Review

Phosphoproteomics and Multi-Omics for Oleanolic Acid Target Deconvolution: From Phosphorylation Signatures to Mechanistic Validation

Andrzej Günther * and Barbara Bednarczyk-Cwynar

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Review

Phosphoproteomics and Multi-Omics for Oleanolic Acid Target Deconvolution: From Phosphorylation Signatures to Mechanistic Validation

Andrzej Günther [1,*] and Barbara Bednarczyk-Cwynar [1,2]

[1] Department of Organic Chemistry, Faculty of Pharmacy, Poznan University of Medical Sciences, Collegium Pharmaceuticum 2 (CP.2), Rokietnicka Str. 3, 60-806 Poznan, Poland

[2] Center of Innovative Pharmaceutical Technology (CITF), Rokietnicka Str. 3, 60-806 Poznan, Poland

* Correspondence: andrzej.gunther@me.pl

Abstract

Oleanolic acid (OA) is a pentacyclic triterpenoid with broad biological activity, but its primary molecular points of engagement remain incompletely resolved. Most available studies describe OA through selected pathway markers, particularly within PI3K/AKT/mTOR, AMPK/mTOR, MAPK, NF-κB, and Nrf2 signaling, without clearly distinguishing direct target engagement from downstream adaptive responses. This limits mechanistic interpretation and weakens translational prioritization. This review examines how phosphoproteomics and integrated multi-omics can support OA target deconvolution. We discuss why phosphoproteomics is particularly informative for capturing early signaling events, how it can be combined with proteomics, transcriptomics, metabolomics, and chemoproteomic approaches, and why orthogonal target-engagement methods remain essential for stronger causal inference. We also organize the current signaling evidence for OA and its derivatives, highlighting the strongest support for AMPK/mTOR-linked regulation of autophagy and apoptosis while identifying major gaps in systems-level validation across other reported pathways. Finally, we propose a stepwise workflow for OA target deconvolution based on time-resolved phosphoproteomics, analysis of informative phosphosite subsets, multi-omics integration, kinase/phosphatase activity inference, and experimental target validation. This framework may help move OA research from descriptive pathway pharmacology toward mechanism-based target prioritization and more rational derivative development.

Keywords: oleanolic acid; phosphoproteomics; multi-omics; target deconvolution; kinases; phosphatases; cell signaling; PTP1B; AMPK/mTOR; mechanisms of action

1. Introduction

Oleanolic acid (OA) is a naturally occurring pentacyclic triterpenoid widely distributed in edible and medicinal plants. Chemically, OA has the molecular formula $C_{30}H_{48}O_3$ and a hydrophobic pentacyclic scaffold bearing a hydroxyl group at C-3, a carboxyl group at C-28, and a double bond between C-12 and C-13. These structural features are relevant because they define the principal sites used for semisynthetic modification and contribute to the biological and pharmacokinetic behavior of OA and its derivatives. The chemical structure of OA, is shown in Scheme 1.

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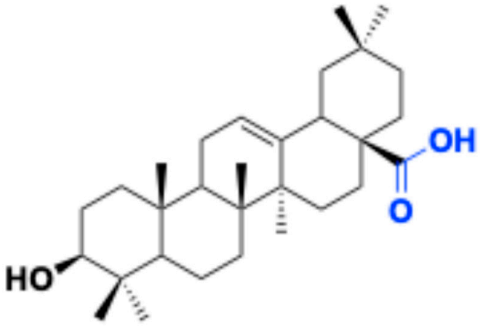

Scheme 1. Chemical structure of oleanolic acid (OA).

OA has attracted considerable attention because of its broad spectrum of biological activities, including anti-inflammatory, anticancer, hepatoprotective, neuroprotective, metabolic, and cytoprotective effects. Interest in OA arises not only from its pleiotropic bioactivity, but also from the fact that it constitutes a chemically tractable scaffold for the generation of semisynthetic derivatives with improved potency, selectivity, or pharmacokinetic properties [1–10].

At the same time, despite the extensive literature on OA, its mechanism of action remains only partially defined. Most studies describe OA through selected proteins or signaling axes, such as PI3K/AKT/mTOR, AMPK/mTOR, MAPK, NF-κB, and Nrf2, but much less often attempt to separate direct target engagement from secondary, adaptive, or compensatory responses [3,5,6,9–14]. As a result, the OA field remains rich in pathway-level observations but comparatively poor in analyses of causal signaling hierarchy.

This challenge is not unique to OA. In the broader field of natural bioactive compounds, target identification and target validation remain among the most methodologically demanding stages of translational development [12,15–21]. Natural products often display polypharmacology, their activity may vary across biological contexts, and single-layer readouts rarely capture the full structure of cause-and-effect relationships. For this reason, integrative strategies combining phosphoproteomics, proteomics, transcriptomics, metabolomics, and network analysis are increasingly being adopted to move from phenotypic description toward mechanism-oriented interpretation [12,13,15–20].

Within this framework, phosphoproteomics is particularly relevant. Phosphorylation is one of the most dynamic and functionally informative post-translational modifications, governing the activity of kinases, phosphatases, adaptor proteins, transcription factors, and entire signaling networks [13,14,17–21]. Because many OA-associated biological effects have been linked to changes in the phosphorylation status of proteins controlling cell survival, autophagy, apoptosis, metabolism, and stress response, phosphoproteomics provides a natural platform for a more systematic readout of OA action [9,11,13,14,17–20].

The aim of this review is to examine how phosphoproteomics and integrated multi-omics approaches can be used for OA target deconvolution. Rather than offering another broad summary of OA biological activities, we adopt a mechanistic and methodological perspective centered on the distinction between primary molecular interactions and downstream secondary responses within signaling networks [3,13,14,18,19]. Such a perspective may help organize the dispersed OA literature, identify the most plausible kinase- and phosphatase-related signaling nodes, and establish a rational workflow for future translational studies.

2. Oleanolic Acid as a Pleiotropic Compound: Biological Relevance and the Limitations of the Classical Mechanistic Approach

OA is among the best-characterized pentacyclic triterpenoids of natural origin. Its presence has been documented in numerous plant species, and the continuing scientific interest in this compound

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stems from both its natural abundance and its favorable pharmacological profile. In recent years, multiple reviews have summarized the activity of OA and its derivatives in cancer, inflammation, oxidative stress, metabolic disorders, and organ injury, underscoring its continuing relevance as a pharmacologically attractive natural-product scaffold [1–9,13,22].

Of particular relevance here is OA's signaling pleiotropy. OA has been linked to modulation of several regulatory axes, including PI3K/AKT/mTOR, AMPK/mTOR, MAPK/JNK, NF-κB, and redox-related pathways [3,5,6,9,22]. This breadth makes OA attractive in multifactorial disease settings, but it also complicates the distinction between early OA-responsive events and downstream network remodeling.

A representative example is provided by studies of OA in colon cancer. Hu et al. showed that OA inhibited proliferation in HCT-116 and SW-480 cells, increased apoptosis, and affected components of the AMPK/mTOR axis [11]. Specifically, OA increased AMPK and proteins associated with autophagy and apoptosis, including TSC2, Beclin 1, LC3B-II, ULK1, BAX, and caspases, while reducing mTOR and p-mTOR, leading the authors to interpret the response as AMPK-dependent induction of autophagy and apoptosis. Similar mechanistic logic has also been reported in other cancer settings in which OA or its derivatives modulate PI3K/Akt/mTOR-associated survival signaling and autophagic responses [3,23,24]. These data are clearly important, but they also illustrate a broader limitation of the OA literature: analyses typically focus on a small number of preselected proteins and do not allow one to determine unequivocally whether OA acts directly on components of the pathway or instead triggers earlier upstream events that only secondarily reshape AMPK/mTOR signaling.

This is where the limits of the classical mechanistic approach become most apparent. Studies based on Western blotting, RT-qPCR, immunofluorescence, and small marker panels remain highly informative for hypothesis testing, but they cannot reconstruct the full architecture of the cellular response [11–13,17,18]. In the case of multi-target compounds such as OA, interpretation based on a few endpoints may therefore lead to an overly simplified conclusion that the compound "acts through" a given pathway, when the observed signal may represent only one layer of a broader systems-level response [12,13].

An additional complication is the strong context dependence of OA activity. The same compound may produce cytoprotective effects in one model and pro-apoptotic effects in another, depending on cell type, dose, exposure time, metabolic state, and coexisting stress conditions [3,5,6,9,11,22,25]. OA should therefore not be viewed simply as an inhibitor or activator of a single protein, but rather as a network-modulating molecule whose biological point of entry may vary across systems. This feature does not diminish its therapeutic potential, but it does mean that more advanced approaches are needed to identify its true molecular points of engagement.

From the perspective of target deconvolution, this requires a shift away from the narrative that OA "regulates many pathways" and toward a more mechanistically useful question: which signaling events occur earliest, which are most reproducible across models, and which are most likely to reflect direct target engagement [12–14,18,19,26]? Classical hypothesis-driven assays alone are poorly suited to answering this question. What is needed instead are methods capable of broad, quantitative, and ideally time-resolved monitoring of signaling changes, combined with other layers of biological information. Taken together, these observations highlight the central conceptual problem in the OA field, namely the difficulty of distinguishing direct target engagement from downstream network remodeling (Figure 1).

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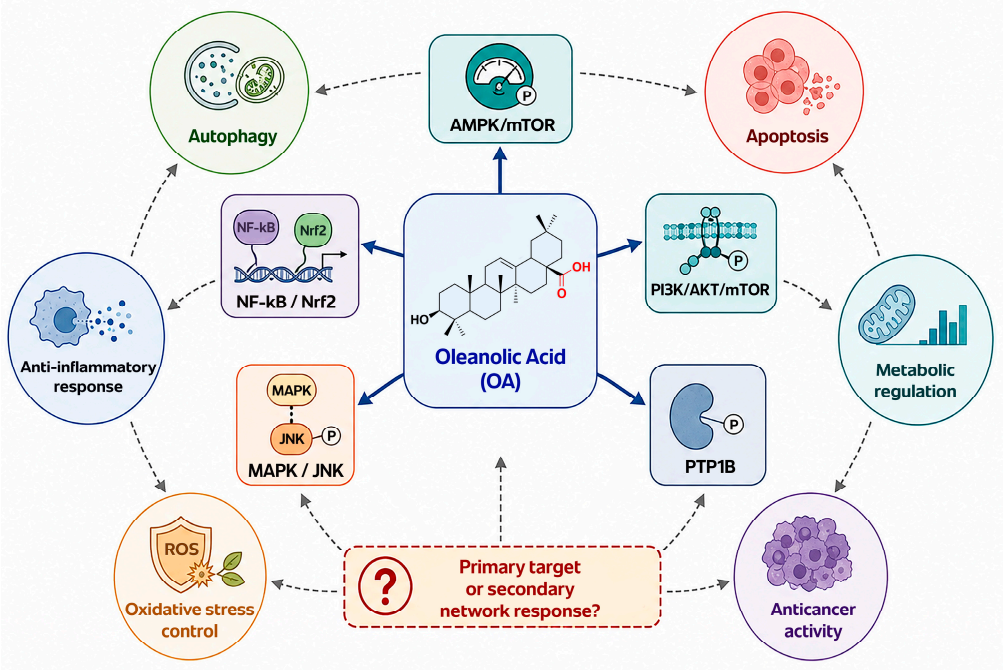

Figure 1. Oleanolic acid as a pleiotropic signaling modulator: from reported biological effects to the target-deconvolution problem. Note: Oleanolic acid has been associated with modulation of several major signaling networks, including AMPK/mTOR, PI3K/AKT/mTOR, NF-κB/Nrf2, MAPK/JNK, and phosphatase-related nodes such as PTP1B [3,9–11]. However, most currently available data are based on marker-level or pathway-centered studies, making it difficult to distinguish primary molecular targets from secondary adaptive responses. The figure highlights the need for a target-deconvolution framework grounded in phosphoproteomics and multi-omics.

3. Phosphoproteomics and Multi-Omics as a Methodological Framework for OA Target Deconvolution.

Phosphoproteomics is one of the most promising tools for investigating the mechanisms of action of compounds that modulate cellular signaling. Its particular value lies in the fact that phosphorylation is a rapid, reversible, and functionally informative modification that is directly coupled to the activity of regulatory proteins and signaling networks [13,14,17–21,27]. Unlike transcriptomics, which mainly captures later consequences of a cellular response, phosphoproteomics enables the detection of early signaling events driven by kinases, phosphatases, and adaptor proteins and is therefore better suited to mapping the initial stages of response to a bioactive compound [13,14,17–20].

Technically, contemporary phosphoproteomics relies primarily on LC-MS/MS following protein digestion into peptides and enrichment of the phosphopeptide fraction. Higgins et al., Gerritsen and White, and Urban emphasize that the success of such analyses depends on careful sample preparation, minimization of sample loss, and efficient phosphopeptide enrichment, most commonly using IMAC or MOAC-based strategies [13,14,17,20,27]. This is particularly important because the phosphoproteome represents only a small fraction of the total proteome, while many highly informative regulatory events are of low abundance. In particular, phosphotyrosine signaling represents only a minor proportion of the global phosphoproteome and may be missed entirely without dedicated enrichment steps [14,17,20,27,28].

This has direct implications for OA research. If OA truly modulates specific kinase or phosphatase nodes, then the earliest and most informative changes may lie precisely in low-abundance phosphorylation events that are invisible in standard nonspecific workflows. For this reason, studies of OA should not rely exclusively on global phosphoproteomics, but should also

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consider the analysis of selected phosphoproteome subsets, such as phosphotyrosine-enriched fractions or phosphomotif-defined kinase substrate classes [13,14,27,28]. Such approaches may increase the likelihood of identifying the regulatory nodes that initiate OA-responsive signaling.

The analytical layer is equally important. Higgins et al. and Gerritsen and White note that the identification of a phosphorylation site alone does not establish its functional significance; robust interpretation requires quantitative comparison across experimental conditions, ideally in a dynamic and well-controlled design [13,14]. DDA, DIA, and targeted approaches each provide different advantages in this respect. Discovery-oriented DDA or DIA workflows may identify candidate OA-responsive phosphosites, whereas targeted methods can then be used to validate those changes more precisely in selected biological systems [13,14,17,20,21].

However, phosphoproteomics alone is not sufficient for robust target deconvolution. A single omics layer captures only one dimension of a complex biological response [12]. In the OA context, phosphoproteomics should therefore be integrated with total proteomics to distinguish phosphorylation-dependent rewiring from changes in protein abundance, with transcriptomics to separate early signaling events from delayed transcriptional adaptation, and with metabolomics to connect signaling changes with cellular metabolic state [12,13,15,16,18–21]. In parallel, candidate mechanisms should be strengthened by direct target-validation approaches such as chemoproteomics, ABPP, DARTS, CETSA, and TPP [12,19–22,29–34].

In practice, this suggests a staged research model for OA. First, time-resolved phosphoproteomics should be applied, including very early time points that maximize the chance of capturing primary signaling events. Second, these data should be integrated with total proteomics, transcriptomics, and metabolomics. Third, the resulting datasets should undergo bioinformatic processing involving clustering, kinase/substrate enrichment analysis, phosphatase-aware interpretation where possible, and network-level reconstruction of signaling architecture [13,14,18,19,26,29]. Only after this should orthogonal validation be undertaken using CETSA, TPP, ABPP, DARTS, or genetic perturbation methods [12,30–35].

This integrated framework is particularly well suited to OA. On the one hand, it allows the pleiotropic biology of OA to be used as a source of information about network-level responses. On the other hand, it reduces the risk of overinterpreting individual markers and permits stepwise narrowing of candidate targets to those that are biologically plausible and consistently supported across data layers [12–14,18–20,26,29]. In this sense, phosphoproteomics and multi-omics should be viewed not simply as additional analytical technologies, but as a framework capable of shifting OA research from descriptive pathway pharmacology toward a more causal understanding of mechanism of action.

Table 1. Contribution of individual omics layers to oleanolic acid target deconvolution.

Omics / analytical layer	Main question addressed	Added value beyond marker-based studies	Main limitation
Phosphoproteomics	Which signaling events occur earliest after OA exposure?	Provides a direct functional readout of signaling-network remodeling and supports kinase/phosphatase-centered interpretation [13,14]	Does not by itself demonstrate direct target binding
Total proteomics	Are phosphorylation changes independent of protein abundance changes?	Distinguishes signaling rewiring from protein abundance shifts	Lower temporal sensitivity for rapid signaling events

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Transcriptomics	Which downstream transcriptional programs are induced by OA?	Helps separate early signaling events from later adaptive responses	Indirect with respect to protein function
Metabolomics	What are the metabolic consequences of OA exposure?	Links signaling changes to cellular metabolic phenotype	Metabolic changes may be downstream and nonspecific
Chemoproteomics / ABPP	Which proteins physically interact with OA or OA-derived probes?	Supports direct target identification and target prioritization [12]	Probe design may alter compound behavior
CETSA / TPP / DARTS	Does OA engage and stabilize candidate targets in a biological context?	Strengthens target-engagement inference [12]	Requires prior candidate selection and careful interpretation
Network analysis / KSEA	Which kinase modules are most consistently reprogrammed?	Translates phosphosite-level data into pathway-level mechanistic hypotheses [13]	Depends on incomplete kinase-substrate annotation
Integrated multi-omics	Which changes are most likely causal rather than secondary?	Enables cross-layer prioritization of candidate targets and mechanisms [12,15,16]	Still remains correlation-based without orthogonal validation

Note: No single omics layer is sufficient for robust OA target deconvolution. Phosphoproteomics is the central discovery layer because it captures early signaling dynamics, but mechanistic interpretation becomes substantially stronger when combined with total proteomics, transcriptomics, metabolomics, and orthogonal target-engagement methods [12–16].

4. Target Deconvolution in the Study of Natural Bioactive Compounds

Identifying the molecular targets of natural products remains difficult because no single readout captures the full mechanism of action. In practice, meaningful target deconvolution requires integration of complementary approaches, including transcriptomics, proteomics, metabolomics, chemoproteomics, and bioinformatic reconstruction of response networks [13,16,26–29,36–39].

In functional terms, target deconvolution may be understood as the process of moving from an observed phenotypic effect to the identification of the true molecular target, or set of targets, that initiates the biological response. In the case of natural bioactive compounds, this task is particularly difficult because such compounds often interact with multiple proteins at once, modulate several signaling axes in parallel, and may additionally induce secondary adaptive changes that blur the boundary between primary effect and downstream response [12,18,19,21,26]. For this reason, target identification cannot rely solely on a single omics readout or a few preselected markers.

This issue is fundamental in the case of OA. OA is a compound with well-described biological pleiotropy and a broad spectrum of activity, including anticancer, anti-inflammatory, antioxidant, metabolic, and hepatoprotective effects. At the same time, recent reviews show that OA and its derivatives have been studied in a wide variety of biological systems, and that mechanisms attributed to OA include NF-κB, STAT, Akt/mTOR, MAPK, Nrf2/HO-1, ferroptosis, PTP1B, and modulation of autophagy [3–7,22]. This diversity of effects confirms the potential of OA, but at the same time illustrates how easily major molecular points of engagement may be confused with secondary remodeling of signaling networks.

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In practice, target deconvolution for OA should include at least several classes of tools. The first class includes genetic and transcriptomic approaches, which allow the response to the compound to be linked to specific genes or transcriptional modules. Zhang et al. note that RNAi, shRNA, and CRISPR may be used to identify genes that condition cellular sensitivity to a given compound, but they also warn that changes in mRNA levels do not always translate into protein activity and that the results may be confounded by off-target effects [12,16,18,19].

The second class is chemoproteomics, which remains one of the most important approaches for direct identification of protein interactors. Strategies based on immobilized probes and click chemistry have been described, as well as photoaffinity labeling and ABPP. Importantly, chemical modification of a natural compound may alter its activity, and the enrichment process itself may generate nonspecific binders [12,18–21,30]. Thus, chemoproteomics provides very strong mechanistic indications, but it requires further orthogonal validation.

The third class of methods includes label-free target-engagement techniques such as DARTS, SPROX, CETSA, and TPP. Their advantage is that they do not necessarily require chemical modification of the investigated compound. These approaches may help distinguish a situation in which OA changes the phosphorylation of a given protein from one in which OA truly binds or stabilizes a specific target [12,30–35].

Finally, target deconvolution in the case of OA should not end with the identification of a single protein. The strength of multi-omics lies not only in identifying targets, but also in distinguishing likely causal interactions from side effects, nonspecific signals, and secondary changes [12,26,28,29,36–40]. This is precisely why the integration of different data layers is more valuable than any individual technique alone.

In the specific case of OA, this logic appears especially justified for three reasons. First, OA is a relatively hydrophobic and pharmacokinetically imperfect compound, meaning that observed biological effects depend not only on target affinity, but also on cellular availability and formulation. OA has very low aqueous solubility, low permeability, and limited oral bioavailability, which may substantially affect the activity profiles observed across models [3,6,25,41,42].

Second, an extensive body of research already exists on OA derivatives, which means that target deconvolution should not be limited to the parent compound. Modifications at C-3, C-12/C-13, C-28, and within rings A and E lead to numerous derivatives with altered biological activity, and the therapeutic potential of these analogues often correlates with improved physicochemical or pharmacokinetic properties [3–7].

Third, available data already suggest that OA may serve as a useful chemical scaffold for targeting specific phosphatases and metabolic enzymes. In particular, OA derivatives with increased activity against PTP1B and other metabolic targets reinforce the view that OA is not merely a pleiotropic natural modulator, but a potential platform for more precise ligand design [3,6,43].

All this leads to an important methodological conclusion: in the case of OA, target deconvolution should not focus on the question of what the single main target is, but rather on the hierarchy of targets and signaling events in a specific biological context. This shift in perspective is crucial for a review based on phosphoproteomics and multi-omics.

5. Why Is Phosphoproteomics Central to OA Mechanistic Deconvolution?

Phosphoproteomics occupies a special place among omics tools because it provides information on signaling-pathway activity at a functional rather than merely descriptive level. Higgins et al. emphasize that phosphorylation is the most frequently studied regulatory post-translational modification and that phosphoproteome analysis provides information on kinase-pathway activity and signaling-network architecture that cannot be directly inferred from genomics or transcriptomics [13,14,17–20,26,27,36,40]. In cancer and other diseases driven by signaling dysregulation, the phosphoproteome therefore represents a direct readout of kinase and phosphatase activity.

From the perspective of OA, this is of fundamental importance. Most biological effects attributed to OA have been described through changes in phosphorylation or activity of proteins belonging to

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the PI3K/AKT/mTOR, AMPK/mTOR, MAPK, NF-κB, or JAK/STAT axes. The problem, however, is that classical studies usually examine only a few phosphoproteins. Phosphoproteomics instead makes it possible to track thousands of phosphorylation sites simultaneously, offering the chance to capture the earliest and most consistent changes induced by OA [13,14,26,27,29,36,40]. Higgins et al. note that the phosphoproteome reveals pathway activation regardless of whether the dysregulation has a genetic, epigenetic, or microenvironmental basis [13]. This is precisely what makes the technique particularly useful in the analysis of a compound such as OA, whose activity is highly context-dependent.

From a technical perspective, both Higgins et al. and Gerritsen and White show that the success of a phosphoproteomic experiment depends on the quality of sample preparation, proper protein digestion, and efficient enrichment of phosphopeptides. IMAC or MOAC/TiO2 are most commonly used for this purpose [13,14,17,27]. Higgins et al. indicate that MOAC is now widely used and may be more stable and selective than classical IMAC, although both methods remain applicable. Gerritsen and White further point out that a single enrichment step is usually sufficient, but that fractionation or selective enrichment of specific phosphoproteome subsets may be used when deeper coverage is required [13,14].

Particularly important for our topic is the fact that standard global phosphoproteomics may fail to capture the most informative events. Gerritsen and White remind us that phosphotyrosine accounts for only 0.1–1% of the total phosphoproteome, so the events most critical from the perspective of receptor tyrosine kinase activation or certain oncogenic nodes may remain invisible without additional enrichment [14,27,28,39]. For this reason, OA studies should consider not only global phosphoproteomics, but also phosphotyrosine enrichment or enrichment of specific kinase substrate motifs.

This leads to an important design conclusion. If OA truly initiates a cellular response through specific kinase or phosphatase nodes, then the most valuable information may lie not in late transcriptional changes, but in very early shifts in phosphorylation of regulatory proteins. Therefore, in the case of OA, a time-course experiment including 5–15 min, 30 min, 2 h, and 8–24 h would be especially informative [13,14,17,26]. Early time points would make it possible to distinguish initiating events from secondary adaptive responses.

Another critical element is the strategy of data acquisition and quantification. Higgins et al. discuss in detail the strengths and limitations of DDA and DIA. DDA enables deep profiling, but is prone to undersampling and missing values, especially in highly complex samples. DIA reduces this problem and improves reproducibility, but generates more complex spectra and requires more advanced computational analysis [13,14,17,20]. In the OA context, this means that the discovery stage may be based on DDA or DIA, but the results should subsequently be validated using more targeted methods.

Biological interpretation is equally important. The identification of a phosphorylation site does not by itself establish functional relevance. This is why kinase-activity inference methods such as KSEA and related tools are useful in OA research: they can move the analysis beyond isolated marker changes toward the coordinated behavior of kinase-responsive substrate modules [13,26,29,36–38,40,44].

Even the best-designed phosphoproteomic experiment, however, does not replace direct target-engagement validation. Phosphoproteomics may indicate dominant signaling axes and prioritize candidates, but techniques such as CETSA, TPP, DARTS, or ABPP are needed to strengthen causal inference and determine whether OA or one of its derivatives truly interacts with a given protein [30–35]. In practice, this means that phosphoproteomics should serve as the core of the discovery model, while target-engagement methods should form the validation layer.

At this point, one additional argument in favor of phosphoproteomics in OA research should be highlighted. OA derivatives have already been designed to affect very different mechanisms, from NF-κB and STAT through Akt/mTOR and ferroptosis to PTP1B and α-glucosidase [3–7]. This means that there is real scope for comparative phosphoproteomic studies: the parent OA compound may be

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compared with structurally directed derivatives to determine which elements of the phosphosignature are shared and which are linked to specific chemical modifications. Such an approach could connect SAR with target deconvolution and represent one of the most modern axes of OA research.

In summary, phosphoproteomics is central to OA deconvolution for at least four reasons. First, it provides a direct functional readout of kinase- and phosphatase-driven network activity. Second, it enables the capture of early signaling events, that is, those most useful for target prioritization. Third, it can be combined with the analysis of phosphoproteome subsets such as pTyr and substrate motifs, thereby increasing biological resolution. Fourth, it provides an ideal point of integration with chemoproteomics, total proteomics, transcriptomics, and metabolomics, thus creating a coherent multi-omics model [3,12–14,26,29,44].

6. What Is Already Known About OA Signaling: Organizing the Current Evidence

6.1. The PI3K/AKT/mTOR Axis as One of the Main Response Nodes to OA

One of the signaling axes most frequently linked to OA and its derivatives is PI3K/AKT/mTOR. Because this pathway regulates growth, survival, and metabolism, it has naturally become one of the central frameworks used to explain the anticancer effects of OA [3,5,6,9,23,24]. In the available literature, OA is repeatedly associated with reduced proliferative activity of cancer cells, growth inhibition, and increased cell death; however, the exact point of entry into this axis remains unclear [3,9,23,24].

Particularly interesting in this regard are data concerning OA derivatives. Some newer OA analogues exhibit anticancer activity associated with inhibition of the Akt/mTOR axis and induction of ferroptosis, suggesting that the OA scaffold may be chemically tuned toward more precise modulation of this signaling network [3–6]. At the same time, it should be emphasized that in many cases mechanistic conclusions are drawn from a limited number of markers, such as p-Akt, p-mTOR, or downstream cell-death effectors, rather than from a system-level map of phosphorylation changes [3,9]. For this reason, the current state of knowledge suggests that PI3K/AKT/mTOR is an important response node to OA, rather than a definitively established direct molecular target [3,9,23,24].

6.2. The AMPK/mTOR Axis and Autophagy: The Best-Documented Mechanistic Direction for OA

Among all signaling axes associated with OA, the AMPK/mTOR system appears to be the most thoroughly documented, especially in the context of autophagy and apoptosis. Hu et al. demonstrated in HCT-116 and SW-480 colon cancer cells that OA reduced cell viability and proliferation while increasing apoptosis. At the molecular level, the authors observed decreased mTOR and Bcl-2 together with increased AMPK and its downstream proteins, including TSC2, BAX, Beclin 1, LC3B-II, and ULK1, which was interpreted as activation of AMPK-dependent autophagy and apoptosis [11]. Importantly, the authors emphasized that OA induced autophagy and apoptosis mainly through AMPK activation. Related metabolic work also supports the broader relevance of PI3K/Akt- and AMPK-linked OA signaling in nonmalignant systems [23].

This area currently represents the strongest anchor for a mechanistic OA narrative. Unlike the more scattered reports concerning other axes, here we have a relatively coherent model: OA promotes AMPK activation, which secondarily attenuates mTOR and shifts the cell from an anabolic and proliferative program toward autophagy and cell death [11]. Nevertheless, caution in interpretation is still required. Even in this best-described system, it remains unclear whether OA acts directly on elements of this axis or instead triggers earlier stress- or metabolism-related events that only secondarily activate AMPK. In other words, the available data document the architecture of the response well, but do not yet resolve the full issue of target deconvolution [11,13,14].

6.3. NF-κB, Nrf2, and Inflammatory/Oxidative Signaling

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A second recurrent area in the OA literature is the regulation of inflammatory and oxidative responses. Both OA and its derivatives have repeatedly been associated with inhibition of NF-κB, reduced expression of pro-inflammatory cytokines, and lower levels of iNOS and COX-2 [3,10,22,43,45]. In some models, this effect coincides with activation of Nrf2/HO-1, suggesting that OA may modulate the balance between pro-inflammatory programming and cytoprotective antioxidant response [3,10,22].

Particularly noteworthy are OA derivatives that, in inflammatory models, simultaneously reduced p-NF-κB, p-p38, p-JNK, p-ERK, and p-Akt expression while increasing Nrf2 and HO-1 [3,43,45]. These findings are especially interesting from the perspective of our review because they show that the OA scaffold may modulate not a single pathway, but an entire stress-inflammatory module comprising NF-κB, MAPK, PI3K/Akt, and Nrf2/HO-1. At the same time, it remains an open question whether these observed changes result from a single dominant upstream mechanism or from broader network remodeling in response to cellular stress [3,10,22,43,45].

For the parent OA compound, the data are similarly convincing at the phenotypic level but less unambiguous in terms of causality. Thus, the available literature suggests that OA consistently affects inflammatory and oxidative pathways, yet we still do not know which of these changes are closest to its primary molecular action. This is precisely the type of problem for which phosphoproteomic studies and multi-layer data integration are needed [3,10,13,14,43,45].

6.4. MAPK/JNK as a Stress-Response and Inflammatory Module

MAPK pathways, especially p38 and JNK, also regularly appear in the OA literature. Data are available indicating that OA may reduce inflammatory response and tissue injury by inhibiting p38 and JNK MAPKs [4,10,30,31]. In addition, in anti-inflammatory models some OA derivatives simultaneously reduced p-p38, p-JNK, and p-ERK levels, indicating that the action of OA on MAPK is not incidental, but rather belongs to recurrent mechanistic motifs in this area [3,43,45].

The interpretation of these findings is not straightforward, however. MAPKs may function both as mediators of cell death and as mediators of adaptive response, and the direction of change may depend on cell type, dose, and exposure time. For this reason, p38/JNK data should be treated as an important part of the OA signaling network, but not as decisive proof of a single linear mechanism of action. From the perspective of the present review, MAPK/JNK should therefore be regarded as an important stress-response module that should be analyzed in parallel with AMPK/mTOR, NF-κB, and PI3K/Akt, rather than in isolation [3,9,43,45].

6.5. PTP1B and Other Phosphatases: The Most Promising but Still Underdeveloped Direction

A particularly interesting and still insufficiently explored area of OA research concerns phosphatases, especially PTP1B. OA derivatives have already been designed as PTP1B inhibitors, and some of them displayed activity clearly stronger than the parent compound [3,6,43]. In the cited studies, selected OA derivatives achieved significant inhibitory activity against PTP1B, and some also demonstrated in vivo effects, including blood-glucose reduction [3].

This is a very important observation for the concept of the present article. Most OA papers focus on broad descriptions of regulation of multiple pathways, whereas PTP1B offers a much more precise entry point into the discussion of target deconvolution and the relationship between a natural compound and a specific class of signaling enzymes. At the same time, the available data suggest that it is specifically OA derivatives, rather than necessarily OA itself, that may provide a better starting point for phosphatase-oriented ligand design [3,6,43]. This raises an important research question: does the parent compound display a genuinely weaker but still mechanistically meaningful interaction with PTP1B, or do appropriate chemical modifications only then reveal the full phosphatase-related potential of the OA scaffold?

Equally importantly, the systems-level validation of phosphatase-related OA signaling remains limited. Compared with kinase-centered studies, far fewer data are available that connect phosphatase inhibition by OA derivatives with broad phosphoproteomic or target-engagement

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validation strategies [3,26,28]. In this sense, PTP1B constitutes one of the strongest arguments for shifting OA research away from general pathway pharmacology toward a more precise biology of kinases and phosphatases.

6.6. What the Current State of Knowledge Suggests: Dominance of Marker-Based Studies and a Clear Phosphoproteomic Gap

After organizing the available data, it becomes clear that the OA literature is rich in biological observations but much poorer in system-level mapping of signaling-event hierarchies. Reports on the activity of OA and its derivatives are still dominated by assessments of proliferation, apoptosis, inflammatory markers, levels of selected phosphoproteins, effector proteins, or transcriptional changes [3,11,13,14,26–29,36–40]. The study by Hu et al. provides a good example of a highly valuable but still low-dimensional mechanistic approach: the authors demonstrate an important role of AMPK/mTOR, but the entire model is based on a limited panel of markers [11].

At the same time, methodological reviews show that phosphoproteomics was developed precisely to solve such problems: to track thousands of phosphorylation sites simultaneously, identify activated kinase modules, analyze signaling dynamics, and draw conclusions from substrate sets rather than from individual proteins [13,14,26–29,36–40]. This means that the juxtaposition of these two domains—rich but dispersed OA biology and modern phosphoproteomic methodology—reveals a clear research gap.

Taken together, the current literature is rich in phenotypic and pathway-level observations but poor in system-level resolution. The key gap is not a lack of biological activity data, but a lack of studies designed to rank OA-responsive events in temporal and mechanistic order. This is the gap that phosphoproteomics, especially when integrated with complementary omics and target-engagement methods, is well positioned to address.

6.7. A Synthetic Conclusion for the Next Part of the Review

The available literature allows one to conclude that OA and its derivatives consistently interfere with several central signaling modules: PI3K/AKT/mTOR, AMPK/mTOR, NF-κB/Nrf2, MAPK/JNK, and—in a more targeted sense—PTP1B [3,9–11,13,14]. However, the available evidence is of uneven quality: the strongest documentation for the parent compound concerns the AMPK/mTOR axis, whereas some of the other directions derive from studies of OA derivatives or from analyses based on limited marker panels [3,11]. The major signaling axes currently associated with OA, together with the predominant evidence types and unresolved mechanistic questions, are summarized in Table 2.

Table 2. Major signaling axes associated with oleanolic acid and the current level of mechanistic evidence.

Signaling axis / node	Representative reported effect	OA or derivative	Typical models	Predominant evidence	Main unresolved issue
AMPK/mTOR	AMPK activation, mTOR suppression, induction of autophagy and apoptosis	Mainly OA	Colon cancer and other cancer models	Marker-based mechanistic studies, autophagy/apoptosis readouts [11]	The direct upstream trigger remains unclear
PI3K/AKT/mTOR	Attenuation of proliferative signaling, reduced survival signaling, growth inhibition	OA and derivatives	Multiple cancer models	Pathway-focused studies using selected phospho-markers [3,9]	The precise point of pathway entry is unresolved
NF-κB/Nrf2	Anti-inflammatory signaling shift, redox reprogramming, reduced	OA and derivatives	Inflammatory and organ-injury models	Cytokine profiling and selected pathway markers [3,10]	It remains unclear whether these changes are

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	inflammatory mediator expression				primary or adaptive
MAPK/JNK	Modulation of stress and inflammatory signaling	OA and derivatives	Inflammation-related and cancer-related models	p-p38, p-JNK, and p-ERK readouts [3,9]	Strong context dependence limits causal interpretation
PTP1B	Enzyme inhibition, metabolic improvement, glucose-lowering potential	Mainly derivatives	Enzyme assays, metabolic models, selected in vivo studies	In vitro inhibition assays with limited in vivo follow-up [3]	Systems-level signaling validation is still limited
Ferroptosis-related signaling	Induction or modulation of ferroptosis-associated pathways	Mainly derivatives	Selected cancer models	Mechanistic marker-based studies [3]	The relationship to direct target engagement remains insufficiently defined

Note: Current evidence indicates that OA and its derivatives modulate several central signaling modules, but the depth of mechanistic validation is uneven. For the parent compound, the most consistent evidence concerns the AMPK/mTOR axis, whereas several other directions are still dominated by pathway-centered or marker-based studies rather than systems-level target deconvolution [3,9–11].

Therefore, the most rational next step is not to add more isolated pathway stories, but to build a research model that organizes these data in time and in functional space. This is the subject of the next section—the proposed phosphoproteomic and multi-omic workflow for OA target deconvolution.

7. A Proposed Phosphoproteomic and Multi-Omic Workflow for the Target Deconvolution of Oleanolic Acid

Given the limitations of marker-based studies, the next logical step for OA research is a layered workflow that combines discovery phosphoproteomics with multi-omics integration and orthogonal target validation [3,12–14,30–35,44]. The aim is not merely to catalogue pathway changes, but to distinguish early OA-responsive events from secondary adaptive remodeling. To address this, we propose the phosphoproteomic and multi-omic workflow shown in Figure 2.

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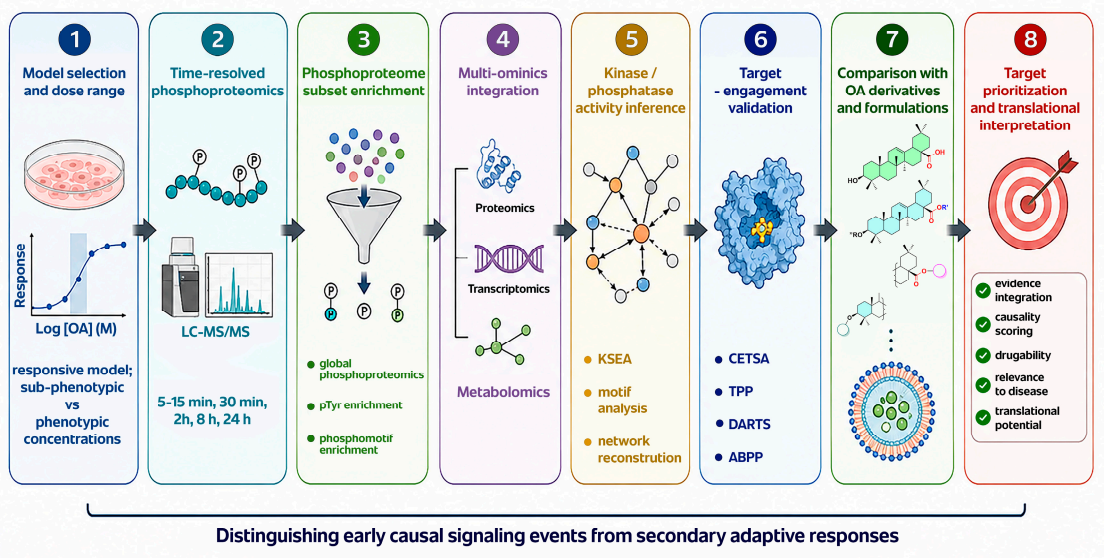

Figure 2. Proposed phosphoproteomic and multi-omic workflow for oleanolic acid target deconvolution. Note: A proposed research framework for systematic deconvolution of OA molecular targets. Rather than relying on endpoint markers, the workflow combines time-resolved phosphoproteomics with multi-omics integration and orthogonal target-engagement validation to distinguish early causal signaling events from secondary network remodeling [3,12–14]. Comparative analysis of the parent compound, OA derivatives, and bioavailability-enhancing formulations may further connect target deconvolution with structure–activity relationships and translational development.

For OA, this approach is especially appropriate. On the one hand, OA produces broad biological effects across cancer, inflammatory, and metabolic systems. On the other hand, its low aqueous solubility, low permeability, and limited oral bioavailability mean that the observed biological output may depend strongly on formulation, dose, and biological model [3,6,25,41,42]. Any experimentally useful workflow must therefore be sensitive not only to signaling biology, but also to compound presentation and context.

7.1. Stage I: Rational Experimental Design and Selection of the Biological Model

The first step should be the deliberate selection of an appropriate biological model. In the case of OA, it is not sufficient simply to choose a cell type that is responsive to the compound, because the goal is not only to confirm a phenotypic effect, but also to define the hierarchy of molecular events. Therefore, the model should fulfill at least three conditions: it should display a measurable OA response; it should have a reasonably well-characterized signaling background; and it should be suitable for downstream chemoproteomic, thermal-shift, or genetic validation.

In practice, good starting systems include models in which the OA response has already been at least partially described, such as HCT-116 and SW-480 colon cancer cells [11]. More broadly, comparison across models with related but nonidentical signaling contexts may be useful for distinguishing conserved OA-responsive signatures from context-restricted effects [3,11,42].

At the design stage, it is also important to distinguish sub-phenotypic from phenotypic concentrations. Sub-phenotypic concentrations do not yet produce major disruption of cellular homeostasis, but may reveal early signaling events. Phenotypic concentrations, by contrast, produce clear changes in viability, autophagy, apoptosis, or inflammation-associated outputs. This distinction is crucial because, from the standpoint of target deconvolution, late cytotoxic responses are less informative than early and selective shifts in signaling.

7.2. Stage II: Time-Resolved Phosphoproteomics as the Core Discovery Experiment

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The core of the workflow should be a time-resolved phosphoproteomic study. Higgins et al. emphasize that the phosphoproteome provides unique information on the activity of signaling pathways and kinase networks that cannot be inferred directly from genomics or transcriptomics, and that phosphorylation itself is highly dynamic, often changing on the scale of seconds to minutes [13]. Gerritsen and White likewise note that many of the most informative regulatory phosphorylation events are low-abundance and transient, making temporal design essential [14].

Accordingly, the proposed OA workflow should include several time points, for example 5–15 min, 30 min, 2 h, 8 h, and 24 h [13,14,17,26,27]. Early time points maximize the chance of capturing events that lie closest to primary compound action, whereas later time points help define secondary adaptation and phenotypic consolidation. Such a design allows initiating events to be separated from downstream network remodeling.

From a technical point of view, the workflow should include rapid quenching of kinase and phosphatase activity, efficient protein digestion, and robust phosphopeptide enrichment [13,14,17,27]. At the discovery stage, DDA and DIA remain the two main acquisition modes. DDA provides deep coverage but is more susceptible to undersampling and missing values, whereas DIA improves reproducibility and quantitative completeness at the cost of greater analytical complexity [13,14,17,20]. In the OA setting, either strategy can be used effectively, provided that the discovery stage is followed by targeted validation.

7.3. Stage III: Enrichment of Informative Phosphoproteome Subsets

In OA studies, global phosphoproteomics alone may not be sufficient. Because phosphotyrosine represents only a minor fraction of the phosphoproteome, many highly regulatory events may remain undetected without dedicated enrichment [14,27,28,39]. For this reason, the most informative OA workflows should not be restricted to a single global readout.

Instead, at least three analytical layers may be considered: global phosphoproteomics to capture the broad signaling response, phosphotyrosine enrichment to reveal low-abundance regulatory events, and phosphomotif-oriented enrichment if a dominant upstream kinase module is suspected [13,14,27,28,39]. In the context of OA, this may be particularly useful when comparing signaling outputs linked to Akt/mTOR, MAPK, or receptor-proximal events.

7.4. Stage IV: Integration with Total Proteomics, Transcriptomics, and Metabolomics

Once phosphoproteomic data have been generated, integration with other omics layers becomes essential. Zhang et al. clearly emphasize that a single analytical layer offers only a partial view of the biological response and that multi-omics is required for more precise target and pathway interpretation [12]. Higgins et al. similarly stress the importance of combining phosphoproteomics with proteomics and transcriptomics [13].

In OA research, such integration should serve at least four purposes: distinguishing phosphorylation changes from changes in protein abundance, separating early signaling from later transcriptional adaptation, linking signaling rewiring to the cellular metabolic phenotype, and building a more credible causal map of OA-responsive biology [12,13,15,16,18–20,41]. In addition, integration across layers may help prioritize signaling nodes that remain consistent despite variation in model system or formulation.

7.5. Stage V: Inference of Kinase and Phosphatase Activities

One of the most important stages of analysis is the transition from a list of phosphosites to a model of signaling-network activity. Higgins et al. describe both substrate-centric and kinase-centric interpretive approaches [13]. For the purposes of OA target deconvolution, kinase-centric methods are particularly valuable because they enable inference of kinase activity from coordinated behavior across substrate sets.

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In the OA context, this makes it possible to distinguish a situation in which a change in p-AKT or p-mTOR is merely a terminal marker of stress from one in which an entire substrate module indicates genuine remodeling of kinase activity [13,26,29,36–38,40,44]. Similar logic may be extended, with more caution, to phosphatases, although the weaker annotation of phosphatase–substrate relationships remains a major limitation [3,13]. Accordingly, kinase and phosphatase inference should be treated as a powerful hypothesis-prioritization tool rather than as stand-alone mechanistic proof.

7.6. Stage VI: Orthogonal Validation of Target Engagement

Following the identification of the most promising signaling nodes, orthogonal validation becomes indispensable. Zhang et al. discuss in detail the importance of methods such as DARTS, CETSA, TPP, photoaffinity labeling, and ABPP in the target-discovery process [12]. More recent work on CETSA and TPP has further strengthened their utility in determining whether candidate targets are engaged under biologically relevant conditions [30–34].

For OA, a practical validation sequence may include: first, confirmation of key phosphosignatures using targeted phosphoproteomic or immunoblot-based methods; second, direct testing of target engagement by CETSA or TPP; third, functional perturbation using genetics or pharmacology; and finally, comparison of the parent compound with OA derivatives displaying more selective or more potent activity [12,30–35].

7.7. Stage VII: Comparative Profiling of OA, Its Derivatives, and Its Formulations

One of the most promising extensions of the proposed workflow is the explicit comparison of OA with its derivatives and bioavailability-enhancing formulations. Structural modifications at C-3, C-12/C-13, and C-28, as well as in rings A and E, have generated numerous analogues with enhanced anticancer, anti-inflammatory, or metabolic activity [3–7,42]. From the perspective of this review, such comparisons are important because they connect structure–activity relationships with target deconvolution.

Comparative phosphoproteomic analysis of the parent compound, selected derivatives, and formulation-enhanced OA could reveal which elements of the OA response are scaffold-intrinsic, which are modification-dependent, and which correlate with improved biological or translational performance [3–7,25,41,42]. This may prove especially important when evaluating shifts toward stronger modulation of Akt/mTOR, NF-κB, ferroptosis-associated pathways, or phosphatase-related biology.

7.8. Proposed Final Model

Taken together, the following experimental framework can be proposed for OA target deconvolution: selection of responsive models and relevant concentration ranges; time-resolved phosphoproteomics; enrichment of informative phosphoproteome subsets; integration with proteomics, transcriptomics, and metabolomics; inference of kinase and phosphatase activity; orthogonal target-engagement validation; and comparative profiling of OA, its derivatives, and its formulations [3,12–14,30–35,44].

In this model, phosphoproteomics functions as the central sensor of signaling-network remodeling, multi-omics provides the integrative layer, and chemoproteomic and biophysical validation methods narrow the list of candidates to true molecular targets. Such a workflow enables OA research to move beyond descriptive pathway pharmacology and toward systems-level causal biology.

8. Methodological and Interpretive Challenges in OA Research Using Phosphoproteomics and Multi-Omics

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Phosphoproteomics and multi-omics can substantially improve mechanistic resolution in OA research, but only if their limitations are handled explicitly. In this context, the main risks concern polypharmacology, context dependence, imperfect target annotation, and the tendency to overinterpret correlative datasets [3,12–14].

8.1. OA Polypharmacology and the Problem of Distinguishing the Primary Target from Secondary Response

The most important challenge remains the distinction between the primary target and secondary changes that arise in the signaling network after OA exposure. Natural bioactive compounds often affect several targets in parallel or indirectly modulate multiple pathways at once, which makes it difficult to assign a single dominant causal interaction [12]. In the case of OA, this issue is especially pronounced because the literature attributes effects on AMPK/mTOR, PI3K/AKT/mTOR, NF-κB, MAPK, Nrf2, PTP1B, and related modules, sometimes within the same experimental setting [3,9–11].

In practical terms, this means that an observed change in phosphorylation of a given protein or the inferred reprogramming of a kinase module does not by itself establish that the protein or kinase is a direct target of OA. The same signal may reflect secondary metabolic stress, redox imbalance, cytoskeletal remodeling, autophagy-associated adaptation, or compensatory signaling. For this reason, OA studies must distinguish clearly between direct target engagement, early signaling response, downstream network remodeling, and the final phenotypic outcome.

8.2. Dependence of OA Response on Dose, Time, and Biological Model

A second major limitation is the strong dependence of OA activity on experimental conditions. Phosphorylation is highly dynamic, and signaling responses may change within minutes [13]. At the same time, OA may generate distinct biological outputs depending on cell type, concentration, exposure time, and metabolic state [3,9,11,25,41].

A practical consequence of this is the risk of conflating several biological layers within a single experiment. Very early time points may reveal only a small number of regulatory events, whereas later time points may already reflect broad stress response, metabolic disturbance, altered transcription, and activation of cell-death programs. If these phases are not explicitly separated, phosphoproteomic interpretation may overestimate the number of direct OA-responsive targets.

Concentration is equally critical. Because OA has poor aqueous solubility and limited bioavailability, experiments performed at high concentrations may capture generalized perturbation rather than physiologically meaningful target engagement [3,6,25,41,42]. This is one reason why formulation-aware interpretation is important in OA research.

8.3. Technical Limitations of Phosphoproteomics

Phosphoproteomics provides a uniquely informative view of signaling-network activity, but it is also technically demanding. The phosphoproteome is difficult to analyze because phosphorylation often shows low stoichiometry, wide dynamic range, and uneven distribution across serine, threonine, and tyrosine residues [13,14,17,20,27,31,46–51]. Many critical regulatory events are of very low abundance and may be missed without dedicated enrichment strategies.

In OA studies, this creates several practical problems. Standard global analyses may fail to capture key upstream events, especially if they are phosphotyrosine-driven. Different sample-preparation, enrichment, and data-acquisition strategies may produce markedly different phosphoproteome coverage. In addition, some observed differences may reflect technical or analytical variability rather than biology. These challenges become even more pronounced when moving toward small-input, nanoscale, or single-cell workflows [31,46–51].

8.4. Limitations in the Interpretation of Kinase and Phosphatase Activity

One of the greatest strengths of phosphoproteomics is the possibility of inferring kinase activity, but this is also one of its main interpretive risks. Activity-inference tools such as KSEA and related

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methods are based on current knowledge of kinase–substrate relationships, and that knowledge remains incomplete and strongly biased toward well-characterized kinases [13,26,29,36–38,40,44]. Accordingly, kinase-activity inference is highly useful for prioritizing mechanistic hypotheses, but it cannot be considered independent proof of mechanism.

The problem is even greater for phosphatases. Substrate annotations for phosphatases remain substantially less developed than for kinases, which makes phosphatase-centered interpretation considerably more difficult [3,13]. This is particularly relevant for OA research in relation to PTP1B and other phosphatase-oriented hypotheses.

8.5. The Problem of Overinterpreting Omics Correlations

Another major pitfall is the tendency to treat omics-level correlation as equivalent to causal mechanism. This is a common problem in the literature on bioactive small molecules and is especially relevant for OA because of its pleiotropic signaling profile [3,9,12]. The observation that OA changes the phosphorylation of a kinase substrate set or shifts the activity of a signaling module does not yet mean that the kinase itself is directly targeted.

This issue becomes even more complex in multi-omics studies. Integration of phosphoproteomics, proteomics, transcriptomics, and metabolomics increases interpretive power, but may also generate apparently coherent models that remain fundamentally correlation-based. Therefore, multi-omics should be used primarily as a framework for prioritizing and organizing mechanistic hypotheses, not as stand-alone proof of causality [12,13,31–35,44,52].

8.6. Chemical and Biological Heterogeneity of OA and Its Derivatives

A further complication is that the term “oleanolic acid” increasingly refers not only to the parent compound, but also to a family of semisynthetic derivatives and optimized formulations. Advances in OA chemistry have produced numerous analogues with distinct biological properties, pharmacokinetic behavior, and therapeutic potential [3–7,41,42]. While this is a major research opportunity, it also complicates interpretation of the literature, because mechanistic observations made for one derivative cannot automatically be transferred to the parent compound.

8.7. Standardization and Reproducibility in Future OA Studies

If phosphoproteomics and multi-omics are to move OA research to a higher mechanistic level, greater experimental standardization will be essential. Phosphoproteomic data are highly sensitive to sample preparation, timing of lysis, phosphosignal stability, enrichment strategy, acquisition method, and data-processing pipeline [13,17,31,34,47,48,51,52]. In future OA studies, it will therefore be important to report exposure time, concentration and solvent system, enrichment strategy, acquisition mode, number of biological replicates, normalization procedures, activity-inference workflow, and validation strategy for candidate targets.

The major methodological and interpretive challenges in OA phosphoproteomic studies, together with practical strategies to address them, are summarized in Table 3.

Table 3. Major methodological challenges in OA phosphoproteomic studies and practical strategies to address them.

Challenge	Why it matters in OA research	Consequence for interpretation	Practical strategy
Polypharmacology	OA may influence several signaling modules simultaneously	Difficult to separate direct targets from secondary responses	Combine time-resolved phosphoproteomics with orthogonal target-engagement validation

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Dose dependence	High concentrations may induce generalized cellular perturbation	Overestimation of direct mechanistic relevance	Distinguish sub-phenotypic from phenotypic dose ranges
Time dependence	Early and late responses may reflect different biological layers	Mixing causal and adaptive events	Include multiple early and late time points
Low-abundance phosphosites	Critical regulatory events, especially pTyr, may be missed	Incomplete or distorted signaling interpretation	Add pTyr enrichment or phosphomotif-focused enrichment [14]
Incomplete kinase/ phosphatase annotation	Activity inference depends on prior knowledge databases	Bias toward well-characterized signaling nodes	Combine activity inference with network analysis and functional validation
Formulation sensitivity	OA bioavailability is poor and formulation-dependent	Cross-study inconsistency and variable signaling outputs	Compare free OA, optimized formulations, and selected derivatives within the same platform
Model dependence	OA effects vary across cell types and biological contexts	Limited generalizability of mechanistic conclusions	Use more than one responsive model and compare conserved signatures
Correlation-driven interpretation	Omics integration may generate plausible but non-causal models	Mechanistic overinterpretation	Treat multi-omics as a prioritization tool, not as proof without validation

Note: The main challenge in OA mechanistic studies is not the lack of measurable biological effects, but the difficulty of assigning those effects to a causal hierarchy of molecular events. For this reason, experimental design, temporal resolution, and orthogonal validation are essential for reliable target deconvolution [3,12–14].

8.8. Synthetic Conclusion

In summary, the principal challenge in OA phosphoproteomic research is not the lack of measurable biological effects, but the difficulty of arranging those effects within a credible causal hierarchy. OA is a multidimensional compound chemically, biologically, and in signaling terms. Phosphoproteomics can substantially improve mechanistic resolution, but only when embedded in a workflow that combines careful model selection, time-resolved sampling, integration across omics layers, and independent target-engagement validation [3,12–14,17,26,35].

9. Translational Relevance and Future Research Directions

The translational value of phosphoproteomic and multi-omic approaches in OA research lies in their ability to convert broad biological observations into testable mechanistic models relevant to therapeutic development [3,9,11–14,30–34,42]. In this framework, OA can be viewed not only as a candidate bioactive compound, but also as a mechanistic probe for mapping disease-relevant signaling states [13].

A major translational advantage of OA is that it provides a chemically versatile scaffold for derivative development. Available studies indicate that selected OA derivatives may exert stronger or more directed effects on Akt/mTOR, NF-κB, ferroptosis-related pathways, or PTP1B than the parent compound [3–7,42]. In this context, phosphoproteomics and multi-omics may support

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mechanism-guided medicinal chemistry by linking chemical modification with shifts in phosphosignatures and signaling output [3,13].

Bioavailability remains a major translational limitation of OA. Changes in formulation may alter not only absorption and systemic exposure, but also the amplitude and architecture of OA-responsive signaling [3,6,22,25,41,42]. Future mechanistic studies should therefore compare free OA, formulation-optimized OA, and selected derivatives within the same experimental platform.

Importantly, OA signaling output is likely shaped not only by intrinsic target affinity, but also by scaffold modification and formulation-dependent bioavailability (Figure 3).

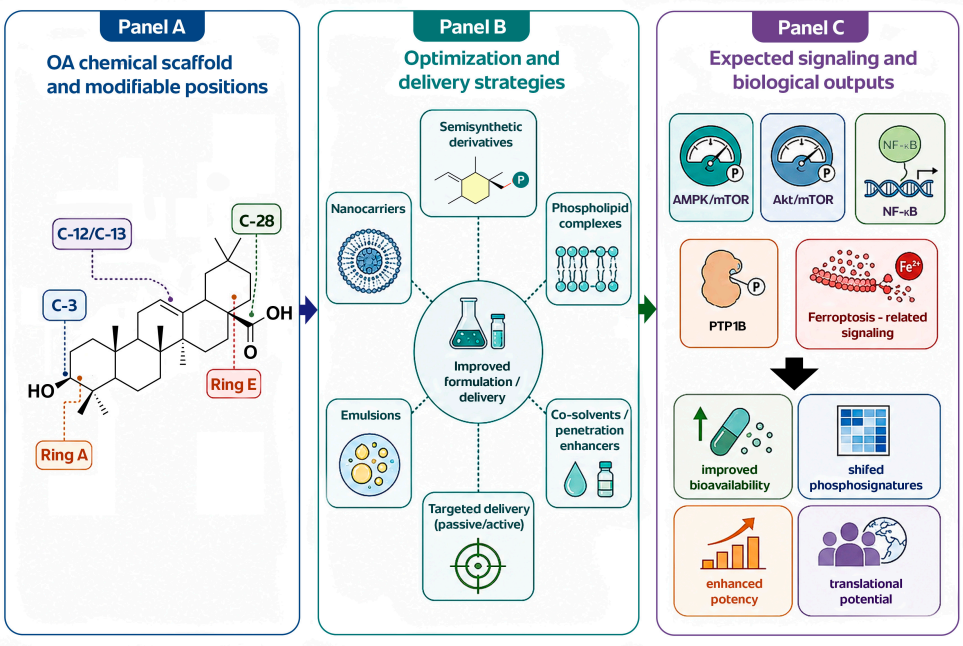

Figure 3. Linking OA chemical space, bioavailability, and signaling output.

Note: OA bioactivity is shaped not only by intrinsic target affinity, but also by physicochemical properties, formulation, and chemical modification of the parent scaffold [3]. Structural optimization and formulation strategies may alter both bioavailability and signaling output, creating a framework in which phosphoproteomics can be used to compare the parent compound with its derivatives and delivery systems at the level of functional pathway remodeling.

Phosphoproteomics may also support biomarker development and response stratification. Because phosphorylation signatures can reveal biologically meaningful pathway states that are not apparent at the genomic or transcriptomic level, they may help identify contexts in which OA is most active, most selective, or most synergistic [13,20,44,46–52]. In practical terms, this may enable the definition of phosphosignatures predictive of OA sensitivity, derivative-specific response, or combination-therapy benefit.

Combination therapy represents another promising translational direction. Although OA's polypharmacological profile may be a limitation from the perspective of classical single-target discovery, it may also prove advantageous when the aim is to overcome compensatory signaling and pathway redundancy. In such settings, OA or one of its derivatives may function less as a primary cytotoxic agent and more as a signaling-network modulator that sensitizes cells to another therapy [3,12,13,42,49].

For translational progress, however, target-engagement validation remains indispensable. Methods such as CETSA, TPP, DARTS, and ABPP are increasingly important because they help determine whether candidate targets are truly engaged under biological conditions and allow stronger discrimination between on-target and off-target effects [12,30–34]. In OA research, this is particularly important because many mechanistic claims still rely primarily on pathway markers.

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Safety and tolerability also require a more mechanistic treatment than is usually given to OA. Rather than relying on the general assumption that OA is relatively safe, future translational work should integrate efficacy-oriented phosphoproteomic studies with broader proteomic and metabolomic profiling of adverse-response signatures [3,22,41,42].

Several priorities emerge from the current literature: time-resolved phosphoproteomic studies in responsive OA models; direct comparison of OA, its derivatives, and optimized formulations; expansion of phosphatase-oriented work, particularly in relation to PTP1B; and closer integration of phosphoproteomics with target-engagement methods [3,7,12–14,22–24,30–35,42,44,46–48,50–52]. A further important direction will be the adaptation of these workflows to low-input, nanoscale, and eventually single-cell settings. Together, these efforts could provide a much stronger mechanistic and translational foundation for OA research. The main translational priorities for future OA research are summarized in Table 4.

Table 4. Translational priorities for future OA research.

Research priority	Rationale	Recommended direction
Time-resolved phosphoproteomics of OA	Current evidence is dominated by endpoint marker studies	Perform early- and late-phase phosphoproteomic mapping in responsive models
Comparative profiling of OA and derivatives	The OA scaffold can be chemically tuned toward specific activities [3]	Compare phosphosignatures across the parent compound and selected semisynthetic analogues
Integration with target-engagement methods	Most mechanistic claims remain indirect	Combine phosphoproteomics with CETSA, TPP, DARTS, or ABPP
Phosphatase-oriented studies	PTP1B represents one of the most promising targeted directions [3]	Expand from enzyme inhibition assays to systems-level signaling validation
Formulation-aware signaling studies	Bioavailability may shape both potency and signaling output	Compare free OA with delivery-enhanced formulations in the same biological system
Biomarker-oriented modeling	OA activity is likely context dependent	Define phosphosignatures predictive of response, resistance, or synergy
Combination-therapy mapping	OA may function as a network modulator rather than a stand-alone cytotoxic agent	Identify synergistic partners using pathway-level and phosphoproteomic readouts
Safety-oriented multi-omics profiling	Translational development requires mechanistic toxicology, not only efficacy	Integrate proteomic, phosphoproteomic, and metabolomic profiling of adverse-response signatures

Note: Future OA research should prioritize studies that move beyond descriptive pathway modulation toward mechanistically anchored and translationally actionable models. In this context, comparative profiling of OA, its derivatives, and its formulations may be especially important for linking target deconvolution with medicinal chemistry and therapeutic development [3,12–14].

10. Conclusions

Oleanolic acid is a biologically versatile triterpenoid, but its primary molecular points of engagement remain incompletely resolved. The current literature most strongly supports OA-associated remodeling of the AMPK/mTOR axis, whereas evidence for other pathways is more

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heterogeneous and often marker-driven [3,9,11–14,16]. This uneven mechanistic resolution limits both causal interpretation and translational prioritization [12,14,15,19,20,26].

Phosphoproteomics, especially when combined with complementary omics and orthogonal target-engagement assays, offers a practical route from descriptive pathway pharmacology to mechanism-based target prioritization [12,14,15,19–22,29–34]. In this context, the key task is not simply to show that OA affects multiple pathways, but to determine which OA-responsive events occur earliest, which are most reproducible, and which are most likely to reflect biologically meaningful target engagement [14,15,26,35,44].

On this basis, OA should be viewed both as a promising scaffold for derivative development and as a useful model system for studying how integrated phosphoproteomics and multi-omics can clarify the action of pleiotropic natural products [5,8,12,14,15,19,20,26,35,42].

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Abbreviations

The following abbreviations are used in this manuscript:

ABPP	Activity-Based Protein Profiling
Akt	protein kinase B
AMPK	AMP-activated protein kinase
CETSA	Cellular Thermal Shift Assay
DARTS	Drug Affinity Responsive Target Stability
DDA	data-dependent acquisition
DIA	data-independent acquisition
ERK	extracellular signal-regulated kinase
IMAC	immobilized metal affinity chromatography
JNK	c-Jun N-terminal kinase
KSEA	kinase-substrate enrichment analysis
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MAPK	mitogen-activated protein kinase
MOAC	metal oxide affinity chromatography
mTOR	mechanistic target of rapamycin
NF-κB	nuclear factor kappa B
Nrf2	nuclear factor erythroid 2-related factor 2
OA	oleanolic acid
PI3K	phosphoinositide 3-kinase
PTP1B	protein tyrosine phosphatase 1B
pTyr	phosphotyrosine
ROS	reactive oxygen species
SAR	structure–activity relationship

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STAT	signal transducer and activator of transcription
TPP	thermal proteome profiling
ULK1	Unc-51-like autophagy activating kinase 1

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