
Research article

Halogen substitution strengthens Z944 binding to the human Cav3.1 calcium channel pore domain: Insights from molecular dynamics simulations

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Abstract: Voltage-gated T-type calcium channels (Cav3.1) are important therapeutic targets for neurological and cardiovascular disorders; the selective blocker Z944 is among the most potent inhibitors currently available. However, the molecular basis underlying the contribution of its halogen substituents to ligand recognition remains poorly understood. In this study, all-atom molecular dynamics simulations totaling 4.8 μ s were performed to investigate the binding dynamics of the human Cav3.1 pore domain in the apo state and in complex with Z944 or a dehalogenated analogue lacking the chlorine and fluorine substituents (mZ944). Ligand binding preserved the overall pore architecture while locally stabilizing the blocker-binding cavity without inducing large-scale conformational changes. Relative MM/GBSA binding-energy calculations showed more favorable calculated binding energetics for Z944 than for mZ944, primarily because of greater dispersion interactions, with domain II residues, particularly Phe956, Asn952, Leu920, and Thr921, forming the dominant energetic contributions. Dynamic interaction analyses further revealed that Z944 maintains a broader protein–ligand interaction network, whereas mZ944 displayed greater positional and conformational variability within the binding cavity. Because mZ944 was initialized from the experimentally determined Z944 pose, its persistence within the same binding cavity over the simulated timescale does not exclude

alternative binding modes. Moreover, because chlorine and fluorine were removed simultaneously, the observed differences reflect their combined removal rather than their individual contributions. Analysis of ligand conformational dynamics showed that the halogenated ligand occupies a more restricted conformational ensemble, favoring well-defined torsional states compatible with stable binding. Overall, these findings indicate that the presence of the chlorine and fluorine substituents is associated with more favorable calculated binding energetics, more favorable dispersion interactions, differences in direct and water-mediated interaction patterns, and reduced ligand conformational heterogeneity. This work provides atomistic insight into the molecular mechanism of Z944 recognition and establishes structural principles that may guide the rational design of next-generation Cav3.1 inhibitors.

Keywords: T-type calcium channel; Cav3.1; Z944; channel blocker; molecular dynamics

1. Introduction

Voltage-gated calcium (Cav) channels mediate calcium influx in response to changes in membrane potential and play fundamental roles in numerous physiological processes, including muscle contraction, neurotransmitter release, hormone secretion, and gene expression. Among the three major Cav channel families (Cav1–Cav3), the Cav3 subfamily (Cav3.1–Cav3.3), also known as T-type calcium channels, is distinguished by its low-voltage activation threshold and transient current kinetics, properties that underlie the designation “T-type”. These channels regulate pacemaker activity, neuronal excitability, and rhythmic firing throughout both the central and peripheral nervous systems [1–4].

Cav3.1 is the prototypical member of the T-type calcium channel family and plays a critical role in thalamocortical oscillations and neuronal excitability. Dysregulation of Cav3.1 has been associated with numerous pathological conditions, including epilepsy, neuropathic pain, psychiatric disorders, autism spectrum disorders, spinocerebellar ataxia, and cardiovascular diseases. Encoded by the *CACNA1G* gene, Cav3.1 contributes to neuronal firing, cardiac pacemaker activity, and vascular tone through its low-threshold activation properties, which permit burst firing in excitable cells [5–9].

Structurally, the human Cav3.1 channel adopts the conserved architecture characteristic of voltage-gated calcium channels (Figure 1A–D). The channel consists of a single $\alpha 1$ subunit containing four homologous domains (DI–DIV), each composed of six transmembrane helices (S1–S6) (Figure 1A). Within each domain, helices S1–S4 form the voltage-sensing domain (VSD), whereas helices S5 and S6, together with pore helix 1 (PH1), the selectivity filter (SF), and pore helix 2 (PH2), constitute the pore domain (PD) (Figure 1B,C). The four pore domains assemble into a central ion-conducting pathway containing Ca^{2+} -binding sites within both the selectivity filter and the central cavity that govern calcium permeation (Figure 1D) [1,3,10].

The development of selective Cav3 inhibitors has therefore emerged as an important therapeutic strategy for disorders associated with abnormal T-type calcium channel activity. Among the currently available compounds, Z944 is one of the most potent and selective Cav3 blockers. Preclinical studies have shown that Z944 delays seizure progression in experimental models of temporal lobe epilepsy, suggesting therapeutic potential beyond symptomatic seizure suppression [11–15]. Structurally, Z944 contains chlorine and fluorine substituents (Figure 1E), two common medicinal chemistry

modifications used to optimize ligand affinity, hydrophobicity, metabolic stability, and target selectivity. In protein–ligand complexes, halogen atoms can enhance binding not only through favorable van der Waals interactions but also by influencing ligand conformation, hydration, and local interaction networks. Despite the widespread application of halogen substitution in drug design, its specific contribution to the molecular recognition and dynamic stabilization of Z944 within the Cav3.1 channel remains poorly understood [16–19].

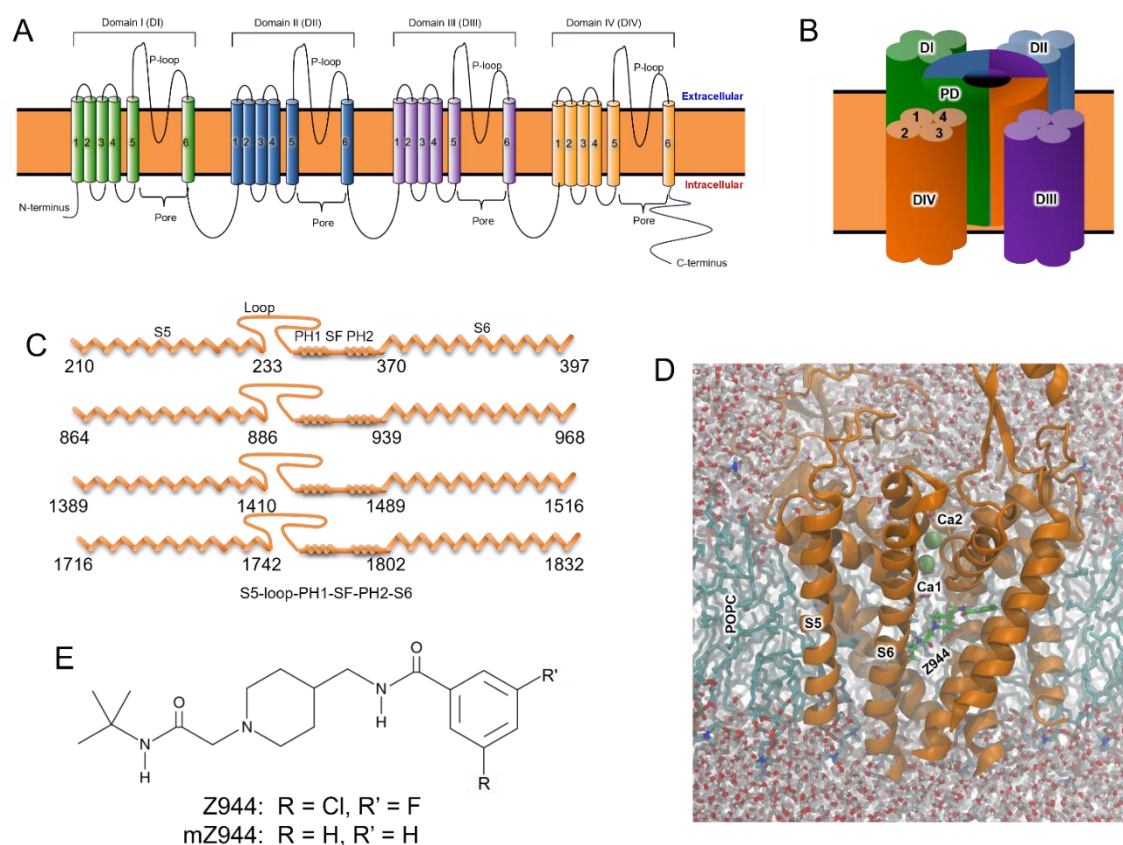


Figure 1. Structural organization of the human Cav3.1 channel and Z944. (A) Transmembrane topology of the Cav3.1 $\alpha 1$ subunit comprising four homologous domains (DI–DIV), each containing six transmembrane helices (S1–S6). Helices S1–S4 form the voltage-sensing domain (VSD), whereas S5–PH1–SF–PH2–S6 constitute the pore domain (PD). (B) Schematic representation of the four-domain transmembrane architecture. (C) Secondary-structure topology of the pore domain showing the S5–PH1–SF–PH2–S6 architecture in each domain. (D) Cryo-EM structure of the Cav3.1 pore domain in complex with Z944 (PDB ID: 6KZP), showing Z944 bound beneath the selectivity filter and two Ca^{2+} ions (Ca1 and Ca2) within the ion-conducting pathway. (E) Chemical structures of Z944 and mZ944. In mZ944, the chlorine ($\text{R} = \text{Cl}$) and fluorine ($\text{R}' = \text{F}$) substituents of Z944 were replaced with hydrogen atoms ($\text{R} = \text{H}$ and $\text{R}' = \text{H}$).

Cryo-electron microscopy (cryo-EM) studies have resolved the structures of human Cav3.1 in both the ligand-free (apo) and Z944-bound states at 3.3 and 3.1 Å resolution, respectively [20]. The Z944-bound structure revealed that the ligand occupies the central cavity beneath the selectivity filter,

with its wider end extending into the fenestration between domains II and III, thereby providing a structural framework for understanding Cav3.1 inhibition (Figure 1D). The original study proposed Lys1462, a residue conserved among T-type channels, as an important determinant of Z944 subtype selectivity [20]. However, an Author Correction published in 2024 reported that this conclusion resulted from deficiencies in the original electrophysiological protocol and analysis [21]. Using a corrected protocol, K1462F and K1462G did not significantly reduce Cav3.1 sensitivity to Z944, whereas Phe956 was identified as a key determinant of Z944 subtype selectivity [21,22]. This revised interpretation is particularly relevant to the present study because both Phe956 and Lys1462 contribute to the ligand interaction environment examined in our simulations. However, because cryo-EM captures only static conformational states, it cannot reveal how the halogen substituents influence ligand stability, hydration, the surrounding interaction network, or the dynamic behavior of the binding pocket. Consequently, the molecular consequences of halogen substitution for Z944 recognition remain incompletely understood.

Molecular dynamics simulations complement cryo-EM by providing atomic-resolution information on protein motions and ligand behavior in an explicit membrane environment. In the present study, we employed the cryo-EM structures of the apo and Z944-bound Cav3.1 pore domains as the starting models for all-atom MD simulations. To examine the consequences of combined halogen removal, we generated a doubly dehalogenated analogue (mZ944) by replacing both the chlorine and fluorine substituents with hydrogen while retaining the experimentally determined Z944 coordinates as the initial binding pose, and compared the apo, Z944-bound, and mZ944-bound systems. Structural stability, binding energetics, protein–ligand interactions, water-mediated interactions, and ligand conformational dynamics were analyzed to characterize differences between Z944 and its doubly dehalogenated analogue within the Cav3.1 pore domain. Because chlorine and fluorine were removed simultaneously, this comparison assesses their combined removal and does not distinguish their individual contributions. Taken together, these findings provide atomistic insight into differences associated with halogen substitution in Z944 recognition and establish structural principles that may facilitate the rational design of next-generation Cav3.1 blockers [23,24].

2. Computational methods

2.1. Structure models

The cryo-EM structures of human Cav3.1 in the apo state and in complex with the selective T-type calcium channel blocker Z944 (PDB IDs: 6KZ0 and 6KZP, respectively) [20] were obtained from the RCSB Protein Data Bank (RCSB PDB) [25]. The transmembrane orientation of the channel was assigned according to the Orientation of Proteins in Membranes (OPM) database [26,27], and the OPM-defined orientation was maintained throughout system construction to ensure proper alignment of the protein within the lipid bilayer. The apo and Z944-bound structures, determined at resolutions of 3.3 and 3.1 Å, respectively, both represent the channel in a closed-state conformation, with Z944 occupying the central cavity beneath the selectivity filter. The simulation model comprised the resolved pore-domain structural elements, including the S5 helix, extracellular pore loop, pore helices (PH1 and PH2), selectivity filter (SF), and S6 helix from each of the four homologous domains. The residue boundaries of these structural elements and the residues forming the Z944-binding pocket are summarized in Table S1. Unresolved residues were not modeled and were therefore excluded from all

simulations. Missing side chains and atoms within the resolved regions were reconstructed according to the CHARMM36m topology definitions, whereas Z944 was retained in its experimentally resolved binding conformation throughout system preparation.

2.2. System preparation

The simulation systems were prepared using a workflow adapted from our previously published molecular dynamics studies of ion channels and protein–ligand complexes, with modifications specific to the Cav3.1–Z944 system [28–30]. Briefly, hydrogen atoms were added, and protein structure files (PSF) were generated, using the psfgen plugin implemented in VMD with the CHARMM36m force field [31]. Missing side-chain atoms within the resolved regions of the cryo-EM structures were reconstructed according to the CHARMM36m topology definitions, whereas unresolved residues absent from the deposited structures were not modeled. Protonation states of titratable residues (Asp, Glu, His, Lys, and Arg) were assigned based on PROPKA predictions at pH 7.0 to represent physiological conditions [32].

Force field parameters and topology files for Z944 were generated using the CHARMM General Force Field (CGenFF) through the CHARMM-GUI Ligand Reader & Modeler, ensuring compatibility with the CHARMM36m force field used for the protein and membrane [33,34]. For the mZ944 analogue, the chlorine and fluorine substituents were replaced by hydrogen atoms while preserving the experimentally resolved binding pose, and the corresponding topology and parameter files were generated using the same protocol.

Three simulation systems were prepared: the apo Cav3.1 pore domain, the Cav3.1–Z944 complex, and the Cav3.1–mZ944 complex. Each system was embedded in a pre-equilibrated palmitoyl-oleoyl-phosphatidylcholine (POPC) lipid bilayer while maintaining the transmembrane orientation defined by the OPM database. The membrane–protein complexes were solvated with TIP3P water molecules in rectangular periodic simulation boxes with approximate dimensions of $154 \times 154 \times 105 \text{ \AA}^3$, providing a minimum solvent buffer of approximately 15 Å between the protein and each box boundary. Sodium and chloride ions were subsequently added to neutralize the systems and achieve a physiological salt concentration of 0.15 M NaCl. The compositions and dimensions of all simulation systems are summarized in Table S2. Finally, each system was subjected to energy minimization and equilibration prior to production molecular dynamics simulations.

2.3 Molecular dynamics simulations

All molecular dynamics (MD) simulations were performed using NAMD [35] with the CHARMM36m force field for proteins, lipids, and ions. A 2-fs integration time step was employed, and all covalent bonds involving hydrogen atoms were constrained using the SHAKE algorithm. Long-range electrostatic interactions were calculated using the particle mesh Ewald (PME) method with a real-space cutoff of 12 Å, whereas van der Waals interactions were smoothly switched between 10 and 12 Å. Simulations were carried out in the isothermal-isobaric (NPT) ensemble at 310 K and 1 atm using Langevin dynamics for temperature regulation and the Langevin piston Nosé–Hoover method for pressure control [36–39]. Three systems were simulated: (i) the Cav3.1–Z944 complex, (ii) apo Cav3.1, and (iii) the Cav3.1–mZ944 complex, in which the chlorine and fluorine substituents of Z944 were replaced by hydrogen atoms.

Before production simulations, each system underwent a five-stage energy minimization and equilibration protocol (Table S3). Initially, harmonic positional restraints were applied to the protein, lipid molecules, and ligand (where applicable) to allow solvent relaxation. The lipid restraints were subsequently released while maintaining restraints on the protein and ligand. In the third stage, restraints were retained only on the protein backbone, ligand, and the two pore Ca^{2+} ions. During the fourth stage, restraints were applied exclusively to the N- and C-terminal residues and the pore Ca^{2+} ions. A final 2-ns equilibration was then performed with restraints applied only to the terminal residues before commencing the production simulations.

Production simulations were performed with weak harmonic restraints ($0.5 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$) applied only to the terminal residues to minimize translational drift while allowing unrestricted conformational fluctuations throughout the pore domain. No restraints were applied to the ligand during production simulations. For each system, three independent simulations were performed, consisting of one 600-ns trajectory and two additional 500-ns replica simulations, yielding a cumulative simulation time of $1.6 \mu\text{s}$ per system and $4.8 \mu\text{s}$ across all three systems.

2.4. Trajectory analysis

Trajectory analyses were performed using VMD 1.9.4, in-house Tcl scripts, and Python scripts utilizing the NumPy, MDAnalysis, and Matplotlib libraries [40,41]. Unless otherwise stated, analyses were performed using only the equilibrated portions of the production trajectories. For the primary 600-ns simulation, the final 300 ns were analyzed, whereas the complete 500-ns trajectories were analyzed for the two independent replica simulations, unless otherwise specified in the corresponding subsections.

2.4.1. Root-mean-square deviation and fluctuation

The structural stability and conformational flexibility of the Cav3.1 pore domain were evaluated using the root-mean-square deviation (RMSD) and root-mean-square fluctuation (RMSF), respectively. For each independent simulation, every analyzed trajectory frame was least-squares-fitted to the first frame of the corresponding analyzed trajectory interval using the $\text{C}\alpha$ atoms of the pore-domain transmembrane region. The fitting selection comprised S5 residues 210–233, 864–886, 1389–1410, and 1716–1742, together with the extended S6/pore-region segments 340–397, 908–968, 1448–1516, and 1764–1832 (Table S1). The resulting single rigid-body transformation was applied to all atoms in each frame to remove overall translational and rotational motion. Following this common alignment, RMSDs were calculated for the specified atom selections without additional fitting of individual structural regions. Protein backbone atoms were used for the whole-protein and structural-subregion RMSDs, whereas ligand non-hydrogen atoms were used for ligand RMSD. RMSD values were calculated from the $\text{C}\alpha$ atoms of the pore domain to assess structural convergence and overall stability during the simulations. Per-residue RMSF values were subsequently calculated from the aligned trajectories using $\text{C}\alpha$ atoms, with each $\text{C}\alpha$ atom referenced to its average position over the analyzed trajectory. Domain-specific RMSF profiles were generated separately for domains I–IV to compare regional flexibility among the apo, Z944-bound, and mZ944-bound systems.

2.4.2. Relative MM/GBSA binding-energy calculation

Relative binding energies of Z944 and mZ944 to the Cav3.1 pore domain were estimated using the molecular mechanics/generalized born surface area (MM/GBSA) method applied to equilibrated MD trajectories [42,43]. The calculations were performed to compare the relative MM/GBSA binding energies of the two ligands and identify residues contributing to ligand stabilization within the binding pocket. Snapshots extracted from the equilibrated production trajectories were used for the MM/GBSA calculations after removing explicit water molecules and ions. The MM/GBSA binding-energy expression was evaluated according to [44,45].

$$\Delta G_{bind} = \Delta H - T\Delta S \quad (1)$$

where ΔH is calculated as

$$\Delta H = \Delta E_{vdW} + \Delta E_{elec} + \Delta G_{solv} \quad (2)$$

where ΔE_{elec} and ΔE_{vdW} are the gas-phase electrostatic and van der Waals interaction energies, respectively, and ΔG_{solv} is the total solvation free energy,

$$\Delta G_{solv} = \Delta G_{GB} + \Delta G_{nonpolar} \quad (3)$$

where ΔG_{GB} is the polar solvation free energy calculated using the generalized born implicit solvent (GBIS) model implemented in NAMD with protein and solvent dielectric constants of 1.0 and 78.5, respectively. The nonpolar solvation free energy was estimated from the solvent-accessible surface area (SASA),

$$\Delta G_{nonpolar} = \gamma \times \Delta SASA + b \quad (4)$$

where γ is the surface tension coefficient ($0.00542 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$), and b is the empirical offset ($0.92 \text{ kcal mol}^{-1}$) used in the standard GB/SA parameterization. The SASA term was evaluated using the GB/SA implementation in NAMD. In the NAMD GBIS implementation, the reported electrostatic (ELECT) term includes both the gas-phase electrostatic interaction energy and the implicit solvation contribution,

$$\Delta E_{ELECT} = \Delta E_{elec} + \Delta G_{solv} \quad (5)$$

such that the MM/GBSA binding-energy expression can be written as

$$\Delta G_{bind} = \Delta E_{vdW} + \Delta E_{ELECT} - T\Delta S \quad (6)$$

Configurational entropy ($T\Delta S$) was not explicitly calculated because of its high computational cost and the limited reliability of entropy estimation for large biomolecular systems. Accordingly, although ΔG_{bind} is retained in Eqs. (1–6) as the conventional notation used in the MM/GBSA formulation, the values reported here do not represent complete Gibbs binding free energies. Because the primary objective was to compare the chemically similar Z944 and mZ944 ligands using an identical

computational protocol, these values are interpreted as relative MM/GBSA binding energies that describe differences in the calculated energetic components of binding.

To examine the energetic differences associated with the combined removal of the chlorine and fluorine substituents, the relative MM/GBSA binding-energy estimate was further decomposed into van der Waals and electrostatic contributions. The van der Waals term corresponds to the Lennard–Jones interaction between the protein and ligand, whereas the electrostatic term includes both the Coulomb interaction and the associated implicit solvation contribution calculated using the GBIS model. Consequently, the reported electrostatic contribution represents the net electrostatic effect on ligand binding and may be positive when the desolvation penalty exceeds the favorable Coulomb interaction formed upon binding. In addition, per-residue energy decomposition analysis was performed to quantify the energetic contribution of individual residues and identify residues contributing to the differences in relative binding energetics between the Z944- and mZ944-bound complexes. Because chlorine and fluorine were removed simultaneously in mZ944, these energetic differences reflect their combined removal and cannot be assigned to either substituent individually. All MM/GBSA calculations were performed using NAMD version 3.0.2 with the CHARMM36m force field.

2.4.3. Protein–ligand interaction network analysis

Dynamic protein–ligand interactions were characterized using the Protein–Ligand Interaction Fingerprints (ProLIF) package [46] in conjunction with MDAnalysis [40]. Separate analyses were performed for the Cav3.1–Z944 and Cav3.1–mZ944 systems using the final 300 ns of each production trajectory. Protein and ligand structures were converted into ProLIF molecular representations, and interaction fingerprints were calculated for each sampled frame. ProLIF was used to identify hydrogen-bond donor and acceptor interactions, hydrophobic contacts, van der Waals contacts, π -stacking, π -cation interactions, and other supported interaction types. For each residue–interaction pair, occupancy was calculated as the percentage of analyzed frames in which a given interaction was detected. Occupancies were evaluated independently for each interaction type because multiple interactions can occur simultaneously within a single frame. Interactions with occupancies $\geq 20\%$ were considered high-occupancy contacts and were included in the subsequent analyses [20].

Ligand-centered interaction networks were generated separately for the Z944- and mZ944-bound systems. Network nodes represent the ligand and interacting Cav3.1 residues, whereas edges denote the detected interaction types and their occupancies. For clarity, only the principal binding-site residues were retained in the network visualizations. Because the analyzed frames were extracted from continuous MD trajectories and may therefore exhibit temporal correlation, the reported occupancies were interpreted as descriptive measures of interaction persistence rather than statistically independent observations.

2.4.4. Water-mediated protein–ligand interaction analysis

Water-mediated protein–ligand interactions were analyzed separately for the Cav3.1–Z944 and Cav3.1–mZ944 systems using VMD 1.9.3 and in-house Tcl scripts. The final 300 ns of each production trajectory were analyzed, with trajectory frames extracted every 0.1 ns, yielding approximately 3100 frames per system. A single-water bridge was defined when the same explicit water molecule

simultaneously formed hydrogen bonds with both the ligand and a Cav3.1 residue within the same trajectory frame. Hydrogen bonds were identified using a donor–acceptor distance cutoff of ≤ 3.5 Å and a D–H $\cdots$ A angle cutoff of $\geq 120^\circ$ [28]. Both protein-to-water and ligand-to-water donor orientations were considered. Ligand donor and acceptor assignments were obtained from the CHARMM topology. Protein donor atoms comprised nitrogen, oxygen, and sulfur atoms covalently bonded to hydrogen, whereas protein acceptors included chemically eligible oxygen and sulfur atoms together with unprotonated histidine nitrogen atoms.

Water-bridge occupancy was calculated as the percentage of analyzed frames in which a given protein residue, protein–ligand atom pair, or water molecule participated in at least one single-water bridge. When multiple water bridges involving the same residue or atom pair occurred within a single frame, the frame was counted only once for occupancy calculations. Accordingly, occupancies represent descriptive measures of how frequently a given water-mediated interaction is sampled within an individual trajectory rather than probabilities derived from statistically independent observations. Water-mediated interaction patterns were subsequently compared between the Z944- and mZ944-bound systems to evaluate how removal of the chlorine and fluorine substituents altered the hydration network surrounding the ligand-binding cavity.

To investigate the origin of replica-dependent water-bridge pathways, frame-wise bridge occurrence was compared with ligand center-of-mass distances, ligand dihedral angles, and local pore-geometry coordinates. Associations were evaluated separately for each independent trajectory. Because the trajectory observations are temporally correlated and this analysis was exploratory, the resulting correlations were interpreted descriptively, with consistency in direction and magnitude across independent simulations considered when assessing reproducibility of the structural relationships.

The continuity and exchange of individual bridging waters were further characterized using identity-resolved bridge episodes. An uninterrupted bridge episode was defined as consecutive analyzed frames in which the same water molecule connected the same protein residue and ligand atom. Episode duration was determined from the number of consecutive observations and the trajectory sampling interval. The number of distinct water identities contributing to each residue-level pathway was also determined. These episode durations quantify the continuity of a specific water-mediated bridging geometry at the available temporal resolution and should not be interpreted as general residence times of water molecules within the binding cavity, because waters present in the cavity without participating in a protein–water–ligand bridge were not included.

3. Results

3.1. Structural stability of the Cav3.1 pore domain

The structural stability of the Cav3.1 pore domain was first evaluated using the representative production simulation (Run 1; Figure 2). To assess the reproducibility of the observed structural behavior, two additional independent simulations were performed for each system. The corresponding RMSD time series and summary statistics are presented in Figures S1–S3 and Table S4. Unless otherwise stated, Figure 2 illustrates the representative trajectories, whereas the supplementary analyses confirm the reproducibility of the observed trends. Structural stability was assessed using RMSD calculations for the protein backbone, pore domain (PD), S6 helices, selectivity filter (SF),

blocker-binding pocket, and bound ligand.

The representative RMSD trajectories (Figure 2A) show that the protein backbone exhibited the largest structural fluctuations, with RMSD values of approximately 2.0–4.2 Å throughout the simulations. Although the three systems displayed slightly different fluctuation patterns, none exhibited abrupt structural transitions, indicating that the overall channel architecture remained stable. The Z944-bound system showed a gradual increase in backbone RMSD during the latter stages of the simulation, whereas the apo and mZ944-bound systems displayed comparable fluctuations. Analysis of the three independent simulations yielded similar backbone RMSD values (Table S4), demonstrating that the overall structural stability of the channel was reproducible across independent trajectories.

In contrast, the pore-forming regions remained considerably more rigid than the protein backbone. The pore domain, blocker-binding pocket, S6 helices, selectivity filter, and blocker-binding pocket exhibited RMSD values close to 1 Å throughout Run 1 (Figure 2A), indicating that the architecture of the ion-conducting pathway was well preserved irrespective of ligand binding. The supplementary replica analyses (Figures S1–S3 and Table S4) further confirmed this high level of structural stability. Although the differences were modest, the apo system consistently exhibited slightly larger fluctuations within the blocker-binding pocket than either ligand-bound system. The Z944-bound complex also showed relatively low run-to-run variation, whereas the mZ944-bound complex exhibited somewhat greater variability, suggesting that ligand binding influences local pocket dynamics without substantially perturbing the overall pore architecture.

The ligand RMSD profiles revealed greater replica-dependent variability for mZ944 than for Z944 (Figures 2A and S2–S3). In Run 1, mZ944 exhibited substantially higher RMSD values than Z944, indicating a larger deviation from its initial binding pose. Across the three independent simulations, Z944 generally converged toward similar RMSD ranges during the later portions of the trajectories, whereas mZ944 showed greater variation among replicas (Table S4). Despite these differences, both ligands remained within the blocker-binding cavity throughout the simulations. Thus, the higher and more variable RMSD of mZ944 reflects greater positional and/or conformational variability within the cavity rather than ligand dissociation.

Structural superposition of representative conformations further supports these observations (Figure 2B). The apo, Z944-bound, and mZ944-bound systems adopted nearly identical transmembrane architectures when viewed from both the membrane and extracellular sides, with only minor deviations confined to the extracellular loops surrounding the pore entrance. Likewise, Z944 and mZ944 occupied essentially the same binding position beneath the selectivity filter, indicating that the two ligands differed in their positional dynamics while remaining within the same blocker-binding cavity over the simulated timescale.

To characterize local conformational flexibility, residue-wise RMSF profiles were calculated separately for each homologous domain (Figure 2C). Domain I exhibited the largest fluctuations in all three systems, primarily within the extracellular S5–PH1 loops containing unresolved cryo-EM residues, indicating that these highly flexible regions are largely independent of ligand binding. By contrast, domain II was the most rigid, with only a localized increase in flexibility near residues 885–895 observed for the mZ944-bound system. Local differences among the three systems were also evident in domains III and IV. In domain III, the apo system displayed greater fluctuations around residues 1418–1435 than either ligand-bound system, suggesting that ligand binding suppresses local backbone mobility in this region. Domain IV exhibited complementary behavior: the apo system

showed broader fluctuations near residues 1788–1800, whereas the Z944-bound system displayed a localized increase around residues 1745–1750, accompanied by reduced flexibility in the distal extracellular loop.

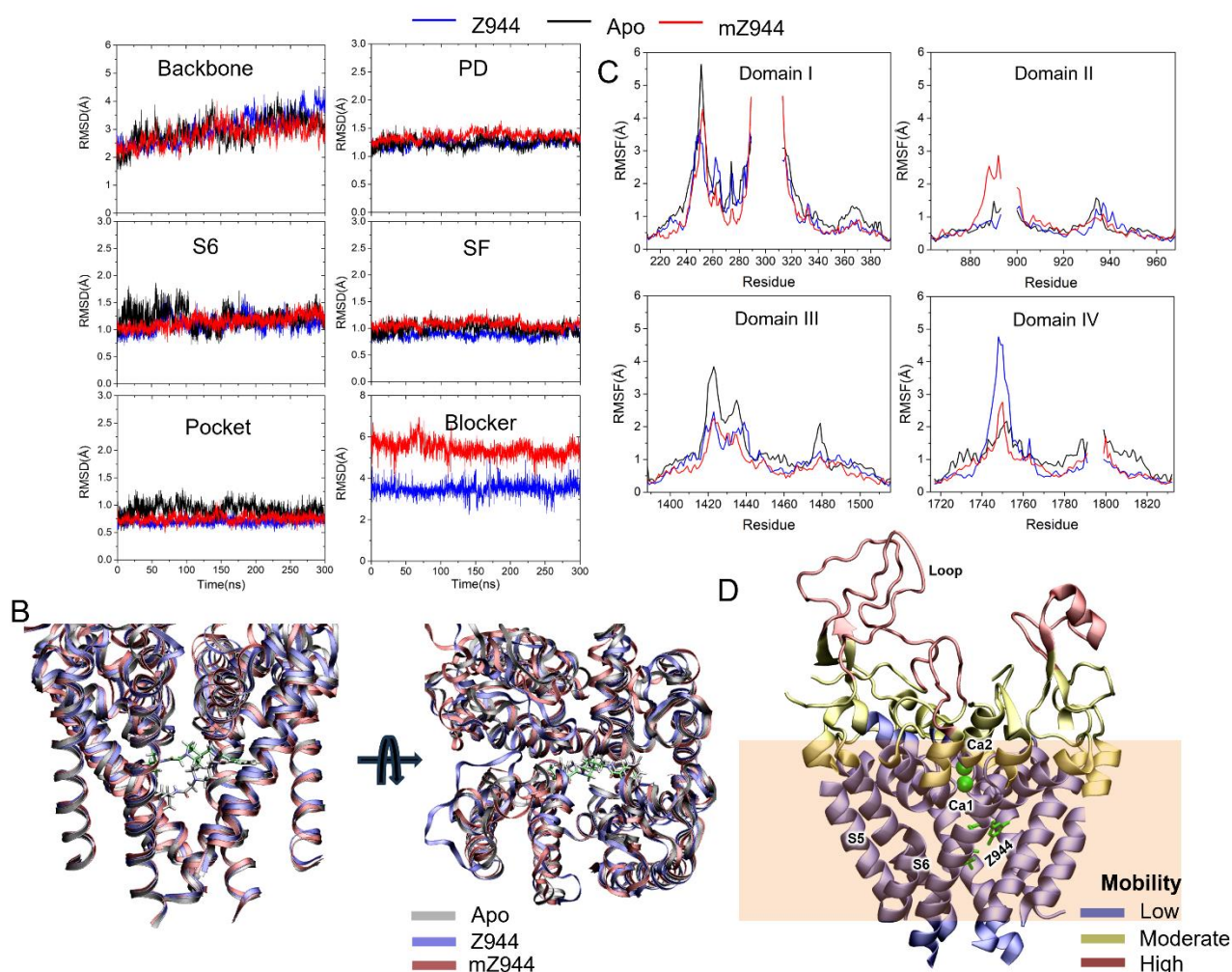


Figure 2. Structural stability and conformational flexibility of the Cav3.1 pore domain in the apo, Z944-bound, and mZ944-bound systems during the final 300 ns of the MD simulation Run 1. (A) RMSD profiles of the protein backbone, pore domain (PD), S6 helices, selectivity filter (SF), blocker-binding pocket, and bound blocker. (B) Superposition of representative structures from the three systems viewed from the membrane (left) and extracellular (right) sides. (C) Per-residue RMSF profiles for the pore-forming regions of domains I–IV. Discontinuities in the RMSF traces correspond to unresolved residues absent from the cryo-EM structures. (D) Representative Cav3.1 structure colored according to residue mobility derived from RMSF values: low (<0.8 Å, blue), moderate (0.8–1.5 Å, yellow), and high (>1.5 Å, red).

Mapping residue mobility onto the Cav3.1 structure provides a structural overview of these flexibility patterns (Figure 2D). Low-mobility residues are concentrated within the transmembrane helices, including the S5 and S6 segments that define the blocker-binding cavity, whereas the

extracellular loops exhibit substantially greater flexibility. Notably, the central pore containing the bound blocker and the two pore Ca^{2+} ions remained among the most structurally stable regions of the channel. Collectively, these results demonstrate that ligand binding preserves the overall architecture of the Cav3.1 pore domain while locally stabilizing the blocker-binding cavity, with the halogenated Z944 exhibiting modestly lower local structural variability than mZ944.

3.2. Relative MM/GBSA binding energy and residue interaction analysis

The structural analyses presented in Figure 2 show that ligand binding stabilizes the blocker-binding cavity without inducing substantial conformational changes in the Cav3.1 pore domain. To investigate the energetic basis of this stabilization and the role of halogen substitution, relative MM/GBSA binding energies were evaluated using MM/GBSA calculations from three independent MD simulations together with replica-averaged per-residue energy decomposition and protein–ligand interaction analyses (Figure 3).

Across the three independent simulations, the Z944-bound complex consistently exhibited a more favorable relative MM/GBSA binding energy than the mZ944-bound complex (-23.30 ± 1.36 versus -21.01 ± 4.16 kcal/mol, Figure 3A). This difference indicates more favorable calculated binding energetics for the native halogenated ligand within the present MM/GBSA framework. The difference in calculated binding energetics was primarily associated with more favorable dispersion interactions for Z944 (-35.50 ± 1.22 versus -29.38 ± 2.34 kcal/mol). By contrast, the electrostatic-related contribution remained unfavorable for both ligands because it includes the generalized Born solvation term, although the magnitude of this penalty differed only modestly between the two systems. Notably, the relative MM/GBSA binding energy of Z944 showed considerably smaller variation among the three independent simulations than mZ944, suggesting that Z944 exhibits not only more favorable binding energetics but also lower replica-to-replica energetic variability.

Replica-averaged MM/GBSA decomposition revealed that favorable binding contributions are distributed throughout the blocker-binding cavity but are dominated by residues from domain II (Figure 3B). Phe956, Asn952, Leu920, and Thr921 consistently provided the largest favorable contributions in both ligand-bound systems, identifying these residues as the principal energetic hotspot for blocker recognition. Additional stabilization was provided by Phe917, Gln922, Lys1462, and several residues from domains I, III, and IV, although these peripheral contributions were generally smaller and exhibited greater variability. Decomposition into van der Waals and electrostatic-related components further showed that the favorable energy contributions arise predominantly from van der Waals interactions, whereas electrostatic contributions remained comparatively small. Lys1462 showed substantial replica-dependent variability, particularly for mZ944, for which the apparently favorable replica-averaged contribution was strongly influenced by Run 1. The corresponding contributions in Runs 2 and 3 were much smaller, indicating that the energetic contribution of Lys1462 was not consistently more favorable for mZ944 across the independent simulations.

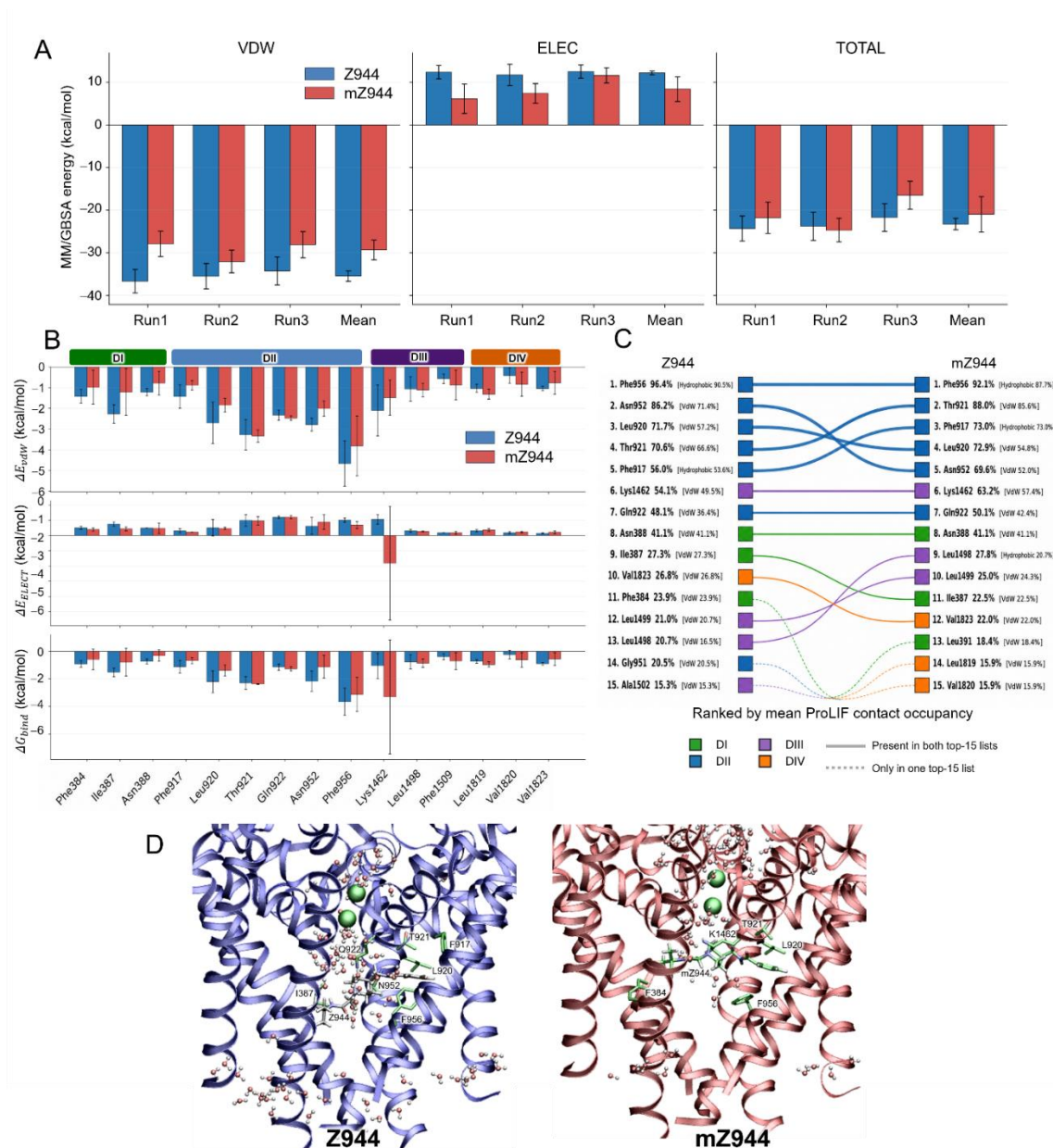


Figure 3. Relative MM/GBSA binding-energy and residue interaction analyses of Z944 and mZ944 bound to the Cav3.1 pore domain. (A) MM/GBSA energy components from three independent MD simulations. Bars for Runs 1–3 represent the mean $\pm$ SD over the final 300 ns of each simulation, and the final bars show the mean $\pm$ SD of the three replica means. (B) Replica-averaged per-residue MM/GBSA decomposition showing van der Waals (ΔE_{vdw}), electrostatic-related (ΔE_{elec}), and total (ΔG_{bind}) energy contributions. Residues are grouped by domain (DI–DIV). Error bars indicate the SD among the three replica means. (C) Alluvial plot showing the correspondence of the dominant residue–ligand interactions ranked by mean ProLIF contact occupancy. (D) Representative binding poses of Z944 (left) and mZ944 (right), highlighting the major residues contributing to ligand stabilization.

Protein–ligand contact analysis further showed that Z944 and mZ944 retain a common high-occupancy interaction core dominated by domain II residues (Figure 3C). Phe956, Asn952, Leu920, and Thr921 consistently ranked among the highest-occupancy interacting residues for both ligands, although their relative rankings and mean occupancies differed. Phe956 showed the highest occupancy for both Z944 and mZ944, while Asn952 was more frequently contacted by Z944, and Thr921 and Phe917 showed higher occupancies for mZ944. Beyond this conserved core, differences were observed in lower-ranked contacts involving residues from domains I, III, and IV. At a mean-contact occupancy threshold of $\geq 20\%$, 14 residues were identified for Z944 and 12 for mZ944, indicating a modest difference in the number of frequently contacted residues rather than a uniformly less extensive interaction network for mZ944.

Representative structures illustrate the spatial organization of these interactions (Figure 3D). Both ligands are surrounded by contacts centered on the conserved domain II residues, while differences are observed in the occupancies and distribution of contacts with surrounding residues. Together, these findings indicate that both ligands retain a conserved domain II interaction core, with more modest differences in the surrounding residue-contact patterns.

3.3. Protein–ligand contacts stabilized by halogen substitution

The structural analyses (Figure 2) demonstrated that occupation of the blocker-binding cavity stabilizes the local binding environment relative to the apo channel, whereas the MM/GBSA calculations (Figure 3) showed that Z944 binds more favorably than its dehalogenated analogue, mZ944. To identify the molecular interactions responsible for this energetic advantage, residue-specific protein–ligand contacts were first analyzed using the representative production trajectory (Run 1), which provides the detailed contact timelines, contact probability differences, and interaction occupancy profiles shown in Figure 4A–C. To evaluate the reproducibility of these interaction patterns, identical ProLIF analyses were subsequently performed for two additional independent simulations of each ligand. The resulting interaction occupancies from all three replicas are summarized in Tables S5a and S5b and integrated into the alluvial comparison shown in Figure 4D.

The representative contact timelines (Figure 4A) demonstrate that both Z944 and mZ944 maintain persistent interactions with a common set of residues lining the blocker-binding cavity, indicating that both ligands remained within the same blocker-binding cavity over the simulated timescale following initialization from the common Z944-derived pose. Nevertheless, the persistence and spatial distribution of these contacts differ markedly between the two complexes. In the Z944-bound system, residues including Phe917, Leu920, Thr921, Gln922, Asn952, Phe956, Leu1499, and Lys1462 maintain continuous interactions over most of the trajectory, producing an interaction network that extends across multiple pore domains. In the representative Run 1 trajectory, the mZ944 complex exhibits a more localized interaction pattern dominated by Thr921, Leu920, Phe956, and Lys1462, whereas several interactions observed for Z944 become weaker or more intermittent. Thus, the representative trajectory indicates differences in the persistence and spatial distribution of contacts following the removal of the halogen substituents, rather than disruption of the overall binding mode.

This redistribution of residue interactions is illustrated more clearly by the residue-wise contact probability differences shown in Figure 4B. Rather than uniformly weakening ligand binding, dehalogenation selectively alters the persistence of individual residue contacts throughout the binding cavity. Contacts involving Phe917, Leu920, Gln922, Asn952, Phe956, Leu1499, and Val1823 are

generally more persistent for Z944, whereas interactions involving Lys1462 together with several neighboring residues become more pronounced in the mZ944 complex. These complementary gains and losses indicate that peripheral interactions differ between the two ligand-bound systems.

The contact occupancy analysis (Figure 4C), together with the three independent simulations summarized in Tables S5a and S5b, identifies a highly conserved interaction core centered on domain II. Across all replicas, Phe956, Thr921, Asn952, and Leu920 consistently ranked among the highest-occupancy interaction residues for both ligands, demonstrating that these residues constitute the reproducible core of the blocker-binding site. Outside this conserved core, secondary interactions involving domains I, III, and IV displayed greater replica-dependent variability. In the Z944 simulations, Phe917 and Gln922 remained prominent interaction partners in Runs 1 and 2, whereas Lys1462 became increasingly important in Run 3. The mZ944 simulations similarly retained the domain II interaction core across all replicas while exhibiting enhanced participation of Lys1462 in Runs 1 and 3. These observations suggest that the principal binding determinants are conserved between Z944 and mZ944, whereas peripheral interactions differ between the two ligand-bound systems.

The comparison shown in Figure 4D provides an integrated view of the residue–ligand interaction networks across the three independent simulations. Although the ranking and occupancy of individual contacts vary between replicas, the dominant interaction contributions consistently remain centered on domain II for both ligands. Compared with mZ944, however, Z944 reproducibly maintains a broader interaction network that extends beyond this conserved core to include neighboring residues from domains I, III, and IV. A representative binding pose (Figure 4D) further illustrates how these additional contacts surround the ligand without altering its overall binding orientation. Together, these results indicate that Z944 exhibits a broader interaction network while retaining the same conserved domain II interaction core as mZ944.

In addition to direct protein–ligand contacts, all three independent simulations revealed an extensive but dynamic network of single-water bridges linking Z944 with residues surrounding the blocker-binding cavity (Figure 4E and Table S6). At least one protein–water–ligand bridge was present in 87.5%, 96.9%, and 91.5% of the analyzed frames in Runs 1–3, respectively, indicating that solvent-mediated interactions were frequently sampled across all three simulations. However, the identities and occupancies of individual water-bridge pathways varied among replicas. Ser383, Asn957, and Lys1462 participated in water-mediated interactions in all three simulations, with occupancies ranging from 33.5% to 62.4%, 18.5% to 52.3%, and 24.2% to 41.2%, respectively. The most highly occupied pathway differed among replicas, with Asn957 contributing most prominently in Run 1 (52.3%), Ser383 in Run 2 (62.4%), and Thr921 in Run 3 (49.6%). Thr921 displayed pronounced replica-dependent behavior, with its occupancy increasing from only 0.8% in Run 1 to 35.1% and 49.6% in Runs 2 and 3. The representative pathways shown in Figure 4E illustrate examples of the sampled solvent-mediated interactions, whereas the complete replica-specific occupancy statistics are summarized in Table S6.

Further analysis showed that high residue-level occupancy did not reflect prolonged participation of a single water molecule (Figure S6). Each major residue pathway involved numerous distinct bridging-water identities, and uninterrupted identity-resolved bridge episodes were predominantly short-lived, with median durations of 0.1, 0.2, and 0.2 ns and 90th-percentile durations of 0.3, 0.6, and 0.4 ns in Runs 1–3, respectively. Thus, the high occupancies observed for individual residues arose primarily from repeated formation of water-mediated connections by exchanging water molecules

rather than persistence of a single bridging water. Frame-matched analyses further showed trajectory-specific associations of bridge formation with ligand-position, torsional, and local pore coordinates, but no single structural coordinate consistently explained the pathway differences across all three replicas. Together, these results indicate that the relative populations of water-mediated pathways are associated with the local protein–ligand conformational ensemble sampled within each trajectory, while exchange of individual bridging waters allows favorable residue-level connections to form repeatedly.

To assess whether dehalogenation preserved the same water-mediated pathways, water-bridge occupancies were compared between Z944 and mZ944 in the matched Run 1 simulations (Figure S7). At least one protein–water–ligand bridge occurred in 87.5% and 99.2% of the analyzed frames for Z944 and mZ944, respectively, indicating that removal of the halogen substituents did not reduce overall water-mediated connectivity in this trajectory. However, the sampled residue-level pathways differed between the two ligands. Ser383 (42.9% versus 95.2%), Asn952 (21.8% versus 53.2%), Gln1816 (3.8% versus 46.8%), and Thr921 (0.8% versus 15.0%) showed higher occupancies for mZ944, whereas Asn957 (52.3% versus 0.0%), Lys1462 (41.2% versus 22.7%), and Gln922 (11.3% versus 0.2%) showed lower occupancies. Thus, the matched Run 1 comparison indicates reorganization of the sampled water-mediated interaction network rather than a simple loss of hydration-mediated contacts following dehalogenation. Because this comparison is limited to a single matched trajectory, these residue-specific differences are interpreted descriptively.

To further characterize ligand positioning within the Cav3.1 pore, the center-of-mass (COM) distances between the ligand and the blocker-binding pocket, as well as between the ligand and the selectivity filter (SF), were analyzed using pooled samples from the final 300 ns of all three independent simulations (Figure 4F and Figure S5). The pooled distributions in Figure 4F provide a descriptive representation of the sampled conformational ensembles; because frames within each trajectory are temporally correlated, they were not treated as independent observations. Replica-specific means were therefore examined separately to assess reproducibility across simulations. For the pocket-to-ligand distance, Z944 exhibited smaller mean distances than mZ944 in all three replicas (2.985, 2.554, and 3.497 Å for Z944 versus 6.224, 3.612, and 3.665 Å for mZ944), giving replica-mean summaries of 3.012 ± 0.472 and 4.500 ± 1.493 Å, respectively (mean $\pm$ SD, $n = 3$ independent simulations). Thus, Z944 remained closer to the blocker-binding pocket across the three simulations, although the magnitude of the difference was strongly replica-dependent. In contrast, the ligand–SF distance showed substantial replica-to-replica variability (11.139 ± 0.413 Å for Z944 and 10.232 ± 1.541 Å for mZ944), with the apparent separation in the pooled distributions arising primarily from Run 1 and not being consistently reproduced in Runs 2 and 3. We therefore do not interpret the ligand–SF distance as evidence for a general ligand-dependent positional difference. Overall, the replica-resolved COM analysis supports a more consistent association of Z944 with the blocker-binding pocket, while also emphasizing substantial ensemble variability, particularly for mZ944. Together with the MM/GBSA, direct-contact, and water-mediated interaction analyses, these results indicate that the presence of the halogen substitution is associated with more favorable calculated binding energetics and a broader network within the Cav3.1 blocker-binding cavity, while the extent of ligand positional differences remains replica-dependent.

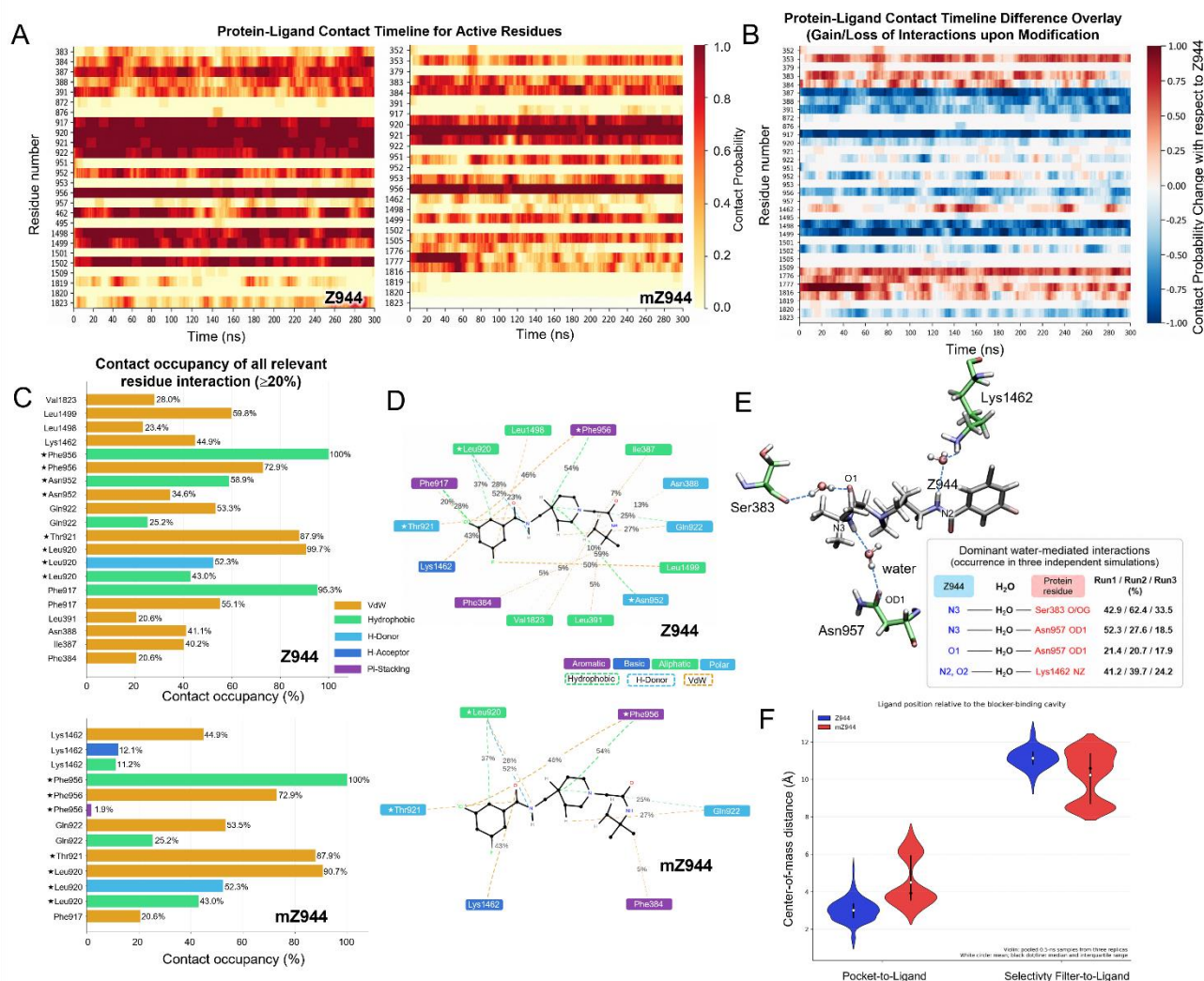


Figure 4. Dynamic protein–ligand interactions in the Cav3.1–Z944 and Cav3.1–mZ944 complexes during the final 300 ns of the representative MD simulation (Run 1). (A) Protein–ligand contact timelines for the Z944 (left) and mZ944 (right) complexes. (B) Difference map of residue contact probabilities between the two complexes. (C) Contact occupancies of the major protein–ligand interactions ($\geq 20\%$) for the Z944 (top) and mZ944 (bottom) complexes. (D) Representative protein–ligand interaction networks for the Z944 (top) and mZ944 (bottom) complexes. Residues marked with a star (★) constitute the conserved domain II interaction core identified across the three independent MD simulations. (E) Representative water-mediated interactions between Z944 and Cav3.1 residues (left). The table (right) summarizes the dominant water-bridge pathways identified across the three independent MD simulations. (F) Violin plots of the center-of-mass distances between the ligand and the blocker-binding pocket (left) and the selectivity filter (right). Distributions were obtained from pooled samples collected every 0.5 ns over the final 300 ns. White circles indicate the mean, whereas black dots and vertical lines represent the median and interquartile range, respectively. Replica-resolved means and the mean $\pm$ SD across the three independent replica means are shown in Figure S5.

3.4. Dehalogenation alters ligand conformational dynamics

The structural and energetic analyses presented in Figures 2–4 demonstrated that Z944 forms a more favorable interaction network and adopts a more stable binding mode than the dehalogenated analogue, mZ944. To determine whether the differences in protein–ligand interactions were accompanied by differences in ligand conformational behavior, the time evolution of the seven principal ligand dihedral angles (χ_1 – χ_7) was examined separately for each of the three independent MD simulations (Figure S8). Probability distributions were then generated from equal-weight pooled samples from the final 300 ns of the three simulations for each ligand (Figure 5), with replica-resolved distributions shown in Figure S9. Because frames within each continuous trajectory are temporally correlated, the pooled distributions are used only as descriptive representations of the sampled conformational ensembles and are not treated as statistically independent observations. Comparison of the replica-resolved distributions showed that some torsional preferences were consistently reproduced across the three simulations, whereas others exhibited substantial trajectory-dependent variation.

The χ_1 torsion showed highly consistent behavior across replicas, with both Z944 and mZ944 strongly concentrated near the trans boundary ($\pm 180^\circ$), indicating that this torsion is largely constrained by the molecular scaffold and is relatively insensitive to dehalogenation. The χ_6 torsion similarly remained predominantly trans-like for both ligands across the three simulations. In contrast, χ_2 exhibited substantial replica-to-replica heterogeneity. Both ligands sampled multiple χ_2 conformational states, but the relative populations of these states varied among the independent trajectories. Although the pooled distributions differ between Z944 and mZ944, the replica-resolved distributions indicate that these differences should be interpreted as differences in the overall sampled ensembles rather than as a uniformly reproduced shift in a preferred rotameric state. The χ_3 distributions were comparatively similar between the two ligands, with only modest differences in their sampled angular ranges and some replica-dependent variation.

The χ_4 , χ_5 , and χ_7 torsions showed additional ligand- and replica-dependent differences. For χ_4 , Z944 sampled an additional population in the negative-angle region in some trajectories, whereas both ligands retained substantial populations near the trans boundary. The χ_5 distributions also differed between Z944 and mZ944, but the relative populations of the sampled states varied among replicas, indicating that these conformational differences are not uniformly reproduced across all trajectories. For χ_7 , Z944 remained predominantly concentrated near the trans boundary, whereas mZ944 additionally sampled a distinct population near 0° . Replica-resolved analysis showed that this alternative mZ944 state was populated principally in Runs 1 and 3 rather than uniformly across all three simulations. Thus, the χ_7 behavior indicates greater conformational heterogeneity of mZ944 within the sampled ensemble, while also demonstrating that the population of this alternative state is trajectory-dependent.

Taken together, the pooled and replica-resolved analyses indicate that dehalogenation is associated with changes in the conformational sampling of the bound ligand, but the magnitude and reproducibility of these changes vary among individual torsional degrees of freedom. The χ_1 and χ_6 torsions show similar and reproducible conformational preferences for both ligands, whereas χ_2 and several other torsions exhibit greater replica-dependent heterogeneity. The additional χ_7 state sampled by mZ944 provides a clear example of increased conformational diversity, although its population varies among independent trajectories. These observations are consistent with differences in ligand conformational dynamics between Z944 and mZ944 within the sampled simulations, while

emphasizing that individual rotamer populations are dynamic, ensemble-dependent features rather than uniformly persistent characteristics of the ligand-bound complexes.

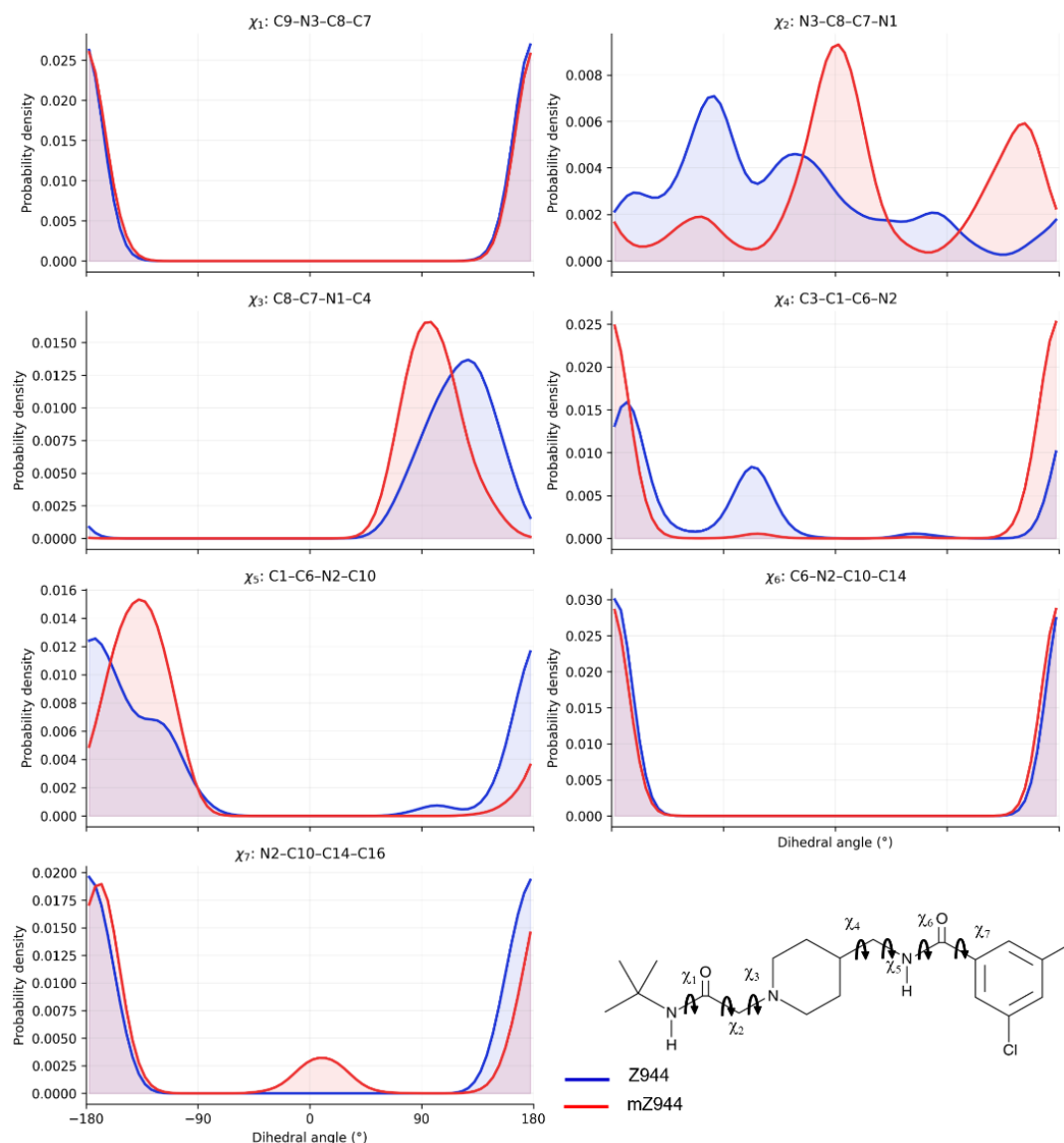


Figure 5. Equal-weight pooled probability distributions of the seven principal ligand dihedral angles (χ_1 – χ_7) for Z944 and mZ944, calculated from the final 300 ns of three independent MD simulations. The pooled distributions provide descriptive representations of the sampled conformational ensembles; individual trajectory frames are temporally correlated and are not treated as independent observations. Replica-resolved time series and probability distributions are shown in Figures S8 and S9, respectively. The corresponding rotatable bonds defining χ_1 – χ_7 are shown on the ligand structure.

4. Discussion

The present MD simulations provide atomistic insight into how the selective T-type calcium channel blocker Z944 is stabilized within the Cav3.1 pore domain and reveal the molecular

consequences of removing its halogen substituents. Both Z944 and its dehalogenated analogue (mZ944) remained within the blocker-binding cavity throughout the simulations following initialization from the Z944-derived pose and retained a conserved domain II interaction core. Ligand binding produced only subtle changes in the overall pore architecture, indicating that occupation of the blocker-binding cavity does not require large-scale structural rearrangements of the pore domain. Instead, the principal effect of ligand binding is local stabilization of the binding cavity, as reflected by the reduced pocket RMSD, decreased residue fluctuations surrounding the ligand, and persistent ligand occupancy. These trends were consistently reproduced across the three independent simulations, extending the static cryo-EM structures by demonstrating that the experimentally observed binding pose remains dynamically stable within a membrane environment.

The MM/GBSA calculations indicate that the more favorable calculated binding energetics of Z944 are primarily associated with more favorable dispersion interactions rather than large changes in electrostatic interactions. mZ944 exhibited less favorable dispersion interactions while modestly increasing the effective electrostatic penalty associated with ligand binding. Per-residue energy decomposition further identified Domain II as the principal energetic hotspot, with residues such as Phe956, Asn952, Leu920, and Thr921 contributing most strongly to ligand stabilization, whereas residues from domains I, III, and IV provided complementary interactions. The prominent contribution of Phe956 is consistent with the corrected electrophysiological evidence identifying this residue as an important determinant of Z944 subtype selectivity [21,22]. In contrast, Lys1462 participated in both direct and water-mediated interactions and showed a larger favorable mean energy contribution for mZ944, although this contribution varied substantially among replicas. These observations support a role for Lys1462 as a dynamic component of the local ligand interaction network but do not imply that it determines the subtype selectivity of Z944. Instead, the more favorable calculated binding energetics of Z944 appear to arise from distributed contributions across the binding cavity, particularly from the domain II interaction network.

The dynamic contact analyses further support this interpretation. Both ligands retained the conserved domain II interaction core identified by the MM/GBSA calculations. Following initialization from the Z944-derived pose, combined chlorine/fluorine removal did not cause mZ944 to leave the blocker-binding cavity during the simulated timescale, and the conserved domain II interaction core remained detectable. However, this observation does not exclude alternative equilibrium binding poses for mZ944. Compared with Z944, the doubly dehalogenated analogue showed differences in the occupancies and spatial distribution of contacts with surrounding residues. This difference was accompanied by greater positional variability of mZ944 within the blocker-binding cavity, whereas Z944 remained more closely associated with the experimentally observed binding position across the independent simulations. Together, these findings indicate that the combined removal of chlorine and fluorine is associated with differences in the persistence and spatial organization of protein–ligand interactions and in ligand positional dynamics within the sampled binding cavity.

Two design limitations constrain the interpretation of the Z944–mZ944 comparison. First, mZ944 was constructed by replacing the chlorine and fluorine substituents with hydrogen while retaining the experimentally determined Z944 binding coordinates. This approach provides a controlled starting geometry but may bias conformational sampling toward the Z944-derived pose. The ligand was unrestrained throughout the production simulations, and the larger, replica-dependent mZ944 RMSD and center-of-mass displacement indicate appreciable relaxation and positional variability relative to

the starting configuration. Nevertheless, conventional 500–600 ns trajectories may not cross barriers separating distinct binding modes; consequently, persistence within the same blocker-binding cavity should not be interpreted as evidence that Z944 and mZ944 adopt identical or uniquely preferred binding poses. Independent pose generation combined with enhanced-sampling or free-energy approaches would be required to assess alternative mZ944 binding modes more rigorously. Second, mZ944 lacks both the chlorine and fluorine substituents. The observed differences therefore represent the combined effect of replacing both halogens with hydrogen and cannot distinguish the contribution of either substituent, determine whether their contributions are additive, or establish cooperativity between them. Simulations and experimental characterization of singly dehalogenated analogues would be required to resolve their individual contributions.

Water molecules constitute an additional component of this interaction network. Ser383, Asn957, and Lys1462 repeatedly participated in single-water bridges across all three independent simulations, although their occupancies varied among trajectories. Other pathways were more strongly replica-dependent; for example, Thr921 occupancy increased from 0.8% in Run 1 to 35.1% and 49.6% in Runs 2 and 3, respectively. This replica dependence can be understood from the geometric sensitivity of water-mediated interactions: formation of a protein–water–ligand bridge requires favorable positioning and orientation of the ligand, nearby protein groups, and the intervening water molecule. Small variations in ligand position, torsional state, or local protein geometry can therefore alter the accessibility of individual hydration pathways. Consistent with this interpretation, frame-matched analyses revealed trajectory-specific associations between bridge formation and ligand or local pore coordinates, but no single structural coordinate consistently explained the pathway differences across all three replicas, suggesting that hydration is coupled to the multidimensional local protein–ligand conformational ensemble rather than to a single conformational switch. Moreover, identity-resolved analysis showed that individual bridge episodes were predominantly short-lived and involved numerous distinct water molecules (Figure S6), indicating that high residue-level occupancy arises from repeated formation of favorable bridging geometries by exchanging waters rather than persistence of a particular water molecule. Water-mediated interactions can complement direct hydrogen bonds by accommodating local geometric fluctuations and maintaining interaction pathways when direct contacts are transient. Removal of the halogen substituents altered the sampled hydration pathways without eliminating the extensive solvent-mediated interaction network, suggesting a reorganization rather than a uniform loss of water-mediated interactions. Because these water bridges are inherently dynamic and show substantial replica dependence, they are best interpreted as ensemble properties rather than deterministic interactions. Together with the energetic and direct-contact analyses, these observations indicate that hydration represents an additional, dynamic component of the ligand interaction environment.

An additional insight provided by this study is that halogen substitution influences the intrinsic conformational landscape of the ligand itself. Several rotatable bonds adopted reproducibly different torsional preferences in Z944 and mZ944, with the halogenated ligand generally occupying a narrower conformational ensemble. This conformational restriction is consistent with the tighter confinement of Z944 within the blocker-binding cavity and its broader interaction network. Rather than functioning solely through direct contacts with protein residues, the chlorine and fluorine substituents are associated with altered conformational preferences of the bound ligand that are more compatible with the geometry of the Cav3.1 binding site. Such conformational restriction is associated with reduced conformational heterogeneity in the bound state and provides a plausible structural explanation for the

more favorable binding energetics predicted by the MM/GBSA calculations. Together, these observations suggest that the more favorable binding energetics of Z944 results from a combination of enhanced intermolecular interactions and reduced ligand conformational freedom within the binding cavity.

The broader physiological relevance of T-type calcium-channel modulation is also supported by recent functional studies. Zhao et al. reported that pharmacological inhibition of T-type calcium channels with mibefradil alleviated anxiety-like behavior in MPTP-treated mice and was accompanied by reduced burst firing and calcium signals in substantia nigra dopaminergic neurons [47]. These findings link T-type-channel activity to neuronal excitability and disease-relevant behavioral outcomes, highlighting the potential therapeutic significance of this channel family. Although Zhao et al. did not examine Z944, halogen substitution, or the specific molecular interactions investigated here, their findings provide complementary functional context for understanding the molecular basis of T-type-channel inhibitor recognition. In this context, the present simulations provide molecular-level insights into Z944 recognition and differences associated with removal of its halogen substituents, which may help inform future functional studies of Cav3.1 inhibition and the investigation of modified Z944 analogues.

Several limitations should be considered when interpreting these findings. The simulations were performed using the isolated pore domain rather than the full-length Cav3.1 channel. Although this reduced model enables a controlled comparison of Z944 and mZ944 within the same local structural environment, it does not reproduce the complete conformational and electromechanical environment of the intact channel. In particular, exclusion of the voltage-sensing domains (VSDs) removes the long-range coupling between voltage sensing and the pore. VSD-dependent conformational changes may influence the geometry and dynamics of the pore-forming helices and thereby alter the local environment of the ligand-binding cavity. The absence of this coupling could therefore affect cavity fluctuations as well as the positional dynamics, residue-interaction networks, and hydration environment sampled by the bound ligands. VSD-dependent changes in pore conformation could also influence ligand accessibility to the binding cavity, an aspect that cannot be evaluated directly from the present prebound simulations. Moreover, because Z944 and mZ944 were initially positioned within the binding cavity, the present simulations primarily characterize the local dynamics of preformed ligand-channel complexes and do not address ligand-access pathways, association or dissociation kinetics, voltage dependence, state-dependent binding, or coupling between ligand binding and channel gating. The observed differences between Z944 and mZ944 should therefore be interpreted as comparative behavior within the modeled prebound pore environment rather than as a complete description of their interactions with the full-length channel.

In addition, the MM/GBSA calculations provide relative binding-energy estimates and do not explicitly account for configurational entropy; therefore, these values should not be interpreted as absolute binding free energies or direct quantitative predictions of experimental binding affinity. Although three independent simulations yielded highly consistent structural, energetic, and interaction trends, experimental validation through site-directed mutagenesis of the predicted key residues, particularly those within domain II, together with electrophysiological characterization of halogen-substituted analogues, will be important for testing the proposed molecular mechanism. Despite these limitations, the combined structural, energetic, interaction, hydration, and conformational analyses consistently indicate that the presence of the chlorine and fluorine substituents is associated with more favorable calculated binding energetics, differences in the surrounding interaction and hydration

networks, and altered ligand conformational sampling within the Cav3.1 blocker-binding cavity.

5. Conclusions

In this study, all-atom molecular dynamics simulations were used to investigate the dynamic binding mechanism of the selective T-type calcium channel blocker Z944 within the human Cav3.1 pore domain and to evaluate the combined effects of its chlorine and fluorine substituents on ligand stabilization. Comparison of the apo, Z944-bound, and dehalogenated mZ944 systems demonstrated that ligand binding preserves the overall pore architecture while locally stabilizing the blocker-binding cavity. The combined structural, energetic, interaction, hydration, and conformational analyses consistently showed that the presence of the chlorine and fluorine substituents is associated with more favorable van der Waals interactions, a broader protein–ligand interaction network, altered water-mediated contacts, and differences in ligand conformational flexibility within the binding pocket. Together, these effects are consistent with the more favorable relative MM/GBSA binding energetics predicted for Z944 compared with the dehalogenated analogue. Both ligands remained within the same blocker-binding cavity and retained a conserved domain II interaction core after initialization from the experimental Z944 pose. However, because this shared initial geometry may bias finite-timescale sampling, alternative mZ944 binding poses cannot be excluded. Furthermore, because chlorine and fluorine were removed simultaneously, the observed differences reflect their combined removal and cannot distinguish the contribution of either halogen individually. Beyond elucidating the molecular basis of Z944 recognition, this study identifies key interaction hotspots and structural features that may facilitate the rational design of next-generation Cav3.1 inhibitors. More broadly, the findings highlight how halogen substitution is associated with differences in protein–ligand interactions, hydration, and ligand conformational dynamics, providing useful insights for structure-based drug design targeting voltage-gated calcium channels.

Data and software availability

The data supporting the conclusions of this study are provided in the article and its Supporting Information. Initial structures, representative NAMD and MM/GBSA configuration files, analysis scripts, and one-frame example trajectories for the apo, Z944-bound, and mZ944-bound systems are openly available in Zenodo at <https://doi.org/10.5281/zenodo.21636217>. The source code is also maintained at <https://github.com/Thepcuchem/cav31-z944-md-analysis>, and the version used for this study is archived as release v1.0.0 in Zenodo. Because of their large file sizes, the complete production trajectories are not included in the compact repository package but are available from the corresponding author upon reasonable request.

Supporting information

Residue boundaries of the Cav3.1 pore-domain structural elements and blocker-binding pocket (Table S1); compositions, dimensions, and simulation lengths of the molecular dynamics systems (Table S2); equilibration and production protocols (Table S3); replica-specific RMSD statistics (Table S4); residue-specific protein–ligand contact occupancies for Z944 and mZ944 (Tables S5a and S5b); water-mediated protein–Z944 interaction pathways (Table S6); RMSD time series for the apo, Z944-

bound, and mZ944-bound systems (Figures S1–S3); representative Z944 binding pose (Figure S4); replica-resolved ligand–pocket and ligand–selectivity-filter center-of-mass distances (Figure S5); replica-resolved water-bridge pathways and bridging-water exchange (Figure S6); matched Run 1 Z944–mZ944 water-mediated interaction comparison (Figure S7); ligand-dihedral time series from the three independent simulations (Figure S8); and pooled and replica-resolved ligand-dihedral distributions (Figure S9).

Limitations of this study

This study has several limitations. First, the simulations were performed using the resolved Cav3.1 pore domain rather than the full-length channel; therefore, possible effects of the voltage-sensing domains and other unresolved regions on ligand binding were not examined. Second, mZ944 was generated by simultaneously replacing the chlorine and fluorine substituents of Z944 with hydrogen while retaining the experimentally determined Z944 binding pose. Consequently, the simulations assess the combined effect of halogen removal and do not distinguish the individual contributions of chlorine and fluorine or exclude alternative binding modes for mZ944. Third, the MM/GBSA calculations provide relative binding-energy estimates and do not include configurational entropy; therefore, they should not be interpreted as absolute binding free energies. Finally, although three independent simulations were analyzed for each system, the accessible conformational space remains limited by the finite simulation timescale. These limitations should be considered when interpreting the molecular mechanisms proposed here.

Use of generative-AI tools declaration

Generative AI tools were used to assist with the preparation of some figures, specifically Figures 3A–C, 4, and 5. These figures were prepared from our original data obtained from molecular dynamics simulations. The underlying MD trajectories and scientific data were generated independently using conventional molecular dynamics simulations and were not generated by AI. The authors reviewed and verified the data, analyses, and final figures. AI tools were also used in an assistive manner for language editing and improving the clarity and readability of the manuscript. All scientific interpretations and conclusions were made and verified by the authors, who take full responsibility for the content of the manuscript.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

T.F.K. performed the molecular dynamics simulations, conducted the computational analyses, and interpreted the data. P.L. and P.B. contributed to data analysis. T.F.K., P.B. and P.S. conceived the study, wrote the manuscript, and prepared all figures. R.B.P. provided critical discussions and intellectual input. All authors reviewed and approved the final manuscript.

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