

Comparative Docking Suggests Peripheral Association of Tirapazamine with Bovine Serum Albumin

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Abstract

Molecular docking was used to investigate the interactions between tirapazamine (a bioreductive anticancer drug) and bovine serum albumin (BSA), with emphasis on binding site localization and geometry. Docking simulations were performed using SwissDock with the AutoDock Vina 1.2.0 scoring engine. Warfarin (a canonical Sudlow Site I ligand) was docked under the same conditions as a reference to provide a relative comparison of binding orientation within Subdomain IIA. Docking results indicate that tirapazamine occupies a peripheral entrance-region position within Subdomain IIA, with limited polar interactions and substantial solvent exposure. In contrast, warfarin adopts a more enclosed binding orientation consistent with canonical Site I burial. UV–visible measurements across three independent titration experiments yielded apparent association constants of $2.30 \pm 1.05 \times 10^4 \text{ M}^{-1}$ (mean $\pm$ SD), consistent with a weak interaction regime and supporting the docking results. Geometric analysis further shows that tirapazamine maintains greater minimum ligand–residue distances from core Site I residues than warfarin, consistent with reduced burial. This interpretation is further supported by the physicochemical profile of tirapazamine, which is characterized by low lipophilicity and high polarity, properties that are incompatible with deep binding in hydrophobic pockets (as seen with warfarin). Short-timescale molecular dynamics simulations (2 ns, triplicate) confirmed the stability of the docked poses, with tirapazamine exhibiting markedly lower ligand RMSD variability ($0.247 \pm 0.002 \text{ \AA}$) than warfarin ($0.578 \pm 0.163 \text{ \AA}$), consistent with a stable peripheral binding geometry. Together, these findings support a model in which tirapazamine associates weakly and peripherally with serum albumin, consistent with non-canonical Site I binding.

Keywords: tirapazamine (TPZ); bovine serum albumin (BSA); molecular docking; protein–ligand interactions; warfarin; molecular dynamics (MD)



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1. Introduction

Tirapazamine (TPZ) (3-amino-1,2,4-benzotriazine-1,4-dioxide) is a hypoxia-activated prodrug that undergoes enzymatic one-electron reductive activation under hypoxic conditions (such as those in tumor environments) leading to DNA damage [1,2]. This reduction is catalyzed by intracellular reductases, producing a short-lived benzotriazinyl radical [3]

that can induce DNA damage including strand breaks and base modifications [1,2]. Once reduced to its active metabolite, tirapazamine acts as a potent topoisomerase II poison [1,2]. Due to tumor hypoxia-associated therapeutic resistance, tirapazamine has been intensively studied as a prototype hypoxia-targeted anticancer agent [1,4]. More recently, tirapazamine has been used in conjunction with non-thermal plasma (NTP) as a promising novel combination therapy [5,6]. Despite promising preclinical and early-phase clinical results, tirapazamine has not demonstrated consistent efficacy in late-stage clinical trials. Various methods, including nanoparticle-based and targeted delivery strategies for tirapazamine, have been explored to improve tumor-selective drug delivery [7–9]. This gap between pre-clinical promise and late-stage clinical trial efficacy raises the question of how best to deliver tirapazamine to the appropriate site of action.

Understanding how tirapazamine interacts with serum albumin is thus relevant to predicting its pharmacokinetic behavior and free-drug availability during systemic transport. Serum albumin is the most abundant plasma protein and plays a central role in drug binding and transport in the bloodstream [10,11], with spectroscopic and clinical evidence continuing to refine understanding of how physiological and pathological states modulate this binding capacity [11]. Consequently, it strongly influences the pharmacokinetics of drugs that interact with it. Structurally, serum albumin is organized into three homologous domains, each comprising two subdomains; the principal drug-binding regions, Sudlow Site I (subdomain IIA) and Sudlow Site II (subdomain IIIA), accommodate structurally distinct classes of ligands and are broadly conserved across mammalian albumins. Bovine serum albumin (BSA), sharing approximately 76% sequence identity with human serum albumin, is widely used as a model protein in mechanistic and structural studies of drug–albumin interactions [12]. Comparative crystallographic studies across mammalian serum albumins have shown that while overall binding-site architecture is broadly conserved, species-specific structural variations can measurably influence ligand accommodation and relative binding affinity [13].

Molecular docking offers a structure-based method for predicting ligand–protein binding modes and analyzing interaction patterns [14,15]. In this study, molecular docking was used to investigate tirapazamine–BSA interactions and compare them with those of warfarin–BSA, thus aiding in binding-site identification and mechanistic understanding. Specifically, this study examines whether tirapazamine adopts a deeply buried binding geometry analogous to canonical Sudlow Site I ligands such as warfarin, or instead occupies a more peripheral, weakly stabilized position within the same binding region.

2. Materials and Methods

2.1. Protein Structure Preparation

The crystal structure of bovine serum albumin (PDB ID: 4F5S; 2.47 Å resolution) [12] was used as the docking target. This structure was selected as it provides a complete, unliganded Site I cavity suitable for docking, consistent with its use as the reference BSA structure in prior comparative albumin structural work [12]. UCSF ChimeraX version 1.11.1 (University of California, San Francisco, CA, USA) [16] was employed for initial protein processing prior to docking; chain A was retained, crystallographic water molecules and non-native ligands were removed, and native disulfide bonds were preserved. Docking calculations (using the processed protein) were performed with the SwissDock 2024 web server (Swiss Institute of Bioinformatics, Lausanne, Switzerland) [17].

2.2. Ligand Preparation

Tirapazamine and warfarin (chemical structures shown in Figure S4) were prepared using SwissDock’s automated workflow with explicit non-polar hydrogens. Partial charges

were computed internally by the AutoDock Vina 1.2.0 scoring function. Full ab initio (quantum mechanical) geometry optimization of the ligands was not performed, as it is not standard practice for docking studies of this scale and is not expected to materially affect docking outcomes for these rigid, small-molecule ligands. Tirapazamine was docked in its neutral amino form, which predominates at physiological pH (~7.4), and thus was selected for docking. Alternative protonation states were not considered. Warfarin was represented without explicit stereochemical specification (racemic), as the study focuses on comparative binding geometry rather than stereoselective behavior. Enantiomer-specific docking was not considered and would represent useful refinement for future work.

While charged radical intermediates and alternate protonation states are associated with hypoxic metabolism and non-physiological pH, the neutral molecule is the main species available for albumin association under normoxic conditions.

2.3. Docking Protocol

Docking simulations were carried out using the SwissDock web server (2024 release) incorporating the AutoDock Vina 1.2.0 scoring function [15,17]. A semi-targeted docking strategy was employed, in which the search space was centered on Subdomain IIA but sufficiently large to sample adjacent interfacial regions. The docking region was centered at coordinates $-4.70, 31.01, 102.24$, corresponding to Subdomain IIA. These coordinates were obtained by selecting residues corresponding to Subdomain IIA (approximately residues 190–300, based on structural domain organization of albumin) and determining the geometric center of the selected region in ChimeraX. A cubic search space of $20 \times 20 \times 20 \text{ \AA}$ was used, and sampling exhaustivity (implemented in SwissDock's AutoDock Vina docking mode) was set to 16. This region was selected to capture both canonical Sudlow Site I binding and potential peripheral entrance-region interactions. Minimum heavy-atom separations were evaluated post-docking using the Contacts analysis tool in UCSF ChimeraX. A targeted rather than blind search strategy was adopted because Sudlow Site I is the well-established, crystallographically characterized warfarin-binding site in serum albumin. This choice was further corroborated by an unconstrained whole-protein blind docking control (AutoDock Vina, exhaustiveness = 32), in which the dominant, most consistent pose cluster (6 of the top-ranked poses) independently converged on Subdomain IIA/Sudlow Site I, involving contact residues (Tyr149, Arg256, Arg198, Arg217, His241) consistent with those identified in the targeted analysis.

2.4. UV–Visible Absorption Measurements

UV–visible absorption measurements were performed at room temperature (approximately 298 K) using a Varian Cary 300 UV–visible spectrophotometer (Varian Inc., Palo Alto, CA, USA). Tirapazamine powder (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) and subsequently diluted in PBS pH7.4 (Gibco, Grand Island, NY, USA), with final DMSO concentrations maintained well below 1% (*v/v*) in all samples. Bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) was prepared in aqueous buffer (Gibco PBS, pH 7.4) at a fixed concentration of 30 μM and titrated with tirapazamine at nine concentrations from 1 to 100 μM (1, 2, 5, 10, 20, 30, 50, 75, and 100 μM). UV absorption spectra were recorded to monitor concentration-dependent spectral changes arising from protein–ligand association. Baseline-corrected absorbance changes (including subtraction of ligand-only absorbance) were analyzed as a function of ligand concentration using standard linear binding models (e.g., Benesi–Hildebrand-type models), as commonly applied to spectroscopic studies of weak protein–ligand interactions [18,19]. These measurements were intended to provide order-of-magnitude estimates

of weak albumin association and were not intended to provide thermodynamic parameters (i.e., binding constants).

2.5. In Silico Physicochemical Analysis

Physicochemical and ADME properties of tirapazamine were evaluated in silico using SwissADME (Swiss Institute of Bioinformatics, Lausanne, Switzerland) [20] web server. The SMILES representations of both ligands were submitted to calculate basic properties, including lipophilicity and topological polar surface area (TPSA), which were used to support the interpretation of binding behavior.

2.6. Molecular Dynamics Simulations

Short-timescale molecular dynamics (MD) simulations were performed to assess the positional stability of the top-ranked docking poses for each ligand. All simulations were carried out using GROMACS 2025.4 [21] with the AMBER99SB-ILDN force field [22] and TIP3P explicit water model [23] at 310 K. Ligand topologies were generated with ACPYPE [24] using the GAFF2 force field and AM1-BCC partial charges. The simulation box was solvated and ionized to a physiological NaCl concentration of 0.15 M (109 Na⁺, 93 Cl[−] ions). Each system was energy-minimized, equilibrated under NVT and NPT ensembles, and subjected to 2 ns of NPT production dynamics. Three independent simulations were performed per ligand, each initiated from Pose 1 of a separate, independent docking run, thus constituting a pose-independent convergence test [25,26]. Backbone and ligand RMSD were computed using GROMACS analysis tools. Ligand RMSD was calculated using a ligand-on-ligand fitting scheme, which reflects intrinsic positional stability of the ligand relative to its own starting coordinates rather than global protein drift.

3. Results

3.1. Docking Energetics and Pose Distribution

Docking of tirapazamine yielded multiple low-energy poses, of which the three top-ranked poses exhibited predicted docking scores of -6.555 , -6.539 , and -6.398 kcal·mol^{−1}. This represents a narrow score spread of approximately 0.16 kcal·mol^{−1} (Table 1). Warfarin docking exhibited more favorable predicted docking scores across its three top-ranked poses, with values of -7.515 , -7.478 , and -7.431 kcal·mol^{−1}. The limited score dispersion (observed for both ligands) reflects assignment of similar scores to multiple low-energy poses within the same general binding region rather than convergence to a single, uniquely defined binding pose [14,15].

Table 1. Predicted docking scores for the three top-ranked poses of tirapazamine and warfarin generated using the SwissDock platform.

Ligand	Pose	Predicted Docking Score (kcal·mol ^{−1})
Tirapazamine	Pose 1	-6.555
Tirapazamine	Pose 2	-6.539
Tirapazamine	Pose 3	-6.398
Warfarin	Pose 1	-7.515
Warfarin	Pose 2	-7.478
Warfarin	Pose 3	-7.431

3.2. Binding Site Localization and Representative Pose

All top-ranked poses localized to a shallow, interfacial region of Subdomain IIA. Pose 1, which exhibited the most favorable predicted docking score, was selected as the representative model. In this pose, tirapazamine occupies a partially exposed region near

the entrance of the Sudlow Site I cavity rather than the deeply buried interior typically characteristic of high-affinity ligands (Figure 1).

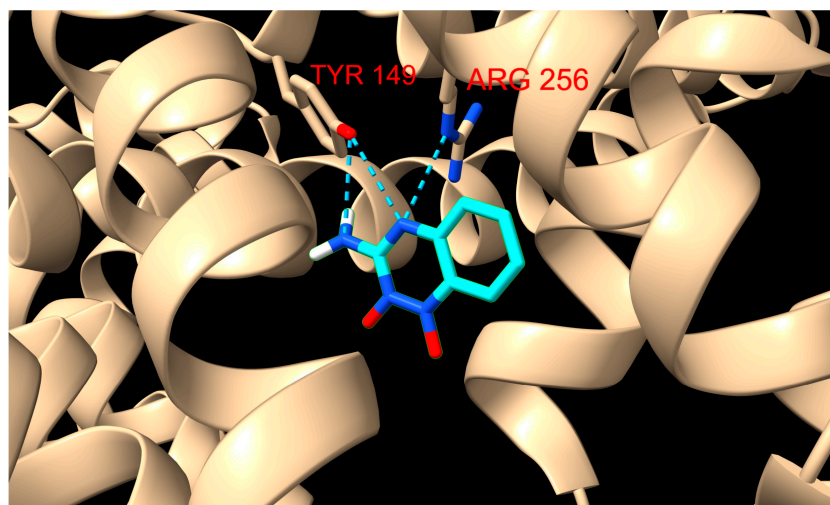


Figure 1. Representative docking pose (Pose 1) of tirapazamine bound to bovine serum albumin (PDB ID: 4F5S). Tirapazamine is shown with cyan carbon atoms, blue nitrogen atoms, and red oxygen atoms and localizes within Subdomain IIA near the entrance of the Sudlow Site I region between adjacent α -helices (tan cartoon). Selected polar contacts with nearby residues (e.g., Tyr149 and Arg256) are shown for visualization; however, these contacts vary across poses and do not form a consistent interaction network. The ligand's positioning and the absence of a cooperative hydrogen-bond network are consistent with limited structural stabilization.

As shown in Figure 2, the binding geometry places tirapazamine at the interface between subdomains IB and IIA. This peripheral localization distinguishes its binding geometry from canonical Site I ligands and suggests limited structural encapsulation and substantial inferred solvent exposure.



Figure 2. Representative docking pose (Pose 1) of tirapazamine bound to bovine serum albumin (PDB ID: 4F5S). Tirapazamine (cyan sticks) localizes at the entrance region of Subdomain IIA (red), adjacent to Subdomain IB (yellow), occupying a shallow, peripheral position at the IB–IIA interface with significant inferred solvent exposure and limited local interactions.

Solvent exposure was inferred from the geometry of the docking poses and partially exposed protein surface representations (Figure S3). All top-ranked poses localized to the same entrance region of Subdomain IIA, indicating spatial convergence rather than distinct alternative binding sites.

3.3. Residue-Level Interactions

In the representative docking pose (Figure 1), tirapazamine engages in short polar contacts with Tyr149, located at the Subdomain IB–IIA interface, and Arg256, which is located within Subdomain IIA. Hydrophobic contacts with Ile289 and Ala260 are also observed in this pose. Residue numbering in this study is based on bovine serum albumin (BSA). Because of a known sequence deletion at position 116, amino acid numbering in BSA beyond residue 115 is shifted by -1 compared to human serum albumin (HSA). Consequently, BSA Tyr149 and Arg256 correspond respectively to HSA Tyr150 and Arg257, as reported previously [27]. According to established residue locations of Sudlow Site I [28], Arg256 (corresponding to Arg257/HSA) is a component of Subdomain IIA, supporting localization of TPZ interactions within this region. However, these contacts occur at residues positioned toward the entrance and cavity wall and do not extend to deeper core residues characteristic of canonical Site I ligands.

Both polar and hydrophobic contacts are observed across the top-ranked docking poses; however, they vary between configurations and do not form a continuous or cooperative interaction network. Contact patterns were verified across three independent docking runs (Table S2), confirming that the pose-dependent variability is reproducible and not an artifact of a single calculation. Among the top-ranked poses, similar patterns of weak, non-cooperative interactions are observed, with variations arising primarily from minor rotational and orientational differences within the same shallow binding region.

The lack of consistent tight packing and extensive stabilization involving canonical Sudlow Site I core residues (e.g., Trp213/214 and Arg217/218, BSA/HSA numbering) further supports a peripheral binding geometry for tirapazamine. Across all three independent docking runs (Table S2), warfarin contacts Arg217 in all 10 top-ranked poses (minimum heavy-atom distances 2.69–3.44 Å), whereas tirapazamine contacts Arg217 only in Pose 6 of each run (3.08–3.25 Å)—a single pose at the upper boundary of the contact distance criterion. Similarly, warfarin contacts Trp213 in multiple poses across all three runs, while tirapazamine shows no Trp213 contacts in any run. These contact frequency differences, together with the predominance of entrance-region and cavity-wall residues (Tyr149, Arg256, His241) in TPZ interactions, support the interpretation that tirapazamine adopts a more peripheral and weakly stabilized binding geometry rather than a deeply buried Site I configuration.

3.4. Comparative Binding Site Overlap Between Tirapazamine and Warfarin

Superposition of the docked poses indicates that both tirapazamine and warfarin are localized within the Sudlow Site I region of Subdomain IIA (Figure 3). Despite occupying the same macroscopic region, the two ligands assume distinct binding orientations. Warfarin adopts an internally oriented configuration that aligns with the canonical Sudlow Site I structure, as outlined in crystallographic studies [29], characterized by deep burial and close packing against core residues such as Trp214 and Arg218 [28,29]. This is reflected quantitatively in the contact analysis (Table S2): warfarin contacts Arg217 in all 10 top-ranked poses across all three independent docking runs, whereas tirapazamine contacts Arg217 in Pose 6 only. Warfarin additionally contacts Trp213 in multiple poses across all three runs; tirapazamine makes no Trp213 contacts in any run. These residue-level

differences support the use of warfarin as a reference ligand for contextual interpretation of binding orientation within Site I rather than as a formal validation of docking accuracy.

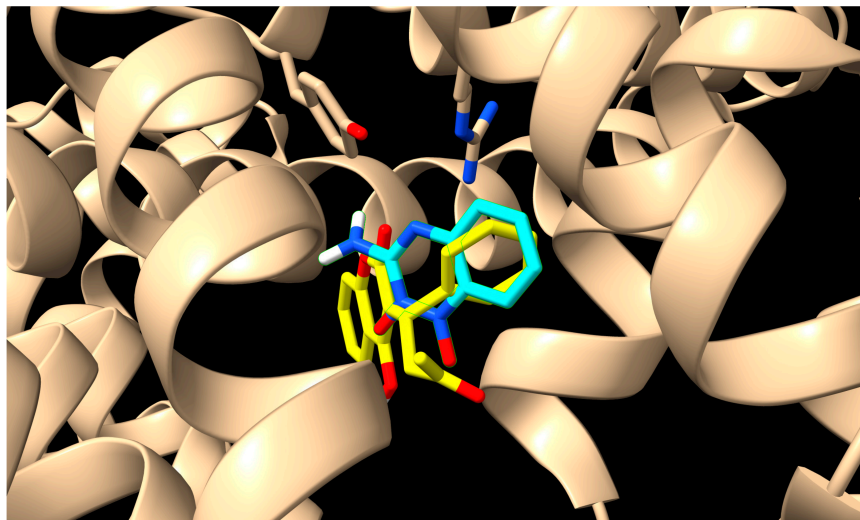


Figure 3. Superposed docking poses of warfarin (yellow carbon atoms) and tirapazamine (cyan carbon atoms) within bovine serum albumin (PDB ID: 4F5S), illustrating their distinct binding geometries within Subdomain IIA (Sudlow Site I region). Both ligands localize to the same macroscopic region but adopt distinct binding orientations. Across the 10 top-ranked poses (Table S2), warfarin exhibits more frequent and often multi-residue polar contacts, including repeated engagement with Arg217, whereas tirapazamine displays more variable and often absent polar contacts, most commonly involving Tyr149 and Arg256. Albumin is shown as tan ribbon helices; differences in burial depth and inferred solvent exposure are further illustrated by surface representations (Figure S3).

Conversely, tirapazamine is located in a more peripheral region near the IB–IIA interface, exhibiting geometry consistent with greater inferred solvent exposure and a simpler interaction network. These differences stem from variations in binding geometry and stabilization rather than site selectivity, demonstrating albumin’s capacity to interact with different ligands within the same region [27].

Although tirapazamine lies spatially within Subdomain IIA (Figure 1), geometric proximity alone does not imply deep Site I anchoring. To further quantify differences in binding geometry, minimum heavy-atom separations were evaluated between each ligand and residues lining Sudlow Site I. Warfarin frequently exhibits shorter contact distances, consistent with tighter packing and deeper enclosure within the binding cavity, and often forms multiple simultaneous contacts with neighboring residues. In contrast, tirapazamine more commonly displays longer or absent contacts, reflecting looser packing and reduced structural encapsulation. These geometric differences reinforce the interpretation that tirapazamine occupies a more weakly stabilized, peripheral position relative to canonical Site I ligands.

3.5. Consistency Between Computed Binding Energetics and Experimental Association Estimates

UV–visible absorption measurements were performed to independently assess tirapazamine–albumin association, and not to determine the association constants. Three independent titration experiments yielded apparent association constants of $2.30 \pm 1.05 \times 10^4 \text{ M}^{-1}$ (mean $\pm$ SD) (Table 2), corresponding to experimentally derived binding free energies of approximately -5.66 to $-6.18 \text{ kcal}\cdot\text{mol}^{-1}$ at 298 K (-5.89 to $-6.42 \text{ kcal}\cdot\text{mol}^{-1}$ at 310 K).

Table 2. Experimental association estimates and the derived binding free energies used to assess consistency with docking predictions. Association constants were measured at room temperature (298 K). ΔG values are reported at both 298 K and 310 K (physiological conditions) and are derived from K_a using $\Delta G = -RT \ln K_a$. The reported association estimates and derived ΔG values are intended as approximate, order-of-magnitude indicators for qualitative comparison with docking results and should not be interpreted as rigorous thermodynamic parameters.

Parameter	Value	Units	Notes
K_a (run 1)	1.26×10^4	M^{-1}	UV-Vis, 298 K
K_a (run 2)	3.35×10^4	M^{-1}	UV-Vis, 298 K
K_a (run 3)	2.30×10^4	M^{-1}	UV-Vis, 298 K
K_a (mean $\pm$ SD)	$2.30 \pm 1.05 \times 10^4$	M^{-1}	UV-Vis, 298 K
ΔG (experimental)	−5.66 to −6.18	kcal·mol ^{−1}	Derived at 298 K
ΔG (experimental)	−5.89 to −6.42	kcal·mol ^{−1}	Converted to 310 K

The bold was used to highlight the mean value derived from the three runs.

The predicted docking scores for tirapazamine fall within a similar range. However, docking energies derived from empirical scoring functions are not rigorous thermodynamic free energies and neglect explicit solvent, entropic contributions, and protein flexibility [14,15,30]. Therefore, the correspondence between docking and experimental estimates is best interpreted at the level of the binding regime. Both approaches independently suggest that tirapazamine associates weakly with albumin.

For comparison, canonical Sudlow Site I ligands (such as warfarin) have higher association constants than those estimated here for tirapazamine. Warfarin binds to bovine serum albumin with experimentally determined association constants ranging from 0.8×10^5 to $1.2 \times 10^5 M^{-1}$ [31]. This categorizes warfarin as a high-affinity binder, in contrast to the weaker association estimated here for tirapazamine.

A similar moderate-affinity interaction was recently reported for another Sudlow Site I ligand, cathepsin B inhibitor II, with human serum albumin ($K_a = 6.13 \pm 0.10 \times 10^5 M^{-1}$) [32], placing the weaker association estimated here for tirapazamine within the broader range of Site I binding affinities documented in the literature.

It should also be noted that experimentally derived binding constants between ligands and serum albumins can vary significantly depending on the analytical method and experimental conditions used [33]. This variability reflects the sensitivity of analytically derived binding parameters to underlying assumptions and data treatment, as discussed previously [34–36].

3.6. Short-Timescale Molecular Dynamics Pose Stability

To assess the positional stability of the representative docking poses, three independent 2 ns MD simulations were performed for each ligand (Table S3). TPZ exhibited markedly consistent ligand RMSD values across all three runs (0.249, 0.246, and 0.245 Å; mean $\pm$ SD = 0.247 ± 0.002 Å), indicating that the docked pose is geometrically stable throughout the simulation and does not undergo significant positional drift. In contrast, WAR showed considerably greater inter-run variability (0.573, 0.744, and 0.418 Å; mean $\pm$ SD = 0.578 ± 0.163 Å), consistent with known conformational mobility of warfarin within the deep Site I cavity, as previously described for BSA–warfarin MD systems [37]. Backbone RMSD values were comparable between the two systems (BSA–TPZ: 1.953 ± 0.159 Å; BSA–WAR: 1.897 ± 0.267 Å), indicating equivalent global protein stability and confirming that differences in ligand RMSD reflect ligand-specific behavior rather than differential protein dynamics (full per-run RMSD summary: Table S3; RMSD-versus-time trajectories for all six runs: Figure S5). These simulations are interpreted strictly as short-timescale pose stability assessments and do not independently establish the peripheral

binding geometry of TPZ; rather, they confirm that the geometry proposed by the docking analysis is dynamically stable over this timescale [25].

4. Discussion

In this study, molecular docking was employed as a comparative structural modeling tool to examine the interaction of tirapazamine with bovine serum albumin relative to a canonical Sudlow Site I ligand. Warfarin, a canonical Sudlow Site I binder as established by crystallographic studies [29], was used to contextualize binding orientation and degree of enclosure within Site I, rather than as a formal validation of the docking protocol.

The docking results indicate that tirapazamine and warfarin are positioned in the same general region of Subdomain IIA, but their binding geometries differ markedly. Warfarin adopts an enclosed orientation characteristic of high-affinity Site I ligands, whereas tirapazamine occupies a more peripheral, solvent-exposed position characterized by weak polar contacts. This difference is further supported by the verified contact analysis across three independent docking runs: warfarin contacts the canonical Site I core residues Arg217 and Trp213 consistently and across multiple poses, whereas tirapazamine contacts Arg217 in Pose 6 only and makes no Trp213 contacts in any run or pose. Warfarin also exhibits substantially more hydrophobic contacts per pose (Leu218, Leu237, Leu259, Ile263, Ala290, and others), reflecting tighter packing and greater burial within the binding cavity. These geometric distinctions are consistent with established principles of albumin–ligand binding [10,29] and with the approximate experimental association constants derived from UV–visible measurements [19].

These observations are further supported by the physicochemical properties of the ligands. Tirapazamine is characterized by low lipophilicity (consensus $\log P \approx -0.74$) and relatively high polarity (TPSA $\approx 90 \text{ \AA}^2$), whereas warfarin exhibits a more lipophilic profile (consensus $\log P \approx 2.8$) and lower polarity (TPSA $\approx 67 \text{ \AA}^2$). Such differences are consistent with their distinct interaction behaviors. Lipophilic compounds like warfarin occupy more deeply buried hydrophobic pockets within serum albumin, while more polar molecules such as tirapazamine instead favor more peripheral/less hydrophobic modes of association. This physicochemical contrast is in agreement with the docking results, which indicate deeper insertion for warfarin and a more exposed, peripheral position for tirapazamine. Moreover, it is consistent with the experimental observations of weaker interactions between tirapazamine and serum albumin.

Since serum albumin is a major drug carrier in the bloodstream, weaker albumin association generally corresponds to a higher unbound fraction (f_u) in plasma and reduced protein-bound drug fraction. The comparatively weak, peripheral interaction observed for tirapazamine is thus qualitatively consistent with greater free-drug availability compared to more strongly bound, canonical Site I ligands such as warfarin. However, because static structural models and order-of-magnitude association estimates do not capture dynamic *in vivo* processes, direct conclusions regarding clinical pharmacokinetics cannot be drawn from these data alone.

Because docking scoring functions rely on experimental approximations and neglect explicit solvent reorganization and entropic contributions, predicted docking energies should be regarded as qualitative indicators of relative interaction strength instead of specific free energy values [14,15,30]. Consequently, the docking and experimental results presented here indicate a weakly stabilized, peripheral interaction between tirapazamine and bovine serum albumin [28]. This behavior is consistent with previous studies of ligand–albumin interactions, in which multiple non-covalent interaction types, including polar and hydrophobic contacts, contribute to the observed association [38], with molecular dynamics evidence indicating that the relative hydrophobicity of the local protein microenvironment

and the bulk solvent governs ligand retention and dissociation energetics in serum albumin complexes [39], and with reports of ligand-dependent differences in affinity between bovine and human serum albumins [40,41]. Short-timescale MD simulations (Section 3.6) provide supporting evidence for the geometric stability of the proposed binding poses, though they do not independently establish binding mode or thermodynamic parameters. Therefore, conclusions drawn from this study are restricted to comparative binding geometry and spatial localization and do not imply definitive binding stability, thermodynamic affinity, or dynamic accommodation within the binding site.

The current study emphasizes bovine serum albumin's ability to accommodate chemically diverse small molecules within a single binding region through variations in burial depth and stabilizing interactions [10,29]. Thus, tirapazamine adopts a more peripheral, solvent-exposed binding geometry relative to the deeply enclosed canonical Sudlow Site I orientation observed for ligands such as warfarin [28]. These findings are consistent with prior biophysical studies demonstrating that binding geometry and solvent exposure, rather than binding site identity alone, play key roles in governing albumin–ligand interactions [10,28,31].

5. Conclusions

Tirapazamine appears to associate peripherally with bovine serum albumin at the entrance region of Subdomain IIA, exhibiting looser geometric packing relative to canonical Sudlow Site I ligands such as warfarin. Although its predicted docking score is only modestly less favorable than that of warfarin, its solvent-exposed binding geometry and limited interaction network support a more weakly stabilized interaction. This interpretation is further supported by the physicochemical properties of tirapazamine, which are consistent with weaker interactions and limited insertion within the hydrophobic core of canonical binding sites. These findings provide a basis for further studies examining tirapazamine pharmacokinetics and interactions with other plasma proteins.

This study has several limitations. The docking analysis employs a rigid protein model and does not account for conformational flexibility, solvent reorganization, or entropic effects. Consequently, the reported binding geometries represent static structural models rather than dynamic binding equilibria. Additionally, the experimental and computational components of this study focused on bovine serum albumin (BSA), a widely used model protein for albumin–ligand interaction studies and a protein that shares significant structural homology with its human counterpart, human serum albumin (HSA). Nonetheless, direct extrapolation of the present BSA-derived findings to HSA pharmacokinetics should be interpreted with caution until experimentally validated with HSA. Moreover, the docking calculations were performed using a single crystallographic BSA receptor structure and thus do not evaluate receptor conformational heterogeneity across multiple albumin crystal structures. Future docking studies could further assess the robustness of predicted tirapazamine binding geometries across different albumin structures and species. Furthermore, because serum albumin contains multiple solvent-exposed and potentially low-affinity ligand-binding regions, rigid receptor docking results should be interpreted as comparative structural models rather than definitive predictors of binding affinity, binding site occupancy, and dynamic binding equilibria. Short-timescale molecular dynamics simulations (2 ns, triplicate; Section 3.6) were performed to assess the positional stability of the docked poses; however, the simulation timescale is insufficient to characterize long-timescale conformational changes or equilibrium binding behavior. Future studies employing longer MD simulations, flexible receptor docking, or thermodynamic measurements would provide additional insight into equilibrium binding behavior.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/biophysica6040073/s1>, Figure S1: UV-visible absorption spectra of bovine serum albumin (BSA) recorded in the absence and presence of increasing concentrations of tirapazamine (0–100 μ M); Figure S2: Benesi–Hildebrand-type linear plot derived from UV-visible absorption titration data for the bovine serum albumin–tirapazamine (BSA–TPZ) system; Figure S3: Partially exposed surface representation of bovine serum albumin illustrating differences in binding depth of warfarin and tirapazamine within Subdomain IIA; Figure S4: Chemical structures of tirapazamine (top) and warfarin (bottom) used in the docking analysis; Figure S5: Protein backbone and ligand RMSD over the 2 ns production trajectory for each of the three independent BSA–warfarin (red) and BSA–tirapazamine (blue) molecular dynamics simulations. Table S1: SwissDock docking scores for tirapazamine and warfarin binding to albumin; Table S2: Pose-dependent contacts between tirapazamine (TPZ) and warfarin (WAR) with bovine serum albumin (BSA) across three independent molecular docking runs (10 top-ranked poses per run); Table S3: RMSD summary for all six MD runs (BSA–TPZ and BSA–WAR triplicates); Supplementary Note S1: Docking Reproducibility; Supplementary Note S2: Interpretation of UV-visible binding estimates; Supplementary Note S3: Molecular Dynamics Simulation Details.

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Abbreviations

The following abbreviations are used in this manuscript:

TPZ	Tirapazamine
BSA	Bovine serum albumin
NTP	Non-thermal plasma
MD	Molecular dynamics

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