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# Potent Antifungal Synergy of Phthalazinone and Isoquinolones with Azoles Against *Candida albicans*

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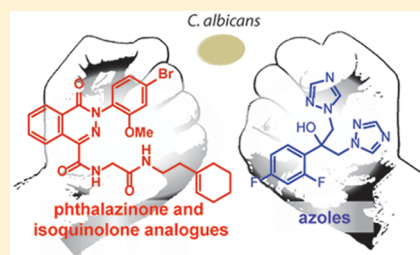
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## S Supporting Information

**ABSTRACT:** Four phthalazinones (CIDs 22334057, 22333974, 22334032, 22334012) and one isoquinolone (CID 5224943) were previously shown to be potent enhancers of antifungal activity of fluconazole against *Candida albicans*. Several even more potent analogues of these compounds were identified, some with EC<sub>50</sub> as low as 1 nM, against *C. albicans*. The compounds exhibited pharmacological synergy (FIC < 0.5) with fluconazole. The compounds were also shown to enhance the antifungal activity of isavuconazole, a recently FDA approved azole antifungal. Isoquinolone 15 and phthalazinone 24 were shown to be active against several resistant clinical isolates of *C. albicans*.

**KEYWORDS:** *Candida albicans*, antifungal agents, fluconazole, synergy, phthalazinone, isoquinolone



Azoles continue to be the drug of choice for many types of invasive fungal infections, acting on a key enzyme, sterol 14 $\alpha$ -demethylase, in the ergosterol biosynthesis pathway. The azole family of antifungals has evolved continuously since the initial introduction of ketoconazole in the 1980s<sup>1</sup> with the goal of achieving high affinity toward the fungal P450 14 $\alpha$ -demethylase, low affinity toward human CYP enzymes,<sup>2,3</sup> and, more recently, evasion of fungal resistance mechanisms.<sup>4</sup> Fluconazole, introduced in 1990, proved well-tolerated in patients and has been used ubiquitously to treat invasive candidiasis.<sup>1</sup> The emergence of fluconazole resistance has led to the increasing use of echinocandins and the development of third-generation azoles (voriconazole, posaconazole, isavuconazole) with higher affinity.<sup>5</sup> Of the third-generation drugs, posaconazole and voriconazole work against a broader range of fungal pathogens but are more expensive and have other disadvantages: posaconazole has a less flexible dosing and absorption profile than fluconazole; voriconazole may be ineffective against strains that have already developed resistance toward fluconazole.<sup>6</sup> Newer azole drugs like albaconazole and fosravuconazole are still in development.

The development of improved azoles has been paralleled by the search for small molecules that enhance the antifungal effect of existing azoles,<sup>7–10</sup> but the efforts have been met with limited success.<sup>11,12</sup> A wide range of azole enhancers have been shown to exhibit antifungal synergy;<sup>13</sup> two approved drugs, flucytosine<sup>14</sup> and calcineurin inhibitors,<sup>15–19</sup> have been shown to synergize with fluconazole against at low concentrations (<10  $\mu$ g/mL) against strains of *C. albicans* but have limitations for general use. Against other species,<sup>20</sup> flucytosine instead antagonizes the effect of fluconazole, so the benefits of flucytosine-azole combinations are not clear.<sup>21</sup> Calcineurin inhibitors such as sirolimus and tacrolimus depend on human CYP enzymes for metabolic

clearance; azole drugs like fluconazole exert off-target effects on these human CYP enzymes. Buildup of these calcineurin inhibitors in plasma increases risk of nephrotoxicity and, as immunosuppressants, may increase rates of infection from other pathogenic fungal species.<sup>22–24</sup> Given the limitations of these existing azole synergizers, new potent alternatives are needed.

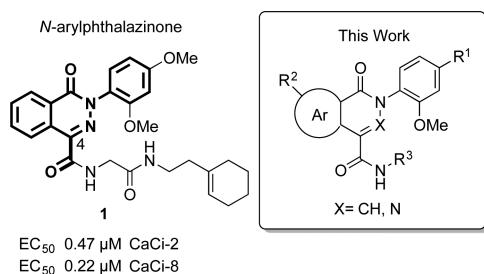
Lindquist, Schreiber, and co-workers carried out a screening campaign to identify small molecules that could enhance the antifungal effect of fluconazole against *C. albicans*.<sup>25,26</sup> After an initial screen of over 300,000 compounds and a subsequent rescreening, 296 compounds were found to enhance the antifungal effect of fluconazole against a partially resistant clinical strain CaCi-8 without cytotoxicity against mammalian cells. Three of those compounds<sup>27,28,26</sup> were selected for further optimization, but none of the resulting compounds (ML189, ML212, and ML229) were active below 0.7  $\mu$ M against CaCi-8. Following up on another hit from the Lindquist–Schreiber screen, we recently reported an analogue called synazo-1 that could enhance the antifungal effect of fluconazole with an EC<sub>50</sub> of 300 pM.<sup>29</sup> Encouraged by the discovery of potent fluconazole synergizers we set out to identify new lead compounds, optimize their structures, study their synergy with other azoles, and assess their selectivity for *C. albicans*.

Among the compounds initially screened by Lindquist and co-workers were 233 structurally related *N*-arylphthalazinones (X = N, Figure 1) and *N*-arylisoquinolones (X = CH). Four phthalazinones and one isoquinolone were active in the initial screen. The most active compound of the five was phthalazinone

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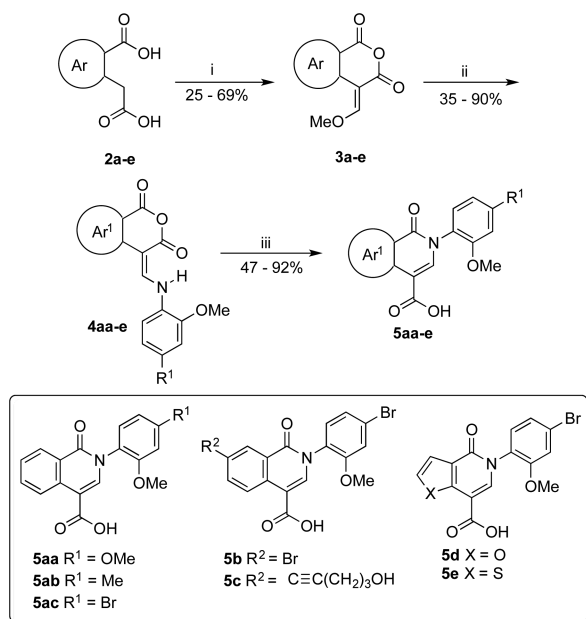
**Figure 1.** Structure of lead phthalazinone (CID 22334057) and representative structure of analogues synthesized in this study.

**1** (CID 22334057), which showed an EC<sub>50</sub> below 0.5 μM for *C. albicans* clinical isolates CaCi-8 and CaCi-2.

An initial analysis of the Lindquist data suggested that the biological activity of phthalazinones was sensitive to substituents on the *N*-phenyl ring and the C4 carboxamide side chain (Figure 1). In particular, a *para* substituent on the *N*-phenyl ring was favorable for activity, and an *ortho* substituent seemed essential. Additionally, the glycine linker was present in all of the active phthalazinones. The effect of modifications within the bicyclic core, at the atom X within the heterocycle or the fused benzo ring, were unclear. Analogues of the isoquinolone and phthalazinone compounds were therefore synthesized to explore chemical modification at three sites: (a) modification of the bicyclic core, including substitution of X (N versus CH), substitution of the fused benzo ring, or replacement of the benzo ring with a heterocycle; (b) substitution at the *para* position of the *N*-phenyl ring; (c) variation of the C4 carboxamide side chain.

All analogues were synthesized by coupling heterocyclic carboxylic acids with amines. Isoquinolone analogues were synthesized from the corresponding dicarboxylic acids **2a–e** following a route developed by Wolfbeis (Scheme 1).<sup>30</sup> A one-

### Scheme 1. Synthesis of Isoquinolone Carboxylic Acids<sup>a</sup>

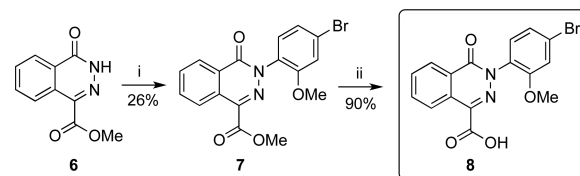


<sup>a</sup>Reagents and conditions: (i) 1.0 mol-equiv of CH(OCH<sub>3</sub>)<sub>2</sub>, Ac<sub>2</sub>O, 140 °C, 5 min–2.5 h; (ii) 1.0 mol-equiv of ArNH<sub>2</sub>, 1,4-dioxane, 23 °C, 1–4 h; (iii) 5.0 mol-equiv of NaOH, EtOH, 80 °C, 1–4 h.

step anhydride formation and formylation generated enol ethers **3a–e**. Conjugate substitution of the methoxy group with various anilines generated the corresponding enamines **4aa–e**. Ethoxide catalyzed the Wolfbeis rearrangement of anhydrides **4aa–e** to isoquinolones **5aa–e**.<sup>31</sup>

The original Dieckmann synthesis<sup>32</sup> of *N*-arylphthalazinones was problematic due to the capricious reactivity of electron-rich arylhydrazines. *N*-Arylphthalazinone **7** was instead synthesized using a Cu-promoted cross-coupling of phthalazinone **6** with an arylboronic acid (Scheme 2). Ester **7** was saponified to afford carboxylic acid **8**.

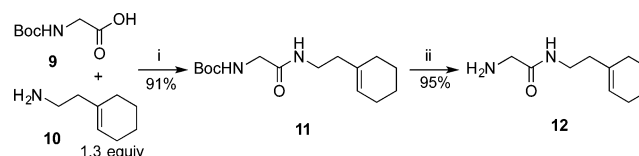
### Scheme 2. Synthesis of Phthalazinone Carboxylic Acid<sup>a</sup>



<sup>a</sup>Reagents and conditions: (i) 2 mol-equiv of Cu(OAc)<sub>2</sub>, 5 mol-equiv of pyridine, CH<sub>2</sub>Cl<sub>2</sub>, 23 °C, 24 h; (ii) 3.8 mol-equiv of LiOH, 3:1 THF/H<sub>2</sub>O, 23 °C, 16 h.

Several 2-amino acetamide fragments were synthesized in order to couple with carboxylic acids **5aa–e** and **8** (Scheme 3).

### Scheme 3. Representative Synthesis of 2-Amino Acetamide Analogues<sup>a</sup>

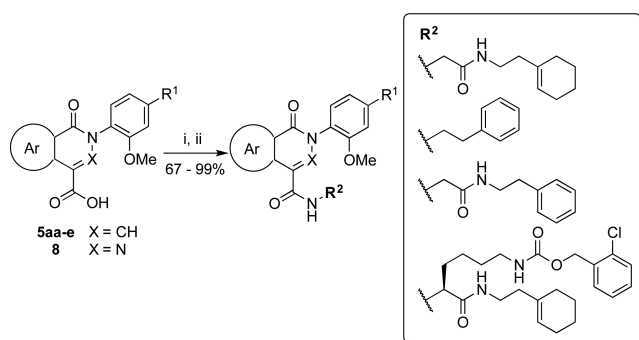


<sup>a</sup>Reagents and conditions: (i) 1.3 mol-equiv of EDC, 1.7 mol-equiv of HOBT, 5.5 mol-equiv of DIPEA, THF, 23 °C, 16 h; (ii) H<sub>2</sub>O, 100 °C, 16 h.

For illustrative purposes, the synthesis of amine **12** is described in Scheme 3. *N*-Boc-glycine **9** was coupled with 2-(1-cyclohexenyl)ethylamine **10** through a carbodiimide coupling reaction to afford amide **11** in good yield.<sup>33</sup> Deprotection of the Boc group in the presence of the cyclohexenyl group, however, was found to be challenging. Common methods such as treatment with trifluoroacetic acid resulted in complete degradation of the starting material due to protonation of the alkene. Consequently, a thermal deprotection was employed to generate amine **12**. Compound **11** was deprotected by heating at reflux in water to remove the Boc group.<sup>34</sup> However, related compounds with limited solubility in water were instead heated under reflux in ethylene glycol.<sup>35</sup>

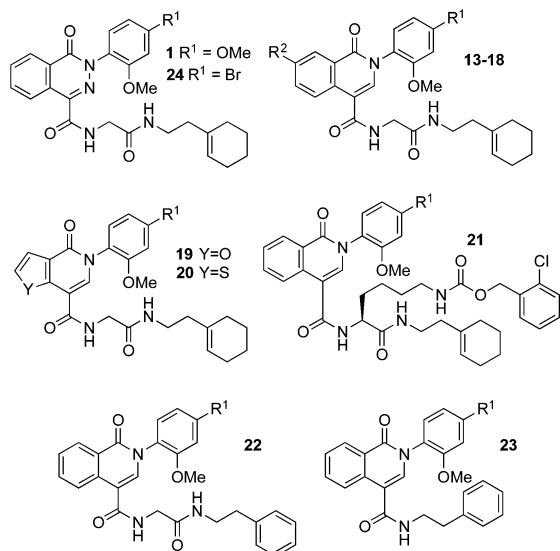
Carboxylic acids **5aa–e** and **8** were converted to the corresponding acid chlorides under Vilsmeier conditions and then directly coupled with amines in the presence of triethylamine (Scheme 4) to afford analogues **13–15** and **17–24** in good yields (Table 1).<sup>36</sup>

To test the steric demands around the *N*-phenyl group the bromine substituent of isoquinolone **15** was converted to the corresponding *ω*-hydroxyalkyne using a palladium-catalyzed Sonogashira coupling (Scheme 5).

Scheme 4. Coupling of Carboxylic Acid and Amine Fragments<sup>a</sup>

<sup>a</sup>Reagents and conditions: (i) 1.5 mol-equiv of oxalyl chloride, 10 mol % DMF, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C, 2 h; (ii) 1.2 mol-equiv of Et<sub>3</sub>N, CH<sub>2</sub>Cl<sub>2</sub>, 0–23 °C, 30 min.

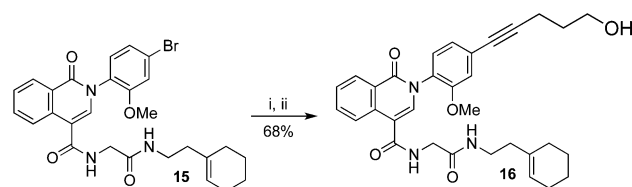
**Table 1. Antifungal Activities for Phthalazinone and Isoquinolone Analogues Against *C. albicans* in the Presence of Fluconazole<sup>a</sup>**



| compd | R <sup>1</sup>                        | R <sup>2</sup>                        | EC <sub>50</sub> (μM) |
|-------|---------------------------------------|---------------------------------------|-----------------------|
| 1     | OMe                                   |                                       | 0.015 ± 0.002         |
| 13    | OMe                                   | H                                     | 0.033 ± 0.005         |
| 14    | Me                                    | H                                     | 0.065 ± 0.02          |
| 15    | Br                                    | H                                     | 0.003 ± 0.001         |
| 16    | C≡C(CH <sub>2</sub> ) <sub>3</sub> OH | H                                     | >100                  |
| 17    | Br                                    | Br                                    | 0.003 ± 0.001         |
| 18    | Br                                    | C≡C(CH <sub>2</sub> ) <sub>3</sub> OH | >100                  |
| 19    | Br                                    |                                       | >10                   |
| 20    | Br                                    |                                       | 0.230 ± 0.01          |
| 21    | Br                                    |                                       | 0.023 ± 0.008         |
| 22    | Br                                    |                                       | 0.020 ± 0.006         |
| 23    | Br                                    |                                       | 0.220 ± 0.05          |
| 24    | Br                                    |                                       | 0.001 ± 0.0003        |

<sup>a</sup>Cells incubated with 0.25 μg/mL fluconazole.

We determined the enhancement of antifungal activity for all of the new analogues against a susceptible strain of *C. albicans*, HLY4123, derived from the common laboratory strain CAI4.<sup>29</sup> In the presence of a constant, nonlethal concentration of fluconazole (0.25 μg/mL), the analogues inhibited fungal cell

Scheme 5. Derivatization of Analogue 15 via Sonogashira Coupling<sup>a</sup>

<sup>a</sup>Reagents and conditions: (i) 1.2 mol-equiv of 4-pentyn-1-ol, 5 mol % Pd(Cl)<sub>2</sub>(PPh<sub>3</sub>)<sub>2</sub>, 5 mol % CuI, 3:2 DMF/Et<sub>3</sub>N, 100 °C, 45 min.

growth in a dose-dependent fashion, from which EC<sub>50</sub> values were determined (Table 1).

Against HLY4123, phthalazinone **1** demonstrated promising activity (EC<sub>50</sub> 0.015 μM) in the presence of 0.25 μg/mL fluconazole. The isostere, isoquinolone **13**, exhibited a similar EC<sub>50</sub> of 0.033 μM suggesting that both the isoquinolone and phthalazinone cores are competent scaffolds for eliciting antifungal activity. Only one of five active compounds in the initial Lindquist–Schreiber screen possessed an isoquinolone core, so it was unclear which structural features most influenced antifungal activity. We set out to examine the isoquinolone core further by making modifications to the *N*-phenyl ring and benzo ring of the core, as well as the C4 carboxamide side chain.

First, we explored the effect of substitution at the *para* position of the *N*-phenyl ring (R<sup>1</sup>). A decrease in activity was observed when methoxy (isoquinolone **13**) was replaced by a methyl group (isoquinolone **14**) at this position.

A bromine atom at the *para* position led to a surprising improvement in activity, with bromo-analogue **15** exhibiting 10-fold better activity than methoxy-analogue **13**, and also better activity than phthalazinone **1**. Substitution at R<sup>1</sup> with an alcohol-terminated alkyne abolishes activity, suggesting that larger or more rigid substituents are detrimental at this position.

After establishing the importance of the bromine substituent in isoquinolone **15**, we proceeded to evaluate the arene ring fused to the central heterocycle. Substitution with bromine at the C7 position of the benzo ring (R<sup>2</sup>) resulted in activity of analogue **17** comparable to that of **15**. As with R<sup>1</sup>, substitution at R<sup>2</sup> with an alcohol-terminated alkyne abolished activity. The sensitivity to substituents at R<sup>1</sup> and R<sup>2</sup> suggests that both positions play an important role in binding to the biological target.

Attempts to replace the benzo group with a smaller furano (analogue **19**) or thiopheno (analogue **20**) group resulted in a significant loss of potency. Even so, the thiophene analogue **20** showed significantly better activity than its electron-rich furan isostere **19**.

Neither contraction of the benzo ring nor expansion by substitution improved activity. Therefore, we directed our attention to the C4 carboxamide side chain. Using analogue **15** again as a reference point, we evaluated the effect of substitution at the glycine α-carbon. The activity of ClCbz-lysine analogue **21** was only slightly attenuated relative to glycine analogue **15**, revealing some tolerance for bulky substituents at this position of the side chain. We then prepared phenethyl amide **22** to test the importance of the cyclohexenylethyl group. The activity of cyclohexenylethyl analogue **15** was superior to that of the aromatic phenethyl analogue **22**. Shortened analogue **23**, missing the glycine linker, was 70-fold less active than full-length analogue **15**.

Table 2. Strains Used in This Study

| species   | strain           | relevant characteristics or genotype |                                |        |                       | ref                           |
|---|------------------|--------------------------------------|--------------------------------|--------|-----------------------|-------------------------------|
| <i>Candida albicans</i>                           | HLY4123          | ERG3-GFP                             |                                |        |                       | Premachandra <sup>29</sup>    |
| <i>Candida glabrata</i>                           | BG2              | WT                                   |                                |        |                       | Fidel <sup>39,40</sup>        |
| <i>Cryptococcus neoformans</i> var. <i>grubii</i> | H99              | WT                                   |                                |        |                       | Broad Institute <sup>41</sup> |
| <i>Candida albicans</i>                           | clinical isolate | mutations                            | expression (fold) <sup>a</sup> |        | Flowers <sup>37</sup> |                               |
|   | 17               | ERG11A114S,Y257H                     | ERG11                          | CDR1   |                       |                               |
|   | 26               | ERG11G307S,G448R                     | 1.6                            | 2.7    |                       |                               |
|   | 33               | ERG11F145L,E266D                     | 23.9                           | 0.8    |                       |                               |
|   | 36               | UPC2Y642F                            | 1.0                            | 0.9    |                       |                               |
|   | 45               | ERG11E266D,G464S, UPC2A646V          | 1.7                            | 16.2   |                       |                               |
|   |                  | 12.6                                 | 6.8                            | 1465.6 | 4.1                   |                               |
|   |                  | 3.5                                  | 4.8                            | 201.5  | 1.5                   |                               |
|   |                  |                                      |                                | 147.8  | 15.5                  |                               |

<sup>a</sup>Fold increase relative to expression levels averaged across three unrelated fluconazole-susceptible strains.

Phthalazinone **24** was slightly more active than the isosteric bromophenyl isoquinolone **15**. With an EC<sub>50</sub> of 1 nM, bromophenyl phthalazinone **24** was the most active of all compounds tested.

Lead compound **1**, isoquinolone **15**, and phthalazinone **24** showed potent activity even at fluconazole concentrations of 0.05 μg/mL, 5-fold lower than that used in Table 1. The EC<sub>50</sub> values were measured as 49, 24, and 7 nM, respectively.

Next, highly active bromophenyl analogues **15** and **24** were tested against five fluconazole-resistant clinical isolates of *C. albicans* (Table 2). Resistance in these isolates has been linked to multiple factors, including overexpression and point mutations in *ERG11*,<sup>37</sup> the gene encoding sterol 14α-demethylase. Point mutations in *ERG11* may contribute to fluconazole resistance by reducing binding affinity to the enzyme active site.<sup>38</sup> In some cases, the isolates also exhibit gain-of-function mutations in *UPC2*, a transcription factor regulating *ERG11* expression. The isolates also show varying levels of *CDR1*, *CDR2*, and *MDR1* expression, implicating efflux pumps as an important source of fluconazole resistance.

In dose-dependent broth microdilution assays, analogues **15** and **24** showed potent activities (EC<sub>50</sub> < 1 μM) against all resistant isolates. The EC<sub>50</sub> values were determined by varying the analogue concentration while keeping the fluconazole concentration fixed. The fluconazole MIC<sub>50</sub> was used and was determined for each isolate individually. Compound **24** exhibited comparable or better activity than compound **15** against the isolates tested (Table 3). At present, the origin of fluconazole synergy is uncertain. Many factors contribute to fluconazole resistance in these isolates, and we did not observe a correlation of compound activity with one specific resistance mechanism, such as levels of *CDR1* expression, among the isolates we tested here. Therefore, we are unable to attribute the synergistic effect

Table 3. Antifungal Activities of Analogues **15** and **24** against Resistant Isolates of *C. albicans* in the Presence of Fluconazole

| isolate | [Flu] <sup>b</sup> | EC <sub>50</sub> <sup>a</sup> |           |
|---------|--------------------|-------------------------------|-----------|
|         |                    | <b>15</b>                     | <b>24</b> |
| 17      | 12                 | 74 ± 10                       | 39 ± 3    |
| 26      | 32                 | 11 ± 1                        | 17 ± 2    |
| 33      | 64                 | 371 ± 23                      | 72 ± 6    |
| 36      | 64                 | 32 ± 4                        | 20 ± 2    |
| 45      | 96                 | 170 ± 20                      | 65 ± 15   |

<sup>a</sup>EC<sub>50</sub> (nM) expressed as arithmetic mean ± SD of three independent experiments. <sup>b</sup>Measured MIC<sub>50</sub> values for fluconazole alone; EC<sub>50</sub> values were determined at the fluconazole concentrations listed.

of compounds **15** and **24** with fluconazole to any single variable. Investigation into the possible causes of fluconazole synergy is ongoing.

Compounds **1**, **15**, and **24** were also tested against *Candida glabrata* BG2<sup>39,40</sup> and *Cryptococcus neoformans* var. *grubii* H99<sup>41</sup> and showed no activity up to 30 μM at their MIC<sub>50</sub> values (128 and 4.0 μg/mL, respectively). It is not clear why analogues **15** and **24** are active against *C. albicans* but not *C. glabrata* or *Cryptococcus neoformans*. We are currently investigating this issue.

Isavuconazole is one of the newest azole drugs, approved by FDA in 2015. We tested the potency of compounds **15** and **24** against HLY4123 and the highly resistant CaCi-45 in the presence of isavuconazole (Table 4). Compounds **15** and **24** showed potent antifungal activity with isavuconazole at much lower concentrations than was required for fluconazole.

Table 4. Antifungal Activities<sup>a</sup> of Analogues **15** and **24** against *C. albicans* in the Presence of Isavuconazole

| compd     | isolate                                    |  |
|-----------|--|--|
|           | HLY4123 ([isa] = 0.001 μg/mL) <sup>b</sup> | CaCi-45 ([isa] = 0.5 μg/mL) <sup>b</sup> |
| <b>15</b> | 6 ± 0.2                                    | 110 ± 20                                 |
| <b>24</b> | 4 ± 0.2                                    | 13 ± 4                                   |

<sup>a</sup>EC<sub>50</sub> (nM) expressed as arithmetic mean ± SD of three independent experiments. <sup>b</sup>MIC values

At 30 μM or below, the analogues do not significantly affect fungal growth in the absence of azoles. Checkerboard assays revealed that compounds **15** and **24** are true synergizers with fluconazole, exhibiting fractional inhibitory concentration indices of less than 0.17 and 0.12, respectively. These indices are well below the upper-limit of 0.5 for pharmacological synergy (Supporting Information, 29–30).

All analogues were also tested for cytotoxicity against 3T3 mammalian fibroblasts. No cytotoxicity was observed up to 10 μM, indicating high selectivity for inhibiting *C. albicans* cell growth over that of mammalian fibroblasts.

In order to assess the potential for compounds **15** and **24** as drug leads, the physicochemical properties were calculated using FAF-Drugs3 (Supporting Information, 32). The bromine atom is beneficial for activity but puts the molecular weight over 500. Both compounds exhibit favorable characteristics according to other criteria including tPSA, predicted bioavailability, and predicted induction of phospholipidosis but were slightly lipophilic based on logD.

In conclusion we have designed, synthesized, and studied phthalazinone and isoquinolone analogues of lead compound **1** (CID 22334057), which was previously shown to be active



against *C. albicans* in the presence of fluconazole. Most of the analogues synthesized showed highly potent activities against a susceptible strain of *C. albicans*, HLY4123. Two compounds, isoquinolone analogue **15** and the other a phthalazinone analogue **24**, were selected for further studies and shown to be active against clinical isolates with high resistance to fluconazole. Checkerboard assays confirmed compounds **15** and **24** as true synergizers of fluconazole. Also, compounds **15** and **24** were shown to increase the efficacy of the new azole drug, isavuconazole.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.6b00355.

Complete experimental details on synthetic and bioassay methods (PDF)

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<sup>†</sup>A.D.M. and I.D.U.A.P. contributed equally to this work.

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### Notes

The authors declare no competing financial interest.

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