DISTILLATION №66 · ALEMBIC LABS

HUMANIN — DOUBLE SUBSTITUTION SER-7 → CYS AND LEU-11 → CYS, INTRODUCING AN I,I+4 INTRAMOLECULAR DISULFIDE BRIDGE ACROSS ONE HELICAL TURN (RESIDUES 7-11) WITHIN THE CENTRAL BAX-ENGAGING SEGMENT. NATIVE CYS-8 IS PRESERVED AS A FREE THIOL; SELECTIVE OXIDATION OF THE ENGINEERED 7-11 PAIR IS ASSUMED (KINETICALLY FAVORED BY I,I+4 GEOMETRY OVER THE LONGER-RANGE 8-X OPTIONS).

generated 2026-05-04T13:59:07.553665+00:00

PROMISING | LONGEVITY

DOUBLE SUBSTITUTION SER-7 → CYS AND LEU-11 → CYS, INTRODUCING AN I,I+4 INTRAMOLECULAR DISULFIDE BRIDGE ACROSS ONE HELICAL TURN (RESIDUES 7-11) WITHIN THE CENTRAL BAX-ENGAGING SEGMENT. NATIVE CYS-8 IS PRESERVED AS A FREE THIOL; SELECTIVE OXIDATION OF THE ENGINEERED 7-11 PAIR IS ASSUMED (KINETICALLY FAVORED BY I,I+4 GEOMETRY OVER THE LONGER-RANGE 8-X OPTIONS).

APOPTOSIS REGULATOR BAX

AVERAGE CONFIDENCE	PTM / IPTM	VERDICT
55.8%	0.479 / 0.579	PROMISING

TARGET	UNIPROT	BINDING PROBABILITY
Apoptosis regulator BAX	Q07812	—

TLDR

Fold #66 installs an i,i+4 disulfide staple between engineered Cys-7 and Cys-11 in Humanin, aiming to pre-organize the central α-helical turn for tighter BAX groove engagement while preserving native Cys-8. Boltz-2 predicts a defined peptide–BAX interface (ipTM 0.58) but per-residue confidence is modest (pLDDT 0.56), insufficient to confirm helix-tightening at the stapled residues. The result is PROMISING rather than REFINED — consistent with the pattern seen in Fold #22's i,i+6 disulfide variant — and the case for the i,i+4 geometry remains biologically rational but structurally unresolved. Wet-lab validation and the cytosolic-redox liability represent the dominant barriers to advancing this design.

EXECUTIVE SUMMARY

Humanin i,i+4 disulfide staple (S7C/L11C): ipTM 0.58 places peptide in a defined BAX groove, but pLDDT 0.56 cannot confirm helix-tightening at the stapled turn. PROMISING — architecturally stronger than Fold #22's i,i+6 variant, but cytosolic redox liability and Leu→Cys hydrophobic loss need wet-lab adjudication.

DETAILED ANALYSIS

Humanin (HN) is a 24-residue mitochondria-derived peptide with well-characterized cytoprotective activity mediated primarily through direct physical engagement of the pro-apoptotic protein BAX. Its anti-apoptotic mechanism involves sequestering BAX into fibrillar complexes that prevent mitochondrial outer-membrane permeabilization (MOMP), and the functional dependence on HN's α-helical structure is established by both CD spectroscopy and site-directed mutagenesis data showing that helix-disrupting mutations abrogate both anti-apoptotic activity and fibrillar BAX complex morphology (Morris et al., 2019). The central segment, approximately residues 5–15, is the experimentally implicated BAX-binding pharmacophore, though the precise helical register in the bound state has not been crystallographically resolved.

Fold #66 tests the hypothesis that installing a disulfide bridge between engineered Cys-7 and Cys-11 — a canonical i,i+4 one-turn helical staple — will pre-organize this pharmacophoric segment into its bioactive α-helical conformation prior to target encounter. The rationale is stereochemically grounded: i,i+4 cysteine pairs enforce

φ/ψ angles in the α-helical region across one complete turn, a constraint better suited to helix nucleation than the longer i,i+6 span explored in Fold #22. Both Cys-7 and Cys-11 are predicted to be surface-exposed on the solvent face of the helix, leaving the BAX-contact face unoccluded. Native Cys-8 is deliberately retained as a free thiol, building on the lesson from Fold #22 which paired Cys-8 with an engineered Cys-14 — the tighter geometry here is designed to produce cleaner helical drive.

Structural prediction by Boltz-2 yields an ipTM of 0.58 and a pTM of 0.48, placing the peptide in what appears to be a defined groove on BAX rather than non-specific surface contact. This is a non-trivial result: an ipTM above 0.5 for a 24-residue peptide against a globular protein is within the range where Boltz-2 has demonstrated meaningful pose discrimination in benchmark studies. However, the per-residue pLDDT averages only 0.56 across the full sequence, and critically the helical segment spanning residues 6–13 — where the conformational hypothesis is most specific — is not resolved with the precision needed to confirm the predicted helix-nucleating effect of the 7–11 staple. The disulfide geometry itself cannot be verified from the pLDDT profile alone. No Chai-1 corroboration was obtained, and the Boltz-2 affinity module produced no numerical output, leaving binding affinity change as purely conjectural.

This result sits within a coherent narrative across the Humanin sub-series in this lab. Fold #22 (S14C/Cys-8 disulfide, i,i+6) was also rated PROMISING with nearly identical pLDDT (0.56), suggesting that the modest confidence ceiling is a property of the peptide–BAX system rather than a specific failure of the staple design. Fold #37 (S7A) was DISCARDED at pLDDT 0.62, paradoxically with higher confidence but no productive structural model. Fold #59 (N-terminal myristoylation) failed outright due to the non-canonical lipid chemistry. The present fold therefore represents the most structurally coherent Humanin modification in the disulfide series, and the step from i,i+6 to i,i+4 is architecturally justified even if the predictors cannot yet resolve the difference.

The literature context adds both support and meaningful cautions. The S14G substitution (HNG), which enhances potency ~1000-fold, demonstrates that HN tolerates residue substitutions in the central region — a favorable precedent for the S7C/L11C double substitution. However, the Leu-11 → Cys change introduces a smaller, more polar side chain at a position potentially involved in hydrophobic contacts with the BAX groove, a change that could reduce affinity independently of any helical-organization benefit. This is a significant chemical liability that structural prediction at this confidence level cannot adjudicate. Separately, Luciano et al. (2005) showed HN also engages BimEL through the same functional surface; rigidifying the central helix might selectively favor one binding geometry, potentially disrupting the BimEL inhibitory axis.

The most substantive biological challenge is the cytosolic-redox environment. BAX resides in the cytosol in its inactive conformation, maintained at reducing potential

by glutathione (~1–10 mM). An engineered disulfide staple would be expected to be reduced in this compartment, abolishing the conformational constraint precisely where it is needed. The literature is silent on disulfide-stapled peptides targeting cytosolic BAX, and this represents a critical unresolved liability for the in-cell efficacy of this design. A cell-permeable, reduction-resistant analog — for example, a thioether or hydrocarbon staple at equivalent positions — would be the logical next design iteration if the BAX-binding hypothesis is validated biochemically.

Heuristic sequence-based profiling predicts moderate-to-low aggregation propensity (0.295), moderate stability (0.512), and a reasonable estimated half-life in the moderate-to-long range (~1–6 hours). BBB penetration is predicted as low (0.222), consistent with the peptide's size and polarity. These estimates are derived from sequence composition alone and carry no mechanistic weight. Overall, Fold #66 is a scientifically well-reasoned design that produces a structurally plausible but unconfirmed result — precisely the PROMISING category it deserves — and represents a meaningful advance over Fold #22 in architectural logic, if not yet in prediction confidence.

RESEARCH BRIEF

DISTILLATION №66 — HUMANIN S7C/L11C I,I+4 DISULFIDE STAPLE

Verdict: PROMISING | Target: BAX (Q07812) | Class: LONGEVITY

> **Disclaimer:** All findings are in silico predictions only. No wet-lab validation has been performed. Heuristic properties are sequence-derived estimates. This is not medical advice.

MECHANISM OF ACTION

Humanin (HN) is a 24-residue mitochondria-derived peptide that directly engages BAX (BCL-2-associated X protein), the central executor of the intrinsic apoptotic pathway. BAX resides in the cytosol in an inactive, monomeric conformation and, upon apoptotic signaling, undergoes conformational activation, oligomerization, and insertion into the mitochondrial outer membrane — triggering MOMP and cytochrome c release. HN physically binds BAX and sequesters it into fibrillar complexes that prevent this translocation (Guo et al., 2003; Morris et al., 2019). The central segment of HN (approximately residues 5–15) is the experimentally implicated BAX-binding pharmacophore, and HN's α-helical structure in this region is functionally load-bearing: mutations disrupting helical integrity abolish both anti-apoptotic activity and the characteristic fibrillar BAX-sequestration morphology.

Beyond BAX, HN also engages BimEL, a BH3-only proapoptotic protein, through the same functional surface, indicating that the central helical segment must accommodate structurally distinct binding partners.

PERFORMANCE APPLICATIONS

Humanin analogs with enhanced BAX-binding affinity are of interest in the context of: - **Cytoprotection and longevity biology:** HN expression declines with age and is inversely correlated with age-related cell loss in neuronal and cardiomyocyte populations. A conformationally stabilized, higher-affinity BAX inhibitor could extend the anti-apoptotic window in post-mitotic cells. - **Ischemia-reperfusion injury:** The S14G HN analog (HNG) has demonstrated cardioprotective and renoprotective effects in preclinical I/R models, and a conformationally locked variant could offer improved potency in similar paradigms. - **Neurodegeneration research:** HN was originally isolated from surviving neurons in Alzheimer's disease tissue, and BAX-mediated apoptosis is implicated in neurodegenerative cell loss. Research tools with tighter BAX engagement would be valuable for dissecting this mechanism.

Note: These are research contexts, not therapeutic indications. No clinical data exist for this specific variant.

MODIFICATION RATIONALE

The native Humanin sequence contains partial α-helical structure in solution, with the central segment (residues 5–15) being only incompletely organized in the free peptide — a conformational liability that limits affinity for BAX by imposing an entropic cost on folding-upon-binding. This fold introduces a disulfide staple between engineered **Cys-7** and **Cys-11**, separated by exactly one α-helical turn (i,i+4 spacing), to pre-organize this pharmacophoric turn into its bioactive helical conformation before target encounter.

The i,i+4 geometry is the canonical spacing for α-helical turn stabilization via disulfide bridges: it enforces φ/ψ angles in the α-helical region across precisely one turn (~100° rotation, ~5.4 Å rise), maximizing the helix-nucleating geometric match. This is architecturally tighter and more directionally specific than the i,i+6 disulfide explored in **Fold #22** (S14C/Cys-8 pair), which spanned a longer loop with weaker helical drive and received a PROMISING verdict at identical pLDDT (0.56). The step from i,i+6 to i,i+4 is the key structural refinement tested here.

Native **Cys-8** is deliberately preserved as a free thiol. It sits at the i+1 position relative to engineered Cys-7, making it the most geometrically proximate potential mispairing partner. Selective oxidation of the 7–11 pair is assumed to be kinetically favored by the i,i+4 geometry, but this is an assumption that requires experimental validation (see Caveats).

Positions 7 and 11 were selected as surface-exposed residues on the predicted solvent face of the helix, not on the BAX-contact face. The native Ser-7 → Cys substitution removes a small polar residue; the Leu-11 → Cys substitution replaces a hydrophobic side chain with a smaller, polar one — the latter is the most significant chemical liability of the design and is discussed in Limitations.

Modified sequence: `MAPRGFCCLLCLTSEIDLPVKRRA` (Bold residues: engineered Cys-7 and Cys-11; native Cys-8 between them is preserved)

PREDICTED PROPERTIES — WHERE SIGNAL IS MODERATE

Parameter	Value	Context
Boltz-2 pLDDT	0.558	Below the 0.7 threshold for confident side-chain placement
pTM	0.479	Moderate global fold confidence
ipTM	0.579	Moderate interface confidence — consistent with defined groove docking
Chai-1 agreement	Not obtained	Single-model prediction only
Affinity module	No output	Binding ΔΔG cannot be predicted
Aggregation propensity (heuristic)	0.295	Low-to-moderate
Stability score (heuristic)	0.512	Moderate
BBB penetration (heuristic)	0.222	Low — expected for a 24-mer
Half-life estimate (heuristic)	Moderate-to-long (~1–6 h)	Sequence-derived estimate only

Where the signal is meaningful: An ipTM of 0.58 for a 24-residue peptide against a globular protein places this prediction in the range where Boltz-2 demonstrates meaningful pose discrimination in benchmarks — the peptide is predicted to occupy a defined groove rather than making non-specific surface contact. This is consistent with the literature placing HN's central segment at the BAX interface.

Where the signal is insufficient: Per-residue pLDDT of 0.56 across residues 6–13 — the segment where the conformational hypothesis is most specific — means that the helical register and side-chain placement in the stapled region cannot be confirmed. The helix-tightening effect predicted by the i,i+4 geometry cannot be

distinguished from the i,i+6 variant of Fold #22 at this resolution. The disulfide bond geometry itself is not verifiable from the pLDDT profile.

All heuristic values are sequence-based computational estimates, not experimental measurements.

WHAT WOULD STRENGTHEN THIS SIGNAL

Additional computational predictions: 1. **Chai-1 ensemble prediction** with explicit disulfide bond encoding at positions 7–11: comparison of Chai-1 vs. Boltz-2 ipTM and pose RMSD would provide the most immediately actionable confidence upgrade. Convergent poses across two independent models would substantially strengthen the PROMISING verdict. 2. **Free-energy perturbation (FEP) or MM-GBSA rescoring** of the Boltz-2 pose to estimate ΔΔG relative to native HN and the Fold #22 variant — this would be the most direct computational test of whether the i,i+4 staple provides a binding affinity advantage. 3. **MD simulation** of the stapled peptide in isolation to confirm that the 7–11 disulfide drives φ/ψ angles into the α-helical region for residues 6–13, and does not introduce strain at Cys-8 (adjacent free thiol). 4. **Disulfide selectivity modeling:** Explicit computational assessment of the competing 7–8 (i,i+1) and 8–11 (i,i+3) mispairings would quantify the kinetic and thermodynamic favorability assumptions of the design.

Wet-lab experiments that would adjudicate this hypothesis: 1. **Surface plasmon resonance (SPR) or ITC** with purified recombinant BAX and the chemically synthesized S7C/L11C Humanin peptide (with 7–11 disulfide selectively oxidized by copper(II) catalysis or DMSO-mediated aerial oxidation in mildly acidic conditions to favor the i,i+4 pair): measure KD vs. native HN and the Fold #22 variant to test the affinity-enhancement hypothesis directly. 2. **CD spectroscopy** comparing the helical content of S7C/L11C-stapled HN vs. native HN vs. Fold #22 variant in oxidized and reduced conditions — this would directly test whether the i,i+4 staple increases α-helical content as predicted. 3. **Mass spectrometry with differential alkylation** of the three cysteines (7, 8, 11) to map disulfide connectivity in the oxidized product — confirming that the 7–11 pair forms preferentially over 7–8 or 8–11 mispairs. 4. **Cell-based apoptosis protection assay** (e.g., staurosporine-treated HEK293T or neuroblastoma cells, with TUNEL or caspase-3 readout) comparing the stapled variant vs. native HN vs. a reduced (DTT-treated) stapled control — this would test whether the disulfide constraint is functionally beneficial and whether cytosolic reduction abolishes the benefit. 5. **Thioether analog synthesis** (replacing the 7–11 disulfide with a non-reducible thioether or hydrocarbon staple) as a follow-up if the disulfide staple shows in vitro benefit but fails cell-based assays due to cytosolic reduction — directly testing the redox-liability hypothesis.

LAB NARRATIVE & CROSS-FOLD CONTEXT

This fold continues the Humanin disulfide stapling series initiated in **Fold #22**, which introduced an i,i+6 Cys-8/S14C disulfide and received a PROMISING verdict at pLDDT 0.56 — strikingly identical confidence to the present fold. The architectural progression is meaningful: the i,i+4 geometry tested here provides a stronger stereochemical rationale for helix nucleation than the longer i,i+6 span, and positions 7 and 11 are predicted to be on the solvent face rather than the BAX-contact face, avoiding the occlusion risk present in Fold #22 where the S14C mutation was closer to the C-terminal end of the pharmacophoric segment. The fact that both disulfide variants converge on pLDDT ~0.56 and ipTM ~0.58 suggests this confidence ceiling may reflect the limitations of predicting a partially disordered peptide against BAX's dynamic surface rather than a failure specific to either design.

Fold #37 (S7A, DISCARDED, pLDDT 0.62) provides a useful control: removing Ser-7's hydroxyl without introducing a staple gave higher per-residue confidence but no productive interface, suggesting that position 7 tolerates substitution but that the staple geometry — not just the residue change — is doing chemical work here.

Fold #59 (N-terminal myristoylation, FAILED) reinforces that AlphaFold-family tools struggle with non-canonical chemical modifications, which is relevant context: the disulfide bond in the present fold is at least encodable within the standard amino-acid alphabet, giving it a structural prediction advantage over lipidated variants even if the confidence is modest.

The next logical step in this series — if Chai-1 corroboration supports the pose — would be a thioether-stapled analog at the same 7–11 positions (replacing the reduction-labile disulfide with a non-reducible covalent constraint), directly addressing the dominant cytosolic-redox liability identified by the literature agent.

SEQUENCES

NATIVE

```
MAPRGFSCLLLLTSEIDLPVKRRA
```

MODIFIED

```
MAPRGFCCLLCLTSEIDLPVKRRA
```

CAVEATS

- In silico prediction only — requires wet-lab validation before any conclusions about biological activity can be drawn
- Single-run Boltz-2 prediction — not ensembled; no Chai-1 corroboration obtained for this fold
- pLDDT 0.56 is below the 0.7 threshold for confident side-chain placement; helical register and disulfide geometry in residues 6–13 cannot be confirmed from this model
- Boltz-2 affinity module produced no output — no predicted ΔΔG binding change; affinity claims are speculative
- Predicted properties (aggregation, stability, BBB penetration, half-life) are heuristic sequence-based estimates, not experimental measurements
- The selectivity of 7–11 disulfide oxidation over competing 7–8 (i,i+1) or 8–11 (i,i+3) mispairings with native Cys-8 is an unvalidated assumption — three-cysteine disulfide selectivity requires experimental mapping by differential alkylation MS
- Cytosolic glutathione (~1–10 mM) would be expected to reduce the engineered disulfide in vivo, potentially abolishing the conformational staple at the site of BAX engagement — this is a critical unresolved biological liability
- Leu-11 → Cys substitution replaces a hydrophobic side chain with a smaller, polar residue at a position potentially involved in hydrophobic contacts with the BAX groove — this could reduce affinity independently of any helical-organization benefit
- The exact BAX binding groove and helical register of HN in its bound state are not crystallographically resolved; the assumption that residues 7–11 are on the solvent face (not the contact face) is inferred from homology models, not experimental structures
- This is research context only — not medical advice

CITATIONS

1. **PMID —** (2019) — — Humanin induces conformational changes in the apoptosis regulator BAX and sequesters it into fibers, preventing mitochondrial outer-membrane permeabilization
2. **PMID —** (2003) — — Humanin peptide suppresses apoptosis by interfering with Bax activation
3. **PMID —** (2004) — — Humanin: after the discovery
4. **PMID —** (2005) — — Cytoprotective peptide humanin binds and inhibits proapoptotic Bcl-2/Bax family protein BimEL
5. **PMID —** (2004) — — Unravelling the role of Humanin

6. **PMID —** (2021) — — Humanin: A mitochondrial-derived peptide in the treatment of apoptosis-related diseases
7. **PMID —** (2022) — — Humanin and Alzheimer's disease: The beginning of a new field
8. **PMID —** (2022) — — Cardio-protective role of Humanin in myocardial ischemia-reperfusion
9. **PMID —** (2026) — — Renoprotective Effect of S14G-Humanin on Renal Ischemia/Reperfusion Injury by Activation of STAT3 and ERK 1/2 Signal Transduction Pathways in Rats
10. **PMID —** (2016) — — Humanin: Functional Interfaces with IGF-I

SOLANA SIGNATURE 2bVWoJNt4nbNEEMSAF5Rut9JU1jeyLtkjXsRifCHLEcaFz4XpkWvmcWS97EwnMFXMc9k5FraVqGieDbF27CLk125
DATA SHA-256 14a9b83264bae8109f03dc3f76b63e35bae15b37be9c27cfd07c3fde9eabe33c
VERIFY https://solscan.io/tx/2bVWoJNt4nbNEEMSAF5Rut9JU1jeyLtkjXsRifCHLEcaFz4XpkWvmcWS97EwnMFXMc9k5FraVqGieDbF27CLk125