Repurposing the FDA-approved anticancer agent ponatinib as a fluconazole potentiator by suppression of multidrug efflux and Pma1 expression in a broad spectrum of yeast species

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Introduction

Fungal infections have emerged as a major global threat to human health because of the increasing incidence and mortality rates every year. The emergence of drug resistance and limited arsenal of antifungal agents further aggravates the current situation resulting in a growing challenge in medical mycology. Here, we identified that ponatinib, an FDA-approved antitumour drug, significantly enhanced the activity of the azole fluconazole, the most widely used antifungal drug. Further detailed investigation of ponatinib revealed that its combination with fluconazole displayed broad-spectrum synergistic interactions against a variety of human fungal pathogens such as Candida albicans, Saccharomyces cerevisiae and Cryptococcus neoformans. Mechanistic insights into the mode of action unravelled that ponatinib reduced the efflux of fluconazole via Pdr5 and suppressed the expression of the proton pump, Pma1. Taken together, our study identifies ponatinib as a novel antifungal that enhances drug activity of fluconazole against diverse fungal pathogens.

Summary

Fungal infections have emerged as a major global threat to human health because of the increasing incidence and mortality rates every year. The emergence of drug resistance and limited arsenal of antifungal agents further aggravates the current situation resulting in a growing challenge in medical mycology. Here, we identified that ponatinib, an FDA-approved antitumour drug, significantly enhanced the activity of the azole fluconazole, the most widely used antifungal drug. Further detailed investigation of ponatinib revealed that its combination with fluconazole displayed broad-spectrum synergistic interactions against a variety of human fungal pathogens such as Candida albicans, Saccharomyces cerevisiae and Cryptococcus neoformans. Mechanistic insights into the mode of action unravelled that ponatinib reduced the efflux of fluconazole via Pdr5 and suppressed the expression of the proton pump, Pma1. Taken together, our study identifies ponatinib as a novel antifungal that enhances drug activity of fluconazole against diverse fungal pathogens.

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were identified to effectively inactivate the ergosterol synthase (cytochrome P<sub>450</sub>) and block ergosterol synthesis, resulting in the accumulation of C-14 methyl sterol (Shekhar-Guturja et al., 2016b). As for the potential side-effect of this drug family, studies have shown that metabolized azoles in the liver are mainly excreted from bile which easily results in hepatotoxicity.

Notably, the development of new antifungal turns out to be expensive, laborious and time-consuming and cannot be relied on during the recurrent and emerging challenge of fungal infections. For example, it took nearly 30 years for echinocandins, the most recent class of antifungals, to develop and obtain success from bench to bedside (Basso et al., 2020). There is a pressing need to develop new antifungal therapeutic agents, and recent studies have strongly suggested that drug repurposing provides an attractive solution for antifungal development, with apparent advantages including the validated information about the knowledge base for the pharmacokinetics and pharmacodynamics, a dramatic shortening of the Research & Development (R&D) cycle and the huge cut of the R&D costs to achieve maximal utilization of the medical resources. There have been several examples of development of new antifungals based on this strategy. For instance, a previous study showed that sertraline, which is a selective inhibitor of central serotonin reuptake with well-established antidepressant and anxiolytic activity, exhibits a synergistic effect with fluconazole (FLC) against Cryptococcus neoformans in a Galleria mellonella model (Spitzer et al., 2011). More recently, clofazimine, a lipophilic riminophenazine antibiotic compound which has been in clinical use for almost 40 years, but almost nothing was known about its mechanism of action, acts synergistically with fluconazole against diverse fungal species (Robbins et al., 2015). In addition, the natural product beauvericin, also a cyclohexadepsipeptide mycotoxin, was found to effectively potentiate the activity of fluconazole against some major human fungal pathogens (Shekhar-Guturja et al., 2016b). These studies will boost the development of more systemic approaches to repurposing compounds for tackling the rising risk of fungal infection.

In this study, we provided strong evidence that ponatinib significantly potentiated fluconazole efficacy and exhibited a broad-spectrum antifungal effect against diverse fungal pathogens, including C. albicans, S. cerevisiae and C. neoformans. More importantly, synergy testing of ponatinib and fluconazole in resistant C. albicans strains resulted in a reversal of fluconazole resistance. Following the elucidation of the mechanism of action, we finally concluded that ponatinib potentiates the antifungal efficacy of fluconazole, providing clues for developing new therapeutic strategies against fungal infections.

Results

The combination of ponatinib with fluconazole exerts a broad-spectrum synergistic and fungicidal activity

Ponatinib, a multiltargeted receptor tyrosine kinase (RTK) inhibitor, was identified through screening an FDA-approved drug library (L4200, TargetMol) (Mei et al., 2020). We observed that a combination of ponatinib (Pona) and fluconazole (FLC) exhibited a much stronger inhibitory effect on the growth of the wild type C. albicans strain SC5314, when compared with ponatinib or fluconazole alone (Fig. 1A–D, Fig. S1). To test whether the function of ponatinib is conserved, we examined its ability to potentiate the activity of fluconazole against a variety of other fungal pathogens. As expected, ponatinib significantly enhanced the antifungal activity of fluconazole against S. cerevisiae (Fig. 1E–H) and the major human fungal pathogen C. neoformans (Fig. 1I–L). The synergistic activity of ponatinib with fluconazole was further confirmed by the checkerboard assay (FICI < 0.5), showing a powerful antifungal combination with broad-spectrum activity against diverse human fungal pathogens (Odds, 2003; Jansen et al., 2009; Spitzer et al., 2011; Robbins et al., 2015; Shekhar-Guturja et al., 2016b) (Table 1). Moreover, we found through a lactate dehydrogenase (LDH) assay that a concentration of ponatinib at 16 µM or lower was considered non-toxic after incubation with the endothelial cells (Fig. S2).

Next, we performed time-kill curve analysis to determine if ponatinib renders fluconazole fungicidal. Candida albicans cells were subjected to fluconazole treatment with or without ponatinib in liquid medium, and the CFU ml<sup>-1</sup> of the suspension from each treatment was counted after plating onto the YPD medium. With the increment of ponatinib concentration in the combination group, we observed that the number of survival colonies decreased gradually with time, revealing that ponatinib can transform fluconazole from fungistatic into fungicidal (Fig. 2A). The ability to switch between yeast and hyphal morphology is critical for C. albicans pathogenicity (Witchley et al., 2019). To examine whether the combinatorial antifungal effect could be attributed to an impact

Fig. 1. Combination of ponatinib and fluconazole synergistically inhibits growth of C. albicans, S. cerevisiae and C. neoformans. C. albicans (SC5314) (A–D), S. cerevisiae (BY4742) (E–H) and C. neoformans (H99) (I–L) isolates were subjected to twofold serial dilutions of fluconazole, ponatinib or both in YPD medium. OD<sub>600</sub> was measured every 15 min at 30°C, and FICI was calculated using the reference guidelines for CLSI broth microdilution method (M38-A).
Repurposing ponatinib as a fluconazole potentiator
on filamentation, we supplemented the hyphae-inducing media with fluconazole, ponatinib or both and evaluated the hyphal growth of *C. albicans*. The media we used in the assay include M199, Spider or YPD medium with 10% serum, which are all traditional hyphae-inducing media and have been widely used to assess the yeast–

Table 1. Interaction between fluconazole and ponatinib against diverse human fungal pathogens by checkerboard microdilution assay.

| Strain   | MIC alone FLC (µg ml⁻¹) | MIC alone Pona (µM) | MIC combination FLC (µg ml⁻¹) | MIC combination Pona (µM) | FICI for combination | Mode of interaction |
|----------|--------------------------|---------------------|-------------------------------|---------------------------|----------------------|---------------------|
| SC5314  | 0.5                      | 16                  | 0.0625                        | 0.0625                    | 0.13                 | Syn                 |
| BY4742  | 16                       | 128                 | 4                             | 0.25                      | 0.25                 | Syn                 |
| H99      | 4                        | 32                  | 0.5                            | 0.125                     | 0.13                 | Syn                 |
| CCC49   | 64                       | 16                  | 2                             | 0.0625                    | 0.04                 | Syn                 |
| CCC80   | 32                       | 8                   | 2                             | 0.0625                    | 0.07                 | Syn                 |

Syn, Synergistic.

Fig. 2. Ponatinib transforms fluconazole from fungistatic into fungicidal and suppresses hyphal formation. A. *C. albicans* (SC5314) cells were inoculated into YPD liquid medium supplemented with or without indicated compounds (DMSO, 32-128 µg/ml fluconazole, 32-128 µM ponatinib or both). The survival colony-forming units (CFUs) of SC5314 were counted after incubation at indicated periods of time (0, 24 and 48 h).

B. *C. albicans* (SC5314) cells were revived and resuspended in each of three different hyphae-inducing media (M199 buffered to pH8 with 50 mM MOPS; Spider; and YPD with 10% serum) containing DMSO, fluconazole (2 µg ml⁻¹), ponatinib (5 µM) or the combination. Cells were incubated at 37 °C, and hyphal morphology was checked under light microscope after 4 h of incubation.

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hyphae morphological change in *C. albicans* (Toenjes et al., 2005; Liu et al., 2017; Lim et al., 2020; Yang et al., 2020). As shown in Fig. 2B, the inhibitory effect was much more significant in the combinatorial group. Thus, ponatinib potentiates fluconazole activity against diverse fungal pathogens and exerts a fungicidal activity with fluconazole.

**Ponatinib prevents the emergence of azole resistance in *C. albicans***

Fluconazole resistance in the pathogenic yeast *C. albicans* poses significant challenges for the treatment and prevention of *Candida* infections frequently confronted by patients in clinic. We therefore investigated whether the combination of fluconazole and ponatinib can also be effective against the fluconazole-resistant clinical isolates of *C. albicans*. The minimal inhibitory concentration (MIC) values of fluconazole against two resistant isolates were 64 and 32 μg ml⁻¹, respectively, which were about 128- and 64-fold higher than that of the standard laboratory strain SC5314 (Fig. S3). Ponatinib combined with fluconazole was very efficient in the inhibition of the resistant strains, as we documented that ponatinib increased the susceptibility of resistant strains to fluconazole by up to eightfold and the effective concentration of fluconazole decreased from 64 to 8 μg ml⁻¹ (Fig. 3B and F). In order to better evaluate the drug–drug interaction between ponatinib and fluconazole, we used the checkerboard assay as previously described (Rand et al., 1993) and calculated the FICI for the combination using a formula described in Experimental procedures. As shown in Table 1, ponatinib was highly synergistic with fluconazole with FICI = 0.04 for CCC49 and 0.07 for CCC80, highlighting its clinical applicability against fluconazole-resistant *C. albicans* isolates.

The above results prompted us to ask whether ponatinib could prevent the emergence of drug resistance, given that this event is often considered as a canonical case of evolution by natural selection (Shekhar-Guturja et al., 2016b; Vincent et al., 2016; Fransen et al., 2017). We plated 3 × 10³ *C. albicans* cells onto YPD plates supplemented with 32 μg ml⁻¹ fluconazole, 32 μM ponatinib or both. A plate without supplementation was used as a control. As shown in Fig. 3I, the emergence of resistance was significantly induced by fluconazole alone. However, the induction was potently suppressed by the combination of ponatinib. Our data further indicated that the desired final concentrations of fluconazole and ponatinib, which confer synergistic activity against drug-resistant *C. albicans* isolates, are 104.48 μM (32 μg ml⁻¹) and 32 μM respectively. Collectively, our results demonstrated that ponatinib not only enhances the antifungal activity against fluconazole-resistant isolates of *C. albicans* but also prevents the emergence of fluconazole resistance.

**Ponatinib reduces efflux of fluconazole via Pdr5 and promotes the intracellular hyperaccumulation of fluconazole**

Drug efflux is one of the most intensive investigated mechanisms for fluconazole resistance (Hampe et al., 2017). To test whether ponatinib might affect drug efflux to enhance the antifungal activity of fluconazole, we first screened the sensitivity of each of 16 *S. cerevisiae* mutants lacking individual ATP-binding cassette (ABC) transporter to ponatinib (Suzuki et al., 2011). The results showed that only the pdr5Δ mutant was hypersensitive to ponatinib treatment, indicating that ponatinib might interact with Pdr5 (Fig. 4A and B).

Pdr5 is an ABC transporter which pumps out a series of structurally unrelated compounds, including azoles and rhodamine (Prasad and Goffeau, 2012; Shekhar-Guturja et al., 2016b; de Moraes et al., 2020). Consistent with previous studies (Lamping et al., 2007; Prasad and Goffeau, 2012), we also confirmed that fluconazole is a substrate of Pdr5. First, we compared the vegetative growth of wild-type and pdr5Δ mutant cells in the absence and presence of fluconazole and the results showed that the mutant lacking PDR5 exhibited hypersensitivity to fluconazole when compared to the wild type (WT). Next, we treated the WT with increasing concentrations of fluconazole and the cells were stained with Rhodamine 6G, which is a commonly used fluorescent dye for mimicking fluconazole efflux as both molecules share the same transporters in yeast (Maesaki et al., 1999). The assay measured the efflux activities in the yeast cells being incubated with fluconazole and ponatinib at increasing concentrations showing synergism based on the intensity of intracellular fluorescence (red colour). Beauvericin, known to effectively potentiate the activity of fluconazole against yeasts (Shekhar-Guturja et al., 2016a; Shekhar-Guturja et al., 2016b), was used as a control. Interestingly, the intracellular accumulation of Rhodamine 6G was found to correlate with the level of fluconazole (Fig. 4C, E). To assess the effect of ponatinib on Pdr5 activity, we examined rhodamine-6G efflux in wild-type *S. cerevisiae* strain BY4742 in incubation with beauvericin or fluconazole/ponatinib at the concentrations of ponatinib MIC showing synergism. We observed in Fig. 4D that the combinatorial use of fluconazole and ponatinib resulted in a dose-dependent accumulation of red fluorescence, suggestive of an effective inhibition of the efflux of rhodamine-6G. The results also supported a close association of the antifungal effects of the fluconazole/ponatinib combination with the function of efflux pumps in the yeast. Finally, qRT-
PCR analysis revealed that ponatinib treatment had no effect on the transcript levels of *PDR5* in *S. cerevisiae*, suggesting that ponatinib may only perturb the activity of Pdr5 instead of its transcription (Fig. S4). Taken together, our data clearly demonstrate that ponatinib improves the efficacy of fluconazole by stimulating intracellular fluconazole accumulation through inhibition of Pdr5.

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Whole-genome sequencing identifies Pma1 as the putative target of ponatinib

Whole-genome sequencing is a technology widely exploited to identify the targets of antifungal agents in the model yeast Saccharomyces cerevisiae (Shekhar-Guturja et al., 2016b). Our results in Fig. 1 showed that Ponatinib alone also harbours the inhibitory activity against the growth of yeast cells, implying the presence of additional targets. We therefore sought to identify the
target of ponatinib using the method of whole-genome sequencing. To do that, the predominant efflux effect of 16 ABC transporters, which are highly expressed in the yeast cells, has to be minimized or excluded firstly. We therefore conducted a screen for ponatinib-resistant isolates in the background of a specific S. cerevisiae mutant lacking all 16 ABC transporter-encoding genes (ABC16 strain; strain 2336) (Suzuki et al., 2011; Shekhar-Guturja et al., 2016b). The parental strain RY0566 was used as WT control. Specifically, about $3 \times 10^7$ cells ml$^{-1}$ of ABC16 strain were plated onto YPD medium supplemented with 64 $\mu$M ponatinib and we randomly isolated and sequenced ten ponatinib-resistant colonies (Fig. 5A–D).

Our whole-genome sequencing of the 10 isolates revealed that nine out of 10 mutants harboured the same missense mutation, T558S, in Pma1 (Fig. 5E and F). Interestingly, it has been reported that the P-type H$^+$-ATPase Pma1 is an essential proton pump able to regulate cytosolic pH homeostasis (Zhang et al., 2010), partly validating our screening results.

To further evaluate the relationship between ponatinib compound and Pma1 expression, we first quantified and compared the transcript levels of PMA1 in the absence or presence of ponatinib. As shown in Fig. 5G, ponatinib significantly repressed the transcriptional expression of PMA1 in a dose-dependent manner. Moreover, we found that Pma1 protein expression could also be modulated by ponatinib, as the immunoblot analysis clearly showed that the Pma1 protein levels were decreased dramatically by treating the cells with ponatinib, and the reduction is also dose-dependent (Fig. 5H). In microbial organisms, the extrusion of protons by the electron transport chain causes an electrochemical gradient of protons, known as the proton motive force (PMF), which is generated across the cell membrane (Mitchell, 2011). Given the role of ponatinib as an essential proton pump in regulation of cytosolic pH, we ask there may exist a possible relationship between PMF and the accumulation of intracellular fluconazole by ponatinib, in other words, the proton motive force (PMF) and drug efflux pumps may play a central role in the drug combination effect.
Our results showed that the enhancement of proton transport by increasing the proton motive force (PMF) after treatment with fluconazole, could be suppressed by supplementation of ponatinib (Fig. S5), further supporting the notion that ponatinib shows synergistic interaction when combined with fluconazole. Taken together, the results shown here highly support that Pma1 is the potential target of ponatinib (Fig. 6).

**Discussion**

The global incidence of fungal infection has increased over the last few decades due to the expanded ageing population, widespread azole resistance and the marked increase in the number of immunocompromised patients. Due to the fact that invasive fungal infections and fungal sepsis are rapidly increasing with considerable morbidity and mortality, especially in the intensive care unit (ICU), combinatorial antifungal therapy has been becoming a promising therapeutic strategy to enhance drug effectiveness and ameliorate the emergence of drug resistance and of course, has been gaining increasing attention within both industry and academia. Ponatinib is an FDA-approved oral tyrosine kinase inhibitor (Mitchell et al., 2018) and has been evaluated to impede tumour progression, including tumours in thyroid, breast, ovary and lung, neuroblastoma, rhabdoid tumours, gastrointestinal stromal tumours, et al (Musumeci et al., 2018). In this study, we discovered a novel function of this compound, which acts as a potentiator of azole activity against diverse human fungal pathogens, as well as the highly fluconazole-resistant isolates of *C. albicans*. Moreover, our work illustrated that the mechanism of the synergistic combinations that potentiate the antifungal fluconazole is associated with the inhibitory activity of ponatinib, as shown by inhibiting efflux of fluconazole via Pdr5 and suppressing the expression of the P-type H⁺-ATPase Pma1. We provided evidence that ponatinib may execute two levels of actions in the synergism: First, it reduces efflux of fluconazole by inhibiting Pdr5 and thus promotes the intracellular hyperaccumulation of fluconazole; Second, it may interfere with the cytoplasmic pH homeostasis by affecting the expression of the putative target Pma1, as the P-type H⁺-ATPase Pma1 has been reported to be an essential proton pump operating to regulate the cytosolic pH homeostasis (Zhang et al., 2010). Importantly, the observation that the effective concentration of ponatinib appears to be non-toxic towards

![Diagram of the role of ponatinib in potentiation of the antifungal efficacy of fluconazole](https://example.com/diagram.png)

**Fig. 6.** Model depiction of the role of ponatinib in potentiation of the antifungal efficacy of fluconazole by inhibition of fluconazole efflux via Pdr5 and suppression of Pma1 expression. Ponatinib (PubChem CID: 24826799) promotes intracellular fluconazole (PubChem CID:3365) accumulation by binding to Pdr5. On the other hand, ponatinib perturbs the cytosolic pH homeostasis by inhibiting the proton pump Pma1 located at the plasma membrane. Eventually, the dual action of ponatinib through these two pathways leads to cell death.

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endothelial cells greatly assists in dealing with the concern of the potential drug side-effect, which may limit its application in future translational studies. Of course, the interaction between ponatinib and fluconazole identified so far is certainly only the tip of the iceberg and awaits more systemic analysis.

Currently, the most urgent problem related to fungal infections in clinic is the emergence of multidrug resistance due to the fungistatic activity of antifungals like fluconazole. Identification of a novel antifungal candidate which can transform the fungistatic effect of fluconazole to fungicidal will be an effective strategy for clearance of fungal pathogens. Our results indicate that ponatinib not only inhibits the growth of a broad-spectrum of fungal pathogens but also converts fluconazole from fungistatic to fungicidal which prevents the emergence of azole resistance, providing a promising therapeutic strategy for antifungal development in the future.

The ABC (ATP-binding cassette) superfamily contains membrane proteins that transport a wide variety of substrates, such as metabolic products, lipids and sterols, and drugs, across extra- and intracellular membranes. In microbial organisms, the multidrug efflux pumps, which belong to members of the ABC superfamily, act to effectively pump drugs out of cells, significantly reduce the intracellular concentration of antifungals and prevent the emergence of drug resistance (Nakamura et al., 2001; Coste et al., 2006; Shapiro et al., 2011). Among them, Pdr5 is the most abundant ABC transporter in S. cerevisiae and shares sequence homology with Cdr1 in C. albicans (Kontoyiannis and Lewis, 2002). Studies have revealed that Pdr5 is able to squeeze out hundreds of structurally independent hydrophobic compounds to pass through the plasma membrane (Kolaczkowski et al., 1996; Egner et al., 1998; Golin et al., 2003). Intriguingly, Pdr5 was the only target that came out of the screen when we used the 16 ABC transporter mutants to evaluate the efflux effect of ponatinib, suggesting that Pdr5 could be the substrate of Ponatinib and by inactivating the activity of Pdr5, the compound prevents the efflux of fluconazole from the fungal cells. In the future, more evidence is required to support our hypothesis, especially those implicated in assaysing direct interaction between Pdr5 and ponatinib.

The cytosolic pH homeostasis plays a key role in cell growth (Gillies et al., 1981, Casey et al., 2010, Dechant et al., 2010, Dechant et al., 2014, Martinez-Munoz and Kane, 2017; Saliba et al., 2018). The ability to regulate intracellular pH enables C. albicans to survive in both extremely acidic and alkaline microenvironments (Casey et al., 2010; Cytet and Philpott, 2013; Felmanova et al., 2017; Martinez-Munoz and Kane, 2017; Saliba et al., 2018; Xu et al., 2019). The critical elements determining pH homeostasis are as follows: proton pumps, exchangers and buffers (Casey et al., 2010). Proton pumps, such as Pma1 in the plasma membrane, are required to establish certain pH gradient (Ferreira et al., 2001; Orij et al., 2011; Martinez-Munoz and Kane, 2017). Exchangers can transport ions and solutes against the gradient by using the energy stored in pH gradients or ion gradients to determine the final pH (Brett et al., 2005; Ohgaki et al., 2011; Kondapalli et al., 2014). Buffers protect the cells or organelles from the disturbance of short-term pH fluctuation (Casey et al., 2010; Poznanski et al., 2013). Pma1, which is independent of ion pumps, is a structural component of the plasma membrane accounting for 20–40% of the total plasma membrane proteins (Monk et al., 1991). Strikingly, this P-type H+-ATPase plays a key role in cytosolic pH regulation (Serrano, 1988; van der Rest et al., 1995) by pumping the proton out of the cells and consuming an impressive amount of intracellular ATP which accounts for approximately a quarter of total ATP consumption (Giacomello et al., 2013), highlighting the potential of Pma1 as an antifungal target. Interestingly, harnessing whole-genome sequencing and RT-PCR analysis, we argued that ponatinib may target Pma1 to perturb the pH homeostasis in the cytoplasm, eventually leading to cell death. Supporting this proposition requires more evidence; however, the effective inhibitor of Pma1 has not been determined in clinic (Stewart et al., 1988; Monk et al., 1995; Perlin et al., 1997; Monk et al., 2005; Chan et al., 2007; Billack et al., 2009; Ottolie et al., 2018). Thus, alternative strategies have to be considered.

Numerous studies have demonstrated that fluconazole is widely used in clinic for the treatment of fungal infection by selectively interfering with the activity of cytochrome P-450 and inhibiting the biosynthesis of ergosterol in the plasma membrane. However, targeting a single drug target is usually difficult to achieve the desired effect and prone to drug resistance. In our work, we found that ponatinib acts as a fluconazole potentiator through suppression of not only multidrug efflux via Pdr5 but also Pma1 expression in vitro, supporting that the multi-target drug ponatinib could enhance the effectiveness of fluconazole via two action modes. Compared with the single drug administration, drug combinatorial therapy has demonstrated great advantages in overcoming drug resistance and improving therapeutic efficacy, since this therapy harbours multi-target activities and confers high information-processing capacity and functional diversity, especially the simultaneous regulation of multiple inputs into the signalling network. Moreover, the drug combinatorial therapy allows many parts of the network to be activated at once and has been applied in the treatment of many chronic diseases such as cancer and thus has drawn intensive attention from researchers and pharmaceutical enterprises. Of course, it has to be
noted that the main problem faced by repurposing the existing multi-target drugs is their potential adverse effects. In addition, the optimal drug concentration for each target is often different, and it is difficult for a multi-target drug to achieve the optimal pharmacological effect for each target, which may affect its synergistic effect. Thus, testing clinically relevant pharmacodynamics and pharmacokinetics of the prioritized drug combinations needs to be seriously considered for identification, development and optimization of efficacious combinatorial drug treatments.

**Experimental procedures**

**Strains and culture condition**

The strains used in this study (Table 2) were stored at −80°C and routinely grown in yeast peptone dextrose (YPD) medium.

**Minimal Inhibitory Concentration (MIC) Assay**

MIC assay was evaluated in 96-well microtitre plates using the broth dilution testing reference method M27-A3/S4, as recommended by the Clinical and Laboratory Standards Institute (CLSI) (Rex, 2008). Twofold serial dilutions of fluconazole (HY-B0101; MedChemExpress, Shanghai, China) or ponatinib (S1490; Selleck, Shanghai, China) were prepared along the columns or rows of a 96-well plate. Overnight cultures were diluted to an OD$_{600}$ of 1.0, followed by another 100-fold dilution to reach a final OD$_{600}$ of ~0.01 (Chen et al., 2011; Chen and Noble, 2012). The plates were incubated at 30°C, and the OD$_{600}$ was measured every 15 min. The fractional inhibitory concentration index (FICI) of each drug combination was determined according to the standard CLSI protocol, and FICI < 0.5 was defined as synergy (Brown et al., 2014). The formula is calculated as follows: $FICI = \frac{(MIC_{B in combination} - MIC_{A alone}) + (MIC_{B in combination} - MIC_{B alone})}{MIC_{A in combination}}$.

**Filamentation assay**

Optimal induction of filamentous growth in *C. albicans* was achieved by incubating the yeast cells on hyphal-inducing medium, following the procedures described previously (Flanagan et al., 2017).

**Emergence of drug resistance in vitro**

For emergence of drug resistance in vitro, fresh overnight yeast cultures were washed twice in PBS and diluted into an optical density (OD$_{600}$) of 0.5. After 10-fold serial dilutions, cells were spread onto appropriate agar plates supplemented with 32 µg ml$^{-1}$ fluconazole, 32 µM ponatinib or both.

**Time-kill curve analysis**

Overnight culture of *C. albicans* was diluted to a final OD$_{600}$ of 0.05 in liquid YPD medium containing fluconazole (32, 64, 128 µg ml$^{-1}$), ponatinib (32, 64, 128 µM) or both in 96-well plates with a final volume of 200 µl. The suspension was diluted at 1:250 000 and plated on YPD plates at indicated periods of time (0, 24 h or 48 h). The plates were incubated at 30°C for 24 h before determining the colony-forming units (CFUs) by counting.

**Rhodamine-6G (R6G) staining assay**

Cells grown overnight in YPD liquid medium at 30°C were washed with ice-cold glucose-free phosphate-buffered saline (PBS) and incubated at 30°C for 1 h under starvation to reduce the ATP-binding cassette (ABC) efflux pumps activity. The collected cells were then washed and diluted to 10$^6$ cells ml$^{-1}$ in ice-cold PBS, which were exposed to 4, 8, 16, 32, 64 µM ponatinib or microdilution method (M38-A2) from the Clinical and Laboratory Standards Institute (CLSI) (Alexander, 2008). The procedures were performed as described in a previous study (Hsieh et al., 1993). Briefly, each compound was serially diluted in two-fold in a 96-well plate, either across columns of the plates (fluconazole) or rows of the plates (ponatinib). Overnight cultures were diluted to an OD$_{600}$ of 1.0, followed by another 100-fold dilution to reach a final OD$_{600}$ of ~0.01 (Chen et al., 2011; Chen and Noble, 2012). The plates were incubated at 30°C, and the OD$_{600}$ was measured every 15 min. The fractional inhibitory concentration index (FICI) of each drug combination was determined according to the standard CLSI protocol, and FICI < 0.5 was defined as synergy (Brown et al., 2014). The formula is calculated as follows: $FICI = \frac{(MIC_{B in combination} - MIC_{A alone}) + (MIC_{B in combination} - MIC_{B alone})}{MIC_{A in combination}}$.
Beauvericin at a concentration of 16 $\mu$g ml$^{-1}$ was added as a positive control with dimethyl sulfoxide (DMSO) as the negative control. All samples were incubated for another 2 h at 30°C. After treatment with 10 $\mu$M (final concentration) R6G, cells were incubated for another 1.5 h at 30°C. The external R6G was then removed by washing with PBS, and 2% glucose was added to the samples to reactivate the ABC efflux pumps. After incubation at 30°C for 1 h, the reactivated cells were washed and observed under fluorescent microscopy to monitor intracellular R6G accumulation.

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Table 2. Strains used in this study.

| Strain name | Parent | Genotype | Strain background/construction | References |
|-------------|--------|----------|---------------------------------|------------|
| C. albicans |        |          |                                 |            |
| SC5314      | Wild type | Fluconazole-resistant clinical isolates | This work |
| CCC49       | Wild type | Fluconazole-resistant clinical isolates | This work |
| CCC80       | Wild type | Fluconazole-resistant clinical isolates | This work |
| S. cerevisiae |        |          |                                 |            |
| BY4742      | Wild type |                           | Suzuki et al. (2011) |
| adp1.1      | BY4742 | adp1.1 transformed with WT | Suzuki et al. (2011) |
| snq2.1      | BY4742 | snq2.1 transformed with WT | Suzuki et al. (2011) |
| ycf1.1      | BY4742 | ycf1.1 transformed with WT | Suzuki et al. (2011) |
| pdr15.1     | BY4742 | pdr15.1 transformed with WT | Suzuki et al. (2011) |
| yor1.1      | BY4742 | yor1.1 transformed with WT | Suzuki et al. (2011) |
| vmr1.1      | BY4742 | vmr1.1 transformed with WT | Suzuki et al. (2011) |
| pdr11.1     | BY4742 | pdr11.1 transformed with WT | Suzuki et al. (2011) |
| nft1.1      | BY4742 | nft1.1 transformed with WT | Suzuki et al. (2011) |
| kpt1.1      | BY4742 | kpt1.1 transformed with WT | Suzuki et al. (2011) |
| ybt1.1      | BY4742 | ybt1.1 transformed with WT | Suzuki et al. (2011) |
| pdr18.1     | BY4742 | pdr18.1 transformed with WT | Suzuki et al. (2011) |
| yol075c.1   | BY4742 | yol075c.1 transformed with WT | Suzuki et al. (2011) |
| aus1.1      | BY4742 | aus1.1 transformed with WT | Suzuki et al. (2011) |
| pdr5.1      | BY4742 | pdr5.1 transformed with WT | Suzuki et al. (2011) |
| pdr10.1     | BY4742 | pdr10.1 transformed with WT | Suzuki et al. (2011) |
| pdr12.1     | BY4742 | pdr12.1 transformed with WT | Suzuki et al. (2011) |
| BY4741      | Wild type |                           | Suzuki et al. (2011) |
| RY0566      | BY4741 |                           | Suzuki et al. (2011) |
| 2336        | BY4741 |                           | Suzuki et al. (2011) |
| R1          | 2336   | Ponatinib resistant isolate | This work |
| R8          | 2336   | Ponatinib resistant isolate | This work |
| R9          | 2336   | Ponatinib resistant isolate | This work |
| R10         | 2336   | Ponatinib resistant isolate | This work |
| R11         | 2336   | Ponatinib resistant isolate | This work |
| R12         | 2336   | Ponatinib resistant isolate | This work |
| R13         | 2336   | Ponatinib resistant isolate | This work |
| R16         | 2336   | Ponatinib resistant isolate | This work |
| R17         | 2336   | Ponatinib resistant isolate | This work |
| R18         | 2336   | Ponatinib resistant isolate | This work |
| C. neoformans |        |          |                                 |            |
| H99         | Wild type |                           | This work |

4, 8, 16, 32, 64 $\mu$g ml$^{-1}$ fluconazole respectively. Beauvericin at a concentration of 16 $\mu$g ml$^{-1}$ was added as a positive control with dimethyl sulfoxide (DMSO) as the negative control. All samples were incubated for another 2 h at 30°C. After treatment with 10 $\mu$M (final concentration) R6G, cells were incubated for another 1.5 h at 30°C. The external R6G was then removed by washing with PBS, and 2% glucose was added to the samples to reactivate the ABC efflux pumps. After incubation at 30°C for 1 h, the reactivated cells were washed and observed under fluorescent microscopy to monitor intracellular R6G accumulation.
Whole-genome sequencing of ponatinib-resistant isolates

The parental strain ABC16 and 10 selected ponatinib-resistant isolates were separately cultured on solid YPD plates for DNA extraction. Then, the mutations in PMA1 of the above 11 isolates were validated by PCR and sequencing to identify the specific mutation (Heitman et al., 1991; Shekhar-Guturja et al., 2016b; Vincent et al., 2016; Sukheja et al., 2017; Wang et al., 2020).

The PCR reaction mixture is comprised of 10xHi-fidelity PCR buffer (2 µl), 2 mM dNTPs (1.2 µl), 50 mM MgSO4 (0.6 µl), 10 mM primers (0.4 µl), 5 µM Taq Enzyme (0.1 µl), DNA template (2 µl) and sterile water up to 40 µl. For the PCR program, the conditions are followed by 95°C 5 min; 94°C 30 s, 55°C 30 s and 68°C 6 min and 40 s for 30 cycles; 68°C 10 min. The genomic sequences of all strains can be accessed through NCBI accession number PRJNA649097.

qRT-PCR

Overnight cell cultures in YPD at 30°C were diluted to OD600 of 1.0. Cells were treated with DMSO, fluconazole, ponatinib or both and grown at 30°C for 3 h. The RNA was then extracted by hot phenol method and further treated with RT reagent with gDNA Eraser (Takara #RR047A, Beijing, China). The PCR was performed using Universal SYBR Green Supermix (Bio-Rad #1725121) with the following program: 95°C for 30 s; 95°C for 5 s; and 60°C for 30 s, for 40 cycles. Primers are listed in Table 3. The data were analysed by the 2−ΔΔCt method, in which ΔΔCt = (Ct value of target gene − Ct value of reference gene)sample − (Ct value of target gene − Ct value of reference gene)control, as described previously (Livak and Schmittgen, 2001; Huang et al., 2007).

Western blot

The cell lysis, protein extraction and Western blot procedures were performed as described in a previous study (Liu et al., 2017). The antibodies are listed in Table 4. For densitometry, Image J software (https://imagej.net/Downloads) was used as in a previous study (Flanagan et al., 2017).

Proton pump in plasma membrane

The BY4741 strain was cultured overnight in YPD liquid medium at 30°C, and 5 × 10^8 cells were used for extraction of IOV (In-side out), which was treated with fluconazole (16 µg ml⁻¹), ponatinib (8 µM) or both in 96 well black plates. The fluorescence intensity at excitation wavelength 490 nm and emission wavelength 530 nm was measured every 5 min for 1 h. The N-ethylmaleimide (NEM) (10 µM) and orthovanadate (OV) (100 µM) were added as a positive control with dimethyl sulfoxide (DMSO) as the negative control (Van Dyke et al., 1985, Kaunitz and Sachs, 1986). IOV (In-side out) was extracted using Fungus/Yeast Membrane Vesicle RSOV/IOV Prep Kit (GMS 10169.3 v.A; Shanghai CHENGONG Biotechnology Co., Ltd, Shanghai, China). The proton pump in the plasma membrane was mea-

Table 3. Primers used in this study.

| Primer name | Purpose | Sequence 5’ to 3’ |
|-------------|---------|------------------|
| NL50        | Forward to amplify PMA1 | ACATTCAAAAGAAAGAAAAATAATACCACCCAGCTAGTT-AAAAGAAAAATATTGTTAACGAGAGACTCCACAGCTAGTT-AAAAGAAAAATAATACCACCCAGCTAGTT-AAAAGAAAAATA | |
| NL51        | Reverse to amplify PMA1 | TTGATATAAAAAATTAAAATTAAAATTAGAAAAATTAAACCAGAAAATATCAATTTGATATTAAAATGTGACAAAAATTATGATTAAATGTTACTTCAACAGGAGAATTCGAGCTCGTTCGTTAATAAAC |
| NL220       | Forward for qRT-PCR of PMA1 | GCCCTGCTAAGACTTACGGAGACGC |
| NL221       | Reverse for qRT-PCR of PMA1 | TCCACCAGGGCCAAAACCTGGAC |
| NL222       | Forward for qRT-PCR of ACT1 | ATATATATATATATATATATATATATATATATATATATATAAATAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
assured using Proton Transport (P ATPase dependent) Assay Kit (GMS 10159.1.2 v.A; Shanghai CHENGONG Biotechnology Co., Ltd).

**Statistics**

All data were shown as the mean ± SDs in three independent experiments. All results were calculated from the means of three separate experiments. Statistical analysis was performed using GraphPad Prism 7, San Diego, CA, USA with the one-way ANOVA and Tukey multiple comparison analysis at a *P < 0.05*, ***P < 0.001* or ****P < 0.0001 level of significance.

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**Conflict of interests**

The authors declare no conflicts of interest.

**Author contribution**

NNL, HW and CC conceived and designed the study; LL, NNL, HW and CC performed data analysis and wrote the manuscript; LL, TJ, YM, JZ, JL, JT, LW, JL and PY conducted all experiments and performed the statistical analysis of the data; LL, YP, HW, LZ, J.L.L.-R., RSS, CC, NNL and HW discussed the experiments and results.

**Data Availability Statement**

The data sets generated from the current study have been deposited in the US National Center for Biotechnology Information (NCBI). The accession number for the gene expression profiling raw data reported in this paper is NCBI PRJNA: 649097. The Submission ID is SUB7844246. The link is provided in https://submit.ncbi.nlm.nih.gov/subs/bioproject/SUB7844246/overview.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Combination of ponatinib and fluconazole synergis
tically inhibits growth of C. albicans. C. albicans (SC5314) was subjected to 4 μM ponatinib combined with two-fold serial dilutions of fluconazole in YPD medium. OD600 was measured every 15 min at 30°C.

Fig. S2. Ponatinib is non-toxic at 16 μM or lower by lactate dehydrogenase assay. Endothelial cells (NCM460) were exposed to two-fold serial dilutions of ponatinib in DMEM at 37°C for 1.5 h.

Fig. S3. The visible MIC of fluconazole or ponatinib on C. albicans, S. cerevisiae, C. neoformans and fluconazole-re
sistant clinical C. albicans isolates. (A) Two-fold serial dilu
tions of fluconazole or ponatinib in YPD without fungi. (B) C. albicans was exposed to two-fold serial dilutions of flucona
zole or ponatinib in YPD. (C) S. cerevisiae was exposed to two-fold serial dilutions of fluconazole or ponatinib in YPD. (D) C. neoformans was exposed to two-fold serial dilutions of fluconazole or ponatinib in YPD. (E) Fluconazole-resistant clinical C. albicans isolates (CCC49) was exposed to two-fold serial dilutions of fluconazole or ponatinib in YPD. (F) Fluconazole-resistant clinical C. albicans isolates (CCC80) was exposed to two-fold serial dilutions of fluconazole or ponatinib in YPD. The plates of C. albicans, S. cerevisiae and fluconazole-resistant clinical C. albicans isolates were incubated at 30°C for 24h, the plate of C. neoformans was incubated at 30°C for 72h and all the plates were pho
tographed by ChemiDoc MP (Bio-Rad).

Fig. S4. Transcriptional expression of PDR5 in S. cerevisiae was quantified under treatment with ponatinib. Cells were subjected to DMSO or ponatinib in YPD at 30°C for 4 h. Experiments were performed in biological triplicates.

Fig. S5. The proton pump in the membrane was enhanced by fluconazole but inhibited when combined with ponatinib. IOV (in-side out) of 4741 was subjected to DMSO, N-ethylmaleimide (NEM) (10 μM) + Orthovanadate (OV) (100 μM), fluconazole (16 μg ml⁻¹), ponatinib (8 μM) or fluconazole (16 μg ml⁻¹) + ponatinib (8 μM) for 1 h. Experiments were performed in biological triplicates.