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When Polypharmacology Is Real and When It Is Annotation Noise: An Evidence-Grading Model for Multi-Target Natural-Product Mechanisms

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ABSTRACT

Natural products are frequently described as polypharmacological because multiple proteins, pathways, or phenotypes become associated with a single compound. Yet target multiplicity can arise from fundamentally different evidentiary processes, including database aggregation, computational prediction, assay interference, nonspecific activity, proteomic target engagement, or functionally consequential interactions. Treating these states as mechanistically equivalent risks converting annotation density into apparent biological complexity. This article develops an original mechanistic evidence model for evaluating multi-target natural-product claims. The analysis distinguishes target nomination from experimental engagement and separates experimentally observed promiscuity from functional multi-target action. The proposed model treats confidence as an evidence-escalation problem rather than a target-count problem: stronger mechanistic interpretation requires progressively greater control of data provenance, assay artifacts, physical target engagement, orthogonal confirmation, functional perturbation, and biological context. Importantly, confidence is assigned separately to individual target claims and to the higher-order claim that several targets jointly contribute to a phenotype. This prevents one well-supported target from legitimizing weak secondary annotations while preserving genuine polypharmacology when multiple interactions withstand mechanistic testing. The framework also accommodates difficult cases, including concentration-dependent target switching, covalent reactivity, indirect proteomic responses, metabolite-mediated activity, and one dominant target producing distributed downstream effects. The model is proposed as an evidence-organizing architecture rather than a prospectively validated scoring system. Its value therefore lies in clarifying what available evidence can establish, what remains inferential, and what additional experiments are required before multi-target natural-product mechanisms should be interpreted as functionally real.

Keywords: Natural products, Polypharmacology, Multi-target mechanisms, Mechanistic evidence, Target engagement, Evidence grading

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Introduction

Natural-product mechanism studies increasingly move beyond phenotype description toward molecular target identification, but the evidentiary meaning of a reported "target" depends strongly on how it was obtained. Labeled and label-free strategies can interrogate binding, stability, competition, enrichment, or other target-associated observables, and each strategy carries distinct chemical and analytical constraints [1]. This heterogeneity matters especially for natural products, whose structural complexity, reactivity, permeability, metabolism, and limited opportunities for derivatization may determine which target-identification technologies are feasible.

Consequently, the accumulation of several reported proteins cannot by itself establish that a compound operates through several mechanistically relevant molecular targets.

Computational polypharmacology adds another layer of complexity. Ligand similarity, chemogenomic relationships, machine-learning models, and target-space representations can expand the set of plausible protein partners, but their outputs remain dependent on reference chemistry, target coverage, annotation quality, and validation design [2]. A predicted multi-target profile is therefore better treated as a hypothesis space than as an observed mechanism. This distinction is easily obscured when predicted targets, database annotations, biochemical hits, and cellular target-engagement measurements are combined in one network and subsequently described using a common mechanistic vocabulary.

The opposite error is also possible. Extensive target engagement is not inherently evidence of poor selectivity or assay noise. Large-scale chemical-proteomic profiling of kinase inhibitors has demonstrated that compounds can exhibit broad, experimentally measurable interaction landscapes that differ substantially from simplified nominal-target descriptions [3]. Although kinase-centered evidence cannot be extrapolated quantitatively to all natural-product chemical space, it establishes an important logical boundary: high target multiplicity may be experimentally real even when the functional contribution of each interaction remains unresolved. An adequate framework must therefore discriminate false multiplicity without defining multiplicity itself as failure.

This article proposes that natural-product polypharmacology should be evaluated as an evidence-escalation problem. The relevant question is not simply how many targets are associated with a compound, but how each association was produced, what competing explanations have been excluded, whether physical engagement is supported, and whether more than one engaged target contributes to the observed biological response. The proposed model consequently separates target-specific confidence from mechanism-level confidence. A compound may have one strongly supported functional target plus numerous weak annotations, several experimentally engaged but functionally silent proteins, or multiple targets whose contributions become credible only after orthogonal and perturbational testing. These states should not share a common mechanistic grade.

The problem of apparent polypharmacology

Apparent polypharmacology can originate before any genuine multi-target mechanism has been demonstrated. Small molecules may generate misleading activity through detection interference, chemically reactive behavior, nonspecific inhibition, or colloidal aggregation; orthogonal testing has also shown that nominally selective tool compounds can carry substantial assay-dependent and off-target liabilities [4–6]. These mechanisms are analytically important because repeated activity across unrelated assays may superficially resemble biological promiscuity. Artifact exclusion should therefore precede any attempt to interpret target multiplicity mechanistically, while recognizing that the presence of an interference risk does not prove that every observed activity is artifactual.

Computational nuisance-compound detection provides useful triage but does not solve this problem experimentally. Machine-learning approaches such as frequent-hitter classifiers can identify chemical patterns associated with repeated assay activity and prioritize compounds for scrutiny [7]. Such classifications are probability-bearing warnings, not demonstrations of aggregation, redox cycling, fluorescence interference, covalent reactivity, or another specific mechanism. Treating a predicted nuisance profile as definitive would reproduce the same inferential error that occurs when a predicted pharmacological target is presented as validated biology.

Apparent multiplicity can also arise through data provenance. ChEMBL integrates bioactivity observations collected across different assay formats, target definitions, measurement types, experimental periods, and curation states [8]. This breadth is indispensable for drug-discovery informatics, but a database-derived target list is not a single standardized experiment. Two proteins attached to the same compound record may have been measured under different conditions, with different endpoints and different evidentiary depth. The number of annotated targets therefore reflects both biology and the architecture of the evidence collection process.

Even apparently simple compound-target pair counts depend on operational choices. Curated comparisons of drugs, clinical candidates, and other bioactive compounds demonstrate that the construction and classification of compound-target relationships shape the resulting target distributions [9]. For mechanistic interpretation, this means that “multitarget” should never function as a provenance-free label. The first analytical task is to reconstruct what produced each target assignment. Only then can assay behavior, physical engagement, and functional consequence be judged separately rather than collapsed into one target count.

Where multi-target claims originate

The weakest mechanistic form of a multi-target claim may begin with computational nomination. Multi-fingerprint similarity approaches can compare a query structure with bioactivity-annotated chemical space and return several plausible target classes [10]. These predictions can be highly useful for hypothesis generation, especially when a natural product lacks an established molecular mechanism, but they inherit the scope and biases of the reference data. Structural resemblance supports plausibility; it does not establish binding, intracellular access, occupancy, or functional contribution.

Validation must therefore be treated as a separate evidentiary operation. Analyses of drug-target interaction prediction methods show that computational evaluation and experimental validation answer different questions and that biological confirmation remains uneven across the prediction literature [11]. A well-performing benchmark can demonstrate that an algorithm generalizes within a defined task, yet it cannot establish that a particular predicted target mediates the phenotype of a specific natural product in a relevant biological context.

Experimental target deconvolution provides stronger contact with the underlying chemistry. Chemoproteomic methods can enrich, compete, stabilize, or otherwise profile proteins associated with a bioactive small molecule, thereby moving the evidentiary state from annotation toward experimentally observed target engagement [12]. Nevertheless, abundance, accessibility, background binding, probe modification, and platform-specific detectability can influence which proteins appear. Proteomic evidence therefore strengthens target attribution without converting every detected protein into a functional target.

Integration across modalities can further increase specificity. Multiplexed approaches combining chemical genomics and chemical proteomics can connect genetic response patterns with experimentally measured protein interactions [13]. Their value lies in convergence across analytically different evidence rather than in simply producing a larger target set. When independent modalities implicate the same protein or mechanistic neighborhood, the interpretation becomes more constrained, although functional causality still requires additional testing.

Photoaffinity-labeling workflows illustrate both the strength and the boundary of direct proteomic target discovery. They can support target engagement and deconvolution at high throughput, but the probe itself introduces chemical modifications and enrichment-dependent background that must be controlled [14]. Thus, the origin of a multi-target claim should remain visible throughout interpretation: database association, computational nomination, biochemical activity, proteomic engagement, and orthogonal functional evidence are related states, not interchangeable ones.

Table 1 classifies these major routes according to what they directly establish and what remains unresolved before a multi-target mechanism can be claimed.

Table 1. Evidentiary meaning of common routes that generate multi-target natural-product claims

Claim origin	Directly observed or produced	Permitted interpretation	Mechanistic limitation
Database annotation	Curated compound–activity–target records	Prior evidence links compound and target	Assay provenance and context may differ
Computational prediction	Ranked or similarity-based target hypotheses	Plausible targets for testing	No physical engagement or functional dependence established
Biochemical activity	Target-associated assay response	Activity under defined assay conditions	Interference or nonspecific behavior may remain
Chemoproteomic engagement	Protein-associated enrichment, competition, or state change	Stronger evidence for molecular interaction	Detectability, probe, or indirect-response effects remain
Orthogonal multimodal evidence	Convergent findings from distinct experimental modalities	Increased confidence in target attribution	Functional contribution still requires perturbational support

Note: The classification is a proposed interpretive organization of the evidence types discussed above; it is not a validated numerical hierarchy.

Distinguishing prediction, promiscuity, and functional multi-target action

Experimental methods do not all interrogate the same property. Thermal proteome profiling measures ligand- or state-associated changes in protein thermal behavior; chemoproteomic-enabled phenotypic strategies connect

cellular responses with candidate molecular interactions; and reactive-site profiling can identify ligand-sensitive residues within accessible proteomic space [15–17]. Their complementarity is important precisely because each modality has its own indirectness and coverage limits. Convergence across different observables can narrow alternative explanations more effectively than repetition within one assay family, but no single positive signal automatically establishes functional dependence.

A target can therefore be experimentally engaged without being necessary for the phenotype. Phenotypic chemoproteomics is especially useful for connecting unbiased biological effects with target hypotheses, yet the resulting relationship still requires mechanistic follow-up [16]. This distinction becomes critical for compounds with large interaction landscapes: several proteins may bind or respond to a compound while only one, or a smaller subset, contributes materially to the measured biological state. In the model proposed here, engagement multiplicity and functional polypharmacology are separate claims.

Site-resolved evidence can strengthen the physical-interaction component of a target assignment. Competitive profiling of reactive cysteines, for example, can localize ligand-sensitive interactions to defined residues and thereby constrain the interpretation of a proteomic signal [17]. However, residue accessibility, intrinsic reactivity, and proteome coverage determine what such experiments can detect. Absence of a competitive site is therefore not proof that no interaction exists, while detection of a site does not by itself demonstrate that altering that site explains the phenotype.

Functional inference additionally depends on the quality of the perturbational tools used to test a target. Chemical-probe guidance emphasizes potency, selectivity, appropriate controls, and fitness for the biological context as prerequisites for interpretable target-validation experiments [18]. Accordingly, the proposed framework does not define functional multi-target action as “two or more positive assays.” It requires evidence that multiple target contributions remain credible after artifact control, engagement assessment, and function-oriented perturbation. Importantly, the evidence may be asymmetric: one target can be strongly validated while another remains predicted. Such a case should not inherit the confidence of the strongest target.

Figure 1 summarizes this proposed separation between target-name accumulation and increasingly constrained evidence for functional multi-target action, while retaining the alternative routes by which apparent or genuine multiplicity can arise.

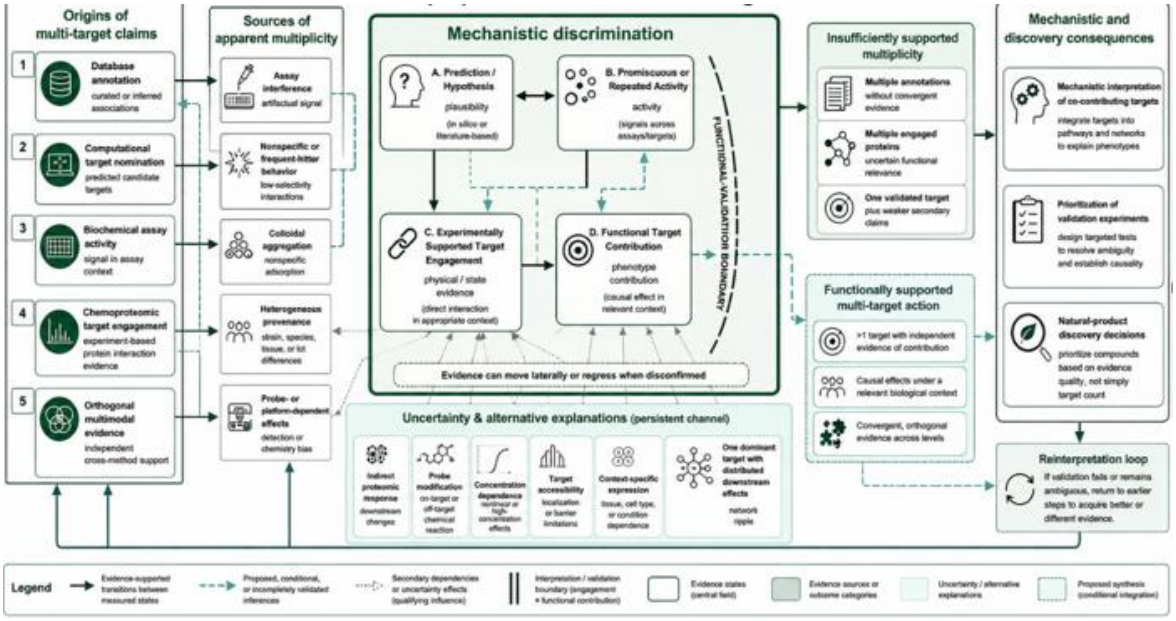

Figure 1. From target-name accumulation to functionally supported polypharmacology: a proposed evidence-constraining architectur

An evidence-grading model

Chemical proteomics makes it increasingly possible to characterize broad interaction landscapes, but an interactome and a functional mechanism remain different evidentiary objects. Contemporary functional chemical-proteomic analysis explicitly treats protein interaction mapping and biological consequence as connected but

nonidentical questions [19]. The proposed model therefore assigns confidence at two levels. The first is target-specific: how strongly does the evidence support a particular compound–target relationship and a functional contribution from that target? The second is mechanism-level: how strongly does the evidence support the claim that more than one target contributes to the same biologically relevant response? A mechanism-level judgment cannot be stronger merely because its target list is longer.

This separation is necessary because genuine broad engagement is experimentally possible. Profiling of clinically used kinase inhibitors revealed interaction landscapes extending beyond simplified nominal-target descriptions [20]. Such evidence prevents an evidence-grading system from treating multiplicity itself as a nuisance signature. The decisive issue is instead whether each proposed contribution is supported at the appropriate level. Several physical interactions may be real while only one is functionally consequential; alternatively, multiple engaged proteins may influence a response through distinct or convergent mechanisms. Target number alone discriminates neither situation.

The framework proposed here uses six evidentiary questions rather than a numerical score. First, provenance asks how the target entered the claim: annotation, prediction, assay activity, or experiment. Second, artifact control asks whether plausible nonmechanistic explanations have been tested. Third, engagement asks whether the parent compound, an adequately characterized probe, or a label-free method supports interaction with the target. Fourth, orthogonality asks whether a materially different modality converges on the same assignment. Fifth, functional perturbation asks whether altering the target changes the compound-associated phenotype in the predicted direction. Sixth, context asks whether engagement and functional contribution coexist under biologically relevant concentrations, cell states, tissues, or exposure conditions. Validation analyses of drug-target prediction emphasize that computational and experimental validation must not be conflated [11], while chemical-probe guidance similarly makes potency, selectivity, controls, and fit-for-purpose use central to interpretable perturbation [18].

These questions define evidence dimensions, not universal checkpoints. A covalent natural product may obtain unusually strong site-level evidence while still requiring discrimination between selective engagement and broad reactivity. A label-free thermal response may support target-associated state change without proving direct binding. Genetic perturbation may strengthen functional attribution yet introduce adaptation or network compensation. The model consequently permits evidence to be asymmetric and nonmonotonic rather than forcing every target through an identical ladder.

Most importantly, the proposed mechanism-level judgment is conditional on the weaker relevant target claims. One highly supported target cannot transfer its confidence to predicted secondary targets. Conversely, several independently supported targets can justify a multi-target interpretation only when their contributions are functionally compatible with the phenotype and biological context. This distinction follows the broader chemical-proteomic requirement to connect interaction maps to functional activity rather than equating the two [19]. It also makes explicit an uncertainty that target-count language obscures: the same compound can simultaneously possess real molecular promiscuity, one dominant mechanism, and several interactions of uncertain consequence.

Figure 2 expresses the proposed grading model as target-specific evidence paths that remain separate until the higher-order multi-target mechanism is evaluated

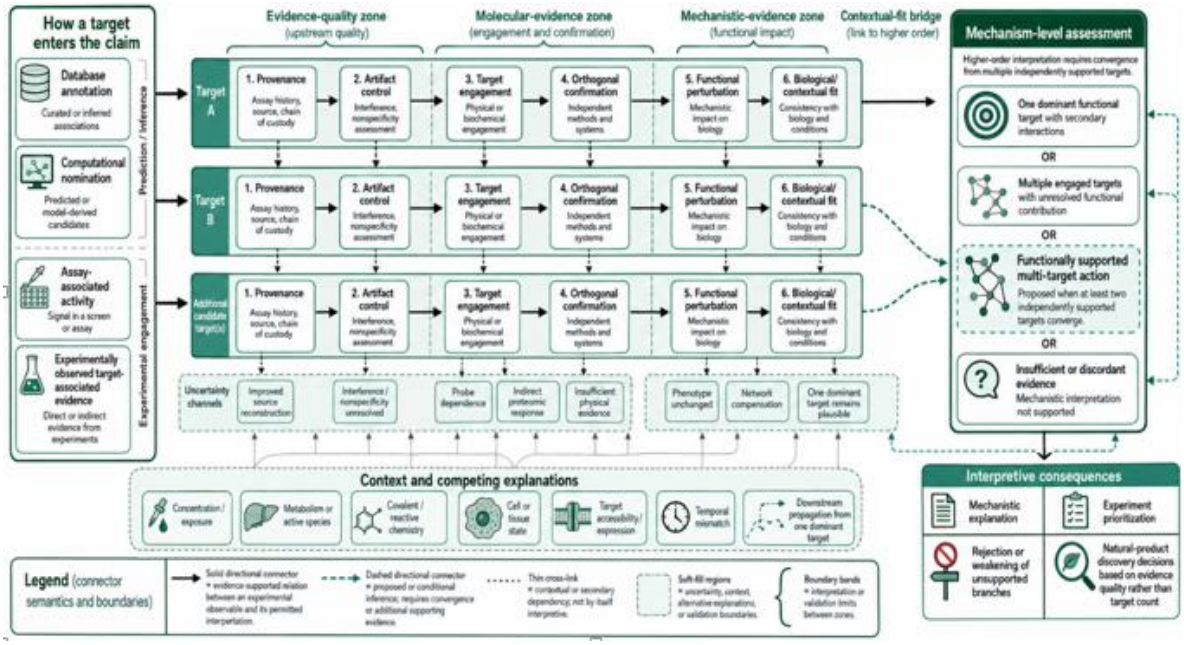

Figure 2. Grading target claims without laundering uncertainty: a proposed two-level model of mechanistic polypharmacology

Mechanistic consequences of genuine polypharmacology

Natural-product studies illustrate why mechanistic confidence should be based on evidence convergence rather than the rhetorical breadth of a target network. Withaferin A has been investigated through a defined interaction with KPNB1 and consequent effects on VCP nuclear behavior in a DNA-damage setting [21]. The relevant lesson for the proposed model is not that this interaction exhausts withaferin A pharmacology. Instead, it shows how a natural-product target claim becomes more interpretable when molecular interaction is connected to a specified cellular process.

A stronger convergence pattern is provided by baicalin research in liver-fibrosis models. Tissue chemoproteomics identified STAT3 as a key candidate, and the study subsequently incorporated binding characterization, genetic loss-of-function, pathway analysis, and in-vivo disease models [22]. This combination supports a substantially more constrained attribution than proteomic nomination alone. It still does not establish that STAT3 is baicalin's only biologically relevant interaction, which illustrates why the proposed framework grades a specific target contribution without turning that contribution into an exclusivity claim.

Other recent examples reach mechanistic attribution through different experimental routes. Stability- and degradation-based proteome profiling was used to connect cannabidiol with the CDC123–eIF2γ axis in colorectal-cancer models, followed by functional investigation [23]. Parthenolide was linked to covalent engagement of TRIM33 and modulation of NF-κB signaling in experimental sepsis, providing a route from reactive chemistry to pathway consequence [24]. These cases are not interchangeable validation templates: their chemical mechanisms, biological models, target observables, and perturbational evidence differ.

A multi-target interpretation also needs temporal and exposure coherence. If one target is engaged only at concentrations that are not compatible with the conditions supporting another target, the two observations should not automatically be combined into one simultaneous mechanism. Likewise, pathway convergence can be additive, redundant, antagonistic, or sequential; none of these relationships follows simply from detecting two protein partners. The proposed model therefore treats co-contribution as a testable relationship among target effects, not as the arithmetic sum of individually credible target assignments. Broad measured target landscapes demonstrate why such relationships are possible, but they do not specify which interactions dominate under a particular biological condition [20].

Genuine polypharmacology requires an additional inference beyond each of these single-target examples. Broad interaction profiling establishes that real molecular multiplicity can exist [20], but a multi-target mechanism requires evidence that at least two target contributions are relevant within a compatible biological and concentration context. Possible consequences include convergent regulation of one pathway, complementary control of different processes, or context-dependent dominance of different targets. These possibilities are

mechanistically plausible outcomes, not effects established universally by target multiplicity. A credible claim must therefore specify which targets contribute, under what conditions, and how their combined interpretation differs from a one-dominant-target explanation.

Table 2 compares the evidentiary patterns that most directly alter the interpretation of a natural-product target claim.

Table 2. How mechanistic evidence changes the interpretation of a natural-product target claim

Evidence pattern	What it adds	Remaining uncertainty
Direct or site-resolved engagement	Constrains compound–protein attribution	Binding may lack phenotypic consequence
Orthogonal target confirmation	Reduces dependence on one assay principle	Shared systematic bias may remain
Selective functional perturbation	Connects target state with phenotype	Compensation or indirect effects may complicate causality
Pathway concordance	Links target perturbation to downstream biology	One target may still dominate the pathway response
Multiple independently supported targets	Supports proposed functional polypharmacology	Co-contribution must coexist in relevant context

Note: These are proposed interpretive states, not validated evidence scores or universal sufficiency criteria.

Implications for natural-product discovery

Natural-product discovery should consequently treat target identification as staged mechanistic inference rather than as a competition to maximize the number of target names. Recent syntheses of natural-product target-identification strategies describe complementary chemical, proteomic, computational, and label-free approaches, each suited to different compound and biological constraints [25]. The practical implication is to choose subsequent experiments according to the uncertainty that remains. A computationally nominated target needs different escalation than a competitively enriched protein; a covalently engaged target needs different controls from a thermal responder; and a phenotype-linked target still requires evidence that the observed contribution is not merely downstream of another dominant interaction.

Technological expansion makes this distinction more important, not less. Current natural-product target-identification platforms broaden access to candidate interactions while retaining modality-specific limitations in labeling, protein coverage, sample requirements, indirectness, and functional interpretation [26]. The proposed evidence model therefore favors complementary evidence when it changes the inference being tested. Repeating related profiling methods can improve reproducibility, but it should not automatically carry the same evidentiary value as adding an orthogonal experiment capable of rejecting a competing explanation.

This approach also changes prioritization. Rather than rank a natural product as mechanistically interesting because a network contains many nodes, discovery programs can identify which target claims are sufficiently supported to justify deeper testing and which remain annotation-level hypotheses. Experimental resources can then be concentrated on discriminating mechanisms: parent-compound competition, orthogonal engagement, structure–activity relationships, selective probes, genetic perturbation, rescue, pathway-specific readouts, or appropriate in-vivo confirmation where feasible. Drug-target prediction methodology already distinguishes computational performance from experimental target validation [11]; the proposed extension is to preserve that distinction after several targets have accumulated around the same compound.

For natural products, experimental sequencing should also respect chemical identity over time. Reactive, unstable, or metabolized molecules may generate target patterns that change with exposure conditions. The evidence grade should therefore follow the molecular species responsible for an observation wherever that distinction can be resolved. A persuasive workflow should avoid merging parent-compound predictions, probe-derived interactomes, and downstream pathway changes into one undifferentiated network. Recent target-identification syntheses emphasize matching method selection to the chemical and biological problem [25]; the proposed framework extends that principle to post-nomination interpretation.

A further implication concerns negative evidence. Failure to confirm a predicted or proteomically nominated target should narrow the mechanism rather than be treated as an inconvenient result to be replaced by another database node. Conversely, failure of one secondary target should not invalidate a well-supported primary interaction. Recording both confirmed and rejected branches would make natural-product mechanism

development more falsifiable and reduce retrospective network construction. The objective is therefore not to privilege single-target pharmacology, but to make multi-target claims earn their mechanistic specificity target by target.

Failure modes and unresolved questions

No evidence architecture eliminates uncertainty inherent to target identification. Label-free approaches avoid structural modification of the parent molecule but infer targets through observables such as stability, protection, or proteome-state changes that have method-specific interpretive limits [27]. An indirect responder may therefore appear target-associated without being a direct binding partner, whereas absence of a detectable response may reflect coverage or protein-state constraints rather than absence of interaction.

Operational definitions also remain a major boundary. Systematic analyses of compounds classified as single- or multitarget show that those categories depend on the underlying activity data and target annotations used to construct them [28]. A dataset-defined multitarget compound is consequently not synonymous with a functionally validated polypharmacological mechanism. The distinction becomes especially difficult when assay concentrations differ substantially, metabolites contribute activity, target expression changes across cell states, or covalent chemistry generates multiple engagements with unequal functional consequences.

A further hard case is the apparently coherent network produced by one dominant target. A single upstream interaction can alter many proteins, transcripts, metabolites, and pathways, creating a systems-level signature that resembles coordinated multi-target pharmacology. Conversely, two genuine binding events may never contribute simultaneously because their effective concentration ranges or cellular locations do not overlap. These alternatives cannot be resolved by network density. They require experiments designed to distinguish direct contribution, downstream propagation, and context-dependent engagement. The grading model therefore treats mechanistic parsimony as a competing explanation rather than assuming either single-target or multi-target causation in advance.

Several experimental ambiguities resist simple grading. Thermal shifts can reflect direct or indirect changes in protein state [15]. Likewise, a broad interactome can contain functional drivers, passengers, and context-dependent interactions [19]. Genetic perturbation may alter network compensation, while chemical probes may lose selectivity as concentration or biological context changes. These problems favor explicit uncertainty rather than artificial precision.

The proposed model therefore requires prospective challenge. Useful tests would begin with compounds whose target claims span different evidence states and ask whether artifact controls, orthogonal engagement, selective perturbation, and context-matched experiments reproducibly discriminate unsupported multiplicity from functionally meaningful co-contribution. Validation should be capable of falsifying target assignments rather than merely accumulating supportive assays [11]. Where chemical probes are used, their potency, selectivity, controls, and fit to the tested context must remain part of that evaluation [18]. Until such testing is performed, the framework should be regarded as an organizing model for mechanistic evidence, not a validated predictor of true polypharmacology.

Conclusion

Polypharmacology is neither intrinsically mechanistic complexity nor intrinsically annotation noise. The term currently spans target lists produced by databases and prediction models, repeated assay activity, experimentally measured engagement, and the much stronger proposition that several targets materially contribute to one biological response. Collapsing those states favors two opposite errors: weak annotations can be promoted into an elaborate mechanism, while genuine broad engagement can be dismissed merely because it is broad.

The model proposed here replaces target counting with evidence grading. It separates the confidence assigned to each compound–target claim from the confidence assigned to the overall multi-target mechanism and asks whether provenance is interpretable, plausible artifacts have been addressed, engagement is supported, orthogonal evidence converges, target perturbation affects the phenotype, and the proposed contributions coexist in a relevant biological context. This architecture allows strong and weak target claims to remain asymmetric and prevents a well-supported primary target from legitimizing unsupported secondary nodes.

A negative result can also be informative. Failure to validate a secondary target should weaken or remove that mechanistic branch, while additional binding partners should expand the model only when their functional relevance is independently supported.

The framework is deliberately nonnumerical and does not define universal thresholds for mechanistic acceptance. Different chemotypes, target classes, experimental modalities, tissues, concentrations, metabolites, and biological states may require different validation routes. It also does not establish that multi-target action is therapeutically preferable to selectivity. Its intended contribution is narrower: to make the evidentiary content of polypharmacology claims explicit and falsifiable. Genuine multi-target mechanisms should emerge when several target contributions survive independent mechanistic scrutiny; where they do not, the scientifically stronger conclusion may be one dominant target, uncertain secondary engagement, or unresolved annotation rather than an overconnected mechanism.

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