Deciphering the Binding Mechanism of Noscapine with Lysozyme: Biophysical and Chemoinformatic Approaches

Damini Sood,†,# Neeraj Kumar,†,# Anju Singh,† Vartika Tomar,† Sujata K. Dass,∥ and Ramesh Chandra,*†§
†Department of Chemistry, University of Delhi, Delhi 110007, India
§Dr. B. R. Ambedkar Center for Biomedical Research, University of Delhi, Delhi 110007, India
∥BLK Super Speciality Hospital, Pusa Road, New Delhi 110005, India

ABSTRACT: Lysozyme is a well-characterized protein in terms of its structure, dynamics, and functions. It has thus emerged as a potential target to understand protein−drug interactions. The aim of our study is to gain a biophysical outlook on the interaction of lysozyme (Lyz), a well-known model protein, with Noscapine, a potent tubulin-binding anticancer drug. Noscapine (Nos) is effective against a wide range of cancer and shows low toxicity and few side effects. We report the underlying mechanism of complex formation between Nos and Lyz using spectroscopic and advanced computational avenues. The spectroscopic techniques, that is, absorption and steady-state and time-resolved fluorescence, proved that Lyz−Nos forms a complex, and the quenching mechanism was of the static type. The binding constant was in the order of 10³ indicative of moderate binding, while the stoichiometry of the protein−drug complex was 1:1 at 298 K. The secondary structural analysis using CD and UV thermal denaturation further confirmed the conformational changes in the protein upon binding with Nos. Molecular dynamics simulation studies confirmed the stable binding with minimum deviations in RMSD. The above conclusions are significant to the development of the pharmacokinetics and pharmacodynamic properties of Nos, and its successful interaction with a versatile protein like Lyz will help in overcoming its previous limitations.

INTRODUCTION
Noscapine (Nos) is a phthalideisoquinoline alkaloid found in the plant family of opium poppy. This non-narcotic drug has been significantly researched due to its potency against various types of cancer. The similarity in structure of Nos (Figure 1) to colchicine and podophyllotoxin led to the exploration of its binding with tubulin proteins. It was successfully demonstrated that the interaction of Nos with tubulins leads to the alteration of the microtubule assembly, causing mitotic arrest. Recently, Nos and its halogenated analogs have been biosynthesized using microbial fermentation, and it is a major step toward industrial production. The drug is emerging as a viable anticancer agent already undergoing clinical trial in phase I/II. Although it has a low-toxicity profile, the high-dosage requirement and low bioavailability have impeded its development as a commercially available drug. Several alternative methods for Nos drug delivery, including nanoparticles, soluble complexes, etc., have been reported, and the investigation is still underway to find the most suitable pathway to overcome the pharmacokinetic limitations. In order to aid the potency of the compound for cancer treatment, drug−protein binding studies are vital to assess its delivery and homing to specific target points. Previously, Nos and its analogues have been explored for their interaction with blood proteins (BSA and HSA) and pharmacological properties using biophysical approaches to aid reports on the potency of the compound for cancer treatment.

Biophysical studies provide deep insight into the drug interaction, in vivo half-life, solubility, efficacy, toxicity, heme metabolism, clearance, and elimination from the body. Therefore, it is necessary to study the biophysical parameters...
of the drug. Considering that fact and a literature survey, in this work, we have performed the binding mechanistic interaction studies of Nos with Lyz abundantly present in the body secretions. Hen egg-white lysozyme, an easily available and low-cost protein, is used as a model system due to its structural similarity (~60% homology) to human lysozyme. Lyz is a small globular monomeric protein (129 amino acid residues, mol wt = 14.3 kDa) with six tryptophan and three tyrosine residues with four disulfide linkages providing structural stability. It has innumerable biologically and pharmaceutically important functions, including anti-inflammatory, antimicrobial, and antiviral functions. One of the most important functions of Lyz is its ability to transport drugs. In view of this property, the binding of Nos with Lyz was deciphered using different spectroscopic and in silico techniques. UV–visible and steady-state and time-resolved fluorescence spectroscopy elucidated the mechanism of binding, while circular dichroism spectroscopy offered an insight into the secondary structural changes upon interaction. Molecular docking has been performed to calculate binding free energy and interaction sites; molecular dynamic simulations have been used to assess the stability of the protein conformations and root-mean-square deviations. Drug dynamics and stability parameters are potential factors to determine the biological functionality of the drug and specific binding of the drug to the binding grove of oncotargets. Since Lyz is extracted from the hen egg white, a boiled egg assay was also performed, an in silico technique to evaluate the gastrointestinal absorption and blood–brain barrier penetration potential. Overall, our current study offers a detailed outlook into the binding of Nos with Lyz, which will be useful in moving closer to realizing its full anticancer potential.

## RESULTS AND DISCUSSION

### Absorption Spectroscopy

UV–visible spectroscopy is one of the most important techniques used to analyze drug–protein interactions. The absorption spectra of Lyz shows a characteristic peak at 281 nm, which is due to the $\pi-\pi^*$ transition of the three aromatic amino acids, tryptophan, tyrosine, and phenylalanine. To elucidate the mechanism of interaction between Lyz and Nos, the fluorescence spectra of Lyz in the absence and presence of the drug was carefully studied. Lyz shows an emission peak at 340 nm (Figure 3) at an excitation wavelength of 280 nm, and this fluorescence was quenched by the successive addition of Nos. Now, it is possible that the decrease in fluorescence can proceed via a static or dynamic mechanism. The two modes responsible can be differentiated on the basis of the value of the quenching constant. In general, static quenching is responsible when there is complex formation, while dynamic quenching comes into play due to collisional encounters. The binding mechanism was assessed by evaluating the fluorescence spectra using the Stern–Volmer equation:

$$\frac{F_0}{F} = 1 + K_v \times [Q] = 1 + K_q \times \tau_0 \times [Q] \tag{1}$$

The symbol $F_0$ denotes the fluorescence intensity of pure Lyz, while $F$ is the intensity in the presence of Nos. $[Q]$ is the
concentration of Nos in M, \( \tau_0 \) is the average lifetime of a biomolecule, and \( K_{sv} \) and \( K_q \) are the Stern–Volmer constant and quenching constant, respectively. \( K_q \) is calculated simply by assuming the value of \( \tau_0 \) to be \( 10^{-8} \) s.32 The value of \( K_q \) (Table 1) was found to be higher than the maximum diffusion rate constant for biomolecules,33 in our case, proteins (\( 2 \times 10^{10} \) L mol\(^{-1}\) s\(^{-1}\)). It can be concluded that the mechanism of quenching was due to the complex formation between Lyz and Nos.

Table 1. Stern–Volmer Quenching Constants and Binding Parameters of the Lyz–Nos System at Room Temperature

| temperature (K) | 298 |
|----------------|-----|
| \( K_{sv} \) (L mol\(^{-1}\)) | \( 3.18 \times 10^3 \) |
| \( K_q \) (L mol\(^{-1}\) s\(^{-1}\)) | \( 3.18 \times 10^{11} \) |
| \( n \) | 0.90 |
| \( K_b \) (L mol\(^{-1}\)) | \( 1.41 \times 10^3 \) |

The fluorescence data was further evaluated to determine the binding constant and the number of binding sites by following the modified Stern–Volmer equation34 (eq 2).

\[
\log \left( \frac{F_0 - F}{F} \right) = \log K_b + n \log[Q]
\]

Here, \( K_b \) is the binding constant and \( n \) represents the number of binding sites.

When eq 2 was used to plot the fluorescence data of the Lyz–Nos interaction, it was seen that the plot was linear and the slope came out to be 0.9, and the intercept gave the value for \( K_b \). The Stern–Volmer and modified Stern–Volmer plots have been depicted in Figure 4. The binding constant was in the order of \( 10^3 \) (L mol\(^{-1}\)) showing a moderate and reasonable binding, while the near-unity value of \( n \) (\( n = 0.9 \)) shows the 1:1 stoichiometry of the Lyz–Nos complex. The UV–visible results were also in coherence with the steady-state fluorescence analysis because if the quenching was dynamic, the absorbance spectra would be unaltered.

**Time-Resolved Fluorescence.** The mechanism of quenching was concluded to be static based on steady-state fluorescence. Time-resolved fluorescence spectra can also be used to differentiate between static or dynamic quenching for any protein–drug interaction. When the quenching is static, the lifetime of the fluorophore remains unchanged in the presence of the ligand, while for dynamic quenching, a change is observed.35,36 On recording the spectra (Figure 5), it was seen that the lifetime of Lyz (1.8 ns) remained the same even in the presence of different concentrations of Nos (Table 2). Thus, the reason for the quenching of Lyz in the presence of the drug can be attributed to a static type.

Table 2. Fluorescence Decay Profile of the Lyz–Nos System at Different Concentrations of Nos

| [Nos] (\( \mu \)M) | \( \tau_1 \) (ns) | \( \tau_2 \) (ns) | \( a_1 \) | \( a_2 \) | \( \tau_{av} \) (ns) |
|-----------------|----------------|----------------|---------|---------|----------------|
| 0               | 1.05           | 2.59           | 51.28   | 48.72   | 1.80           |
| 3               | 1.1            | 2.63           | 55.23   | 44.77   | 1.785          |
| 6               | 1.09           | 2.56           | 52.46   | 47.54   | 1.788          |
| 10              | 1.1            | 2.66           | 54.18   | 45.82   | 1.787          |
| 15              | 1.05           | 2.49           | 48.28   | 51.72   | 1.795          |

**UV-Thermal Denaturation.** UV-Tm experiments have very important implications in interaction studies. It plays a significant role in interpreting the stabilizing/destabilizing effect of the ligand on biomolecules (protein, DNA, etc.). In
In the present study, the UV Tm of Lyz is monitored in the absence as well as in the presence of Nos. Figure 6 displays an inverted melting profile for Lyz in the absence as well as in the presence of a varied concentration of Nos. The thermal melting profile of Lyz is manifested with a melting temperature of 77 °C. On addition of Nos in the varied ratio (i.e., 1:1 and 1:2), a decrease in the melting temperature was observed, that is, 73 °C, which reflects destabilization of the protein. It might be possible that Nos interacts with the protein via intercalation to the hydrophobic pocket of the protein where tryptophan is located, thus distorting the structure of the protein.

**CD Spectroscopy.** Circular dichroism (CD) spectroscopy has been employed as an informative tool to investigate the folding of proteins as well as conformational changes in the protein upon interaction with ligands. Various structural elements (α helix, β sheets, peptide bonds, etc.) manifested characteristic CD signatures. CD spectroscopy of Lyz is carried out in phosphate buffer saline (pH 7.4) at room temperature (298 K). Hen egg protein Lyz has components of α + β with a larger α domain confining four α helices along with a comparatively smaller β domain. CD spectra recorded in the near-UV region (250–300 nm) are very significant in order to explain the folding of the proteins along with the presence of aromatic moieties, disulfide bonds, and prosthetic groups in the protein. When the protein is correctly folded, a prominent signal in the near-UV region is observed. Near-UV CD spectra significantly give information about the tertiary structure and environment of protein, whereas far-UV CD spectra reveal the important characteristic feature of the secondary structure in proteins.

Figure 7 demonstrates CD spectra of Lyz in the absence as well as in the presence of Nos. The far-ultraviolet CD spectrum of Lyz is manifested with two negative minima centered at 208 and 222 nm, reflecting the characteristic CD signature of an α-helical structure. It is in good agreement with a previous report.37 Minima at 208 nm correspond to the π–π* transition of the α helix, whereas minima at 222 nm belong to the n–π* transitions for both the α helix and the random coil. On subsequent addition of Nos, the far-UV CD spectrum of Lyz decreased in intensity with a shift of a 222 nm peak toward 208 nm, indicating secondary-structure conformational changes in the protein.38

To gain more insight into secondary-structure conformational changes, the secondary structure is determined by using Yang software and tabulated in Table 3. The table clearly shows that native Lyz contains 30.5% α helices, 37% turns, and 32.5% random coils. On successive addition of Nos, the content of α helices decreases along with an increase in the content of β sheets for Lyz. Increasing the concentration of Nos at 1:1, 1:2, 1:6, and 1:8 manifested a decrease in the α-helix percentage, that is, 29.2, 24.4, 16, and 3%, respectively, along with an increment in the β-sheet percentage such as 24.1, 40.6, and 91.6% was observed. Herein, it is a clear indication of conformational changes occurring on the interaction of Lyz with Nos. It might be possible that Nos goes inside the hydrophobic pocket of the protein, binds there, and by doing so, induces conformation alteration/disruption of the protein’s native conformation.39 The CD experiment is in good correlation with the UV-thermal denaturation experiment.

**Boiled-Egg Permeation Assay of Nos.** Pharmacokinetics and bioavailability gastrointestinal absorption for high efficacy of the drug were studied using the boiled-egg permeation assay. A boiled-egg model-based intuitive graphical analysis was performed for passive absorption of Nos through intestinal and brain penetration. The result showed that the Nos drug is found to fall inside the white ellipse, which showed that the Nos drug was found to fall very close and partially inside the yellow ellipse (yolk), which showed that the Nos has high values for permeation in the blood–brain barrier (BBB) (Figure 8).

**Retrieval of the 3D structure of Lyz and Preparation for Molecular Docking.** Prior to the molecular docking assay, the 3D structure of Lyz was downloaded from the Protein Data Bank (PDB ID: 2LYZ). Stereorechemical proper-
ties were analyzed by the Ramachandran plot using the Saves server. The Ramachandran plot showed that ∼85% of residues lie in the most favored region, 15% of residues lie in the additionally allowed region, and no residue lies in the outlier region. Furthermore, physicochemical properties of the Lyz structure were evaluated by the Swiss server, and it showed the QMEAN score to be $-1.39$ for the structure, representing the overall absolute parameters, including the torsion angle energy, solvation energy, solvent accessibility, and atom pairwise energy. These results indicated the stability of the structure lying in a region close to the white region of the QMEAN plot. The precision of the structural assessment was determined by QMEANDisCo scores. It showed the consistent interatomic distances of the structure, and the local quality plot showed a high-quality structure by scoring values above 0.6, which was expected for the high-quality structure according to the QMEANDisCo algorithms (Figure 9).

**Molecular Docking Assay.** Molecular docking was performed to assess the binding of Nos to the Lyz protein. The Lyz protein was prepared by removing the water molecule to achieve the dry trajectory, and hydrogen atoms were added to stabilize the protein structure. HEX 8.0 parameters were set for the shape and conformations to analyze the binding in the 3D FFT mode with a whole-protein-range angle sampling method. The grid dimension of 3D Lyz was set to 0.6 Å, and the protein twist range of 360° and translation steps of 0.8 and 25 outputted the interacting complex system by the steric scan. The resulting top-score docked complex showed a strong interaction with a binding score of $-215.32$ kJ mol$^{-1}$ for Nos, and it was found to possess a strong interaction into the binding groove of the Lyz protein with two hydrogen bonds, one at Arg14 of a bond length of 2.85 Å with an angle of 147.48° and a second hydrogen bond at Ile88 of a bond length of 2.97 Å with an angle of 174.14° as well as strong hydrophobic interactions at Phe50, Phe51, and Ala160. Arginine14 was also found to form three water bridges with three different atoms of Nos with a distance of 3.94, 2.71, and 3.85 Å (Figure 10). Notably, Arg14 and His15 residues of Lyz were found to form the salt bridges with a distance of 4.74 and 5.06 Å. These molecular interaction dynamics and high binding scores suggested the potential binding of Nos to the Lyz protein.

**Molecular Dynamics Simulation Studies of the Nos–Lyz Complex.** Molecular dynamics analysis of Nos interactions with the Lyz protein was done for 100 ns-long simulations that were run using the MDWeb modeling suite. Multiple frames for the complex system were retrieved for different structural conformations. Resulting trajectory files of the interacting complex system were taken and analyzed for RMSD, and they showed a minimal deviation in the Lyz

| protein          | concentration | α helix | β sheet | β turn | random coil |
|------------------|---------------|---------|---------|--------|-------------|
| lysozyme         | lysozyme      | 30.5%   | 0.0%    | 37.0%  | 32.5%       |
| lysozyme + Nos (1:1) | 29.2%        | 0.0%    | 36.0%   | 34.9%  |
| lysozyme + Nos (1:2) | 24.4%        | 24.1%   | 24.3%   | 27.2%  |
| lysozyme + Nos (1:6) | 16.0%        | 40.6%   | 20.9%   | 22.5%  |
| lysozyme + Nos (1:8) | 3.0%         | 91.6%   | 0.0%    | 5.4%   |

**Figure 8.** Pharmacokinetics and bioavailability property analysis of Nos. (A) Depiction of physicochemical properties of Nos. It is shown that Nos lies in an optimal range for various properties required for the potential drug. (B) Boiled-egg permeation assay of Nos. It lies inside the white ellipse and yellow ellipse (yolk). (C) Depiction of Nos biological targets.
protein in the range of 0.06 to 0.63 Å. Atomic fluctuation analysis showed the low atomic fluctuations in the range of 0.3 to 11 Å (Figure 11). These outcomes suggested the stable interaction of Nos with the Lyz protein with minimal deviations.

**CONCLUSIONS**

The mechanistic interaction of Nos and Lyz and their corresponding conformational changes have been investigated. The emergence of Nos as a versatile and non-narcotic anticancer drug has encouraged the studies to decipher its interactions with various model proteins. Here, in the current report, UV−vis absorption and steady-state and time-decay fluorescence confirmed the complex formation between Lyz and Nos, while CD spectra showed the decrease in alpha-helicity upon binding. Molecular docking and simulations provided a deeper insight into the phenomenon of binding, while the in silico boiled egg assay showed good gastrointestinal absorption of the drug. All these results are complimentary to each other and support the moderate binding of Nos with Lyz. This study is crucial in developing Nos for its oncological purposes and helps in understanding its pharmacokinetic properties.
Lysozyme (3× crystal, CAS no. 12650-88-3) was purchased from Sisco Research Laboratories (SRL), while Nos was purchased from Sigma-Aldrich, U.S.A. The chemicals used for all experiments were of analytical grade, and no further purification was done.

**METHODS**

**Sample Preparation.** The stock solution of Lyz (25 μM) was prepared in phosphate buffer saline (PBS, 10 mM, pH 7.4) and diluted as per the requirement of the experiment. It was stored in a refrigerator and maintained at a temperature of 2–4 °C. The 10 mM stock solution of Nos was prepared in DMSO. The volume/volume ratio of DMSO/PBS was less than 1% in all experiments as it has been reported that, at this concentration, DMSO does not affect the structure of Lyz. All the experiments were performed at 298 K.

**UV–Visible Spectroscopy.** The UV–visible spectra were recorded using a Thermo Scientific Evolution 300 UV–vis spectrophotometer. The concentration of Lyz was kept constant at 15 μM, while the concentration of Nos was increased from 0 to 120 μM in intervals of 15 μM. The spectra were recorded in the UV range (200–400 nm).

**Steady-State Fluorescence.** The fluorescence emission spectra of Lyz in the absence and presence of Nos was recorded using Horiba PTI QM-8450-11-C. The excitation wavelength was set at 280 nm, and the spectra were recorded between 300 and 550 nm. The excitation and emission bandwidths were 3 nm.

**Inner Filter Effect.** The reason for the quenched fluorescence of the protein can also be attributed to the absorbance of the exciting light such that a less intense flux is encountered by the sample solution. The given equation can be used to correct the spectra for the inner filter effect.

\[
F_{\text{corr}} = F_{\text{obs}} \times e^{(A_{ex} + A_{em})/2}
\]

Here, \( F_{\text{corr}} \) is the corrected fluorescence intensity of the protein, while \( F_{\text{obs}} \) is the originally observed intensity. \( A_{ex} \) and \( A_{em} \) are the absorbances of the drug or ligand at the excitation and emission wavelengths of the protein, respectively.

**UV-Thermal Denaturation.** The transition midpoint (Tm) of Lyz was monitored by the absorbance at 292 nm as a function of temperature with a Varian CARY 300 Conc UV spectrophotometer equipped with a peltier using the software Cary WinUV, thermal. A quartz cuvette of 10 mm path length is used to record the melting of samples. The concentration of Lyz was kept constant at 15 μM, while Nos is used at concentrations of 15 and 30 μM. The analysis of the melting curves for the measurement of TM was carried out using the first derivatives.

**CD Experiment.** CD spectroscopic analysis was carried out using a J-815 spectrophotometer (JASCO, Japan) at room temperature using a quartz cuvette of 10 mm path length. Camphorsulfonic acid was used for CD calibration. Samples were pre-equilibrated at the desired temperature for 15 min, and the scan speed is fixed at 100 nm min⁻¹ with a response time of 1 s and a 1 nm bandwidth. To avoid water condensation in the cuvette chamber, it was flushed with constant purging of a N₂ gas. The CD spectra were collected from 320 to 200 nm at wavelength steps of 1 nm, and the final reported spectra correspond to the average of three scans. The scans of the buffer alone were subtracted from the average scans for each sample for baseline correction. Data were collected in units of millidegrees and were normalized to total protein concentrations. Samples were prepared in 10 mM phosphate buffer saline of pH 7.4. To study the conformational changes of Lyz, a constant concentration of Lyz (0.7 μM) is titrated with increasing concentrations of Nos (0.7–5.6 μM).

The molar ellipticity \( [\theta] \) is calculated from the observed ellipticity \( \theta \) as

\[
[\theta] = 100\left(\frac{\theta}{C \times l}\right)
\]
These experiments were performed for the molar ratio in 1:1, 1:2, 1:6, and 1:8. Secondary structure estimation is done by JASCO software Yang.jwr.

**Boiled-Egg Permeation Assay of Nos.** For potential drug development, the drug is required to possess the high values for pharmacokinetics and bioavailability gastrointestinal absorption for high efficacy of the drug at different stages. To assess gastrointestinal absorption and brain penetration, we have performed the intestinal estimated permeation method (boiled egg) using accurate predictive models. This method is based on determining the lipophilicity ($W \log P$) and polar nature of the drug (TPSA) from a large dataset of drugs with reported 93% of accuracy.

**Molecular Docking Assay.** Molecular docking assay was performed using the HEX 8.0 molecular docking suite. Hex uses the fast Fourier transformation algorithm (FFT). Hex outputs the multiple interacting ligand–protein complexes with the lowest energy scores; out of these results, a complex system with the lowest energy value was considered and further studied.

For docking preparations, the three dimensional (3D) crystal structure of Lyz was retrieved from the Protein Data Bank (http://www.rcsb.org). Lyz was prepared and evaluated to perform molecular dynamics. Stereochemical parameters of Lyz were assessed through the Ramachandran plot and other in silico tools. The Nos compound was prepared for docking by drawing using ChemDraw and saving in the required format. After that, molecular docking parameters were set and docking was performed, and the resulting complex system (Nos–Lyz) was analyzed for involved molecular interactions (hydrogen bonds and hydrophobic bonds), and interacting conformations were analyzed using Chimera molecular modeling software.

**Molecular Dynamics Simulation Studies of the Nos–Lyz Complex System.** The complex of Nos–Lyz was evaluated for its stability through the molecular dynamics simulation analysis using the MDWeb program. The MDWeb suite works by performing the simulations through the NAMD full molecular dynamics set up to provide the molecular interaction trajectories. Simulation steps involve the complex-system cleaning, side-chain fixing, solvent addition, and energy minimization of system. After that, equilibration of the complex system was done by heating the solvent at 300 K, and the resulting dry trajectory was evaluated for RMSD and atomic fluctuations to assess the stability and flexibility of Nos binding to Lyz.

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