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MINI REVIEW

Modes-of-action of antifungal compounds: Stressors and (target-site-specific) toxins, toxicants, or *toxin–stressors*

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Abstract

Fungi and antifungal compounds are relevant to the United Nation's Sustainable Development Goals. However, the modes-of-action of antifungals—whether they are naturally occurring substances or anthropogenic fungicides—are often unknown or are misallocated in terms of their mechanistic category. Here, we consider the most effective approaches to identifying whether antifungal substances are cellular stressors, toxins/toxicants (that are target-site-specific), or have a hybrid mode-of-action as *toxin–stressors* (that induce cellular stress yet are target-site-specific). This newly described '*toxin–stressor*' category includes some photosensitisers that target the cell membrane and, once activated by light or ultraviolet radiation, cause oxidative damage. We provide a glossary of terms and a diagrammatic representation of diverse types of stressors, toxic substances, and *toxin–stressors*, a classification that is pertinent to inhibitory substances not only for fungi but for all types of cellular life. A decision-tree approach can also be used to help differentiate toxic substances from cellular stressors (*Curr Opin Biotechnol* 2015 33: 228–259). For compounds that target specific sites in the cell, we evaluate the relative merits of using metabolite analyses, chemical genetics, chemoproteomics, transcriptomics, and the target-based drug-discovery approach (based on that used in pharmaceutical research), focusing on both ascomycete models and the less-studied basidiomycete fungi. Chemical genetic methods to elucidate modes-of-action currently have limited application for fungi where molecular tools are not yet available; we discuss ways to circumvent this bottleneck. We also discuss ecologically commonplace scenarios in which multiple substances act to limit the functionality of the fungal cell and a number of as-yet-unresolved questions about the modes-of-action of antifungal compounds pertaining to the Sustainable Development Goals.

INTRODUCTION

Against the backdrop of deteriorating planetary health and the unsustainably large human population, fungi and fungicides are implicated in most of the 17 Sustainable Development Goals (SDG) of the United Nations (Sachs et al., 2022). This is because:

- poor regions need to become self-sufficient in terms of agricultural productivity and food security, including safeguarding fungi needed for soil and plant health (Timmis & Ramos, 2021) and effective control of plant-pathogenic and food-spoilage fungi (SDG 1 'No poverty');
- fungi can be used as a food source and effective control of pre- and post-harvest fungi is needed in

relation to crop plants, animal feeds, and human foods (SDG 2 'Zero hunger');
- control of fungal pathogens of humans is needed including *Candida albicans*, *Cryptococcus gattii*, *Cryptococcus neoformans*, and fungi that are implicated in aspergillosis (Paulussen et al., 2017) and seborrhoeic dermatitis (Hay, 2011); fungi provide for bioprospecting (including new antibiotics, new fungicides, and new probiotics such as *Saccharomyces boulardii* var. *cerevisiae*); and fungi can be implicated in microbiome dysbiosis such as inflammatory bowel diseases (Ott et al., 2008; SDG 3 'Good health and well-being');
- microbes, including fungi, can be important as educational tools to increase microbial literacy (McGenity et al., 2020; Timmis et al., 2020; SDG 4 'Quality education');
- fungicides (and other pesticides) and fungal pathogens that can affect humans should be prevented from polluting bodies of water in the environment or otherwise contaminating water supplies (SDG 6 'Clean water and sanitation');
- *Saccharomyces cerevisiae* is useful for the production of bioethanol (SDG 7 'Affordable and clean energy');
- fungi for food and drinks—including alcoholic drinks—and for use in biotechnology (including white biotechnologies, production of fungi as inoculants of soils and plants and fungi as biocontrol agents of insect pests), as well as fungicide production, are economically important commercial sectors (SDGs 8 'Decent work and economic growth' and 9 'Industry, innovation and infrastructure');
- fungi that grow within the indoor environment need to be controlled to protect human health, and those that rot timber and damage other building materials should be controlled through the use of fungicides in paints or other antifungal treatments (SDG 11 'Sustainable cities and communities');
- fungi are a sustainable food source, a source of bioethanol (see above), and can be used to make sustainable construction materials (Jones et al., 2020; SDG 12 'Responsible consumption and production');
- fungi that provide essential services that contribute to soil and plant health are being impacted adversely by climate change and fungal pathogens that infect trees, crop plants, humans, and other animals are being advantaged by climate change (Kespohl et al., 2022; Smith & Casadevall, 2022; SDG 13 'Climate action');
- perturbations of underwater ecosystems due to pollution and/or climate change are damaging microbial communities and, in some cases, advantaging fungal pathogens that attach corals, fish, and other aquatic organisms (Landrigan et al., 2020; Moriarty et al., 2020; SDG 14 'Life below water'); and
- pollution and climate change are also damaging soils and other land-based ecosystems and in some cases favour fungal pathogens (Casadevall et al., 2019; Garcia-Solache & Casadevall, 2010; SDG 15 'Life on land'; see also Anand et al., 2023; Cavicchioli et al., 2019).

In the context of climate change, biodiversity loss, and the deterioration of environmental health, we advocate the development of fungicides that have negligible toxicity beyond their target group and are not environmentally persistent. Furthermore, the United Nations Biodiversity Conference Agreement (Montreal, Canada in December 2022 https://www.cbd.int/conferences/2021-2022/cop-15/documents) aims to reduce the use of chemical pesticides by 50% by 2030. It is therefore imperative to understand the modes-of-action of antifungals to make knowledge-based decisions about those that are safe to use and are environmentally sustainable. It is also necessary to understand modes-of-action to avoid or circumvent problems arising from the development of resistance to antifungals. Recent reviews of antifungal compounds have focused on those active against the Ascomycotina (Braga et al., 2022; Corkley et al., 2021; Zubrod et al., 2019). However, many of the fungi that are problematic in pre-harvest agriculture, feeds and foods, forestry, and in the built environment belong to the Basidiomycota. For example, members of the basidiomycete subdivisions Pucciniomycotina and Ustilaginomycotina are responsible for plant diseases and rot-fungi of the subdivision Agaricomycotina are responsible for wood decay.

Here, we evaluate the diverse approaches to identifying modes-of-action of antifungal compounds by determining whether they are stressors or target-site-specific toxins/toxicants or *toxin–stressors* (a term we use here to describe those inhibitors that have a hybrid mode-of-action) and, where applicable, to determine their target-specific mechanisms-of-action. We consider the use of metabolite analyses, chemical genetics, chemoproteomics, transcriptomics, and the target-based drug-discovery approach, focusing on both ascomycete models and the less-studied basidiomycete fungi. In addition, we discuss the ecophysiological complexity of antifungal substances in Nature, including those found in plants, and consider the implications for anthropogenic approaches to fungal control.

SIMULTANEOUS INHIBITION OF FUNGI BY MULTIPLE ANTIFUNGALS IN NATURAL HABITATS/ CONDITIONS

Even in simple microbial systems with relatively low diversity, such as some food and drink fermentations, microbial cells are subjected simultaneously to a mechanistically diverse array of antimicrobials including organic acids, biosurfactants, and alcohols and other chaotropic solutes (see Box 1), and hydrophobic

BOX 1 Glossary of terms.

Antifungal compounds: a generic term for substances—whether natural or anthropogenic—that inhibit or prevent cell division and growth of fungi (or a subset of fungi), and in some cases damage and kill fungal cells. See also 'Antimicrobial substances' and 'Fungicides'.

Antimicrobial substances: a catch-all term for substances—whether natural or anthropogenic—that inhibit or prevent cell division and growth of microbes (or a subset of microbes), and in some cases damage and kill microbial cells. See also 'Antifungal compounds', 'Biosurfactants', 'Cellular stressors', 'Chaotropic solutes', 'Fungicides', 'Hydrophobic stressors', 'Inhibitory substances', 'Toxic substances', 'Toxicants', '*Toxin–stressors*', and 'Toxins'.

Biosurfactants: microbial metabolites that have a hydrophilic moiety and a hydrophobic moiety, so reduce surface- and interfacial tension by acting as surfactants. Some biosurfactants have antimicrobial activity.

Cellular stress: a phenotypic condition that occurs when the cell is subjected to physical or physicochemical parameter(s)/forces (e.g., those related to stress parameters such as extreme temperature, pH, water activity, or chaotropicity and those related to cell turgor, shear damage or other mechanical forces, rehydration, freeze-thawing, and oxidative damage) that can reduce or prevent cell function(s), impair or damage cell membranes and macromolecules, elicit cellular responses including an increase in energy generation, and may be lethal (Hallsworth, 2018). Some cellular stressors exhibit multiple modes-of-action, such as ethanol, that is chaotropic and reduces water activity, and $MgCl_2$ that is chaotropic and causes osmotic stress (Alves et al., 2015; Cray et al., 2015; Hallsworth & Nomura, 1999). Arguably, all cells are perpetually stressed and there is no such thing as a stress-free condition (Hallsworth, 2018). See also 'Cellular stressors', 'Chaotropic solutes', 'Hydrophobic substances', 'Stress parameter' and '*Toxin–stressors*'.

Cellular stressors: substances that cause cellular stress, including those substances that reduce water activity, modify the cell's osmotic balance, exhibit chaotropic and/or hydrophobic properties, cause oxidative damage, and markedly increase ionic strength (e.g., Figure 1A–C). See also 'Biosurfactants', 'Cellular stress', 'Chaotropic solutes', 'Hydrophobic substances', and '*Toxin–stressors*'.

Chaotropic solutes: are those solutes that are typically less polar than water and increase the entropy of biomacromolecular systems thus increasing disorder in cell membranes and proteins (Figure 1A; Ball & Hallsworth, 2015).

Chaotropicity: the property of the phenomenon whereby biomacromolecules are disordered as described for 'Chaotropic solutes', see also 'Hydrophobic stressors' (Figure 1A,B).

Chemical genetics screening: the study of protein function and signal transduction pathways in cells by screening libraries of small molecules (Kubinyi, 2006).

Chemical proteomics (chemoproteomics): the proteome-wide study of protein targets of antifungal compounds or other biologically active molecules to identify modes-of-action of substances that damage, inhibit the functionality of and/or kill cells (Wright & Sieber, 2016).

Drug discovery: the process of finding new biologically active substances—whether natural or anthropogenic—that can be used as pharmaceuticals or for other purposes (including agricultural fungicides, and other pesticides).

Haploinsufficiency: a phenotypic condition where a dominant genetic trait is expressed in diploid organism that is heterozygous for a function/loss-of-function allele.

Hydrophobic stressors: those cellular stressors that partition into the hydrophobic domains of cell membranes and other biomacromolecular systems, typically increasing their entropic condition (via a chaotropicity-mediated mode-of-action: Figure 1B; Bhaganna et al., 2010; McCammick et al., 2010).

Kosmotropicity: the action of substances that are more polar than water and that entropically stabilise biomacromolecular structures (lower right inserts of Figure 1A,B; Ball & Hallsworth, 2015).

Fungicides: antifungal compounds, commonly of anthropogenic origin, produced to inhibit or prevent the growth of, or kill, fungal pathogens of crop plants and humans, or other animals or to prevent or limit control

fungal growth on surfaces or in materials including paints and construction materials. See also 'Antifungal compounds' and 'Antimicrobial substances'.

Mode-of-action (or mechanism-of-action): the biophysical, physicochemical, or biochemical mechanism via which a biologically active substance (such as a toxin, stressor, drug, or fungicide) exerts its biological effect (see Figure 1 of the current manuscript and Figure 1 of Cray et al., 2015).

Photosensitisers: substances that can be light-activated to produce reactive species via either electron or energy transfer; these reactive species will, in most cases, modify nearby biomacromolecules via oxidative damage (Braga et al., 2022). See also 'Antifungal compounds', 'Cellular stress', 'Cellular stressors', 'Fungicides', and '*Toxin–stressors*'.

Stress: see 'Cellular stress'.

Stress parameter: those thermodynamic, physical, or physicochemical parameters that can induce cellular stress, including. extremes of temperature, pH, water activity, chaotropicity, ionising radiation, and ultraviolet radiation. See also 'Cellular stress'.

Stressors: see 'Cellular stressors'.

Target-specific drug discovery: the process of finding new biologically active substances that interact with a specific molecular target (such as a gene, gene product, or molecular mechanism). See also 'Drug discovery'.

Toxic substances: those substances that poison the cell via a target-specific mode-of-action, usually with a minimum concentration to prevent microbial cell division in the nM or pM range; they include antibiotics, heavy metals, and many fungicides such as ergosterol biosynthesis inhibitors (Figure 1D of the current manuscript and Figure 1 of Cray et al., 2015). See also 'Toxicants' and 'Toxins'.

Toxicants: toxic substances of anthropogenic origin, including many fungicides and other pesticides. See also 'Toxic substances'.

Toxin–stressors: substances that act as site-specific cellular stressors because, like toxic substances, they target a specific site within the cell, but act as stressors (e.g., by causing oxidative damage). One example is hydrophobic photosensitisers that accumulate in the cell membrane and once activated by light or ultraviolet radiation release reactive oxygen species (Braga et al., 2022). The term *toxin–stressor* is not yet established, hence is shown in italics (Figure 1c). See also 'Cellular stress', 'Cellular stressors', 'Photosensitisers', and 'Toxic substances'.

Toxins: toxic substances that are of natural origin, including heavy metals and some plant and microbial metabolites (N.B., most of the antimicrobial compounds produced by microorganisms are stressors rather than toxins; see Cray, Bell, et al., 2013). See also 'Toxic substances'.

substances (Cray et al., 2015; Cray, Bell, et al., 2013; Randazzo et al., 2010). Heterogeneous and highly diverse habitats of fungi, such as soils, present an indeterminate number of challenges to the cell including stress parameters, stressors, and toxins that can vary spatially, temporally, and across different scales (Hallsworth et al., 2023). Individual stressors can also inhibit cells via multiple mechanisms (Alves et al., 2015; Benison et al., 2021; Cray et al., 2015; Hallsworth, 2018). Furthermore, stressors may induce so many cellular changes (including secondary stresses such as oxidative damage; Hallsworth et al., 2003) that these can mask their primary mode-of-action or are misallocated as inducing mechanical injury (e.g., Giannousi et al., 2015) albeit that mechanical injury can also act as a source of stress (e.g., Garduño-Rosales et al., 2022; Hallsworth, 2018; Villalobos-Escobedo et al., 2022).

As a result of the mechanistic diversity of the thermodynamic, biophysical, physicochemical, photochemical, and/or toxic assaults on cells in natural ecosystems (and the dynamic nature of the conditions to which cells can be subjected), the development of resistance to inhibition by a specific substance is somewhat rare. By comparison, the anthropogenic application of individual fungicides, like the clinical use of antibiotics, is a powerful way to select for resistant microbial phenotypes (Larsson & Flach, 2022; Mshana et al., 2021). Furthermore, there is no resistance *per se* to the ultimate failure of cell function at the high chaotropicity or low water activity that can be caused by cellular stressors (Cray et al., 2015; Hallsworth et al., 2007; Stevenson, Hamill, O'Kane, et al., 2017) or at extreme temperatures (Chin et al., 2010; Ferrer et al., 2003; Price & Sowers, 2004). Relatively few fungicide products are

formulated to expose fungi to multiple, mechanistically diverse modes-of-inhibition. One example is Phytex (Pharmasol Ltd), which is based on a formulation of antimicrobial organic acids and substances which are not usually considered active ingredients but are added to confer other properties on the formulation. These include methyl salicylate, ethyl acetate, and methylated spirits that are known to act as chaotropic and/or hydrophobic stressors and thus could contribute to the potency of Phytex (Cray et al., 2014).

Not only are many metabolites within the microbial cell chaotropic or hydrophobic–including antimicrobials produced by microbes themselves–but many naturally occurring and anthropogenic substances are too including some salts (see below), fertilisers such as urea and nitrates, substances within oil, many metabolites of plants, and substances within plant, animal and microbial necromass, faeces, etc. Availability of elements/compounds that are essential to the cell, such as metal cofactors, can also determine the efficacy of an antifungal. This has been demonstrated in *S. cerevisiae*, where iron deficiency increases the cell's susceptibility to terbinafine, a compound that inhibits squalene epoxidase Erg1 and so disrupts the ergosterol biosynthetic pathway. This has been attributed to both a decrease in Erg1 translation and the lanosterol-induced degradation of Erg1 under iron deprivation conditions (Jordá et al., 2022).

Although the current article considers the modes-of-action of individual antifungals, we advocate the use of more-complex antifungal strategies whenever possible that emulate the complexity of constraints that fungi can experience in Nature and minimise the risk of resistance.

ANTIFUNGAL COMPOUNDS ARE STRESSORS AND (TARGET-SITE-SPECIFIC) TOXINS, TOXICANTS, OR *TOXIN–STRESSORS*

The modes-of-action of antifungals can be misunderstood, sometimes for decades, if the nature of their activity is mis-diagnosed. For example, ethanol was long thought of as a toxic substance and its mode-of-action was not understood until it was identified as a cellular stressor with a chaotropicity-mediated stress mechanism (Cray et al., 2015; Hallsworth, 1998; Hallsworth et al., 2003, 2023). Other chaotropes as well as hydrophobic substances can have so many individual stress-induced impacts on the cell (e.g., Rao et al., 2010) that these can be mistaken for site-specific toxicity. Similarly, salts that act as stressors are sometimes mistaken for toxic substances (e.g., Bhargava & Srivastava, 2013). It is therefore important to be able to identify the mechanistic category to which an inhibitory compound belongs: toxin/toxicant or stressor (or whether it has a combination of traits; Box 1; Figure 1).

Toxic compounds have a target-specific mode-of-action and are inhibitory at low concentrations (see below), whereas stressors can impact many/diverse structures or sites in the cell and many are in most of the cases inhibitory in the mM or M concentration range. Some substances, such as some photosensitisers (Box 1), are target-specific in as much as they accumulate in the fungal cell membrane but act as stressors because, once activated, they damage the cell by releasing singlet oxygen that causes oxidative damage; we call these *toxin–stressors* (Figure 1C). It should be noted that chaotropic and hydrophobic stressors (as do osmotic stressors) can inhibit/damage any type of cellular system, whereas toxic substances (including *toxin–stressors*) are typically more taxon-specific. A classification of antimicrobials, according to their modes-of-action, was devised by Cray, Bell, et al. (2013).

A large number of naturally occurring antifungal compounds are not toxins but stressors that exist in the aqueous phase of cells (chaotropic stressors) or the hydrophobic domains of the membranes and other biomacromolecules (hydrophobic stressors) and entropically disorder macromolecular systems (Figure 1A,B; Ball & Hallsworth, 2015; Cray et al., 2015). A decision tree has been constructed to aid the identification of toxins versus chaotropic and hydrophobic stressors (Cray et al., 2015) based in part on the degree of hydrophobicity expressed as the partition coefficient of the compound in a two-phase octanol : water system (log $P_{octanol–water}$) and based in part on the concentration at which the compound is inhibitory to growth because toxins typically prevent growth in the nM or pM concentration range, hydrophobic stressors in the low-mM or the nM range, and chaotropic solutes in the M to mM range (Bhaganna et al., 2010).

Well-known examples of toxic compounds are inhibitors of ergosterol biosynthesis such as clotrimazole and other azoles which act on lanosterol-14α-demethylase (the CYP51/ERG11 protein; Figure 1D). The inhibition of this enzyme affects membrane integrity through the disruption of ergosterol synthesis (Sagatova et al., 2015). Other cell components can be affected by toxic compounds such as the cytoskeleton, which is destabilised by benzimidazoles that target β-tubulin (Albertini et al., 1999), or mitochondria, where the respiration can be altered by succinate dehydrogenase inhibitors and quinone outside inhibitors that target the cytochrome b (Bartlett et al., 2002; Sierotzki & Scalliet, 2013).

One effect of chaotropes such as $MgCl_2$, ethanol, butanol, and the secondary metabolites vanillin and guaiacol or the hydrophobic secondary metabolite cinnamaldehyde is cellular leakage due to an increase of cell membrane permeability, alteration of Ca^{2+} homeostasis, and oxidative stress (Cray et al., 2015; Gao et al., 2021; Huang et al., 2019; Santos et al., 2015; Yang et al., 2021). Both chaotropes and hydrophobic stressors are characterised by lower polarity than water (Bhaganna et al., 2010; Cray, Russell, et al., 2013; McCammick et al., 2010;

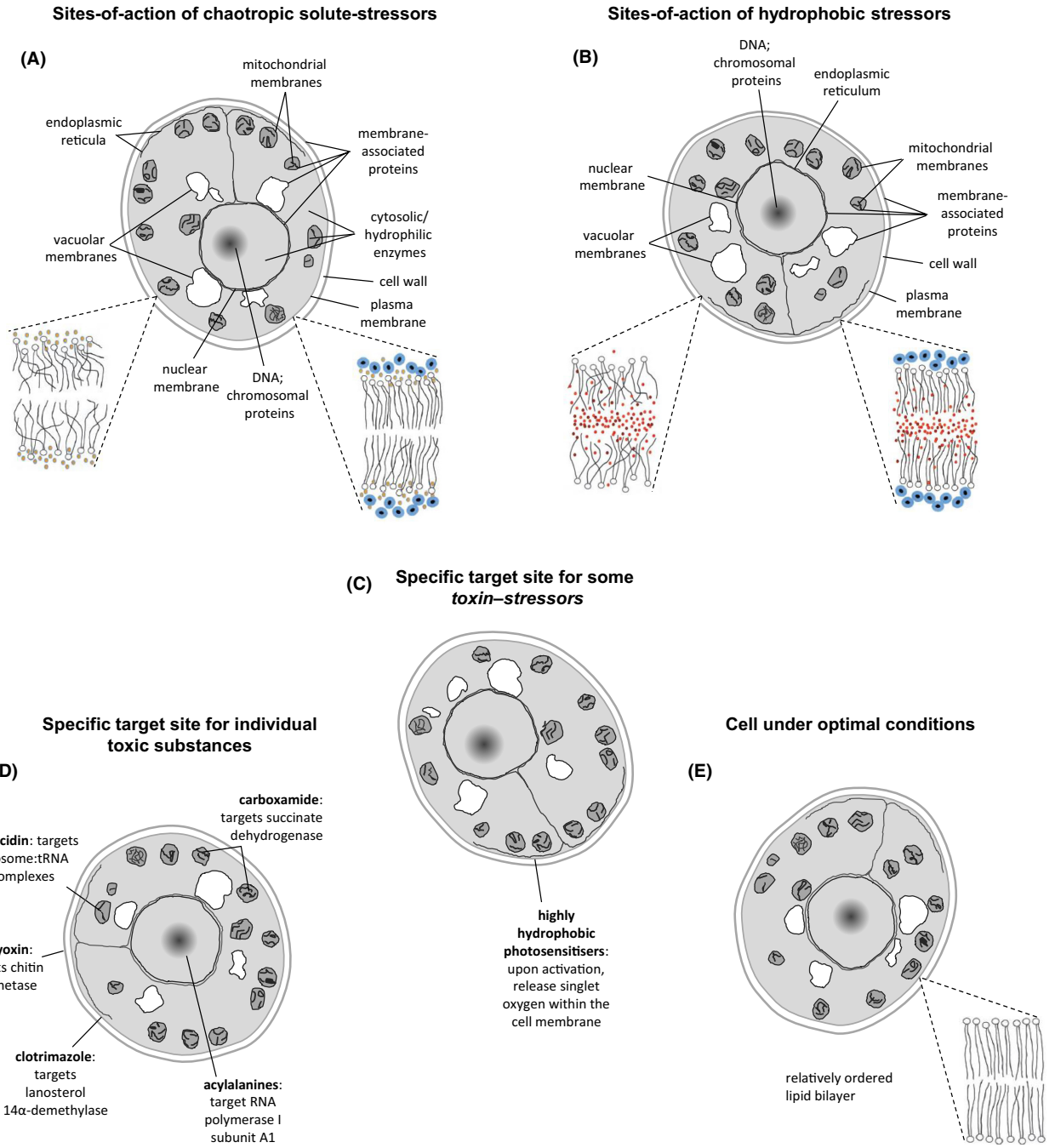

FIGURE 1 Cross-sectional representations of the *Saccharomyces cerevisiae* cell, illustrating sites-of-action for inhibitory substances: (A) soluble chaotropes that enter the cell, such as ethanol; (B) hydrophobic substances (log P > 1.95), such as benzene; (C) some photosensitisers, such as highly hydrophobic phenothiazines and polyacetylenes, that act as *toxin–stressors* with a site-specific location and target-specific activity (within the cell membrane) but a stressor mode-of-action (release of reactive oxygen species that cause oxidative stress; Braga et al., 2022); and (D) named, toxic substances (each with a target-specific mode-of-action), compared with (E) an uninhibited cell. Inserted are cartoons of the lipid bilayer indicating (A, left) structural disorder induced by a chaotropic solute; (A, right) the protective activity of a kosmotropic compatible solute such as trehalose; (B, left) structural disorder induced by a hydrophobic stressor; (B, right) the protective activity of a kosmotropic compatible solute such as trehalose; and (E, right) a relatively unstressed membrane. These lipid-bilayer representations are simplified for clarity; chaotropic solute molecules may enter the hydration shell of kosmotropic compatible solutes (though this has yet to be experimentally determined). This display was modified with permission from the publisher: a version of the figure was published in *Current Opinion in Biotechnology*, volume 33, by Cray et al. 'Chaotropicity: A key factor in product tolerance of biofuel-producing microorganisms', pp. 228–259, Copyright Elsevier (2015). The inserts originally came from 'Hydrophobic substances induce water stress in microbial cells' by Bhaganna et al. (2010) *Microbial Biotechnology* volume 3 issue 6 pp. 701–716.

Suryawanshi et al., 2017). They induce similar cellular responses such as the production of proteins involved in protein stabilisation, the production of compatible solutes (e.g., trehalose), or an alteration of lipid-bilayer composition (Bhaganna et al., 2010; Hallsworth et al., 2003). Although these compounds do not act on specific protein

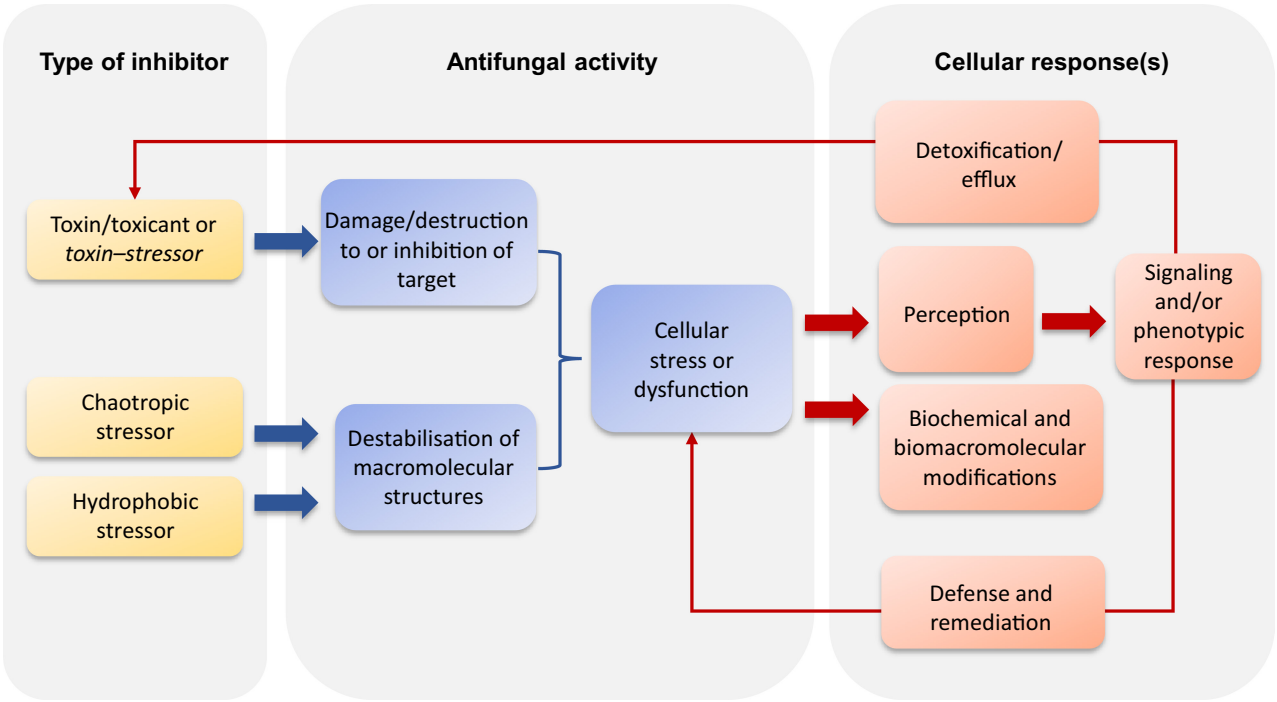

FIGURE 2 Antifungal compounds and cellular responses, assuming that cell death does not occur. Molecules with antifungal activity can be classified according to their modes-of-action. Toxicants act on a specific target so, for example, their interaction may alter protein function via allosteric changes or other inhibitory mechanisms (Figure 1D). Chaotropic or hydrophobic molecules act as stressors that exert adverse impacts on diverse types of biomacromolecules so can inhibit cell functions (Figure 1A,B). In all cases, these activities lead to cellular dysfunctions including inhibition of growth which, if not lethal, induce cellular responses. Remediation consists in limiting the damages in cells and detoxification aims at eliminating the compound.

targets within cells, interactions with different enzymes are reported due to their chaotropic and hydrophobic properties. For example, chaotropic compounds such as ethanol and $MgCl_2$ are able to inhibit β-galactosidase activity and the addition of protective agents (e.g., betaine) can reverse this inhibition depending on the stressor (Bhaganna et al., 2010), a principle confirmed using agar gel-point assays (Alves et al., 2015) and whole-cell systems (Hallsworth et al., 2007; Stevenson et al., 2015; Stevenson, Hamill, Medina, et al., 2017; Williams & Hallsworth, 2009).

Unlike chaotropic solutes, hydrophobic compounds do not seem to exert inhibitory activity on hydrophilic biomacromolecules due to their low solubility and limited interactions with hydrophilic domains (Bhaganna et al., 2010). For example, the hydrophobic compounds benzene, toluene, octanol, xylene, and hexane inhibit the activity of the hexokinase of *S. cerevisiae* by interacting with its hydrophobic domain. Similarly, hydrophobic cinnamaldehyde interacts with various enzymes such as squalene epoxidase, thymidylate synthase, β-1,3-glucan synthase, and chitin synthase 1 (Bang et al., 2000; da Nóbrega Alves et al., 2020). These activities likely result from the generic hydrophobic properties/behaviour of the compound and not from the inhibition of a specific target *per se*.

Whatever the properties of inhibitory substances (toxins/toxicants, chaotropes or hydrophobic compounds), they may secondarily induce oxidative stress and can trigger global responses to cellular dysfunctions, both to limit the cell damages and to mitigate against damage to the cell and its functions and to catabolise/detoxify or expel the compound (Figure 2; Hallsworth et al., 2003). If toxins and toxicants exert their toxicity by causing oxidative damage, then their mode-of-action is best characterised as a *toxin–stressor*. The same is true for those photosensitisers (whether they are plant metabolites or anthropogenic compounds) that preferentially accumulate in the fungal cell membrane but, once activated by light or ultraviolet radiation, release singlet oxygen and other reactive species thus causing oxidative stress/damage that can be lethal (Figure 1C; Braga et al., 2022). Collectively, such factors can complicate determinations of the precise modes-of-action of antifungal compounds.

ANALYSIS OF FUNGAL METABOLITES

Ergosterol biosynthesis is specific to the fungi and some algae (Darnet et al., 2021). This sterol is not only needed for cell membrane fluidity but may also play an important role in glycerol efflux and survival of *S. cerevisiae* cells during osmotic downshock and acts as a fungal hormone that stimulates growth and proliferation (Figure 1D; Jorda & Puig, 2020; Mohid et al., 2022; Szomek et al., 2022; Toh et al., 2001). Sterol profiling has been one of the methods used to identify the targets of azole-based compounds

and other types of ergosterol biosynthesis inhibitors (Müller et al., 2017). By quantifying the amounts of each intermediate in the ergosterol biosynthetic pathway, it is possible to deduce which enzyme has been targeted by a given inhibitory compound that had added to the growth medium. However, this approach requires that the whole pathway is well known in the cultured organism and that the analytic method is sufficiently sensitive to extract and detect all intermediates.

To better understand the sterol biosynthetic pathway in the *Leishmania* protozoan parasite and elucidate the mechanism underlying the differential efficacy of antifungal azoles, a new liquid chromatography coupled to tandem mass-spectrometry (LC–MS/MS) method was recently developed and allowed detection of 16 sterols including intermediates between lanosterol and ergosterol (Feng et al., 2022). One category of ergosterol-biosynthesis intermediates acting as precursors of ergosterol in fungi is the 4-methylsterols (Darnet & Schaller, 2019). By metabolites analysis, it was shown that the resistance of *C. albicans* to fluconazole is due to the synthesis of 14-α methylsterol intermediates that maintain the functionally of the cell membrane (Lu et al., 2022). A synergistic treatment, based on fluconazole and CZ66, a small compound identified from a series of 3-(benzo[1,3]dioxol-5-yl)-N-(substituted benzyl) propanamides, was able to deplete 14-α methylsterols by inhibiting the activity of Erg251, which is the enzyme involved in C4-position demethylation (Lu et al., 2022).

However, this method is suitable for azoles and other ergosterol biosynthesis inhibitors, which exhibit a specific mode-of-action on a given enzyme within the ergosterol biosynthesis pathway. This is not the case for all types of antifungal compounds. To explore the molecular mechanisms of the antifungal compound phenazine-1-carboxamide that inhibits *Rhizoctonia solani*, and discover potential targets-of-action, an integrated analysis of transcriptome and metabolome was performed (Zhang et al., 2022). The study identified a total of 466 metabolites that were unique to either the phenazine-1-carboxamide-supplemented culture or the control (no-phenazine-1-carboxamide-added) culture. Kyoto Encyclopedia of Genes and Genomes (KEGG)-enrichment revealed that the phenylpropanoid-biosynthesis pathway and those pathways involved in metabolism of purine and arachidonic acid were adversely impacted.

CHEMICAL GENETIC SCREENING TO IDENTIFY MODES-OF-ACTION

Screening libraries of haploinsufficient strains and other mutant strains to identify the targets of toxic compounds

Identifying the modes-of-action and molecular targets of antifungal compounds has been possible in *S. cerevisiae* via chemical genetic screening (Box 1). The use of collections of homozygous diploid deletion strains or haploid deletion strains allows the identification of cellular targets processes that are altered by/in response to an antifungal compound depending on the susceptibility of the cells to that antifungal (Xue et al., 2020; Zimmermann et al., 2018). Using a collection of haploid mutants deleted for cell-wall components, it was shown that jervine, a jerveratrum-type steroidal alkaloid that impairs cell-wall biosynthesis in *S. cerevisiae*, acts on the type II membrane proteins Kre6 and Skn1, which are involved in β-1,6-glucan biosynthesis (Kubo et al., 2022). Screening of haploid mutant strains that are susceptible to inhibition by poacic acid, a diferulate commonly found in lignocellulosic hydrolysates, revealed that the Protein Kinase C (PKC) pathway (which is involved in cell-wall-integrity signalling) was the pathway that was the most-vulnerable to inhibition by this compound (Piotrowski et al., 2015). By coupling this screening approach with morphological analyses, the authors found that poacic acid localises to the cell wall where it binds to glucan fibrils that are usually implicated in the extracellular part of β-1,3-glucan synthesis, thus inhibiting this synthesis (Piotrowski et al., 2015).

Working with haploid deletion strains or homozygous diploid deletion strains is only possible for non-lethal mutants whereas working instead with heterozygous diploid deletion strains can bypass that problem. Indeed, in this case, heterozygous diploid deletion strains have had one copy of a gene deleted but the other copy remains present and functional, which facilitates production of the protein (albeit that less is produced than in the wild-type strain) (Figure 3; Giaever et al., 1999, 2004). The haploinsufficiency profiling compares the growth of heterozygous diploid deletion strains and wild-type strain of heterozygous diploid deletion strains that are grown in the presence of an antifungal compound (Figure 3). It allowed the identification of chitin-synthase-1 and chitin-synthase-2 enzymes as the targets of the antifungal plant metabolite 4-cyclopentene-1,3-dione (Mokhtari et al., 2018). Another study identified glutamate dehydrogenase (GDH) and the resistance-to-rapamycin-deletion-2 protein (RRD2) as targets of antofine, a phenanthroindolizidine alkaloid isolated from milkweed (*Asclepias syriaca*), leading to alteration of the target-of-rapamycin (TOR) signalling pathway (Mogg et al., 2019).

Chemical genetic screening for identifying modes-of-action of stressors

Haploinsufficiency screenings with thousands of small molecules (Box 1) that have inhibitory activity against *S. cerevisiae* have also shown that the majority of these molecules do not target a single site because they are stressors that act on multiple sites, bind DNA, or chelate metal ions (Hoepfner et al., 2014). For

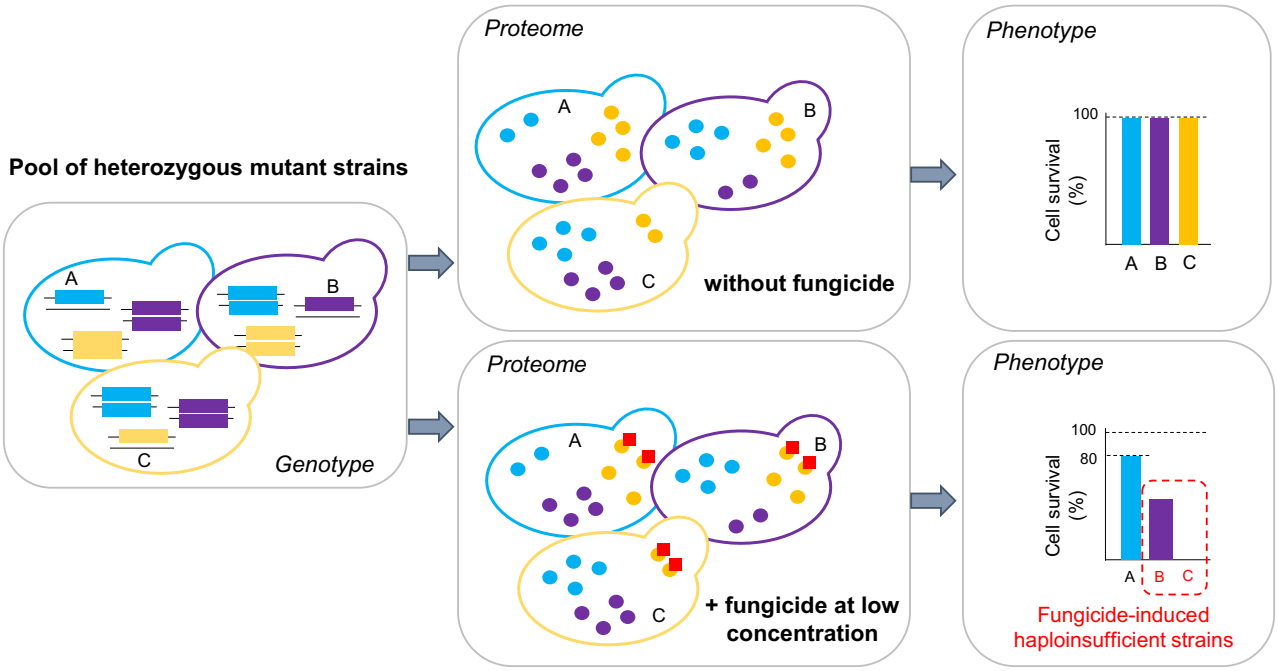

FIGURE 3 Principle of haploinsufficiency profiling. To determine the protein target(s) of an antifungal molecule, a pool of *S. cerevisiae* heterozygous mutant strains is incubated in the absence and presence of a fungicide/antifungal compound. The concentration of this antifungal is determined in advance, such that it causes some lethality but allows a 70-80% survival rate for the wild-type strain (Piotrowski et al., 2015). Since heterozygous mutant strains have been deleted for one copy of a given gene, this results in a decrease in the amount of protein produced by that gene compared to the wild-type strains that each have two copies of that gene, If this specific protein is not a/the target of the antifungal, the same phenotype as the wild type is observable in cells of mutant strains grown in the presence of the antifungal compound (Strain A). By contrast if the protein is a target of the fungicide, the amount of this protein that is produced is not enough to compensate for the toxicity of the fungicide, inducing hypersensitivity of the strain that cannot survive (Strain C). For some mutants, a decrease in cell survival occurs because the antifungal targets more than one protein, or the concomitant decrease in the amount of two proteins is responsible for growth inhibition. This is the case for Strain B, for example, where the amount of the purple-coded protein is reduced because one allele of the gene is deleted and the amount of the yellow-coded protein that are functional is reduced because of the antifungal. Symbols indicate budding cells of S. cerevisiae (coloured, irregular-shaped outlines), three genes (coloured, horizontal bars), the proteins that are coded for by these genes (coloured dots), and percentage survival for each strain (vertical columns on the histograms). In each case, colour coding indicates S. cerevisiae Strain A (blue), Strain B (purple), or Strain C (yellow). Red squares represent the location of the antifungal compound.

example, three *S. cerevisiae* strains that are susceptible to inhibition by α-hederin, a saponin found in the leaves of common ivy (*Hedera helix*), have deletions in genes encoding proteins involved in transcription processes; proteins including a component of the chromatin structure-remodelling complex SWI/SNF (ARP7), a subunit B16 of RNA polymerase II (RPB7), and a component of the remodelling-the-structure-of-chromatin complex RSC58. Moreover, the growth inhibition due to α-hederin was similar to the one observed for several compounds inhibiting β-glucan synthesis such as caspofungin, ergokonin A, ascosteroside, and an echinocandin C derivative (Prescott, Rigby, et al., 2014). This suggests that α-hederin has other modes-of-action in addition to the inhibition of cell-wall synthesis. The growth of *S. cerevisiae* strains deleted for iron permease FTR1, iron oxidoreductase FET3, copper transporting ATPase CC2, and the voltage-gated chloride channel GEF1 was inhibited in presence of gossypol, a phenolic compound. The growth of these strains was also inhibited by the two iron chelators deferasirox and desferricoprogen, and interestingly, the combined presence of Fe^{2+} and gossypol in culture media prevented growth inhibition of these deletion strains by the iron chelators. Thus, gossypol appears to inhibit growth of *S. cerevisiae* through its Fe^{2+}-chelating properties rather than through any direct interaction with a specific protein target (Prescott et al., 2018).

A core collection of 24 homozygous and heterozygous diploid strains, referred to as *signature strains*, was assembled to determine whether each antifungal acts as a stressor or a toxin/toxicant prior to undertaking a large-scale genetic screening from the deletion-strain collections. The antifungal effect of the organosulphur compound allicin, obtained from garlic (*Allium sativum*), was assayed using these signature strains. This facilitated the identification of a mode-of-action of this antifungal via impacts on Cu^{2+} and Zn^{2+} import and homeostasis (Prescott & Panaretou, 2017). The mode-of-action of allicin was then confirmed by haploinsufficiency screening on a library of *S. cerevisiae* deletion mutants (Sarfraz et al., 2020).This screen revealed that growth of three of the mutant strains was inhibited by allicin. These three strains were deletion

mutants that lacked genes coding for proteins involved in DNA replication, chromatid cohesion, or mitochondrial translation. These processes are essential for cell division of *S. cerevisiae* and may thus reflect allicin-induced stress responses rather than a targeted interaction with one of the three proteins identified in the screen. Similarly, a collection of 4786 *S. cerevisiae* haploid deletion mutants were screened for their hypersensitivity to the cell wall-destabilising agents caspofungin, zymolyase, and Congo Red. Forty-three mutants exhibited reduced growth compared to the wild-type, in presence of these compounds and the deleted genes code for proteins involved in cell-wall morphogenesis, transcription, chromatin remodelling, signal transduction and stress response, RNA metabolism, ergosterol biosynthesis, and vesicular trafficking and transport. This set of genes was designated as a 'signature of cell-wall maintenance' (García et al., 2015).

The chemical genetics approach is also used to identify whether an antifungal exhibits modes-of-action other than, or in addition to, the intended target. Calcineurin is a serine/threonine phosphatase which modulates gene expression via the calcineurin pathway (Crabtree, 2001). In *S. cerevisiae*, calcineurin-dependent gene expression is involved in various aspects of the cell's physiology and homeostasis, including maintenance of cell-wall integrity (Stie & Fox, 2008). To determine whether the calcineurin inhibitor 3,4,5-trimethoxybenzyl isothiocyanate has additional antifungal activities, the haploinsufficiency method was employed. Seven deletion strains of *S. cerevisiae* that were sensitive to 3,4,5-trimethoxybenzyl isothiocyanate compared to the wild-type strain were identified. The three deletion strains that were the most-susceptible to growth inhibition by this antifungal were found to have deletions for two genes encoding subunits of RNA polymerase III (RET1 and RPC53) and one gene encoding actin nucleation protein (ARP3; Prescott, Panaretou, et al., 2014).

Limits of chemical genetic methods and alternative strategies to determine modes-of-action of antifungals for non-model filamentous fungi

Although chemical genetics is effective for determining the mechanisms-of-action and/or the molecular target(s) of antifungals molecules, this methodology is primarily used for those Ascomycota, especially *S. cerevisiae*, for which genetic manipulation is relatively easy and the systemic deletion of individual genes is possible. Whereas *S. cerevisiae* has been widely used as a model organism, studies suggest that *S. cerevisiae* data cannot reliably predict targets in *C. albicans* or other fungi (Kelly et al., 2000; Nagahashi et al., 1998). This practice has limited our ability to study pathogenesis and treat fungal diseases of plants and animals, including humans. Thus, multiple types of gene-mutant libraries have been constructed in the diploid ascomycete *C. albicans*, including gene-knockout libraries, transposon-insertion libraries, and the conditional expression libraries, such as the gene replacement and conditional expression (GRACE™) mutant library (Roemer et al., 2003; Wang et al., 2022).

For filamentous basidiomycetes, no mutant library is currently available. This is mainly because genetic transformation has been achieved in only few species such as *Coprinus* sp., *Schizophyllum commune* (Fincham, 1989), *Phanerochaete sordida* (Yamagishi et al., 2007), and *Pleurotus ostreatus* (Xu, Nakazawa, et al., 2022). Whereas random mutagenesis by ultraviolet radiation and/or ethyl methanesulfonate represents an efficient alternative method to chemical genetics that can be used for non-model organisms. Random mutagenesis was originally developed in *S. cerevisiae* to obtain auxotrophic strains (Brandriss, 1979; Little & Haynes, 1979), this method is now mainly used to generate strains with increased/optimal fitness or higher/optimal productivity of compounds of biotechnological interest than a wild-type strain for biotechnological purposes (Ho & Ho, 2015; Zhao, Ma, et al., 2018). This classical genetic strategy, also called a forward genetics approach, was used in the ligninolytic basidiomycete *P. ostreatus*. Combining these mutagenesis methods and whole-genome resequencing revealed that the mutations responsible for impaired lignolysis were located in the putative chromatin modifier CHD1-coding gene and the agaricomycete-specific protein WTR1-coding gene (Nakazawa et al., 2017). Similarly, for the plant-pathogen *Sclerotinia sclerotiorum*, 32 mutants were identified that have a slower growth rate than the wild-type on control media (with no antifungal added) and either do not produce sclerotia or produce sclerotia that are discoloured or malformed (Xu, Ao, et al., 2022). Two of the three mutants that were discoloured were analysed morphologically and genetically and their mutations were found to be localised in the same gene. This gene is an orthologue of the Botrytis cinerea BcSMR1 that codes for a master transcription factor controlling the melanisation of sclerotia.

TRANSCRIPTOMICS FOR ASSESSING CELLULAR DAMAGE AND CELLULAR RESPONSES

The analysis of whole-genome expression in presence of antifungals can be used to determine modes-of-action of these compounds. However, transcriptomics approaches have only on occasion identified the targets of antifungal compounds because it is difficult to know whether the genes regulated in the presence of the antifungal are those which code for the proteins

targeted by the compound, or whether they are regulated as a consequence of the resulting cellular dysfunction. This is the case for a transcriptomic study of *C. albicans* in response to tomatidine, an alkaloid found in tomato (*Solanum lycopersicum*; Dorsaz et al., 2017). The presence of tomatidine results in upregulation of genes involved in ergosterol biosynthesis. Sterol analysis identified an accumulation of zymosterol and ergosta-5,7,24(28)-tetraenol indicating that tomatidine inhibited both C24 sterol methyltransferase (ERG6) and Δ5-sterol-Δ24-reductase (ERG4), respectively. These targets were further validated using a forward genetic approach in *S. cerevisiae* that revealed two non-synonymous mutations in the *ERG6* gene in mutant strains that are resistant to tomatidine (Dorsaz et al., 2017). A study carried out in the ascomycete *Magnaporthe oryzae* identified the pyruvate dehydrogenase E1 (PDHE1) as a target of isobavachalcone, an antifungal compound present in the seed extract of babchi (*Psoralea corylifolia*). A three-dimensional structural model of PDHE1 was used to predict the physical interaction between PDHE1 and isobavachalcone (by performing molecular docking and molecular dynamics simulations) (Li et al., 2021).

Most of the time, the gene regulation pattern, revealed using transcriptomic analysis, is a consequence of both mix between the direct effect of the antifungal compound on gene expression and the secondary effects caused by proteins produced by the former that in some way trigger the expression of other genes. Moreover, determining whether an antifungal acts through (target-specific) toxicity or as cellular stressor can be difficult because cellular damage/dysfunctions can lead to similar cellular responses in both cases (Figure 2). These responses correspond to remediation/mitigation strategies such as metabolic modifications and involvement of the detoxification system (Morel et al., 2013), overexpression of the targeted protein in the case of toxicity, overexpression of many proteins in the case of chaotropicity-induced stress (Hallsworth et al., 2003), oxidative stress responses that can be triggered by both toxicity and stress, and synthesis of specific molecules (e.g., chaperones, kosmotropic molecules—see Box 1) in the case of stress generated by chaotropic or hydrophobic molecules (Cray et al., 2015). This is all the more complicated in the case of plant extracts that can contain both cellular stressors and toxic substances. Transcriptomic analyses of the white-rot basidiomycete *Phanerochaete chrysosporium* revealed the induction of genes coding for proteins belonging to several functional Clusters of Orthologous Groups (COG) categories in response to acetonic extracts of the heartwood of sessile oak, *Quercus petraea*. These oak extractives were a set of molecules mainly composed of hydrolysable tannin-derived molecules and flavanone derivatives and the COG categories of the induced genes were: energy production and conversion, secondary metabolism, post-translational modification, and protein turnover (Fernández-González et al., 2018). Similar types of cellular responses, attributed to the COG category 'energy production and conversion', were observed in P. chrysosporium grown in the presence of the chaotropes vanillic acid or benzoic acid (Matsuzaki et al., 2008; Shimizu et al., 2005). Global gene expression analysis also highlighted that modulation of the secondary metabolism is part of the response of various pathogenic fungi (including *Alternaria alternata* and aspergilli) to diverse substances such as eugenol, magnolol, gallic acid, resveratrol, and other (chaotropic/hydrophobic) compounds such as ethanol and cinnamaldehyde (Lv et al., 2018; Ren et al., 2020; Wang et al., 2015, 2019, 2020; Zhao, Zhi, et al., 2018). However, it is difficult to decipher whether this pathway is specifically targeted by these antifungal compounds or modified/regulated as a consequence of stress. Transcriptomics is thus a useful approach to study the physiological responses of a fungus to antifungal compounds. This said, identifying molecular (intracellular) targets of antifungals is difficult using gene expression regulation alone.

CHEMOPROTEOMICS TO IDENTIFY WHICH PROTEINS ARE TARGETTED BY AN ANTIFUNGAL COMPOUND

Chemoproteomics has emerged as a key type of approach for the determination of the modes-of-action of antifungal compounds by identifying their cellular targets (Box 1; Figure 4; Conway et al., 2021; Drewes & Knapp, 2018; Lenz et al., 2011). Chemoproteomics is an umbrella term to describe techniques that analyse protein-small molecule interactions on a proteome-wide scale. These techniques include affinity-based target identification, that is used to probe structural aspects of protein-small molecule interactions, and activity-based protein profiling that uses specially designed probes (that form a covalent bond with an enzyme's active site) and was developed to monitor the availability of active sites to their endogenous ligands (Adam et al., 2002; Geoghegan & Johnson, 2010; Meux et al., 2013; Tsuboi et al., 2011) (Figure 4). Affinity-based target identification, techniques such as limited proteolysis, thermal proteome profiling, or photoaffinity labelling are used—in conjunction with mass spectrometry—as high-throughput methods to provide low-resolution structural information of non-covalent natural product-protein complexes (Figure 4A) (Cheng et al., 2010). Chemoproteomics was developed for (and has thus-far been primarily used for) drug discovery against cancer cells, with few examples of its use to determine the modes-of-action of antifungals. One of them concerns the ascomycete *Paracoccidioides brasiliensis* that is a human pathogen (Campos et al., 2008; Ferreira

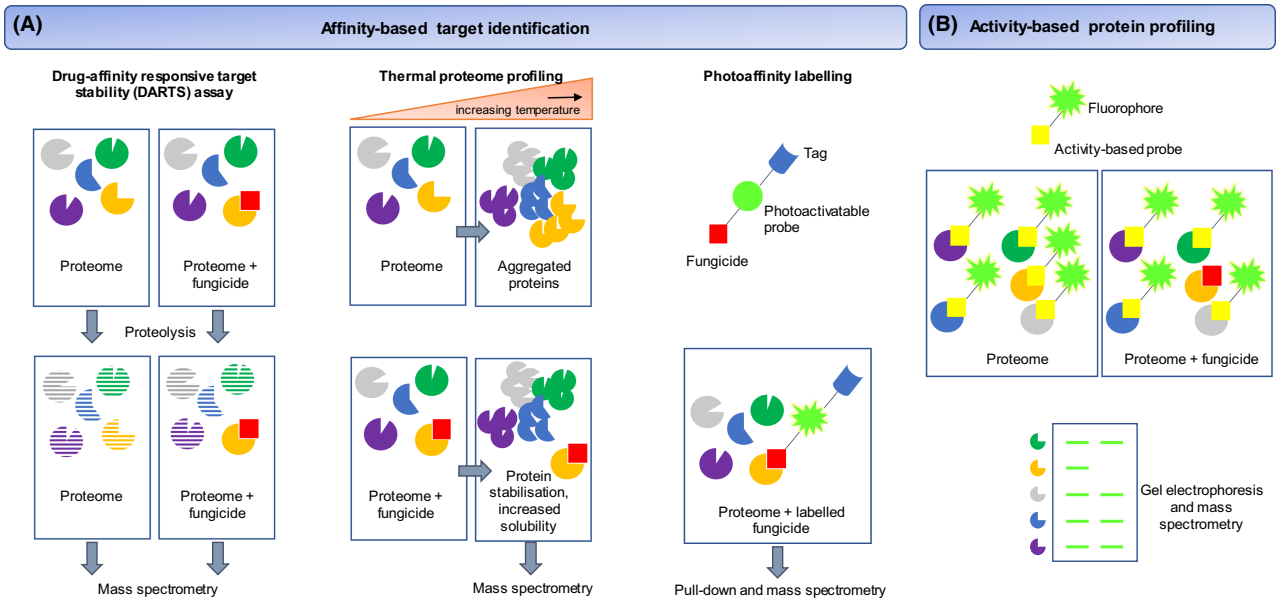

FIGURE 4 Examples of methods used for chemoproteomics. (A) The affinity-based target identification methods are used to compare the stability of proteins upon binding by a ligand (drug-affinity responsive target stability (DARTS) assay) or increased solubility (thermal proteome profiling) and identifying the stabilised proteins by mass spectrometry. In the photoaffinity labelling method, the ligand contains a cross-linking group, which is activated, often with light, labelling the target protein. The presence of a tag allows the labelled (target) protein to be isolated from within proteome by pull-down assay for a definitive identification using mass spectrometry. (B) Activity-based protein profiling uses fluorescent chemical probes to covalently label functional sites of proteins, thus labelling the proteins. Any ligand that physically interacts with a protein could prevent the binding of the probe so, in such cases, no fluorescence is detected. For coloured circles (grey, blue, dark green, yellow, and purple), each colour represents a different protein of the proteome, and the segments that are missing from each circle reflect that each protein has a unique three-dimensional structure. The faded versions of the coloured circles correspond to proteins that have been digested via proteolysis.

et al., 2016). The chemoproteomics approach via affinity chromatography was used to explore the interactions between *P. brasiliensis* proteins and argentilactone, a plant metabolite isolated from Brazilian savannah bushmint *Hyptis ovalifolia* (do Carmo Silva et al., 2020). Most of the proteins that interact with argentilactone are involved in amino-acid metabolism, energy generation, or detoxification.

THE TARGET-BASED DRUG-DISCOVERY STRATEGY

Another way to identify inhibition of a target by a plant-derived compound is to select molecular candidates to be inhibited. The advantage of this (target-based) approach is that it focuses on molecular targets that are unique to fungi (targets not found in other organisms). The target-based drug-discovery strategy has been carried out on azole-resistant strains. Some pathogenic fungi overexpress efflux-pump genes of the ATP-binding cassette (ABC) superfamily and major facilitator superfamily (MFS) resulting in a decrease in intracellular azole concentration and thus conferring resistance to azole fungicides. This has enabled the development of antifungal treatments that combine the use of azole fungicides and plant metabolites (such as thymol, carvacrol, vanillin, tetrandrine, and geraniol) able to inhibit the activity of these efflux pumps and/or causing their mislocalisation within the fungal cell (Ahmad et al., 2013; Saibabu et al., 2020; Singh et al., 2018; Zhang et al., 2009).

Tyrosinases have also been considered potential targets in fungi because they play roles in melanin biosynthesis and pathogenesis (Halaouli et al., 2006). By combining kinetic analysis, molecular docking, and statistical modelling for 44 molecules from the flavonoid family, the characteristics of flavonoid structures that inhibit the catalytic activity of tyrosinase were identified. These characteristics were the presence of hydroxyls at C3 and C7 positions of flavonoids, and the *O*- and *C*-glycosylation, methylation, and acetylation of OH groups (Jakimiuk et al., 2022). This target-based drug discovery strategy was also used to identify compounds that inhibit proteins of the ergosterol biosynthesis pathway, proteins involved in cell-wall construction, or those involved in signalling systems (Ahmad et al., 2010; Yoshimi et al., 2022).

A number of methods are available to study ligand binding onto a known protein target. These include thermal shift assay and affinity-based techniques. The thermal shift assay has been used for large-scale screening of interactions of hundreds of compounds and plant extracts with glutathione transferases, which are part

of the detoxification system of the cell (Barbier et al., 2020; Perrot et al., 2018). The 'ligand fishing' strategy, which is an umbrella term to describe affinity-based techniques, allows a ligand to be isolated from within an extract, using proteins as the bait. Affinity crystallography is an approach to isolate and elucidate the complete three-dimensional structures of low-abundance but high-affinity target ligands from partially fractionated natural product extracts. Solving the three-dimensional structure of the protein/compound complex allows both the identification of the compound and the localisation of its binding site on the protein (Aguda et al., 2016). For example, affinity crystallography revealed that among a partially purified wild-cherry (*Prunus avium*) extract, the flavonoid dihydrowogonin was retained at the substrate binding site (H-site) of the glutathione transferase GSTO3S of the basidiomycete *Trametes versicolor* (Schwartz et al., 2018). Affinity crystallography is an approach that isolates and elucidates the complete three-dimensional structures of high-affinity but low-abundance target ligands from partially fractionated natural product extracts. This method is not suitable for working with several proteins at the same time and requires the empirical determination of optimal conditions to induce the crystallisation of proteins. However, advances in robotics and automation systems have made the processes of crystallisation and X-ray data collection more efficient.

We believe that the target-based drug discovery strategy has the advantages of being easier to execute and faster than other phenotypic approaches. However, antifungals may act on more than one target protein and a recombinant approach carried out in vitro may not represent the situation in vivo where so many proteins and other substances exist and so many interactions/reactions between them can occur.

CONCLUDING REMARKS

Deciphering the modes-of-action of antifungals is in some cases straightforward, but in other cases complex, sometimes due to the diversity of the cellular damage caused and the diversity of responses implemented by fungi to detoxify or expel the compound and/or limit stress-induced damage. This is even more challenging in non-model fungi, for which genetic tools and mutant libraries are not yet available. The use of random mutagenesis combined with next-generation sequencing, or the development of new bioinformatic approaches to predict protein-ligand binding sites, are two promising ways to try to surmount this challenge (Lin et al., 2020; Nakazawa et al., 2017; Piazza et al., 2020; Xu, Ao, et al., 2022). To validate the results of each of these methodologies, effective means of genetic manipulation to make mutant libraries in filamentous fungi must be available to the research community. This will help speed up the discovery and subsequent use of new natural compounds as antifungal agents and benefit to the development of combinatory therapy, as already demonstrated for the antitumour drug ponatinib[1] and the antifungal fluconazole that exhibit synergetic activities against fungal pathogens of humans (Liu et al., 2022).

A number of key scientific questions remain outstanding in relation to the modes-of-action of antifungal compounds. Importantly, how can knowledge of antifungal modes-of-action be most effectively applied towards the United Nations Sustainable Development Goals? For example, how to produce inexpensive, safe, effective antifungal treatments for pre- and post-harvest use and timber preservation in relation to SDGs 1, 2, 6, and 11; what fungicides can be developed with a high specificity to target taxa (negligible activity against non-target organisms) and a short half-life to avoid residues that remain in foods (so do not impact human health—SDG 3) (e.g., MagalhÃes et al., 2023), if antifungals destabilise the dynamics of resource sharing within microbial communities (Timmis et al., 2023) how does this impact fermented foods, biofuel fermentations, soil health, etc (SDGs 2, 9, 12, and 13); feedstock for use in biofuel fermentations (so do not inhibit yeast used for ethanol production—SDGs 7 and 12) or soils (so do not impact soil health—SDG 15); and how can policy-makers minimise the risk of fungicide-induced emergence of human pathogens (SDGs 3, 6, and 13)?

Finally, we believe that further research is also needed to determine what types of antifungals belong to the *toxin-stressors* category other than photosensitisers, and how determinations of modes-of-action can be made more-efficient for basidiomycete fungi or other taxa for which genetic screenings are not applicable.

AUTHOR CONTRIBUTIONS

Mélanie Morel-Rouhier: Conceptualization (equal); investigation (equal); supervision (equal); validation (equal); writing – original draft (equal); writing – review and editing (equal). **Delphine Noel:** Investigation (equal); writing – original draft (equal); writing – review and editing (equal). **John E. Hallsworth:** Conceptualization (equal); Investigation (equal); writing – original draft (equal); writing – review and editing (equal). **Eric Gelhaye:** Investigation (equal); writing – original draft (equal); writing – review and editing (equal). **Sylvain Darnet:** Investigation (equal); writing – original draft (equal); writing – review and editing (equal). **Rodnay Sormani:** Investigation (equal); supervision (equal); writing – original draft (equal); writing – review and editing (equal).

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[1]The systematic name of ponatinib is 3-(2-imidazo[1,2-b]pyridazin-3-ylethynyl)-4-methyl-N-[4-[(4-methylpiperazin-1-yl)methyl]-3-(trifluoromethyl)phenyl] benzamide.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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