

Article

Structure-Based Computational Evaluation of Betulinic Acid-Derived Hybrids as Potential Bcl-2/Bcl-XL Modulators

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Abstract

The anti-apoptotic Bcl-2 protein family, frequently upregulated in a wide range of cancers, contributes to tumor persistence and therapeutic resistance, making these proteins attractive targets for structure-based inhibitor development. Betulinic acid-derived hybrids represent promising scaffolds for apoptosis-oriented anticancer drug discovery due to their reported antiproliferative and pro-apoptotic properties. In this study, a virtual library of 152 betulinic acid-derived hybrids was screened against Bcl-2 and Bcl-XL. This molecular docking study using AutoDock Vina identified BA–Celastrol and BA–Proanthocyanidin B2 as top-ranked ligands, with docking scores ranging from −13.00 to −8.7 kcal/mol. Both compounds were further analyzed by 100 ns molecular dynamics simulation runs, which revealed system-dependent ligand behavior rather than uniform preservation of the initial docked pose across all complexes. BA–Celastrol showed a more compact internal ligand conformation in the ligand property and RMSF analyses, whereas BA–Proanthocyanidin B2 showed greater intramolecular flexibility and conformational adaptability. Ligand displacement relative to the protein differed between targets, with BA–Proanthocyanidin B2 showing a more retained profile in the Bcl-XL model and BA–Celastrol showing more moderate positional behavior in the Bcl-2 model. MM-GBSA calculations resulted in free energy values ranging from −4.95 to −31.82 kcal/mol, indicating protein-dependent energetic differences across the investigated systems. Based on docking performance, molecular dynamics stability, and energetic data, both hybrids were ranked as computational candidates for further exploration against Bcl-2 family targets. The present findings, although confined to computational analysis, underscore the need for prioritizing betulinic acid-based hybrids for subsequent experimental evaluation.



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1. Introduction

Cancer remains a leading cause of morbidity and mortality worldwide, and resistance to classic chemotherapy continues to be a major obstacle in oncology treatment and management [1]. One of the mechanisms that allows cancer cells to survive is the disruption of apoptosis, which favors continued growth and resistance to cell death [2–4]. In the intrinsic mitochondrial apoptotic pathway, the Bcl-2 protein family regulates the balance between cell survival and cell death, while anti-apoptotic members such as Bcl-2 and Bcl-XL have been linked to tumor progression and poor therapeutic response in several malignancies [2,4]. Their involvement in apoptosis resistance has made them relevant targets in anticancer drug discovery [5,6].

Modulation of anti-apoptotic Bcl-2 family proteins has become an important direction in anticancer research because these proteins help tumor cells tolerate apoptotic stress and maintain survival during treatment [7,8]. At the same time, the functional overlap among anti-apoptotic family members may reduce the effectiveness of approaches directed against a single protein, since cancer cells may remain dependent on related pro-survival pathways [9,10]. Against this background, considering both Bcl-2 and Bcl-XL may provide a broader basis for identifying compounds capable of interfering with apoptosis resistance [7,9,10]. Since dysregulation of Bcl-2 family proteins has been reported in multiple malignancies, including leukemia, breast, lung, and colorectal cancers, the present study was designed from a broader anticancer perspective rather than focusing on a single cancer type. This strategy was intended to support the computational identification of betulinic acid-derived hybrid compounds with potential relevance across different apoptosis-resistant tumor models [11].

From a medicinal chemistry perspective, hybrid molecule design represents a useful strategy by combining distinct bioactive fragments within a single molecular framework [12–14] with the aim of improving biological activity, target interaction profiles, or pharmacological properties [14,15]. Betulinic acid is of particular interest in this context because it is a natural pentacyclic triterpene with previously reported anticancer activity, including apoptosis-related effects, and has been regarded in the literature as a suitable scaffold for chemical modification [16]. Previous studies have shown that hybridization of betulinic acid with other bioactive fragments can generate derivatives with improved antiproliferative potential and broader biological profiles compared with the parent compound [16,17]. The incorporation of complementary pharmacophoric fragments into betulinic acid structures may also improve multitarget interactions and pharmacological properties, supporting their investigation as promising candidates for apoptosis-oriented anticancer therapy [18].

Previous studies have suggested a mechanistic relationship between betulinic acid and apoptosis regulation mediated by Bcl-2 family proteins. Betulinic acid has been reported to induce mitochondrial apoptosis through modulation of anti-apoptotic proteins such as Bcl-2 and Bcl-xL, including decreased expression levels and increased mitochondrial membrane permeabilization in different cancer models [19]. In addition, several Betulinic Acid derivatives and structurally related triterpenoids have demonstrated pro-apoptotic activity associated with Bcl-2 family signaling pathways, supporting the relevance of this scaffold in apoptosis-oriented anticancer research [20]. Although direct biochemical evidence regarding selective Bcl-2/Bcl-xL inhibition by betulinic acid hybrids remains limited, these previous findings provide a rationale for investigating such compounds as potential modulators of anti-apoptotic Bcl-2 family proteins.

The present study was conducted to evaluate a virtual library containing betulinic acid-derived hybrid compounds for their potential interactions with Bcl-2 and Bcl-XL using an *in silico*-based workflow. Blind molecular docking was used for the initial assessment of

binding potential, followed by molecular dynamics simulations and MM-GBSA calculations to further examine protein–ligand interaction behavior and estimated binding energy values. In addition, ADMET prediction was used as a preliminary step for the characterization of pharmacological and toxicity-related properties for the selected compounds. The aim of this computational workflow was to identify selected betulinic acid-based hybrids that may be suitable for further biological investigation, rather than to provide experimental confirmation for their biological activity.

2. Materials and Methods

2.1. Ligand Preparation

The molecular library in our created database contains 71 biologically active parent molecules selected from different classes of natural or synthetic organic compounds with antioxidant, antiproliferative/antitumor properties and 152 hybrid molecules. The parent molecules were extracted from the NCBI PubChem [21], DrugBank [22], ChEMBL [23], eMolecules [24], ChemSpider [25], ZINC-22 [26] databases; the molecular hybrids were manually generated using DECIMER.ai, an open-source platform [27], and ChemDraw Ultra 12.00 Software [28]; and the molecular parameters were determined *in silico* using the Molsoft server [29,30]. When designing the database structure, we defined for each drug substance the following fields: Serial number, Name (IUPAC), Molecular formula, Molecular formula in SMILES format, Structural formula in PNG format, Molecular mass, Numb. HBA, Numb. HBD, MolLogP, MolLogS, MolPSA, MolVol, pKa of most Basic/Acid group, BBB score, Numb. of stereo centers, and Drug-likeness model score [31].

2.2. Protein Preparation

Three inhibitor-bound Bcl-2/Bcl-xL-related crystal structures, namely 2W3L, 2YXJ, and 4LVT, were selected from the RCSB Protein Data Bank for molecular docking studies [32]. The three selected structures were chosen according to the following criteria: (i) relevance to human anti-apoptotic Bcl-2 family proteins involved in apoptosis regulation; (ii) availability of an experimentally resolved inhibitor-bound structure; (iii) presence of a co-crystallized small-molecule ligand occupying the canonical BH3-binding groove, allowing binding-site definition and docking protocol validation; (iv) suitable crystallographic resolution (<2.5 Å); and (v) inclusion of complementary Bcl-2, Bcl-xL, and Bcl-2/Bcl-xL-related conformational models. Based on these criteria, 2YXJ was selected as an ABT-737-bound Bcl-xL structure, 4LVT as a navitoclax-bound Bcl-2 structure, and 2W3L as an inhibitor-bound Bcl-2/Bcl-xL chimeric structure. Water molecules and heteroatoms were removed, and the proteins were cleaned using Discovery Studio Visualizer 2024 (BIOVIA, San Diego, CA, USA).

2.3. Molecular Docking

Docking simulations were conducted using AutoDock Vina 1.1.2 integrated in PyRx 0.8 [33–35]. Ligands were converted to PDBQT format [36] and grid boxes were centered around known active site coordinates. Energy minimization of ligand structures was performed prior to docking in order to optimize molecular geometry and reduce steric conflicts. Docking scores were recorded in kcal/mol, and binding interactions were visualized using Discovery Studio [37–40]. Hybrids 96 (BA–celastrol) and 112 (BA–proanthocyanidin B2) showed consistent high affinity across all three targets and were selected for further analysis [40–42].

2.4. Molecular Dynamics Simulation

Molecular dynamics simulations were performed using Desmond (Schrödinger Suite 2025-1) for 100 ns. Complexes were solvated using a TIP3P water model in an orthorhombic

box with a 10 Å buffer and 0.15 M NaCl. Counter ions were added to neutralize the systems before simulation. Simulations were run under NPT ensemble at 300 K and 1 atm using the OPLS_2005 force field. Prior to the production phase, each system underwent energy minimization and equilibration steps to ensure structural stability. Trajectories were analyzed for RMSD, RMSF, and secondary structure elements to assess the conformational stability of each protein–ligand complex [43,44].

2.5. MM-GBSA Binding Free Energy Analysis

The molecular mechanics generalized Born surface area (MM-GBSA) approach implemented in the Prime module of the Schrödinger Suite was used to estimate the binding free energy (ΔG_{bind}) of the investigated protein–ligand complexes. The binding free energy was calculated according to the following equation [45,46]:

$$\Delta G_{\text{bind}} = \Delta E_{\text{MM}} + \Delta G_{\text{solv}} + \Delta G_{\text{SA}}$$

where ΔE_{MM} represents the difference in minimized molecular mechanics energies between the protein–ligand complex and the sum of the energies of the isolated protein and ligand. The ΔG_{solv} term corresponds to the change in solvation free energy calculated using the generalized Born (GB) implicit solvent model. The ΔG_{SA} term represents the change in the nonpolar surface area contribution upon ligand binding. MM-GBSA calculations were performed using representative trajectory frames extracted from the molecular dynamics simulations to estimate the relative binding stability of the investigated complexes.

2.6. In Silico ADMET and Anticancer Activity Prediction

Pharmacokinetic and toxicity profiles of the selected hybrids were assessed using the pkCSM webserver. Canonical SMILES representations of Hyb.96 and Hyb.112 were submitted to the platform, and selected ADMET descriptors, including absorption, distribution, metabolism, excretion, and toxicity-related endpoints, were recorded [47].

In addition, the pdCSM-cancer predictive model was used to estimate the potential antiproliferative activity of the selected hybrids across different cancer cell lines, expressed as predicted GI_{50} values. The obtained results were interpreted as theoretical computational predictions and used only for preliminary compound characterization [48]. These analyses were intended to provide preliminary insight into the pharmacokinetic suitability and potential drug-likeness of the selected hybrid compounds.

3. Results and Discussion

3.1. Molecular Docking

Molecular docking was used as an initial virtual screening approach to evaluate the binding potential of betulinic acid-derived hybrid compounds toward selected anti-apoptotic members of the Bcl-2 protein family. A virtual library comprising 152 betulinic acid-based hybrid molecules was constructed through systematic modification of the parent scaffold in order to explore structural diversity within a synthesizable chemical framework. The library incorporated pharmacologically relevant structures previously reported in the literature. The complete compound library, including the SMILES representations, is provided in the Supplementary Materials (Table S1).

Molecular docking runs were performed using AutoDock Vina implemented in PyRx, and the resulting docking scores were used for comparative ranking of the hybrid ligands across the investigated protein targets. For the process of identifying potential Bcl-2/Bcl-XL dual inhibitors, three PDB structures were selected for this analysis: 2W3L (chimeric Bcl-2/XL), 2YXJ (Bcl-XL), and 4LVT (Bcl-2). The molecular docking search-space parameters used for these structures are provided in the Supplementary Materials (Table S3). To

assess the reliability of the docking protocol, redocking of the co-crystallized ligands was performed for the three protein structures. The calculated RMSD values were 0.373 Å for 2W3L, 1.569 Å for 2YXJ, and 0.732 Å for 4LVT, indicating a good reproduction of the experimental binding poses for each native ligand (Table S3).

Based on the obtained docking results, the top 10 ranked hybrids for each protein target, according to their Vina docking scores, are summarized in Table 1. Because the objective of this study was to identify candidates with potential dual Bcl-2/Bcl-XL inhibitory potential, compound prioritization was based not only on high docking scores for a single structure but also on consistent top-ranking performance across all three models. Hyb.96 and Hyb.112 were chosen in this particular case because they were among the top 10 ranked ligands for the three target proteins (2W3L, 2YXJ, and 4LVT), indicating a greater theoretical potential for inhibiting both Bcl-2 and Bcl-XL. Furthermore, the docking scores of the native ligands were included in Table S2 as protocol-specific reference values, enabling the comparison of the hybrid library with the co-crystallized compounds under identical docking conditions. Many hybrids exhibited docking scores that were comparable to, and in some cases, even more favorable than, those of the native ligands. Nevertheless, Hyb.96 and Hyb.112 were chosen primarily due to their consistent top-ranking performance across all three protein models, which was considered more suitable for the identification of candidates with potential dual Bcl-2/Bcl-XL inhibitory behavior.

Table 1. Top 10 docking scores ($\text{kcal}\cdot\text{mol}^{-1}$) for betulinic acid-derived hybrids against 2W3L, 2YXJ, and 4LVT using AutoDock Vina.

2W3L/Hybrid	Docking Score (kcal/mol)	2YXJ/Hybrid	Docking Score (kcal/mol)	4LVT/Hybrid	Docking Score (kcal/mol)
Hyb.129	−13.10	Hyb.129	−11.60	Hyb.96	−9.3
Hyb.120	−13.10	Hyb.146	−10.9	Hyb.106	−9.1
Hyb.113	−13.00	Hyb.135	−10.8	Hyb.13	−9.0
Hyb.96	−13.00	Hyb.96	−10.7	Hyb.105	−8.8
Hyb.101	−12.70	Hyb.121	−10.7	Hyb.112	−8.7
Hyb.106	−12.70	Hyb.145	−10.7	Hyb.118	−8.6
Hyb.110	−12.60	Hyb.91	−10.6	Hyb.143	−8.5
Hyb.73	−12.50	Hyb.113	−10.6	Hyb.127	−8.4
Hyb.128	−12.50	Hyb.112	−10.4	Hyb.79	−8.3
Hyb.112	−12.50	Hyb.75	−10.2	Hyb.99	−8.2

The two-dimensional chemical structures of the selected hybrids, Hyb.96 (BA–Celastrol) and Hyb.112 (BA–Proanthocyanidin B2), are presented in Figure 1 to facilitate compound visualization. Structure-based molecular docking was used to examine the predicted binding poses of Hyb.96 and Hyb.112 in the anti-apoptotic protein Bcl-2 (PDB ID: 2W3L). As shown in Figure 2, both docked poses were located within the BH3-binding pocket. PLIP (Protein–Ligand Interaction Profiler) analysis indicated that Hyb.96 formed four hydrogen bonds and six major hydrophobic contacts, whereas Hyb.112 formed six hydrogen bonds and eight major hydrophobic contacts in the same binding region (Table 2). For Hyb.96, the hydrogen-bonding pattern involved ARG68, TRP103, and GLY104, while the principal hydrophobic contacts included ARG66, ARG68, ARG69, ALA72, LEU160, and TYR161. For Hyb.112, hydrogen bonds involved ALA59, SER75, GLY104, ASN122, and PRO163, and the main hydrophobic contacts included ARG65, ARG68, PHE71, ALA72, VAL115, ASN122, and TYR161. These results indicate that both ligands occupied the same binding region in the 2W3L model but differed in the number and distribution of predicted polar and hydrophobic contacts.



Figure 1. Two-dimensional chemical structures of Hyb.96 (BA–Celastrol) (A) and Hyb.112 (BA–Proanthocyanidin B2) (B) (Generated using ChemDraw Ultra 12.0).

Table 2. Summary of key protein–ligand interactions between Hyb.96 or Hyb.112 and Bcl-2 (PDB ID: 2W3L), identified by PLIP.

Protein: 2W3L (Bcl-2)	Hyb.96	Hyb.112
Major hydrophobic contacts	ARG66, ARG68, ARG69, ALA72, ASN102, LEU160, TYR161	ARG65, ARG68, PHE71 (three contacts), ALA72, VAL115, ASN122, TYR161
Key hydrogen bonds	ARG68 (two H-bonds), TRP103, GLY104	ALA59, SER75 (two H-bonds), GLY104, ASN122, PRO163
Total number of H-bonds	4	6
Notable interaction features	Hydrophobic contacts with LEU160 and TYR161, together with 4 hydrogen bonds	Larger hydrogen-bonding pattern and multiple hydrophobic contacts within the pocket
General interaction pattern	Mixed hydrophobic and hydrogen-bond contacts	More extensive polar and hydrophobic contact pattern

These results for the 2W3L protein indicate that although both ligands occupy the same BH3-binding region, Hyb.112 establishes a more extensive hydrogen-bonding network, suggesting a stronger contribution of polar interactions to the binding process. This may enhance electrostatic stabilization within the binding pocket. In contrast, Hyb.96 exhibits a more balanced interaction profile, combining hydrophobic contacts with fewer but persistent hydrogen bonds, which may contribute to a more structurally constrained and stable binding mode within the pocket.

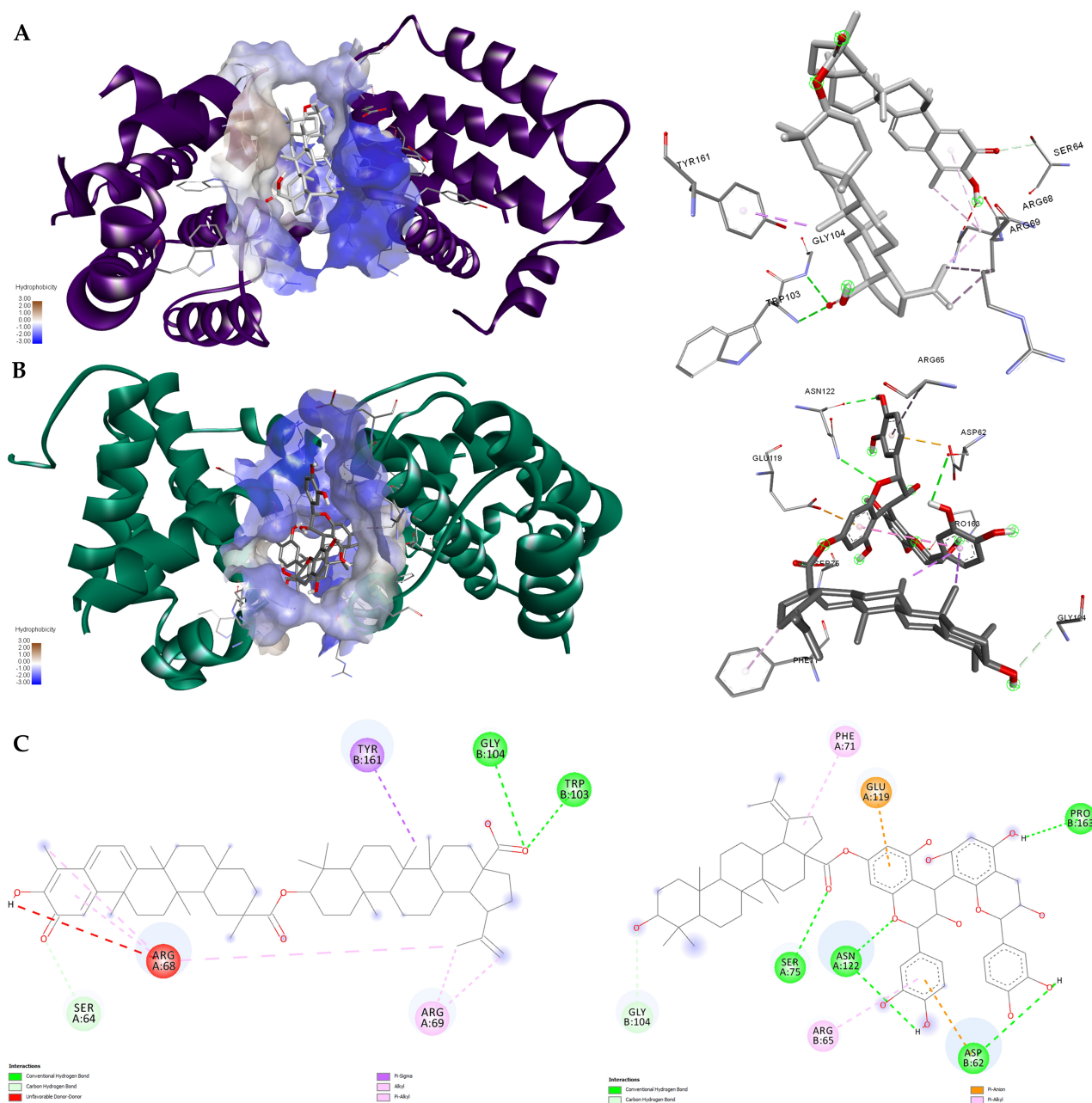


Figure 2. (A) 3D docking pose of the BA-Celastrol hybrid (Hyb.96) within the binding region of Bcl-2 (PDB ID: 2W3L). (B) Three-dimensional docking pose of the BA-Proanthocyanidin B2 hybrid (Hyb.112) in the same 2W3L structure, with the pocket surface representation (left) and the corresponding interaction diagram showing the predicted non-covalent contacts (right). (C) 2D interaction diagrams of the BA-Celastrol (left) and BA-Proanthocyanidin B2 (right) hybrids, illustrating hydrogen bonds, hydrophobic interactions, and additional predicted non-covalent contacts within the 2W3L binding region.

The predicted binding poses of Hyb.96 and Hyb.112 were also examined in Bcl-XL (PDB ID: 2YXJ). Figure 2 indicates that both docked conformations were positioned within the elongated hydrophobic cavity and formed non-covalent contacts with residues lining the pocket.

The PLIP analysis revealed that Hyb.96 formed three main hydrophobic contacts with TRP24, VAL161, and PRO180, as well as three hydrogen bonds that involve SER164 (two contacts) and ASN175, as summarized in Table 3 and illustrated in Figure 3C. Hyb.112 also formed three significant hydrophobic contacts, which involved GLN160, VAL161,

and PRO180. However, it also exhibited five hydrogen bonds, which involved ARG6, VAL135, SER164, TYR173, and HIS177. Consequently, the 2YXJ model indicated that both ligands exhibited the same number of significant hydrophobic contacts. However, Hyb.112 exhibited two additional predicted hydrogen bonds in comparison to Hyb.96.

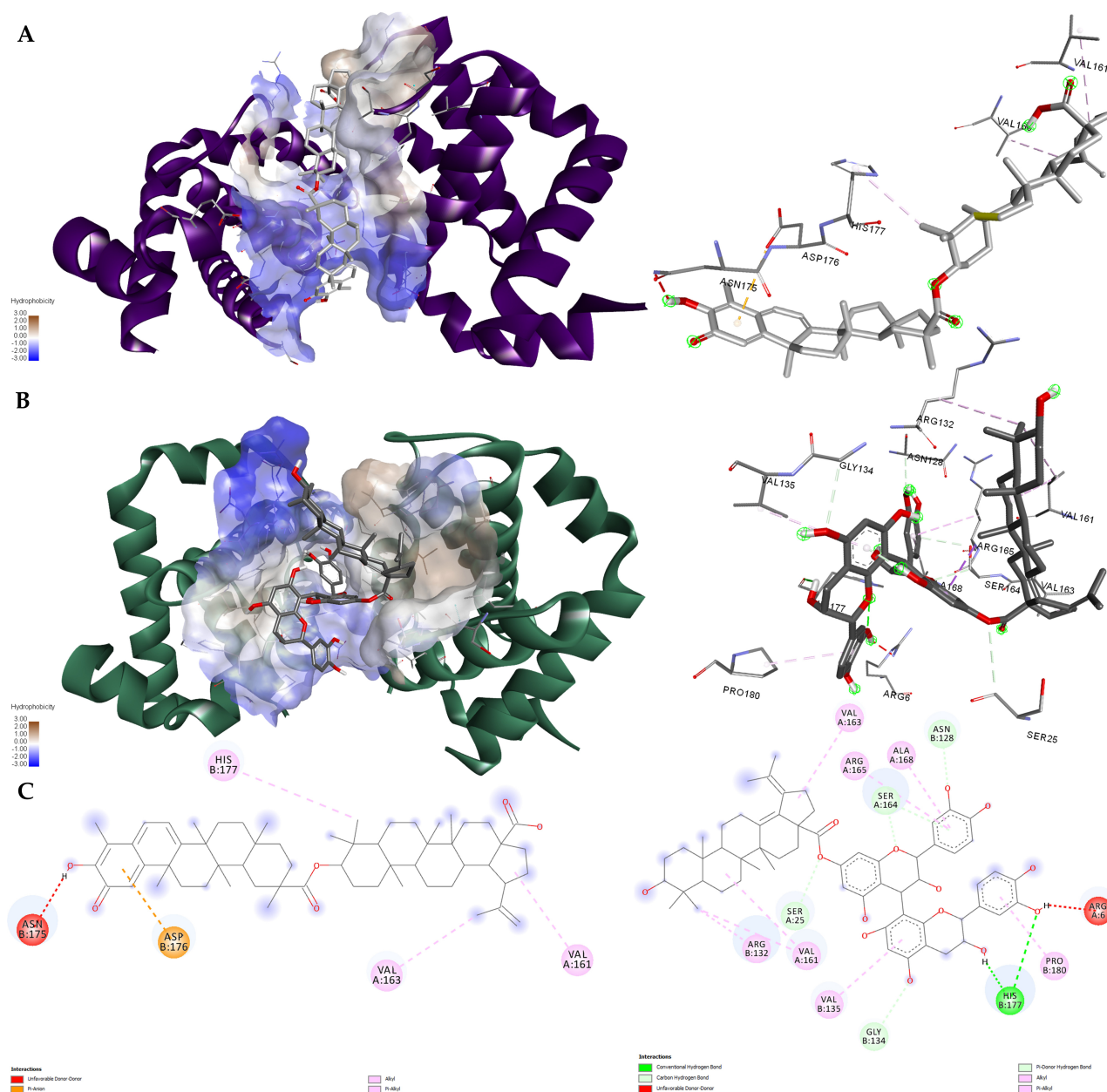


Figure 3. (A) 3D view of the BA–Celastrol hybrid (Hyb.96) docked in Bcl-XL (PDB ID: 2YXJ). (B) Docking pose of the BA–Proanthocyanidin B2 hybrid (Hyb.112) in the same 2YXJ binding pocket. The (left panel) shows the ligand positioned within the cavity surface, and the (right panel) highlights the predicted non-covalent contacts. (C) 2D interaction schemes for BA–Celastrol (left) and BA–Proanthocyanidin B2 (right), summarizing hydrogen bonds, hydrophobic contacts, and additional predicted interactions within the 2YXJ binding region.

The interaction patterns observed for the 2YXJ model suggest that both ligands retain comparable hydrophobic anchoring within the elongated binding cavity. However, the higher number of hydrogen bonds formed by Hyb.112 indicates an increased contribution of polar interactions, potentially enhancing electrostatic stabilization within the binding

site. In contrast, Hyb.96 maintains a more localized and structurally restrained interaction profile, which may favor a more stable and less dynamic binding mode within the pocket.

Table 3. Summary of key protein–ligand interactions between Hyb.96 or Hyb.112 and Bcl-XL (PDB ID: 2YXJ), identified by PLIP.

Protein: 2YXJ (Bcl-XL)	Hyb.96	Hyb.112
Major hydrophobic contacts	TRP24, VAL161, PRO180	GLN160, VAL161, PRO180
Key hydrogen bonds	SER164 (two H-bonds), ASN175	ARG6, VAL135, SER164, TYR173, HIS177
Total number of H-bonds	3	5
Notable interaction features	Hydrophobic contacts centered on TRP24 and PRO180, together with 3 hydrogen bonds	Larger hydrogen-bonding pattern with multiple donor/acceptor contacts
General interaction pattern	Localized hydrophobic and hydrogen-bond contacts	More extended polar contact pattern within the pocket

The predicted binding poses of the BA–Celastrol and BA–Proanthocyanidin B2 hybrids in Bcl-2 (PDB ID: 4LVT) are shown in Figure 4. Both docking poses were located within the surface-defined pocket, where they established non-covalent contacts with residues in the binding region. The interaction panels summarize the predicted contact patterns for the two ligands in the 4LVT model.

For the 4LVT model, the two ligands showed different predicted contact patterns (Table 4). Hyb.96 formed four hydrophobic contacts involving TYR26, VAL159, ARG161, and GLU162, while no hydrogen bonds were detected in the PLIP analysis. In contrast, Hyb.112 showed three hydrophobic contacts involving THR119, ARG161, and GLU162, together with six hydrogen bonds involving LEU116, HIS117, and GLU157, as well as a predicted π –cation interaction with ARG161. Thus, in the 4LVT docking model, both ligands showed the same number of major hydrophobic contacts, whereas Hyb.112 exhibited a larger number of predicted polar interactions than Hyb.96, six relative to zero hydrogen bonds, plus one π –cation interaction.

Table 4. Summary of key protein–ligand interactions between Hyb.96 or Hyb.112 and Bcl-2 (PDB ID: 4LVT), identified by PLIP.

Protein: 4LVT (Bcl-2)	Hyb.96	Hyb.112
Major hydrophobic contacts	TYR26 (two contacts), VAL159, ARG161, GLU162	THR119, ARG161 (two contacts), GLU162
Key hydrogen bonds	— (no H-bonds detected)	LEU116, HIS117, GLU157 (three strong H-bonds), plus additional Glu-mediated contacts
Total number of H-bonds	0	6
π –cation interactions	—	ARG161 (aromatic ring interaction)
Notable interaction features	Predominantly hydrophobic contact pattern, no hydrogen bonds detected	More extensive hydrogen-bonding pattern with an additional predicted π –cation interaction
General interaction pattern	Predominantly hydrophobic interaction profile	Mixed hydrophobic, hydrogen-bonded, and π –cation contacts

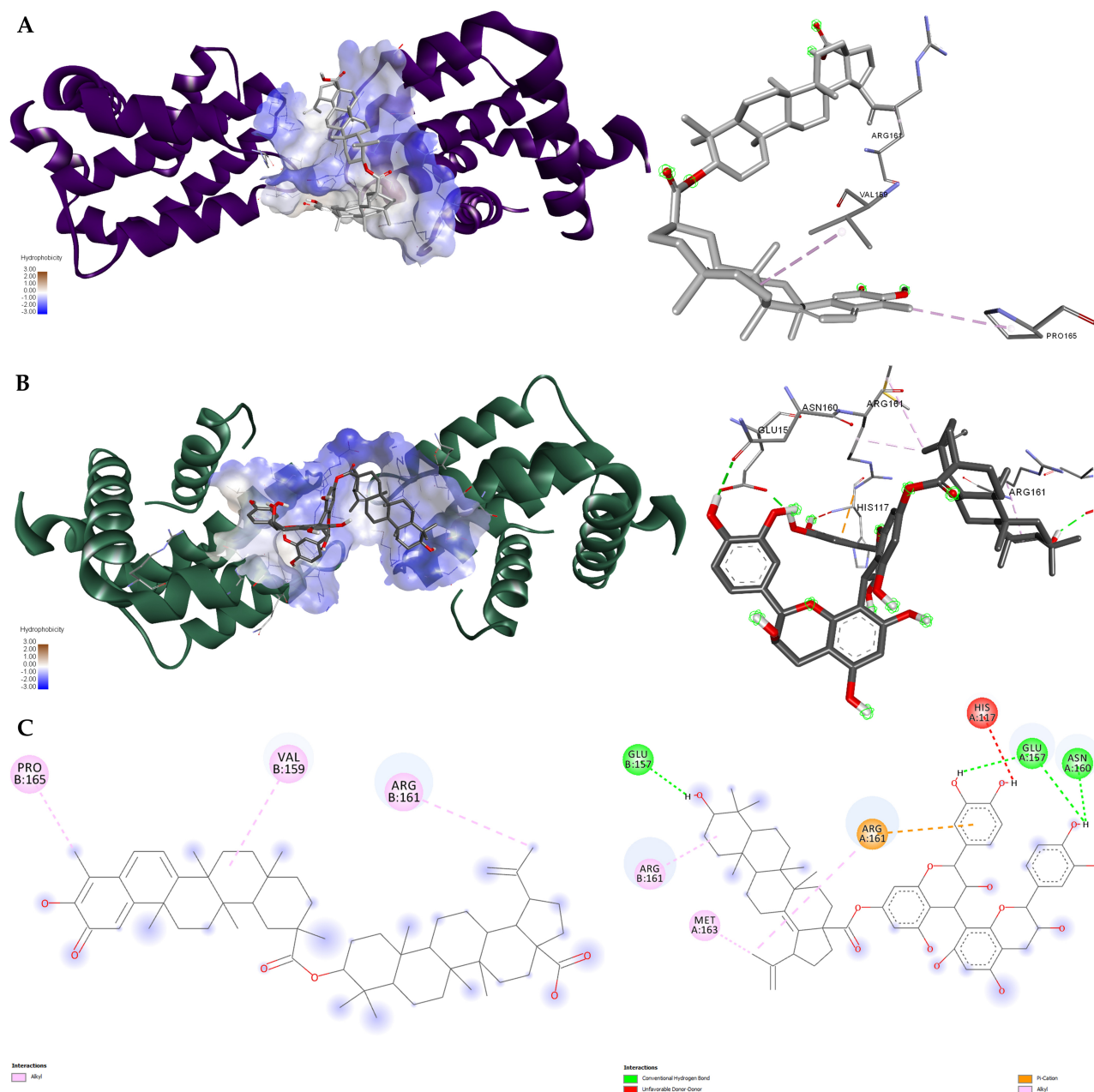


Figure 4. (A) 3D representation of the BA–Celastrol hybrid docked in Bcl-2 (PDB ID: 4LVT). The left panel shows the ligand positioned within the surface-rendered pocket, whereas the right panel summarizes the predicted residue-level contacts. (B) Predicted docking pose of the BA–Proanthocyanidin B2 hybrid in the same 4LVT structure. (C) 2D interaction diagrams of the BA–Celastrol (left) and BA–Proanthocyanidin B2 (right) hybrids, showing hydrogen bonds, hydrophobic contacts, and additional predicted non-covalent interactions within the 4LVT binding region.

In the 4LVT model, the absence of hydrogen bonds for Hyb.96 and its predominantly hydrophobic interaction profile suggests a binding mode driven mainly by dispersion forces. This indicates that ligand stabilization is largely governed by van der Waals interactions within the binding pocket. Conversely, Hyb.112 exhibits a significantly larger number of hydrogen bonds and an additional π -cation interaction, indicating a more complex and polar interaction network. This pattern suggests an increased contribution of electrostatic interactions, which may enhance estimated binding energy but also introduce greater sensitivity to solvation effects.

Overall, the docking analysis indicates that both Hyb.96 and Hyb.112 are able to occupy the BH3-binding groove across all investigated Bcl-2 family structures. Hyb.112 consistently exhibits a more extensive hydrogen-bonding and polar interaction profile, whereas Hyb.96 displays a more balanced interaction pattern characterized by hydrophobic stabilization and fewer but persistent hydrogen bonds. The consistent involvement of key residues such as ARG68, GLY104, and TYR161 supports the biological relevance of the predicted binding modes. Furthermore, the ability of both ligands to maintain top-ranking positions across all three protein models suggests a robust binding behavior that is not dependent on a single receptor conformation. Taken together, these differences indicate distinct binding strategies, with Hyb.112 favoring stronger electrostatic contributions and Hyb.96 exhibiting a more stable and structurally constrained binding mode. This distinction provides a structural basis for the differences observed in the subsequent molecular dynamics simulations.

Hyb.96 (BA–Celastrol hybrid) and Hyb.112 (BA–Proanthocyanidin B2 hybrid) were subsequently submitted to molecular dynamics simulations and binding free-energy calculations.

3.2. Molecular Dynamics Simulation

To evaluate the dynamic stability of the six protein–ligand complexes, 100 ns molecular dynamics simulations were performed, and the resulting trajectories were analyzed in terms of protein C α RMSD (left Y-axis) and ligand RMSD relative to the protein (right Y-axis). Panels A–F (Figure 5) correspond to the complexes 2W3L–Hyb.96, 2W3L–Hyb.112, 2YXJ–Hyb.96, 2YXJ–Hyb.112, 4LVT–Hyb.96, and 4LVT–Hyb.112.

In the 2W3L–Hyb.96 complex, the protein RMSD increases after 20 ns, after which it fluctuates within a range of approximately 7–9 Å. Although relatively high, these values likely reflect global conformational rearrangements of flexible regions rather than structural instability of the protein core. A similar trend is observed in the ligand case, where after fitting the protein, RMSD reaches values between 10 and 14 Å. Overall, the 2W3L–Hyb.96 system appears to undergo coupled protein–ligand rearrangement rather than maintaining its initial binding pose.

The 2W3L–Hyb.112 system exhibits different behavior, characterized by marked structural rearrangement with pronounced RMSD changes during 0–25 ns. The ligand RMSD shows similar behaviour, exhibiting substantial variations in the same interval, indicating major positional changes relative to the initial docked pose. Following this transition phase, the ligand RMSD stabilizes at roughly 8–10 Å, whereas the protein RMSD falls and varies within a smaller range (3–6 Å). This behavior implies that the complex experiences early protein–ligand rearrangement followed by persistence in a rearranged binding state rather than maintaining the original binding orientation. Thus, in the 2W3L system, Hyb.112 seems to adopt a less restricted and more flexible binding mode than Hyb.96.

In the 2YXJ context, the protein dynamic profiles are more moderate than those observed in the 2W3L systems. For the 2YXJ–Hyb.96 complex, the protein RMSD fluctuates within a relatively limited range of approximately 2.5–4.0 Å after the initial phase, indicating moderate structural stability of the protein framework. However, the ligand RMSD calculated after fitting the protein increases sharply and remains very high throughout the trajectory, indicating that the ligand does not preserve its initial docked orientation.

In the 2YXJ–Hyb.112 complex, the protein RMSD remains within a relatively moderate range throughout the simulation, generally around 2.5–4.0 Å, indicating limited structural rearrangement of the Bcl-XL framework. The ligand RMSD calculated after fitting the protein fluctuates within a more moderate interval, approximately 6–8 Å, after the initial phase of the trajectory. This behavior indicates that Hyb.112 does not fully preserve its initial docked orientation but also does not undergo the extensive positional displacement

observed for Hyb.96 in the same protein model. Overall, the 2YXJ–Hyb.112 system is consistent with a retained but dynamically adaptable binding mode, in which the ligand remains associated with the binding region while sampling positional adjustments during the simulation.

The protein RMSD in the 4LVT–Hyb.96 complex shows modest rearrangement of the Bcl-2 framework and stays within a reasonably moderate range throughout the simulation, typically around 2.5–4.0 Å after the initial equilibration phase. After fitting the protein, the ligand RMSD changes mostly within 7.5–10 Å, indicating positional fluctuation with respect to the original docked pose but without the large displacement seen in the less stable systems. All of these findings point to a somewhat flexible binding mode in the 4LVT model and ongoing ligand association with the binding region.

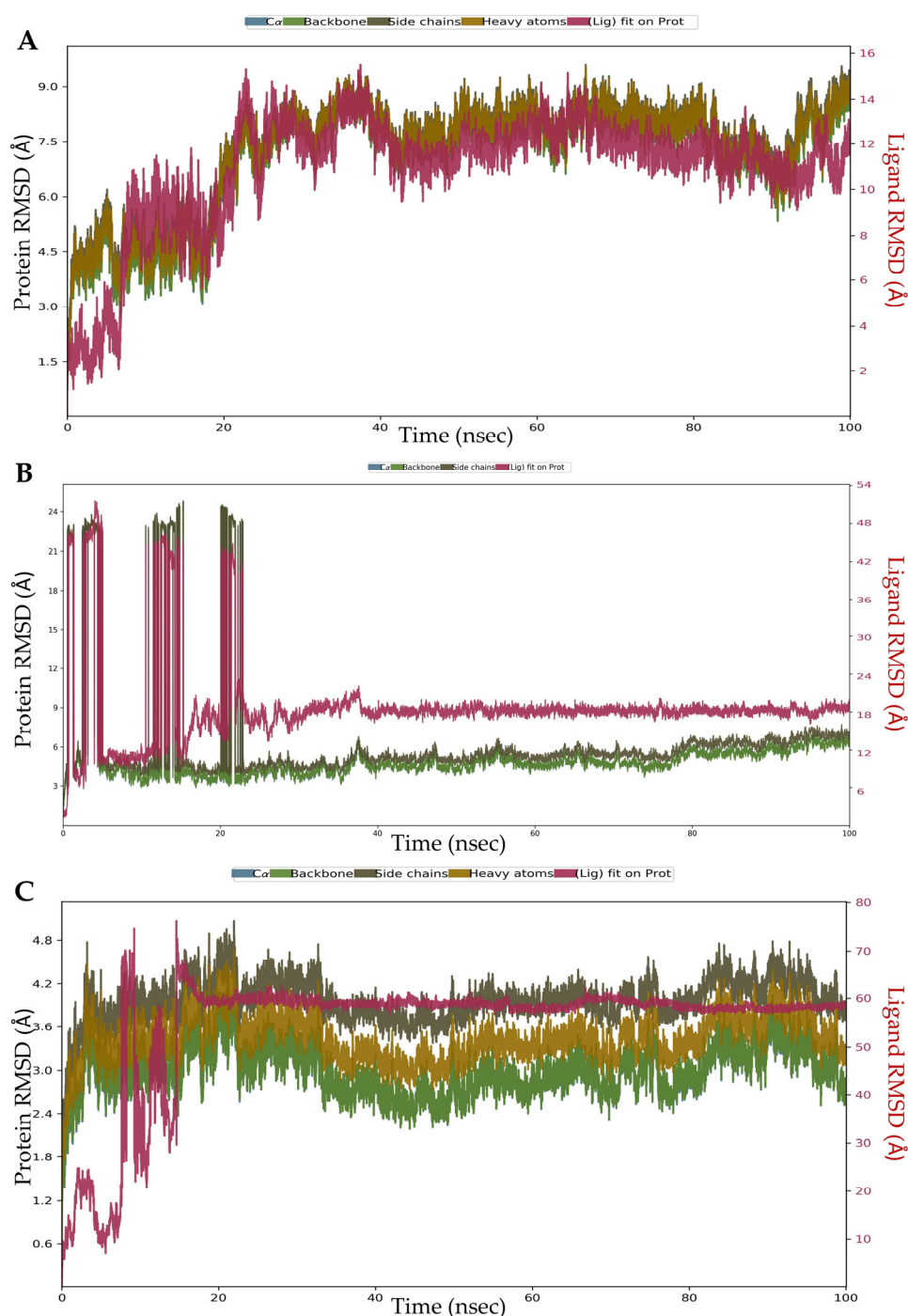


Figure 5. Cont.

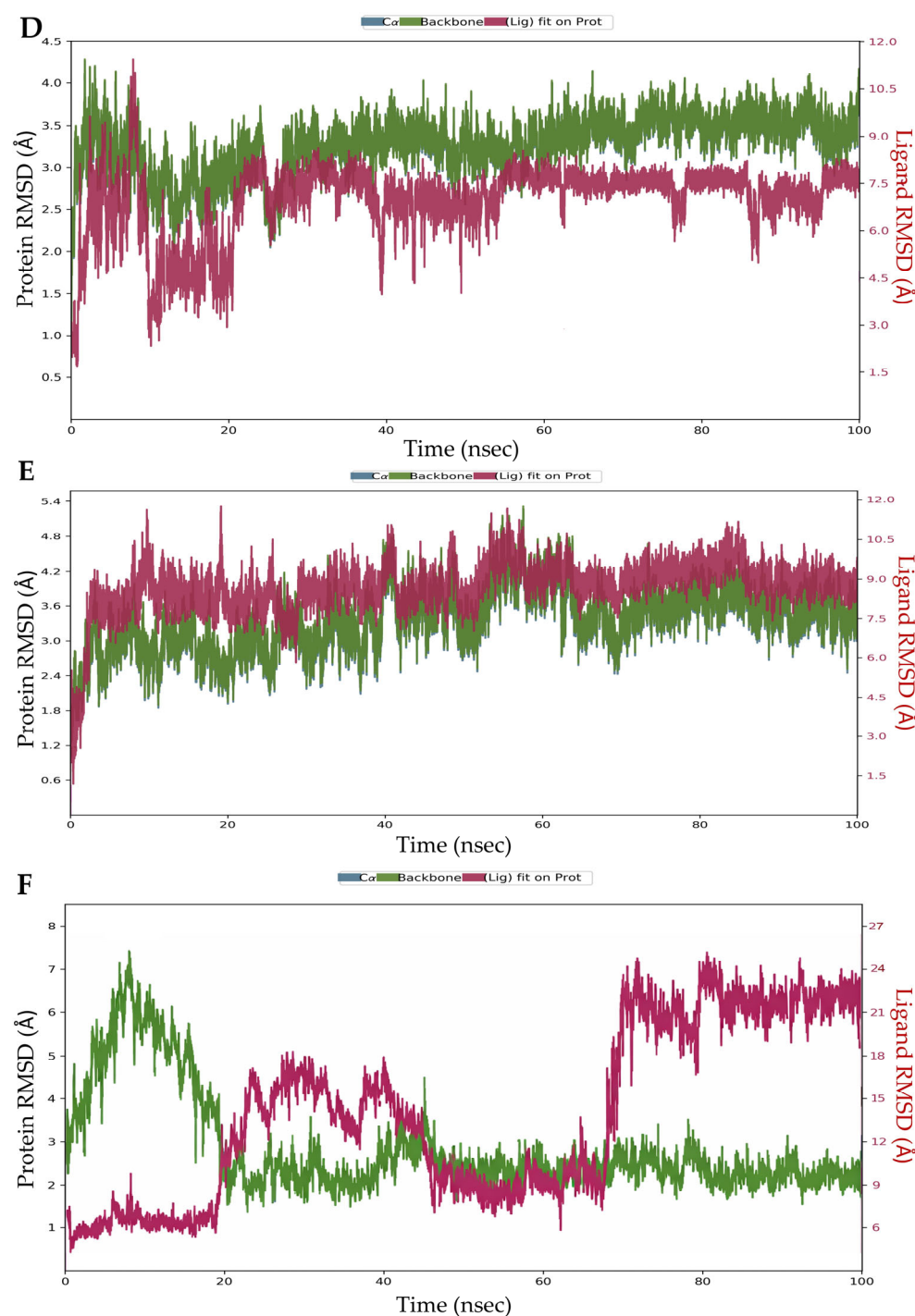


Figure 5. Protein–ligand RMSD trajectories for six complexes over 100 ns MD simulations. The left Y-axis corresponds to protein C α RMSD, whereas the right Y-axis corresponds to ligand RMSD relative to the protein. (A) 2W3L–Hyb.96 complex. (B) 2W3L–Hyb.112 complex. (C) 2YXJ–Hyb.96 complex. (D) 2YXJ–Hyb.112 complex. (E) 4LVT–Hyb.96 complex. (F) 4LVT–Hyb.112 complex.

The protein RMSD in the 4LVT–Hyb.112 complex exhibits a substantial increase in the early stages of the simulation, reaching values above 6 Å before declining after around 20 ns. For the remainder of the trajectory, it fluctuates within a smaller range of roughly 2–3 Å. This pattern points to a more stable dynamic state after the protein undergoes an initial conformational change. After fitting the protein, the ligand RMSD is computed and it also varies significantly over time. It starts out in a low range, rises to around 12–18 Å over the first half of the simulation, briefly drops at about 45–65 ns, and then rises once more to

about 20–24 Å after about 70 ns. These alterations show that Hyb.112 undergoes significant positional rearrangements in relation to the protein rather than maintaining a single binding orientation throughout the trajectory. All things considered, the 4LVT–Hyb.112 system is consistent with a very flexible binding mode that is marked by numerous transitions between different ligand locations throughout the simulation.

Overall, the MD results show that instead of retaining a consistently conserved binding pose throughout all simulations, both Hyb.96 and Hyb.112 experience system-dependent positional rearrangements. In the target-specific models, Hyb.96 exhibited more moderate positional behavior in the 4LVT system, whereas Hyb.112 showed a more retained and dynamically adjustable profile in the 2YXJ system. In the 2W3L chimeric model, on the other hand, both ligands displayed more noticeable protein–ligand rearrangement, which led to a more cautious interpretation.

Figure 6 depicts residue interaction histograms that allow direct comparison of binding patterns across all six protein–ligand complexes. The ligand in the 2W3L–Hyb.96 complex (Figure 6A) mostly interacts with the binding site through water bridges, hydrogen bonds and hydrophobic contacts. The largest interaction fractions are found in the 118–124 residue region, suggesting that Hyb.96 is bound to a specific area of the binding pocket. The high protein RMSD for this system may be attributed to a binding mechanism that allows limited flexibility while preserving persistent connections.

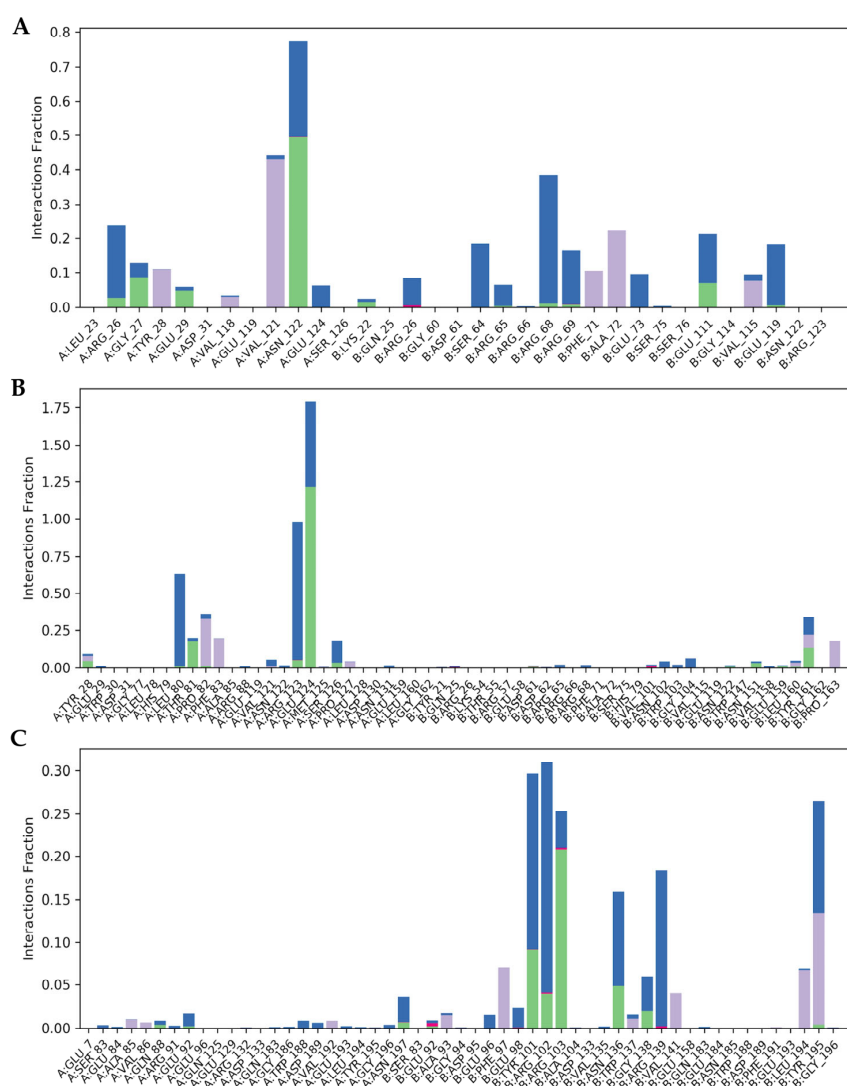


Figure 6. Cont.

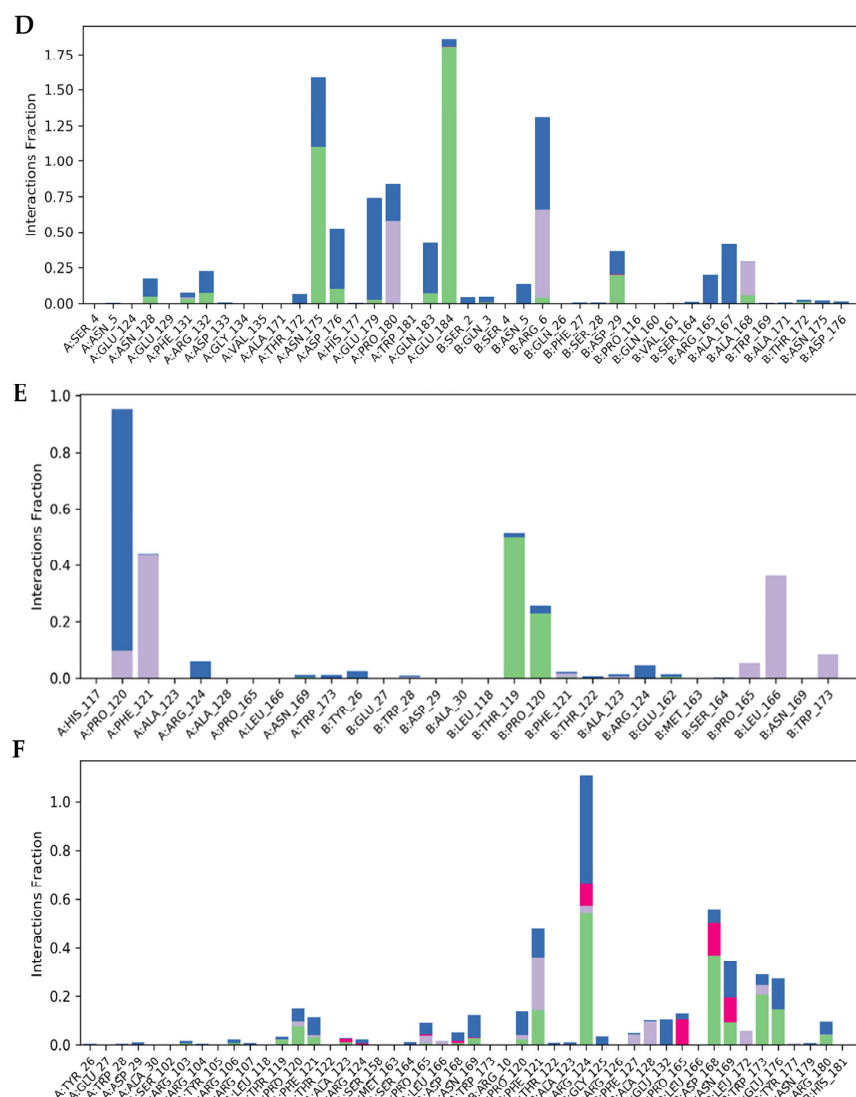


Figure 6. Protein–ligand interaction fractions during 100 ns MD simulations for six hybrid complexes 2W3L–Hyb.96 (A), 2W3L–Hyb.112 (B), 2YXJ–Hyb.96 (C), 2YXJ–Hyb.112 (D), 4LVT–Hyb.96 (E), and 4LVT–Hyb.112 (F). Bars represent the fraction of simulation time during which specific protein residues maintained interactions with the ligand. Interaction types are color-coded as follows: green: hydrogen bonds, purple: hydrophobic contacts, pink: ionic interactions, and blue: water bridges.

The 2W3L–Hyb.112 complex exhibits a more confined interaction pattern (Figure 5B). Specifically, GLU124 is the most frequently contacted residue; other residues like PRO82, PHE83, and LEU80 also contribute but with reduced consistency. A greater frequency of solvent-mediated interactions implies a less restricted binding mechanism and enhanced ligand mobility.

The interaction profile for the 2YXJ–Hyb.96 complex (Figure 6C) is thoroughly dispersed over several residues, with interactions being mostly hydrophobic and water-mediated. The residues TYR101, ARG102, ARG103, ASN136, ARG139, and TYR195 had the most consistent interactions with Hyb.96. These interactions, however, are more consistent with a constantly rearranged binding mode than with rigid retention of the initial docked orientation, given the RMSD behavior.

The 2YXJ–Hyb.112 complex shows a larger interaction network (Figure 6D). A distributed and permanent contact network is supported by the most notable interaction hotspots, which are ASN175, ASN179, PRO180, GLU 184 and ARG6.

The interaction pattern of the 4LVT–Hyb.96 complex (Figure 6E) is very confined, with PRO120, PHE121, THR119, and LEU166 being the most notable interactions. While PHE121 and LEU166 also contribute significant contact fractions, suggesting ongoing ligand association within a specific pocket region, water-mediated interactions involving PRO120 are particularly persistent.

The majority of residues in the 4LVT–Hyb.112 complex show interaction percentages between around 0.2 and 0.5, while fewer residues achieve values between 0.6 and 0.8, suggesting a dispersed interaction pattern (Figure 6F). The dominating interactions are linked to ARG124, PHE121, ASP168, ASN169, and TRP173. This is consistent with a wider interaction network than that seen for Hyb.96 in the same system.

Overall, both ligands retain residue-level connections throughout the simulations, although the distribution of these interactions varies depending on the system. Hyb.112 typically displays a broader and more dynamic interaction network, which supports its higher conformational flexibility seen throughout the simulations. At the same time, Hyb.96 typically displays more confined interaction patterns in certain systems.

The ligand RMSF profiles (Figure 7) describe the intramolecular flexibility of Hyb.96 and Hyb.112 in all three protein systems. The 2W3L–Hyb.96 complex (Panel A) has ligand atoms with RMSF values ranging from 2 to 4 Å, with a few peaks reaching 5–6 Å, indicating considerable flexibility without large-amplitude motions. In contrast, the 2W3L–Hyb.112 complex (Panel B) has significantly higher fluctuations, typically between 11 and 13 Å, with peaks approaching 14 Å, indicating improved intramolecular mobility and fewer positional restrictions inside the binding pocket. A similar pattern is seen in the 2YXJ system. RMSF values for 2YXJ–Hyb.96 (Panel C) range between 2 and 4 Å, with localized maxima around 5–6 Å, indicating moderate and spatially restricted flexibility. Hyb.112 (Panel D) exhibits a wider fluctuation interval of 6–10 Å, with peaks reaching 10–12 Å, indicating improved conformational flexibility across numerous ligand segments. In the 4LVT system, Hyb.96 (Panel E) has constant low RMSF values of 1.5–2.2 Å, with only modest increases for certain residues, indicating a well-restricted conformation. Hyb.112 (Panel F) exhibits high fluctuations, with numerous peaks reaching 9–11 Å, indicating higher intramolecular mobility and conformational flexibility inside the binding region.

Considering all of the above, Hyb.96 has overall lower RMSF values across all systems, indicating a less flexible and more structurally constrained ligand conformation than Hyb.112, whereas the latter shows higher intramolecular flexibility, allowing for more dynamic and adaptable conformational behavior. These RMSF differences represent internal ligand mobility and were evaluated alongside the RMSD analysis, which shows system-dependent conformational behaviour relative to the protein.

The ligand property profiles (Figure 8) describe the evolution of Hyb.96 and Hyb.112 within the binding pockets across the three protein systems, integrating RMSD, radius of gyration, surface descriptors, and intramolecular hydrogen bonding to assess positional stability, compactness, and conformational variability.

In the 2W3L system, Hyb.96 (Figure 8A) exhibits consistently narrow fluctuation ranges across all descriptors, with RMSD values of approximately 1.0–2.0 Å and radius of gyration values around 6.3–7.2 Å, indicating a relatively compact ligand conformation. Surface descriptors show minimal variation, and the absence of intramolecular hydrogen bonds suggests a structurally constrained ligand. In contrast, Hyb.112 (Figure 8B) displays broader fluctuations, with RMSD values of approximately 1.5–3.5 Å and a wider radius of gyration range of 6.0–7.2 Å, indicating increased conformational variability. The presence of intermittent intramolecular hydrogen bonds and variable surface descriptors suggests dynamic structural rearrangements and changing solvent exposure.

A similar trend is observed in the 2YXJ system. Hyb.96 (Figure 8C) maintains relatively narrow fluctuation ranges (RMSD 0.8–2.5 Å; Rg 6.5–7.5 Å) consistent with a generally compact ligand conformation. In contrast, Hyb.112 (Figure 8D) exhibits moderate fluctuations (RMSD 0.5–4.5 Å; Rg 6.5–7.5 Å) with intermittent intramolecular hydrogen bonds and gradual variations in surface descriptors, indicating controlled conformational adaptability.

In the 4LVT system, Hyb.96 (Figure 8E) again displays limited fluctuations (RMSD 1.5–2.3 Å; Rg 6.8–7.4 Å), with stable surface descriptors and no intramolecular hydrogen bonds, reflecting a constrained conformation. Conversely, Hyb.112 (Figure 8F) shows broader variability (RMSD 0.7–3.0 Å; Rg 5.5–7.0 Å), accompanied by intermittent intramolecular hydrogen bonding and increased variation in surface descriptors, indicating enhanced conformational flexibility and dynamic solvent exposure.

Overall, these descriptors consistently indicate that Hyb.96 maintains a more compact and less variable conformation across all investigated systems, whereas Hyb.112 undergoes continuous structural adjustments, reflecting a more flexible and adaptable conformational profile.

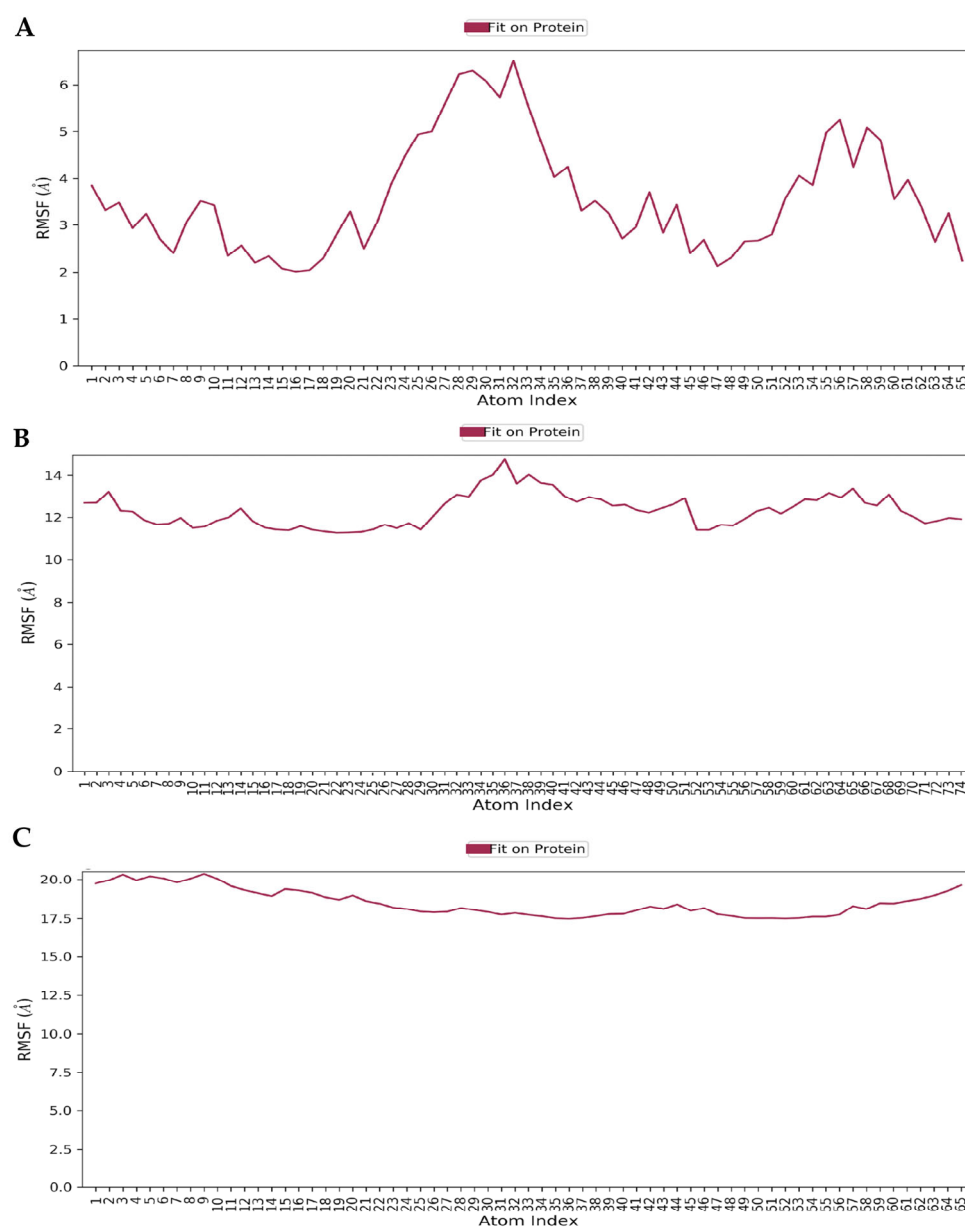


Figure 7. Cont.

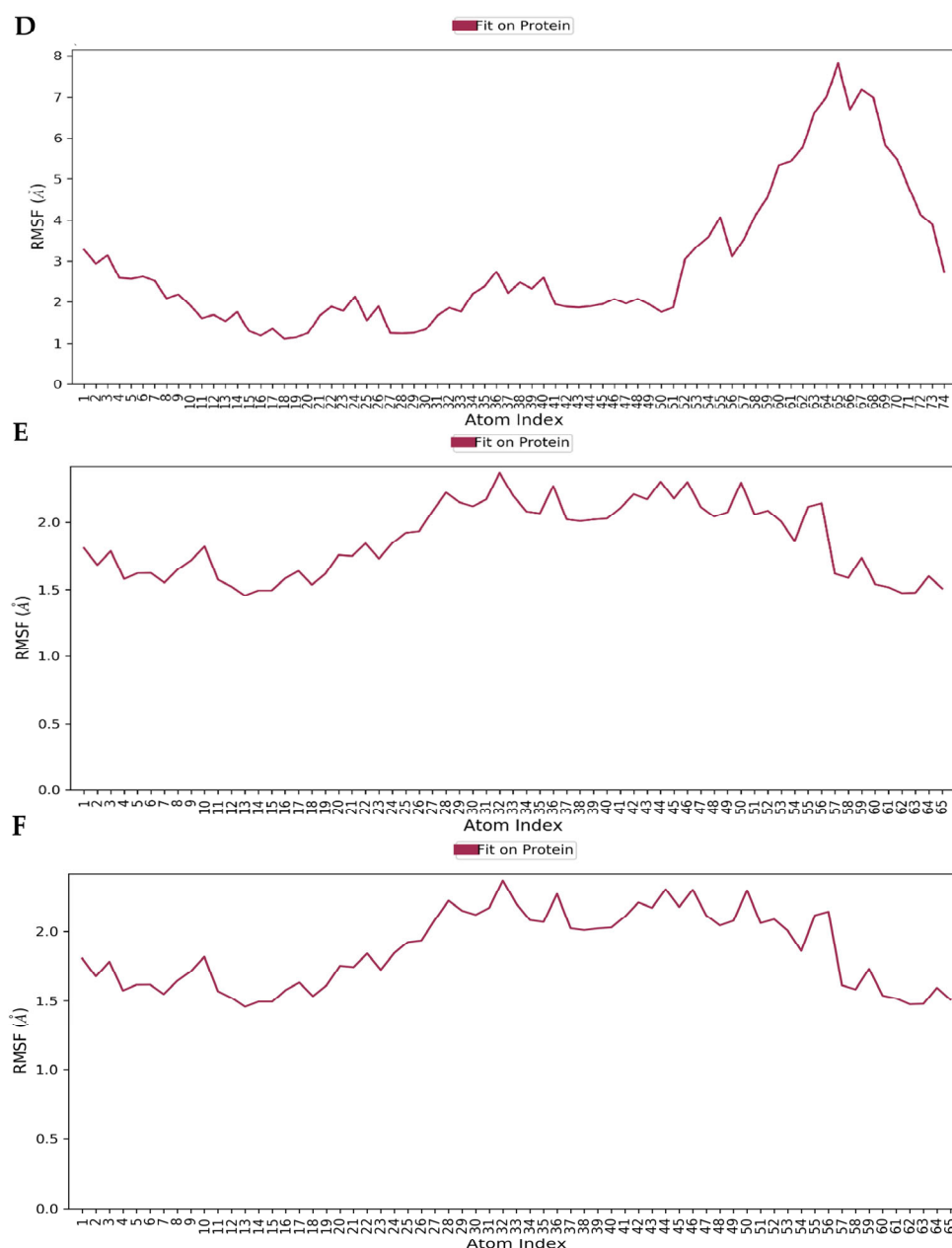


Figure 7. Flexibility and compactness profiles of the hybrid ligands Hyb.96 and Hyb.112 across the two protein targets 2W3L and 2YXJ. Panels A–F correspond to 2W3L–Hyb.96 (A), 2W3L–Hyb.112 (B), 2YXJ–Hyb.96 (C), 2YXJ–Hyb.112 (D), 4LVT–Hyb.96 (E), and 4LVT–Hyb.112 (F), respectively. For each complex, the ligand heavy-atom RMSF (aligned on the protein backbone) is shown together with the protein C α RMSF per residue, where peaks reflect locally flexible regions of the protein.

The comparative assessment of the six hybrid–protein systems, summarized in Table 5, indicates that both Hyb.96 and Hyb.112 display system-dependent dynamic behavior rather than a uniformly retained binding pose across all complexes. In the 2W3L system, both ligands undergo pronounced protein–ligand rearrangement, with Hyb.96 showing ligand RMSD values of approximately 10–14 Å and Hyb.112 stabilizing at approximately 8–10 Å after an initial transition phase. These trends suggest that neither ligand preserves its initial docked orientation in the chimeric model, although Hyb.112 appears to persist in a rearranged binding state after the early conformational adjustment.

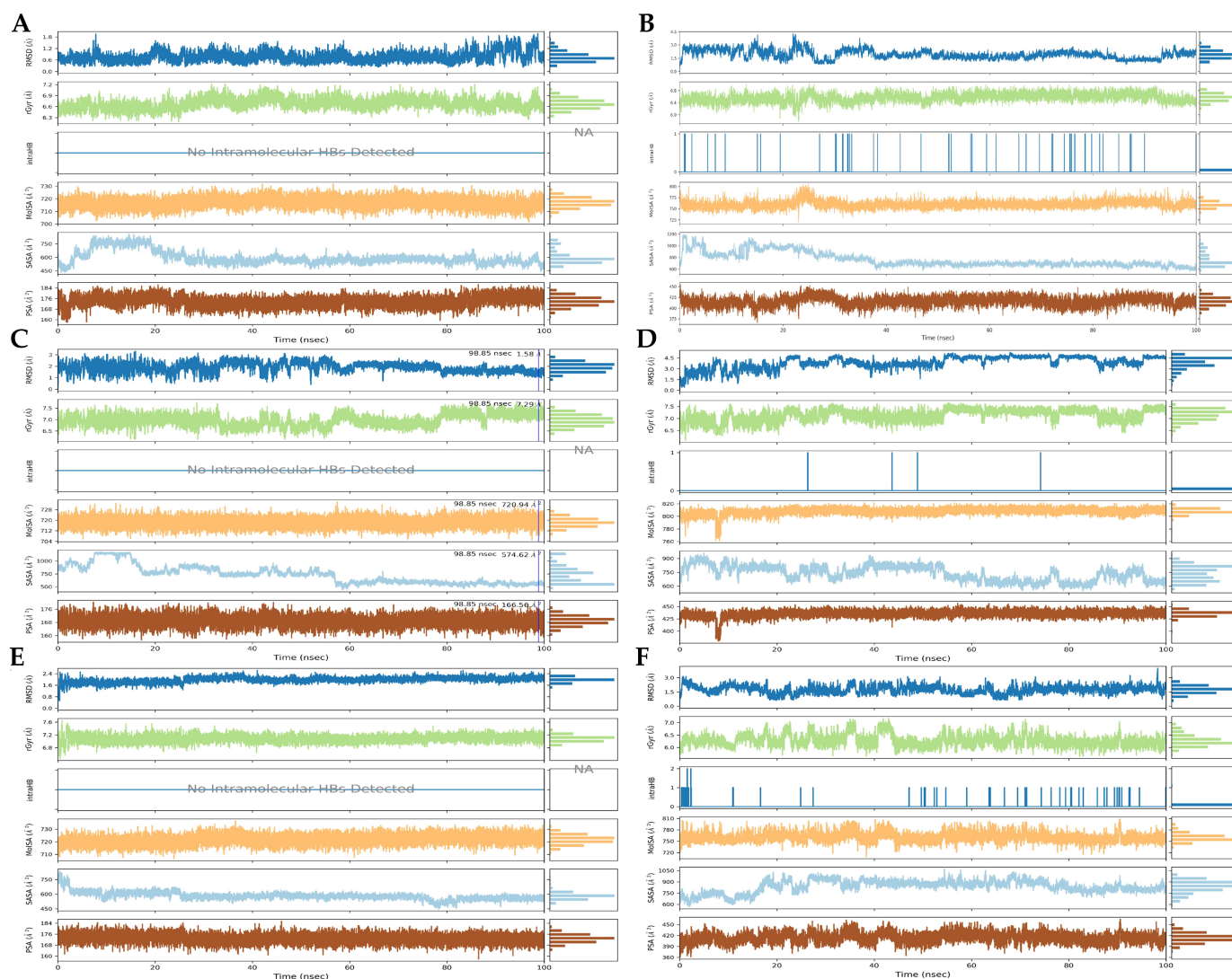


Figure 8. Ligand physicochemical stability and compactness profiles for the hybrid ligands Hyb.96 and Hyb.112 across the 2W3L and 2YXJ protein targets. Panels (A–F) correspond to 2W3L–Hyb.96 (A), 2W3L–Hyb.112 (B), 2YXJ–Hyb.96 (C), 2YXJ–Hyb.112 (D), 4LVT–Hyb.96 (E), and 4LVT–Hyb.112 (F).

In the 2YXJ and 4LVT systems, the dynamic behavior is more differentiated. In the 2YXJ model, Hyb.112 shows the more retained and dynamically adaptable profile, with ligand RMSD values of approximately 6–8 Å, whereas Hyb.96 undergoes extensive positional displacement relative to the protein. In the 4LVT model, the opposite trend is observed: Hyb.96 shows more moderate positional behavior, while Hyb.112 exhibits broader ligand rearrangements and transitions between multiple positions during the trajectory. Thus, the target-specific structures do not support a single global ranking of ligand stability but instead indicate that the dynamic behavior of each hybrid depends on the protein environment.

Both ligands continue to interact at the residue level throughout the simulations, although the distribution and durability of such interactions vary between systems. While Hyb.96 exhibits more confined interaction patterns in specific complexes, Hyb.112 often interacts through a larger and more dynamic network, including many residues. These results, along with the ligand property profiles, show that Hyb.112 exhibits greater conformational plasticity, whereas Hyb.96 tends to maintain a more compact ligand conformation.

Table 5. Comparative summary of MD-derived dynamic parameters for the six hybrid ligands bound to the three protein targets.

Ligand–Protein Complex	Protein RMSD (Å)	Ligand RMSD (Å)	H-Bonds (≥0.1 frac)	Hydrophobic Contacts (≥0.1)	Water Bridges (≥0.1)
2W3L–Hyb.96	7.0–9.0	10.0–14.0	Asn122, Gly27	Val121, Tyr28, Phe71, Ala72	Arg26, Gly27, Asn122, Ser64, Arg68, Arg69, Glu111, Asn122
2W3L–Hyb.112	3.0–6.0 *	8.0–10.0 *	Ser25, Arg23	Phe82, Ala83, Gly162, Pro163	Thr80, Phe82, Glu123, Met124, Pro126, Gly162, Pro163
2YXJ–Hyb.96	2.5–4.0	58.0–62.0	Arg102, Ala103, Asn135	Gly195	Arg101, Arg102, Ala103, Asn136, Val139, Gly195
2YXJ–Hyb.112	2.5–4.0	6.0–8.0	Asn175, Glu184, Pro29, His176	Pro180, Arg6, Ala168	Asn175, His176, Glu179, Pro180, Gln183, Arg6, Pro29, Ala167, Ala168
4LVT–Hyb.96	2.5–4.0	7.5–10.0	Thr119, Pro120	Phe121, Leu166	His117, Thr119, Pro120
4LVT–Hyb.112	2.0–3.0 *	12.0–24.0	Gly125, Asn168, Glu173, Tyr176	Phe121, Glu165, Asp166	Gly125, Phe121, Asn168, Glu173, Tyr176

* For 2W3L–Hyb.112 and 4LVT–Hyb.112, these ranges describe the post-transition regime, while the trajectories also show larger early excursions during the initial rearrangement phase.

3.3. MM-GBSA Binding Free Energy Analysis

Table 6 depicts the MM-GBSA binding free energy breakdown for the 2W3L–Hyb.96 and 2W3L–Hyb.112 complexes. It provides a quantitative overview of the energetic factors that affect ligand binding in the 2W3L binding pocket. In both systems, van der Waals contributions are the most important intermolecular contacts. The Δ VDWAALS values are -28.41 kcal/mol for Hyb.96 and -30.38 kcal/mol for Hyb.112. This means that the binding site has strong dispersion interactions and is densely packed with hydrophobic molecules.

Table 6. Binding free energy (kcal/mol) for Hyb.96 and Hyb.112 against the 2W3L protein (post 100 ns MDS) calculated by MM-GBSA analysis.

Energy Component	$\Delta G(\text{MM-GBSA}) \pm \text{SE}$ 2W3L–Hyb.96	$\Delta G(\text{MM-GBSA}) \pm \text{SE}$ 2W3L–Hyb.112
Δ VDWAALS	-28.41 ± 0.29	-30.38 ± 0.06
Δ EEL	-0.13 ± 0.27	-22.21 ± 0.11
Δ EGB	12.97 ± 0.26	34.45 ± 0.10
Δ ESURF	-3.75 ± 0.04	-3.78 ± 0.01
Δ GGAS	-28.54 ± 0.37	-52.59 ± 0.14
Δ GSOLV	9.22 ± 0.25	30.67 ± 0.09
Δ TOTAL	-19.33 ± 0.24	-21.92 ± 0.07

A marked difference is observed in the electrostatic component, where Hyb.96 exhibits an almost neutral contribution (Δ EEL = -0.13 kcal/mol), whereas Hyb.112 shows a stronger electrostatic stabilization (Δ EEL = -22.21 kcal/mol). This aspect reflects increased involvement of charge-based interactions with residues in the binding pocket. However, the polar solvation term mostly cancels out this electrostatic contribution. This introduces a higher penalty for Hyb.112 (Δ EGB = $+34.45$ kcal/mol) compared to Hyb.96 (Δ EGB = $+12.97$ kcal/mol), reflecting the energetic cost associated with desolvation of polar groups upon complex formation.

The nonpolar solvation contribution (Δ ESURF = -3.75 kcal/mol) for both ligands yields a similar stabilizing effect, aligning with the entrapment of hydrophobic surface

area upon binding. The gas-phase interaction energy is much more favorable for Hyb.112 ($\Delta G_{\text{GAS}} = -52.59$ kcal/mol) than for Hyb.96 ($\Delta G_{\text{GAS}} = -28.54$ kcal/mol). This shows that intermolecular interactions are stronger when there are no solvent effects.

Still, the overall solvation free energy largely cancels out these gas-phase contributions. For example, Hyb.112 ($\Delta G_{\text{SOLV}} = +30.67$ kcal/mol) is much greater than that for Hyb.96 ($\Delta G_{\text{SOLV}} = +9.22$ kcal/mol). This balance between intermolecular interactions and solvation effects prevents Hyb.112 from having a net energy advantage.

Consequently, the total binding free energies of the two systems remains comparable. This indicates that although Hyb.112 forms stronger direct interactions within the binding pocket, its calculated binding free energy is influenced by higher desolvation penalties, resulting in a modest overall energetic outperformance of Hyb.96. These findings are consistent with the dynamic MD behavior of Hyb.112, where it showed increased conformational variability, while Hyb.96 maintained a more constrained binding mode.

The MM-GBSA binding free energy breakdown for the 2YXJ–Hyb.96 and 2YXJ–Hyb.112 complexes is provided in Table 7. A marked difference is observed in the estimated binding free energy of the two ligands, reflected by the total binding free energy values of -4.95 kcal/mol for Hyb.96 and -29.81 kcal/mol for Hyb.112.

Table 7. Binding free energy (kcal/mol) for hybrid ligands Hyb.96 and Hyb.112 against the 2YXJ protein, calculated by MM-GBSA analysis.

Energy Component	$\Delta G(\text{MM-GBSA}) \pm \text{SE}$ 2YXJ–Hyb.96	$\Delta G(\text{MM-GBSA}) \pm \text{SE}$ 2YXJ–Hyb.112
$\Delta \text{VDWAALS}$	-6.73 ± 1.82	-55.54 ± 0.45
ΔEEL	-2.67 ± 0.81	-19.52 ± 1.02
ΔEGB	5.37 ± 1.53	52.81 ± 0.96
ΔESURF	-0.92 ± 0.25	-7.55 ± 0.04
ΔGAS	-9.40 ± 2.62	-75.07 ± 1.11
ΔGSOLV	4.45 ± 1.28	45.26 ± 0.95
ΔTOTAL	-4.95 ± 1.34	-29.81 ± 0.41

The 2YXJ–Hyb.96 complex is characterized by modest stabilization, as indicated by the overall binding free energy. The favorable contributions come from van der Waals interactions ($\Delta \text{VDWAALS} = -6.73$ kcal/mol) and electrostatic interactions ($\Delta \text{EEL} = -2.67$ kcal/mol), reflecting limited intermolecular contacts within the binding pocket. These interactions are partially offset by the polar solvation term ($\Delta \text{EGB} = +5.37$ kcal/mol) and the total solvation free energy ($\Delta \text{GSOLV} = +4.45$ kcal/mol). These terms introduce desolvation penalties that reduce the estimated binding free energy. In addition, the large standard errors associated with ΔGAS and ΔTOTAL suggest sensitivity to conformational sampling, indicating variability in the energetic profile of this system.

The 2YXJ–Hyb.112 complex exhibits a more favorable binding free energy, with a ΔTOTAL value of -29.81 kcal/mol. This difference primarily stems from a dominant van der Waals contribution ($\Delta \text{VDWAALS} = -55.54$ kcal/mol) and a significant electrostatic component ($\Delta \text{EEL} = -19.52$ kcal/mol). These contributions result in a strongly favorable gas-phase interaction energy ($\Delta \text{GAS} = -75.07$ kcal/mol), substantially larger in magnitude than that observed for Hyb.96.

However, this gas-phase stabilization is accompanied by a significant polar solvation penalty ($\Delta \text{EGB} = +52.81$ kcal/mol) and a large total solvation contribution ($\Delta \text{GSOLV} = +45.26$ kcal/mol), reflecting the binding energetic cost associated with desolvation of polar and charged groups.

The MM-GBSA binding free energy decomposition for both 4LVT complexes is depicted in Table 8. The total binding free energy values are very similar for the two systems,

with -31.08 kcal/mol and -31.82 kcal/mol for Hyb.96 and Hyb.112, respectively, indicating comparable predicted energetic stabilization.

Table 8. Binding free energy (kcal/mol) for hybrid ligands Hyb.96 and Hyb.112 against the 4LVT protein, calculated by MM-GBSA analysis.

Energy Component	$\Delta G(\text{MM-GBSA}) \pm \text{SE}$ 4LVT-Hyb.96	$\Delta G(\text{MM-GBSA}) \pm \text{SE}$ 4LVT-Hyb.112
$\Delta \text{VDWAALS}$	-41.35 ± 0.52	-48.27 ± 0.61
ΔEEL	-6.42 ± 0.48	-14.63 ± 0.72
ΔEGB	21.87 ± 0.67	37.12 ± 0.85
ΔESURF	-5.18 ± 0.09	-6.04 ± 0.11
ΔGGAS	-47.77 ± 0.71	-62.90 ± 0.93
ΔGSOLV	16.69 ± 0.74	31.08 ± 0.89
ΔTOTAL	-31.08 ± 0.53	-31.82 ± 0.58

For the 4LVT-Hyb.96 complex, van der Waals interactions ($\Delta \text{VDWAALS} = -41.35$ kcal/mol) induce stabilization, reflecting efficient hydrophobic packing within the binding site. Electrostatic interactions also contribute to binding ($\Delta \text{EEL} = -6.42$ kcal/mol), although to a lesser extent. The resulting gas-phase interaction energy ($\Delta \text{GGAS} = -47.77$ kcal/mol) indicates favorable intermolecular contacts between the ligand and the protein.

In the 4LVT-Hyb.112 complex, both van der Waals ($\Delta \text{VDWAALS} = -48.27$ kcal/mol) and electrostatic contributions ($\Delta \text{EEL} = -14.63$ kcal/mol) are more pronounced, leading to a more favorable gas-phase interaction energy ($\Delta \text{GGAS} = -62.90$ kcal/mol). These values indicate that Hyb.112 outperforms Hyb.96 in terms of direct intermolecular interactions and more extensive contact formation within the binding pocket.

However, the increased interaction strength in the Hyb.112 complex is accompanied by a substantially larger polar solvation penalty ($\Delta \text{EGB} = +37.12$ kcal/mol) and a higher total solvation contribution ($\Delta \text{GSOLV} = +31.08$ kcal/mol), compared to $+21.87$ kcal/mol and $+16.69$ kcal/mol for Hyb.96, respectively.

The combined structural, dynamical, and energetic analyses reveal that binding behaviors for Hyb.96 and Hyb.112 against the three protein targets differed. The MM-GBSA results showed that binding free energies varied depending on the protein model, with values ranging from -4.95 to -31.82 kcal/mol. This variation was most evident in the case of Hyb.96, which showed modest stabilization in the 2YXJ protein ($\Delta \text{TOTAL} = -4.95$ kcal/mol) but clearly more favorable binding in the 4LVT target ($\Delta \text{TOTAL} = -31.08$ kcal/mol). This difference is consistent with Hyb.96's behaviour during MD analysis, where marked conformational displacement occurred in the 2YXJ model, whereas in the 4LVT system its behaviour was more moderate. In contrast, Hyb.112 showed more favorable MM-GBSA values across all three protein targets, particularly in 2YXJ ($\Delta \text{TOTAL} = -29.81$ kcal/mol) and 4LVT ($\Delta \text{TOTAL} = -31.82$ kcal/mol). Nevertheless, these values were accompanied by large polar solvation penalties, especially in the 2YXJ complex. The presented results show that the predicted binding energy values are influenced by the specific protein environment and by the balance between favorable van der Waals/electrostatic interactions and opposing solvation contributions. Therefore, the MM-GBSA values should be interpreted as comparative energetic estimates rather than absolute measures of binding affinity.

Beyond the computational results obtained in the present study, the hybrid design of Hyb.96 and Hyb.112 is supported by previous reports describing betulinic acid- or lupane-type triterpenoid hybrids with other natural-product fragments.

A particularly relevant example is represented by betulinic acid-brosimine B hybrids, in which BA was conjugated with a natural polyphenolic/flavonoid-type moiety. These hy-

brids were investigated as anticancer candidates [49], and a related BA–brosimine B hybrid later showed cytotoxic activity in imatinib-sensitive and imatinib-resistant chronic myeloid leukemia cells, inducing intrinsic apoptosis through caspase-3/caspase-9 activation [50]. This provides a useful precedent for the BA–Proanthocyanidin B2 hybrid evaluated here because both designs combine BA with a polyphenolic natural-product fragment. In addition, lupane–coumarin conjugates, such as betulin esters with coumarin-3-carboxylic acid [51], and other triterpene–coumarin conjugates have been reported, supporting the broader feasibility of linking pentacyclic triterpenoid scaffolds to aromatic/phenolic bioactive fragments [52].

For the BA–Celastrol hybrid, direct experimental evidence for a covalent BA–celastrol derivative appears limited; however, BA and celastrol have been investigated in combination and co-delivery systems, where celastrol was reported to sensitize tumor-associated stromal components and enhance the anticancer efficacy of BA [53]. Taken together, these studies do not demonstrate direct Bcl-2/Bcl-xL inhibition by the specific hybrids evaluated in the present work, but they support the medicinal-chemistry rationale for exploring BA-based hybrids with polyphenolic or triterpenoid fragments as apoptosis-oriented anticancer candidates. It should also be noted that MM-GBSA calculations have intrinsic methodological limitations. The method provides an approximate post-processing estimate of binding free energy and does not fully account for conformational entropy, receptor flexibility, explicit solvent effects, or the complete range of ligand-binding states sampled during molecular dynamics simulations. In addition, the results may depend on the selected trajectory snapshots and on the conformational state of the protein–ligand complex. For this reason, the MM-GBSA values reported here were used to support comparative prioritization of the selected hybrids rather than to provide definitive quantitative binding free energy estimates.

3.4. ADMET Study

The predicted ADMET and toxicity profiles of Hyb.96 and Hyb.112 (Table 9) indicate differences in their predicted pharmacokinetic and biological risk characteristics. At the absorption level, both compounds showed low predicted aqueous solubility together with high predicted intestinal absorption. The predicted Caco-2 permeability was higher for Hyb.96 than for Hyb.112, suggesting that Hyb.96 may have a greater tendency for passive membrane permeation, whereas Hyb.112 may show more limited transcellular transport. Both compounds were predicted to be substrates of P-glycoprotein, indicating that efflux transport may influence intracellular exposure. In addition, Hyb.112 was also predicted to act as a P-glycoprotein inhibitor, pointing to a potentially more complex interaction with transporter-mediated processes.

There were also differences in the expected distribution profiles. Hyb.96 had a lower estimated volume of distribution and a smaller unbound fraction. On the other hand, Hyb.112 had a higher predicted volume of distribution and a higher unbound fraction. These predictions correspond with a more extensive tissue distribution for Hyb.112. There was also a significant difference in blood–brain barrier prediction for both compounds. Hyb.96 was predicted to be BBB-permeable, whereas Hyb.112 was not expected to perform well. This pattern is in line with the CNS-related goals that were predicted for Hyb.96, such as BBB penetration and GABA receptor interaction (Table 9).

Table 9. Selected predicted ADMET properties of Hyb.96 and Hyb.112 with concise pharmacokinetic interpretation.

Parameter	Hyb.96	Hyb.112	Interpretation
Water solubility (log mol/L)	−2.918	−2.892	Both hybrids show low aqueous solubility, suggesting possible formulation-related limitations.
Caco-2 permeability (log P _{app} , 10 ^{−6} cm/s)	0.567	−0.180	Hyb.96 is predicted to have better passive membrane permeability than Hyb.112.
Intestinal absorption (%)	100.0	89.621	Both compounds show high predicted intestinal absorption, although Hyb.96 appears more favorable.
P-gp substrate	Yes	Yes	Both hybrids may be subject to efflux transport, which could reduce intracellular retention.
P-gp inhibitor I	No	Yes	Only Hyb.112 is predicted to inhibit P-gp I, suggesting a higher potential for transporter-mediated interactions.
P-gp inhibitor II	Yes	Yes	Both compounds may interfere with P-gp-related transport processes.
VD _{ss} (human, log L/kg)	−1.228	−0.024	Hyb.112 shows a higher predicted distribution volume than Hyb.96.
Fraction unbound (F _u)	0.196	0.388	Hyb.112 is predicted to have a larger unbound fraction in plasma, whereas Hyb.96 may be more strongly protein-bound.
BBB permeability (logBB)	0.860	−2.529	Hyb.96 may cross the blood–brain barrier, whereas Hyb.112 is predicted to have negligible BBB penetration.
CNS permeability (logPS)	−0.492	−2.844	CNS exposure is predicted to be substantially lower for Hyb.112 than for Hyb.96.
CYP3A4 substrate	Yes	Yes	Both hybrids may undergo CYP3A4-mediated metabolism.
Major CYP inhibition	None predicted	None predicted	Neither compound is predicted to inhibit the main CYP isoforms, suggesting a lower risk of broad CYP-mediated drug–drug interactions.
Total clearance (log mL/min/kg)	−1.702	−2.683	Hyb.112 is predicted to have lower clearance and may therefore persist longer systemically.
AMES toxicity	No	No	No mutagenicity signal was predicted for either compound.
hERG I inhibitor	No	No	No hERG I liability was predicted.
hERG II inhibitor	No	Yes	Hyb.112 shows a potential cardiotoxicity-related warning signal that warrants caution.
Hepatotoxicity	No	No	No hepatotoxicity is predicted.

From a metabolic standpoint, both compounds were expected to be substrates of CYP3A4 and exhibited no inhibitory effects on the principal CYP isoforms included in the model. In addition, both compounds had low anticipated clearance, especially Hyb.112, which could mean that they take longer to be eliminated.

The combined ADME and toxicity predictions indicate that Hyb.96 and Hyb.112 differ in their predicted permeability, distribution, and toxicity-related profiles. Hyb.96

was associated with higher predicted permeability and possible central nervous system exposure, whereas Hyb.112 was associated with lower predicted permeability and broader predicted tissue distribution.

3.5. *pdCSM Anticancer Activity Prediction*

The antiproliferative potential of the two hybrid molecules, Hyb.96 and Hyb.112, was evaluated using the *pdCSM*-cancer predictive model. This *in silico* approach provides estimated GI_{50} values in micromolar units, corresponding to the concentration required to achieve 50% inhibition of tumor cell growth across a panel of human cancer cell lines.

The predicted GI_{50} values presented in Table 10 range from 5.010 to 7.639 μ M for Hyb.96 and from 5.041 to 6.767 μ M for Hyb.112 across the investigated cancer cell lines.

Table 10. *In silico*-predicted GI_{50} values (μ M) for Hybrid 96 and Hybrid 112 across multiple cancer cell lines using the *pdCSM*-cancer platform.

Cancer Type	Cell Line (Hybrid 96)	GI_{50} —Hyb. 96	Cell Line (Hybrid 112)	GI_{50} —Hyb. 112
Breast cancer	Breast T-47D	6.493	Breast MDA-MB-468	6.317
CNS (central nervous system)	CNS SF-539	5.821	CNS XF-498	5.896
Colon cancer	Colon HCT-116	5.419	Colon KM12	5.436
Leukemia	Leukemia P388	7.470	Leukemia P388	6.637
Melanoma	Melanoma MDA-MB-435	6.661	Melanoma MDA-N	5.519
Non-small cell lung cancer	NSCLC HOP-18	5.586	NSCLC A549_ATCC	5.240
Ovarian cancer	Ovarian NCI_ADR_RES	6.382	Ovarian NCI_ADR_RES	5.212
Prostate cancer	Prostate PC-3	5.010	Prostate PC-3	5.133
Renal cancer	Renal SN12K1	7.639	Renal SN12K1	6.767
Small-cell lung cancer	Small Cell Lung DMS-273	5.150	Small Cell Lung DMS-114	5.041

These values show that there is a moderate predicted antiproliferative activity for both compounds. The lowest estimated GI_{50} values for Hyb.96 were 5.010 μ M for prostate PC-3, 5.419 μ M for colon HCT-116, and 5.586 μ M for non-small cell lung cancer HOP-18. The highest values were 7.470 μ M for leukemia P388 and 7.639 μ M for kidney SN12K1. For Hyb.112, the lowest predicted GI_{50} values were found for small cell lung cancer DMS-114 (5.041 μ M), ovarian cancer NCI_ADR_RES (5.212 μ M), and non-small cell lung cancer A549_ATCC (5.240 μ M). The highest values were found for leukemia P388 (6.637 μ M) and renal cancer SN12K1 (6.767 μ M). Both hybrids show a rather stable projected activity profile across different cancer cell lines, with just small differences based on tumor type. The differences between Hyb.96 and Hyb.112 are modest and do not indicate a clear distinction in their predicted antiproliferative effects.

It is important to emphasize that these results are derived exclusively from a computational model and lack experimental validation. Therefore, they should be considered as theoretical predictions that require confirmation through *in vitro* and *in vivo* studies.

4. Conclusions

In the present study, a virtual library of 152 betulinic acid-derived hybrids was evaluated against Bcl-2 and Bcl-XL using an *in silico* workflow including molecular docking, molecular dynamics simulations, MM-GBSA binding free-energy analysis, ADMET prediction, and cancer profiling prediction. Based on their recurrent top-ranking per-

formance across the investigated protein structures, Hyb.96 (BA–Celastrol) and Hyb.112 (BA–Proanthocyanidin B2) were selected for further computational analysis.

Overall, Hyb.96 (BA–Celastrol) and Hyb.112 (BA–Proanthocyanidin B2) showed the most favorable overall computational profiles among the investigated betulinic acid-derived hybrids across molecular docking, molecular dynamics, MM-GBSA, ADMET, and predicted anticancer activity analyses. Molecular dynamics simulations suggested differences in ligand conformational behavior depending on the protein target, while MM-GBSA calculations indicated protein-dependent energetic variability across the investigated systems. In addition, predicted ADMET and pdCSM-cancer profiles suggested moderate antiproliferative potential across multiple cancer cell lines.

Taken together, these findings support the prioritization of Hyb.96 and Hyb.112 as promising computational candidates for further investigation against anti-apoptotic Bcl-2 family proteins. However, since the present study is based exclusively on computational analyses, future experimental validation through in vitro anticancer assays and apoptosis-related mechanistic studies will be necessary to confirm their biological activity, selectivity, and safety.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pr14111707/s1>, Table S1: Chemical structures and SMILES representations of the betulinic acid-derived hybrid library used for molecular docking; Table S2: Molecular docking results of all betulinic acid-derived hybrids against Bcl-2 and Bcl-XL proteins; Table S3: Summary of key protein–ligand interactions identified by PLIP analysis; Figure S1: Interaction profiles (hydrophobic contacts, hydrogen bonds, and water bridges) for selected hybrids.

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Abbreviations

The following abbreviations are used in this manuscript:

ADMET	Absorption, Distribution, Metabolism, Excretion, and Toxicity
AR	Androgen Receptor
AR-LBD	Androgen Receptor Ligand-Binding Domain
BA	Betulinic Acid
BBB	Blood–Brain Barrier
Bcl-2	B-cell lymphoma 2 protein
Bcl-XL	B-cell lymphoma-extra large

BH3	Bcl-2 Homology 3 domain
CNS	Central Nervous System
CPPTRAJ	Coordinate Processing Program for Trajectory Analysis
DECIMER	Deep Learning for Chemical Image Recognition
GB	Generalized Born
GI ₅₀	50% Growth Inhibition Concentration
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
H-bond	Hydrogen Bond
IUPAC	International Union of Pure and Applied Chemistry
LD ₅₀	Median Lethal Dose
LINCS	Linear Constraint Solver
MD	Molecular Dynamics
MDS	Molecular Dynamics Simulation
MM-GBSA	Molecular Mechanics Generalized Born Surface Area
MMP	Mitochondrial Membrane Potential
NaCl	Sodium Chloride
NPT	Constant Number of Particles, Pressure, and Temperature
OPLS	Optimized Potentials for Liquid Simulations
PB	Poisson–Boltzmann
PDB	Protein Data Bank
PDBQT	Protein Data Bank Partial Charge and Atom Type format
PLIP	Protein–Ligand Interaction Profiler
PME	Particle Mesh Ewald
PXR	Pregnane X Receptor
RCSB	Research Collaboratory for Structural Bioinformatics
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation
SMILES	Simplified Molecular Input Line Entry System
TIP3P	Transferable Intermolecular Potential with 3 Points

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