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Exploring the Molecular Level Interactions of Molnupiravir with Serum Albumin- An Analytical Approach

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ABSTRACT

Molnupiravir is an antiviral agent used to treat mild coronavirus infection. The drug has good oral bioavailability and safety profile. The protein binding profile of molnupiravir with albumin protein is not well characterized. Therefore, to provide deeper insights, the binding characteristics of molnupiravir to bovine albumin serum were explored using spectral techniques and thermodynamic analysis. The Uv-vis spectral analysis revealed moderate affinity between molnupiravir and BSA with a binding constant value of 1.84 x 10^4 M^{-1}. Molnupiravir quenched the fluorescence spectrum of BSA via a static quenching mechanism. The negative ΔG (-9.53 $kJ.mol^{-1}$, -11.77 $kJ.mol^{-1}$, -14.28 $kJ.mol^{-1}$) for the molnupiravir-BSA complex confirmed spontaneous and exergonic interactions between the molecules.

Keywords: *BSA, Spectroscopy, Thermodynamic Analysis, Quenching, Binding Affinity*

1. INTRODUCTION

Molnupiravir (Figure 1) is an experimental antiviral agent with a good safety profile. It has a potent antiviral activity profile against several (+) - RNA viruses including the SARs-CoV-2 virus. It inhibits the replication process of the virus by blocking the RNA-dependent RNA polymerase enzyme. The active metabolite of molnupiravir, N4-hydroxy cytidine is responsible for its therapeutic activity. Molnupiravir was first approved by the UK, for emergency use and by the U.S.FDA for treating mild to moderate COVID-19 infection in adults [1-3]. The adverse effects are mild and include back pain, headache, and drowsiness. The World Health Organization recommended the oral use of molnupiravir for individuals with non-severe COVID-19 complications but with active monitoring [4]. Molnupiravir demonstrated no adverse effects when administered in kidney transplant recipients affected by coronavirus [5]. The active metabolite exhibited potential embryotoxic effects in animal studies [6]. Molnupiravir exhibited drug-drug interaction with warfarin in a COVID-19 patient [7].

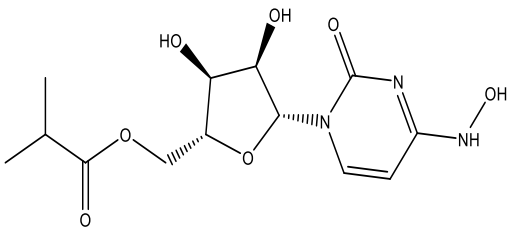


Figure.1 Chemical structure of molnupiravir

Plasma protein binding is crucial for understanding drug distribution patterns, half-life, and predicting possible drug-drug interactions. Albumin is the main plasma protein that binds with hydrophobic and charged drug molecules [8]. The 3D-structural details of albumin are being updated constantly due to its integral role in drug pharmacokinetic studies [9-12]. Human serum albumin (HSA) (585 amino acids) is organized into structural domains (I, II & III). The two major binding sites of HSA are located in domain IIA (site-I/warfarin binding site) and IIIA (site-II/benzodiazepine binding site). Drug-albumin binding strength is influenced by several factors including pH, temperature, the concentration levels of albumin,

glycation of albumin, oxidation of albumin, and hydrophobicity of the drug. Hydrophobicity is a key determinant of a drug's affinity for albumin, with more hydrophobic drugs exhibiting strong interactions with albumin [13]. Recent experimental findings suggested that glycated HSA (GHSA) and oxidized HSA (OxHSA) exhibit altered binding affinities for drugs like NSAIDs and anticancer agents emphasizing personalized drug dosing in diabetic and cancer patients [14, 15]. Bovine serum albumin (BSA) has close structural similarity with HSA (>75%). It is the most preferred one for the preliminary screening of drug-albumin-binding interactions [16].

The interaction between serum proteins and various antiviral agents has been widely studied due to its substantial impact on bioavailability and drug half-life. Lamivudine binds well with HSA at site I through the static mechanism. The interaction was spontaneous and involved an exothermic process [17]. Stavudine also binds well with HSA with the static quenching mechanism. Stavudine interaction induced conformational changes in HSA structure [18]. Lopinavir, favipiravir, and saquinavir preferentially bind with alpha-acid glycoproteins and lipoproteins, the other plasma proteins present in the body [19]. A thorough review of the literature revealed that no drug albumin-binding interactions were carried out for molnupiravir. In this study, an attempt was made to explore drug-BSA interactions using different spectral techniques.

Experimental details

Preparation of BSA and drug solutions

High-purity BSA was acquired from Sigma-Aldrich (USA). The chemical was stored as per the manufacturer's guidelines. Molnupiravir (500 mg) was gifted by Shree Icon Laboratories, Hyderabad. BSA was accurately weighed and dissolved in the phosphate buffer (pH 7.4) to get a 1mg/mL solution. From this stock solution, serial dilutions were carried out to get 20µM/mL. 16.5mg of molnupiravir was dissolved in 50 mL phosphate buffer to get 1000 µM/mL solution. Further, serial dilutions were made to get various concentrations (4, 8, 12, 16, 20, 24, 28, and 32 µM) of molnupiravir solution.

Spectroscopy studies

A double-beam UV-visible spectrophotometer (Shimadzu, UV-1800) was used to record the UV spectrum. Fluorescence measurements were performed by spectrofluorophotometry Shimadzu RF-5301PC having a xenon lamp (150W). Emission spectra were recorded at the wavelength range.

(250-900 nm) and at 295 nm, the excitation wavelength was set. The FT-IR spectra were obtained using a Bruker spectrometer (the ATR method).

To record the UV-visible and emission spectra, equal volumes of BSA in phosphate buffer (20 µM) and molnupiravir solutions (4 to 32 µM) in phosphate buffer were mixed, incubated for two hours at lab temperature (triplicates). For site-specific binding experiments, equal volumes of free BSA (20 µM), Ibuprofen (20 µM), Indomethacin (20 µM), and various concentrations of molnupiravir (4 to 32 µM) were added and incubated for 2 hrs. FTIR spectrum was recorded for the free BSA (20 µM) and drug-BSA complex (4 µM) solutions while DSC thermograms were recorded for free BSA (20 µM) and drug-BSA complex (32µM) solutions.

2. RESULTS AND DISCUSSION:

UV-Visible spectroscopy

It is a valuable tool to probe protein-ligand (non-covalent) interactions. Albumin (tryptophan, tyrosine, and phenylalanine residues) absorbs UV light (200-400 nm). When Albumin interacts with drug molecules, it induces changes in the absorption. These spectral changes reveal the formation of albumin-drug binding [20]. In our results, λmax of the drug-HSA complex was observed at 235nm. When the molnupiravir concentration was increased (4µM-32 µM), the absorbance of the drug-BSA complex gradually increased (Figure 2A & 2B). The K value was calculated using equation (1) [21, 22]. For the molnupiravir-BSA complex, it was found to be 1.84 x 10^{-4} M^{-1} indicating moderate affinity between the molecules (strong binding-10^5-10^8 M^{-1}; moderate binding-10^3-10^5 M^{-1}; weak binding - < 10^3 M^{-1}).

Equation-1= $A0/A0 - A = \varepsilon BSA/\varepsilon B + \varepsilon BSA/\varepsilon B.K \ .1/Cdrug$

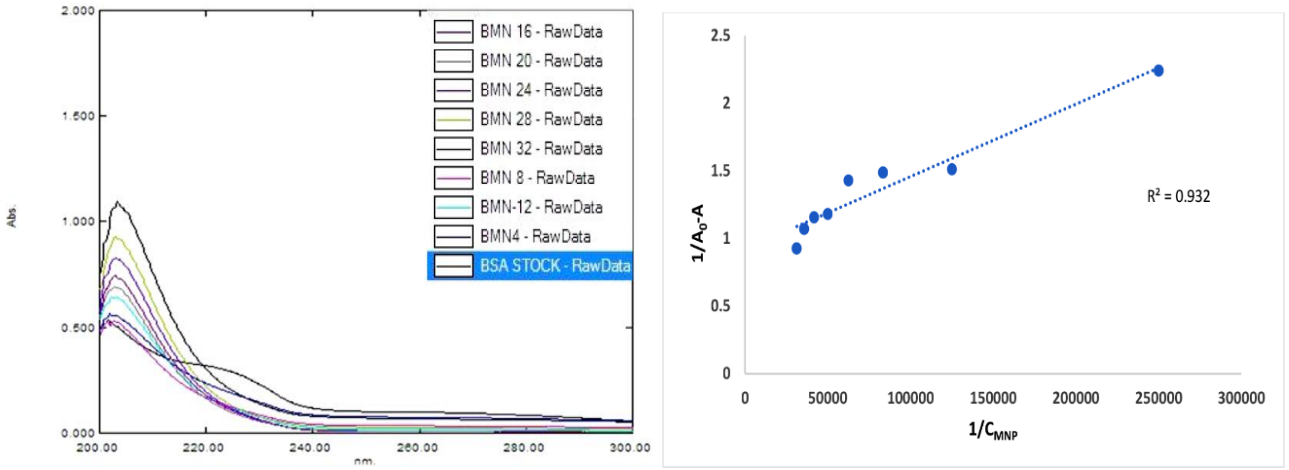


Figure 2:(A) UV-Vis spectral overlay of various concentrations of MNP-BSA complexes (B) The double reciprocal plot of $1/A_0$-A Vs $1/C_{MNP}$

Fluorescence spectroscopy

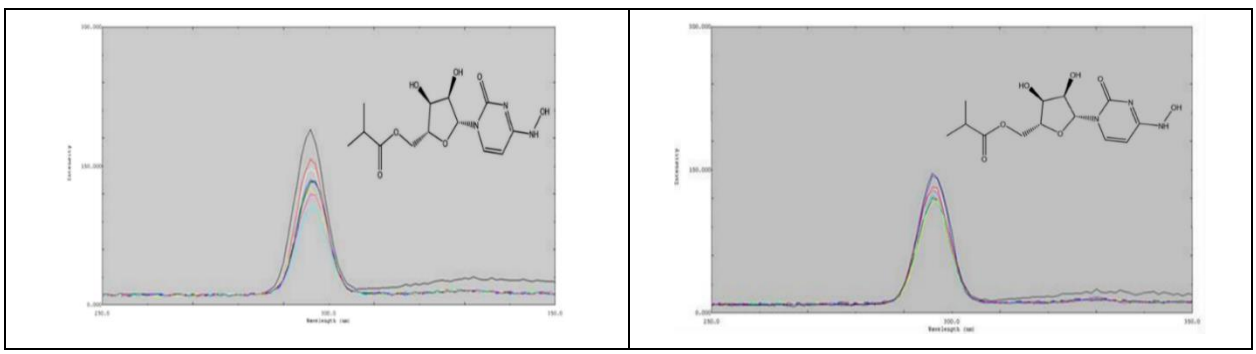
Fluorescence spectroscopy is a highly sensitive technique to investigate molecular-level dynamic changes during protein-ligand interactions, including binding affinity and conformational changes. In albumin structure, the aromatic residues (especially tryptophan 214) act as intrinsic fluorophores, making fluorescence a valuable tool for exploring drug binding. When albumin binds with any small molecule, the fluorescence intensity or emission wavelength undergoes alterations, indicating drug-induced changes in the microenvironment of the protein (quenching). The quenching efficiency (Stern-Volmer quenching constant-K_{SV}) can be obtained from the Stern-Volmer plot, which is a plot of the ratio of the initial to observed fluorescence intensity (F_0/F) as a function of quencher concentration [Q] (Equation-2). The Ksv values indicate the strength of the interaction at different temperatures (higher Ksv indicates stronger quenching) The changes in Ksv with temperature provide insights into the nature of the quenching (static or dynamic). Thermodynamic parameters (ΔH and ΔS) can be calculated via the Van't Hoff Equation (Equation 3&4). Fluorescence-derived binding constants are considered more accurate than those obtained from UV-visible spectroscopy [23].

Equation-2 = $\frac{F0}{F} = 1 + Ksv(Q) = 1 + Kqr0(Q)$

Equation-3 = $\Delta G - \Delta H - T\Delta S = -RTInKa$

Equation-4 = $\frac{\log[(F0-F)]}{F} = logKa + nlog[Q]$

The fluorescence intensity of the molnupiravir-BSA complex exhibited a progressive decrease with increasing drug concentration (4µM-32 µM), suggesting quenched fluorescence due to altered microenvironment around Trp214 (Figure 3A). The K_{SV} values were increased (4.96 x10^4L.mol^{-1}; 5.17 x10^4L.mol^{-1};9.37 x10^4L.mol^{-1}) with rising temperature (298K, 303K, and 310K) (Figure 3A- 3C) indicating a dynamic quenching mechanism (Table 1). These values indicate moderate interaction with BSA (strong interaction ->10^5 L.mol^{-1}; moderate interaction-10^3-10^5 L.mol^{-1}; weak interaction - < 10^3 L.mol^{-1}) The binding constant (Ka) values were also found to increase with temperature (3.15x10^4L.mol^{-1}; 4.12x10^4L.mol-1 and 5.57x10^4L.mol^{-1}) suggesting greater stability of complex at higher temperatures and entropy-driven binding. The binding site number was found to be approximately 1, indicating a single binding site on BSA for molnupiravir (Figure 3D)



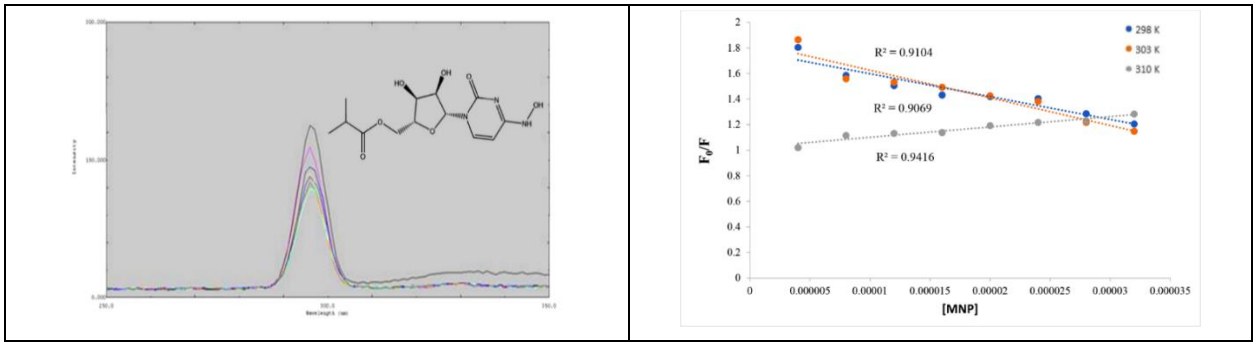


Figure 3. Fluorescence emission spectra of BSA with different concentrations of MNP at (A)298K,(B) 303K, and (C)310K (D) Stern -Volmer plots

Thermodynamic parameters provide insights into molecular-level interactions. A negative Gibbs free energy indicates spontaneous interactions. Negative Gibbs free energy values (ΔG = -9.53kJ.mol^{-1} at 298K, -11.77kJ.mol^{-1} at 303K, -14.28kJ.mol^{-1} at 310K) in our results confirm spontaneous interaction (thermodynamically favorable) and it is also clear that binding strength increases with temperature rise. The negative enthalpy change (ΔH = - 4.37 kJ.mol^{-1}) suggests that binding releases heat. The positive entropy change (ΔS =15.82 kJ.mol^{-1}) suggests the involvement of hydrophobic interactions during binding (Table 2). Overall, the binding interaction between BSA and molnupiravir is exergonic, favored by higher temperatures, and involves hydrophobic forces.

Site-specific competitive binding experiments

BSA facilitates the transport of drug molecules by binding at site I and site II, analogous to HSA. To experimentally prove the primary binding site of molnupiravir, site-specific binding experiments were conducted using indomethacin and ibuprofen, site-I and site-II markers, respectively, in the earlier studies [24, 25]. The binding constant (Ka) of molnupiravir was significantly decreased in the presence of Indomethacin (Ka=1.65 x10^{4}L.mol^{-1}) compared to Ibuprofen (Ka=2.13 x10^{4}L.mol^{-1} (Figure 4A &4B**).** This notable reduction in binding affinity in the presence of **indomethacin,** a **site I ligand,** suggests that molnupiravir primarily binds to **Sudlow's Site I on BSA** (Table 3).

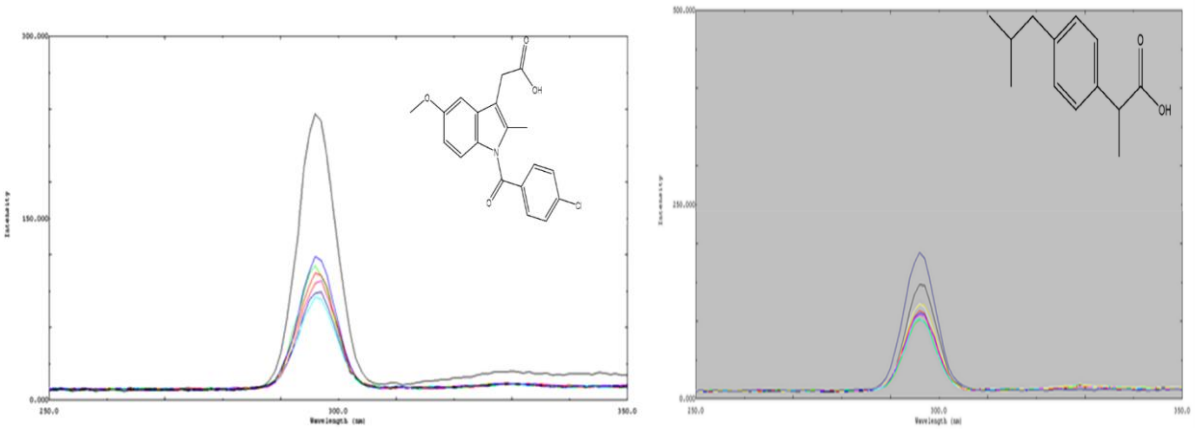


Figure 4: (A) Fluorescence emission Spectra of BSA-IND-MNP complexes (B) BSA-IBF- MNP complexes

Table 1. Stern-Volmer constants and binding constants (Ka) of the system of MNP–BSA complex

T(K)	K_{SV} (×10^{4}L.mol^{-1})	K_q (×10^{12} L.mol.s^{-1})	R	S.D
298	4.96	4.96	0.9069	0.183
303	5.17	5.17	0.9104	0.221
310	9.37	9.37	0.9416	0.338

Table 2: Binding constants (Ka) and relative thermodynamic parameters of the system of MNP–BSA complex

T(K)	K_a ($\times 10^4$L.mol^{-1})	n	R	ΔH (kJ.mol^{-1})	ΔG (kJ.mol^{-1})	ΔS (kJ.mol^{-1})
298	3.15	0.5724	0.9115	-4.37	-9.53	15.82
303	4.12	0.7609	0.9376		-11.77	
310	5.57	1.3597	0.9535		-14.28	

Table 3: Binding constants for the site-specific competitive experiments

Site Maker	K_a($\times 10^4$L.mol^{-1})	R
BSA-MNP	5.572	0.9535
BSA -IND - MNP	1.6515	0.9348
BSA -IBF- MNP	2.1396	0.9171

MNP-Molnupiravir, IND-Indomethacin, IBF-Ibuprofen, R-Correlation coefficient, S.D-Standard deviation

FT-IR

FT-IR spectroscopy can provide structural details of proteins such as the backbone amide bonds (amide-I, II, and III) and secondary structure composition [26]. The comparison of FTIR spectra (peak shifts) obtained from free BSA and BSA-molnupiravir complex demonstrate drug-induced alterations in protein structure. The amide-A band (due to N-H stretching) shifted from 3268.9cm^{-1} in free BSA to 3263.3 cm^{-1} in the complex, a downshift indicating the involvement of the N-H group in the hydrogen bonding interactions. A slight shift was observed in the amide-I band (C=O stretching), from 1638.2 cm^{-1} in BSA to 1636.3 cm^{-1} in the complex, suggesting minor and insignificant conformational changes (localized binding). The absence of amide-III bands (C-N stretching) in the complex suggests alteration or modification in the protein microenvironment (Figure 5A&B).

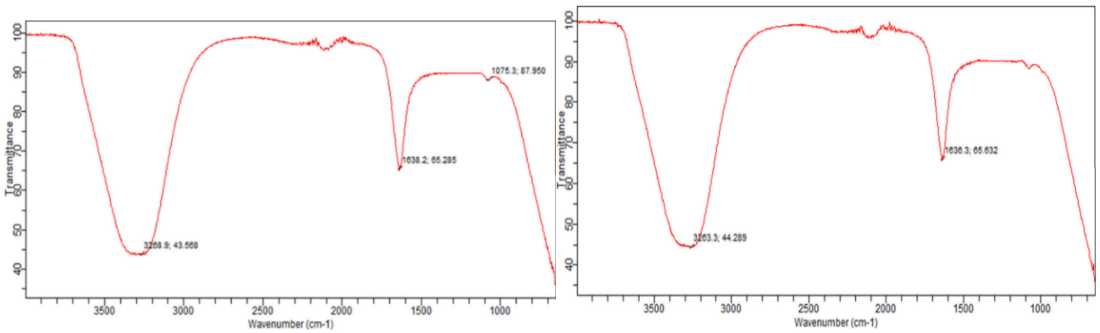


Figure 5: (A) FT-IR spectra of free BSA (20 µM) (B) BSA - MNP complex at drug concentration (4µM)

3. DSC

DSC analysis provides valuable information about the thermal stability of proteins. In DSC analysis, T_m denotes the temperature at which the protein undergoes its major conformational transition, marked by the peak of the endothermic response. A sharp peak denotes single transition and cooperative unfolding of protein while a broader peak represents multi-step transitions or gradual unfolding of protein upon drug binding. The comparison of Tm values obtained for free-BSA and drug-bound BSA provides insights into the effect of drug interaction on protein stability. A shift in T_m toward higher temperatures signifies increased protein stability due to ligand-induced structural reinforcement [27].

In the DSC analysis, two endothermic peaks were observed for free BSA (20µM) at 66.8 ºC and 107.7ºC (Figure 6A). Upon complexation with molnupiravir (32µM), thermal transitions were shifted to 75.2 ºC and 110.2 ºC, respectively (Figure 6B). The first transition or peak associated with the initial unfolding process, showed a significant shift in T_m value (+8.4 ºC), suggesting molnupiravir stabilizes BSA structure. The second peak corresponding to the complete unfolding, was slightly

shifted to a higher temperature (+2.5 ºC), indicating a modest improvement in the overall stability of BSA. These results confirmed localized binding (binding at a specific site or domain) effects rather than a global binding process. Furthermore, the DSC results align with fluorescence spectroscopy data, which identified site-I as the primary binding region for molnupiravir.

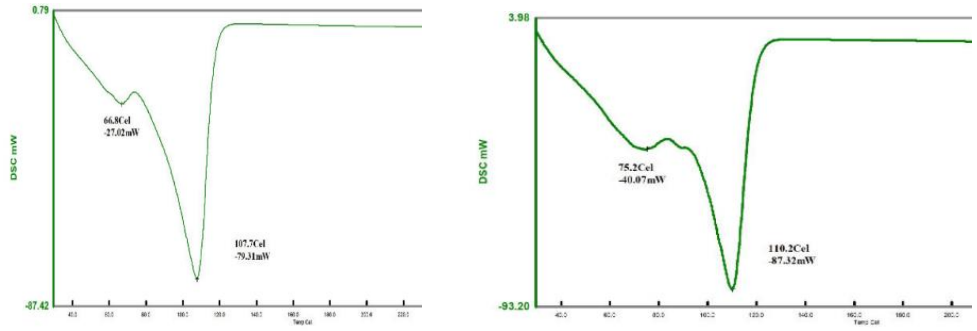


Figure 6 (A). DSC thermogram for free BSA (20 µM) (B) MNP - BSA complex at drug concentration (32µM)

4. CONCLUSION

Molnupiravir formed a stable complex with the BSA structure. Quenching was evident in the fluorescence experiments. The K_{SV} values were increased (4.96 x10^4L.mol^{-1}; 5.17 x10^4L.mol^{-1};9.37 x10^4L.mol^{-1}) when the temperature was constantly increased (298K,303K, and 310K) indicating dynamic quenching of BSA. It was also evident that the binding was spontaneous (ΔG values = -9.53kJ.mol^{-1}, -11.77kJ.mol^{-1}, -14.28kJ.mol^{-1}). The binding process was mainly driven by the hydrogen bonding and hydrophobic interactions. The site-specific competitive binding studies suggested site -I as the primary site if molnupiravir. FT-IR, and DSC results corroborate the formation of a stable BSA-complex.

Conflicts of interest: None

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