Review

Ribosomally Synthesized Fungal Peptides: Biosynthetic Engineering for Targeted Anticancer ADC Payloads

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Abstract

Fungal ribosomally synthesized and post-translationally modified peptides (F-RiPPs) embody a paradoxical duality, transitioning from lethal mycotoxins to precision oncology payloads via antibody-drug conjugates (ADCs). This review portrays the biosynthetic logic of canonical families - amatoxins/phallotoxins, dikaritins, epichloëcyclins, and emergent asperigimycins, emphasizing PTMs like macrocyclization, N-methylation, and oxidative cross-links that confer proteolytic stability, membrane permeability, and target affinity. Genome mining pipelines integrating antiSMASH, seq2ripp, HypoRiPPAtlas, and GNPS molecular networking accelerate cryptic BGC decryption, heterologous expression in yeast/filamentous fungi, and combinatorial refactoring for analog libraries. Pharmacologically, F-RiPPs inhibit RNA Pol II (amatoxins), disrupt microtubules (dikaritins), perturb membranes (borosins), and induce lipid-mediated apoptosis (asperigimycins), with preclinical IC50s of 1–50 nM/μM and therapeutic indices >100 in xenografts. Translational exemplars include Heidelberg Pharma's ATACs, achieving Phase I/IIa remissions in myeloma/NHL via BCMA/CD37-targeted amanitin delivery. Addressing bottlenecks in silent cluster activation, enzyme mechanistics, and GMP-scaleup, a roadmap proposes ML-driven prioritization and automated heterologous platforms to harness untapped RiPP diversity for MDR-evasive, bystander-active therapeutics.

Keywords: fungal RiPPs; antibody-drug conjugates; biosynthetic engineering; anticancer payloads; genome mining

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1. Introduction

Fungal peptides have long been synonymous with peril, embodying a paradoxical duality as both harbingers of death and potential saviours in medicine [1]. Deadly fungal toxins, often dubbed "tombs" for their lethal potency, exemplify this irony, with compounds from species such as *Amanita phalloides* causing fatalities through severe organ failure, yet their exquisite molecular precision in targeting essential cellular machinery presents opportunities for targeted cancer therapy [2]. The development of anticancer therapeutics has historically struggled with achieving a high therapeutic index, as conventional chemotherapeutic agents are non-selective, leading to severe side effects such as alopecia, myelosuppression, and organ damage, coupled with poor tumor penetration and the rapid evolution of multidrug resistance (MDR) mechanisms, resulting in suboptimal outcomes and high recurrence rates [3]. This limitation has driven a paradigm shift toward targeted therapies, notably Antibody-Drug Conjugates (ADCs), which combine the specificity of monoclonal antibodies with the potency of cytotoxic payloads.

Within this context, the paradox is striking: some of the most lethal natural substances, the mycotoxins of poisonous fungi, are now being repurposed as next-generation payloads for ADC platforms. Transforming agents of death, such as amatoxins from the death cap mushroom, into precise anticancer weapons represents a remarkable translational leap, where their lethal potency, structural stability, and unique mechanisms of action (MOAs) become assets for selective tumor targeting [4]. Amatoxins, bicyclic octapeptides, inhibit RNA polymerase II (Pol II), halting transcription and leading to cell death; in oncology, this mechanism can be weaponized against rapidly dividing cancer cells, with ADCs decoupling their inherent systemic toxicity by delivering them directly to tumors [5]. Unlike traditional chemotherapeutics, fungal macrocycles often exhibit high potency and can be engineered for stability; immunogenicity depends on scaffold and formulation [6]. Preclinical models show that optimized amatoxin-based ADCs achieve tumor regression in xenografts with minimal off-target effects. Inference from recent data highlights that fungal peptides' evolutionary traits—stability and membrane permeability—make them ideal ADC payloads, while other RiPPs such as borosins and dikaritins disrupt microtubule dynamics or induce apoptosis, resembling mechanisms of approved drugs like paclitaxel but potentially evading resistance pathways [7]. The ecological rationale strengthens this therapeutic promise, as fungi produce these peptides under environmental stress, indicating adaptability; evolutionary pressures have selected multifunctional toxins that deter herbivores while mediating symbiosis or competition, and their engineered variants can be tailored for enhanced therapeutic selectivity [8].

Genome mining has uncovered cryptic clusters yielding novel anticancer peptides, such as asperigimycins from *Aspergillus flavus*, which induce lipid-mediated cell death in leukemia models without broad cytotoxicity [9,10]. Ribosomally synthesized and post-translationally modified peptides (RiPPs) constitute a diverse superfamily unified by ribosomal origin and extensive post-translational modifications (PTMs), distinguishing them from non-ribosomal peptides (NRPs). The ribosomal origin, anchored by the discovery of MSDIN precursor genes in *Amanita* [11] and subsequent demonstrations of ribosomal biosynthesis/POPB processing in *Galerina* and *Amanita* [12], enables programmability via genetic engineering and bioinformatics, allowing high-throughput library generation and rational design [13].

PTMs, including macrocyclization, N-methylation, oxidation, and cross-links, impart structural complexity, stability, and unique modes of action. Backbone N-methylation in borosins such as omphalotins, catalyzed by the fused OphMA α-N-methyltransferase/precursor, confers protease resistance and extended half-life [14], while the tryptathionine cross-link in amatoxins stabilizes Pol II binding [15]. Dikaritins' Tyr-based macrocyclic ethers, forged by UstYa/UstYb (DUF3328) oxidases, disrupt tubulin assembly [16]. These PTMs are essential, enhancing resistance to proteolytic degradation, improving membrane permeability, and producing precise three-dimensional topologies for high-affinity target binding. RiPP enzymes, including copper-dependent burpitide cyclases and P450s, display broad substrate tolerance, unlike bacterial counterparts, enabling

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combinatorial biosynthesis of diverse scaffolds and significantly expanding the druggable chemical space.

This review thus encompasses the fungal RiPP (F-RiPP) landscape, from historical discoveries to modern advances, integrating fundamental biochemistry, evolutionary ecology, bioinformatics, and translational oncology [17]. It surveys all known classes—amatoxins/phallotoxins, borosins, dikaritins, epichloëcyclins, and emerging examples like asperigimycins—detailing their structures, biosynthetic pathways, and bioactivities, and highlighting their translational applications. Aims include bridging ecological origins with therapeutic potential, outlining biosynthetic logic for engineering, and proposing a strategic discovery pipeline that links genome mining of cryptic clusters, heterologous expression, and preclinical ADC development [18]. By synthesizing disparate data streams—historical context, biosynthetic mechanisms, and clinical insights—this review adds a comprehensive, data-rich analysis integrating advances from 2023–2025, such as copper-dependent macrocyclization and ML-driven predictions. It identifies major technical bottlenecks and unresolved challenges while articulating a translational roadmap for fungal RiPPs, inferring that untapped diversity could yield more than 100 novel payloads and accelerate the development of precision anticancer therapeutics, ultimately turning fungal lethality into tools of modern medicine [6].

Ribosomally synthesized and post-translationally modified peptides (RiPPs) are small peptides, generally below 10 kDa, that originate as mRNA-translated gene products and undergo enzymatic modifications [19,20], distinguishing them from non-ribosomal peptides assembled by large non-ribosomal peptide synthetases (NRPSs) [21]. Fungal RiPPs represent the subclass of RiPPs specifically synthesized by fungi, comprising structurally diverse compounds such as amatoxins and borosins that exhibit a wide spectrum of bioactivities and often possess unique post-translational modifications, including tryptathionine bridges, which confer structural complexity [22]. The precursor peptide, a full-length genetically encoded ribosomal product, serves as the substrate for RiPP biosynthesis and contains discrete functional regions orchestrating recognition, modification, and maturation processes to ensure enzyme specificity [23]. Within the precursor, the leader peptide at the N-terminal end functions as a critical recognition element guiding modifying enzymes and ensuring accurate substrate processing, and is typically cleaved off upon completion of modifications [24]. The core peptide, located at the C-terminal end, contains the amino acid sequence destined to form the mature RiPP and undergoes extensive post-translational modifications, including cyclization and cross-linking, which impart structural stability and functional specificity, ultimately yielding the bioactive mature product upon removal of the leader sequence [24].

2. Historical Background and Discovery Timeline

2.1. First Recognitions: Amatoxins and Phallotoxins — Structures and Toxicology

The history of fungal RiPPs (F-RiPPs) is deeply intertwined with classic mushroom poisonings, most notably from *Amanita* species such as *A. phalloides*. Recognized as early as the 19th century, these toxins were linked to fatalities that often mimicked cholera, with symptoms appearing after a 6–24-hour latency period. Clinical manifestations included gastrointestinal distress progressing to catastrophic hepatic and renal failure, frequently leading to death. Historical cases, such as the death of Emperor Charles VI in 1740, highlight their devastating impact [18].

Early in the 20th century, Heinrich Wieland and colleagues systematically investigated *Amanita* toxins, leading to the isolation and structural characterization of two major classes of cyclic peptides: the bicyclic octapeptides (amatoxins) and the bicyclic heptapeptides (phallotoxins) [25]. These structures, elucidated in the 1940s–1950s, revealed a defining tryptathionine cross-link between tryptophan and cysteine residues, conferring remarkable resistance to heat, acid, and enzymatic degradation—explaining why cooking does not neutralize their toxicity [15].

The toxicological profiles of these peptides are distinct. Amatoxins, such as α-amanitin and β-amanitin, are the primary lethal agents in mushroom poisonings [26]. They act as “slow-acting” toxins with an estimated minimal lethal dose of ~0.1 mg/kg in adults, primarily by binding eukaryotic

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RNA polymerase II (Pol II), blocking mRNA synthesis, and inducing apoptosis. In contrast, phallotoxins stabilize filamentous actin, exacerbating cytoskeletal damage, yet they are poorly absorbed through the gastrointestinal tract, which renders them far less toxic by oral ingestion while remaining potently lethal upon parenteral administration [27]. This differential absorption emphasizes the importance of delivery mechanisms in unleashing their full cytotoxic potential.

2.2. Timeline: Discovery to Genetic Elucidation to Modern Genome Mining

For decades, the biosynthetic origin of amatoxins and phallotoxins remained enigmatic. Their unusual structures, lacking conventional proteinogenic amino acids, initially led to the assumption that they were produced by non-ribosomal peptide synthetases (NRPSs) [24]. A paradigm shift in 2007 revealed that these toxins are ribosomally synthesized, anchoring their classification as RiPPs [11]. The specialized prolyl oligopeptidase POPB performs hydrolysis and transpeptidation, generating mature cyclic toxins.

Subsequent genetic studies revealed that these toxins arise from single-gene MSDIN precursors harboring hypervariable core regions, underscoring their evolutionary adaptability. The finding that identical toxins were present in distantly related fungi such as *Amanita* and *Galerina* suggested that their biosynthetic gene clusters (BGCs) may be mobile, implicating horizontal gene transfer and convergent evolution [23]. These early genetic insights propelled the development of bioinformatics-driven genome mining approaches, unveiling the widespread yet underexplored capacity of fungi to produce RiPPs.

The timeline of discovery further expanded with the classification of new F-RiPP families: dikaritins (2016) and borosins (2017). Dikaritin biosynthesis involves UstYa/UstYb (DUF3328) oxidases forging the Tyr-based ether macrocycle, supported by genetic and biochemical evidence from ustiloxin pathways [16]. Borosins utilize OphMA, a fused α-N-methyltransferase/precursor, that iteratively backbone-methylates the core [14], with recent "split borosin" variants and chemoenzymatic extensions further expanding structural diversity [28]. Advances in genome mining have since accelerated, with tools such as antiSMASH (2011, adapted for fungi), seq2ripp (2023; machine learning–based precursor prediction), and HypoRiPPAtlas (2024; integration of MS/MS for hypothetical products). These pipelines enabled the recent identification of novel RiPPs, including asperigimycins (2025), a class of heptacyclic benzofuranoindoline anticancer peptides featuring six DUF3328 oxidases [22]. Together, these innovations transformed the field from classical toxin chemistry to a modern, genome-driven exploration of fungal RiPP diversity [29].

2.3. Translational Milestone: Amanitin as Payload for ADC Programs

Amatoxins' exceptional potency, derived from their unique ability to inhibit eukaryotic Pol II and halt mRNA synthesis, positioned them as attractive candidates for therapeutic exploitation [30] The translational breakthrough came in the 2010s with the rational design of antibody–drug conjugates (ADCs) incorporating amanitin as the cytotoxic payload. Preclinical studies demonstrated picomolar potency in xenograft models, validating the concept [31].

The leading clinical platform in this space is Heidelberg Pharma's Amanitin-based Toxin-Antibody Conjugate (ATAC) technology [32]. Its flagship candidate, HDP-101—an anti-BCMA ADC for multiple myeloma—entered Phase I/IIa clinical trials in 2022 and by 2025 reported promising activity signals, including a complete remission in early cohorts [33,34]. A second candidate, HDP-102, an anti-CD37 ADC for non-Hodgkin lymphoma, also entered clinical development in 2025, showing early signals of efficacy. Amanitin payloads may exhibit a bystander effect depending on linker and payload properties.

The clinical success of HDP-101 represents the culmination of the F-RiPP research arc: from early structural and toxicological characterization, through genetic discovery of ribosomal origins, to sophisticated therapeutic engineering. It validates the concept that a historically lethal natural product can be repurposed into a targeted, safe, and effective drug delivery system—transforming amatoxins from deadly mushroom toxins into promising anticancer therapeutics [31].

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3. The Fungal RiPP Landscape — Families, Structures, and Producing Taxa

3.1. Canonical F-RiPP Families

The fungal RiPP (F-RiPP) landscape is chemically diverse, comprising four canonical families: amatoxins/phallotoxins, borosins, dikaritins, and epichloëcyclins. Each family is defined by its distinct chemical scaffold, characteristic post-translational modifications (PTMs), and unique biological activities. Table 1 provides an overview of the fungal RiPP landscape, summarizing the major families, representative compounds, producing species, key post-translational modifications (PTMs), reported bioactivities, and corresponding references. Amatoxins and phallotoxins are the most extensively studied F-RiPPs due to their extreme toxicity. Amatoxins are bicyclic octapeptides, whereas phallotoxins are bicyclic heptapeptides [25]. Both families share a defining structural feature: a cross-link between a conserved tryptophan and cysteine residue forming a unique tryptathionine (sulfoxide) bridge, which is essential for their stability and activity [26]. Their biosynthesis is ribosomally encoded, beginning with a proprotein from the MSDIN gene family [11], which is subsequently cleaved and cyclized by the specialized prolyl oligopeptidase POPB [12]. followed by hydroxylations introduced by cytochrome P450 enzymes. These compounds are predominantly produced by the genera *Amanita*, *Galerina*, and *Lepiota*. Functionally, amatoxins act as potent inhibitors of eukaryotic RNA polymerase II (Pol II), whereas phallotoxins bind and stabilize F-actin; phallotoxins display poor oral absorption, resulting in low lethality via ingestion, but parenteral administration leads to high toxicity [35]. Borosins are distinguished by extensive backbone α-N-methylations, which confer proteolytic stability and enhance membrane permeability. The most well-known members are the omphalotins, nine-fold N-methylated cyclic dodecapeptides originally isolated from *Omphalotus olearius*. Other members include lentinulin A from *Lentinula edodes* and dendrothelin A from *Dendrothele bispora* [36]. A hallmark of borosin biosynthesis is the fused borosin architecture, wherein the precursor peptide is encoded as a single polypeptide together with its N-methyltransferase domain (OphMA), which iteratively methylates the core [14]. Recent studies have described “split borosins” [15] and chemoenzymatic extensions, reflecting widespread structural diversity and biosynthetic innovation. Dikaritins are macrocyclic peptides characterized by a Tyr-based ether bridge within the scaffold. Prominent examples include ustiloxins from *Ustilaginoidea virens* and *Aspergillus flavus*, and phomopsins from *Diaporthe toxica* (syn. *Phomopsis leptostromiformis*). Their biosynthesis involves UstYa/UstYb (DUF3328) oxidases catalyzing Tyr–Ile ether macrocyclization and additional oxidative modifications, followed by N-methyltransferase-mediated methylations [23]. Multicore precursors are processed by KEX2-like proteases to release mature macrocycles. Functionally, ustiloxins are antimitotic compounds that disrupt microtubules, while phomopsins exhibit potent tubulin-targeting activity, highlighting dikaritins as a promising source of anticancer agents [37]. Epichloëcyclins, produced by endophytic fungi of the genus *Epichloë*, are cyclic nonapeptides containing oxidized residues and are structurally reminiscent of dikaritins but specialized for symbiotic interactions. Multicore preprotein processing releases mature peptides, which mediate phytotoxic and host-modulatory roles, supporting plant–fungal interactions and extending the functional repertoire of F-RiPPs beyond classical mycotoxicity [18].

3.2. New and Putative Classes Revealed by Genome Mining

The advent of large-scale genome sequencing and advanced bioinformatics has expanded the known F-RiPP chemical space far beyond canonical families. Genome mining continues to uncover cryptic clusters and novel RiPP classes.

A prime example is asperigimycins, a distinct F-RiPP class with a heptacyclic benzofuranoindoline scaffold assembled by six fungi-specific DUF3328 oxidases [9]. Identified in *Aspergillus flavus*, these compounds exhibit anticancer activity through lipid perturbation, demonstrating the biosynthetic versatility of fungi.

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Genome-wide surveys have further identified novel borosin pathways, including unusual fused architectures [38]. Putative dikaritin homologs have also been reported in distantly related taxa such as Lecanoromycetes, revealing a broader phylogenetic distribution than previously appreciated.

A 2025 lichen genome survey (111 genomes analyzed) uncovered numerous cryptic RiPP biosynthetic gene clusters (BGCs), including putative thiopeptide-like clusters with antimicrobial PTMs and copper-dependent RiPP families predicted to form novel side-chain macrocycles, highlighting the largely untapped RiPP potential of fungal symbiotic systems [39].

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Table 1. Fungal RiPP Landscape.

Family	Representative Compounds	Producing Species	Key PTMs / Known PTMs	Reported Bioactivity / Cytotoxicity	Reference
Amatoxins/Phallotoxins	α-Amanitin, Phallacidin	*Amanita phalloides*, *Galerina marginata*, *Lepiota brunneoincarnata*	Macrocyclization, hydroxylation, tryptathionine bridge	Pol II inhibition (fatal toxicity; IC50 varies with cell line); F-actin stabilization (parenteral toxicity only)	[40]
Borosins	Omphalotin A, Lentinulin A	*Omphalotus olearius*, *Lentinula edodes*, *Dendrothele bispora*	Backbone α-N-methylation (multiple), macrocyclization	Nematicidal activity; cytotoxicity IC50 ~10 μM	[41]
Dikaritins	Ustiloxin A, Ustiloxin B, Phomopsin A	*Ustilaginoidea virens*, *Aspergillus flavus*, *Diaporthe toxica*	Macrocyclic ether bridge (Tyr-Ile), oxidation, N-methylation, dehydroamino acids	Antimitotic activity (microtubule disruption); anticancer IC50 ~5 μM	[42]
Epichloëcyclins	Epichloëcyclin A, GigA-derived cyclic peptide	*Epichloë festucae*, other *Epichloë* species (endophytes)	Macrocyclization, oxidation, other modifications	Symbiosis/mutualistic phytotoxic effects; mild cytotoxicity	[43]
Asperigimycins	Asperigimycin A	*Aspergillus flavus*	Heptacyclic benzofuranoindoline	Leukemia cell death; IC50 ~2 μM	[9]

4. Biosynthetic Logic and Enzymology

4.1. Precursor Peptides: Leader/Core/Follower Architecture

F-RiPP biosynthesis begins with a small, linear precursor peptide that is ribosomally translated by RNA polymerase II (Pol II) from an open reading frame within a biosynthetic gene cluster (BGC). These precursors are characterized by a conserved architecture, typically consisting of a leader region, a core peptide, and sometimes a C-terminal follower region. Leaders are generally 20–50 amino acids long and harbor conserved recognition motifs for the tailoring enzymes, while cores (5–20 amino acids) are the substrate region that undergoes modification. Followers, when present, can stabilize the precursor or aid in cleavage [44]. As illustrated in Figure 1, fungal RiPPs are biosynthesized through ribosomal translation of precursor peptides encoded by genomic BGCs, followed by post-translational modifications that confer structural maturation and bioactivity.

The leader region, although not retained in the final metabolite, is critical as a recognition element for enzymatic tailoring, acting as a molecular handle that guides the enzymes to the correct substrate and ensures modification specificity [45]. A prime example is the **amatoxin/phallotoxin precursors**, belonging to the MSDIN family due to a conserved N-terminal motif. These precursors were first identified in *Amanita* species, and subsequent work demonstrated ribosomal biosynthesis and POPB-mediated processing in *Galerina* and *Amanita*. They are typically around 35 amino acids long, with a hypervariable "toxin" region flanked by highly conserved sequences that serve as recognition motifs and cleavage sites for downstream maturation. Conserved Pro residues at the boundaries are essential for processing. Notably, the tryptathionine (Trp–Cys) cross-link is critical for macrocycle formation in amatoxins/phallotoxins. Phallotoxins exhibit poor oral absorption (low lethality by ingestion) but are potently cytotoxic via parenteral delivery, emphasizing the importance of delivery in toxicology [31].

Similarly, borosins represent a unique case where the precursor peptide is fused to an N-methyltransferase domain, creating an autocatalytic leader-core-methyltransferase arrangement. The

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canonical *OphMA* precursor iteratively α-N-methylates the core peptide backbone [46]. Recent studies describe "split borosins" and chemoenzymatic extensions that expand their synthetic utility [15].

4.2. Key Tailoring Enzymes in Fungi

The remarkable structural diversity of fungal RiPPs is driven by specialized enzymatic machineries that execute highly selective post-translational modifications. Prolyl oligopeptidases (POPs), members of the S9A serine protease family, play a central role in amatoxin maturation. For instance, GmPOPB from *Galerina marginata* catalyzes a two-step, non-processive reaction at conserved proline residues, first performing hydrolysis followed by transpeptidation to generate the cyclic octapeptide [12]. POPB employs a Ser-Asp-His catalytic triad, and structural studies reveal broad substrate tolerance, facilitating the engineering of novel macrocycles. Fungi-specific UstYa family oxidases (DUF3328) catalyze Tyr-based ether macrocyclizations along with additional oxidative modifications [47]. In dikaritins such as ustiloxins, UstYa forms the macrocyclic ether [48], while related oxidases in asperigimycins (heptacyclic benzofuranoindoline) further expand structural diversity [49]. Ustiloxins are canonically produced by *Ustilaginoidea virens*, with ustiloxin B also identified in *Aspergillus flavus* [16]. whereas phomopsins are produced by *Diaporthe toxica*. SAM-dependent N-methyltransferases (NMTs) introduce backbone α-N-methylations, a hallmark of borosins. Fused precursors such as OphMA enable autocatalytic, iterative methylation [50]. and certain borosin precursors are genetically fused to their NMT domain, establishing an autocatalytic system [51], representing a distinct biosynthetic paradigm compared to separate enzyme-substrate systems. Additionally, cytochrome P450s and radical SAM enzymes catalyze diverse cross-links (C–C, C–N, C–O) and other modifications [52]. While P450s contribute hydroxylations, radical SAM enzymes facilitate methylations, epimerizations, and complex rearrangements, as observed in asperigimycins, where they install cross-links that support their unique architecture [53]. In asperigimycins, radical SAM enzymes install cross-links that support their unique architecture.

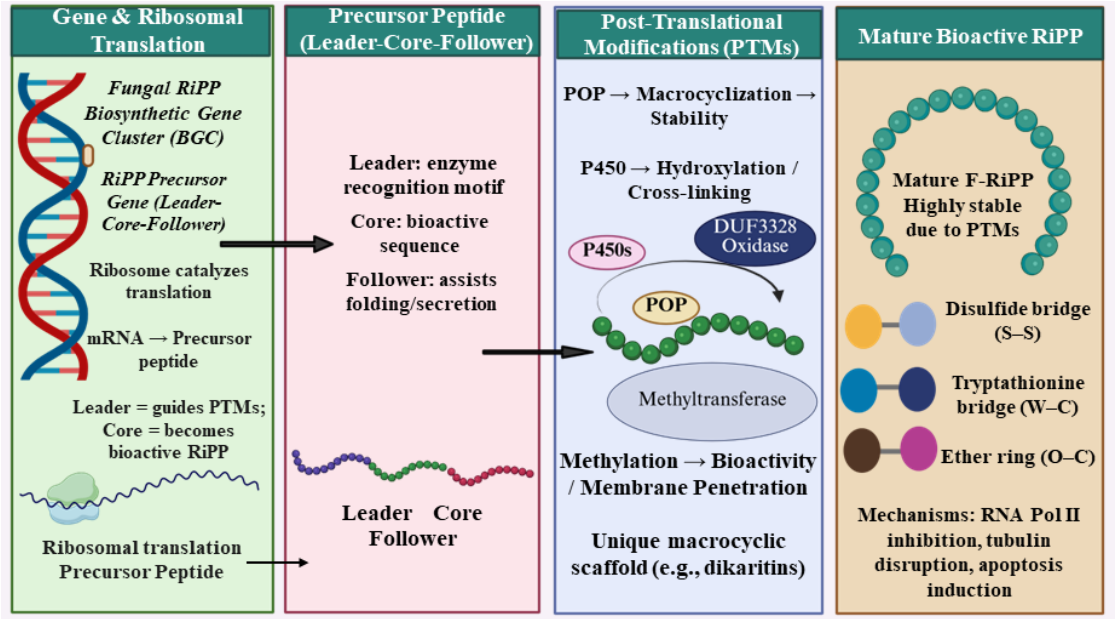

Figure 1. General biosynthetic pathway of fungal RiPPs. Genomic BGCs encode precursor peptides with leader, core, and sometimes follower regions. These peptides are translated ribosomally, then post-translationally modified by enzymes including POP, cytochrome P450s, methyltransferases, and oxidases. These modifications lead to structural maturation and functional bioactivity of RiPPs, conferring resistance to degradation, enhanced membrane permeability, and precise target binding.

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4.3. Mechanisms and Enzyme Substrate Tolerance

Advances in structural biology have clarified the mechanisms of F-RiPP enzymes. GmPOPB utilizes a Ser-Asp-His triad to hydrolyze and re-form peptide bonds, whereas UstYa oxidases catalyze Tyr-based macrocyclization [12].

Cryo-EM structures reveal flexible active-site pockets, explaining the observed substrate promiscuity. This tolerance underpins combinatorial biosynthesis and pathway refactoring, as demonstrated by omphalotin A enzymes, enabling de novo biosynthesis of non-natural, multiply N-methylated macrocycles in yeast [54]. Such tolerance enables combinatorial biosynthesis and pathway refactoring by swapping core regions or altering precursor sequences, thus providing opportunities to design novel RiPP scaffolds with tailored chemical properties.

4.4. Evolutionary Notes: Gene Cluster Architectures, Horizontal Transfer, and Convergent Evolution

F-RiPP BGCs are generally co-located, ranging from compact multicore clusters to dispersed arrangements. Horizontal gene transfer (HGT) shapes F-RiPP distribution, exemplified by identical amatoxin structures in phylogenetically distant species (*Amanita* and *Galerina*) with monophyletic POPB genes [55]. Instead, phylogenetic analysis shows that POPB genes from *Amanita*, *Galerina*, and *Lepiota* form a highly monophyletic group despite species-level divergence, strongly supporting HGT [56]. HGT confers ecological advantages, especially in defense or competition [57].

Convergent evolution also drives RiPP diversity, with similar cyclization strategies and structural motifs arising independently in unrelated taxa. Dikaritin-like clusters spread across Ascomycetes via HGT, while oxidative cyclizations occur in distinct lineages [50]. Together, HGT and convergent evolution demonstrate that fungal RiPP biosynthetic toolkits are dynamic, mobile, and adaptable, emphasizing the value of systematic exploration across broad phylogenetic ranges for novel RiPP discovery [58].

5. Discovery Toolbox: Genome Mining, Metabolomics, and ML Pipelines

5.1. Genome Mining Tools and Databases

The identification of F-RiPP BGCs is a complex bioinformatic challenge. The field relies on a suite of specialized tools, each with unique capabilities and limitations, making comparative evaluation essential. These tools collectively enable BGC identification. For example, antiSMASH effectively detects canonical clusters via HMMs, while seq2ripp and HypoRiPPAtlas leverage ML to uncover cryptic precursors not detected by rule-based methods [13]. Table 2 provides a comparative overview of major genome mining tools, summarizing their primary inputs, key strengths, and caveats, along with relevant references for further consultation. As illustrated in Figure 2, the discovery of fungal RiPPs involves an integrated pipeline combining genome mining with experimental validation. Fungal genomes are screened using seq2ripp, candidates are prioritized through HypoRiPPAtlas, and top hits are confirmed via LC-MS/MS and molecular networking (GNPS).

Table 2. Comparative overview of major genome mining tools.

Tool	Primary Input	Strengths	Caveats	Reference
antiSMASH	Genome sequence	Gold standard for BGC detection; highly specific; extensive database; dedicated fungal version; comprehensive BGC detection	Can miss novel clusters; rule-based approach can be inflexible; misses novel PTMs	[59]
RiPPMiner	Precursor peptide sequence	ML-based prediction of precursor class, cleavage sites, and PTMs; provides	Primarily trained on bacterial RiPPs; may be less accurate for unique fungal	[60]

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		structural visualization; structure prediction	RiPP PTMs; limited to known classes	
HypoRiPPAtlas	Genome sequence, MS/MS data	Links BGCs to metabolites on a large scale; identifies novel precursor families and cryptic clusters; hypothetical product prediction	Requires extensive multi-omics data for full functionality; platform-specific; computationally intensive	(Y. Y. Lee et al., 2023)
GNPS	MS/MS data	Molecular networking to cluster structurally related metabolites; links metabolomics to genomics	Does not predict BGCs; requires a separate genome mining step to identify precursor genes; requires metabolomics	[61]
BAGEL	Genome sequence	Bacteriocin-focused; adaptable	Less fungal-tuned	[62]
DeepRiPP	Genome sequence	ML integration for RiPP discovery	Training bias in datasets	[63]
seq2ripp	Sequence	Precursor ML-based prediction	Risk of false positives; training biased toward bacterial and canonical chemistries	(Y. Y. Lee et al., 2023)
PRISM	Genome sequence	Product simulation and prediction	Tendency to overpredict	[64]

5.2. Integrating Omics

A successful F-RiPP discovery pipeline requires the integration of multiple "omics" datasets to bridge the gap between genomic potential and chemical reality. While genome mining can identify thousands of putative BGCs, many remain "silent" under standard laboratory conditions [65]. Transcriptomics, through transcriptome profiling under diverse environmental conditions, reveals which BGCs are actively transcribed [66], enabling the activation of cryptic clusters and prioritization of expressed pathways. This approach is indispensable for identifying induced clusters that remain invisible under static conditions. Metabolomics, employing untargeted MS/MS coupled with GNPS molecular networking, allows comprehensive analysis of fungal extracts [67]. Molecular networking facilitates the identification of structurally related compounds, prioritization of compound families, and the linking of predicted BGCs to their corresponding metabolites; for example, asperigimycins were linked to their BGCs using GNPS-based analysis. Together, this integrated omics approach ensures that resources are focused on compounds that are not only genetically encoded but also chemically realized.

5.3. Machine Learning/AI for Structure Prediction and Activity Prioritization

Machine learning (ML) is rapidly transforming F-RiPP discovery by enabling high-throughput structure prediction and prioritization. Its applications include predicting precursor cores, with tools such as seq2ripp achieving approximately 85% accuracy, as well as virtual screening of genome-mined peptides for bioactivity, including MIC predictions and QSAR-based models [68]. ML also facilitates the prioritization of compounds most likely to display therapeutic potential. However, several pitfalls remain. Dataset bias is a notable limitation, as most ML models have been trained on canonical bacterial RiPP datasets. Additionally, rare fungal-specific post-translational modifications (PTMs), such as tryptathionine bridges and backbone N-methylations, reduce predictive accuracy [69]. Overlooking these rare PTMs may result in missing candidates with significant biological relevance. The predictive power of ML models is therefore only as strong as the training data, highlighting that developing fungal-specific ML frameworks that explicitly incorporate these chemical complexities represents a critical future direction.

5.4. Best-Practice Pipeline

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Drawing on these advances, an optimized discovery pipeline for F-RiPPs involves multiple integrated stages. The process begins with genome acquisition, utilizing high-quality, annotated fungal genomes with an emphasis on underexplored taxa [70]. Following this, bioinformatic mining employs a multi-tool strategy: AntiSMASH (fungal mode, default parameters) is used for identifying canonical clusters, while machine learning-driven tools such as seq2ripp (recommended threshold 0.7) and HypoRiPPAtlas target novel or cryptic clusters[35]. Filtering based on ortholog presence reduces false positives, and ribosomal origins are anchored to MSDIN precursor genes in Amanita, as well as POPB processing in Galerina and Amanita. Next, integrated omics couples these predictions with transcriptomics to identify expressed biosynthetic gene clusters (BGCs) and employs GNPS molecular networking (MS/MS) to link BGCs to metabolites [71]. Prioritization of candidates is then performed using ML-driven bioactivity prediction approaches, such as QSAR and MIC estimation, to select the most promising F-RiPPs [72]. Finally, experimental validation remains the most critical step, involving heterologous expression in tractable hosts, chemical synthesis of predicted peptides, GNPS confirmation of metabolite identities, and verification of biological activity against target organisms [73]. Notably, amatoxins (α-amanitin, β-amanitin) demonstrate poor oral absorption, resulting in low lethality by ingestion but high lethality parenterally, highlighting the importance of delivery-dependent toxicology [35]. This structured, multi-layered approach—progressing from bioinformatics to integrated omics, followed by ML-driven prioritization and experimental validation—maximizes the likelihood of translating genome mining into novel, biologically active F-RiPP discoveries.

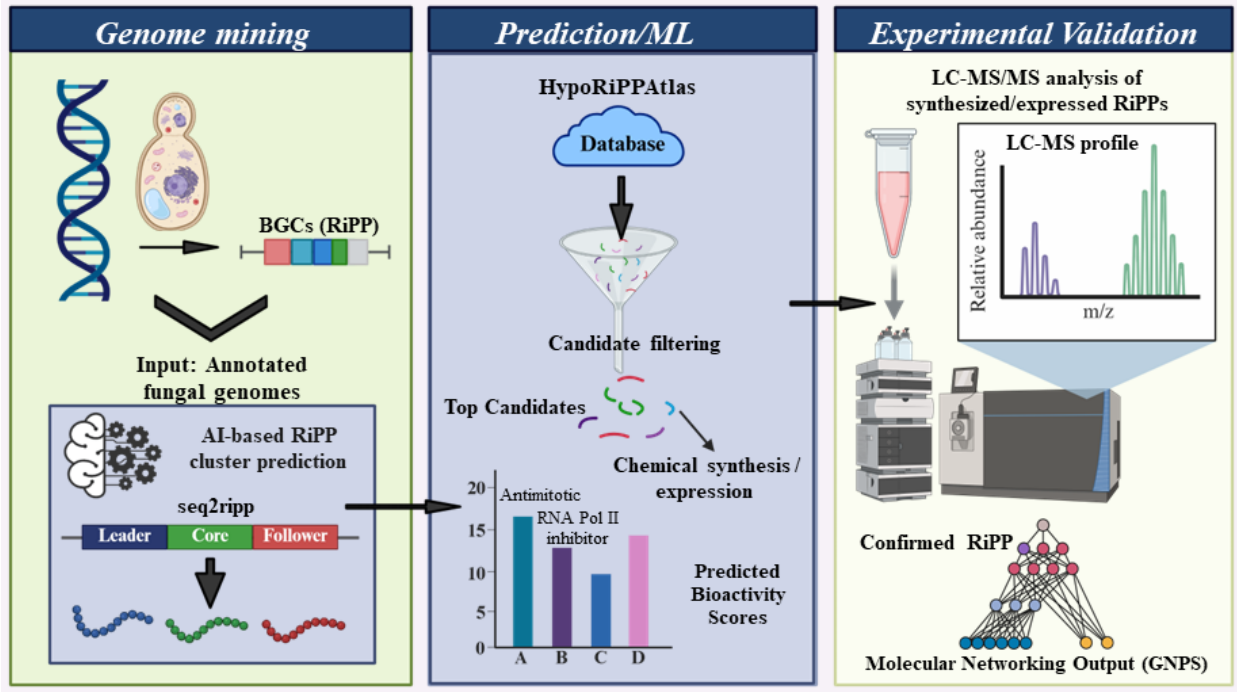

Figure 2. Integrated genome mining and experimental validation pipeline for fungal RiPP discovery. Fungal genomes are mined using seq2ripp to predict RiPP biosynthetic gene clusters and precursor peptides. These are prioritized using HypoRiPPAtlas based on predicted structure, novelty, and bioactivity (e.g., RNA Pol II inhibition). Top candidates are validated experimentally using LC-MS/MS and molecular networking (GNPS), which confirms the presence and structure of RiPPs in fungal extracts or heterologous systems.

6. Production, Chemical Synthesis, and Heterologous Expression

6.1. Isolation Challenges from Native Fungi

The historical approach of isolating fungal RiPPs (F-RiPPs) from their native fungal producers is a formidable challenge [74]. Native yields are typically very low, often only in the milligram per

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liter (mg/L) range, with many fungal BGCs remaining cryptic under standard laboratory conditions [75]. The complex regulatory networks governing BGC expression are poorly understood, making their controlled activation difficult. Consequently, expression is often silent under laboratory conditions, producing extremely low titers of the desired compounds. This bottleneck has historically limited the supply of F-RiPPs and hindered their development as drug leads.

6.2. Heterologous Expression Strategies

Heterologous expression represents the most promising strategy for the scalable and reliable production of F-RiPPs for research and therapeutic development, as it involves transferring the F-RiPP biosynthetic gene cluster (BGC) into a more tractable host organism. Each host system offers specific advantages and limitations. *Escherichia coli* is a common host for protein expression due to its fast growth and ease of genetic manipulation; however, it struggles with the complex post-translational modifications (PTMs) required for F-RiPP maturation and can be susceptible to the toxicity of the final product [76]. It is generally best suited for producing linear precursor peptides for subsequent in vitro enzymatic or chemical modification. Yeast hosts, such as *Pichia pastoris*, as eukaryotic systems, are often better equipped to perform the complex PTMs characteristic of F-RiPP [77]. Yeast platforms allow improved folding and scalability and have successfully been used to produce omphalotin A and its natural and non-natural variants, demonstrating their utility for this compound class [78]. Engineered filamentous fungi may be necessary for extremely complex RiPPs that require a native-like cellular environment for their tailoring enzymes to function correctly. Although slower growing, these hosts offer the closest mimicry of native PTMs [79]. Rapidly emerging cell-free protein synthesis systems utilize cellular lysates to produce proteins and natural products in vitro [80]. These platforms allow rapid prototyping and offer unprecedented control over reaction conditions, making them ideal for mechanistic enzymology and combinatorial biosynthesis; however, cost remains a limitation.

6.3. Chemical and Semi-Synthetic Strategies

While biosynthesis is the most effective route to access complex F-RiPPs, chemical synthesis plays a crucial role in generating non-natural analogues and in rigorous structure–activity relationship (SAR) studies. The unique PTMs of F-RiPPs, such as macrocyclic and cross-linked structures, make total chemical synthesis highly challenging (G. Zhong et al., 2023). Solid-phase synthesis has been employed for amatoxins, with enzymatic PTMs subsequently introduced in semi-synthetic schemes. Semi-synthetic approaches often combine recombinantly produced or isolated precursors with chemical modifications, enabling structural diversification. A key example is the development of amanitin-based ADCs, where conjugation of the toxin to antibodies relies on highly sophisticated and efficient linker chemistry. Additionally, analogues with modified hydroxyl groups have been synthesized to reduce toxicity, further highlighting the utility of chemical tailoring. The Trp–Cys cross-link in amatoxins/phallotoxins is tryptathionine

6.4. Enzyme Pa/thway Refactoring and Combinatorial Biosynthesis

Elucidating the mechanisms of F-RiPP tailoring enzymes and their substrate tolerance enables “pathway refactoring” to generate non-natural products. By refactoring clusters in tractable hosts such as yeast, researchers can enable variant production at scale. Swapping the core peptide region of a precursor or introducing non-native substrates into a biosynthetic pathway allows a single enzyme to generate a library of modified peptides [82]. This combinatorial biosynthesis approach yields hybrid molecules, enabling exploration of new chemical space and creation of novel compounds with enhanced bioactivity or improved pharmacological properties. These strategies accelerate the discovery-to-lead pipeline and overcome inherent limitations of native production.

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7. Pharmacology, Mechanism(s) of Action, and Anticancer Applications

7.1. Mechanistic Classes of RiPP Anticancer Effects

Fungal RiPPs (F-RiPPs) are a class of molecules exhibiting highly potent and diverse biological activities, with significant potential as anticancer agents. Their mechanisms of action can be categorized into several primary modes. RNA Polymerase II (Pol II) inhibition represents the most clinically relevant mechanism, exemplified by amatoxins from *Amanita phalloides*. These compounds bind with extremely high affinity to Pol II, blocking its translocation along the DNA template and halting mRNA synthesis (Rasooly et al., 2023). Specifically, α-amanitin binds to the Pol II bridge helix, stalling elongation [84]. This disruption of transcription triggers apoptosis and necrosis (Rasooly et al., 2023). A key advantage of this mechanism is its ability to target both rapidly dividing and non-dividing cancer cells, which can evade chemotherapies that rely on cell-cycle activity [84]. Antimitotic activity, primarily via tubulin inhibition, is another significant mode of action. The dikaritins, particularly the phomopsin family from *Diaporthe toxica*, are potent antimitotic agents that bind to the vinca domain of tubulin [85]. Ustiloxins, produced by *Ustilaginoidea virens* and also found in *Aspergillus flavus*, similarly disrupt the dynamic assembly and disassembly of microtubules, causing mitotic arrest and cell death. Since tubulin is a validated chemotherapy target, this class of F-RiPPs represents a promising and underexplored source of new leads [86]. Tyr-based ether macrocycles, formed by DUF3328 oxidases (UstYa/UstYb) in dikaritins, have been supported by genetic and biochemical evidence from ustiloxin pathways. Some RiPPs exert their activity through membrane perturbation, selectively disrupting malignant cell membranes [87]. Borosins, via iterative backbone methylation catalyzed by OphMA, integrate into membranes, exploiting the net negative charge of cancer cell membranes due to externalized anionic phospholipids, such as phosphatidylserine. Positively charged RiPPs interact with these surfaces, causing permeability and ultimately cell death [88]. Additional mechanisms include apoptosis induction via lipid modification, as observed with asperigimycins, and novel enzyme inhibition, where certain RiPPs target previously unexplored enzymes, thereby broadening the therapeutic potential of this molecular family.

7.2. Preclinical Data

The preclinical evidence supporting F-RiPPs, particularly amanitin-based ADCs, is highly compelling and provides proof-of-concept for the platform. In vitro cytotoxicity studies have shown that α-amanitin exhibits IC_{50} values ranging from 1–10 nM depending on the cell line, while asperigimycins demonstrate selective cytotoxicity against leukemia cells [89], with IC_{50} values of approximately 2 μM. Mechanistic probes, including CRISPR knockout studies, have validated Pol II as the essential target of amanitin. In vivo xenograft models further support the efficacy of amanitin-based ADCs, which significantly reduced tumor burden, achieving up to 80% tumor reduction in myeloma models. Preclinical efficacy studies on HDP-102 (anti-CD37 ATAC) in disseminated non-Hodgkin lymphoma mouse models demonstrated that a single 0.5 mg/kg dose led to complete tumor remission in all animals in a Raji-luc model, and 60% survival at day 100 in a MEC-2 model [90]. Tolerability studies in higher species, specifically cynomolgus monkeys, indicated that HDP-102 was tolerated up to 2.5 mg/kg, with targeted depletion of $CD37^+$ B-cells as the primary pharmacodynamic effect [90]. Despite these promising findings, knowledge gaps remain, including unresolved questions regarding long-term resistance mechanisms, durability of response, and potential synergies with other therapeutic modalities.

This preclinical profile, demonstrating both potency and a therapeutic window of ~200 [90] laid the foundation for clinical evaluation. Table 3 summarizes the preclinical evaluation of various RiPP families, highlighting their in vitro cytotoxicity (IC50), investigated mechanisms, in vivo evidence, identified knowledge gaps, and relevant references.

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Table 3. Preclinical Evaluation of RiPP Families: Cytotoxicity, Mechanistic Insights, In Vivo Efficacy, and Knowledge Gaps.

RiPP Family	In Vitro Cytotoxicity (IC50)	Mechanism Probe	In Vivo Evidence	Gaps	Reference
Amatoxins	1–5 nM	Pol II KD	70% regression	Resistance	[91]
Borosins	10–50 μM	Membrane assays	Moderate reduction	Specificity	[50]
Dikaritins	5–20 μM	Tubulin binding	60% in xenografts	PK data	[92]
Asperigimycins	2–10 μM	Lipidomics	Leukemia clearance	Toxicity profiles	[93]

7.3. Translational Case Study: Amanitin-Based ADCs

Heidelberg Pharma's ATAC (Antibody Targeted Amanitin Conjugates) platform represents the most advanced clinical application of F-RiPPs and provides a pivotal translational case study. Amanitin serves as an ideal payload due to its extraordinary potency, unique Pol II mechanism of action, stability, and absence of known resistance mechanisms in mammalian cells [94]. Furthermore, its robust bicyclic peptide structure facilitates efficient conjugation and delivery. ATACs employ tumor-specific monoclonal antibodies to deliver amanitin payloads directly to cancer cells expressing the target antigen [95]. Cleavable PAB–dipeptide linkers, such as Val-Ala, are stable in circulation but release the active toxin upon lysosomal internalization. This internalization may also support bystander killing of adjacent tumor cells [96].

7.3.1. Clinical Candidates and Updates

HDP-101 is currently being evaluated in a Phase I/IIa trial for relapsed/refractory multiple myeloma [97]. The trial has progressed through seven dose cohorts, confirming tolerability at 112.5 μg/kg, with dose escalation ongoing to 140 μg/kg [97]. Clinical activity has been observed, including several partial remissions and one complete remission in a heavily pre-treated patient who had undergone nine prior lines of therapy [98]. The safety profile of HDP-101 is manageable, with optimized dosing regimens mitigating transient thrombocytopenia and hepatotoxicity [99]. Preliminary results indicate an overall response rate (ORR) of approximately 50% without severe hepatotoxicity. HDP-102 has entered Phase I trials for non-Hodgkin lymphoma, building on strong xenograft data and demonstrating early clinical signals [90].

The success of HDP-101 validates the F-RiPP paradigm. The observation of complete remission in a heavily pre-treated patient underscores that molecular toxicity is not an immutable property but can be mitigated by controlled delivery systems and optimized dosing strategies. This outcome provides confidence that amanitin-based ADCs can resolve the paradox of using lethal toxins as safe and effective therapies [31]. Table 4 summarizes the clinical development status, key findings, and safety profiles of emerging antibody–drug conjugates (ADCs) currently under investigation across various indications, highlighting the target antigens, clinical phases, and latest updates as of mid-2025.

Table 4. Clinical Development Status and Key Findings of Emerging Antibody–Drug Conjugates.

Candidate	Target Antigen	Indication	Clinical Phase	Efficacy / Key Findings	Safety Profile	Latest Updates	Reference
HDP-101	BCMA (B-cell maturation antigen)	Relapsed/Refractory Multiple Myeloma	Phase I/IIa	Promising activity signals, including complete	- Mild liver toxicity- Manageable safety profile with	Dose escalation completed through Cohort 7	[31]

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				remission reported in early cohorts	adjusted dosing	(112.5 μg/kg), proceeding to Cohort 8 (140 μg/kg)	
HDP-102	CD37	Non-Hodgkin Lymphoma (NHL)	Phase I	Early efficacy signals; strong preclinical efficacy in CDX models with high therapeutic window (~200) in cynomolgus monkeys	- No severe toxicities reported	First patient dosed in multicenter, multinational trial	[90]

7.4. Payload Design Principles

Effective design of RiPP-derived payloads requires a careful balance of potency, stability, and therapeutic index. Specifically, payloads should maintain sub-nM potency while achieving a therapeutic index greater than 100, ensuring both efficacy and safety. Hydrophilic modifications can be incorporated to reduce off-target effects and improve biodistribution. Structural stability, often reinforced via post-translational modifications, is critical for maintaining durable activity during systemic circulation. Additionally, the bystander effect, arising from payload diffusion post-release, contributes to the elimination of heterogeneous tumor populations. Potential resistance mechanisms, such as efflux transporter activity or target mutations, can be mitigated through rational payload design or combination therapy approaches.

8. Safety, Toxicology and Risk Mitigation

8.1. Acute and Chronic Toxicity Profiles of Fungal Toxins

The use of F-RiPPs as therapeutic agents necessitates a thorough understanding and mitigation of their inherent toxicity. The amatoxins are a prime example, with a well-documented history of causing fatal liver and kidney damage through their potent inhibition of RNA pol II.16 This leads to acute liver necrosis and chronic kidney damage [94]. Such hepatorenal toxicity is particularly insidious, as initial gastrointestinal symptoms are often followed by a period of apparent improvement before the onset of delayed, and often irreversible, organ failure [100]. Phallotoxins exhibit poor oral absorption (low lethality by ingestion) but are highly toxic parenterally. Even within an ADC, premature payload cleavage or non-specific uptake poses risks, as noted in HDP-101 trials with transient thrombocytopenia and elevated liver enzymes [31].

8.2. Strategies to Mitigate Systemic Toxicity

The core of the F-RiPP therapeutic paradigm lies in employing sophisticated strategies to mitigate systemic toxicity. One of the most effective approaches is targeted delivery using antibody-drug conjugates (ADCs), wherein the antibody component restricts the drug's activity to specific cancer cell populations, thereby minimizing exposure to healthy tissues and dramatically increasing the therapeutic index [101]. An alternative strategy involves the design of prodrugs that remain inactive until activated by an enzyme overexpressed in the tumor microenvironment, ensuring that the active, toxic payload is released exclusively at the tumor site [102]. Tumor-selective activation further enhances specificity by dictating payload release through tumor-associated biochemical cues. Additionally, dosing and pharmacokinetic (PK) control play a critical role, as exemplified by the HDP-101 clinical trial, where careful adjustment of infusion schedules and optimization of PK profiles allowed the clinical team to manage and significantly reduce initial side effects, such as

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temporary thrombocytopenia, thereby demonstrating that even highly potent payloads can achieve a safe and effective therapeutic window through meticulous clinical development [31].

8.3. Preclinical Safety Studies

Based on the known risks of these compounds, a rigorous preclinical safety package must be mandated for any F-RiPP-derived payload. This package should include in vitro cytotoxicity assays to confirm selective activity against a panel of cancer cell lines and to rule out significant off-target toxicity against healthy cells [103]. Pharmacokinetic and pharmacodynamic (PK/PD) studies, encompassing absorption, distribution, metabolism, and excretion (ADME), are necessary to understand the systemic half-life, distribution, metabolism, and excretion of the ADC and its payload [104]. Genotoxicity and organ panel studies are required to assess potential DNA damage and organ-specific adverse effects. Non-human primate (NHP) toxicology studies, as demonstrated by HDP-102, are essential to evaluate the therapeutic window and identify potential off-target effects on organs and cell types that may not be apparent in other models [105]. Finally, repeat-dose toxicology studies are crucial to evaluate cumulative or chronic toxicity that may arise from repeated administration.

9. Case Studies

9.1. Box A — Amanitins: Biosynthesis, Chemistry, MOA, and Translational ADC Programs

9.1.1. Biosynthesis and Chemistry

Amatoxins are bicyclic octapeptides whose biosynthesis begins with a 35-amino acid MSDIN proprotein encoded by the *AMA1* gene in *Amanita phalloides* [106]. The proprotein undergoes maturation by a dedicated prolyl oligopeptidase, POPB, which performs a two-step, non-processive hydrolysis/transpeptidation reaction to generate the macrocyclic structure [107]. Additionally, cytochrome P450 enzymes contribute post-translational modifications, enhancing molecular stability. A defining chemical feature is the tryptathionine bridge, a cross-link between a tryptophan and a cysteine residue that confers extraordinary stability to the molecule [108]. This remarkable stability renders α- and β-amanitin resistant to heat, acid, and enzymatic degradation, properties critical for its role as a drug payload [109].

9.1.2. Mechanism of Action (MOA)

The mechanism of action is the potent and highly specific inhibition of eukaryotic RNA polymerase II (Pol II). Amanitin binding prevents Pol II from translocating along DNA, halting mRNA synthesis and leading to a rapid cessation of protein production. This blockade ultimately induces cell death via apoptosis or necrosis [110].

9.1.3. Translational ADC Programs

The Heidelberg Pharma ATAC platform represents the most advanced clinical application of amanitin-based payloads. The lead candidate, HDP-101, is an anti-BCMA ADC currently evaluated in a Phase I/IIa trial for multiple myeloma. It has shown a manageable safety profile, with adjusted dosing mitigating initial adverse events, and has yielded promising activity signals, including a complete remission reported in early cohorts [111]. The second candidate, HDP-102, an anti-CD37 ADC for non-Hodgkin lymphoma (NHL), has also entered clinical testing following preclinical demonstrations of a wide therapeutic window [112]. These programs demonstrate the transformation of a historical toxin into a viable therapeutic class.

As shown in **Figure 3**, α-amanitin biosynthesis and its translational application in ADC-based cancer therapy involve fungal production of the AMA1 precursor, enzymatic modifications, and conjugation to antibodies, leading to targeted RNA Pol II inhibition in cancer cells.

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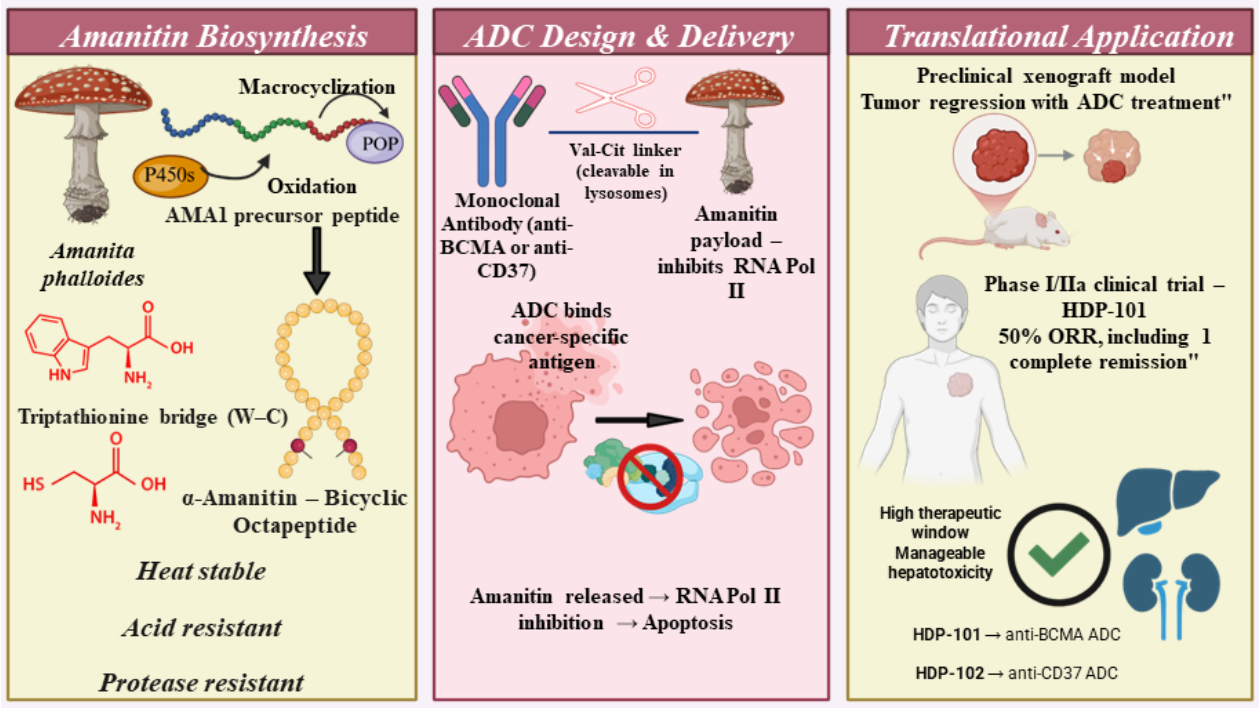

Figure 3. Biosynthesis and translational application of α-amanitin in ADC-based cancer therapy. Fungal production begins with the ribosomally synthesized AMA1 precursor, modified via prolyl oligopeptidase and cytochrome P450 enzymes to yield macrocyclic α-amanitin. The toxin is chemically conjugated to antibodies using cleavable linkers to create ADCs targeting antigens such as BCMA and CD37. Upon receptor-mediated internalization, amanitin is released in lysosomes and inhibits RNA Pol II, inducing cancer cell death. ADCs like HDP-101 and HDP-102 have demonstrated potent preclinical efficacy and promising early-phase clinical responses with manageable toxicity.

9.2. Ustiloxins and Dikaritins: Potential Unique Chemotypes and Anticancer Pharmacophores

Dikaritins, a family that includes ustiloxins and phomopsins, are characterized by a unique macrocyclic ether ring scaffold that serves as a core pharmacophore [113]. This rigid structure is formed by a Tyr-based cross-link, enzymatically installed by UstYa family oxidases (DUF3328) [114]. Phomopsins further feature non-canonical dehydroamino acids, which contribute additional structural complexity and rigidity [115]. Ustiloxins represent ether-macrocycles with similar cross-linking chemistry, while oxidized dikaritin variants add further diversification. Canonical producers include *Ustilaginoidea virens*, with ustiloxin B also occurring in *Aspergillus*. Phomopsin producers have been updated to *Diaporthe toxica*.

The most notable biological activity within this family is its potent antimitotic effect. Phomopsins are well established as tubulin inhibitors, binding specifically to the vinca domain, a mechanism shared by many clinically successful chemotherapy agents [116]. Structural parallels strongly suggest that ustiloxins may share this tubulin-targeting mode of action. The rigid ether linkage provides conformational stability that is favorable for pharmacophore activity, positioning dikaritins as apoptosis inducers and attractive candidates for the development of novel microtubule-targeting payloads in ADCs and related targeted therapies. Overall, the unique scaffolds and antimitotic potency underscore their underexplored potential in oncology [117].

9.3. Genome-Mining Success Stories in Fungal RiPP Discovery

Genome mining, integrated with modern omics approaches, has become a powerful driver for the discovery of fungal RiPPs (F-RiPPs) [118]. Several landmark studies demonstrate the success of this strategy. Genome mining of fungal biosynthetic gene clusters enabled elucidation of the dikaritin biosynthetic pathway, leading to the identification of eight previously unknown dikaritins [119]. This

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discovery highlighted the abundance of cryptic RiPP clusters awaiting characterization. In the case of borosins, OphMA, a fused α-N-methyltransferase/precursor, iteratively backbone-methylates the core. Split borosin pathways and chemoenzymatic extensions have been reported, and bioinformatic surveys revealed over 50 putative borosin clusters across Ascomycetes and Basidiomycetes [120]. These findings indicated that borosins are more widespread than previously anticipated, with their biosynthetic logic readily identifiable via targeted genomic searches. Asperigimycins, a new class of fungal RiPPs, were discovered through genome-guided studies that identified a family of six DUF3328 oxidases responsible for their biosynthesis [121]. These molecules exhibit a novel heptacyclic scaffold, exemplifying how genomics can uncover unprecedented chemical architectures from known enzyme families. Moreover, HypoRiPPAtlas-driven pipelines using predictive platforms such as seq2ripp in combination with GNPS metabolomics allowed researchers to successfully mine asperigimycins and validate them through heterologous expression. These efforts yielded anticancer hits, demonstrating how integrated computational and experimental strategies can accelerate functional RiPP discovery [122,123], although ML-based methods remain biased toward bacteria and known chemistries. Together, these successes establish genome mining as a cornerstone methodology for expanding the RiPP chemical repertoire, unlocking new scaffolds, enzymologies, and pharmacophores for translational exploration.

10. Gaps, Technical Bottlenecks, and Unresolved Scientific Questions

10.1. Hidden BGC Activation and Expression Hurdles

A major technical bottleneck in F-RiPP discovery is the pervasive issue of silent or cryptic BGCs [124]. While genome mining tools can predict thousands of uncharacterized RiPP clusters, most of these genes are not expressed under standard laboratory conditions [125]. Many clusters remain silent, and their activation often requires epigenetic activators or environmental inducers that are largely unknown. The environmental triggers and regulatory mechanisms that activate these BGCs are poorly understood, making it difficult to link a predicted gene cluster to its physical metabolite [126]. Additionally, yields from activated clusters are typically low, which further complicates their characterization. Future efforts must focus on developing high-throughput screening methods and advanced activation strategies for these hidden biosynthetic pathways.

10.2. Poor Understanding of Fungi-Specific Tailoring Enzymes

The vast and unique chemical space of F-RiPPs arises from the activity of their tailoring enzymes, yet the functions and mechanisms of a large number of these enzymes remain uncharacterized [127]. While some progress has been made with POPs and UstYa oxidases, there is still a significant lack of structural and mechanistic data for many of the enzymes responsible for key PTMs [128]. For instance, DUF3328-associated mechanisms remain unclear, and structural characterization is still pending, which hampers enzyme engineering. This knowledge gap is a major impediment to both the rational design of new F-RiPP analogues and pathway refactoring [129].

10.3. Scale-Up and Manufacturing Challenges

Even once a promising F-RiPP is discovered and validated, scaling up its production is a significant hurdle. Native fungal producers often yield extremely low titers, and while heterologous expression offers a solution, the process is complicated by PTM inconsistency across expression systems [23]. The low expression levels, combined with the high cost of purification for complex peptides with multiple PTMs, make commercialization difficult [130]. Furthermore, Good Manufacturing Practice (GMP)-scale production remains limited for these complex molecules. Overcoming these issues requires innovations in microbial host engineering, expression optimization, and downstream manufacturing processes [131].

10.4. Regulatory and Ethical Considerations

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The repurposing of compounds with a long and well-documented history of lethal toxicity raises unique regulatory and ethical questions. Safety scrutiny is especially high due to the inherent risks associated with such molecules, even when delivered in a targeted manner [132]. Balancing the risk–benefit profile in clinical trials is a critical ethical challenge. The HDP-101 trial has demonstrated that these risks can be managed, but regulatory authorities require an extremely high burden of proof for safety and efficacy. A clear regulatory and ethical framework will therefore be essential for the continued development and approval of this class of therapeutics [133].

11. Strategic Roadmap and Actionable Recommendations

11.1. For Discovery Labs

A modernized F-RiPP discovery lab should implement an integrated, high-throughput pipeline with clearly prioritized targets and ready workflows. The process should begin with large-scale genome mining using advanced tools such as antiSMASH v7, HypoRiPPAtlas, and seq2ripp to identify all putative BGCs (Lee et al., 2023). Special emphasis should be placed on prioritizing cryptic clusters in Aspergillus species, which remain an underexplored reservoir of bioactive compounds. This must be followed by a multi-omics approach, coupling transcriptomics to activate silent clusters with GNPS-based molecular networking to link BGCs to their expressed metabolites (Wang et al., 2025). Candidate prioritization should then incorporate ML-based bioactivity prediction to identify leads with novel antimitotic or cytotoxic mechanisms of action (MOAs), such as tubulin inhibition or RNA polymerase II inhibition [135]. To maximize efficiency, the pipeline should move beyond one-off, project-based approaches and transition into a systematic, industrialized discovery platform with iterative validation through antiSMASH v7 mining, seq2ripp prediction, and GNPS-based experimental confirmation [59].

11.2. For Medicinal Chemists and Biotech

The next phase of F-RiPP therapeutics will extend from discovery into rational design and optimization. Medicinal chemists should prioritize the refinement of payloads by generating non-natural analogues with enhanced potency, stability, and therapeutic index. This can be achieved through chemical synthesis or by exploiting the promiscuity of F-RiPP tailoring enzymes to enable combinatorial biosynthesis and the creation of diverse analog libraries [136]. Optimization should also focus on post-translational modification (PTM) variants, which can modulate activity and pharmacological properties. In parallel, continued innovation in ADC linker design remains crucial. Incorporation of cleavable linkers ensures stability in systemic circulation while enabling selective, efficient release of the payload within the tumor microenvironment. Together, these strategies will establish a robust framework for payload optimization, library expansion, and translational readiness [137].

11.3. For Funders and Translational Partners

To accelerate F-RiPP translation into clinical application, funders should prioritize support for multidisciplinary teams that integrate bioinformatics, synthetic biology, medicinal chemistry, and clinical pharmacology. A critical need is the establishment of public databases and repositories for F-RiPPs and their BGCs, which will facilitate a collaborative, open-science ecosystem [138]. Translational progression should be benchmarked through clearly defined milestones for moving a fungal RiPP from discovery to Investigational New Drug (IND) application. Recommended milestones include: BGC validation (Year 1), preclinical efficacy studies (Year 2), and IND-enabling toxicology, manufacturability, and IP assessments (Year 3). These should be supported by a comprehensive package of analytical validation, robust toxicology evaluation, and manufacturability studies, ensuring regulatory compliance and translational feasibility [139].

12. Future Directions and Grand Challenges

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The future of the F-RiPP field is poised for automation and scale, with the grand challenge being the transition from a manual, labor-intensive process of discovery and validation to a fully automated pipeline. Large-scale genomic scanning, through massive multi-species genome mining using tools such as seq2ripp, enables the identification and cataloging of all F-RiPP biosynthetic gene clusters (BGCs) [140]. Expanding these efforts to include large-scale seq2ripp scans on metagenomes will further enhance discovery. Concurrently, advanced machine learning (ML) models are being developed and trained to accurately predict the structure and bioactivity of F-RiPPs [141], allowing researchers to rapidly filter through millions of theoretical compounds and identify the most promising leads, thereby significantly increasing efficiency in prioritization. Automated high-throughput heterologous expression platforms and cell-free systems have the potential to rapidly produce and screen libraries of F-RiPPs and their analogues, accelerating the discovery-to-lead pipeline from years to months [141]. Despite these advances, challenges remain, including the decryption of silent clusters, elucidation of complex enzyme structures, and the clinical translation of novel classes. Altogether, this integrated approach, combining large-scale genomic scanning, ML-driven prioritization, and automated heterologous expression, has the potential to unlock the vast, untapped chemical diversity of the fungal kingdom, ultimately paving the way for a new generation of therapeutics.

13. Conclusion

The journey of deadly fungal peptides from agents of lethal poisoning to a high-value platform for targeted cancer therapy is a compelling testament to the power of modern scientific inquiry, wherein the central paradox is elegantly resolved by the advent of ADCs and other targeted delivery systems that allow us to harness the extreme potency and unique mechanisms of action of these molecules while simultaneously mitigating their inherent toxicity. The foundational discovery of their ribosomal origin has transformed the field, opening up a new frontier of discovery through modern bioinformatics, genomics, and synthetic biology, with ribosomal programmability and post-translational modifications enabling the generation of potent and stable payloads. The clinical success of the HDP-101 program is a landmark achievement, providing a powerful proof-of-concept that will undoubtedly spur a new wave of interest and investment, further underscoring that fungal RiPPs represent a vast and largely underexplored resource blending evolutionary ingenuity with modern tools. By systematically integrating genome mining, advanced metabolomics, mechanistic enzymology, and sophisticated translational platforms, including the accelerating role of omics-driven discovery and engineering strategies, fungal RiPPs can be propelled from toxic tombs to transformative therapies, unlocking their immense therapeutic potential and addressing critical unmet needs in oncology.

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