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RNAi Technology: A Novel Approaches Against Fungal Infections

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Abstract

Despite the introduction of new antifungal agents, resistances to antifungal therapy continue to increase and outcome of invasive fungal infections treatment is frequently suboptimal. A large amount of the recent effort in antifungal drug discovery has focused on a limited set of targets with functions known or expected to be important for fungal viability and virulence. A variety of techniques can be used to identify fungal genes of interest. Gene expression profiling, RNA mediated gene silencing and insertional mutagenesis are three main molecular genetics technologies used to identify and validate antifungal drug targets. The term of RNA interference (RNAi) refers to a cellular process by which a sequence-specific double-stranded RNA (dsRNA) inhibits the expression of a gene. This mechanism is strongly conserved in eukaryotes and has been documented to be existed in different fungal species such as *Candida albicans*, *Aspergillus nidulans* and *Penicillium marneffei*. Many vital and virulence genes have been successfully knocked down using RNAi technology. RNAi would be regarded as a promising approach for discovery of new gene targets for the design of fungus-specific antifungal agents. Here we discuss about a novel approach and its application in designing new molecular antifungal targets.

Keywords: RNAi; Fungal infections; siRNA; Antifungal drugs

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Introduction

Changing patterns of invasive fungal infection (IFI) epidemiology and drug susceptibility would result in complication in management (1, 2). Moreover, a general lack of available or well standardized diagnostic techniques for early detection and identification of IFI make management more elaborated (3-5). Currently available antifungal drugs are different in their spectrum of activity, dosing, safety profiles, pharmacokinetic/pharmacodynamic (PK/ PD) properties, and cost (6); however, they are few and their targets are mainly restricted to the cell membrane and cell wall (7). Additionally, these drugs have several limitations including toxicity, resistance and high cost (7). In large scale, matching the patient and fungal disease with an antifungal regimen as well as delayed initiation of antifungal treatment would affect IFI management (8-10). Depending on their antifungal properties, these drugs are used in specific clinical situations and for specific fungal species (11). Nevertheless, this antifungal therapy is not always effective. So that fungal cells are able to overcome the growth inhibitory action of antifungals by the development of various resistance mechanisms which will allow them to remain alive at a higher drug concentration (11). Therefore, new drugs that aim at novel pathways, other than the currently available antifungal drugs, are greatly needed (7). Basically, good fungal drug targets are either gene which their products are essential for viability or virulence / pathogenicity of fungi. To find the targets, a variety of RNA-mediated gene silencing or knockout methods that inhibit genes at the post-transcriptional level have been identified in different organisms including fungi (12).The most common forms involve the introduction of antisense RNA, double-stranded RNA (dsRNA) (also known as RNA interference or RNAi) and sense transgenes (also known as co-suppression in plants or quelling in

fungi) (12). RNAi has attracted considerable attention as a means of inhibiting the expression of specific genes and thus as a potential tool for identifying and validating drug targets. The story of RNAi in fungi began with a finding by Romano and Machino in 1992 (13), whereby the expression of the *al-1* gene, an endogenous gene involved in carotenoid biosynthesis, was attenuated by a transformation with homologous al-1 sequences in the fungus *Neurospora crassa*. This phenomenon was designated as quelling. A series of remarkable studies on quelling-deficient mutants of *N. crassa* has revealed the involvement of RNA dependent RNA polymerase (RdRP) and argonaute protein in the pathway, providing molecular evidence that quelling belongs to a broad category of RNA-mediated post-transcriptional gene silencing, as typified by RNAi (14). Therefore, here we reviewed an alternative approach for effective, specific and safe treatment of IFI known as post transcriptional gene silencing.

Trend in antifungal resistance in fungi

Despite the introduction of novel antifungal agents, resistances to antifungal therapy increased dramatically and outcome of invasive fungal infections (IFI) treatment is frequently suboptimal (2, 6). Available antifungal agents used for the management of IFIs include polyenes (liposomal and deoxycholate amphotericin B), triazoles (fluconazole, itraconazole, voriconazole, and posaconazole), echinocandins (caspofungin, micafungin, and anidulafungin), and flucytosine (15, 16). However, there has been no such ideal agent that can be used broadly in different and complex patient so far.Resistance to antifungal drugs, which is characterized by elevated level of minimal inhibition concentration measured in reference susceptible organisms (17), has been reported in clinical situations for three antifungal categories which prescribed the most: the polyenes, pyrimidine analogues (5-fluorocytosine) and the azoles (Table 1) (18).

In the most clinical cases, antifungal resistant isolates have been reported in the class of azoles. Acquisition of azole resistance in fungal species has been observed mostly in *Candida* species including *C. albicans*, *C. glabrata*, *C. dubliniensis*, *C. tropicalis* and, less frequently, in *Cryptococcus neoformans* (19). Acquisition of azole resistance in filamentous fungi such as *Aspergillus fumigatus*, which causes invasive aspergillosis,has also been observed in a hematopoietic stem cell transplant recipient after azoles therapy (20). Toxicity is another important issue which must to be considered when individualizing antifungal therapy. For example, deoxycholate amphotericin B and, to a lesser extent,lipid formulations of amphotericin B are associated with nephrotoxicity (21, 22) and this information should be considered taking medication in a patient with significant renal insufficiency. Similarly, cyclodextrin in the IV formulation of voriconazole has been shown to accumulate in patients with moderate to severe renal dysfunction (23).

Novel molecular targets for antifungal drugs

A large amount of the recent effort in antifungal drug discovery has focused on a limited set of targets with functions known or expected to be important for fungal growth (24). Currently used antifungal drugs have essentially four molecular targets: sterol-14a-demethylase (azoles), ergosterol (amphotericin B), β-1, 3-glucan synthase (echinocandins), DNA and RNA synthesis (flucytosine).

To address the problems of emerging drug-resistance and high toxicity of some of the current drugs, it is now generally believed that the development of new drugs directed against novel genomics-based targets is critical (25).

A variety of techniques can be used to identify fungal genes of interest. There are basically two classes of targets to evaluate: genes essential for viability and genes essential for virulence/ pathogenesis factors (12, 26). Although the virulence factors are usually pathogen-specific and not found in the human host, chance of finding a virulence factor that is conserved among all fungal pathogens is low. Hence, it seems to be complicated to find suitable virulence factor for designing a broad-spectrum antifungal agent (12). There are three main molecular genetics technologies used to identify and validate antifungal drug targets: gene expression profiling, RNA mediated gene silencing and insertional mutagenesis (12). Here we limit ourselves to RNA mediated gene silencing.

RNA mediated gene silencing approach

Several methods that inhibit genes at the post-transcriptional level have been identified in different organisms. In 1998, Andrew Fire and Craig Mello showed that double-stranded RNA molecules could inhibit the expression of homologous genes in eukaryotes (27-29). The most common forms of post-transcriptional gene silencing (PTGS) methods involve the introduction of antisense RNA, double-stranded RNA (dsRNA) (also known as RNA interference or RNAi) and sense transgenes (also known as co-suppression in plants or quelling in fungi) (12).

The term RNA interference (RNAi) refers to a cellular process by which a sequence-specific double-stranded RNA (dsRNA) inhibits the expression of a gene (Figure 1). This mechanism is strongly conserved in eukaryotes and probably acts as a protection tool against virus mobile genetic elements such as transposons (29). In *A. nidulans*, mycovirus

defense was demonstrated via the RNAi pathway (30) Similarly, The expression of the RNAi component genes was up-regulated by long dsRNA and upon viral infection in *N.crassa* and *Cryphonectria parasitica* (31, 32). Originally, RNAi mechanism was observed in plants (29), but correctly described for the first time in the late 1990s for the nematode *Caenorhabditis elegans* (33) by Andrew Fire and Craig Mello. (27, 28).

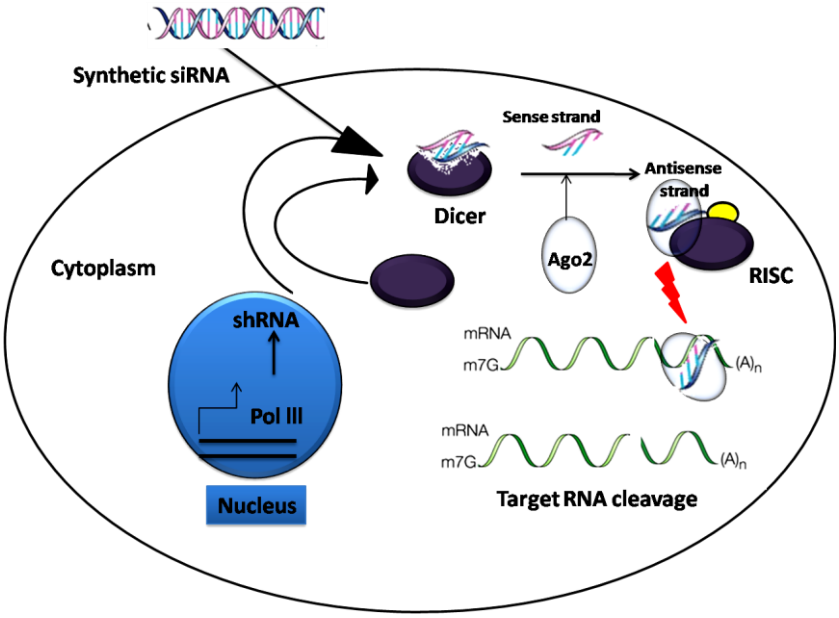

Figure 1. Simplified model of the RNAi mechanism. After uptake of the chemically synthesized siRNAs into the cells, Dicer-mediated cleavage takes place. One strand of the siRNA duplex (the guide strand) is loaded onto an Argonaute protein at the core of an RNA-induced silencing complex (RISC). (For simplicity, in the figure, the RISC is represented just by an Argonaute protein.) Argonaute loading takes place in the RISC-loading complex, a ternary complex that consists of an Argonaute protein, Dicer and a dsRNA-binding protein (known as TRBP in humans). During loading, the non-guide (passenger) strand is cleaved by an Argonaute protein and ejected. The Argonaute protein then uses the guide siRNA to associate with target RNAs that contain perfectly complementary sequence and then catalyses the slicing of these targets. After slicing, the cleaved target RNA is released, and the RISC is recycled for another round of slicing.A longer term inhibition of gene expression can be accomplished when a shRNA is expressed intracellular instead of by the exogenous application of a siRNA.

RNAi poses several advantages which differentiated it from other gene knockout methods. In contrast to gene disruption, RNAi does not rely on the locus for gene silencing, instead, it depends on the sequence of the target and owing to this fact; post-transcriptional gene silencing (PTGS) is considered as an attractive alternative (34). Moreover, the sequence-specific characteristic of RNAi is highlighted in gene family studies. RNAi suppresses gene expression in a sequence manner; moreover, family members sharing high sequence similarities, so a functionally redundant gene family, such as aflatoxin biosynthesis pathway, can be simultaneously silenced by a single RNAi construct (35, 36). For fungi or fungi-like species that have multinuclear heterokaryotic mycelia, RNAi possibly offers a valuable gene analysis tool since the RNAi machinery is known to degrade cognate mRNA in the cytoplasm and, therefore, is likely to be operative against any mRNA in multinuclear heterokaryotic mycelia. Inter-nuclear transfer of gene silencing was shown in heterokaryotic *N. crassa* strains and in the multinuclear heterokaryotic Oomycete *Pythophthora infestans* (37, 38).

The History of RNAi in Fungi

After the discovery of RNAi in 1998 (29), attempts were made to employ this technology for controlling gene expression in a variety of fungal species. Consequently, suppression of gene expression by a dsRNA-expressing plasmid or related-system has been shown in many fungal species in different order like Ascomycota, Basidiomycota, and Zygomycota (34, 39-44), and as well as the fungus-like Oomycota (37). Involvement of typical RNA silencing protein components such as dicer in the silencing phenomena was shown in *A. nidulans*, *Magnaporthe oryzae* (*M. grisea*) and *N. crassa* (40, 45-47), and biogenesis of siRNA was detected in *A. nidulans*, *M. oryzae*, *Mucor circinelloides*, *N. crassa*, and *Schizosaccharomyces pombe* (40, 41, 48-50). Therefore, the fundamentals for RNA silencing seem to be conserved in most of fungal species with some exceptions (Table 2).

Table 1. Activity of antifungal agents against principal fungal pathogens

Spectrum	Antifungals
Broad activity against *Candida* spp., (except *C. lusitaniae*), *Cryptococcus neoformans* and filamentous fungi (except in the *Aspergillus* spp. *A. terreus* and *A. flavus*).	Polyenes - Amphotericin B
Active against *Candida* spp. and *Cryptococcus* spp., however rapid emergence of resistance can appear when 5-FC is used as monotherapy.	Pyrimidines analogues - 5-fluorocytosine (5-FC)
Active against *Candida* spp. and *Cryptococcus* spp., less active against *C. glabrata* and no activity against *C. krusei*. No activity against filamentous fungi.	Azoles - Fluconazole
Like fluconazole, but enhanced activity against filamentous fungi	- Itraconazole
Like fluconazole, but enhanced activity against filamentous fungi, including *Aspergillus* and *Fusarium* spp.	-Voriconazole
Active against most dermatophytes, poor activity against *Candida* spp.	Allylamines - Terbinafine
Active against most dermatophytes, poor against *Candida* spp.	Morpholines - Amorolfine
Active against *Candida* spp. with fungicidalactivity and moderately active against *Aspergillus* spp., poor activity against *Cryptococcus neoformans*.	Echinocandins - Caspofungin

RNAi has attracted considerable attention as a potential tool for identifying and validating drug targets. The basic process involves a dsRNA that is cleaved into small (double-stranded) interfering RNAs (siRNAs) that guide recognition and targeted cleavage of homologous mRNA (51). The siRNAs are thought to provide the sequence information that allows a specific mRNA to be targeted for degradation. During the recent years, significant progress has also been made towards the application of PTGS in the clinically important fungi such as *C. albicans*, *Cryptococcus neoformans* (42, 52) and *Aspergillus* spp (53, 54).Both antisense and RNAi-based functional genomics approaches thus become feasible in the pathogen *C. neoformans* (55).Expression of long dsRNAs corresponding to portions of the cryptococcal *CAP59* and *ADE2* genes results in reduced mRNA levels for those genes, with phenotypic consequences similar to that of gene disruption (42). In 2001, De Backer et al (56) reported an anti-sense based functional genomics approach for the identification of genes which are critical for growth in *C. albicans.* To support the functionality of the approach, many known essential *C. albicans* genes were isolated in this screen. Accordingly, more than half of the open reading frames (ORFs) that were identified had a completely unknown function and were selected as potential novel targets for antifungal drugs. Further improvements in, for example, transformation efficiency (12) might significantly facilitate this type of genome-wide functional screen in *C. albicans*. Other researchers demonstrated a rapid, cost-effective, easy delivery system for siRNA entrance (57). In *C. albicans*, one of the most important genes which play a significant role in yeast pathogenesis has been knocked down by using RNAi technology (57-59). A true genome-wide antisense-based screen to identify novel drug targets in *Cryptococcus* has not been reported yet. However, it has been reported that two essential genes, calcineurin A (*CNA1*) and laccase (*LAC1*) were successfully silenced by this approach in *C. neoformans* (52). Antisense RNA-mediated inhibition of gene expression has also been demonstrated in pathogenic fungi *A. nidulans*, but not as yet with the aim to identify new drug targets or to devise a genome-wide functionality screen (53).

The proteins that are crucial for the RNAi pathway are present in *S. pombe*. Indeed, it has recently been documented that RNAi machinery is required for heterochromatic gene silencing in fission yeast (49) and is associated with small interfering RNAs (siRNA) homologous to these centromeric regions (60). In addition, it has been shown that the formation of heterochromatin at the silent mating type region (61) and accurate chromosomal segregation during mitosis requires the RNAi machinery. Although involvement of dsRNAs has not yet been directly demonstrated in gene regulation in this model yeast system, it has been shown that this mechanism does exist and involve in gene regulation in the yeast *C. albicans.* It has been documented that *EFG1* gene, an essential regulator of morphogenesis and virulence in *C. albicans*, was successfully knocked-down using synthetic dsRNAs (57).

Accordingly, a strong *EFG1* gene silencing (>80% reduction) induced in a large number of transfected yeasts and consequently decreasing in Efg1 protein (Efg1p) level in the cells led in a significant reduction in yeast germination and in *SAP5* and *ALS3* gene expression (>95% reduction) (59).

Table 2: Various types and methods applied for gene silencing in fungi and fungus-like organisms

Species	RNAi trigger	Transformation	Reference
Coprinus cinereus	IR*	Lithium acetate method	(42)
Neurospora crassa	Homologous transgene	PEG-mediated method	(13)
Schizophyllum commune	IR	PEG-mediated method	(68)
Magnaporthe oryzae	IR	PEG-mediated method	(41)
Mucor circinelloides	Homologous transgene	PEG-mediated method	(48)
Mortierella alpina	IR	Microparticle bombardment	(87)
Phytophthora infestans	Homologous transgene	PEG-mediated method	(88)
P. infestans	Homologous transgene	Electroporation	(89)
P. infestans	dsRNA	Lipofectin-mediated transfection	(90)
Dictyostelium discoideum	IR	Electroporation	(91)

* IR, hairpin RNA or inverted repeat RNA-expressing plasmid.

A *lacZ* fission yeast model has previously been employed to investigate features of antisense RNA technology in vivo (62). It has been shown that gene inhibition is dependent on the dose of antisense RNA while co-localisation of antisense and target genes does not affect the level of target gene suppression in this system (63). Additionally, the size of the antisense transcript (64, 65) and the region to which it is targeted (66) can affect the efficacy of target gene inhibition. Table 3 summarized the genes silenced by RNAi technology.

RNAi delivery systems used for fungal cell

A hairpin RNA (hpRNA)-expressing plasmid

The first example of fungal RNAi by an hpRNA-expressing plasmid was demonstrated by Liu et al. in *C. neoformans* (42). To date, plasmid constructs expressing hpRNA or intron-containing hpRNA (ihpRNA) are the most prevalent and reliable platforms to induce RNAi in fungi (41, 67-70).

To facilitate the construction of an ihpRNA-expressing plasmid for cloning, the versatile vector pSilent-1 (available from the Fungal Genetic Stock Center (http://www.fgsc.net/) was developed for Ascomycete (58). In addition, the Gateway technology into pSilent- 1 in order to establish the high-throughput RNAi vectors such as pTroya and pFANTAi4 have been recently introduced (71, 72). Those vectors are applicable for large-scale functional genomics in yeasts and filamentous fungi.

RNAi using an opposing-dual promoter system

While an ihpRNA-expressing plasmid is useful for inducing RNAi in fungi, its applicability is generally limited because the construction of such vectors normally requires two steps of orientated cloning. As mentioned above, the Gateway technology is a solution for this limitation. As an alternative, RNAi vectors with an opposing-dual promoter system, which enables vector construction with a single, non-orientated cloning step, have been developed for *Histoplasma capsulatum*, and *C. neoformans* (73) (I Bose, TL Doering, unpublished data). In these systems, sense and antisense RNA of the target gene, which is expected to form dsRNA in the cell, are transcribed independently under the control of the two opposing RNA polymerase II. Recently, pSilent–Dual1 (pSD1), carrying opposing *trpC* and *gpd* promoters, has been constructed and used in *M. oryzae* (74).

However, similar to the case of *H. capsulatum*, the efficacy of gene silencing by pSD1 was generally lower than that exhibited by ihpRNA expressing vectors. A fragment of *eGFP* gene has been introduced into pSD1to facilitate efficient screening for strongly silenced transformants. In this screening system GFP fluorescence provides an effective indicator to select transformants in which interference was operating. This cosilencing-based screening has been successfully demonstrated in *C. neoformans*, *Venturia inaequalis*, *Acremonium chrysogenum*, and *Blastomyces dermatitidis* using *GFP*, *DsRed* or endogenous maker genes (39, 42, 72, 75).

Direct delivery of siRNA/dsRNA into the fungal cells

Even though the direct delivery of synthetic siRNA to cultured cells is a common method to introduce RNAi in mammalian systems, to date; such applications have been very rarely reported in fungi. This delivery system has been successfully used in *C. albicans* and *A. nidulan* (57, 71, 76).

In *A. nidulan*, ornithine decarboxylase (*ODC*), a key polyamine biosynthesis gene was specifically silenced by treating germinating spores with synthetic 23 nt siRNA duplex with 2 nt overhangs at the 3'-end (76). EFG1 gene which is responsible for encoding Efg1p, an essential factor for filamentous growth in *C. albicans*, was successfully silenced with synthetic 19 nt siRNA duplex with 2 nt overhangs at the 3'-end (57).

Table 3: Genes targeted for RNAi technology applied in pathogenic fungi

Species	RNAi trigger	Transformation	Target gene(s)	Target function	Reference
Histoplasma capsulatum	IR*	Electroporation	*AGS1*	Alpha-(1,3)-glucan synthase, a cell wall polysaccharide	(73)
	IR	Electroporation	*YPS3*	A cell wall protein produced only during the pathogenic yeast phase	(92)
Aspergillus nidulans	IR	PEG-mediated method	*CreA*	The major carbon catabolite repressor involve in a substantial increase in the levels of glucose-repressible enzymes	(53)
Aspergillus nidulans	Synthetic siRNA	Uptake from culture medium	*SidB*	Septation, conidiation and vegetative hyphal growth	(71)
Aspergillus nidulans	Synthetic siRNA	Uptake from culture medium	*ODC*	Ornithine decarboxylase	(76)
Aspergillus flavus *Aspergillus parasiticus*	Synthetic siRNA	Lipofection	*aflD*	Involve in aflatoxin production	(93)
Candida albicans	Synthetic siRNA	Lithium acetate method	*EFG1*	Involve in morphogenesis and pathogenesis	(57)
Penicillium marneffei	IR	Not mentioned	*acuD*	Involve in glyoxylate cycle	(94)
Penicillium chrysogenum	IR	Protoplast	*cefEF*	Cephalosporin production	(95)
Blastomyces dermatitidis	IR	Not mentioned	*Cdc11* homolog	Septin; Role in morphogenesis and Sporulation	(96)
	IR	Not mentioned	*BAD1*	Blastomyces adhesin 1	
Cryptococcus neoformans	IR	Electroporation	*CAN1*	Calcineurin A	(42)[6]
	IR	Electroporation	*LAC1*	Laccase	

* IR, hairpin RNA or inverted repeat RNA-expressing plasmid.

Efg1p has been discovered as a key regulator of a number of cellular processes in the human fungal pathogen *C. albicans*.

It is required for the development of a true hyphal growth form which isessential for interactions with human host cells and the yeast's pathogenesis as well as for chlamydospore formation (77-80). Reduced levels of *EFG1* expression suppress hyphal rather than psudohyphal formation. Not only does Efg1p regulates the hyphal formation, but also regulates the expression of several genes that play leading roles in host cells invasion or biofilm formation. For instance, the expression of the *SAP4*, *SAP5* and *SAP6* proteases, which are involved in host cells invasion, are upregulated by *EFG1* during hyphal growth in vitro (81, 82). Furthermore, adhesions Als1p, Als3p and HWp1p are required for biofilm formation and their expression is reliable on *EFG1* gene expression (83-85). The other notable role of *EFG1* gene is its direct involvement in phenotype switching. Over expression of this gene in strain WO-1 stimulates opaque-phase cells to switch to the white phase (86). Accordingly, *EFG1* gene may be considered as a novel target for antifungal drug. Those results indicated that siRNA duplex could be taken up by germinating fungal spores from the culture medium, providing a rapid and convenient method to induce RNAi in fungal cells.

Conclusion

RNAi is a potentially powerful tool for a wide variety of gene silencing applications. As described, the RNAi and gene disruption methods differ in principle, and therefore, have their own strengths and limitations. From the point of view of accuracy, gene destruction methods generally give better data than RNAi. The drawbacks of RNAi, such as incomplete repression and possible unintended targets, are often described for pharmacological studies. RNAi should be proven useful for the studies of functional genomics in fungi which can provide novel and rapid gene analysis applications that gene disruption methods cannot offer. To make RNAi a better tool for gene function analysis in fungi, the next challenges are to know the extent of off-target effects in fungal cells. Another point is to develop an inducible RNAi system with a combination of a strictly controlled promoter and a convenient inducer which can be used for a wide range of fungi. In conclusion, RNAi can be regarded as a promising approach for discovery of

new gene targets for the design of fungus-specific antifungal agents.

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