FOXO4-DRI — N-TERMINAL PALMITOYLATION: ATTACH A C16 PALMITOYL FATTY ACID VIA AN AMIDE BOND TO THE ALPHA-AMINO GROUP OF LEU-1, OPTIONALLY THROUGH A SINGLE LYS SPACER (PAL-LYS-LTLRK...), TO DRIVE NON-COVALENT ALBUMIN BINDING

generated 2026-05-03T06:23:28.324216+00:00

DISCARDED | LONGEVITY | N-TERMINAL PALMITOYLATION: ATTACH A C16 PALMITOYL FATTY ACID VIA AN AMIDE BOND TO THE ALPHA-AMINO GROUP OF LEU-1, OPTIONALLY THROUGH A SINGLE LYS SPACER (PAL-LYS-LTLRK...), TO DRIVE NON-COVALENT ALBUMIN BINDING | CELLULAR TUMOR ANTIGEN P53

AVERAGE CONFIDENCE	PTM / IPTM	VERDICT
58.4%	0.311 / 0.128	DISCARDED

TARGET	UNIPROT	BINDING PROBABILITY
Cellular tumor antigen p53	P04637	—

TLDR

DISTILLATION №27 tests N-terminal palmitoylation of FOXO4-DRI — a senolytic peptide targeting the FOXO4–p53 protein–protein interaction — with the goal of extending plasma half-life via albumin anchoring, mirroring the liraglutide/semaglutide pharmacokinetic strategy. Structural prediction returned a global pLDDT of 0.58 and a critically low ipTM of 0.13, insufficient to draw reliable conclusions about whether the palmitoyl-Lys extension disrupts or tolerates the CR3-mimetic helix. The fold is discarded on structural grounds, but the

pharmacokinetic hypothesis itself remains scientifically interesting and is undermined more by fundamental mechanistic incompatibilities — albumin binding vs. CPP-driven membrane penetration — than by the prediction failure alone. Literature analysis reveals that the CPP tail directly contacts p53TAD2 (2025 NMR data), adding a second reason to question whether albumin sequestration of the palmitoyl anchor would impair both cell entry and target engagement simultaneously.

EXECUTIVE SUMMARY

Palmitoyl-Lys FOXO4-DRI: pLDDT 0.58, ipTM 0.13 — too low to assess the modification's structural impact. Beyond the prediction failure, 2025 NMR data reveal the CPP tail directly contacts p53TAD2, meaning albumin-complex formation may simultaneously impair cell entry and target engagement.

DETAILED ANALYSIS

FOXO4-DRI is an 18-residue D-retro-inverso senolytic peptide designed to mimic the CR3 domain of FOXO4, displacing FOXO4 from its interaction with the p53 transactivation domain (p53TAD2) and thereby restoring p53-mediated apoptosis in senescent cells. The peptide is notable for its dual-domain architecture: an N-terminal CR3-mimetic helical region that engages p53 directly, and a C-terminal polycationic cell-penetrating peptide (CPP) tail (the RPPPRRRQRRKKRG stretch) that drives membrane penetration and nuclear translocation. Fold #12 in this lab previously established that truncating this CPP tail collapses structural prediction confidence to pLDDT 0.56, reinforcing that the tail is structurally and functionally indispensable. The current fold attempts a conceptually opposite intervention: leaving the CPP intact while adding a palmitoyl-Lys unit at the opposite, N-terminal end to drive reversible albumin binding and extend plasma exposure.

The pharmacokinetic rationale is straightforward and well-validated in incretin pharmacology. Liraglutide (C16 palmitoyl) and semaglutide (C18 with a short PEG-γGlu linker) both achieve multi-hour to multi-day half-lives by binding serum albumin non-covalently, shielding the peptide from renal filtration and proteolytic degradation. FOXO4-DRI, with its highly cationic character and relatively small size, is plausibly subject to rapid renal clearance, and all published in vivo studies use cyclic intraperitoneal dosing every few days — an implicit acknowledgment of limited persistence. Extending exposure through albumin anchoring is therefore a scientifically motivated hypothesis, even if the specific pharmacokinetic liabilities of FOXO4-DRI have never been formally characterized in the literature.

The structural prediction, however, provides a weak foundation for this design. The predicted pLDDT of 0.58 reflects the known difficulty of predicting disordered, cationic, all-D-amino-acid peptides with AlphaFold-class models trained

predominantly on globular L-amino acid proteins. The ipTM of 0.13 is particularly uninformative — near the floor for a meaningful peptide–protein complex — and cannot distinguish whether the palmitoyl-Lys extension is truly non-disruptive to helix formation or whether the model simply lacks the parameterization to resolve it. No Chai-1 cross-validation was available to corroborate or challenge the ESMFold/AF output, and the Boltz-2 affinity module returned no values. The heuristic stability score of 0.385 is modest, and the long half-life estimate in the heuristic profile reflects the palmitoyl modification's expected albumin-anchoring contribution — but this is a sequence-based heuristic, not a real pharmacokinetic measurement.

The 2025 NMR study (PMID:40593617) by Bourgeois et al. introduces a critical complication that was not anticipated at the time of design: the cationic CPP tail does not merely deliver FOXO4-DRI to the nucleus — it directly contacts p53TAD2 as part of the binding interface. This means the CPP tail is bifunctional, serving both as a cell-penetration appendage and as a target-engagement element. This finding has direct implications for the palmitoylation strategy: albumin is a 67 kDa protein that binds fatty acids within a hydrophobic cleft, and the geometry of albumin–palmitoyl engagement would orient the attached FOXO4-DRI in a manner that could sterically occlude or conformationally constrain the CPP tail's ability to simultaneously contact p53TAD2 in free solution. The palmitoyl group is at the opposite (N-terminal) end from the CPP tail, but the peptide is only 18 residues before the Pal-Lys spacer — not long enough to guarantee that albumin binding at one end leaves the other end fully accessible.

A second mechanistic incompatibility concerns cell entry. FOXO4-DRI penetrates cells via its CPP-driven direct membrane translocation mechanism, a biophysical process that depends on the cationic tail's electrostatic interaction with anionic membrane phospholipids and its ability to disrupt lipid bilayer integrity transiently. Albumin-bound peptide complexes (~70 kDa) do not penetrate cell membranes by this mechanism; they rely instead on endosomal uptake, receptor-mediated transcytosis (notably via FcRn for albumin), or passive dissociation before membrane contact. Whether the palmitoyl–albumin Kd (approximately 1–10 μM for C16) is sufficiently weak to allow efficient dissociation and free peptide release in relevant tissue compartments is unknown. If dissociation is rate-limiting, the sustained plasma exposure benefit could be offset by reduced intracellular bioavailability at the nuclear target.

Compared to Fold #25 (MOTS-c N-terminal myristoylation, PROMISING, pLDDT 0.63), which targeted membrane association for a cytoplasmic-acting peptide, the present fold faces a more complex challenge: FOXO4-DRI must not only cross the membrane but reach the nucleus, and its mechanism of nuclear access is entirely CPP-dependent. MOTS-c's myristoylation improved membrane partitioning for a peptide whose target is intracellular but not nuclear — a qualitatively different barrier. This distinction explains in part why the lipidation-for-pharmacokinetics hypothesis is less straightforward for FOXO4-DRI than it might appear from the incretin analogy.

The safety dimension also warrants mention. PMID:36515093 documented that FOXO4-DRI worsened pulmonary hypertension in animal models by eliminating senescent cells that were performing a protective role in that vascular context. A sustained-exposure formulation would, by design, extend the window of systemic senolytic activity — potentially amplifying this context-dependent harm in patients with pulmonary or vascular disease. This is not a prediction failure; it is a biological risk that the palmitoylation strategy would need to address through either tissue-targeted delivery or carefully controlled release kinetics. In its current form, Pal-Lys-FOXO4-DRI does not include a targeting element, making the sustained-exposure concern a genuine translational liability.

In summary, DISTILLATION №27 is discarded because the structural predictors could not generate usable affinity or interface data at the confidence thresholds required, and because the biological rationale — while internally coherent — carries two compounding mechanistic risks that the literature now flags more clearly than when the hypothesis was formed: albumin sequestration may impair CPP-mediated cell entry, and prolonged systemic senolysis may be harmful in specific disease contexts. The pharmacokinetic goal of extended half-life remains valid and worth pursuing, but through a mechanistically compatible strategy — such as a cleavable linker design, PEGylation at a non-CPP site, or nanoparticle encapsulation — rather than direct palmitoylation.

RESEARCH BRIEF

DISTILLATION №27 — DISCARDED

FOXO4-DRI · N-TERMINAL PALMITOYL-LYS CONJUGATE · PHARMACOKINETIC EXTENSION VIA ALBUMIN ANCHORING

MECHANISM OF ACTION (BACKGROUND)

FOXO4-DRI is a D-retro-inverso senolytic peptide that mimics the CR3 domain of the transcription factor FOXO4. In senescent cells, FOXO4 forms a complex with p53 that sequesters p53 in the nucleus and suppresses p53-driven apoptosis — a key mechanism by which senescent cells resist clearance. FOXO4-DRI competes with endogenous FOXO4 for binding to the p53 transactivation domain 2 (p53TAD2), displacing FOXO4 and restoring p53-mediated apoptotic signaling. The downstream axis — nuclear exclusion of p53, BCL-2 downregulation, Caspase-3 activation — has been documented in senescent Leydig cells, endothelial cells, chondrocytes, keloid fibroblasts, and cancer-associated fibroblasts across multiple independent studies.

A critical structural insight from a 2025 NMR study (PMID:40593617, Bourgeois et al.) reframes the peptide's architecture: the C-terminal cationic CPP tail (RPPPRRRQRRKKRG) is not merely a cell-penetration appendage — it makes direct contact with p53TAD2 as part of the binding interface. The CPP tail is therefore bifunctional: it drives membrane translocation AND contributes to target engagement. This finding is central to evaluating any modification that might alter the CPP tail's conformation, charge, or accessibility. **Fold #12** in this lab confirmed the structural indispensability of this region: truncating the CPP tail collapsed pLDDT to 0.56 and yielded a DISCARDED verdict.

MODIFICATION HYPOTHESIS (WHAT WE TESTED)

This fold attached a C16 palmitoyl fatty acid to the N-terminus of FOXO4-DRI via a Lys spacer (Pal-Lys-LTLRK...), mirroring the albumin-anchoring strategy used in liraglutide (C16 palmitoyl) and semaglutide (C18 with linker). The hypothesis:

- FOXO4-DRI's highly cationic structure and small size likely drive rapid renal clearance, limiting tissue exposure after bolus dosing
- N-terminal palmitoylation would enable reversible albumin binding (Kd ~1–10 μM for C16), converting the peptide from a flash-exposure agent to a sustained-exposure depot
- Attaching the lipid at the N-terminus — maximally distal from the C-terminal CPP tail — would minimize steric disruption to either functional domain
- The all-D-amino-acid backbone (proteolytic stability already ensured by the DRI design) would make albumin binding the primary PK extension mechanism

The design rationale was sound at the level of analogy: liraglutide/semaglutide demonstrate that N-terminal fatty acid conjugation can extend half-life from minutes to hours-to-days. The cyclic IP dosing used in all FOXO4-DRI in vivo studies implicitly acknowledges short persistence. Extended half-life would directly increase the time the peptide is available in senescent-cell-rich tissues where p53 phosphorylation (pS15) enhances binding affinity.

WHY THE PREDICTION WAS UNINFORMATIVE (TECHNICAL ANALYSIS)

Structural confidence metrics: - pLDDT: **0.58** — below the threshold for reliable secondary structure assignment in a peptide of this complexity - ipTM: **0.13** — near the floor of meaningful interface scoring; essentially no confident peptide–protein docking information was generated - Chai-1 cross-validation: **not available** — single-model output cannot be treated as a reliable structural prediction - Boltz-2 affinity module: **no values returned** — no quantitative binding change predicted

The low confidence scores are not surprising given the target system. FOXO4-DRI is an all-D-amino-acid peptide — structure predictors trained on L-amino acid protein databases have fundamentally degraded performance on DRI peptides. The modified sequence also includes a non-standard palmitoyl-Lys N-terminal extension that is chemically outside the training distribution of most protein structure models. The disordered nature of the CPP tail further limits confidence globally, as unstructured polycationic regions consistently produce low pLDDT regardless of the helical core's actual folding.

What the model could and could not tell us: The prediction confirms that the CR3-mimetic helix is present as a short helical segment and that the palmitoyl-Lys extension appears as a flexible appendage without obvious clashes. However, at pLDDT 0.58 and ipTM 0.13, this is not a reliable structural prediction — it is a low-confidence sketch. Absence of visible aberrant lipid–helix contacts in the model does not constitute evidence of structural tolerance; the model lacks the resolution and the parameterization to detect subtle hydrophobic insertion or helix-destabilizing contacts in this sequence context.

Comparison with **Fold #25** (MOTS-c myristoylation, PROMISING, pLDDT 0.63) is instructive: a slightly higher pLDDT and a shorter, simpler target peptide allowed that fold to clear the PROMISING threshold. FOXO4-DRI's greater length, disordered CPP tail, and all-D backbone make it a systematically harder prediction target, and the ipTM gap between the two (0.13 here vs. not reported but inferred higher for Fold #25) reflects this.

WHAT THIS TELLS US (NEGATIVE RESULTS ARE DATA)

1. The structure predictor limitation is specific, not general. The prediction failure here is not evidence that palmitoylation disrupts FOXO4-DRI's structure — it is evidence that current in silico tools cannot reliably model all-D, long, polycationic peptides with non-canonical N-terminal modifications. This is an important distinction: the fold is discarded for informational failure, not biological failure.

2. The pharmacokinetic hypothesis has a more fundamental problem than the prediction failure. Literature analysis surfaces two compounding mechanistic incompatibilities that the structural prediction cannot resolve but which should govern future design decisions:

- Albumin anchoring may impair CPP-mediated cell entry. Albumin-bound peptide complexes (~70 kDa) cannot cross cell membranes via the direct electrostatic/CPP mechanism that FOXO4-DRI relies on for nuclear translocation. If the palmitoyl–albumin Kd is not sufficiently weak to allow rapid dissociation at the cell surface, the sustained plasma half-life benefit would come at the cost of reduced intracellular bioavailability.

- Albumin orientation may sterically restrict the CPP tail's target engagement. The 2025 NMR finding (PMID:40593617) that the CPP tail directly contacts p53TAD2 means that albumin binding at the N-terminal palmitoyl anchor could geometrically restrict the CPP tail's freedom to engage p53 in the free-peptide state post-dissociation.

3. Context-dependent safety risk for sustained senolysis. PMID:36515093 documents that FOXO4-DRI worsened pulmonary hypertension by eliminating senescent cells performing a vascular-protective role. A sustained-exposure formulation amplifies this risk by extending the senolytic window beyond the intended dosing interval. This is not a prediction-derived caveat — it is a literature-derived contraindication for the sustained-exposure design strategy in vascular disease contexts.

4. The PK premise is unverified. No published data characterize FOXO4-DRI's actual plasma half-life, renal clearance rate, or dominant elimination pathway. The assumption that rapid renal clearance is the primary PK liability — rather than hepatic clearance, tissue sequestration, or receptor-mediated endocytosis — is biologically plausible but unsubstantiated. Palmitoylation is the right solution only if renal filtration is the rate-limiting clearance mechanism.

ALTERNATIVE HYPOTHESES TO TEST (AVOIDING THE FAILURE MODE)

The pharmacokinetic extension goal remains valid. The following strategies avoid the specific failure modes identified here:

1. PEGylation at a non-CPP, non-helix site via Lys spacer in the linker region. The GGK linker between the CR3 helix and CPP tail (around residues 28–30) provides a candidate attachment point. PEG20–40k extends half-life without driving albumin-complex formation, preserving CPP-driven membrane translocation while reducing renal filtration. This is structurally testable with higher confidence than palmitoylation.

2. Cleavable albumin-binding linker (pH- or protease-sensitive release). A palmitoyl group attached via a protease-cleavable dipeptide (e.g., Val-Cit, cleaved by cathepsin B in endosomal/lysosomal compartments or at tumor/senescent-tissue sites) would provide the sustained plasma PK benefit while releasing free FOXO4-DRI intracellularly where it can engage the CPP-driven nuclear pathway. This design has precedent in ADC (antibody-drug conjugate) chemistry.

3. Nanoparticle encapsulation without covalent modification. Lipid nanoparticles or PLGA microparticles loaded with unmodified FOXO4-DRI would provide controlled release and extended local exposure without any risk of CPP-tail

interference. This preserves the native peptide sequence entirely and avoids all the structural concerns raised here.

4. Half-life extension via D-amino acid stapling or macrolactamization at a non-functional site. If the pharmacokinetic goal is secondary to improving stability and structural rigidity, hydrocarbon stapling or macrolactamization within the helical CR3 region (tested with D-amino acid stapling chemistry compatible with the all-D backbone) could increase proteolytic half-life and receptor residence time without driving albumin sequestration. The Fold #22 approach on Humanin (disulfide bridge, PROMISING) provides a conceptual precedent in this lab.

5. Direct pharmacokinetic characterization before further modification. Before engineering solutions, measure FOXO4-DRI's actual plasma half-life, renal clearance fraction, and tissue distribution in a rodent model. If half-life is already >2 hours (as the all-D backbone and highly cationic character might produce via tissue sequestration), the PK problem may be smaller than assumed, and the modification priority should shift to affinity or selectivity.

In silico prediction only. All structural and property data are computational estimates. This report does not constitute medical advice. Requires wet-lab validation before any biological conclusions can be drawn.

SEQUENCES

NATIVE

```
LTLRKEPASEIAQSILEAYSQNGWANRRSGGKRPPPRRRQRRKKRG
```

MODIFIED

```
Pal-K-LTLRKEPASEIAQSILEAYSQNGWANRRSGGKRPPPRRRQRRKKRG
```

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
- all-D-amino-acid peptides are systematically underperforming in AlphaFold-class structure predictors trained on L-amino acid protein databases — pLDDT and ipTM values for DRI peptides should be interpreted as lower bounds on actual structural order

- no Chai-1 cross-validation was available; single-model ipTM of 0.13 is below the threshold for any reliable interface conclusion
- the heuristic half-life estimate (long, >6 hours) reflects the palmitoyl modification's albumin-anchoring potential in isolation and does not account for the CPP-driven internalization competition or tissue-specific distribution
- the albumin-anchoring strategy is extrapolated from GLP-1 incretin pharmacology (liraglutide/semaglutide) and has not been validated for all-D, polycationic, nuclear-targeted peptides
- FOXO4-DRI pharmacokinetics (plasma half-life, renal clearance fraction, tissue distribution) have not been formally characterized in published literature — the premise of rapid renal clearance as the dominant PK liability is biologically plausible but unsubstantiated
- sustained senolytic exposure via albumin anchoring carries a context-dependent safety risk: PMID:36515093 documents disease worsening in pulmonary hypertension models with FOXO4-DRI treatment

CITATIONS

1. **PMID —** (2025) — — The disordered p53 transactivation domain is the target of FOXO4 and the senolytic compound FOXO4-DRI
2. **PMID —** (2022) — — Identification of Hotspots in Synthetic Peptide Inhibitors of the FOXO4:p53 Interaction
3. **PMID —** (2025) — — FOXO4-DRI induces keloid senescent fibroblast apoptosis by promoting nuclear exclusion of upregulated p53-serine 15 phosphorylation
4. **PMID —** (2025) — — FOXO4-DRI regulates endothelial cell senescence via the P53 signaling pathway
5. **PMID —** (2023) — — Eliminating Senescent Cells Can Promote Pulmonary Hypertension Development and Progression
6. **PMID —** (2020) — — FOXO4-DRI alleviates age-related testosterone secretion insufficiency by targeting senescent Leydig cells in aged mice
7. **PMID —** (2024) — — FOXO4-DRI improves spermatogenesis in aged mice through reducing senescence-associated secretory phenotype secretion from Leydig cells
8. **PMID —** (2021) — — Targeting senescence-like fibroblasts radiosensitizes non-small cell lung cancer and reduces radiation-induced pulmonary fibrosis
9. **PMID —** (2021) — — Senolytic Peptide FOXO4-DRI Selectively Removes Senescent Cells From in vitro Expanded Chondrocytes

SOLANA SIGNATURE 3vFgwa1bpMXZY39M84BsdWdVGtddyV9bsoVBEHv6Qq9Vc3hJaJGf8swQuvYwpgvmt2fLkhU9nEta66vHoh8iWipe

DATA SHA-256 d419f2cdd4a30b4c3b12373317b85db3a31a8173ec097a9b116b28c577395878

VERIFY https://solscan.io/tx/3vFgwa1bpMXZY39M84BsdWdVGtddyV9bsoVBEHv6Qq9Vc3hJaJGf8swQuvYwpgvmt2fLkhU9nEta66vHoh8iWipe