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Mechanism Claims in Molecular Pharmacognosy Need an Evidence Ladder: Linking Phytochemical Identity, Target Engagement, Pathway Perturbation, and Phenotypic Relevance without Docking Inflation

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ABSTRACT

Mechanistic language in molecular pharmacognosy often compresses several distinct evidentiary questions into a single claim: whether the phytochemical entity is correctly identified, whether a molecular target is merely predicted or physically engaged, whether perturbation of that target changes a relevant pathway, and whether the pathway contributes to the observed phenotype. This perspective examines why those questions should remain analytically separate and proposes a graded evidence ladder for natural-product mechanism claims. The analysis integrates phytochemical authentication, docking and target nomination, chemical-probe quality, chemoproteomics, target deconvolution, cellular target-engagement methods, network-level interpretation, and phenotype-oriented validation. The central proposal is that stronger mechanistic language should require closure of specific inferential gaps rather than accumulation of experiments that all interrogate the same level. Computational prediction can prioritize hypotheses but cannot substitute for target engagement; engagement can strengthen target attribution but does not establish functional necessity; pathway changes can demonstrate biological perturbation without proving target-specific causation; and phenotypic activity can remain compatible with polypharmacology, metabolites, mixture effects, or context-dependent biology. The proposed ladder is therefore intended as an interpretive and reporting framework, not a validated score or universal ranking system. Its purpose is to make unresolved links visible, preserve alternative explanations, and encourage mechanism statements whose strength is proportional to the evidence actually obtained. Important boundaries include uncertain phytochemical identity, assay-specific artifacts, indirect engagement signals, heterogeneous biological states, multiple active constituents, and incomplete exposure relevance.

Keywords: Molecular pharmacognosy, Natural products, Target engagement, Chemical proteomics, Mechanistic evidence, Molecular docking

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Introduction

Natural products continue to supply structurally and biologically distinctive starting points for drug discovery, while advances in analytical chemistry, genomics, biosynthetic methods, and chemical biology have expanded the routes by which their activities can be investigated [1]. Yet molecular pharmacognosy also carries an evidentiary burden that is unusually easy to underestimate. A mechanism claim may begin with a crude or partially characterized material, progress through a tentative constituent assignment, move into computational target nomination, and end with pathway or phenotypic observations. Each transition can be scientifically informative, but none is equivalent to the next. When those transitions are collapsed, the resulting narrative can sound

mechanistically complete while still containing unresolved questions about what molecule was present, what protein was engaged, and what event actually caused the phenotype.

That problem starts before target biology. Plant metabolomes contain chemically dense and highly variable specialized-metabolite spaces, and complementary analytical techniques are often required to improve annotation and structural confidence [2]. In herbal materials, untargeted and pseudotargeted metabolomics combined with tandem mass spectrometry and chemometrics can discriminate authentic from adulterated material, illustrating how analytical identity and sample provenance can materially alter the object of biological inference [3]. Fingerprinting approaches likewise address quality and adulteration across Chinese herbal medicines, although fingerprint similarity should not be treated as proof that every constituent has been structurally resolved or that two preparations are pharmacologically equivalent [4]. A target-specific mechanism cannot be stronger than the chemical entity to which that target claim is assigned.

The distinction is especially consequential when a mechanism is assigned to an isolated constituent from evidence originally generated with a chemically broader preparation. Analytical confidence in the material, confidence in the constituent assignment, and confidence that the tested exposure corresponds to that constituent are related but separate questions. A mechanistic narrative should therefore state what chemical object was actually tested and avoid silently transferring evidence between extract, fraction, isolated molecule, metabolite, or analogue.

The same separation is necessary downstream. A computationally plausible protein–ligand relationship, a biochemical binding signal, a thermal stabilization event in cells, a pathway change, and a disease-relevant phenotype represent different kinds of evidence. They answer different questions and have different alternative explanations. Molecular pharmacognosy therefore needs a vocabulary that distinguishes hypothesis generation from interaction evidence, target engagement from functional dependence, and pathway perturbation from causal explanation. The central problem is not that one method is inherently weak and another inherently decisive. Rather, mechanistic inflation occurs when evidence produced at one level is described using language appropriate to a stronger level.

This perspective proposes an evidence ladder for organizing those distinctions. The ladder is not a numerical score and does not assume that all studies must proceed through identical assays. Instead, it treats mechanistic confidence as conditional on which inferential gaps have actually been addressed. Chemical identity, target plausibility, direct or cellular engagement, functional perturbation, pathway coherence, and phenotypic relevance can contribute cumulatively, but unresolved uncertainty at an earlier level remains visible rather than being erased by abundant downstream data. This design is particularly important for natural products because polypharmacology, metabolites, complex mixtures, concentration-dependent promiscuity, and context-specific biology can all make apparently coherent pathways compatible with more than one causal account.

The article therefore asks a narrower question than whether a natural product is “mechanistically active.” It asks what kind of mechanism statement is justified by a given pattern of evidence. By separating analytical validity, molecular interaction, biological perturbation, and phenotype linkage, the proposed framework aims to support more calibrated interpretation without dismissing exploratory computational approaches or demanding an unrealistic single experimental template.

Why mechanistic language often exceeds the evidence

Docking illustrates the distinction particularly clearly. Large-scale molecular docking is a legitimate structure-based method for sampling poses, ranking candidates, and prioritizing molecules for further testing [5]. Its value lies in hypothesis generation under specified structural and scoring assumptions. The problem begins when a predicted pose or favorable score is rewritten as evidence that a natural product binds a target in the relevant biological system, inhibits that target at the tested exposure, or explains a phenotype. Those are different propositions requiring different experiments.

Critical analyses of docking-based virtual screening show how methodological choices, protein preparation, ligand state, scoring limitations, and inadequate validation can create false confidence in apparently precise predictions [6]. This does not make docking scientifically useless. It means that docking should retain the semantic status of a prediction until independent evidence closes the relevant inferential gap. “Predicted to interact with,” “prioritized against,” or “structurally compatible with” are therefore more defensible descriptions than “targets” or “acts through” when the only evidence is computational.

Inflation can also arise through repetition at the same evidentiary level. Several docking engines, multiple predicted target databases, or concordant pathway-enrichment outputs may create an appearance of convergence while remaining dependent on related structural assumptions, annotations, or input data. Such replication can improve robustness within a computational hypothesis space, but it does not become experimental confirmation simply because several outputs agree. The important question is therefore not how many analyses point toward the same target, but whether a later experiment interrogates a genuinely different uncertainty, such as physical interaction, cellular engagement, or functional dependence.

Mechanistic inflation is not restricted to computational work. Cell-based experiments can also generate overconfident target claims when compounds are used at poorly justified concentrations, lack appropriate selectivity, or are not accompanied by adequate controls. A systematic assessment of chemical-probe use found substantial problems in how probes were selected and applied in biomedical research, showing that an experimental perturbation can be technically real yet mechanistically ambiguous if the perturbing molecule is unsuitable for the claim being made [7]. The presence of a wet-laboratory assay does not itself confer causal validity.

Target assessment is therefore better treated as a multidimensional evidence problem. The GOT-IT recommendations emphasize that target validity depends on convergent information rather than a single favorable observation [8]. For natural products, this principle has an additional analytical layer: the molecular entity itself may need confirmation before target evidence can be interpreted confidently. The consequence is a two-sided discipline. Computational predictions should not be promoted prematurely into validated mechanisms, but experimental findings should also not receive automatic causal status merely because they occur in cells or animals. What matters is whether each method resolves the specific uncertainty assigned to it.

Different meanings of mechanistic evidence

One source of confusion is the tendency to classify all target-related experiments as “mechanistic.” Chemical-probe research shows why this is inadequate. Potency, selectivity, cellular activity, and the quality of the available evidence all affect whether a small molecule can serve as an interpretable perturbation of a biological target [9]. Probe quality therefore conditions the meaning of downstream pathway or phenotype data; a strong cellular effect produced by a promiscuous compound may be biologically important without being diagnostically specific for the nominated target.

Selecting a small molecule for target validation likewise requires attention to fit-for-purpose controls, concentration, selectivity, and the biological context in which the target is being interrogated [10]. In molecular pharmacognosy, those requirements should be treated as evidentiary qualifiers rather than imported as universal numerical cutoffs. Natural products may have different physicochemical properties, multiple legitimate targets, metabolites, or limited probe analogues. The relevant question is not whether they conform to one standardized probe profile, but whether the experimental perturbation is sufficiently interpretable to support the proposed target claim.

Chemoproteomics provides a different type of evidence by enabling systematic discovery of proteins that interact with bioactive molecules [11]. Target deconvolution becomes more persuasive when complementary methods compensate for the limitations of any single strategy [12]. Even so, identification of an enriched, captured, or otherwise responsive protein is not identical to showing that this protein is functionally necessary for the phenotype. Target discovery and target causation should remain separate analytical categories.

This distinction is directly relevant to natural medicines. Chemical-proteomic approaches can connect bioactive natural products with candidate proteins through probe synthesis, target fishing, and protein identification [13]. Such data can substantially improve a mechanism claim compared with docking alone, but derivatization of a natural product may alter its behavior, nonspecific capture can occur, and a physically associated protein may still be biologically incidental. The evidentiary contribution is therefore real but bounded.

Cellular target-engagement methods add another layer. CETSA and thermal proteome profiling have been developed for target identification and drug discovery with natural products, providing evidence that compound exposure changes protein thermal behavior in a cellular context [14]. Contemporary chemical proteomics further expands the range of natural-product target-discovery strategies [15], while labeled and label-free approaches offer complementary ways to interrogate interactions without assuming one universally superior method [16]. Thermal evidence itself also requires interpretation: deep thermal profiling demonstrates that thermal behavior

can reflect proteoform states, modifications, and protein-context effects rather than a simplistic one-compound/one-protein event [17]. Expert resources for chemical-probe assessment similarly reinforce the need to evaluate the quality of the perturbing tool rather than infer validity from assay type alone [18]. Mechanistic evidence is thus best understood as a set of distinct evidentiary functions, not a single ascending list of technologies.

A graded evidence ladder for molecular pharmacognosy

A useful hierarchy must preserve both phenotype-first and target-first routes. Phenotypic and target-focused drug discovery can provide complementary information, and neither route is inherently closer to causality simply because of where it begins [19]. A natural product may first be recognized through a disease-relevant phenotype and only later undergo target deconvolution, or it may begin with a candidate protein and proceed toward phenotypic validation. The proposed ladder therefore organizes evidence by the inferential question resolved, not by the chronological order of experiments.

At the systems level, the distinction between association and causation becomes equally important. Network pharmacology can organize multicomponent and multitarget relationships, but causal mechanisms require more than network proximity, centrality, or pathway enrichment [20]. Likewise, single-cell and multiomic approaches can add valuable context by resolving heterogeneous cell states and linking target-identification efforts to broader response patterns in natural-product research [21]. These methods can reveal where and when a response occurs, but contextual resolution should not be mistaken for proof that a nominated molecular interaction is necessary for that response.

The framework proposed here therefore treats mechanism claims as transitions across several evidentiary functions. First, the investigated chemical entity must be defined with sufficient confidence for the intended claim. Second, computational nomination or structural plausibility can generate a target hypothesis. Third, biochemical or proteomic evidence can support physical interaction, and cellular engagement can show that the interaction occurs in a biological environment. Fourth, target-directed perturbation can test whether changing the candidate target alters the downstream effect. Fifth, pathway-level measurements can establish coherent biological consequences. Sixth, phenotype-level evidence can connect those consequences to a relevant cellular, tissue, or organismal outcome. These functions can branch, recur, or remain incomplete.

Crucially, the ladder does not convert evidence accumulation into a single score. It also does not assume that each evidentiary function is a mandatory checkpoint. Some questions may be answered by alternative experimental routes, while other studies may appropriately stop at target nomination or pathway association. The framework instead asks authors to identify their strongest supported claim and the nearest unresolved link. In this sense, a "higher" position is not a reward for methodological complexity; it is a statement about which alternative explanations have been constrained for a particular causal proposition.

Stronger mechanistic language should depend on closure of the particular inferential gap being claimed. Ten pathway measurements cannot substitute for untested target engagement if the sentence asserts direct target action; conversely, convincing engagement does not prove that the engaged protein causes the phenotype. Rescue, competition, genetic perturbation, resistant-target designs, temporal ordering, and exposure-compatible testing may strengthen specific links, but the necessary design depends on the biological system and natural-product context.

Complex extracts and polypharmacological compounds require an additional safeguard. The framework permits a study to occupy different evidentiary positions for different claims. A preparation may have strong phenotype-level evidence while its active constituent remains uncertain; an isolated compound may have convincing target engagement but incomplete pathway causation. This asymmetric classification is intentional. It prevents a manuscript from being labeled globally "mechanistic" when only some of its links have been resolved.

Table 1 summarizes the proposed evidence functions and, importantly, the claim boundaries that should remain visible when moving between them.

Table 1. Proposed evidence functions and claim boundaries for natural-product mechanism statements

Evidence function	What it can establish	What remains unresolved	Appropriate claim language
Chemical identity	Defined material or constituent context	Biological target and function	identified; characterized

Computational nomination	Plausible target or binding hypothesis	Physical interaction; cellular relevance	predicted; prioritized
Interaction evidence	Molecular association or binding support	Cellular engagement; causal role	interacts; binds in assay
Cellular engagement	Target is engaged in biological context	Functional necessity	engages; target-associated
Functional perturbation	Target-linked change affects downstream biology	Full pathway attribution	functionally implicated
Pathway coherence	Consistent downstream biological response	Target exclusivity; causal sufficiency	modulates; is consistent with
Phenotypic linkage	Mechanism is connected to relevant outcome	Human effectiveness; universality	contributes to; supports involvement

Note: The classification is a proposed interpretive framework, not a validated quantitative scale. Evidence functions may branch, coexist, or remain incomplete; later evidence does not automatically erase unresolved earlier uncertainty.

Moving from prediction to biological causation

Natural-product mechanism studies become more persuasive when successive experiments address genuinely different uncertainties rather than repeatedly supporting the same hypothesis. Trishizukaol A illustrates an intermediate case: experimental findings support involvement of the TRAF6/MAPK pathway in its anti-inflammatory activity, extending the argument beyond computational nomination alone [22]. Such evidence can justify language of pathway or target involvement, but it does not automatically establish that one molecular interaction is necessary, sufficient, or exclusive. Residual targets, pathway convergence, and model-specific effects remain plausible.

A stronger evidentiary transition occurs when target nomination is followed by cellular engagement and perturbational tests. JaponiconeA was investigated in bortezomib-sensitive and -resistant myeloma models using approaches including transcriptomic target nomination, CETSA, DARTS, and functional experiments implicating IKKβ [23]. In the proposed ladder, the important feature is not the number of techniques but their different inferential functions: nomination identifies a candidate, engagement assays test whether the candidate responds to compound exposure in a relevant biological environment, and perturbation asks whether that target contributes materially to the phenotype.

Usenamine A provides another example of evidence convergence. Its relationship with Myosin-9 was investigated using mass spectrometry, surface plasmon resonance, CETSA, structural modeling, and functional experiments connecting the interaction with actin-dependent cytoskeletal remodeling and hepatoma-cell responses [24]. Orthogonal physical and cellular approaches reduce some alternative explanations because they do not rely on a single measurement principle. They nevertheless leave questions about off-target activity, biological context, and whether the nominated target explains every downstream effect.

The evidence chain can also cross levels of biological organization. Ginsenoside Rh2 was linked experimentally to ERp5 inhibition and altered natural-killer-cell surveillance in breast-cancer models [25]. Such a result is stronger than demonstrating pathway-marker changes alone because a molecular interaction is connected to an immune-cell phenotype. It remains inappropriate, however, to translate an experimental oncology mechanism directly into claims of human therapeutic effectiveness.

The general lesson is that target discovery, engagement, and functional validation should be interpreted together but not conflated. Natural-product chemical proteomics, CETSA/thermal profiling, and complementary target-identification strategies provide different routes toward this convergence [13–15]. Their agreement can strengthen attribution, while disagreement is also informative because it exposes unresolved biology rather than merely lowering an imagined score.

Figure 1 integrates these distinctions into a proposed evidence architecture, showing how mechanism claims can gain specificity through orthogonal validation while retaining unresolved chemical, biological, and causal boundaries.

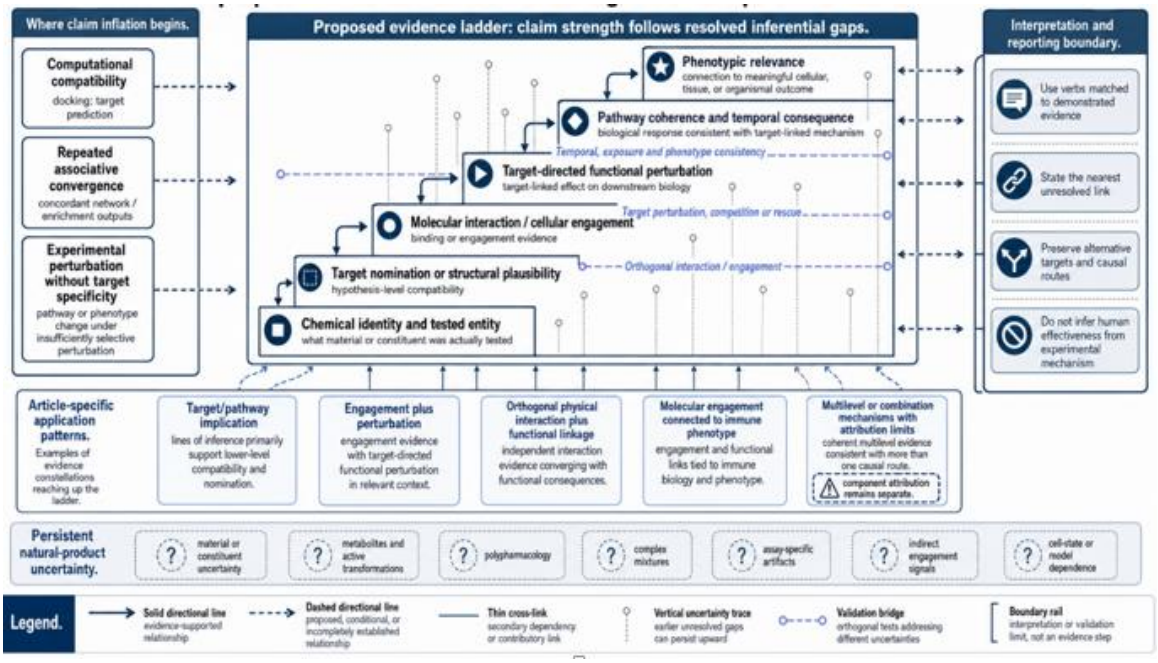

Figure 1. From natural-product mechanism hypotheses to evidence-bounded causal claims: a proposed ladder with validation bridges and interpretation boundaries

Applying the ladder to natural-product mechanism claims

Application of the proposed ladder should begin with the exact sentence an investigator wants to defend. If the intended statement is that a compound "binds" a protein, pathway changes are not substitutes for interaction evidence. If the intended statement is that a target "mediates" a phenotype, binding or engagement alone is insufficient. The framework therefore classifies claims rather than papers: the same study may support a relatively strong target-engagement claim and a weaker causal-pathway claim, or strong phenotypic activity while leaving the active constituent unresolved.

Wedelolactone illustrates why this claim-specific approach matters. Experimental work links the compound with an FXR–bile acid–NF-κB/NRF2 axis in cholestatic liver injury and integrates molecular, pathway, inflammatory, oxidative-stress, and organism-level observations [26]. This convergence supports a substantial mechanistic interpretation within the studied model. It does not require the stronger assertion that FXR is the sole molecular target or that every observed benefit results exclusively from one linear pathway. A pathway can be mechanistically important without being mechanistically exhaustive.

Combination studies create an even sharper attribution problem. Combined ursolic acid and solasodine treatment was associated with inhibition of colorectal-cancer progression through an AKT1/ERK1/2–GSK-3β–β-catenin signaling axis [27]. Evidence supporting a mechanism of the combination should not automatically be redistributed to each constituent separately. Additivity, synergy, sequential effects, pharmacological interaction, or different target contributions can generate the same downstream pathway signature. The ladder therefore requires the chemical object named in the mechanism statement to match the chemical object actually tested.

Computational evidence can still make an important contribution at earlier stages. Docking can prioritize structurally plausible hypotheses, while critical methodological analysis demonstrates why those hypotheses require experimental qualification before they are described as biological mechanisms [5, 6]. In a well-designed natural-product study, computational nomination may determine which targets are tested experimentally, explain structural hypotheses after interaction has been demonstrated, or guide discriminating follow-up experiments. Its role changes from evidentiary endpoint to hypothesis-generating component.

The framework also rejects a binary division between "validated" and "unvalidated" mechanisms. A target may be reproducibly engaged but not necessary for the phenotype. A pathway may change reproducibly while the upstream target remains uncertain. A compound may legitimately affect multiple targets, making exclusive-target language biologically inappropriate rather than merely underpowered. Thermal behavior can additionally reflect proteoform and protein-context complexity [17], while network-level relationships can remain associative unless

causal dependencies are directly tested [20]. These are not failures of mechanism research; they are reasons to describe the achieved evidence state precisely.

Accordingly, application of the ladder should end by identifying both the strongest justified statement and the most consequential unresolved alternative. That practice prevents a persuasive narrative from becoming stronger than its experiments while preserving the scientific value of partial but informative mechanistic evidence.

Implications for interpretation and reporting

Reporting should distinguish evidence verbs as carefully as experimental methods. “Predicted,” “prioritized,” “interacts with,” “engages,” “functionally implicated,” “modulates,” and “contributes to” describe different evidentiary positions. The proposed terminology is not intended as a compulsory nomenclature, but it embodies a useful rule: the verb should not imply resolution of an inferential step that was never tested. Multidimensional target assessment similarly supports the principle that confidence should arise from convergent evidence rather than a single favorable observation [8].

Small-molecule perturbation deserves particular transparency. Evidence on chemical-probe quality, target-validation practice, and expert probe assessment shows why potency, selectivity, concentration, appropriate controls, and fit-for-purpose tool choice materially affect interpretation [9, 10, 18]. For natural products, authors should report these considerations without pretending that one universal probe standard applies to every scaffold. When selectivity is uncertain or multiple targets are biologically plausible, the mechanism statement should preserve that uncertainty rather than conceal it behind a pathway diagram.

Reports should also separate what was measured from what was inferred. A docking score is a computational output; protein enrichment is an interaction-related observation; a thermal shift is engagement-related evidence requiring context; transcriptomic or phosphoproteomic change is a perturbational response; and a cellular or organismal phenotype establishes an outcome. Mechanistic interpretation emerges from relationships among these findings, not from relabeling any one of them as causal proof.

This distinction has practical consequences for figures and abstracts, where overstatement is especially easy. Arrows should not imply direct regulation when the evidence demonstrates only association. Network edges should not silently change from database-derived predictions into validated interactions. A pathway should not terminate in a therapeutic claim when the evidence ends with an experimental phenotype. Similarly, a chemically complex preparation should not be represented by a single molecular structure unless constituent attribution has actually been established.

Future evaluation of the proposed ladder should test whether independent readers can classify the same evidence consistently and whether the resulting claim categories predict later replication or successful mechanistic refinement. Testing should span isolated natural products, metabolites, standardized mixtures, and chemically complex preparations, because the inferential problems are not identical. Single-cell and multiomic approaches may help reveal state-specific responses and hidden heterogeneity [21], but they should be integrated with intervention rather than treated as substitutes for it.

Most importantly, stronger reporting does not require every natural-product study to become an exhaustive target-validation program. Exploratory studies can remain exploratory; computational studies can remain computational; engagement studies can report engagement. The scientific gain comes from matching language to evidence and identifying what would need to be tested next. Such calibration preserves useful uncertainty while making genuine mechanistic advances easier to recognize.

Conclusion

Mechanism claims in molecular pharmacognosy become difficult to evaluate when chemical identity, target prediction, physical interaction, cellular engagement, pathway perturbation, and phenotypic relevance are compressed into a single evidentiary category. The proposed evidence ladder separates these functions and treats mechanistic strength as a property of resolved inferential links rather than of methodological quantity. It therefore allows a computational prediction to remain scientifically useful without being mistaken for target validation, an engagement result to remain important without being promoted automatically to causal necessity, and a phenotype to remain compelling without being assigned prematurely to a single molecular explanation.

This framework is deliberately nonnumeric and claim-specific. It does not rank laboratories, compounds, assays, or papers, and it does not prescribe one mandatory sequence of experiments. Natural-product mechanisms may be branched, polypharmacological, metabolite-dependent, mixture-dependent, or highly context sensitive. Consequently, different claims within the same study may occupy different evidentiary positions, and uncertainty at one level need not invalidate evidence obtained at another.

The practical recommendation is correspondingly modest but consequential: define the tested chemical entity, state what each method actually demonstrates, use orthogonal evidence to address different uncertainties, distinguish engagement from functional dependence, and match mechanistic verbs to the strongest resolved link. Where alternatives remain credible, they should remain visible in the interpretation.

The ladder should itself be treated as a proposed scholarly framework requiring further evaluation. Its value will depend on whether it improves consistency of interpretation across diverse natural-product systems without becoming a rigid checklist that suppresses legitimate polypharmacology or exploratory discovery. Mechanistic credibility in molecular pharmacognosy is therefore not achieved by eliminating uncertainty from the narrative. It is achieved by locating uncertainty precisely, preventing unsupported inferential jumps, and making clear why a particular mechanism statement is warranted—and why a stronger one is not.

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