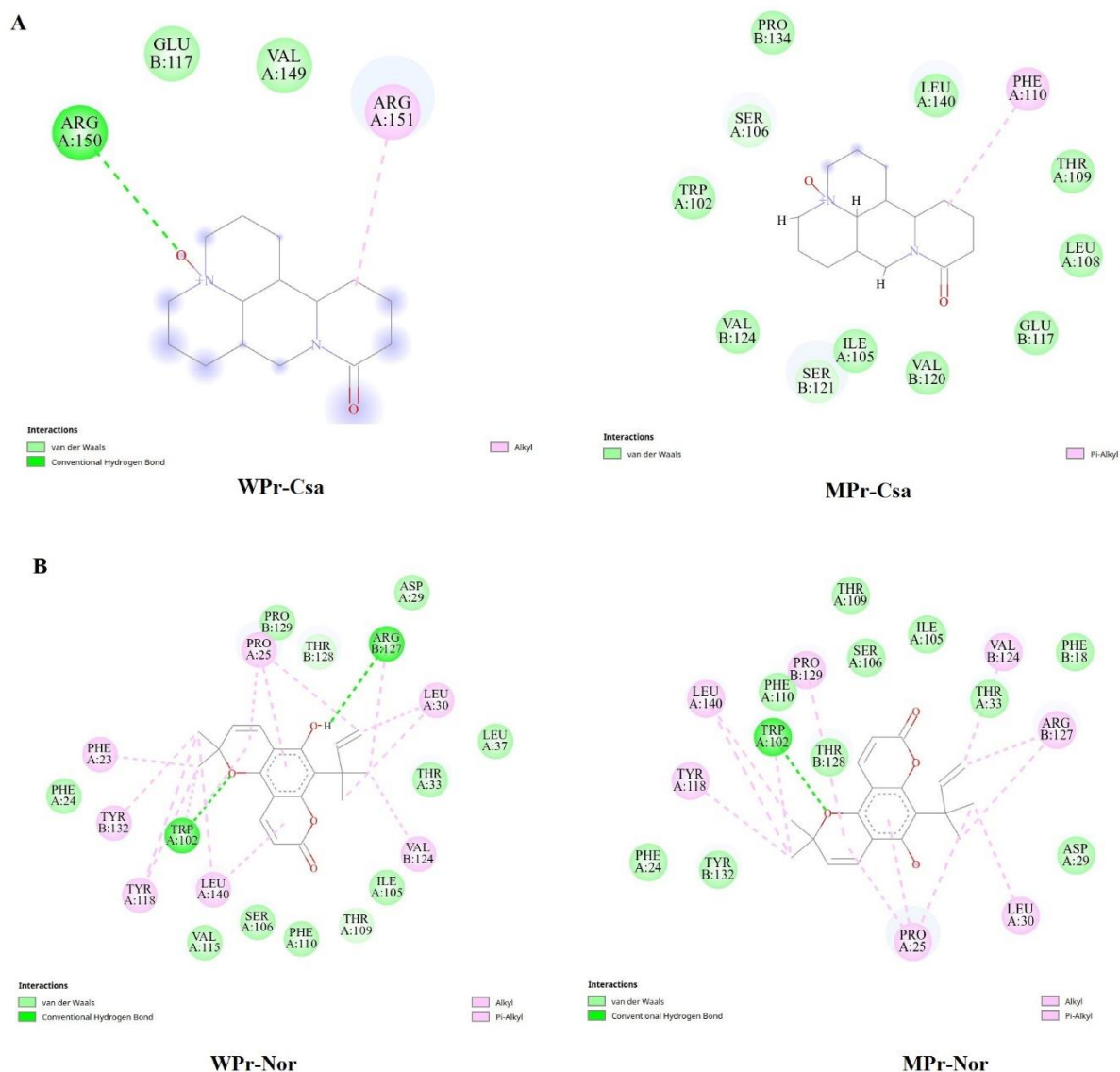


Fig. 5. Interaction patterns of Csa and Nor with WPr and MPr (P135Q) HBcAg after 500 ns of MD simulations. The interaction maps show stable hydrogen bonds and key alkyl contacts observed in the final snapshot. In the mutant complex, Nor appeared to form more selective interactions compared to Csa.

3.7. Binding Free Energy Analysis

The binding free energies of the HBcAg–ligand complexes were calculated over the last 50 ns of molecular dynamics simulations using the MM-PBSA method. The calculated binding free energies for all four complexes are presented in Table 3. All complexes exhibited negative binding free energy values.

The total binding free energy values were -240.97 ± 88.61 kJ/mol for WPr–Csa, -148.97 ± 10.33 kJ/mol for WPr–Nor, -414.79 ± 13.49 kJ/mol for MPr–Csa, and -131.81 ± 10.40 kJ/mol for MPr–Nor. The relatively large standard deviations observed for some complexes (e.g., WPr–Csa) indicate that the apparent energetic differences between systems should be interpreted with caution. The decomposition of

the total binding energy into van der Waals, electrostatic, polar solvation, and nonpolar

solvation (SASA) components is also reported in Table 3.

Table 3. MM/PBSA binding free energy components (kJ/mol) for HBcAg–ligand complexes.

Energy Component (kJ/mol)	WPr-Csa	WPr-Nor	MPr-Csa	MPr-Nor
van der Waals energy	-43.097 ±30.260	-206.673±9.355	-154.812±9.769	-184.914± 10.127
Electrostatic energy	-242.208±85.044	-12.486± 3.501	-373.345± 19.573	-11.339 ±3.875
Polar solvation energy	49.500± 55.846	87.622±5.788	128.317±16.019	82.320±8.139
SASA energy	-5.162±3.559	-17.431±0.638	-14.947±0.649	-17.878±0.674
Binding energy	-240.968±88.607	-148.968±10.328	-414.786±13.489	-131.812±10.396

3.8. Visualization of Molecular Dynamics Snapshots

To assess the ligand behavior within the binding pocket and evaluate the structural stability of the complexes throughout the simulations, representative snapshots were extracted at regular intervals over the 500 ns MD trajectories (Fig. S1, Supplementary File).

In the WPr–Csa complex, the ligand dissociated from the binding pocket during the early stages of the simulation and only reapproached the binding site after approximately 400 ns, remaining near it until the end of the trajectory.

In contrast, the ligands in the MPr–Nor, MPr–Csa, and WPr–Nor complexes remained positioned within the binding pocket throughout the simulation, showing only minor conformational rearrangements, such as limited rotational adjustments.

Both WPr and MPr (P135Q) HBcAg experienced conformational fluctuations during the simulation, while their overall tertiary structures remained conserved across all systems.

4. DISCUSSION

Hepatitis B remains a significant global health challenge due to its high prevalence and severe hepatic consequences, including chronic inflammation, cirrhosis, and hepatocellular

carcinoma [1]. HBcAg plays a central role in HBV replication and antigenic presentation, making it an attractive target for inhibiting viral replication and reducing cccDNA load [3, 6].

In this study, virtual screening of natural compounds, followed by pharmacological and ADMET filtering, identified 18 candidates for docking. Among them, Carnosic acid (Csa) and Nordentatin (Nor) were selected for detailed MD analysis and MM/PBSA calculations based on their binding energies and pharmacokinetic profiles. Based on a multi-criteria scoring system (Table S5, Supplementary File), Carnosic acid was chosen as the best overall candidate due to its favorable binding affinity ($\Delta G = -8.77$ and -8.85 kcal/mol for WPr and MPr, respectively), acceptable ADMET profile, non-mutagenic nature, and lack of BBB permeability. Nordentatin was selected despite its positive Ames test and BBB permeability, owing to its superior binding affinity ($\Delta G = -9.70$ and -9.84 kcal/mol) and chemical diversity as an alkaloid scaffold. Other compounds were excluded primarily due to weaker binding affinity, BBB permeability, mutagenicity, carcinogenicity, or P-glycoprotein inhibition. For instance, Gingerol, despite having a slightly better binding affinity than Nordentatin ($\Delta G = -9.89$ vs. -9.84 kcal/mol for MPr), was excluded due to BBB permeability and P-glycoprotein inhibition, which

outweigh its marginal energetic advantage. Similarly, Populene D and Sophocarpine were excluded due to carcinogenicity and BBB permeability. This aligns with previous reports suggesting that natural compounds, despite some limitations, show favorable binding properties toward HBV targets [6, 24]. Importantly, previous studies have shown that structural modifications of coumarin-based scaffolds can significantly reduce genotoxicity while preserving biological activity [25]. Therefore, the mutagenicity of Nordentatin does not necessarily preclude its development as a drug lead, as targeted chemical modifications could potentially eliminate or reduce its genotoxic properties.

MD analyses revealed that the P135Q mutation slightly increased RMSD in MPr compared to WPr, suggesting a possible reduction in structural stability. Ligand binding appeared to have context-dependent effects: Nor was associated with lower RMSD in MPr, while Csa showed lower RMSD in WPr. RMSF analysis indicated that ligand binding reduced residue fluctuations in WPr, potentially reflecting increased structural rigidity. In MPr, Csa decreased flexibility, while Nor slightly increased residue mobility. Rg and SASA analyses suggested that Csa induced structural compaction, whereas Nor was associated with greater flexibility. These findings are consistent with previous reports showing that natural compounds can modulate protein stability by altering structural dynamics [6, 16, 26, 27]. However, these observations are based on a single MD trajectory per system. While the extended 500 ns simulation provides valuable insights into the binding behavior, additional independent simulations would further strengthen the statistical robustness of these findings.

PCA results suggested that the P135Q mutation may alter the dynamic landscape of HBcAg. In WPr, ligand binding concentrated dynamics into fewer modes, whereas in MPr, ligand binding was associated with increased cumulative variance.

Qualitatively, Csa was associated with a more confined conformational ensemble in MPr, whereas Nor allowed sampling of a broader region, consistent with RMSF analysis. These observations are descriptive rather than mechanistic, as PCA does not establish causality between ligand binding and specific functional changes.

Ligand-protein interaction analysis revealed that Nor in mutated HBcAg formed stable hydrophobic and hydrogen-bond networks with key residues. In contrast, Csa lost its hydrogen bond with Ser106 after mutation, interacting mainly through alkyl contacts. This suggests that the P135Q mutation may alter ligand binding pathways, requiring adaptive interactions for stability. These results align with previous studies showing that mutations can significantly change hydrogen-bond patterns and ligand binding modes [16, 26].

Notably, in the WPr–Csa complex, the ligand fully dissociated from the binding pocket during the early stages of the simulation and only re-associated after approximately 400 ns. This observation suggests that Csa may not be a stable binder for the wild-type HBcAg, challenging its candidacy for wild-type targeting. In contrast, Csa remained more stably bound to the mutant form, while Nor maintained stable binding in both forms. The differential behavior of Csa and Nor can be attributed to their distinct physicochemical properties. Csa, a polar diterpenoid containing phenolic and carboxylic groups, primarily stabilizes WPr through electrostatic and hydrogen-bond interactions, leading to structural compaction. Nor, a hydrophobic coumarin derivative, interacts mainly via van der Waals and hydrophobic forces, allowing better adaptation to the conformational flexibility introduced by the P135Q mutation. These differences suggest that ligand polarity, interaction type, and adaptability to structural changes may be important factors in modulating HBcAg–ligand dynamics and stability.

MM/PBSA calculations showed that all complexes had negative binding energies, with MPr–Csa having the most negative value (–414.79 kJ/mol). Van der Waals, electrostatic, and nonpolar (SASA) interactions favored binding, while polar solvation energy was unfavorable. In the Csa complexes, electrostatics appeared to dominate, whereas Nor binding seemed to be primarily governed by van der Waals forces. However, the relatively large standard deviations observed in the binding energy estimates (e.g., ± 88.6 kJ/mol for WPr–Csa) indicate that the apparent energetic differences between systems are not statistically significant. Therefore, any conclusions regarding ligand binding preferences based solely on these MM/PBSA values should be considered preliminary. Statistical support, such as replica simulations and appropriate tests (e.g., t-test or ANOVA), would be needed to confirm these findings in future studies [6, 14, 15]. Comparison with clinically approved anti-HBV drugs (Ent, Lam, Ade) revealed that Csa and Nor showed comparable binding affinities. Nordentatin demonstrated binding affinity similar to Entecavir, while Csa performed comparably to Lamivudine. Notably, Nordentatin uniquely targeted Thr109—a residue not engaged by approved drugs—suggesting a distinct binding mechanism. A comprehensive comparison of our computational approach and findings with previous *in silico* studies on HBcAg inhibitors is provided in Table S1 (Supplementary File). This observation is consistent with previous structural and functional studies that have identified Thr109 as a critical residue located near the capsid assembly modulator (CpAM) binding pocket, where its mutation can significantly impact viral replication and sensitivity to antiviral compounds [28, 29].

Recent computational studies have further confirmed HBcAg as a viable target for *in silico* drug discovery. For instance, Itani (2023) employed molecular docking and ADMET screening to identify benzofuranone derivatives as potential HBcAg binders, while Mohebbi et al.

(2025) utilized a computer-aided drug repurposing approach, combining pharmacophore modeling and molecular dynamics simulations, to identify FDA-approved drugs (such as ciclopirox olamine and voriconazole) with potential anti-HBV activity [30, 31]. These studies underscore the growing utility of computational methods in identifying novel HBcAg inhibitors. However, neither study specifically addressed the clinically relevant P135Q mutant or employed extended simulation times (500 ns) combined with MM/PBSA analysis. Our work complements these efforts by providing a more detailed and system-specific investigation of ligand binding to the P135Q mutant, offering deeper mechanistic insights into mutation-induced conformational changes and binding preferences.

According to the literature, Capsid assembly modulators (CAMs) are a promising class of anti-HBV agents that target the HBcAg dimer–dimer interface, disrupting capsid formation and viral replication. Based on their mechanism of action, CAMs are generally divided into two classes: Class I CAMs (e.g., phenylpropenamides and sulfamoylbenzamides) induce the formation of morphologically normal but genome-free capsids, whereas Class II CAMs (e.g., heteroaryldihydropyrimidines, HAPs) lead to the assembly of aberrant, non-capsid structures [11, 12]. Recent computational studies have employed molecular docking and dynamics simulations to identify novel CAM candidates, highlighting the utility of *in silico* approaches in this area [30, 31].

Our docking and MD simulation results suggest that both Csa and Nor bind to the dimer–dimer interface, consistent with the binding site of known CAMs [11, 12]. However, the precise structural outcome of this binding—whether it leads to empty capsids (Class I) or aberrant aggregates (Class II)—cannot be determined from our current data. Qualitatively, the binding of Nor to the mutant form was associated with stable hydrogen-bond networks and hydrophobic contacts, which may resemble the interaction patterns observed for some Class I

CAMs. In contrast, Csa's binding was more dependent on electrostatic interactions and structural compaction, potentially aligning with Class II-like behavior. Nevertheless, these interpretations remain speculative without experimental validation.

Therefore, whether Csa and Nor act as Class I or Class II CAMs remains an open question that warrants further experimental investigation, for instance via size exclusion chromatography, transmission electron microscopy, or capsid assembly assays [12, 17]. Such studies would be essential to confirm the mechanism of action and to guide future optimization of these compounds as anti-HBV agents.

In summary, this study suggests a potential role for plant-derived scaffolds in anti-HBV drug development. Csa may be a candidate for mutant HBcAg, while Nor shows promise for both forms despite mutagenicity concerns. However, these findings are purely computational and require experimental validation. Future studies should include experimental validation, replica simulations, and expanded compound libraries to confirm and extend these findings.

Therefore, our findings should be considered hypothesis-generating rather than conclusive, and the compounds should be regarded as potential candidates for further experimental investigation rather than confirmed anti-HBV agents. As with any computational study, the results are subject to limitations inherent to the methods used, including the use of a single MD trajectory (which limits statistical sampling for PCA and MM/PBSA), the relatively large standard deviations observed in the MM/PBSA binding energy estimates (e.g., ± 88.6 kJ/mol for WPr-Csa), the absence of explicit pKa calculations (we used default AMBER99SB protonation states at pH 7.4), and the reliance on a homology model. Nevertheless, the extended 500 ns simulations, combined with MM/PBSA and PCA analyses, provide useful initial insights into the binding behavior of Csa and Nor. Future

experimental validation will be essential to confirm these predictions and to determine the precise mechanism of action, including whether these compounds act as Class I or Class II CAMs. Despite these limitations, our computational analysis provides preliminary insights into the potential interactions of Csa and Nor with wild-type and P135Q mutant HBcAg, and the distinct binding mechanisms observed warrant further experimental investigation.

5. CONCLUSION

This computational study suggests that the natural compounds Carnosic acid (Csa) and Nordentatin (Nor), through distinct binding mechanisms, may serve as candidate compounds for HBcAg binding. According to our MD simulations, Csa stabilized the wild-type protein primarily via electrostatic interactions and structural compaction, whereas Nor, in the mutated form (P135Q), relied on van der Waals forces and stable hydrogen-bond networks. These findings suggest that the P135Q mutation not only alters HBcAg structural stability but also may reshape ligand binding pathways. Such insights could potentially inform the rational design of novel compounds targeting HBcAg, though experimental validation is required. Overall, Csa and Nor may serve as starting points for further optimization and experimental validation, but further *in vitro* and *in vivo* studies are necessary to confirm their actual efficacy and safety.

Supplementary files

Supplementary file 1 contains Fig. S1 and Tables S1–S5.

Declaration of interest

There are no conflicts to declare. The authors report no conflicts of interest. Also, the authors are responsible for the writing and content of this article.

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Supplementary:

Fig. S1. Time-resolved structural snapshots of HBcAg–ligand complexes during molecular dynamics simulations. Superimposed snapshots were extracted at 100 ns intervals over the 500 ns trajectory to monitor conformational stability of the ligand-bound complexes (WPr–Csa, WPr–Nor, MPr–Csa, and MPr–Nor). In most systems, both Carnosic acid and Nordentatin retained stable binding poses with minimal displacement or rotation, suggesting persistent ligand–protein interactions. However, in WPr–Csa, the ligand displayed initial instability and left the binding site, before re-establishing interactions near the pocket after approximately 400 ns, where it remained stable until the end of the simulation.

Table S1. Comparison of the present computational study with previous in silico investigations on HBcAg inhibitors.

Table S2. Comparison of phytochemical ligands and FDA-approved drugs (Entecavir, Lamivudine, and Adefovir) based on Lipinski's rule of five.

Table S3. Comparison of phytochemical ligands and FDA-approved drugs (Entecavir, Lamivudine, and Adefovir) based on pharmacodynamic analysis.

Table S4. Comparison of phytochemical ligands and FDA-approved drugs (Entecavir, Lamivudine, and Adefovir) based on pharmacokinetic analysis.

Table S5. Multi-criteria scoring for compound prioritization and selection of Carnosic acid (Csa) and Nordentatin (Nor) for detailed MD analysis.



مطالعه محاسباتی ترکیبات طبیعی و تأییدشده توسط FDA در اتصال به HBcAg نوع وحشی و جهش یافته P135Q

سمیه فرهنگمند^{۱*}، مبینا اشتری^۲، رضا حاجی حسینی^۳، غلامرضا بخشی خانیکی^۴

چکیده

عفونت ویروس هپاتیت ب (HBV) همچنان یکی از چالش‌های جدی سلامت در جهان است و جهش در آنتی‌ژن هسته (HBcAg) می‌تواند اثربخشی داروهای ضدویروسی موجود را کاهش دهد. در این مطالعه، با به‌کارگیری یک رویکرد جامع محاسباتی (in silico)، تلاش شد تا ترکیبات طبیعی و داروهای تأییدشده توسط سازمان غذا و داروی آمریکا (FDA) که قادر به هدف‌گیری نوع وحشی (WPr) و نوع جهش‌یافته P135Q HBcAg (MPr) هستند، شناسایی شوند. ترکیبات طبیعی ابتدا با استفاده از قانون پنج لیپینسکی و ارزیابی ADMET غربالگری شدند. سپس داکینگ مولکولی با نرم‌افزار AutoDock Vina نسخه x1.2 انجام شد و کمپلکس‌های برتر به مدت ۵۰۰ نانوثانیه با استفاده از GROMACS2023 و میدان نیروی AMBER99SB تحت شبیه‌سازی دینامیک مولکولی قرار گرفتند. تحلیل‌های ساختاری و انرژی شامل RMSF، RMSD، شعاع چرخش (Rg)، سطح قابل دسترسی به حلال (SASA)، پیوندهای هیدروژنی، تحلیل مؤلفه اصلی (PCA) و محاسبه انرژی آزاد اتصال با روش MM/PBSA بود. از میان ۵۰۰۰ ترکیب گیاهی غربال‌شده، اسید کارنوزیک (Csa) و نوردنتاتین (Nor) تمایل اتصال مطلوبی (به ترتیب -8.85 و -9.84 ΔG = کیلوکالری بر مول) و پروفایل ADMET قابل‌قبولی نشان دادند. شبیه‌سازی دینامیک مولکولی نشان داد که این دو ترکیب مکانیسم‌های اتصال متفاوتی دارند؛ Csa از طریق برهمکنش‌های الکترواستاتیک، پروتئین وحشی را تثبیت می‌کند، در حالی که Nor با ایجاد پیوندهای هیدروژنی پایدار و برهمکنش‌های واندروالسی با باقیمانده‌های Trp102، Ser106 و Tyr118، فرم جهش‌یافته را تثبیت می‌نماید. نتایج تحلیل انرژی آزاد اتصال نیز نشان داد که Csa تمایل بیشتری به فرم جهش‌یافته دارد، در حالی که Nor نوع وحشی را ترجیح می‌دهد. جهش P135Q تأثیر چشم‌گیری بر دینامیک اتصال لیگاندها و الگوهای برهمکنش داشته و تفاوت‌های ساختاری بین دو فرم پروتئین را آشکار ساخته است. یافته‌های ما حاکی از آن است که Csa و Nor می‌توانند به‌عنوان ترکیبات کاندید برای اتصال به HBcAg در نظر گرفته شوند؛ با این حال، تأیید این نتایج محاسباتی نیازمند اعتبارسنجی تجربی برای اثبات فعالیت بیولوژیکی واقعی آنها است.

واژه‌های کلیدی

اسید کارنوزیک، جهش P135Q، دینامیک مولکولی، ضد HBV، نوردنتاتین، HBcAg.

۱. استادیار گروه زیست‌شناسی، دانشگاه پیام نور، تهران، ایران.
۲. کارشناس ارشد گروه زیست‌شناسی، دانشگاه پیام نور، تهران، ایران.
۳. استاد گروه زیست‌شناسی، دانشگاه پیام نور، تهران، ایران.
۴. استاد گروه زیست‌شناسی، دانشگاه پیام نور، تهران، ایران.

نویسنده مسئول:

سمیه فرهنگمند

رایانامه: s.farahmand@pnu.ac.ir

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