Advances in Biological Regulation xxx (xxxx) 100891

Contents lists available at ScienceDirect

Advances in Biological Regulation

journal homepage: www.elsevier.com/locate/jbior

New strategies for combating fungal infections: Inhibiting inositol lipid signaling by targeting Sec14 phosphatidylinositol transfer proteins

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ARTICLE INFO

Keywords:
Fungal infections
Phosphoinositides
Lipid kinases
Sec14 PITPs
anti-Fungal drugs

ABSTRACT

Virulent fungi represent a particularly difficult problem in the infectious disease arena as these organisms are eukaryotes and therefore share many orthologous activities with their human hosts. The fact that these activities are often catalyzed by conserved proteins places additional demands on development of pharmacological strategies for specifically inhibiting target fungal activities without imposing undesirable secondary effects on the host. While deployment of a limited set of anti-mycotics has to date satisfied the clinical needs for treatment of fungal infections, the recent emergence of multi-drug resistant fungal 'superbugs' now poses a serious global health threat with rapidly diminishing options for treatment. This escalating infectious disease problem emphasizes the urgent need for development of new classes of anti-mycotics. In that regard, Sec14 phosphatidylinositol transfer proteins offer interesting possibilities for interfering with fungal phosphoinositide signaling with exquisite specificity and without targeting the highly conserved lipid kinases responsible for phosphoinositide production. Herein, we review the establishment of proof-of-principle that demonstrates the feasibility of such an approach. We also describe the lead compounds of four chemotypes that directly target fungal Sec14 proteins. The rules that pertain to the mechanism(s) of Sec14 inhibition by validated SMIs, and the open questions that remain, are discussed – as are the challenges that face development of next generation Sec14-directed inhibitors.

1. Introduction

Fungi typically engage in commensal relationships with their human hosts. While such a relationship benefits both organisms, inappropriate expansions of commensal fungal populations lead to opportunistic superficial infections. Such infections are typically not life threatening if treated rapidly, but their progression to systemic infections is another matter. Systemic fungal infections claim some two million lives annually worldwide (Bongomin et al., 2017), and the associated mortality rates are in many cases of a magnitude similar to those associated with other deadly viral and bacterial pathogens (Dagenais and Keller, 2009; Pappas et al., 2018). Moreover, fungal infections contracted in hospital settings are increasing at alarming rates and are particularly problematic to im-

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https://doi.org/10.1016/j.jbior.2022.100891
Received 11 February 2022; Accepted 22 February 2022
2212-4926/

munocompromised individuals (Dupont et al., 2009; Delaloye and Calandra, 2014). The rapid escalation in the incidence of nosocomial infections has elevated fungal diseases to the level of a major health crisis.

The great majority of deaths attributed to fungal disease result from infections by the filamentous fungus *Aspergillus* and by yeast species of the genera *Candida*, *Cryptococcus* and *Pneumocytis* (Monk and Goffeau, 2008; Brown et al., 2012; Denning and Bromley, 2015). The *Candida* genus includes some 20 different species that are considered virulent. Of those, *C. albicans* is the primary human pathogen as this organism accounts for nearly 50% of *Candida* infections in the United States (Pfaller et al., 2014). However, the incidence of dangerous *C. glabrata* infections is rising steadily, and the recently discovered *C. auris* is garnering serious attention as an emerging fungal 'superbug' that the Centers for Disease Control have recently elevated to serious global health threat status (https://www.cdc.gov/fungal/candida-auris/c-auris-antifungal.html). This designation acknowledges the recovery of *C. auris* clinical isolates resistant to all classes of available anti-mycotics approved for use by the Food and Drug Administration (Sears and Schwartz, 2017; Tortorano et al., 2021). As such, *C. auris* threatens to become the fungal version of the methicillin/multi-drug resistant *Staphylococcus aureus* MRSA bacterium that has proven so problematic in the infectious disease arena (Enright et al., 2002; Chambers and Deleo, 2009).

2. Multidrug-resistant *Candida* and the pipeline for anti-mycotics

Virulent *Candida* form biofilms (Cavalheiro and Teixeira, 2018; Eix and Nett, 2020). These biofilm communities frequently contaminate catheter tubes and therefore represent major reservoirs of infection in hospital settings. Formation of *Candida* biofilms also occur in the infected host and, once established, are exceedingly difficult to clear. As a result, the window of treatment between initial infection and invasive candidiasis is often uncomfortably short (Zeng et al., 2001; Hirano et al., 2018; Wang et al., 2019). This feature of the *Candida* lifestyle, when coupled with the increased incidence of nosocomial *Candida* infections and the emergence of multidrug resistant clinical isolates of *C. auris* (Brown et al., 2012; Denning and Bromley, 2015; Sears and Schwartz, 2017), paints an alarming picture. Time is of the essence for effective treatment.

Unfortunately, it now appears that fungal infectious diseases have not attracted adequate attention in past years from the standpoint of development of anti-mycotics (Perfect, 2017). To some extent, the consequence of this indifference stems from the historical effectiveness of the four classes of workhorse anti-mycotics (i.e. the polyene amphotericin B, the azoles, the echinocandins, and pyrimidine analogs) approved by the FDA for treating fungal infections (Woolley, 1944; Hazen and Brown, 1950; Galgiani, 1990; Sanglard, 2002; Khan et al., 2021). However, the pipeline for new anti-mycotics is now critically thin (Ostrosky-Zeichner et al., 2010; Perfect, 2017), and there is a heightened urgency in developing new classes of anti-fungal drugs. This effort requires identification and exploitation of novel fungal targets for inhibition (Tortorano et al., 2021).

Fungi, like their human hosts, are eukaryotes. As a result, both pathogen and host execute a large number of common biochemical activities that are catalyzed by orthologous proteins (Zeng et al., 2001). Thus, new efforts in anti-fungal drug discovery require development of agents that either: (i) potently inhibit fungal targets with exquisite specificity while remaining indifferent to cognate host activities, or (ii) target the more limited set of activities that are unique to the pathogen (Tortorano et al., 2021). We focus our discussion on the second approach. Herein, we review current progress in the identification and validation of four new classes of anti-fungal compounds first discovered by exploitation of the yeast *Saccharomyces cerevisiae* model. These small molecule inhibitors (SMIs) target, with exquisite specificity, an unusual and highly conserved fungal activity -- the Sec14 phosphatidylinositol/phosphatidylcholine exchange protein (PITP). Sec14 is essential for biologically sufficient execution of inositol lipid-based phosphoinositide signaling pathways required for fungal viability and pathogenesis. The collective studies make the case that fungal Sec14 PITPs represent attractive, yet essentially unexplored, targets for rational development of new classes of anti-mycotics (Nile et al., 2014; Khan et al., 2016, 2021; Filipuzzi et al., 2016; Pries et al., 2018).

3. Phosphoinositide signaling at a glance

Phosphatidylinositol (PtdIns) is the metabolic precursor of a cohort of phosphoinositides that represent derivatives of PtdIns phosphorylated at specific positions on the inositol headgroup. Known modifications occur at the 3-OH, 4-OH and/or 5-OH positions of the inositol ring and can occur as mono-phosphorylations at a single position (PtdIns-3-phosphate; PtdIns-4-phosphate; PtdIns-5-phosphate), mono-phosphorylation at two positions (PtdIns-3,4-bisphosphate; PtdIns-3,5-bisphosphate; PtdIns-4,5-bisphosphate), or a mono-phosphorylation at three positions (PtdIns-3,4,5-trisphosphate) (Fig. 1). The production of each stereoisomer, with the possible exception of PtdIns-5-phosphate, is governed by the activities of positionally-specific PtdIns kinases (Fruman et al., 1998; Martin, 1998; Di Paolo and De Camilli, 2006; Strahl and Thorner, 2007). The turnover of these molecules is similarly governed by the activities of phosphoinositide phosphatases that exhibit some measure of positional-specificity (Guo et al., 1999; Rivas et al., 1999; Liu and Bankaitis, 2010), and by phospholipases (Bill and Vines, 2020). Whereas mammals produce all seven of these phosphoinositides, fungi produce fewer. Generally, these organisms produce PtdIns-3-phosphate, PtdIns-4-phosphate, PtdIns-3,5-bisphosphate, and PtdIns-4,5-bisphosphate. Of these, PtdIns-4-phosphate and PtdIns-4,5-bisphosphate are essential for cell viability in yeast. The 3-phosphoinositides, while important for fitness, are dispensable except under various conditions of environmental stress (Strahl and Thorner, 2007; Sardana and Emr, 2021).

Phosphoinositides are present in all eukaryotes where these molecules collectively represent core components of a major intracellular signaling system(s). The seven phosphoinositide stereoisomers have intrinsic functions as cofactors required for the activities of key signaling proteins (Hammond et al., 1997; Lee et al., 2007; Mizuno-Yamasaki et al., 2010) and ion channels (Hille et al., 2015), and as specific platforms for appropriate spatial and temporal recruitment of signaling effector proteins to membrane surfaces

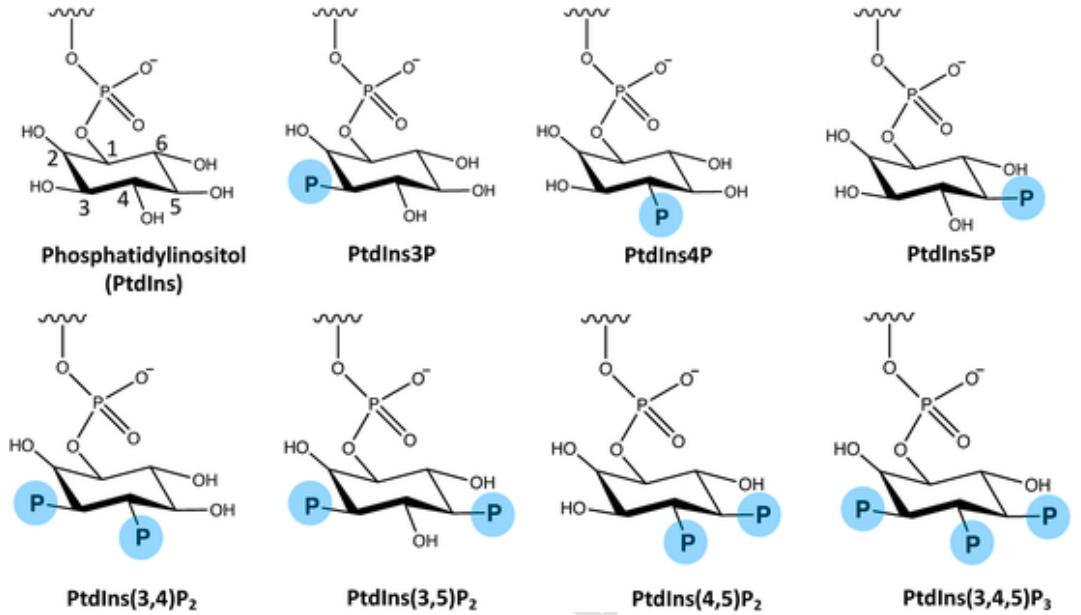

Fig. 1. The phosphoinositides. The headgroup moieties of phosphatidylinositol (PtdIns) and the seven naturally-occurring phosphoinositides are shown. These are chemically unique and are distinguished by the position and the number of phosphorylations of the headgroup inositol (Ins) ring.

(Lemon, 2003; Overduin and Kervin, 2021). Moreover, some phosphoinositides are precursors for the generation of a broad cohort of soluble inositol-phosphate/polyphosphate stereoisomers that execute various second messenger functions. The classic example is the regulation of agonist-stimulated calcium release from intracellular stores by inositol-1,4,5-trisphosphate, which is produced via phospholipase C-catalyzed hydrolysis of PtdIns-4,5-bisphosphate (Cullen, 2003; Putney and Tomita, 2012). Soluble inositol phosphates/polyphosphates also serve as structural co-factors that regulate the folding and/or stability of important intracellular proteins (Macbeth et al., 2005; Hatch et al., 2017).

4. Unforseen avenues for chemical intervention of PtdIns-4-phosphate signaling

PtdIns-4-phosphate (PtdIns-4-P) is the product of a phosphorylation of PtdIns at the 4-OH position of the Ins headgroup by two functionally non-redundant and structurally distinct classes of type II and type III PtdIns 4-OH kinases (PI4Ks). The type III enzymes produce the bulk of the cellular PtdIns-4-P (Audhya et al., 2000: Strahl and Thorner, 2007; Clayton et al., 2013). Yeast PI4KIIIα (Stt4) and PI4KIIIβ (Pik1) activities produce plasma membrane and trans Golgi network (TGN)/endosomal pools of PtdIns-4-P, respectively (Strahl and Thorner, 2007). Both PI4Ks represent essential activities in cells, and their functional non-redundancies reflect their specific roles in producing these spatially distinct pools of PtdIns-4-P. While PtdIns-4-P is the primary metabolic precursor of PtdIns(4,5)P_2, this phosphoinositide holds intrinsic signaling power. PtdIns-4-P is essential for cell viability in part because it is a core signaling component that regulates the activities of the trans-Golgi network (TGN) and endosomal membrane trafficking machineries in all eukaryotic cells studied thus far (Di Paolo and De Camilli, 2006; Strahl and Thorner, 2007; Graham and Burd, 2011; Clayton et al., 2013). Moreover, PtdIns-4-P-containing vesicles derived from the Golgi system promote mitochondrial fission at mitochondrial/endoplasmic reticulum membrane contact sites (Nagashima et al., 2020).

An unusual aspect of type III PI4Ks is that these are intrinsically biologically insufficient enzymes (Schaaf et al., 2008; Bankaitis et al., 2010; Grabon et al., 2015). That is, the core enzymes themselves are not efficient lipid kinases in vivo and cannot generate sufficient PtdIns-4-P to elicit productive signaling responses in the face of antagonistic activities. These include metabolic activities such as PtdCho synthesis via the CDP-choline pathway and PtdIns-4-P degradation by Sac1 phosphatases, and the actions of PtdIns-4-P sequestering proteins such as Kes1/Osh4-like members of the oxysterol binding protein family (Cleves et al., 1989, 1991; Fang et al., 1996; Guo et al., 1999; Rivas et al., 1999; Li et al., 2002; de Saint-Jean et al., 2011; Mousley et al., 2012). Nature's solution to this obstacle is to couple PI4Ks with the activities of PtdIns transfer proteins (PITPs). PITPs have no catalytic capacity of their own, but it is proposed these proteins allow PI4Ks to solve an interfacial catalysis problem via a PtdIns 'presentation' mechanism that renders PtdIns a superior substrate for the PI4K on a membrane surface (Ile et al., 2006: Schaaf et al., 2008: Bankaitis et al., 2010). In so doing, PITPs stimulate PI4K activities to the extent (~2.5-fold) that sufficient PtdIns-4-P is generated to induce signaling in the face of the PtdIns-4-P signaling antagonists. The proposed mechanism for PtdIns presentation is a heterotypic lipid exchange cycle where a second lipid ligand is being exchanged from the single lipid-binding cavity in the interior of the PITP-fold for membrane-embedded PtdIns. This process is envisioned to expose the PtdIns headgroup in a manner that facilitates its recognition by PtdIns 4-OH kinases (Fig. 2A).

The unique feature of the functional collaboration between PITPs and PtdIns 4-OH kinase is that evolution has taken advantage of the Sec14-fold to engineer a set of PtdIns-binding modules with distinct second lipid ligand specificities (Schaaf et al., 2008; Bankaitis et al., 2010; Tripathi et al., 2019; Khan et al., 2020a,b). In this manner, the biochemical diversity of Sec14-like PITPs is translated into

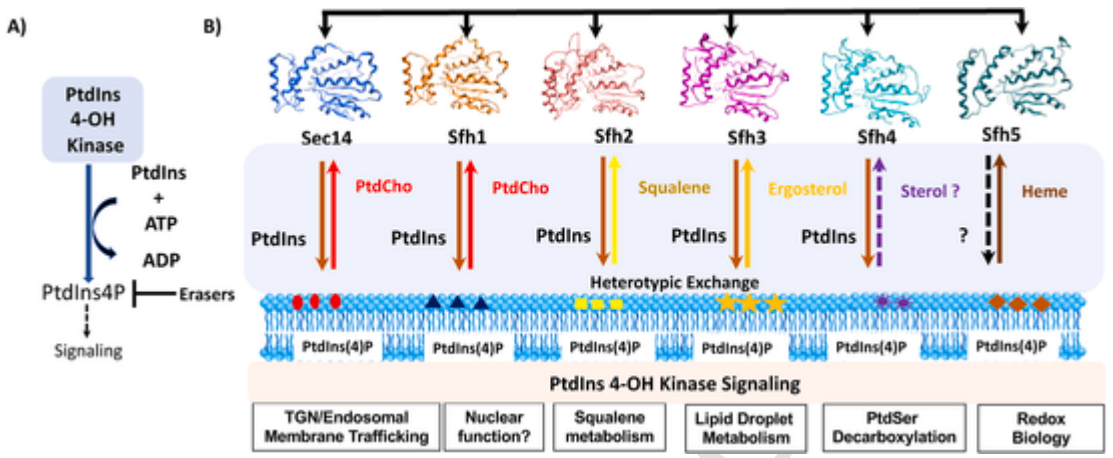

Fig. 2. The PITP-PtdIns 4-OH kinase partnership. **(A)** PtdIns 4-OH kinases are biologically insufficient enzymes in cells and do not produce sufficient PtdIns-4-P to overcome the opposing activities of erasers of PtdIns-4-P signaling such as the Sac1 phosphoinositide phosphatase and the Kes1-like PtdIns-4-P-binding members of the oxysterol binding protein superfamily. **(B)** Sec14-like PITPs utilize a heterotypic PtdIns-second ligand exchange cycle to render PtdIns a better substrate for PtdIns 4-OH kinase. This 'presentation' mechanism potentiates PI4K activities such that these enzymes produce sufficient product for temporally and spatially regulated PtdIns-4-P signaling. Studies with yeast Sec14 and its five yeast homologs (Sfh1 – Sfh5) indicate pool specificity for PtdIns-4-P production rests with the nature of the Sec14-like PITP-PI4K partnership (Schaaf et al., 2008; Bankaitis et al., 2010). For each PITP-PI4K combination, the resultant PtdIns-4-P pool produced is committed to a distinct biological outcome (identified at bottom). As the Sec14-like PITPs exhibit distinct specificities for the second ligand, the PITPs input specific metabolic information into production of the unique PtdIns(4)P pools. PtdCho represents the second ligand for Sec14 and Sfh1, squalene for Sfh2, ergosterol for Sfh3, likely a sterol-like molecule for Sfh4, and heme for Sfh5 (Schaaf et al., 2008; Tripathi et al., 2019; Khan et al., 2020a,b; Wang et al., 2020). It remains unclear whether Sfh4 and Sfh5 function as bona fide PITPs in cells, however (Khan et al., 2020; Wang et al., 2020).

diversity of biological outcomes for PtdIns-4-P signaling from an otherwise homogeneous chemical code (Schaaf et al., 2008; Bankaitis et al., 2010; Grabon et al., 2015). That is, it is the nature of the PITP/PtdIns 4-OH kinase pair that specifies the biological signaling outcome from the corresponding PtdIns-4-P pool (Fig. 2B). This concept expands a simple view of PtdIns-4-P as a unique chemical code to a more nuanced and sophisticated signaling program. Thus, PITPs identify a unique vulnerability of PtdIns-4-P signaling to specific pharmacological intervention of PI4K activity without targeting the highly conserved lipid kinases directly. Previous demonstrations that the major Sec14 PITP is highly conserved amongst the fungi, and is essential for the viability of *S. cerevisiae* and *C. albicans* and for the dimorphic switch in other yeast species (i.e. a switch in cell-division mode that is characteristic of many pathogenic fungi upon infection of the host), further testify to the attractiveness of fungal Sec14 PITPs as new drug targets (Lopez et al., 1994; Monteoliva et al., 1996; Klein and Tebbets, 2007).

5. Sec14 orthologs in fungal pathogens

The founding member of the Sec14 superfamily is Sec14, the major PITP of *S. cerevisiae*. Sec14 activity is essential for the viability of this organism (Bankaitis et al., 1989, 1990). The essential nature of Sec14 function rests on the cellular requirement for Sec14 activity to stimulate the activities of the yeast PI4KIIIα and PI4KIIIβ enzymes so they produce PtdIns-4-P amounts that exceed the threshold required for signaling. In so doing, Sec14 coordinates multiple branches of lipid metabolism with PtdIns-4-P production, and therefore PtdIns-4-P signaling (Ile et al., 2006; Schaaf et al., 2008; Bankaitis et al., 2010; Grabon et al., 2015). The available data report that it is PtdIns-4-P-regulated membrane trafficking within the TGN/endosomal system that is critical for cell viability. However, Sec14 activity might not be limited to regulation of membrane trafficking. Sec14 activity (and PtdIns-4-P signaling) also controls passage of cells through the G1 and G2 stages of the cell cycle and the timing of nuclear division (Mousley et al., 2012; Huang et al., 2018; Maitra et al., 2022), although whether these phenotypes derive from some as yet unidentified compromise in membrane trafficking remains to be determined.

Of direct relevance to this review, Sec14 is highly conserved across the *Eukaryota*, and Sec14 orthologs are present throughout the fungal kingdom. It has long been appreciated that Sec14 might represent an attractive target for the development of new classes of anti-mycotics. This perspective derived not only from the work on *S. cerevisiae* Sec14, but also from subsequent studies in dimorphic yeast and other virulent yeast species. Those studies demonstrated Sec14 is also essential for the viability of pathogenic yeast such as *C. albicans*, and for the transition of dimorphic fungi from a yeast mode of growth to a filamentous growth mode (Lopez et al., 1994: Monteoliva et al., 1996; Riggle et al., 1997). In the case of *C. albicans*, even reductions in Sec14 activity that remain compatible with cell viability strongly compromise virulence due to the inability of those hypomorphic mutants to form a mycelium (Monteoliva et al., 1996; Riggle et al., 1997).

With regard to membrane trafficking pathways, Sec14-dependent secretion of cryptococcal phospholipases and metalloproteases (and likely other cargos) is required for the virulence of *Cryptococcus neoformans* (Djordjevic, 2010; Chayakulkeeree et al., 2011; Vu et al., 2014). This encapsulated yeast is a significant pathogen. It is the causative agent of lung infections that often develop into life-threatening encephalomeningitis in individuals with compromised immune systems (Charlier et al., 2008). As described below, we

now understand that it is through inhibition of Sec14 PITPs that fungal phosphoinositide metabolism and membrane trafficking can be targeted with a specificity that is unlikely to be achieved by directly targeting the highly conserved PI4Ks.

6. First generation lead compounds for Sec14-Directed anti-mycotics

High throughput chemogenomic small molecule inhibitor screens are very useful in identifying potential, albeit unvalidated, candidates for SMIs directed against individual yeast activities. These screens cross-correlate a combination of *S. cerevisiae* deletion mutant and gene overexpression libraries tested against individual compounds in chemical libraries of various origins (Hoon et al., 2008; Lee et al., 2014; Filipuzzi et al., 2016; Pries et al., 2018). Via this general approach, and in combination with a strict validation platform (Nile et al., 2014), SMIs that target *S. cerevisiae* Sec14 have been discovered and validated. These SMIs fall into four distinct chemotypes: nitrophenyl(4-(2-methoxyphenyl)piperazin-1-yl)methanones (NPPMs), picolinamides/benzamides, ergolines, and the alkaloid natural product himbacine (Nile et al., 2014; Filipuzzi et al., 2016; Pries et al., 2018, Fig. 3). Himbacine is an effective Sec14 inhibitor in vitro, but not in vivo. While this property does not disqualify the himbacine scaffold from further development of Sec14-targeted anti-mycotics, the remainder of this review discusses SMIs of the other three chemotypes. These chemotypes are effective Sec14 inhibitors both in vitro and in vivo. Moreover, NPPM, ergoline and picolinamide-based SMIs inhibit Sec14 as the sole essential target in yeast cells and show outstanding in vitro and in vivo specificities amongst PITPs of the Sec14 superfamily -- even when tested against close paralogs and homologs (Nile et al., 2014; Pries et al., 2018). To date, the NPPM and picolinamide scaffolds have yielded the most potent Sec14-directed SMIs with IC_{50}~200 nM. Molecular docking and emerging crystallographic studies show these SMIs inhibit PtdIns or PtdCho binding and exchange by forming a network of non-covalent interactions in the Sec14 lipid binding pocket (Nile et al., 2014; Pries et al., 2018).

7. SMI specificity and sensitivity signatures of Sec14 PITPs

Sensitivity of Sec14 PITPs to NPPM, picolinamide and ergoline SMIs is forecast by two Sec14 signatures. The first is the PtdCho-binding 'barcode'. The barcode concept originated from the analysis of high-resolution 3D structures of Sec14 and Sec14-like PITPs and refers to the amino acid motifs that determine the ligand specificities of those proteins (Schaaf et al., 2008; Bankaitis et al., 2010). These barcodes predict all Sec14-like proteins bind PtdIns but that the great majority fails to bind PtdCho. As the tyrosine cage that forms the PtdCho headgroup-coordinating substructure is a critical binding motif for the SMIs, Sec14-like PITPs that cannot bind PtdCho are completely SMI-resistant (Nile et al., 2014; Pries et al., 2018; Khan et al., 2021). Importantly, no mammalian Sec14-like proteins exhibit this PtdCho-binding barcode and these homologs are therefore expected to be resistant to inhibition by the SMIs discussed herein (Schaaf et al., 2008; Nile et al., 2014). All fungal Sec14 PITPs conserve this PtdCho barcode, however (Fig. 4A).

The second predictive signature is a di-valine (VV)-motif that contributes to the contours of the PtdCho barcode environment (Khan et al., 2016, 2021). Sec14 PITPs with a VV-motif are typically inhibited by SMIs. Some virulent fungi preserve this signature in their Sec14 PITPs (e.g. *C. glabrata*). However, most virulent fungal species express Sec14 PITPs with polymorphisms in the VV-motif (Fig. 4B). These polymorphisms involve amino acid substitutions with bulkier side chains (e.g. Met-Cys, Ala-Phe) that alter their PtdCho-binding barcode environments compared to that of the *S. cerevisiae* Sec14. As a result, the aryl-halide moieties of NPPMs and picolinamides cannot be accommodated in the Sec14 lipid binding cavities of these pathogens as the *C. albicans* example illustrates (Fig. 4C). PtdCho-binding is not compromised by these polymorphisms, thereby emphasizing the highly specific effect of these polymorphisms on the interactions of SMIs with Sec14 PITPs. Reconstitution of the VV-motif into SMI-resistant Sec14 PITPs of virulent fungi is typically sufficient to completely restore SMI-sensitivity to these normally SMI-resistant PITPs. Reciprocally, trans-placement of the polymorphisms present in virulent fungal Sec14 PITPs into the *S. cerevisiae* Sec14 context confers SMI-resistance to this normally SMI-

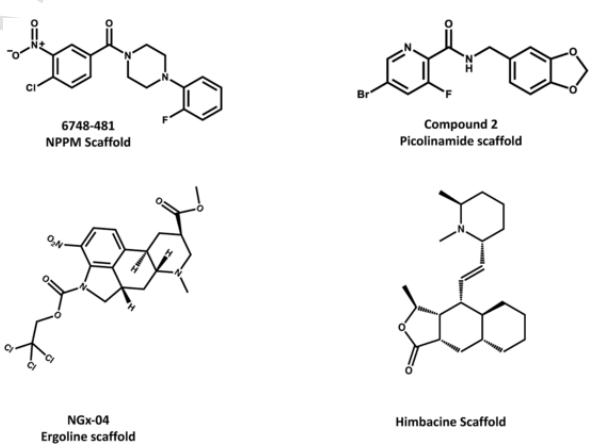

Fig. 3. The validated Sec14-directed SMIs define at least four chemotypes. The chemical scaffold structures for an example of each chemotype are shown and identified below. NPPM: nitrophenyl(4-(2-methoxyphenyl)piperazin-1-yl)methanone.

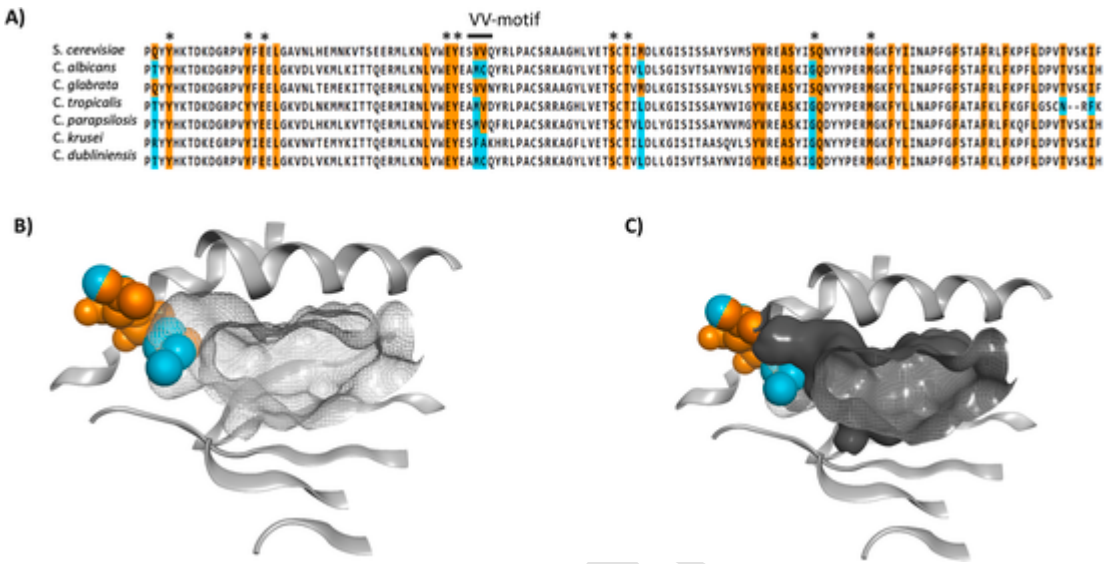

Fig. 4. Phosphatidylcholine binding barcode and VV-motif. A) The primary structures of the PtdCho binding regions of Sec14 PITPs from *S. cerevisiae* and the indicated pathogenic *Candida* species are aligned. Residues whose side chains line the Sec14 lipid-binding cavity are highlighted in orange. Residues that constitute the PtdCho-binding barcode are identified by the asterisks at top, and polymorphisms at those positions are highlighted in cyan. The VV-motif is highlighted by the black bar at top. **B)** The Sec14 cavity envelopes of the PtdCho-binding barcode region are compared. The *S. cerevisiae* VV-motif side chains configurations are highlighted in orange and those of the *C. albicans* VV → MC polymorphism are in cyan. The associated structural differences are illustrated by the more expansive gray mesh envelope of the *S. cerevisiae* lipid-binding pocket relative to the inner gray mesh that represents the shape envelope of the *C. albicans* Sec14 lipid-binding cavity. Bound SMI poses are omitted for purpose of clarity. The net effect of the VV→ MC polymorphism in the *C. albicans* Sec14 is that the PtdCho-headgroup binding space is predicted to be contracted relative to that of the *S. cerevisiae* Sec14. Although both Sec14 proteins can accommodate PtdCho-binding, the contracted binding space cannot accommodate the validated SMIs of the NPPM, picolinamide or ergoline chemotypes. **C)** The same depiction as in B with the exception that the *S. cerevisiae* cavity envelope boundaries are depicted in dark gray surface render while the contracted *C. albicans* cavity envelope is shown in gray mesh. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

sensitive PITP (Khan et al., 2016, 2021; Pries et al., 2018). Thus, the VV-motif polymorphisms are a primary basis for SMI resistance in Sec14 PITPs that preserve the PtdCho-binding barcode.

The collective data indicate that the dual presence of a functional PtdCho-binding barcode and the VV-motif in the primary structure of an uncharacterized Sec14 PITP is a reliable predictor of its sensitivity to these NPPM, picolinamide and ergoline lead compounds. The data also outline the challenges in rational design of the next generation of Sec14-targeted antifungal compounds. While all available data indicate that the distal regions of these lead compounds are accommodated by the Sec14 PITPs of virulent fungi, the A-ring warheads of at least the NPPM and picolinamide SMIs must be redesigned to address the steric clashes that come with Sec14 VV-motif polymorphisms. How binding of the ergoline chemotype is influenced by VV-motif polymorphisms remains to be determined.

8. Outstanding questions and applicability of 19-fluorine NMR to interrogation of Sec14/SMI interactions

The conformational dynamics by which Sec14 executes the lipid exchange cycle --wherein the PITP exchanges a bound PtdIns or PtdCho for a membrane incorporated PtdIns or PtdCho molecule -- remain essentially uncharacterized. Current thoughts regarding the requisite conformational transitions are described elsewhere (Sha et al., 1998; Schaaf et al., 2008; Grabon et al., 2015). These details are relevant to the mechanism by which SMIs inhibit Sec14 function, as it is presumably via the exchange cycle that SMIs invade the Sec14 lipid-binding cavity. This begs the outstanding question of precisely how do SMIs arrest the Sec14 lipid-exchange cycle? There are other central mechanistic issues that remain unresolved. It is proposed that SMI intoxication of Sec14 occurs at the membrane interface where membrane incorporated SMI (these molecules exhibit $C_{log}P$ values of ~3) is exchanged into the Sec14 lipid-binding cavity during the course of a 'standard' crank of the lipid exchange cycle. While this is a reasonable proposal, there are no direct lines of evidence to support this or any alternative mechanism. Resolution of these key questions regarding mechanism of Sec14 inhibition by SMIs requires a detailed understanding of protein, lipid and SMI dynamics during the intoxication process. In that regard, given fluorine is a constituent of NPPM-based lead compounds, and can be incorporated into the other SMI scaffolds, we anticipate that application of fluorine-19 Nuclear Magnetic Resonance (^{19}F-NMR) spectroscopy will prove particularly informative in addressing these questions.

^{19}F NMR offers key advantages with regard to dissecting drug-target interactions (Marsh and Suzuki, 2014; Dalvit and Vulpetti, 2019; Divakaran et al., 2019; Kang 2019), and is particularly suited for resolving the complex SMI-Sec14 interactions in ternary membrane systems. The first is the high sensitivity of the basic measurement. ^{19}F is the second most sensitive stable NMR-active nucleus and therefore can be detected even in low-concentration samples of high-molecular-weight proteins and their complexes. The second advantage is the absence of natural ^{19}F background in biological samples, which facilitates the site-specific assignment of ^{19}F

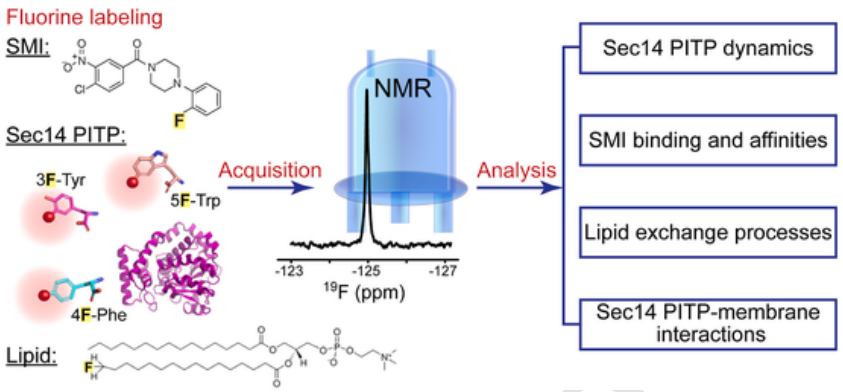

Fig. 5. Fluorine labeling strategies for the Sec14 PITPs systems and their ligands. Information about Sec14 PITP dynamics, membrane interactions, lipid-exchange processes, and interactions with SMIs can be obtained by using appropriate ^{19}F NMR techniques.

resonances in the NMR spectra. Third, NMR itself as a technique is exceptionally versatile and can be applied to characterize both thermodynamics and kinetics of protein-ligand interactions. In particular, ^{19}F NMR chemical shifts are exquisitely sensitive to the changes in the electronic environment and give rise to easily interpretable signatures that report on weak, intermediate and tight binding regimes. Of special significance to the studies of Sec14 PITPs is the availability of the NMR-compatible membrane mimics that would enable the measurements to be conducted on the ternary protein-ligand-membrane systems.

Given that ^{19}F-labeled amino acids can be incorporated into proteins in a site-specific manner, and that ^{19}F-labeled PtdCho is a commercially available reagent, the ^{19}F-NMR approach offers an expansive capacity to collectively monitor specific aspects of Sec14, SMI and PtdCho interactions (Fig. 5). It is these types of analyses that are required for construction of a thermodynamic and mechanistic picture for how SMIs inhibit the Sec14 lipid exchange cycle. A detailed understanding of that process will inform further design and optimization of next generation Sec14-directed SMIs, and will also provide unprecedented insights into the mechanics of the PITP-mediated lipid exchange cycle itself.

Uncited references

Fisher et al., 2018, Michell, 2008.

Declaration of competing interest

The authors declare no competing interests.

Acknowledgments

This work was supported by National Institutes of Health grant R35 GM131804 and BE0017 from the Robert A. Welch Foundation to VAB, and National Institutes of Health grant R01 GM108998 and National Science Foundation grant CHE-1905116 to TII. The authors declare no competing interests.

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