DISTILLATION №22 · ALEMBIC LABS

HUMANIN — INTRODUCE AN INTRAMOLECULAR DISULFIDE BRIDGE BY SUBSTITUTING SER-14 → CYS, PAIRING WITH THE NATIVE CYS-8 TO FORM AN I,I+6 DISULFIDE LOOP SPANNING THE CENTRAL HELICAL REGION (RESIDUES 8-14)

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PROMISING LONGEVITY

INTRODUCE AN INTRAMOLECULAR DISULFIDE BRIDGE BY SUBSTITUTING SER-14 → CYS, PAIRING WITH THE NATIVE CYS-8 TO FORM AN I,I+6 DISULFIDE LOOP SPANNING THE CENTRAL HELICAL REGION (RESIDUES 8-14)

APOPTOSIS REGULATOR BAX

AVERAGE CONFIDENCE	PTM / IPTM	VERDICT
55.8%	0.470 / 0.357	PROMISING

TARGET	UNIPROT	BINDING PROBABILITY
Apoptosis regulator BAX	Q07812	—

TLDR

DISTILLATION №22 introduces a disulfide-cyclized variant of Humanin (S14C, pairing with native Cys-8) designed to pre-organize the central α-helical segment for BAX engagement. Structural prediction yields a partially helical central region with Cys8–Cys14 positioned in an i,i+6 register consistent with the intended disulfide geometry, but interface confidence (ipTM 0.36) is too low to confirm productive BAX groove docking. The fold earns a PROMISING verdict based on suggestive helix pre-organization, not a confirmed binding pose. A key biological complication — the reducing intracellular environment where BAX inhibition is most needed — remains an unresolved challenge for any disulfide-dependent strategy.

EXECUTIVE SUMMARY

S14C-Humanin: disulfide loop across Cys8–Cys14 predicted to partially pre-organize the BAX-binding helix (pLDDT 0.558), but ipTM 0.357 leaves the interface unresolved. PROMISING signal — redox sensitivity and the HNG flexibility paradox are the critical next tests.

DETAILED ANALYSIS

Humanin (HN) is a 24-amino acid mitochondrial-derived peptide with one of the most thoroughly documented anti-apoptotic mechanisms in the longevity peptide literature. Its primary axis of action is direct physical engagement with BAX, preventing its conformational activation, cytosolic-to-mitochondrial translocation, and downstream mitochondrial outer membrane permeabilization (MOMP). The foundational Guo et al. (2003) work established this as a bona fide physical interaction, and Morris et al. (2019) extended this to biophysical characterization showing HN and BAX co-form fibers with associated BAX conformational changes. Critically, HN mutations that alter anti-apoptotic potency also alter fiber morphology — implicating HN's own conformation as a determinant of BAX engagement efficacy and providing a rational basis for the conformational pre-organization strategy tested here.

The native HN sequence contains a single cysteine at position 8 (Cys-8), embedded within the FSCL stretch. DISTILLATION №22 introduces a minimal single-point mutation — Ser-14 → Cys — to create a second cysteine capable of forming an intramolecular disulfide bridge with Cys-8 in an i,i+6 register. Spanning approximately two turns of an α-helix, this geometry is well-established in the stapled peptide and constrained peptide literature as effective at nucleating and stabilizing helical conformations by reducing conformational entropy. The modified sequence becomes MAPRGFSCLLLLTCEIDLPVKRRA, with only one amino acid change from wild-type. The LLLL hydrophobic tract (residues 9–12) and adjacent acidic residues are preserved, maintaining the amphipathic character hypothesized to be important for BAX groove engagement.

Structural prediction via AlphaFold (with BAX as the target context) yields a pLDDT of 0.558 for the peptide — moderate, and notably higher than the collapsed scores seen in recent LONGEVITY folds including Fold #21 (Epitalon amidation, pLDDT 0.34) and Fold #6 (D-Ala Epitalon). The pTM of 0.470 reflects reasonable overall fold confidence for a short peptide, but the ipTM of 0.357 is the critical limiting value: it indicates the predicted interface between S14C-Humanin and BAX is not confidently modeled, and the docking pose cannot be taken as reliable. The central segment (residues 8–14) shows partial helical character, and the Cys8–Cys14 Cα–Cα distance in the predicted structure is consistent with i,i+6 disulfide geometry. However, because current structure prediction tools do not explicitly model disulfide bonds as

covalent constraints, the SS bond itself is inferred from geometry rather than directly represented, adding epistemic uncertainty to the pre-organization claim.

The literature context introduces a notable complication for this modification strategy. Position 14 in HN is the site of the S14G substitution that produces HNG, the most widely studied high-potency HN analog. Glycine at position 14 is specifically selected for its flexibility and minimal steric bulk — the opposite of the conformational constraint introduced by Cys and a disulfide bond. This raises a genuine mechanistic question: if HNG's enhanced potency partly derives from local flexibility at position 14 enabling an induced-fit engagement with BAX (or conformational rearrangement into the fibrillar interaction mode described by Morris et al.), then rigidifying this position may be counterproductive. The disulfide strategy is well-reasoned by canonical α-helix stabilization logic, but HN's engagement with BAX may not follow a simple lock-and-key helix docking model.

A second unresolved complication is the redox context. BAX inhibition is most consequential in the cytosol and at the cytosolic face of the mitochondrial outer membrane — environments that are generally reducing, maintained by glutathione at millimolar concentrations. A disulfide-bridged peptide delivered extracellularly or administered systemically may undergo disulfide reduction before reaching its intracellular target, effectively reverting to the unconstrained wild-type-like sequence. This does not negate the fold's value as a structural hypothesis test, but it is a critical translational constraint that would need to be addressed through cell-penetrating delivery with disulfide-reducing pathway bypass, or through replacement of the disulfide with a non-reducible hydrocarbon staple or lactam bridge in subsequent iterations.

The heuristic peptide property profile shows moderate aggregation propensity (0.342) and stability (0.476), consistent with a peptide that is neither highly aggregation-prone nor robustly stable. BBB penetration is predicted low (0.242), appropriate for a peptide targeting intracellular BAX rather than CNS targets. Half-life is estimated moderate-to-long (1–6 hours), though this will depend heavily on whether the disulfide remains intact in vivo. No Boltz-2 affinity module values or Chai-1 agreement scores were available for this fold, limiting the depth of interface analysis.

In the context of the lab's running LONGEVITY series, this fold represents the first cyclization-class modification and the first conformation-focused hypothesis in the recent rotation (following Epitalon terminal amidation in Fold #21, MOTS-c K13R substitution in Fold #19, SS-31 naphthylalanine substitution in Fold #17, and FOXO4-DRI tail truncation in Fold #12). The FOXO4-DRI work (Fold #12) is thematically adjacent — both target apoptosis-regulatory mechanisms — though FOXO4-DRI operates via p53 engagement rather than direct BAX inhibition. The present fold advances the lab into new modification chemistry territory. The PROMISING verdict is appropriate: the structural signal is suggestive but not

confirmatory, and the biological rationale carries both strong supporting evidence and genuine mechanistic complications that keep this from REFINED status.

RESEARCH BRIEF

DISTILLATION №22 — PROMISING

HUMANIN S14C: DISULFIDE-BRIDGED CYCLIZATION TO PRE-ORGANIZE THE BAX-BINDING HELIX

Peptide: Humanin (modified) — MAPRGFSCLLLLT**C**EIDLPVKRRA
Class: LONGEVITY
Target: BAX (UniProt Q07812)
Modification: Ser-14 → Cys, forming an intramolecular disulfide with native Cys-8 (i,i+6 register)
Verdict: PROMISING
pLDDT: 0.558 | **pTM:** 0.470 | **ipTM:** 0.357

MECHANISM OF ACTION

Humanin (HN) is a 24-amino acid mitochondrial-derived peptide that directly binds the pro-apoptotic BCL-2 family protein BAX and prevents its activation cascade. Under apoptotic stress, BAX undergoes conformational activation, exposes its N-terminus and BH3 domain, translocates from the cytosol to the mitochondrial outer membrane, oligomerizes, and drives MOMP — releasing cytochrome c and committing the cell to caspase-dependent death. HN intercepts this cascade upstream: Guo et al. (2003, PMID:12732850) demonstrated by co-immunoprecipitation and functional assay that HN physically binds BAX, blocks its conformational change, and prevents mitochondrial translocation. This is a direct, physical anti-apoptotic mechanism — not a downstream signaling effect.

Morris et al. (2019, PMID:31690630) extended this picture using CD spectroscopy, fluorescence, and negative-stain EM, revealing that HN and BAX co-form fibers with BAX undergoing secondary and tertiary structural rearrangements upon engagement. Critically, HN mutations that alter anti-apoptotic potency also alter fiber morphology — directly linking HN's own conformation to its functional output. Luciano et al. (2005, PMID:15661735) demonstrated HN also inhibits BimEL and other BCL-2 family pro-apoptotic members, but the BAX axis is the most mechanistically resolved and is the focus of this distillation.

MODIFICATION RATIONALE

Native HN is largely disordered in solution. Its anti-apoptotic function requires the central amphipathic segment — spanning approximately residues 7–17, encompassing the LLLL hydrophobic tract and the EIDL acidic region — to adopt an α-helical conformation competent to engage the BAX hydrophobic groove or fiber interface. This conformational disorder imposes an entropic cost upon binding: HN must fold-upon-binding, reducing the effective affinity relative to a pre-organized helix.

The S14C modification exploits the presence of native Cys-8 to introduce a disulfide bridge with minimal mutational footprint — only a single substitution is required. An i,i+6 disulfide spans approximately two turns of an α-helix (Cα–Cα ~9–10 Å, compatible with SS geometry) and is well-established in the constrained peptide literature as an effective helix-nucleating constraint. Ser-14 was selected because it sits on the solvent-facing, polar face of the helix in the predicted helical model, away from the hydrophobic Leu-rich face hypothesized to contact BAX — minimizing disruption to the binding interface while enforcing backbone geometry.

This modification also opens a new experimental avenue for the lab: DISTILLATION №22 is the first **cyclization-class** modification in the LONGEVITY series, complementing the substitution strategies explored in Fold #19 (MOTS-c K13R) and Fold #17 (SS-31 Nal), and providing a structural pre-organization hypothesis not previously tested on any peptide in the recent rotation.

PREDICTED PROPERTIES — WHERE THE SIGNAL IS MODERATE

Metric	Value	Interpretation
pLDDT	0.558	Moderate — partial helical order predicted in central segment
pTM	0.470	Reasonable overall fold topology
ipTM	0.357	**Low** — interface pose not confidently modeled
Cys8–Cys14 geometry	i,i+6, Cα–Cα consistent with SS	Suggestive of disulfide compatibility
Hydrophobic face (Leu8–12)	Partially solvent-exposed	Not unambiguously docked into BAX groove
Aggregation propensity (heuristic)	0.342	Moderate — manageable

Metric	Value	Interpretation
Stability score (heuristic)	0.476	Moderate
BBB penetration (heuristic)	0.242	Low — appropriate for intracellular target
Half-life estimate (heuristic)	Moderate-to-long (~1–6 hr)	Redox-dependent caveat applies

The structural prediction places S14C-Humanin near BAX with a partially helical central segment and a Cys8–Cys14 register consistent with the intended disulfide geometry. This is the predicted structure's most encouraging signal: the folding algorithm independently arrives at a helical register that would accommodate the proposed SS bond, without being explicitly told to enforce it. However, current AF/Chai tools do **not** model disulfide bonds as covalent constraints — the SS bond is inferred from geometry, not enforced, which means the actual constrained peptide could behave differently.

The low ipTM (0.357) is the primary limiting factor: the docking interface between peptide and BAX is not confidently resolved. The hydrophobic Leu face is partially exposed but not unambiguously positioned in the BAX hydrophobic groove. The PROMISING verdict reflects genuine structural signal in the peptide itself, not a confirmed binding event.

WHAT WOULD STRENGTHEN THIS SIGNAL

Computational next steps:

1. **Ensemble prediction with explicit SS constraint:** Run the S14C variant with explicit disulfide bond modeling in tools that support covalent constraint definition (e.g., RosettaFold with constraint files, or Schrödinger's CovDock). A single unconstrained prediction cannot capture the true effect of the covalent loop on backbone geometry.
2. **MD simulation of constrained vs. unconstrained HN:** Molecular dynamics with the disulfide bond explicitly enforced (GROMACS/AMBER with SS bonding) would quantify the change in helical content and conformational entropy of residues 8–14, directly testing the hypothesis that the bridge pre-organizes the helix.
3. **Chai-1 / Boltz-2 affinity module re-run:** This fold lacked Chai-1 agreement scores and Boltz-2 affinity values. A second-model agreement run would substantially increase or decrease confidence in the predicted pose.

4. **Comparison fold — lactam analog:** Run the lactam-bridged equivalent (Lys-8, Glu-14 → lactam, or vice versa) as a non-reducible helix staple at the same i,i+6 position. If the lactam variant shows higher ipTM and similar helix geometry, it would both validate the pre-organization concept and sidestep the redox limitation (see below).

5. **Wild-type Humanin baseline fold:** A direct structural comparison fold of native HN against BAX would establish a pLDDT/ipTM baseline, allowing the S14C modification to be assessed as a delta rather than in isolation.

Critical biological question to resolve computationally:

The S14G substitution (HNG) is the most potent known HN analog, and Gly at position 14 specifically increases local flexibility — the opposite of disulfide constraint. A direct structural comparison fold of HNG (S14G) vs. S14C-Humanin against BAX, under identical prediction conditions, would test whether the predictor favors the flexible or rigid variant for BAX engagement. If HNG scores higher ipTM than S14C-HN, the flexibility hypothesis would be supported and the disulfide strategy would warrant reconsidering. This comparison fold is the single most informative next experiment available without wet lab resources.

Wet lab validation priorities (if resources allow):

1. Oxidative folding and HPLC verification of disulfide bond formation in the purified S14C peptide.
2. CD spectroscopy comparing S14C-HN vs. native HN helical content in buffer — directly testing the pre-organization hypothesis.
3. BAX pull-down or SPR binding assay under non-reducing conditions (to maintain disulfide) vs. reducing conditions (to demonstrate disulfide-dependence of any affinity gain).
4. Cell-based apoptosis assay (staurosporine or H_2O_2 challenge) in reducing intracellular context — the critical test of whether disulfide-mediated pre-organization survives to the relevant cellular compartment.

UNRESOLVED COMPLICATIONS

Two issues carry enough weight to be flagged prominently:

1. The HNG paradox. Position 14 is the defining residue of HNG, the most potent HN analog — but HNG's Gly substitution increases flexibility, not rigidity. If enhanced potency at position 14 is mechanistically linked to local conformational freedom (enabling induced-fit engagement or fiber-competent rearrangement), the disulfide constraint is working against the grain of the SAR data at this exact position. This is not a reason to discard the hypothesis — it may be that HNG's potency derives from

reduced steric clash rather than increased flexibility — but it is a genuine contradicting signal that the next experiments should be designed to resolve.

2. Redox context. BAX inhibition is required in the cytosol — a reducing environment maintained by millimolar glutathione. A disulfide-bridged peptide delivered extracellularly would need to reach the cytosol with its SS bond intact, which is not guaranteed. If disulfide reduction occurs before intracellular delivery, the modification provides no advantage over native HN. This is a fundamental translational constraint. Non-reducible staples (hydrocarbon, lactam) at the same i,i+6 position would be the rational next iteration if wet lab data confirms pre-organization benefit.

> **In silico only. All properties are computational predictions, not experimental measurements. This report does not constitute medical advice. Heuristic peptide properties (aggregation, stability, BBB, half-life) are sequence-based estimates, not validated assay results. Disulfide bond geometry is inferred from predicted Cα positions; the SS bond is not explicitly modeled by the prediction tools used.**

SEQUENCES

NATIVE

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MAPRGFSCLLLLTSEIDLPVKRRA
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MODIFIED

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MAPRGFSCLLLLTCEIDLPVKRRA
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CAVEATS

- In silico prediction only — requires wet lab validation
- Single-run prediction (not ensembled)
- Predicted properties may not reflect real-world biological behavior
- This is research, not medical advice
- Disulfide bond geometry inferred from predicted Cα–Cα distances; the SS bond is NOT explicitly modeled as a covalent constraint by AlphaFold or related tools — actual constrained peptide behavior may differ substantially
- ipTM 0.357 is below reliable threshold for confident interface interpretation — BAX docking pose is suggestive, not confirmatory

- Heuristic peptide properties (aggregation 0.342, stability 0.476, BBB 0.242, half-life estimate) are sequence-based estimates, not experimental measurements
- Redox caveat: disulfide bridge may be reduced in the cytosolic/mitochondrial environment where BAX inhibition is functionally required, potentially negating the pre-organization benefit in vivo
- The S14G (HNG) analog data introduces a competing SAR hypothesis — local flexibility at position 14 may enhance rather than impair BAX engagement, which is the opposite of the constraint strategy tested here
- No Chai-1 agreement score or Boltz-2 affinity module values available for this fold — second-model validation is absent

CITATIONS

1. **PMID —** (2003) — — Humanin peptide suppresses apoptosis by interfering with Bax activation
2. **PMID —** (2019) — — Humanin induces conformational changes in the apoptosis regulator BAX and sequesters it into fibers, preventing mitochondrial outer-membrane permeabilization
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 —** (2026) — — Renoprotective Effect of S14G-Humanin on Renal Ischemia/Reperfusion Injury by Activation of STAT3 and ERK 1/2 Signal Transduction Pathways in Rats
8. **PMID —** (2023) — — Humanin and Its Pathophysiological Roles in Aging: A Systematic Review
9. **PMID —** (2022) — — Humanin and Alzheimer's disease: The beginning of a new field
10. **PMID —** (2022) — — Cardio-protective role of Humanin in myocardial ischemia-reperfusion

SOLANA SIGNATURE 5pR8AkbZWKnQNkxXdRNAoeDV3pBhrwUrGKgR3obT4omfzW3BWWVmNWuoC4AhJWtpqggYXZfZgTPs2MDig7EvATRW
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