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Trends in Microbiology

CellPress

Opinion

RNA-based therapeutics to treat human fungal infections

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In recent decades, RNA-based therapeutics have transitioned from a near impossibility to a compelling treatment alternative for genetic disorders and infectious diseases. The mRNA vaccines against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are truly groundbreaking, and new adaptations are already being proposed to fight other microbes. Unfortunately, the potential of RNA-based therapeutics to treat human fungal infections has remained mostly absent from the conversation, despite the fact that invasive fungal infections kill as many per year as tuberculosis and even more than malaria. Here, we argue that RNA-based therapeutics should be investigated for the treatment of human fungal infections and discuss several major roadblocks and potential circumventions that may allow for the realization of RNA-based therapies against human fungal pathogens.

Highlights

Fungal infections are a major problem affecting over one billion people annually. Emerging pathogens and increasing resistance rates are complicating the situation, while new therapeutic options remain slow to materialize.

RNA-based therapeutics are changing the way we treat both genetic and infectious diseases, building on several decades of basic research. The most notable advancement in RNA-based therapeutics came as the mRNA vaccines used to limit severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection.

Experiments with fungal plant pathogens have revealed that RNA interference can be leveraged as a tool to limit phytopathogens from destroying crops, providing a proof-of-principle that RNA-based therapeutics can fight fungi.

Barriers remain to using RNA-based therapeutics against fungal infections, namely, delivery across complicated fungal cell wall structures. Extracellular vesicles may provide one solution.

Emerging fungal pathogens and rising resistance rates necessitate new therapeutic approaches

Fungi (see Glossary) remain important global pathogens of organisms across the kingdoms Plantae and Animalia [1]. Emerging outbreaks of chytrid fungi are devastating amphibian populations worldwide, and fungi like *Magnaporthe oryzae* and *Botrytis cinerea* routinely hinder crop production [2]. In humans, *Cryptococcus gattii* has proven to be a dangerous, infectious pathogen of immunocompetent individuals with poorly defined risk factors [3,4], *Aspergillus fumigatus* remains an ever-present danger to the immunocompromised [5], and *Candida auris* is threatening patients in hospitals with exceedingly high rates of **antifungal resistance** (Figure 1, Key figure) [6,7]. More generally, invasive fungal infections caused by the genera *Aspergillus*, *Candida*, *Pneumocystis*, and *Cryptococcus* are estimated to kill upwards of 1.5 million people per year [8]. In the clinic, the rise of antifungal resistance is further complicating treatment of these difficult infections.

Existing therapeutic options for systemic human fungal infections now center on five classes of drugs, the azoles, echinocandins, polyenes, allylamines, and antimetabolites, which have been extensively reviewed previously [9,10]. Although effective in many instances, a combination of high toxicity and increasing resistance limits their therapeutic efficacy [11]. The limited scope of available antifungals necessitates the development of an expanded repertoire of new drugs [9]. Numerous strategies are being undertaken to develop novel antifungals, including modifications to existing drugs, combination therapies, and altogether new approaches. One strategy that has received only cursory attention thus far is the use of RNA-based therapeutics to target human fungal pathogens.

RNA-based therapeutics are proving useful against both genetic disorders and infectious diseases

Traditional treatment approaches often rely on the use of small molecules to induce a therapeutic effect against target proteins [12–14]. Unfortunately, this approach has some underlying

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limitations, including few 'druggable' target proteins and a tremendous workload to screen for strong affinity drugs [14–16]. Nucleic-acid 'biologics' have emerged as a compelling alternative to address many of these limitations [13,14]. The variety of RNA species belonging to this group offers options that not only influence the activity of a target protein but can also modulate the genetic information, transcription, and even translation of the target gene (Figure 2).

The most notable, although not first, success of **RNA-based therapeutics** came as the mRNA vaccines against SARS-CoV-2 (Figure 2A). mRNA therapeutics have been investigated widely over the past 30 years, including for immunization, cancer treatments, and even as protein substitution therapies [14,17]. Development of functional mRNA therapeutics was initially hampered by a variety of issues regarding administration, translational regulation, stability, and immunogenicity, but these obstacles were mostly resolved by progress in **RNA modifications**, transfection technologies, and expression regulation systems [13,14,17]. It is no wonder then, that many mRNA-based therapeutics are now being investigated in clinical trials [14]. The mRNA therapeutics provide an intriguing example of how RNA biologics can be employed to enhance an existing host response against an invading microbe.

In addition to the mRNA-based approaches, a number of reports have described manipulation of target expression at the transcriptional or translational level by the employment of small single- or double-stranded RNAs, like **short activating RNAs (saRNAs)**, **antisense oligonucleotides (ASOs)**, **small interfering RNAs (siRNAs)**, and **microRNA (miRNA)** mimics. All of these have proven to be useful tools for regulating expression [14], but siRNAs and miRNA mimics may represent the most interesting options in fighting fungal infections by inducing an immune response or directly targeting the pathogen.

MiRNAs and small interfering RNAs are core molecules of the well-conserved **RNA interference** (RNAi) pathway, which regulates gene expression by post-transcriptional gene silencing (Figure 2B). After processing of long double-stranded precursor molecules, the resulting small RNAs are loaded into an **Argonaute** (**AGO**)-containing RNA-induced silencing complex [14,18–20]. The activated complex is then directed to a target mRNA, resulting in a block of translation. Complete hybridization of siRNAs leads to a cleavage of the target transcript by AGO, whereas partial hybridization by miRNAs prompts steric hindrance of the translation process and eventual mRNA degradation [14,18,19]. The RNAi pathway has been frequently investigated for therapeutic use and numerous clinical trials have reached varying stages of success in treating a wide range of diseases, including viral infections and cancers [14,18,21].

Using RNAi to fight fungal pathogens has advanced more quickly in the agricultural sector [22]. Phytopathogenic fungi release extracellular small RNAs that exploit the RNAi machinery of their host plant to weaken host defense, while the host is able to send short RNA molecules to silence virulence genes of the pathogen in return [22]. This molecular interplay termed **cross-kingdom RNA interference** was leveraged to limit pathogen infection by silencing virulence genes with small RNAs generated from either transgenic plants expressing these small RNAs (host-induced gene silencing) or by spray-application of RNA onto the host plant (spray-induced gene silencing) [22]. The success of this procedure depends on the RNA uptake efficiency of the pathogen as not all plant pests efficiently import RNA [23]. Nonetheless, this example demonstrates that RNAi-based applications are a feasible way to fight fungal pathogens and that the spray-induced gene silencing principle might be adaptable as nasal sprays or salves to treat human fungal pathogens in the future [24].

Despite bearing strong similarities to siRNAs and miRNAs, saRNAs induce, rather than repress, gene expression (Figure 2C) [25–27]. **RNA activation** occurs when an AGO protein transports

Glossary

Antifungal resistance: the ability of a fungus to grow in a concentration of drug that would kill a wild-type organism.
Antisense oligonucleotides (ASOs): small, single-stranded nucleic acids that bind a target to trigger gene silencing, typically via RNase H activity.
Argonaute (AGO) protein: a well-conserved protein clade belonging to the 'P-element-induced wimpy testis' (PIWI) superfamily and specializing in binding small RNAs for interaction with target nucleic acids. Most isoforms bear slicing capacity that is guided by small RNAs.
CRISPR/Cas: a bacterial system for defending against viral infection that has been repurposed for genetic engineering and therapeutic development.
Cross-kingdom RNA interference: the occurrence of small RNAs sent from an organism of one kingdom (e.g., Plantae) to one of another kingdom (e.g., Fungi) to silence gene expression via RNA interference.
Extracellular vesicles: small lipid-bilayer-enclosed containers released from nearly all cells that contain protein, nucleic acid, and lipid cargo molecules.
Fungus: any member of the kingdom Fungi, including yeasts, molds, rusts, mildews, smuts, and mushrooms. Fungi are eukaryotes, heterotrophic, and typically contain chitin in their cell walls.
Hypha: a branching, filamentous fungal structure; hyphae are collectively referred to as a mycelium.
MiRNA: a small, single-stranded noncoding RNA of about 22 nucleotides that can function in gene-silencing and post-transcriptional gene regulation.
Ribozymes: entities that include RNA species capable of executing catalytic activities comparable with those of enzymes.
RNA activation: a mechanism that employs short activating RNAs and Argonaute 2 for inducing the transcription of specific genes.
RNA aptamer: an RNA oligonucleotide with a high specificity and affinity for a specific target, such as a protein of interest.
RNA-based therapeutics: an emerging class of drugs that use RNA molecules to elicit a therapeutic effect in a recipient organism.
RNA interference (RNAi): the process in which small interfering RNA and miRNA species bring about

saRNAs into the nucleus to interact with the target promoter sequence or transcript [14,25,28]. This mechanism was anticipated to be an excellent therapeutic possibility for applications where a disease-suppressed gene needs to be increased in expression [25]. In fact, this proved successful in a recent Phase I clinical trial for the saRNA MTL-CEBPA, which treats advanced hepatocellular carcinoma [29]. In this case, the researchers selected and validated specific candidate-binding RNAs that activated the target gene *CEBPA* (CCAAT/enhancer-binding protein alpha), then incorporated a 5′ inverted abasic modification to the sense strand and 2′-O-methyl moieties on the antisense strand, and finally encapsulated the RNA in liposomal nanoparticles called SMARTICLES to improve delivery and prevent immune activation [26,30]. Building on studies like these, a situation could easily be imagined in which a saRNA might be leveraged to promote host defense against a fungal pathogen.

RNA aptamers offer a completely different mode of action and are designed to directly bind a target molecule instead of modulating transcription or translation (Figure 2D). These single-stranded oligonucleotides can be easily selected using approaches like the 'systematic evolution of ligands by exponential enrichment' (SELEX) technology. Over the years, this approach has provided an efficient way of screening extensive libraries of nucleic acids for their ligand capability in a high-throughput manner [31]. The evolved DNA and RNA aptamers generally bind to their target in a picomolar or nanomolar range, respectively, and bear various advantages over conventional antibodies [32]. Only one aptamer drug has been approved for therapeutic use – for the treatment of age-related macular degeneration (Pegaptanib); however, numerous clinical trials are now underway [14,32]. Development of aptamer-based diagnostics to identify pathogens or pathogen-secreted toxins are also in various stages of commercial availability, for instance, to find mycotoxins such as aflatoxins or ochratoxin A in food [32,33].

Other RNA-based approaches have also been considered for the treatment of a broad spectrum of diseases. Short, single-stranded ASOs consisting of either deoxy- or ribonucleotides are capable of binding a target mRNA (seemingly) unassisted where they can exert a variety of effects [14,34]. Early variants of antisense oligonucleotides consisted of DNA and were designed to interact with mRNA to form **RNase H**-degradable DNA–RNA hybrids [14,34]. The RNA-based ASOs added more mechanisms of regulation to our therapeutic toolkit, for example, translation impedance by hybridization close to the translation start site, masking of upstream open reading frames, and modulation of transcript splicing to correct faulty protein production [14,34].

The most recent addition to RNA therapeutics is represented by the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated proteins (Cas) system. **CRISPR/Cas** offers the possibility to precisely modify genetic information to directly tackle the cause of a variety of diseases [35] by employing guide RNAs. The therapeutic use of this system is currently limited to *ex vivo* approaches, which are conducted by retransplantation of genetically modified patient cells [14,35]. Several studies as well as clinical trials are now investigating the potential of this system as a treatment for different cancer variants, HIV, diabetes, and other severe (genetic) diseases [35–39].

Considering the progress in the development of RNA therapeutics, it appears to be only a matter of time before some, if not all, of these RNA-based tools are employed in antifungal therapies.

The current roadblocks to fighting human fungal pathogens with RNA

Significant hurdles exist in adapting these existing strategies for the treatment of eukaryotic fungal pathogens with RNA-based therapeutics (see Outstanding questions). In fact, many of the same problems that hinder current efforts to treat genetic disorders and cancers, including rapid

sequence-specific suppression of gene expression.

RNA modifications: post-transcriptional alterations to RNA molecules that often convey a change in function, immune reactivity, or localization.

RNase H: type H endoribonuclease; it recognizes and degrades DNA–RNA hybrids.

Short activating RNAs (saRNAs): small, double-stranded RNAs that induce transcription by targeting gene promoters.

Small interfering RNAs (siRNAs): small, double-stranded noncoding RNAs, typically 20–27 nucleotides, that operate within the RNA interference pathway to silence gene expression.

Spore: a sexual or asexual reproductive unit particularly suited to surviving harsh conditions and adapted for efficient dispersal.

Key figure

Human fungal pathogens remain a major therapeutic challenge

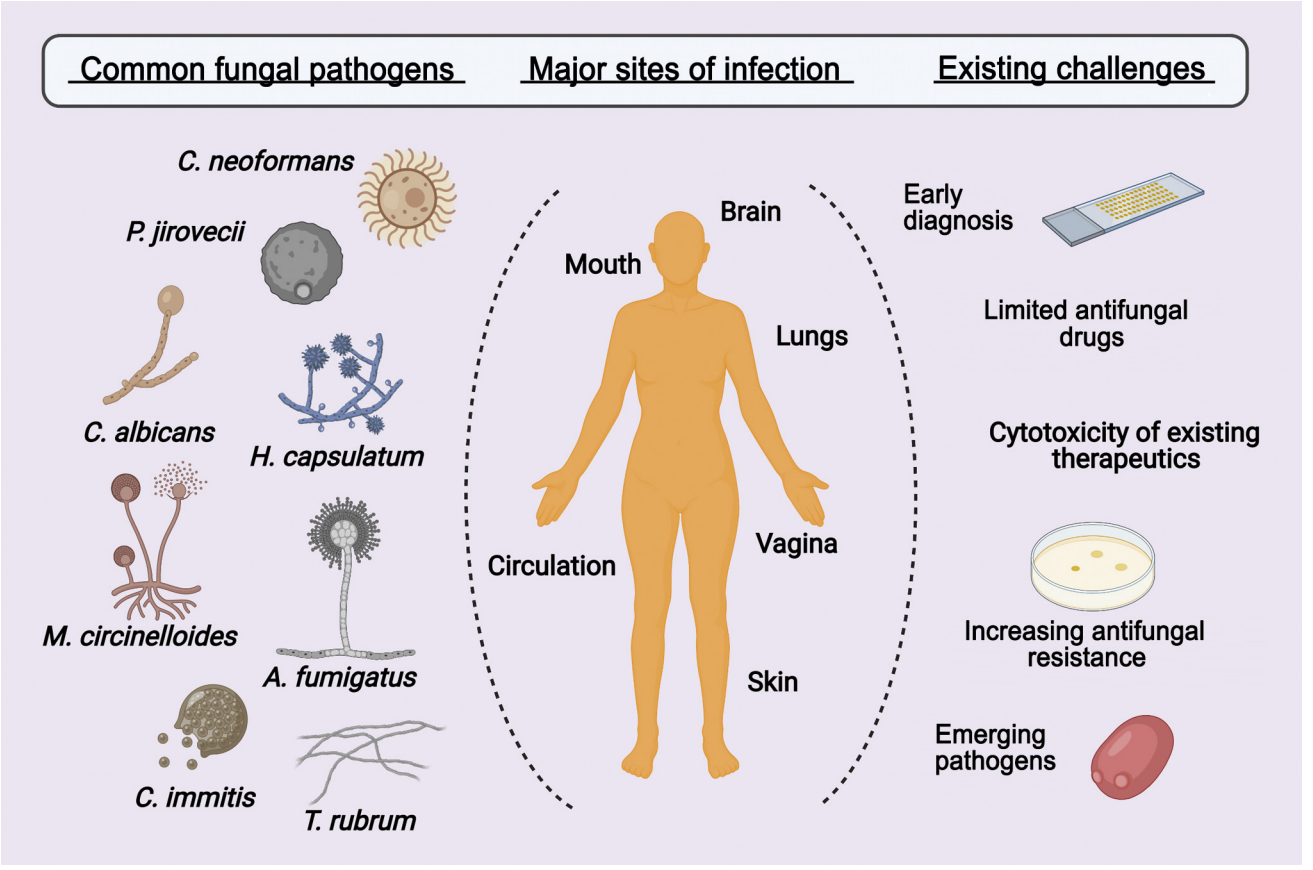

Figure 1. Human fungal infections are caused by a range of pathogens, including yeasts such as *Candida albicans*, *Pneumocystis jirovecii*, and *Cryptococcus neoformans*; molds such as *Mucor circinelloides* and *Aspergillus fumigatus*; and dimorphic fungi such as *Histoplasma capsulatum* and *Coccidioides immitis*. These organisms infect a variety of body sites, including the lungs, skin, and brain, where they can cause allergic, chronic, or even invasive infections. Dermatophytes such as *Trichophyton rubrum* cause immense numbers of skin infections across the globe annually. In immunocompromised patients, fungal infections can be particularly dangerous, with over a million deaths attributed to them each year. Treatment remains a major challenge due to limited drugs, increasing resistance, and difficulties in early diagnosis. Emerging pathogens such as *Candida auris* and *Cryptococcus gattii* are adding additional layers of complexity to the clinical situation. The figure was created with BioRender.com.

therapeutic degradation and difficulties in promoting RNA escape from the endosome, will also make treatment of fungal infections more difficult [14,40–42].

The first challenge is to define appropriate targets for RNA-based therapies that will specifically inhibit the fungal pathogen without detrimentally altering the host or commensal organisms (Figure 3). Studies of model organisms such as *Saccharomyces cerevisiae* and *Neurospora crassa* have provided a wealth of knowledge into RNA regulation in fungi, yet our understanding of many of these same systems in fungal pathogens such as *Candida albicans* and *A. fumigatus* is still lagging. We do know that there can be differences between RNA regulatory pathways in pathogens and non-pathogens. For example, the model filamentous fungus *Aspergillus nidulans* has a functional

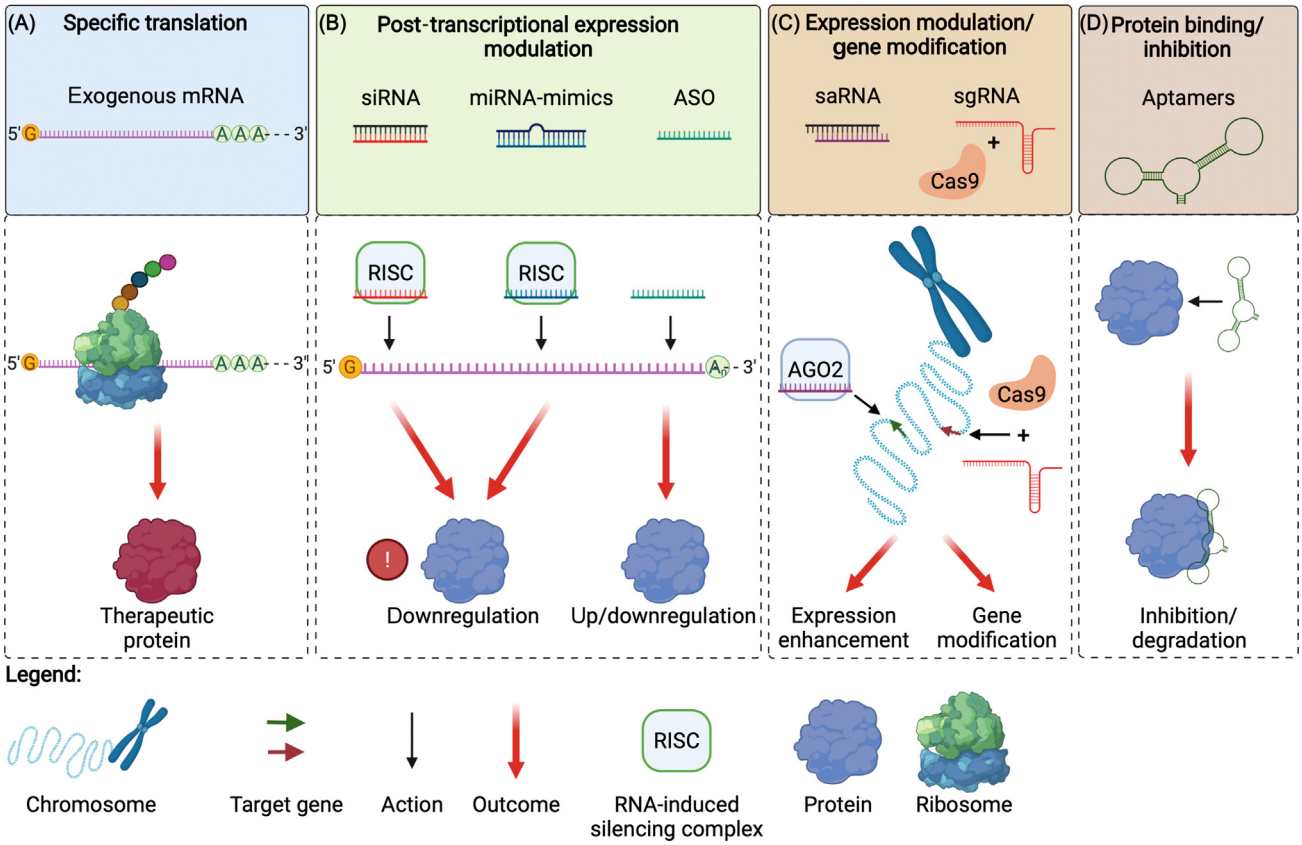

Figure 2. Common mechanisms of RNA-based therapeutics. Listed are four mechanistic examples of common approaches applied in RNA-based therapeutics. RNA-based therapeutics are currently used, in a number of forms, to introduce an mRNA for translation of a specific product, to modify gene expression, and even inhibit protein activity by direct binding. Panel (A) shows an example of specific translation in which an exogenously delivered mRNA can be used to produce a therapeutic protein, such as the spike protein of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Panel (B) depicts post-transcriptional modulation by small interfering RNAs (siRNAs), microRNA (miRNA) mimics, or antisense oligonucleotides (ASOs). Panel (C) shows how RNA-based therapeutics can be used to promote gene expression with short activating RNAs (saRNAs) or modify genes with clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9. Finally, Panel (D) depicts how RNA aptamers can be used as therapeutics to trigger inhibition or degradation of a target protein. Together, these provide just a few examples of how RNA-based therapeutics are revolutionizing treatment of disease. The figure was created with BioRender.com.

RNAi system, as does its close relative, *A. fumigatus*; however, several RNAi proteins in *A. nidulans* are truncated compared with those of *A. fumigatus* [43]. The practical importance of these differences in RNAi functionality remain incompletely described in these systems. *A. nidulans* is not the only example of a modified RNAi system. In fact, the model yeast *S. cerevisiae* has actually lost the RNAi machinery in favor of maintaining a killer virus that provides a growth advantage [44]. Other yeasts, such as *C. albicans* and *Cryptococcus neoformans*, typically have a functional RNAi system [45], but examples of RNAi loss do exist in pathogenic yeast as well [46]. Studies across the fungal kingdom have revealed that the RNAi system appears relatively plastic and can be repurposed or supplemented with additional proteins to provide additional functionality [47,48]. The biological importance and conservation of the RNAi system in many fungal pathogens, including organisms such as *Mucor circinelloides* [49] and *C. neoformans* [46], suggests that it may be a suitable target to exploit for RNA-based therapeutics in the future.

Production of fungal long noncoding RNAs and their downstream regulatory functions also provide an attractive ingress for RNA-based therapeutic intervention. Long noncoding RNAs are

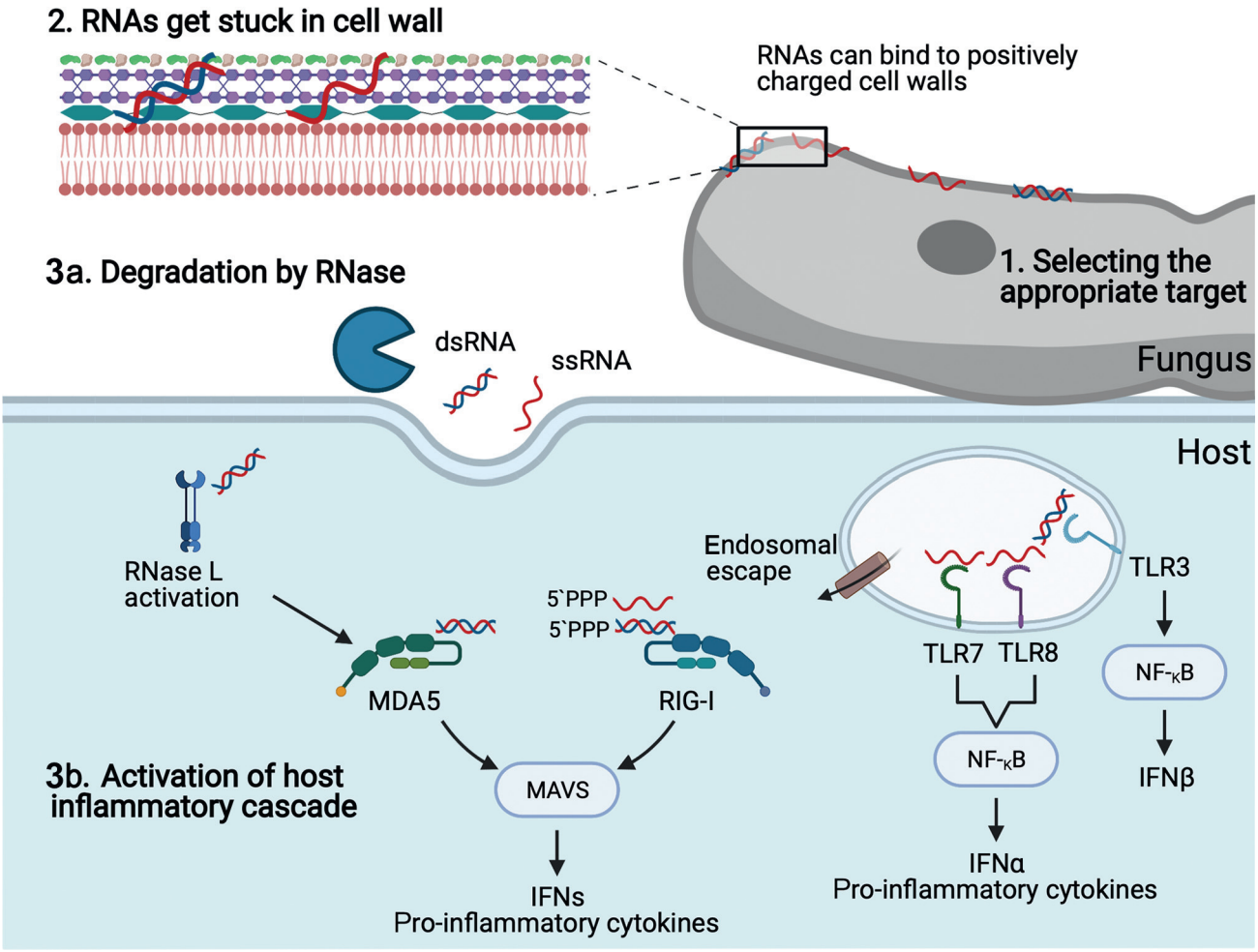

Figure 3. Model of barriers associated with treating fungi with RNA. Several key barriers exist to the development of RNA-based therapeutics against human fungal pathogens. (1) We need to improve our understanding of RNA regulation in human fungal pathogens to facilitate identification and exploitation of potential targets. (2) RNA must be directed to the appropriate site of infection and limit activation of host inflammatory cascades. (3) The RNA therapeutic must be resistant to degradation by host- or pathogen-produced RNases and be capable of entry into the fungal cell across what is oftentimes a robust cell wall. Ultimately, specific targets within the fungal pathogen must be targeted to limit off-target effects and promote growth arrest or death of the target pathogen. Despite these challenges, numerous solutions are available that make RNA-based therapeutics a promising way forward in treating human fungal infections. The figure was created with BioRender.com. Abbreviations: dsRNA, double-stranded RNA; IFN, interferon; MAVS, mitochondrial antiviral-signaling protein; MDA5, melanoma differentiation-associated protein 5; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; RIG-I, retinoic acid-inducible gene I; ssRNA, single-stranded RNA; TLR, Toll-like receptor.

known to be highly sequence divergent, even between closely related species, making them intriguing targets for sequence-specific RNA-based drugs. Additionally, these long noncoding RNAs often exhibit important regulatory functions despite frequently being transcribed at low copy numbers per cell. Although poorly studied so far in human pathogens, there are some hints that long noncoding RNAs can contribute significantly to regulation of fungal pathogenesis. As just one example, the long noncoding RNA RZE1 of *C. neoformans* was previously shown to be a potent regulator of the morphological switch between yeast and hyphae – a feature with obvious importance for virulence [50]. Efforts are now underway in many organisms to better define the long noncoding RNA repertoire and uncover the diversity of regulatory cascades that remain hidden in this varied set of pathogens. Clearly, more mechanistic research into fungal RNA

biology is essential to expand our understanding of appropriate targets and determine the potential of existing classes of RNA for future RNA-based therapeutics.

The second major roadblock to delivery of antifungal RNAs is the diverse cell wall and capsule structures of fungal pathogens. Fungi come in many forms, ranging from yeasts to **hyphae**, to impermeable, stress-resistant **spores**. As one example, the hyphal cell wall of *A. fumigatus*, composed primarily of positively charged galactosaminogalactan, rapidly binds free, negatively charged nucleic acids and provides a strong barrier against nucleic acid uptake [51]. Ultimately, some RNA does enter fungal hyphae, and these small RNAs have a limited ability to silence gene expression. In addition to the cell wall as a barrier, some fungi promote formation of even more complicated 3D structures in the host, such as nodules, biofilms on catheters, or even granulomas composed of host immune cells. These forms remain clinically challenging even under the best circumstances. Delivery of RNA into fungal pathogens may limit the effectiveness of RNA therapeutics to a subset of fungal pathogens that readily take up RNA, as in plant fungal pathogens [23].

The third set of obstacles relates to difficulties in delivering RNA into specific human sites of infection without stimulating an unwanted immune reaction. Fungal infections can spread throughout the human body, but most commonly begin on the skin, lungs, and gastrointestinal tract (Figure 1). In addition to getting an RNA therapeutic specifically to the site of infection, the RNA must be modified or protected to prevent activation of sensitive host pathogen recognition receptors such as Toll-like receptor (TLR) 3, retinoic acid-inducible gene I (RIG-I), and melanoma differentiation-associated protein 5 (MDA-5), which typically recognize double-stranded RNA molecules to promote an aggressive inflammatory response [52]. Delivery of a therapeutically active RNA into a pathogen at the site of infection without triggering an overreactive immune response will be challenging, yet advances in RNA delivery, modification, and synthesis suggest that there is, in fact, a path forward for treating human fungal disease with RNA-based drugs.

The way forward in fighting human fungal pathogens with RNA

Despite the challenges, RNA-based therapeutics do offer unique advantages for the treatment of fungal infections. In particular, RNA is easy to synthesize, fast to adapt to evolving pathogens or after development of antifungal resistance, and it can offer specificity against targets at the sequence level, where variation can exist even in highly conserved pathways. Although barriers are in place, numerous innovative biochemical and biological solutions are already in development in related systems as described earlier – solutions that might be adapted for the treatment of fungal infections. Replacement of uridine with modified N1-methyl-pseudouridine in the mRNA vaccines against SARS-CoV-2 limits recognition by host nucleic acid sensors and aberrant activation of host inflammatory cascades [53–55]; the nucleic acid phosphate backbone is modifiable with 2′-O-methylation to minimize immunogenicity [52]; and advances in RNA synthesis and *in vitro* transcription can limit production of unwanted immunogenic byproducts such as double-stranded RNA [56]. Combined, these developments seem likely to change the way we treat all sorts of diseases in the future, but additional research is required to see how they can be applied to the treatment of human fungal pathogens.

The fungal cell wall remains a formidable obstacle. To facilitate delivery of RNA into fungal pathogens, new approaches will have to be developed to cross this barrier. The protection of RNA in modified liposomes and nanoparticles, or conjugation to peptide aptamers and antibodies, has improved delivery to both mammalian cells [14] and bacterial pathogens [57,58], but the utility of these approaches in crossing more complicated fungal cell walls remains unclear. Synthesized nanoparticles coupled to RNA can be produced in a variety of potential formulations [59] but few

studies exist testing these particles for uptake by fungi. Decoration of nanoparticles with protein receptors or viral proteins is another option that has been considered but only a few extracellular mycoviruses are known at this time that might be leveraged for these purposes [60]. In other systems, researchers are turning to naturally evolved delivery mechanisms for inspiration, such as **extracellular vesicles**. New approaches using engineered extracellular vesicles are compelling [61], but additional study is required to identify their full potential as therapeutics. We do know that the host produces extracellular vesicles in response to infection with *A. fumigatus*, *C. albicans,* and *C. neoformans* that can modulate both fungal and host behavior [62–64]. Finally, coupling RNA-based therapeutics to enzymes or compounds that can degrade the cell wall may serve as an additional strategy for delivery.

If delivery of an RNA into fungi proves too challenging, alternative host-directed strategies could still be advantageous. Vaccines have been attempted against many fungal pathogens with little success [65,66], but the rapid advances in mRNA vaccines could provide a new tactic [67]. Alternatively, manipulation of the host to recruit additional immune cells, or modulate behavior, remain interesting [68]; however, we still have much to learn about the immunological response to fungal pathogens in humans before these host-directed strategies will come to fruition. A recent report investigated RNA tertiary structures, namely, group II intron **ribozymes**, as potential target sites for chemical antifungal therapeutics. Target specificity and catalytic activity inhibition of the ribozyme was demonstrated, which suppressed growth of the pathogen *Candida parapsilosis* [69]. This finding may offer new opportunities for RNA-based treatments by enlarging the repertoire of possible target sites in fighting fungal infections.

Concluding remarks

RNA-based therapeutics are rapidly changing the way we treat disease, from siRNAs-based drugs to treat liver diseases to mRNA vaccines that provide protection against SARS-CoV-2. As these treatment options mature, opportunities will become available to extrapolate these advances to the treatment of human fungal infections. We hope that by bringing attention to the possibility of using RNA-based therapeutics to treat fungal infections, the fungal research community and clinical infrastructure will be poised to adapt and quickly advance these gains into meaningful treatment options for this often devastating, but certainly under-studied class of difficult-to-treat infections.

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Declaration of interests

There are no interests to declare.

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Outstanding questions

How can we select appropriate targets for RNA-based therapeutics against human fungal pathogens?

Will promoting a more robust immune response, or production of additional immune effector cells, serve as a host-directed strategy towards treating fungal infections?

How can the targeting of RNA therapeutics be improved to direct treatment to specific tissues, organs, or pathogen cells?

How do we facilitate RNA passage across the diverse fungal cell wall structures?

Will strategies used to make RNA immunologically inert still allow these modified RNAs to inhibit/modulate fungal gene expression pathways?

Can RNA-based therapeutics be coupled to, or loaded into, nanoparticles or extracellular vesicles to promote delivery into fungal pathogens?

Is it possible to adapt existing strategies designed and optimized for genetic diseases, cancers, or viral infections to target specific fungi instead?

Will facilitating endosomal escape of RNA from fungal endosomes or endosome-like structures prove as difficult as it has for human cells?

Can RNA-based therapeutics be made cost-effective enough for low-income countries?

Will we be able to design robust RNA-based therapeutics against currently unculturable pathogens from genome sequences alone, for example, *Pneumocystis jirovecii*?

How will the high adaptability of RNA-based therapeutics impact the drug approval process? Does every sequence/nucleotide change justify a revisiting of the clinical trials for a previously approved pharmaceutical?

How fast can fungal pathogens evolve new strategies to circumvent the effect of RNA-based therapeutics?

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Are enzymatically active RNAs (e.g., ribozymes, riboswitches) a useful addition to the repertoire of RNA-based therapeutics? What could their roles be in therapeutic context?

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