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Article

AI-designed mini-protein targeting claudin-5 to enhance blood–brain barrier integrity

Sreyan Biswas[1], Debjani Roy[2]
[1] Hillsdale High School, San Mateo, California
[2] University of California, Berkeley, California

SUMMARY

The blood–brain barrier (BBB) is a highly selective interface composed of endothelial cells, astrocytes, and pericytes that maintains central nervous system (CNS) homeostasis and guards against circulating toxins and pathogens in the brain. Disruption of the BBB has been associated with several neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis. Among the junctional components that maintain the BBB integrity, claudin-5 (CLDN5) is the principal endothelial tight-junction tetraspan transmembrane protein that maintains paracellular permeability, and its dysregulation is present across multiple neurodegenerative diseases, thereby associating BBB breakdown with specific tight-junction alterations. We hypothesized that strengthening the tight junctions of the BBB by enhancing CLDN5 binding affinity using a computationally designed mini-protein could counteract the disruption of CLDN5. We have performed computational simulations and machine learning techniques to identify the mini-protein capable of binding the CLDN5 protein and tightening the BBB. The CLDN5 and mini-protein structures were predicted via AlphaFold 3. As an extracellular β-barrel transport protein with exceptional ligand-binding capacity Lipocalin-1 (LCN1) was selected and computationally mutated to generate a library of mini-proteins. The mini-proteins were docked on CLDN5 utilizing the HDOCK2.0 software, and the resulting mini-protein-bound CLDN5 structures were analyzed. The mini-proteins were computationally evaluated based on the binding energy formed between the CLDN5 and mini-proteins and the accessible binding location with CLDN5. In addition, hydrogen bonds formed between the mini-protein and CLDN5 were analyzed to ensure reliable CLDN5 binding with the mini-protein and to minimize the likelihood of protein complex dissociation. Considering binding strength, hydrogen bond analysis, and accessible binding location, mini-protein X was the most suitable candidate for further investigation. This research highlights the potential of computationally engineered mini-proteins to stabilize BBB integrity and slow the progression of neurodegenerative diseases.

INTRODUCTION

The blood–brain barrier (BBB) is a highly selective mechanism that protects the central nervous system (CNS) by tightly regulating the movement of molecules, ions, and cells between the bloodstream and brain tissue (1, 2). This barrier is composed of endothelial cells joined by tight junctions, which are protein complexes that seal intercellular spaces and restrict paracellular diffusion, which is the passage of substances between adjacent cells (3, 4). Cellular components such as astrocytes, pericytes, and neurons collaborate in the neurovascular unit and maintain CNS homeostasis (1, 2, 8, 9). Advancements in computational modeling, such as AlphaFold 3 for protein structure prediction and HDOCK for protein–protein docking, have expanded the ability to investigate these molecular interactions at high resolution (5, 7). Lipocalins, which are flexible proteins with β-barrel structures and high binding capacity, have been identified as promising proteins for computational binding analysis (6).

Neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis disrupt BBB function by weakening the tight junctions, allowing harmful toxic substances to enter the brain (11, 12). BBB integrity is essential for CNS homeostasis (13). Cerebral capillaries make up about 85% of the brain's vascular network, exist in an estimated 1:1 ratio with neurons, and form an endothelial surface area estimated between 12–18 m^2, along with facilitating molecular exchange while protecting neurons (13-15).

Claudin-5 (CLDN5) is a tight junction protein in the BBB endothelium, critical for maintaining barrier integrity and selective permeability (4, 10). Its two extracellular loops seal junctions, regulate ion flux, and mediate paracellular diffusion, the passage of molecules between adjacent cells (16, 18). Through homotypic and heterotypic strand formation, CLDN5 preserves barrier selectivity, and structural studies show that the two loops contribute distinct functions to junction assembly (17, 19). CLDN5 also regulates immune cell trafficking across the BBB, linking structural maintenance to immune control (20, 23). Reduced CLDN5 expression has been observed in several neurodegenerative conditions. In Alzheimer's disease, inflammatory cytokines such as TNF-α suppress CLDN5 through NF-κB signaling, weakening the barrier (22). In multiple sclerosis, diminished CLDN5 is associated with tight junction breakdown and increased immune cell infiltration (23, 24). Stabilizing CLDN5 expression has been shown to improve cognitive outcomes in Alzheimer's disease models (25). Although no disease-related mutations in CLDN5 have been identified, several studies have reported reduced expression and mislocalization of this protein in pathological conditions

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(3, 10-12, 26, 27). These observations provide strong support for targeting CLDN5 stabilization as a therapeutic strategy to preserve BBB integrity in neurodegenerative diseases such as Alzheimer's disease and multiple sclerosis (21, 25, 28).

Targeting CLDN5 for stabilization with computationally designed mini-proteins can restore tight junction function, allowing researchers to better study how improving BBB integrity influences neurodegenerative disease progression (25). The mini-proteins used in this study were derived from LCN1, which is part of the lipocalin family and has a flexible structure, making it possible to redesign it to bind with the CLDN5 protein (30). BBB leakiness is both a cause and consequence of neurodegenerative diseases such as Alzheimer's disease and multiple sclerosis (31). Its disruption worsens neurodegenerative disease progression by allowing toxins into the brain, though disease pathology may further impair BBB integrity (12, 26, 27, 31). An excessively tight BBB may cause trouble in drug delivery, emphasizing the need to balance protection and permeability (28, 29).

In this computational research, we analyzed the structures of CLDN5 and computationally designed mini-proteins to improve their binding affinity. We hypothesized that strengthening the tight junctions of the BBB by enhancing CLDN5 binding affinity using a computationally designed mini-protein could counteract the disruption of CLDN5. This work identifies a computationally designed mini-protein with strong binding affinity to CLDN5 at an accessible site and highlights its potential to restore tight junction stability, providing a foundation for future *in vitro* and *in vivo* experimental validation.

RESULTS

A set of LCN1-based mini-protein variants was generated through targeted computational sequence edits; each variant was docked to CLDN5 with HDOCK, and the ten best-scoring candidates were selected for detailed analysis. For context, an overview schematic of healthy versus disrupted BBB junctions and their stabilization by a CLDN5-binding mini-protein is shown **(Figure 1).** To identify the most promising mini-protein candidate, we evaluated all computationally designed mini-proteins based on binding affinity, accessible binding location, and hydrogen bonds. The mini-protein X may attach to the accessible extracellular region of CLDN5 (**Figure 2A-B**).

Electrostatic interaction analysis

Electrostatic analysis revealed a negatively charged CLDN5 surface that aligns with the basic residues in mini-protein X, supporting electrostatic compatibility (**Figure 2C**) (32). Electrostatic surface mapping of the CLDN5 model shows distinct potential across the protein, with an overall negative extracellular domain and an overall positive cytoplasmic domain (**Figure 2C**). The extracellular side is mostly negative (indicated in red color), while the inside cytoplasmic side is mostly positive (indicated in blue color) (**Figure 2C**). The plasma membrane bilayer acts as a boundary, with each side having distinct electrostatic properties (**Figure 2C**). This charge separation across the plasma membrane creates distinct electrostatic environments, allowing the extracellular domain to attract ligands carrying positively charged residues on the mini-protein (32).

Binding Energy Data Analysis

A set of ten computationally mutated mini-proteins derived from LCN1 was designed to bind with CLDN5, and their binding affinities were evaluated and analyzed. For each variant, we submitted the top-ranked HDOCK pose to the PRODIGY web server to estimate binding energy (kcal/mol), where more negative values indicate stronger predicted binding (7, 37, 38). The mini-protein with the highest binding affinity showed binding energy of -8.1 kcal/mol (**Figure 3**), which was higher than the average binding energy of -6.69 kcal/mol. The remaining mini-proteins displayed binding energies ranging from -5.7 to -7.4 kcal/mol (**Figure 3**). The standard deviation of the binding energies was 0.67 kcal/mol, indicating consistency in binding strength. Additionally, the mean absolute deviation was 0.529 kcal/mol, highlighting a low fluctuation of individual binding energies relative to the mean. The identification of the high-affinity mini-protein X suggests that the specific computational mutation (**Table 1**) is promising and could be used for further research analysis.

Hydrogen Bond Data Analysis

We wrote an in-house script that reads each docked protein file, looks for nitrogen or oxygen atoms from CLDN5 and the mini-protein that sit within 4.0 Å of each other, and

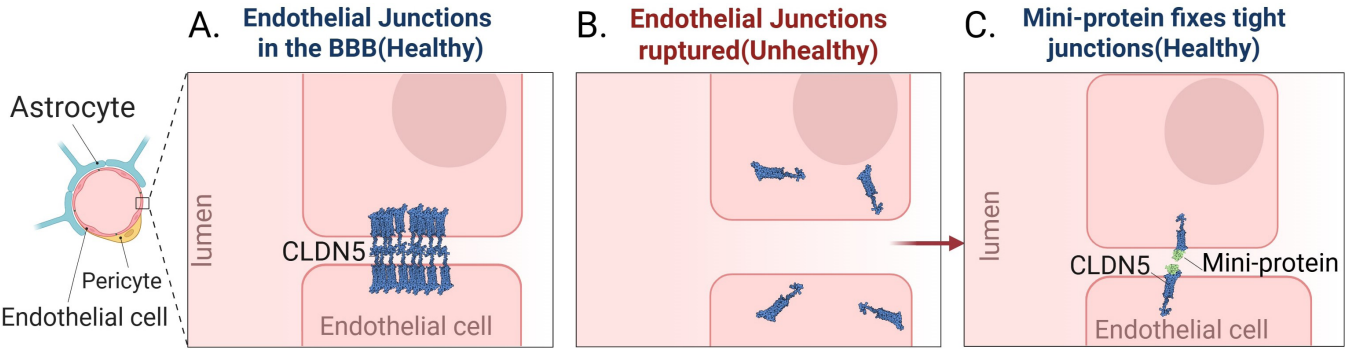

Figure 1: Role of Claudin-5 in endothelial junctions of the blood-brain barrier. The states of the blood-brain barrier (BBB) in healthy, unhealthy, and treated individuals. **(A)** In the healthy BBB, Claudin-5 (CLDN5) proteins form tight junctions between endothelial cells, preventing unwanted molecules from passing through. **(B)** In the unhealthy BBB, these junctions are disrupted, creating gaps that allow harmful substances to enter the brain. **(C)** The treated BBB shows the mini-protein binding to the junction proteins, stabilizing the assembly and restoring barrier integrity. Figure created using biorender.com (54).

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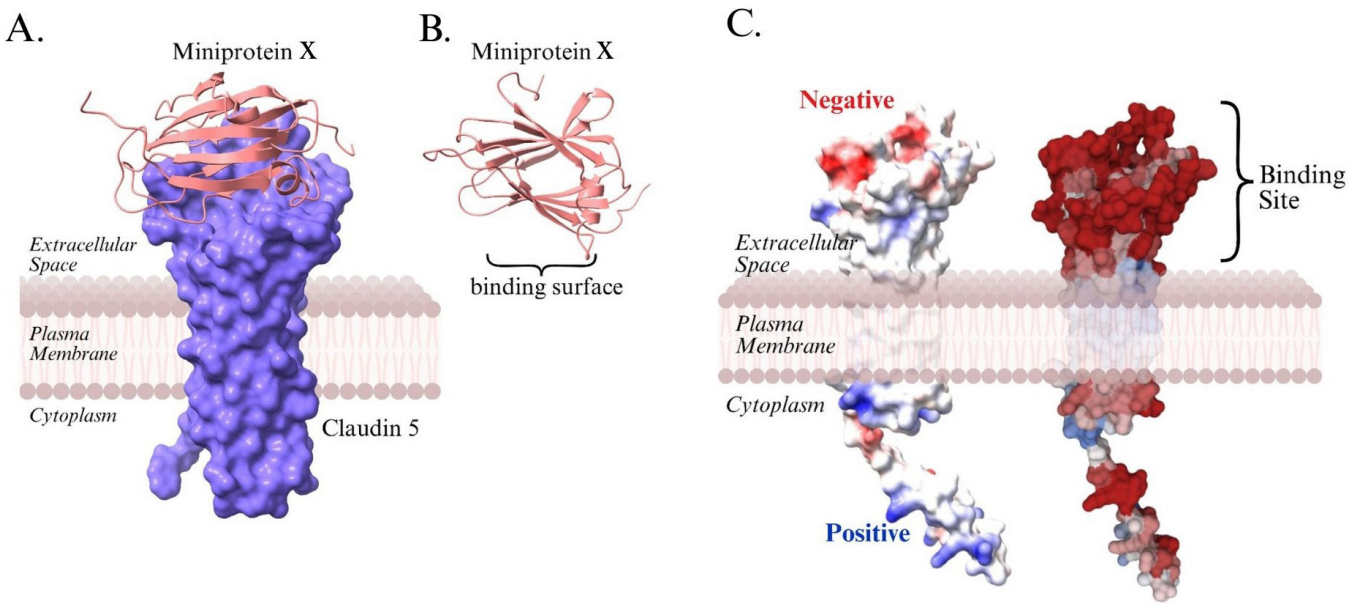

Figure 2: Structural representation of mini-protein binding to CLDN5 in the BBB. (A) CLDN5 (purple) is nestled within the plasma membrane, stretching from the cytoplasm out to the extracellular space. The mini-protein X (pink) attaches itself to the extracellular domain of CLDN5, which could potentially affect tight junction stability. **(B)** Close-up view of the mini-protein, showcasing its binding surface. **(C)** Electrostatic properties and binding site of CLDN5 in the BBB. The electrostatic surface shows negative (red) and positive (blue) regions, with a predominantly negative extracellular domain and positive cytoplasmic side, the extracellular binding site (dark red) is an accessible target for mini-protein X binding. This image was created using Chimera-X.

saves a table of those pairs and their distances to Excel. Analysis of hydrogen bond formation at the CLDN5–mini-protein interface across ten computationally mutated mini-proteins was performed using a 4.0 Å N/O distance cutoff and revealed a wide range of variability. The number of hydrogen bonds ranged from 5 to 43, with mini-protein X exhibiting the highest count of 43 **(Figure 4**). Given that hydrogen bond counts are discrete integers, we round summary statistics to the nearest whole number to avoid implying fractional bonds. Under this convention, the mean is 19 and the standard deviation is 13. This data indicates deviation in hydrogen bond counts across the mini-proteins. Mini-protein X stands out with a higher number of hydrogen bonds, suggesting a stronger candidate for BBB tight junction stabilization. More hydrogen bonds may help strengthen binding, but other factors, such as binding energy and binding site accessibility, play a role in how well a mini-protein interacts with CLDN5. High hydrogen bond count paired with strong affinity suggests enhanced interaction stability between mini-protein X and CLDN5 compared to the other mini-proteins (**Figure 4**) (28). These combined features identified mini-protein X as the most structurally and functionally promising candidate for further investigation.

DISCUSSION

This study explored the binding of CLDN5 with computationally designed mini-proteins to find the optimal candidate that has a strong binding affinity, favorable hydrogen bonding, and favorable electrostatic interactions at the accessible region of the extracellular domain of CLDN5. Structural predictions using AlphaFold 3, molecular docking via HDOCK2.0, and binding energy evaluation through PRODIGY led to the identification of a promising candidate (5, 7, 37, 38). Among the ten computational mini-proteins, both binding energy and hydrogen bonds were evaluated and analyzed to determine the overall binding strength and potential physiological relevance. Mini-protein X showed the highest binding energy of –8.1 kcal/mol, and the highest number of hydrogen bonds of 43, suggesting a highly stable and specific interaction with CLDN5 (33, 37). Hydrogen bonding contributes significantly to protein stability, and an increased number of hydrogen bonds is typically associated with improved binding affinity and complex formation (32). However, excessive hydrogen bonds may not always be the ideal scenario for protein flexibility (33, 39). Taken together, these results indicate mini-protein X as the optimal candidate.

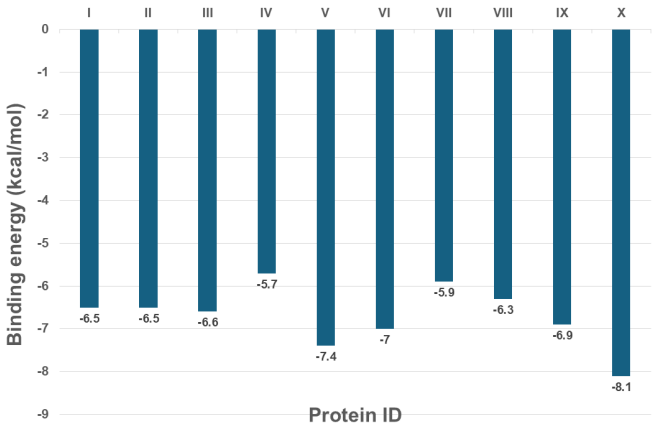

Figure 3: Binding energy analysis of computationally mutated mini-proteins. The bar graph shows the binding energy outcomes of the computationally mutated mini-proteins. The weakest binding energy was from mini-protein IV with -5.7 kcal/mol. Strongest binding energy came from mini-protein X with -8.1 kcal/mol. Mean binding energy was -6.69 kcal/mol. Standard deviation was 0.67 kcal/mol.

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Protein ID	Original Residue	Position	Mutated Residue	Mutation Notation	Rationale for Substitution
I	Glycine (G)	67	Glutamate (E)	G67E	Added negative charge to strengthen electrostatic interaction with Claudin-5.
II	Glycine (G)	67	Aspartate (D)	G67D	Added a negative charge to improve binding affinity.
III	Asparagine (N)	68	Leucine (L)	N68L	Tested changing to a residue that avoids water to see if it helps the proteins stick better.
IV	Asparagine (N)	68	Lysine (K)	N68K	Added a positive charge to attract nearby negative charges and strengthen protein binding.
V	Glycine (G)	62	Glutamate (E)	G62E	Added negative charge to strengthen electrostatic interaction with Claudin-5.
VI	Glycine (G)	62	Aspartate (D)	G62D	Added a negative charge to improve binding affinity.
VII	Glutamate (E)	56	Aspartate (D)	E56D	Tested shortening the acidic side chain to adjust binding affinity.
VIII	Aspartate (D)	38	Glutamate (E)	D38E	Tested extending the acidic side chain to enhance interaction with Claudin-5.
IX	Aspartate (D)	120	Glutamate (E)	D120E	Tested extending the acidic side chain to enhance interaction with Claudin-5.
X	Aspartate (D)	37	Glutamate (E)	D37E	Tested making the acidic part longer to reach new spots on Claudin-5, which improved binding.

Table 1: Computationally mutated mini-protein mutations. This table summarizes the computationally designed ten mini-proteins that were generated by computationally mutating Lipocalin-1 (LCN1). For each mini-protein, the original residue, mutation position, substituted residue, mutation notation, and a brief justification explaining the purpose of each modification are listed.

Having identified mini-protein X as the optimal *in silico* candidate, we now analyzed these findings in a disease context, where Vascular Endothelial Growth Factor (VEGF) driven CLDN5 loss compromises BBB junctions and lacks targeted therapy (25, 34). Despite extensive research, there is currently no approved therapy that directly targets CLDN5 disruption at the BBB (25). Elevated levels of VEGF, secreted by endothelial and immune cells during inflammation, downregulate CLDN5 expression and weaken tight junctions (34). Under normal conditions, VEGF expression is low and CLDN5 levels are high, maintaining healthy tight junctions at the BBB. During inflammation, elevated VEGF secreted by the endothelial and immune cells promotes CLDN5 downregulation through internalization and degradation, resulting in weakened tight junctions and increased BBB permeability (3, 34). As the BBB becomes more permeable, immune cells can enter the brain and release pro-inflammatory cytokines, which can worsen the progression of neurodegenerative diseases (35).

Translating these in silico findings into practice will require addressing key constraints in delivery, stability, and off-target effects. Our computationally designed mini-protein aims to stabilize CLDN5. While our results are promising at the computational level, several important challenges would need to be addressed before this approach could move forward, including how the protein could be delivered into the brain, whether it would remain stable in the body, and how to minimize off-target effects, including unintended immune activation or interactions with non-target tissues (36).

Electrostatic surface analysis of CLDN5 revealed a clear charge separation between the extracellular (negatively charged) and cytoplasmic (positively charged) domains. The extracellular domain, being both structurally accessible and negatively charged, may facilitate electrostatic interactions with positively charged regions on the mini-protein (32). However, because these analyses rely solely on computational models, confirmatory *in vitro* and *in vivo* experiments are required to validate whether these predicted interactions perform as per expectations in real-world conditions.

The wide range of variations in hydrogen bonding interactions demonstrates that there is no predicted pattern for hydrogen bonding within the mini-proteins. Structural analysis further indicated that only mini-protein X binds with

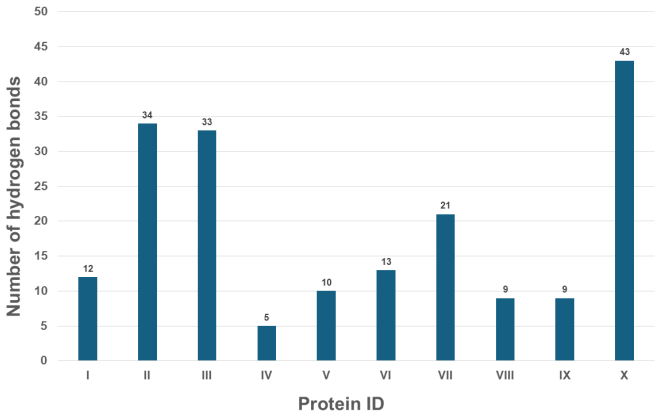

Figure 4: Hydrogen bond analysis of computationally mutated mini-proteins. The bar graph above represents mutated mini-proteins and their respective number of hydrogen bonds formed with CLDN5. The lowest amount of hydrogen bonds came from mini-protein IV with 5 hydrogen bonds. The highest number of hydrogen bonds came from mini-protein X with 43 hydrogen bonds. The mean hydrogen bond count was 19 hydrogen bonds.

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CLDN5 effectively at its accessible extracellular domain, where therapeutic targeting is most viable because a candidate can be delivered to this outside surface without crossing the plasma membrane, thereby helping stabilize BBB tight junctions (40). Notably, although mini-protein V exhibited the second strongest binding energy of –7.4 kcal/mol, it only had 10 hydrogen bonds and failed to bind within the accessible region, making it not an ideal candidate for further investigation. Mini-proteins II and III formed a considerably high number of hydrogen bonds, 34 and 33, respectively, but showed weaker binding energies and no advantage in structural accessibility. These contrasting cases, where mini-protein V has more favorable binding energy but few hydrogen bonds at the wrong site, and mini-proteins II and III with sufficient hydrogen bonds but weaker binding energy and poor binding sites, show that neither metric alone predicts effective CLDN5 binding. These findings suggest effective CLDN5 binding requires all three features: more negative binding energy, enough well-distributed hydrogen bonds, and a proper binding location on the extracellular surface (40, 42, 43). Considering all factors, mini-protein X is the most suitable candidate for further investigation. *In vitro* experiments are needed to test whether the predicted protein interactions work in real biological conditions (41).

This study identifies an LCN1-derived computational mini-protein predicted to bind with CLDN5, a key tight junction protein disrupted in early Alzheimer's disease and multiple sclerosis (44). These computationally mutated mini-protein analyses helped refine the interactions with CLDN5 and enhance binding stability. Together, these findings lay the foundation for future experimental validation to evaluate the mini-protein's real-life impact on BBB disruption. Future *in vitro* and *in vivo* studies assessing safety, stability, and delivery will test whether CLDN5-binding mini proteins can restore BBB integrity.

MATERIALS AND METHODS

Designing the mini-proteins

Proteins that bind to the CLDN5 protein were searched on the protein data bank (PDB) (46). A bacterium known as *Clostridium perfringens* produces an enterotoxin (CPE) that binds with the CLDN5 protein and disrupts the claudin-claudin interactions (48). However, CPE is highly toxic to the human body, so we searched for proteins that have similar structures to CPE but are non-toxic (49, 50). We looked for mini-proteins with a well-characterized structure and stability that would be able to bind small hydrophobic molecules together. For our study, the ideal mini-protein should firmly bind to CLDN5 and maintain a flexible structure while not disturbing the fluid environment around it. LCN1 (PDB ID: 5T43) is a flexible protein from the lipocalin family and can be engineered to bind specific targets, including CLDN5 (51–53). LCN1 was selected as the base protein for computational mutation based on its compact β-barrel structure, intrinsic ligand-binding capability, and established ability to interact with hydrophobic molecules (6). LCN1 met all the requirements of the ideal mini-protein, so it was chosen as a mini-protein that can bind to the CLDN5 protein and maintain the BBB integrity. The amino acid sequences of CLDN5 and the LCN1 protein were obtained from the UniProt database and folded using AlphaFold 3 to visualize and analyze the three-dimensional structure (5, 45). Computational mutations in the amino acid sequence of the LCN1 **(Table 1)** were performed to generate ten mini-proteins.

Exploring mini-protein interactions with CLDN5

To explore the interactions between CLDN5 and each mini-protein, molecular docking simulations were performed using the HDOCK 2.0 server (7). Molecular docking simulation uses computer models to predict when a ligand will bind to a receptor (7). The analysis included calculating hydrogen bonds, which are interactions between an electropositive hydrogen atom and an electronegative atom such as oxygen or nitrogen, using an in-house script by identifying donor and acceptor atoms based on interatomic distance and bond angle. For each mutated LCN1, we submitted the HDOCK-derived CLDN5–LCN1 complex to the PRODIGY web server to compute binding energy (37, 38). The interaction between CLDN5 and the mini-protein was visualized using Chimera X (47).

In-house Python Script for Hydrogen-Bond Counting

The full code of the hydrogen-bond counts reported in Figure 4 can be found in the Appendix. The script operationalizes a simple, distance-based criterion (N/O atom pairs within 4.0 Å) on docked CLDN5–mini-protein complexes and outputs per-pair distances as well as a file level summary.

Using Python 3.10+, packages Biophython (Bio.PDB), NumPy, and pandas, the script takes input one or more PDB files of docked two-chain complexes (chain A is CLDN5 and chain B is mini-protein) and outputs an Excel file (hydrogen_bonds.xlsx) with the columns PDB File, Residue 1, Atom Name 1, Residue 2, Atom Name 2, and Distance (Å).

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APPENDIX

Usage

Place the following script and your .pdb files in the same folder. Open a terminal in that folder and run python file HydorgenBond-Table.py. When it finishes, the output file hydrogen_bonds.xlsx will be in the same folder.

In house python script

```
# HydorgenBond-Table.py
from Bio import PDB
import numpy as np
import os
import pandas as pd

def calculate_distance(atom1, atom2):
    """Calculate the Euclidean distance between two atoms."""
    diff_vector = atom1.coord - atom2.coord
    return np.sqrt(np.sum(diff_vector * diff_vector))

def get_hydrogen_bonds(chain1, chain2, cutoff=4.0):
    """Get hydrogen bonds between two chains within a cutoff distance."""
    hydrogen_bonds = []
    for residue1 in chain1:
        for atom1 in residue1:
            if atom1.element in ('N', 'O'):  # Consider only N and O atoms
                for residue2 in chain2:
                    for atom2 in residue2:
                        if atom2.element in ('N', 'O') and atom1 != atom2:
                            distance = calculate_distance(atom1, atom2)
                            if distance < cutoff:
                                hydrogen_bonds.append({
```

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```
                    'Residue 1': f"{residue1.get_resname()}
{residue1.get_id()[1]}",
                    'Atom Name 1': atom1.get_name(),
                    'Residue 2': f"{residue2.get_resname()}
{residue2.get_id()[1]}",
                    'Atom Name 2': atom2.get_name(),
                    'Distance (Å)': round(distance, 2)
                })
    return hydrogen_bonds

def process_pdb_file(pdb_file):
    parser = PDB.PDBParser(QUIET=True)
    structure = parser.get_structure('protein', pdb_file)
    chains = list(structure.get_chains())
    if len(chains) < 2:
        print(f"The PDB file '{pdb_file}' does not contain two
separate chains.")
        return []
    chain1 = chains[0]
    chain2 = chains[1]
    return get_hydrogen_bonds(chain1, chain2)

def main():
    pdb_files = [file for file in os.listdir('.') if file.endswith('.pdb')]
    if not pdb_files:
        print("No PDB files found in the current directory.")
        return
    all_data = []
    for pdb_file in pdb_files:
        print(f"Processing {pdb_file}...")
        hydrogen_bonds = process_pdb_file(pdb_file)
        for bond in hydrogen_bonds:
            bond['PDB File'] = pdb_file
            all_data.append(bond)
    if all_data:
        df = pd.DataFrame(all_data)
        output_file = "hydrogen_bonds.xlsx"
        df.to_excel(output_file, index=False)
        print(f"Hydrogen bond data has been saved to '{output_
file}'.")
    else:
        print("No hydrogen bonds found in the PDB files.")

if __name__ == "__main__":
    main()
```