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RESEARCH ARTICLE

Binding Behavior of Anticancer Agent Trametinib Across Different Mammalian Serum Albumins: A Comparative Perspective

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SUMMARY

Understanding the binding interactions between drugs and serum albumins is crucial for evaluating pharmacokinetics, bioavailability, and species-specific therapeutic responses. In this study, the binding behavior of the anticancer agent trametinib (TMB) using serum albumins derived from four distinct mammals, namely human (HSA), bovine (BSA), goat (GSA), and sheep (SSA)—was investigated using steady-state fluorescence spectroscopy. TMB caused a concentration-dependent quenching of intrinsic albumin fluorescence, indicating complex formation. Analysis of Stern–Volmer and double logarithmic plots revealed that the quenching mechanism was predominantly static in nature, as supported by high bimolecular quenching rate constants (k_q ~10^{12} M^{-1} s^{-1}), which exceeded the diffusion-controlled limit. Binding parameters, including the association constant (K_a) and binding stoichiometry (n), were calculated, with all albumins exhibiting a 1:1 binding ratio. Among the studied proteins, BSA displayed the strongest affinity for TMB, followed by GSA, HSA, and SSA. Thermodynamic evaluation via Gibbs free energy change (ΔG^0) confirmed the spontaneity of the interaction, with ΔG^0 values ranging from −25.2 ± 0.10 to −28.9 ± 0.10 kJ mol^{-1}. These results not only provide molecular-level insight into TMB–albumin interactions but also support the rational selection of animal models in preclinical pharmacological studies based on their albumin binding profiles.

Keywords: *Trametinib, Serum albumin, Fluorescence spectroscopy, Protein–ligand interaction, Pharmacokinetics.*

Antikanser Ajan Trametinib'in Farklı Memeli Serum Albüminlerine Bağlanma Davranışı: Karşılaştırmalı Bir Bakış

ÖZ

İlaçların serum albüminleriyle olan bağlanma etkileşimlerinin anlaşılması, farmakokinetik, biyoyararlanım ve türlere özgü terapötik yanıtların değerlendirilmesi açısından büyük önem taşımaktadır. Bu çalışmada, antikanser ajan trametinib'in (TMB) insan (HSA), sığır (BSA), keçi (GSA) ve koyun (SSA) olmak üzere dört farklı memeli türüne ait serum albüminleriyle olan bağlanma davranışı, kararlı durum floresans spektroskopisi kullanılarak araştırılmıştır. TMB, albüminlerin içsel floresansında konsantrasyona bağlı olarak bir sönümlemeye neden olmuş ve bu durum kompleks oluşumunu işaret etmiştir. Stern–Volmer ve çift logaritmik grafiklerin analizi, sönümleme mekanizmasının ağırlıklı olarak statik karakterde olduğunu göstermiştir; bu durum, difüzyon kontrollü sınırın üzerinde olan yüksek biyomoleküler sönümleme hız sabitleriyle (k_q ~10^{12} M^{-1} s^{-1}) desteklenmiştir. Bağlanma parametreleri, yani bağlanma sabiti (K_a) ve stokiyometri (n) hesaplanmış; tüm albüminlerin 1:1 bağlanma oranı sergilediği bulunmuştur. İncelenen proteinler arasında BSA, TMB'ye en yüksek afiniteyi göstermiştir; bunu sırasıyla GSA, HSA ve SSA takip etmiştir. Gibbs serbest enerji değişimi (ΔG^0) üzerinden yapılan termodinamik değerlendirme, bağlanma etkileşimlerinin kendiliğinden gerçekleştiğini doğrulamış ve ΔG^0 değerlerinin −25,2 ± 0,10 ile −28,9 ± 0,10 kJ mol^{-1} arasında değiştiği gösterilmiştir. Bu sonuçlar, TMB–albümin etkileşimlerine moleküler düzeyde ışık tutmakla kalmayıp, preklinik farmakolojik çalışmalarda albümin bağlanma profillerine dayalı olarak uygun hayvan modelinin rasyonel seçimini de desteklemektedir.

Anahtar Kelimeler: *Trametinib, Serum albümin, Floresans spektroskopisi, Protein–ligand etkileşimi, Farmakokinetik.*

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INTRODUCTION

Trametinib (TMB; Figure 1) is a targeted anticancer agent belonging to the class of mitogen-activated protein kinase kinase (MEK) inhibitors. TMB has gained significant importance in oncology, particularly for the treatment of cancers with B-Raf proto-oncogene (BRAF), serine/threonine kinase V600E (Valine → Glutamic acid) or V600K (Valine → Lysine) mutations, most notably metastatic melanoma. Approved by the FDA in 2013, TMB is often used as monotherapy or in combination with BRAF inhibitors such as dabrafenib to enhance therapeutic efficacy and overcome resistance mechanisms (Corcoran et al., 2015; Infante et al., 2013; Odogwu et al., 2018).

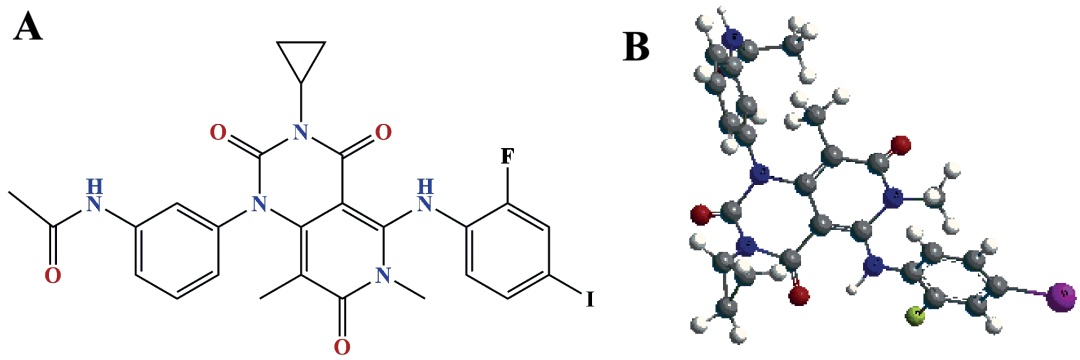

Figure 1. 2D (A) and 3D (B) chemical structures of TMB.

Pharmacologically, TMB selectively inhibits MEK1 and MEK2, critical kinases in the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway. This pathway regulates cellular proliferation, differentiation, and survival. By targeting MEK, TMB effectively reduces the downstream activation of ERK, thereby inhibiting tumor cell proliferation and promoting apoptosis (Blumenschein et al., 2015; Caunt et al., 2015). TMB is administered orally, with a bioavailability of approximately 72% and a relatively long half-life of 3–4 days, allowing once-daily dosing. It is metabolized primarily via deacetylation and is not extensively processed by cytochrome P450 enzymes, which reduces the likelihood of certain drug–drug interactions (Lugowska et al., 2015; Marani et al., 2023; Tan et al., 2023).

Clinically, TMB has demonstrated improved progression-free and overall survival in patients with advanced melanoma. However, its pharmacokinetic profile, including distribution and bioavailability, may be influenced by binding interactions with serum albumins, which play a crucial role in drug transport and bioavailability (Voon et al., 2022). Understanding TMB's binding affinity to serum albumins can provide valuable insights into its therapeutic efficacy, toxicity, and potential drug–drug interactions, highlighting the importance of albumin-binding studies in the context of personalized medicine and targeted therapy. Therefore, examining drug–albumin binding characteristics is essential in preclinical studies. However, interspecies variability in albumin structure and binding behavior may lead to divergent pharmacological outcomes between animals and humans. Consequently, the selection of appropriate animal models that closely mimic human serum albumin binding patterns is crucial for accurately predicting drug behavior and optimizing translational relevance in preclinical research (Feroz et al., 2014).

Elucidating drug–protein interactions requires highly sensitive and specific analytical techniques, and fluorescence spectroscopy has emerged as one of the most powerful tools in this context. Its exceptional sensitivity, rapid response, and non-destructive nature make it particularly well-suited for monitoring subtle molecular changes associated with ligand binding. In the case of serum albumins, this technique

allows precise detection of alterations in intrinsic fluorescence, primarily originating from tryptophan residues, which are highly responsive to microenvironmental shifts upon drug interaction. Beyond its sensitivity, fluorescence spectroscopy also provides valuable quantitative data, such as binding constants and quenching parameters, enabling detailed characterization of drug–protein interactions at the molecular level (Erkmen & Kabir, 2024; Lakowicz, 2006).

In this study, the binding interactions between the anticancer agent TMB and serum albumins from four different mammalian species—human, bovine, goat, and sheep—were examined for the first time in a comparative framework. By examining the binding affinities and interaction profiles across these distinct albumins, this work provides valuable insights into species-specific pharmacological behaviors. Therefore, the results generated through this investigation may contribute to the rational selection or design of animal models that closely mimic the biological conditions regarding drug–protein interactions, thereby supporting more predictive preclinical evaluations in drug development.

MATERIALS AND METHODS

Chemicals

Fatty acid-free albumins sourced from human (HSA), bovine (BSA), goat (GSA), and sheep (SSA) origins, together with the anticancer agent trametinib, were procured from Sigma-Aldrich and utilized directly without undergoing any additional purification steps. All other chemicals and solvents applied throughout the study were of analytical grade and employed as received.

Preparation of solutions

The proteins were initially dissolved in a 60 mM phosphate buffer solution (PBS, pH 7.4) to prepare stock solutions. The concentrations of HSA, BSA, and SSA were determined spectrophotometrically at 280 nm using their respective molar extinction coefficients: 35,700 M^{-1} cm^{-1} (HSA), 43,827 M^{-1} cm^{-1} (BSA), and 42,925 M^{-1} cm^{-1} (SSA) (Khan, Chaturvedi & Khan, 2013). For GSA, which lacked established extinction coefficients, protein concentrations were estimated using the colorimetric method described by Lowry et al. (Lowry et al., 1951).

TMB stock solution was prepared by dissolving an appropriate amount of the compound in 1 mL of dimethyl sulfoxide (DMSO), followed by dilution with 60 mM PBS (pH 7.4) to a final volume of 10 mL. The prepared solution was stored in the dark at 4°C until use to maintain stability. All absorbance measurements were carried out using a Shimadzu model UV-2600 double-beam spectrophotometer with 1 cm path length quartz cuvettes.

Analytical procedures for the TMB–albumin interaction

The interaction of TMB with serum albumins derived from multiple mammalian species was examined through fluorescence quenching-based titration, following a previously established protocol (Duman et al., 2023; Tayyab et al., 2019). Varying concentrations of TMB (1.5–15 μM) were incrementally added to a constant concentration (3 μM) of albumin in a final volume of 3 mL, prepared using 60 mM PBS (pH 7.4). The mixtures were incubated at 25 °C for 1 hour to allow equilibrium binding. Fluorescence spectra were recorded using a JASCO FP-8300 spectrofluorometer equipped with a 1 cm path length quartz cuvette. The excitation wavelength was set at 280 nm, and emission was scanned in the range of 300–420 nm.

Fluorescence data processing and binding analysis

To account for inner filter interference, fluorescence measurements were corrected in accordance with the equation proposed by Lakowicz (Lakowicz, 2006):

$$F_{cor} = F_{obs} 10^{(A_ex + A_em)/2} \qquad (1)$$

In this equation, F_{cor} corresponds to the fluorescence value corrected for inner filter effects, whereas F_{obs} is the observed intensity. The absorbance at excitation (A_{ex}) and emission (A_{em}) wavelengths is incorporated to compensate for potential ligand-induced

spectral distortions.

Subsequent analysis of the adjusted fluorescence values was carried out through the Stern–Volmer approach, enabling calculation of the K_{SV} constant and clarification of the quenching type involved:

$$F_0/F = 1 + K_{SV}[Q] = 1 + k_q\tau_0[Q] \quad (2)$$

Here, the fluorescence signals recorded in the absence (F_0) and presence (F) of TMB, the quencher, are denoted as such; [Q] indicates the concentration of this quencher; k_q is the bimolecular quenching rate constant, and τ_0 is the average lifetime of the fluorophore in the absence of the TMB, assumed to be 10^{-8} s for serum albumins (Lakowicz, 2006).

To determine the binding parameters, the modified Stern–Volmer equation was applied to calculate the association constant (K_a) and the number of binding sites (n):

$$log(F_0\text{-}F)/(F = n\ logK_a\text{-}n\ log\ [(1)/([L_T]\text{-}(F_0\text{-}F)\ [P_T]/F_0)] \quad (3)$$

Where $[L_T]$ and $[P_T]$ denote the total concentrations of the ligand and protein, respectively.

Finally, the standard Gibbs free energy change (ΔG^0) for the interaction was calculated using the following thermodynamic relationship:

$$\Delta G^0 = -RTlnK_a \quad (4)$$

Where R is the universal gas constant (8.3145 J mol^{-1} K^{-1}) and T is the absolute temperature in Kelvin.

RESULTS AND DISCUSSION

Fluorescence quenching of serum albumins triggered by TMB

Among various analytical techniques, fluorescence spectroscopy is frequently employed to investigate the binding interactions between macromolecules and their ligands, offering valuable insight into the underlying mechanisms. In proteins, particularly serum albumins, intrinsic fluorescence is notably sensitive to structural and environmental changes triggered by the binding of low-molecular-weight compounds. When a small molecule, such as drugs, pesticides, and ions, interacts with these proteins, it often results in a measurable decrease in fluorescence intensity, indicative of quenching phenomena and conformational alterations (Lakowicz, 2006).

Firstly, to evaluate and correct for possible inner filter effects, the UV–Vis absorption spectra of TMB in the presence of different serum albumins were recorded (Figure 2). Figures 2A–D show the spectra for HSA, BSA, GSA, and SSA, respectively, with protein concentration fixed at 3 μM and TMB concentration gradually increased from 0 to 15 μM. In all cases, the absorbance intensity increased progressively with rising TMB concentration, particularly in the range of 260–300 nm, which corresponds to the characteristic absorption bands of TMB. This increase reflects the additive contribution of TMB absorption to the intrinsic absorption of the albumins. Importantly, the absorbance values at both excitation (280 nm) and emission (~340 nm) wavelengths were significantly influenced by TMB concentration, confirming the necessity of applying inner filter effect corrections in the fluorescence analyses. Accordingly, all fluorescence intensities were corrected following Lakowicz's method using the measured absorbance values at excitation and emission wavelengths, ensuring accurate determination of quenching and binding parameters (Lakowicz, 2006).

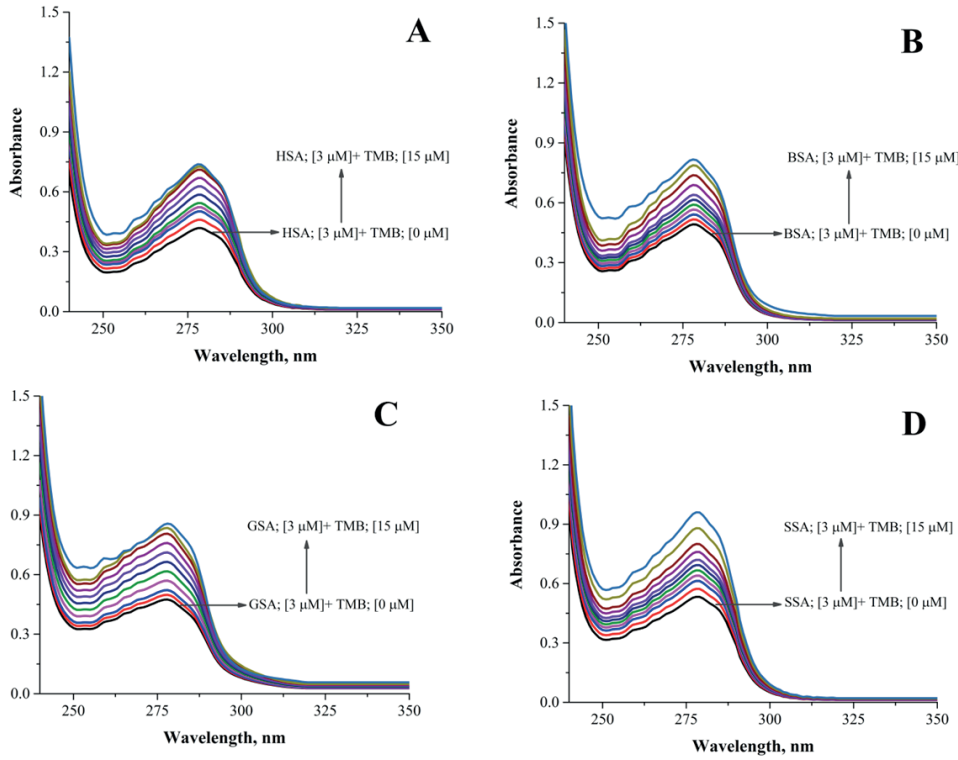

Figure 2. UV–Vis absorption spectra for various serum albumins obtained at increasing TMB levels. Figures correspond to: (A) HSA, (B) BSA, (C) GSA, and (D) SSA.

Figure 3 presents the fluorescence emission profiles of four different mammalian serum albumins (each at a concentration of 3 μM): HSA (Figure 3A), BSA (Figure 3B), GSA (Figure 3C), and SSA (Figure 3D). These spectra were obtained both in the absence and the presence of progressively increasing concentrations of TMB, ranging from 1.5 to 15 μM. Table 1 summarizes the native fluorescence characteristics of different albumins, including their emission intensities and peak wavelengths. As illustrated, significant differences were observed in the fluorescence intensities of the studied proteins, following the trend: SSA>GSA>BSA>HSA. In contrast, the maximum fluorescence emissions for these proteins were confined to a relatively narrow range between 339 and 342 nm. These findings are consistent with previously reported data regarding the fluorescence behavior of different serum albumins (Boon Kim, Abdul Kadir & Tayyab, 2008; Feroz et al., 2014; Ishtikhar et al., 2018). The intrinsic fluorescence of proteins is predominantly attributed to their aromatic residues, especially tryptophan and tyrosine, as reported in prior studies (Lakowicz, 2006). Therefore, the observed variations in emission spectra among these albumins are likely influenced by both the quantity and spatial arrangement of tryptophan and/or tyrosine residues within the protein's tertiary structure. Notably, in class B albumins, tryptophan residues are the predominant contributors to the overall fluorescence signal (Lakowicz, 2006). Table 1 provides information regarding the number and sequence positions of tryptophan residues in the primary structures of the examined albumins. Notably, the position of Trp residues appears to be highly conserved across albumins from different species. As illustrated, albumins containing two tryptophan residues—such as BSA, SSA, and GSA—exhibited both stronger fluorescence signals and greater maximum emission wavelengths than those possessing a single tryptophan residue—such as HSA.

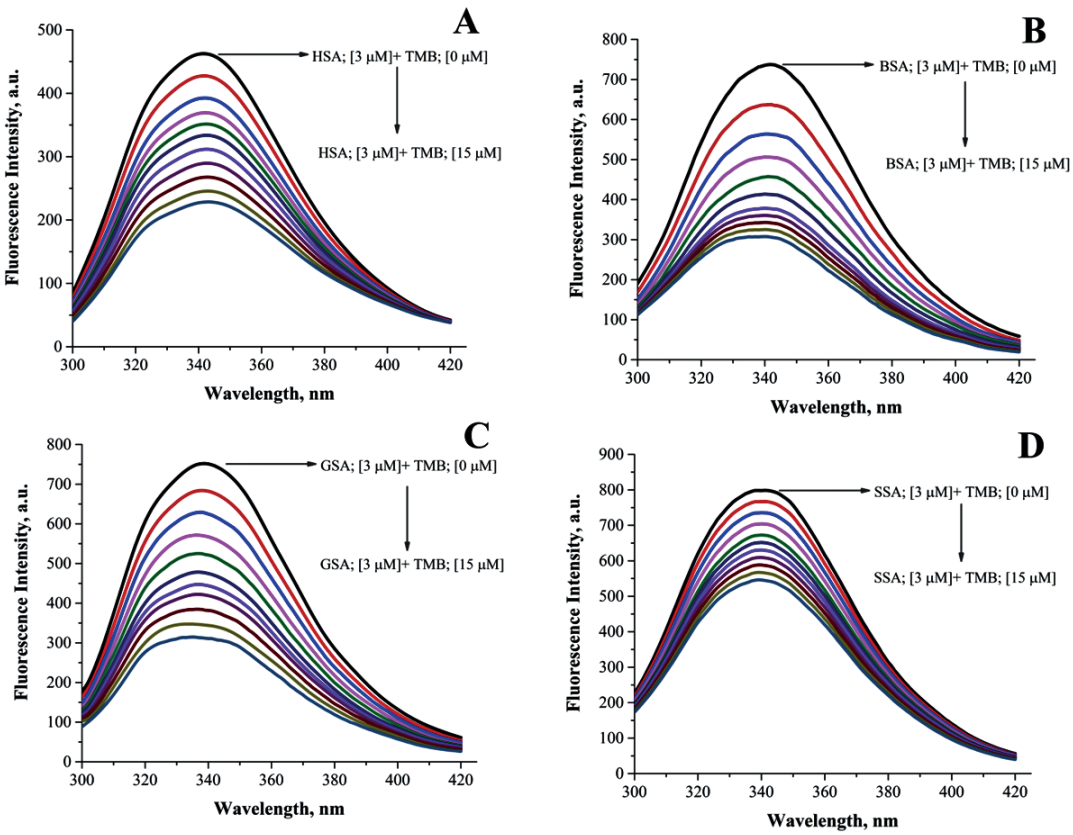

Figure 3. Fluorescence emission profiles for various serum albumins obtained at increasing TMB levels. Figures correspond to: (A) HSA, (B) BSA, (C) GSA, and (D) SSA.

Table 1. Analysis of fluorescence responses of mammalian albumins in both the presence and absence of TMB.

Albumin	*Intensity (a.u.)*	*Emission maximum (nm)*	*Trp residu*[a*]	*% Quenching*
BSA	737.03	341	Trp-134, Trp-213	59
GSA	751.96	339	Trp-134, Trp-213	55
HSA	462.53	342	Trp-214	48
SSA	798.57	341	Trp-134, Trp-213	32

a:* Obtained from the Univesal Protein Resource (Uniprot) database at www.uniprot.org

Titration of albumin solutions with increasing concentrations of TMB resulted in a marked reduction in the intrinsic fluorescence of all albumin types examined. As shown in Figure 4A, this quenching effect intensified progressively with higher TMB levels. Nevertheless, the extent of fluorescence suppression varied among the different albumins. At a TMB-to-protein molar ratio of 5:1, BSA, GSA, and HSA exhibited the most significant decreases in fluorescence—59%, 55%, and 48% respectively—whereas the remaining albumin, SSA, showed approximately 32% quenching. The observed decline in fluorescence intensity upon TMB addition strongly supports the occurrence of a ligand–protein interaction (Ladokhin, 2000).

Fluorescence-based investigation of the interaction mechanism between TMB and serum albumins

The reduction of protein fluorescence intensity caused by a ligand (quencher) can occur via different mechanisms, primarily classified as collisional (dynamic), static, or a combination of both (mixed)

quenching (Lakowicz, 2006). Collisional quenching involves transient interactions where the quencher collides with the fluorophore while it is excited, leading to non-radiative energy dissipation. In contrast, static quenching arises from the formation of a non-fluorescent ground-state complex between the ligand and the protein, which prevents the fluorophore from being excited (Braslavsky, 2007). The mixed quenching mechanism involves contributions from both static complex formation and collisional encounters, often characterized by more complex fluorescence decay behavior.

To elucidate the binding mechanism between TMB and serum albumins, fluorescence titration results were analyzed using the Stern–Volmer formalism (Eq. 2). The resulting Stern–Volmer curves for the various TMB–albumin systems are presented in Figure 4B, and the corresponding K_{SV} are summarized in Table 2. Since the slope of the Stern–Volmer plot corresponds to the K_{SV}, it reflects the magnitude of fluorescence quenching. The relatively high K_{SV} values determined for HSA, BSA, and GSA are consistent with the significant decrease in fluorescence values observed for these albumins at a TMB-to-albumin molar ratio of 5:1. Moreover, using the K_{SV} values obtained from the Stern–Volmer analysis (Eq. 2), the bimolecular quenching rate constants (k_q) for the various TMB–albumin systems were calculated and determined to be approximately 10^{12} M^{-1} s^{-1}. In general, collisional (dynamic) quenching is characterized by k_q values around 10^{10} M^{-1} s^{-1}, which corresponds to the diffusion-controlled limit in aqueous media. When the calculated k_q values exceed this value, it typically suggests the involvement of a static quenching mechanism. Therefore, given that the calculated k_q values for the TMB–albumin systems were on the order of 10^{12} M^{-1} s^{-1}—well above the diffusion-controlled limit—it can be inferred that the quenching mechanism is predominantly static in nature, suggesting complex formation between TMB and the albumin proteins. The occurrence of static quenching in the TMB–HSA system had previously been validated through temperature-dependent studies, where an inverse relationship between quenching efficiency and temperature was observed (Suo et al., 2018). Our findings are consistent with these observations, as the calculated quenching parameters and the high bimolecular quenching constants further support a static quenching mechanism in the TMB–albumin interactions.

Binding parameters, including the K_a and the number of binding sites (n), for the TMB–albumins interaction were derived from fluorescence quenching results by Eq. 3. The applied analytical approach offers an advantage over traditional fluorescence-based methods, as it does not require prior assumptions about the concentrations of free or bound ligand species (Sahoo, Hennig & Nau, 2006). Figure 4C presents the linearized double-logarithmic curves corresponding to the different TMB–albumin complexes, from which the K_a and n were determined. The corresponding numerical results are summarized in Table 2. Considering the determined K_a values, the albumins examined in this study can be grouped into two categories: GSA, BSA, and HSA exhibited comparatively stronger binding affinities for TMB, while the remaining protein, SSA, demonstrated weaker interaction. Among them, BSA displayed the highest affinity toward TMB, whereas SSA showed the lowest binding strength.

The comparative analysis revealed clear differences in the binding affinity of TMB toward the four albumins, following the order BSA>GSA>HSA>SSA. These variations can be attributed to structural and sequence-specific factors of the proteins. For instance, BSA and GSA possess two tryptophan residues (Trp-134 and Trp-213), strategically located within hydrophobic pockets that often correspond to drug-binding regions. This structural feature may create a more favorable microenvironment for stabilizing TMB through hydrophobic and van der Waals interactions, resulting in its higher binding constants. In contrast, HSA contains only a single tryptophan residue (Trp-214), which may partially limit the extent of fluorescence quenching and decrease the over-

all binding strength. The lowest affinity observed for SSA suggests that subtle conformational or surface accessibility differences, despite also containing two tryptophan residues, may reduce the stability of the TMB–protein complex. Notably, the binding stoichiometry (n) for all TMB–albumin systems was approximately 1.0, indicating that a single TMB molecule interacts with each albumin molecule in a 1:1 binding ratio. Overall, from a pharmacological standpoint, these protein-binding differences are highly relevant. Stronger binding to BSA and GSA suggests that TMB may exhibit altered free drug fractions and distribution profiles in bovine and goat models compared to humans. While BSA has long been used as a surrogate for HSA in binding studies due to its availability and structural similarity, our results indicate that its higher binding affinity may lead to an underestimation of the pharmacologically active free fraction of TMB. Conversely, the relatively weaker binding in SSA indicates that sheep models may not adequately mimic human binding behavior. Therefore, GSA, which demonstrated an intermediate affinity closer to HSA, could represent a more suitable preclinical model for predicting TMB's pharmacokinetic behavior in humans.

The binding process was further evaluated through the evaluation of the ΔG^0 using Equation 4. The obtained ΔG^0 values ranged from -25.2 ± 0.10 to -28.86 ± 0.10 kJ mol^{-1} (Table 2), indicating that the interaction between TMB and serum albumins is thermodynamically favorable and occurs spontaneously under physiological conditions. The magnitude of these negative ΔG^0 values reflects moderate to strong non-covalent binding forces, typically associated with hydrogen bonding, hydrophobic interactions, and van der Waals forces. These interactions are consistent with those commonly observed in small-molecule–protein complexes. Furthermore, the similarity in ΔG^0 values across different albumins suggests that TMB binds comparably, possibly targeting structurally conserved domains such as Sudlow's sites I or II. These findings are also in agreement with previous reports (Suo et al., 2018) on TMB–HSA interaction, where spontaneous binding with similar ΔG^0 ranges has been reported, supporting the role of serum albumins as efficient transporters of anticancer agents.

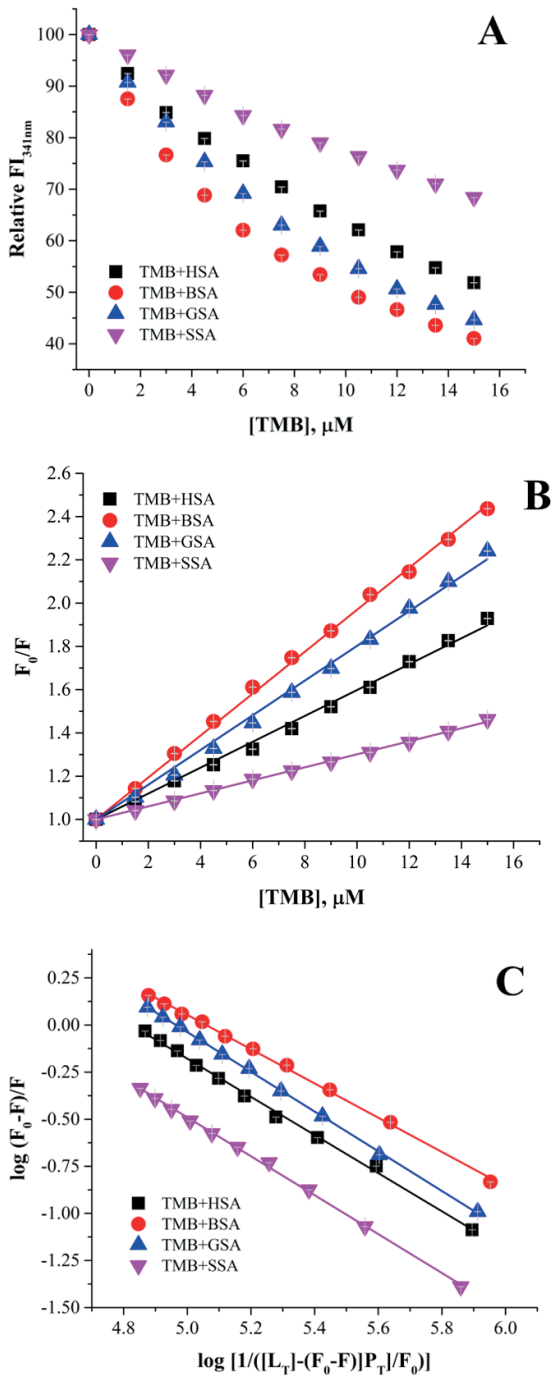

Figure 4. (A) Relative decrease in fluorescence intensity of various serum albumins upon TMB interaction. Analysis of fluorescence quenching titration data: (B) Stern–Volmer plots and (C) double logarithmic plots illustrating the binding interaction between TMB and various serum albumins (n = 3).

Table 2. Binding properties between TMB and various serum albumins, as obtained in PBS 7.4 at 298 K (n = 3).

Systems	$K_{sv} \times 10^4$ *(M⁻¹)*	$K_a \times 10^4$ *(M⁻¹)*	*n*	R^2	ΔG^0 *(kJ mol⁻¹)*
TMB–BSA	9.47 ± 0.09	11.46 ± 0.09	0.92	0.998	– 28.86 ± 0.10
TMB–GSA	8.49 ± 0.08	9.22 ± 0.09	1.05	0.997	– 28.32 ± 0.10
TMB–HSA	6.26 ± 0.06	6.67 ± 0.07	1.01	0.999	– 27.52 ± 0.10
TMB–SSA	3.06 ± 0.05	3.35 ± 0.06	1.03	0.999	– 25.82 ± 0.10

CONCLUSION

This study provides the first comparative analysis of TMB interactions with serum albumins derived from four different mammalian species—HSA, BSA, GSA, and SSA—using steady-state fluorescence spectroscopy. The fluorescence quenching observed upon titration of TMB into albumin solutions indicates the formation of non-fluorescent complexes, suggesting significant binding interactions. Stern–Volmer analysis and calculated bimolecular quenching constants revealed that the quenching mechanism is predominantly static, consistent with complex formation in the ground state. All albumins demonstrated a 1:1 binding stoichiometry with TMB, indicating that one drug molecule associates with one albumin molecule.

Among the species studied, BSA exhibited the highest binding affinity, followed by GSA, HSA, and SSA. Thermodynamic evaluation through ΔG^0 values confirmed that the interactions are spontaneous and thermodynamically favorable, with binding likely driven by non-covalent interactions such as hydrogen bonding and hydrophobic forces. The comparable ΔG^0 values across species also imply a structurally conserved binding environment.

These findings enhance our understanding of how trametinib interacts with albumins from different species and underscore the relevance of protein-binding studies in drug development. Importantly, they support the careful selection of preclinical animal models based on albumin binding similarity to humans, thereby improving the translational accuracy of pharmacokinetic predictions and therapeutic outcomes.

AUTHOR CONTRIBUTION STATEMENT

The author was responsible for developing the hypothesis, conducting literature research, performing experiments, and preparing and reviewing the manuscript (CE).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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