TB-500 — LYS-2 → ORNITHINE (ORN) SUBSTITUTION; SIDE CHAIN SHORTENED BY ONE METHYLENE WHILE RETAINING A PRIMARY AMINE AND POSITIVE CHARGE

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PROMISING REGENERATIVE

LYS-2 → ORNITHINE (ORN) SUBSTITUTION; SIDE CHAIN SHORTENED BY ONE METHYLENE WHILE RETAINING A PRIMARY AMINE AND POSITIVE CHARGE

BETA-ACTIN

AVERAGE CONFIDENCE	PTM / IPTM	VERDICT
80.2%	0.818 / 0.702	PROMISING

TARGET	UNIPROT	BINDING PROBABILITY
Beta-actin	P60709	—

TLDR

Fold #16 tests whether replacing Lys-2 in TB-500's LKKTETQ sequence with ornithine — a one-methylene-shorter lysine isostere — can resist trypsin-like proteolytic cleavage at the dibasic K2-K3 motif while preserving actin-binding electrostatics. The structural predictors returned a pLDDT of 0.80 and reasonable complex scores, meaning the tools functioned correctly, but the absence of Chai-1 agreement data, no Boltz-2 affinity output, and no predicted binding change left the central pharmacological question — whether actin contact is maintained — unanswerable from this run. The verdict is DISCARDED: not because the hypothesis is wrong, but because the prediction pipeline did not produce the discriminating signal needed to evaluate it.

EXECUTIVE SUMMARY

Fold #16 — Lys2→Orn in TB-500 — returned pLDDT 0.80 and a plausible complex geometry, but no affinity readout or Chai-1 cross-validation. The protease-resistance hypothesis remains biologically sound; the prediction tools could not discriminate at single-residue resolution.

DETAILED ANALYSIS

TB-500 (LKKTETQ, commercially sold as the acetylated form Ac-LKKTETQ) is a heptapeptide fragment of thymosin β4 (Tβ4, residues 17–23) that recapitulates much of the parent protein's regenerative biology — actin sequestration, cell migration, wound healing, and angiogenesis. Its mechanism centres on G-actin binding through an amphipathic alpha-helical conformation, with the two consecutive lysine residues (K2 and K3) making electrostatic contacts with acidic actin surface residues in subdomains 1 and 3. This makes TB-500 an interesting but pharmacologically fragile peptide: the very dibasic motif that anchors it to actin is also a canonical trypsin-family cleavage site, driving rapid plasma degradation. Rahaman et al. (2024, PMID:38382158) confirmed this directly, identifying Ac-LK as the dominant metabolic fragment in human serum in vitro, with Ac-LKK persisting as a long-term species — placing the primary cleavage event squarely within or immediately after the K2-K3 stretch.

The modification hypothesis for Fold #16 was therefore mechanistically sound: substitute Lys-2 with ornithine (Orn), a non-proteinogenic amino acid that retains the primary amine and positive charge of lysine but carries a shorter delta-amino side chain (four carbons vs. five). Trypsin's S1 specificity pocket is optimised for the five-carbon lysine or six-carbon arginine side chain; ornithine's four-carbon chain is a well-established isostere that disrupts S1 recognition while maintaining cationic character. The strategy complements Fold #7, which addressed exopeptidase vulnerability at the N-terminus via acetylation and refined with pLDDT 0.87. Fold #16 was designed to tackle the orthogonal endopeptidase liability — a logical extension of that earlier work, targeting internal cleavage rather than terminal degradation.

The structural prediction run yielded a pLDDT of 0.80 and a pTM of 0.82, with an ipTM of 0.70 for the peptide-actin complex — scores that fall in the moderate-to-acceptable range and indicate the predictor produced a geometrically plausible output rather than collapsing into noise. For a short seven-residue peptide, these values are not alarming in isolation. However, the absence of Chai-1 agreement data means there is no orthogonal structural model to cross-validate the backbone geometry. More critically, the Boltz-2 affinity module returned no values, and the predicted binding change field is empty — meaning the run produced no quantitative estimate of whether Orn-2 preserves, weakens, or abolishes actin

affinity relative to either native LKKTETQ or the Ac-LKKTETQ benchmark from Fold #7.

This is the core reason for the DISCARDED verdict. The prediction pipeline functioned — it did not crash or produce nonsensical geometry — but it failed to generate the discriminating pharmacological signal the hypothesis required. To evaluate whether Orn-2 maintains actin contact, we needed either a binding affinity delta or a convergent structural prediction from multiple engines showing the ornithine side chain occupying a geometry consistent with the native Lys-2 contact. Neither materialised. A pLDDT of 0.80 tells us the backbone is plausible; it tells us nothing about whether the shorter delta-amine reaches the actin electrostatic surface in the same register as the epsilon-amine of lysine.

The literature context adds important nuance to this null result. Rahaman et al.'s metabolite data, while supporting K2-K3 as a cleavage region, identifies Ac-LKK as a long-lived species — implying that the dominant proteolytic event may occur after Lys-3 (generating Ac-LKK + TETQ) rather than after Lys-2 (generating Ac-LK + KTETQ). If K3 is the primary cleavage site, a Lys-2 substitution alone may offer less half-life extension than anticipated, and a Lys-3 modification or a dual Orn-2/Orn-3 variant would be a more targeted approach. This ambiguity was present before the fold and is not resolved by the structural output.

The heuristic peptide profile is worth noting: aggregation propensity of 0.0 (low risk for a short, charged, hydrophilic peptide), stability score of 0.6 (moderate), and a half-life estimate of 15–45 minutes — consistent with the rapid proteolytic clearance documented in the literature. BBB penetration probability of 0.018 is appropriately low for a charged heptapeptide. These heuristic values are sequence-derived estimates, not experimental measurements, but they are internally consistent with TB-500's known pharmacological profile and do not raise new red flags about the Orn-2 variant itself.

Scientifically, this fold is not a refutation of the Orn substitution strategy. The hypothesis remains chemically reasonable, the metabolite literature supports the target liability, and the isostere logic is validated in other peptide systems. What the fold demonstrates is that short peptide-protein complexes of this type — flexible, charge-dependent, without a solved reference crystal structure — are at the edge of what current in silico tools can discriminate at single-residue resolution. Ornithine is not a standard amino acid in most prediction training sets, and the absence of affinity readouts likely reflects tool limitations rather than biology. The negative result here is informative: it establishes the boundary of what AlphaFold-class predictions can tell us about this modification, and redirects the strategy toward either better computational tools (free energy perturbation, explicit-solvent MD) or direct experimental approaches.

RESEARCH BRIEF

FOLD #16 — LYS2→ORN SUBSTITUTION IN TB-500

Verdict: DISCARDED | Peptide: L-Orn-KTETQ | Target: G-actin (ACTB, P60709) | Class: Regenerative

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MECHANISM OF ACTION (BACKGROUND)

TB-500 (LKKTETQ) is the minimal pharmacophore of thymosin β4, a 43-amino acid actin-sequestering protein expressed in most human tissues. Within the full-length Tβ4 structure, residues 17–23 form a short amphipathic alpha-helix that inserts into G-actin's hydrophobic cleft between subdomains 1 and 3. The dibasic Lys-2/Lys-3 segment contributes electrostatic interactions with acidic actin residues (including Glu-167 and Asp-25), while the C-terminal TETQ portion contacts the nucleotide-binding cleft. By sequestering G-actin monomers, TB-500 shifts the G-actin/F-actin equilibrium, suppresses actin polymerisation, and activates downstream integrin and PI3K/Akt signalling cascades associated with cell survival, migration, angiogenesis, and tissue repair.

The Ac-LKKTETQ form (commercially sold as TB-500) was characterised in doping-control literature (Ho et al., PMID:23084823; Esposito et al., PMID:22962027) and confirmed to retain the core biological activity. Rahaman et al. (2024, PMID:38382158) demonstrated that TB-500 is rapidly metabolised in human serum, with Ac-LK as the primary metabolite and Ac-LKK as a long-term fragment detectable up to 72 hours — placing proteolytic degradation squarely at the K2-K3 dibasic motif.

MODIFICATION HYPOTHESIS (WHAT WE TESTED)

Fold #16 proposed replacing Lys-2 with ornithine (Orn), a non-proteinogenic amino acid structurally identical to lysine except for one fewer methylene group (δ-amine vs. ε-amine). The hypothesis had two components:

1. **Protease resistance:** Trypsin's S1 specificity pocket is optimised for lysine (5C) and arginine (6C) side chains. Ornithine's 4C side chain is a validated isostere that disrupts S1 recognition while preserving cationic character — a strategy with precedent in protease-resistant peptide design broadly, though never specifically applied to TB-500.

2. **Actin contact preservation:** The positive charge at position 2 was expected to be maintained by Orn's δ-amine, satisfying the electrostatic geometry of the native K2 contact with acidic actin surface residues — albeit with a shorter reach.

This fold was designed as an orthogonal complement to **Fold #7**, which addressed N-terminal exopeptidase vulnerability via acetylation (Ac-Leu1, refined with pLDDT 0.87). Where Fold #7 capped the terminus against aminopeptidase attack and refined confidently, Fold #16 targeted internal endopeptidase cleavage — a distinct and arguably dominant degradation liability given Rahaman et al.'s metabolite profile.

WHY THE PREDICTION WAS UNINFORMATIVE

The structural predictors ran without technical failure and returned geometrically plausible outputs:

- **pLDDT: 0.80** — moderate-to-acceptable backbone confidence for a short polar heptapeptide
- **pTM: 0.82 / ipTM: 0.70** — suggesting the complex prediction is structurally reasonable, though ipTM at 0.70 is at the lower boundary for confident interface interpretation
- **Chai-1 agreement: None** — no orthogonal model was generated to cross-validate backbone geometry
- **Boltz-2 affinity module: no values** — the discriminating pharmacological readout (ΔΔG of binding, Orn-2 vs. Lys-2) was not produced
- **Predicted binding change: None** — no quantitative estimate of whether the ornithine substitution preserves, weakens, or enhances actin affinity

The absence of Boltz-2 affinity values and Chai-1 agreement is the central problem. The hypothesis lives or dies on whether the Orn δ-amine can reach and engage the actin electrostatic surface in the same spatial register as the Lys ε-amine — a question that requires either a binding energy estimate or convergent multi-model structural agreement. Neither was produced. A pLDDT of 0.80 confirms the backbone is plausible; it does not discriminate between a functionally equivalent and a functionally compromised side-chain contact geometry.

A contributing factor is likely the non-standard amino acid problem: ornithine is not a canonical residue in most AlphaFold-class training datasets, which may have degraded the confidence of side-chain placement and suppressed the affinity module's ability to score the interface. This is a tool limitation, not a biological verdict.

WHAT THIS TELLS US (NEGATIVE RESULTS ARE DATA)

What this fold rules in: The backbone conformation of L-Orn-KTETQ is not predicted to be grossly destabilised — pLDDT 0.80 is consistent with a folding-competent, helical-prone short peptide. The modification does not appear to induce structural collapse.

What this fold rules out: Nothing definitive about actin binding or protease resistance can be concluded from this run. The prediction was uninformative on the pharmacological question.

What the metabolite literature adds: Rahaman et al.'s identification of Ac-LKK as a long-term metabolite (up to 72h) alongside Ac-LK as the primary early metabolite raises the possibility that the dominant cleavage event is after Lys-3 (K3↓T4), not after Lys-2. If K3 is the primary endopeptidase site, a K2-only substitution may yield less half-life extension than anticipated. A dual Orn-2/Orn-3 variant, or a Lys-3-priority substitution, may be pharmacologically more impactful — but this question also cannot be resolved by the current in silico approach alone.

What the heuristic profile adds: The sequence-derived profile for L-Orn-KTETQ is unremarkable: aggregation propensity 0.0, stability 0.6, half-life 15–45 min (similar to native TB-500), BBB penetration 0.018. No new safety flags emerge from the modification at the heuristic level, but these estimates do not incorporate the Orn residue's actual physicochemical contribution.

ALTERNATIVE HYPOTHESES TO TEST

To avoid the failure mode of this fold, future experiments should be structured around tools capable of discriminating single-residue side-chain contacts in short peptide-protein complexes:

1. **Lys-3 → Orn substitution (Fold #17 candidate):** Given that Rahaman et al. suggest cleavage may occur after K3, test the L-K-Orn-TETQ variant. Compare heuristic and structural outputs to this fold and to Fold #7 to map the K2 vs. K3 protease liability.
2. **Dual Orn-2/Orn-3 variant (L-Orn-Orn-TETQ):** The most aggressive endopeptidase-resistance strategy. Risks reducing helical propensity and actin affinity, but tests the ceiling of protease resistance achievable through this isostere approach.
3. **Molecular dynamics simulation (MM-GBSA or FEP):** AlphaFold-class tools are not designed to compute ΔΔG for side-chain truncations. A short explicit-solvent MD run with a TB-500/actin model (built from the Fold #7 prediction as a starting point) would give a binding free energy estimate for Orn-2 vs. Lys-2 — directly testing the actin contact hypothesis.

4. **Protease susceptibility assay (in vitro):** Incubating native and Orn-2 TB-500 with purified trypsin or human plasma and measuring degradation by LC-MS/MS is a relatively low-cost experiment that would directly validate the protease-resistance component of the hypothesis, independent of the actin-binding question.
5. **Lys-2 → Arg substitution:** Arginine is also poorly cleaved by most trypsin-family proteases (despite being an S1 substrate) when the adjacent residue is not optimal, and has a longer, guanidinium-capped side chain with potentially stronger electrostatic engagement of actin. This represents an alternative single-substitution strategy at position 2.

SEQUENCES

NATIVE

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LKKTETQ
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MODIFIED

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L-Orn-KTETQ
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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
- ornithine is a non-canonical amino acid underrepresented in AlphaFold-class training data — side-chain placement confidence may be systematically reduced
- Boltz-2 affinity module returned no values; no quantitative binding change estimate was produced for this fold
- Chai-1 agreement not available — no orthogonal structural model for cross-validation
- heuristic property estimates (aggregation, stability, half-life, BBB) are sequence-derived approximations that do not account for non-standard residue physicochemistry
- the dominant TB-500 cleavage site (K2 vs. K3) is not definitively established in the literature, limiting interpretation of a single-position substitution strategy

- no randomised controlled human trial evidence exists for native TB-500 efficacy; modification strategies are preclinically premature from a translational standpoint
- Verdict reclassified: DISCARDED → PROMISING. Raw metrics (pLDDT/pTM/ipTM) permit at least the higher tier; the original LLM discard reflected modification chemistry the predictor cannot represent (D-AA, lipid moiety, non-canonical residue). Per the metric-floor rule this is a caveat, not a verdict downgrade. Report text below pre-dates the rule and may still describe the fold as DISCARDED — the structural verdict shown is the authoritative one.

CITATIONS

1. **PMID —** (2024) — — Simultaneous quantification of TB-500 and its metabolites in in-vitro experiments and rats by UHPLC-Q-Exactive orbitrap MS/MS and their screening by wound healing activities in-vitro
2. **PMID —** (2012) — — Doping control analysis of TB-500, a synthetic version of an active region of thymosin β₄, in equine urine and plasma by liquid chromatography-mass spectrometry
3. **PMID —** (2012) — — Synthesis and characterization of the N-terminal acetylated 17-23 fragment of thymosin beta 4 identified in TB-500, a product suspected to possess doping potential
4. **PMID —** (2026) — — Therapeutic Peptides in Orthopaedics: Applications, Challenges, and Future Directions
5. **PMID —** (2026) — — Injectable Peptide Therapy: A Primer for Orthopaedic and Sports Medicine Physicians
6. **PMID —** (2026) — — Safety and Efficacy of Approved and Unapproved Peptide Therapies for Musculoskeletal Injuries and Athletic Performance
7. **PMID —** (2014) — — Analytical approaches for the detection of emerging therapeutics and non-approved drugs in human doping controls
8. **PMID —** (2017) — — Adsorption effects of the doping relevant peptides Insulin Lispro, Synachten, TB-500 and GHRP 5

SOLANA SIGNATURE 2GhbkdfZgYygK5i9M9sBScZXnvq3WhG2R3nmMsMSXpS3yGnZU9vHYfFjnqmKa1ip5MfWPVkzs3TKuBi7LZyWhayh
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