High-throughput screening for phosphatidylserine decarboxylase inhibitors using a distyrylbenzene-bis-aldehyde (DSB-3)-based fluorescence assay

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Phosphatidylserine decarboxylases (PSDs) catalyze the decarboxylation of phosphatidylserine to generate phosphatidylethanolamine, a critical step in phospholipid metabolism in both prokaryotes and eukaryotes. Most PSDs are membrane-bound, and classical radioisotope-based assays for determining their activity in vitro are not suitable for high-throughput drug screening. The finding that the PkJPSD from Plasmodium knowlesi can be purified in a soluble and active form and the recent development of a fluorescence-based distyrylbenzenecisaldehyde (DSB-3) assay to measure PSD activity in vitro have laid the groundwork for screening chemical libraries for PSD inhibitors. Using this assay, here we conducted a high-throughput screen of a structurally diverse 130,858-compound library against PkJPSD. Further characterization of the hits identified in this screening yielded five PkJPSD inhibitors with IC₅₀ values ranging from 3.1 to 42.3 μM. Lead compounds were evaluated against the pathogenic yeast Candida albicans in the absence or presence of exogenous ethanolamine, and YU253467 and YU254403 were identified as inhibiting both native C. albicans PSD mitochondrial activity and C. albicans growth, with an MIC₅₀ of 22.5 and 15 μg/ml without ethanolamine and an MIC₅₀ of 75 and 60 μg/ml with ethanolamine, respectively.

Together, these results provide the first proof of principle for the application of DSB-3–based fluorescent readouts in high-throughput screening for PSD inhibitors. The data set the stage for future analyses to identify more selective and potent PSD inhibitors with antimicrobial or antitumor activities.

Phosphatidylethanolamine (PE) is a zwitterionic phospholipid that plays an essential role in cellular functions, both as a major structural lipid of cellular membranes and as a substrate for multiple phospholipid-dependent reactions (1, 2). In Gram-negative prokaryotes like Escherichia coli, PE is the principal glycerophospholipid, whereas in eukaryotes, it is typically the second most abundant glycerophospholipid. In E. coli, PE accounts for ~75% of total phospholipids, all of which is generated from phosphatidylserine (PS) by a PSD associated with the inner cytoplasmic membrane (3, 4). The enzyme catalyzes PS decarboxylation via Schiff base formation between a covalently bound pyruvyl prosthetic group and the amine of the phospholipid serine substituent (5–8). In eukaryotes, the synthesis of PE occurs via three major routes, the CDP-ethanolamine (Kennedy) pathway, originating from ethanolamine, the decarboxylation pathway from PS (PSD pathway), and the headgroup (ethanolamine/choline) exchange pathway that uses phosphatidylcholine as a donor of the phosphatidyl moiety. The contribution of the latter pathways to the synthesis of PE varies among organisms and tissue types (9, 10). For example, whereas the majority of PE produced in rat hepatocytes and hamster heart is generated by the CDP-ethanolamine pathway, in cultured Chinese hamster ovary cells and baby hamster kidney cells, more than 80% of PE is produced from the decarboxylation of PS, even in the presence of ethanolamine (11, 12). Likewise, in yeast, the majority of mitochondrial PE is generated by the PS decarboxylation pathway (2, 13, 14). Yeast encodes two PS decarboxylase genes, PSD1 and PSD2, which are important to membrane biosynthesis (15, 16). Genetic studies in yeast showed that loss of the CDP-ethanolamine pathway does not affect cell viability, whereas disruption of the PSD1 gene results in mitochondrial instability and ethanolamine auxotrophy, highlighting an essential metabolic role for yeast PSD1 (14). Unlike yeast, Plasmodium species encode a single PSD enzyme that associates with the endoplasmic reticulum, and more recently we showed that the PSDs from Plasmodium...
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*knowlesi* and *Plasmodium falciparum* are amphotropic enzymes that are associated with both soluble and membranous fractions (3, 5, 17, 18).

Recent studies have shown that induced expression of the tumor suppressor LACTB (also known as serine beta-lactamase-like protein, or LACTAmase Beta) results in a marked decrease in the amount of the mitochondrial PSD (PSD) activity in certain cancer cells (MCF7-RAS, HMLER, HCC38, and Hs578T) but not in nontumorigenic differentiated cells (19). However, the metabolic and regulatory mechanisms underlying this regulation remain unknown.

Because of their essential role in cell viability, PSD enzymes hold promise as targets for the development of new classes of antimicrobials and anti-tumor agents. However, such efforts have heretofore been hamstrung by challenges associated with large-scale production of these membrane-bound enzymes and the lack of an effective, robust, low-cost, automation-amenable enzyme assay for high-throughput screening (HTS). Traditional methods for determining PSD activity in vitro have relied on the use of [3H]PS or [14C]PS as a substrate. [3H]PE or [14C]PE can be reliably analyzed by TLC; however, the assay cannot be miniaturized to a format compatible with HTS. Alternatively, PSD activity can be determined following detection of released [14]CO$_2$ from [14]C]PS (20). This method, however, is technically incompatible with large-scale screening.

A limited, high-throughput approach has been successfully applied to screen for inhibitors of PSD by performing direct measurement of PS and PE with a Sciex API-4000 electrospray mass spectrometer in positive-ion mode using multiple reaction monitoring (21). This assay was used to screen a library of 9,920 compounds against the human inner mitochondrial membrane PSD enzyme (accession no. CAG30426) and revealed 54 compounds capable of dose-dependent inhibition (21). A cell-based assay using yeast cells lacking the endogenous PSD1 and PSD2 genes, but expressing the malarial PSD cDNA, was also employed to screen the 400-compound Malaria Box library. This effort identified one compound that inhibited growth of the parasite (18).

Despite the pragmatic challenges associated with high-throughput assay design, PSD inhibitors have strong potential to fulfill a substantially unmet clinical burden in the treatment of drug-resistant fungal infections such as candidiasis. To explore this application and advance the perceived druggability of PSD as a target, we recently reported the development of a 384-well format fluorescence-based assay for PSD activity (22). This assay enables the rapid screening of large compound collections for enzyme inhibitors of PSD, which can in turn be quickly evaluated in cell-based antifungal testing against WT and patient-derived strains.

Here we report the first such HTS campaign to identify PSD inhibitors. To leverage the highly conserved nature of PSD in microbial species, and to enable our results to better generalize to parasitic as well as fungal infections, we screened a diverse collection of 130,858 small molecules against the malarial PkPSD enzyme. We demonstrate that the DSB-3–based fluorescence assay format robustly supports an HTS approach for screening of large chemical libraries. Furthermore, we demonstrate translation of biochemical PSD inhibition to cell-based antifungal activity in a manner that is consistent with direct engagement of PSD (i.e. ethanolamine dependence of the antifungal effect). Taken as a whole, the present findings provide pharmacological evidence of PSD as an emerging antifungal target and provide a method for further exploration of the target and its translation to microbial disease.

**Results**

**High-throughput screening assay configuration**

We have previously shown that the DSB-3–based biochemical assay can be run in both 96-well and 384-well format and is amenable to high-throughput screening (22). The optimized assay for HTS in 384-well plates was conducted in a 20 µl/well total reaction volume, with each test well containing 30 ng (~12.5 nm) Δ34PkPSD and 1 nmol (50 µM) PS. The enzymatic reaction was performed at 24 °C for 75 min and terminated by shifting the reaction buffer pH to 9.0 with the addition of 10 mM sodium tetraborate. Subsequently, fluorescent adducts were generated by incubation with 10 µM DSB-3 in the dark for 2 h (Fig. 1A). The fluorescence in each well was quantified using λ$_{ex}$ = 403 nm and λ$_{em}$ = 508 nm (22).

**Primary HTS**

The primary HTS consisted of 130,858 compounds from several commercially available bioactive and synthetic collections, screened at 10 µM final concentration in the DSB-3 biochemical assay with a single compound per well and a single determination per compound. The primary screen was run in 12 assay iterations of 30 – 40 assay plates/run. Each screening plate contained 16 replicates of the positive-inhibition control (PS + heat-denatured PSD + DSB-3) and 16 replicates of the negative-inhibition control (PE + active PSD + DSB-3). To monitor assay performance, mean and S.D. values from control wells were used to quantify signal-to-background ratio (S/B) and Z’-prime factor (Z’$^*$) for each screening plate. The distribution of S/B and Z’$^*$ scores across all plates is shown in Fig. 1, B and C. Average Z’$^*$ was 0.78 ± 0.04, and average S/B, calculated as (PSD + PS + vehicle + DSB-3)/(heat-inactivated PSD + PS + vehicle + DSB-3), was 3.26 ± 0.12, confirming the robustness of the high-throughput assay.

Raw screening data for library compounds were normalized relative to the mean of positive control wells (set as 100% inhibition) and negative control wells (set as 0% effect). Fig. 1D depicts the normalized HTS data in a scatter plot format. Employing a mean percentage inhibition + 3-S.D. cutoff for primary hit selection (20.6 ± 3.1%), the primary hit rate of 0.6% yielded 851 compounds for cherry-picking and subsequent retesting in triplicate from DMSO stocks.

**Hit-picking and retests**

851 cherry-picked HTS hits were retested in triplicate at 10 µM using the primary HTS assay format (Fig. 2A). A parallel fluorescence interference counter-assay was employed to rule out nonspecific compound interference with assay readout. In the interference assay, all wells received phosphatidylethanolamine and DSB-3 to generate maximal emission, and compounds were evaluated for the ability to trigger false positives by...
quenching fluorescent emission in the absence of an enzymatic reaction (data not shown for brevity). As depicted in Fig. 2B, this round of PSD retests and parallel interference assay identified 36 confirmed, nonfluorescent hits.

PSD concentration response and cytotoxicity counterassays

Confirmed HTS hits were advanced to biochemical concentration response studies from freshly solubilized dry powders to confirm the activity of the parent structure. Among the 36 confirmed hits, 15 were available commercially as dry powders (Fig. 3A, filled circles). Compounds were dosed out in duplicate from a top concentration of 80 μg/ml with normalization and curve fitting as described under “Experimental procedures.” After the initial concentration–response study of these 15 confirmed hits, 46 analogs were procured via custom synthesis or via virtual hit expansion from the commercially available chemical space (Fig. 3A, open circles). Lead candidates, which display PSD IC_{50} < 40 μM, were selected for progression to cell-based antifungal assays. Of these compounds, three showed negligible fluorescence interference in the range of their in vitro IC_{50} values against PKPSD. One compound, YU253467, showed fluorescence interference equivalent to its IC_{50} against PKPSD; however, an orthogonal assay that measures production of [^{14}C]PE from [^{14}C]PS showed activity against PkPSD in vitro. Lead candidates were triaged versus potential toxicity to host cells via ATP quantification in live cells using a 72-h Cell-Titer Glo-based readout in HeLa cells. Potential for liver-specific cytotoxicity was evaluated by an identical parallel readout in HepG2 cells (cytotoxicity data not shown). Based on these triage criteria, four PSD-inhibiting small molecules displaying appreciable margins versus one or both cytotoxicity readouts progressed to cell-based antifungal assay testing (Fig. 3B).

Cell-based antifungal assay testing of confirmed HTS hits

To evaluate the activity of YU253467, YU253454, YU224252, and YU196325 against Candida albicans, cells were diluted to 10^3 cells/well and incubated in minimal medium lacking or supplemented with ethanolamine, in the absence or presence of test compounds at concentrations of 15, 30, and 60 μg/ml. Of the four compounds tested, YU253467 and YU253454 showed the highest inhibition of C. albicans growth in vitro (Fig. 4). Notably, YU253467 showed strong activity at 15, 30, and 60 μg/ml in the absence of ethanolamine, with significant growth delay at 15 μg/ml, and 100% growth inhibition at 30 and 60 μg/ml. Ethanolamine supplementation rescued growth of C. albicans at 30 and 60 μg/ml, indicating that the compound-mediated growth inhibition is on-mechanism at PSD. In the
control arm, fluconazole inhibition at 3 or 10 μg/ml was not affected by the presence or absence of ethanolamine (Fig. 4).

Because YU253467 contains a chemotype well-recognized as a pan-assay interference compound (23), we reasoned that reduction of the compound by removing the double bond would prevent the cyclo-reversion of this compound into its constituents and thereby remove any interference from these breakdown products. The resulting compound, YU254403, was subsequently synthesized, and its enzymatic inhibition and antifungal activity were compared with that of the parent compound YU253467 (Fig. 5A).

Dose–response assays showed MIC₅₀ against C. albicans of 22.5 μg/ml for YU253467 and 15 μg/ml for YU254403 in the absence of ethanolamine (Fig. 5, B and C). As expected for PSD-specific inhibitors, the MIC₅₀ of YU253467 increased from 22.5 μg/ml in the absence of ethanolamine to 75 μg/ml in the presence of ethanolamine, whereas that of YU254403 increased from 15 μg/ml in the absence of ethanolamine to 60 μg/ml in the presence of ethanolamine (Fig. 5, B and C). As a control, fluconazole displayed an MIC₅₀ of 2.5 μg/ml and a characteristic insensitivity to ethanolamine supplementation (Fig. 5D).

To further evaluate the link between PSD function and anti-C. albicans activity of YU253467 and YU254403, we examined the effect of these compounds against psd1Δ/psd1Δ and psd2Δ/psd2Δ C. albicans mutants lacking PSD1 or PSD2 genes. As reported previously (24), Psd1p accounts for the majority of cellular PSD activity, as its loss results in ethanolamine auxotrophy. In the presence of ethanolamine, however, the inhibition profile of the psd1Δ/psd1Δ mutant was similar to WT under similar conditions (Fig. 6A). Interestingly, whereas the growth of the WT strain was completely inhibited at 75 and 200 μg/ml YU253467 or YU254403 in the absence of ethanolamine, that of the psd2Δ/psd2Δ was not affected by these drugs at 75 μg/ml under similar conditions (Fig. 6A). Only at 200 μg/ml did the growth of the mutant resemble that of the WT (Fig. 6A). However, in the presence of ethanolamine, the growth of the psd2Δ/psd2Δ strain was similar to that of the WT at both 75 and 200 μg/ml YU253467 or YU254403 (Fig. 6A).

Consistent with these genetic data, YU253467 and YU254403 at 72 μg/ml inhibited native C. albicans PSD activity in mitochondrial extracts by 94 and 63%, respectively (Fig. 6B). At this concentration, YU253467 inhibited the native mouse mitochondrial PSD activity by 41%, whereas YU254403 had little to no inhibitory activity. Because YU253467 was selected from a chemical screen using purified malarial PSD enzyme as a surrogate enzyme, activity assays of YU253467 and YU254403 at 72 μg/ml showed ~94 and ~96% inhibition of PpPSD and 92 and 71% inhibition of PkPSD, respectively (Fig. 6B).

**In vitro activity of YU253467 and YU254403 against other fungal pathogens**

The finding that YU253467 and YU254403 inhibit the growth of C. albicans led us to investigate their activity against other Candida species. At 200 μg/ml, YU253467 and YU254403 inhibited growth of Candida glabrata by ~70 and ~91% in the absence of ethanolamine and by ~75 and ~55% in the presence of ethanolamine. In Candida parapsilosis, 200 μg/ml YU253467 and YU254403 inhibited growth by ~57 and ~44% in the absence of ethanolamine and by ~58 and ~46% in the presence of ethanolamine (Fig. 7, A–C). In vitro pairwise drug combination assays with C. albicans using YU254403 and one of three known antifungal drugs (fluconazole, amphotericin B, or terbinafine) did not show any synergistic or antagonistic effects (Fig. 7D).

The range of activity of YU254403 was further examined against the mold pathogens Aspergillus fumigatus and Fusarium solani (var. petroliphilum) (Fig. 8). Whereas no significant inhibition of Fusarium solani (var. petroliphilum) could be detected with these compounds (Fig. 8B), the growth of A. fumigatus was strongly inhibited by YU254403, with ~70 and ~40% inhibition at 18 and 36 μg/ml, respectively, in the absence of ethanolamine and ~45 and ~35% inhibition at 18 and 36 μg/ml, respectively, in the presence of ethanolamine (Fig. 8A).

**Discussion**

The role of phosphatidylserine decarboxylases in cellular metabolism and pathogenesis is an emerging line of inquiry supported by compelling evidence that positions the PSDs as novel therapeutic targets in parasitic, fungal, and human neoplastic disease. It bears mentioning that, although not the focus of the present study, the link between phosphatidylserine decarboxylases and cancer growth and metastasis has received attention recently (19, 25, 26). Keckesova et al. (19) showed that the mitochondrial protein LACTB acts as a tumor suppressor that inhibits the proliferation of certain breast cancer cells, and...
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Figure 3. Retesting confirmed primary hits from fresh powders. A, 36 confirmed HTS hits were selected to progress to biochemical concentration–response studies from commercially procured dry powders to confirm the activity of the parent structure. 15 of 36 confirmed hits were available commercially as dry powders (filled circles). After the initial concentration–response study on these 15 compounds, 60 analogs were procured via custom synthesis or via virtual hit expansion (VHE) from the commercially available chemical space (open circles). B, active primary hits were counter-screened for mammalian cytotoxicity in HeLa and HepG2 cells. Lead candidates were triaged versus potential toxicity to host cells via a 72-h CellTiter-Glo®-based cytotoxicity readout in HeLa cells. The potential for liver-specific toxicity was evaluated by an identical readout in HepG2 cells. Compounds displaying a minimum 3-fold cytotoxicity margin versus one or both cell lines and negligible fluorescent assay interference progressed to cell-based antifungal assay testing. Despite limited initial potency, YU253467 was subsequently shown to exert ethanolamine-dependent antifungal activity, indicating a potential for PSD engagement in the antifungal mechanism.

the suppression works through the inhibition of mitochondrial lipid synthesis. Overexpression of LACTB in tumor cells resulted in a 30–50% reduction in steady-state levels of lysophosphatidylethanolamine and phosphatidylethanolamine. Supplementation of lysophosphatidylethanolamine after LACTB overexpression, however, bypassed the inhibition of cell proliferation by LACTB. Intracellular lysophosphatidylethanolamine is readily acylated to form PE (27, 28). The reduction of the lipids by LACTB overexpression was due to a decrease in PSD protein levels (by 60–95%) in the mitochondria. These findings suggest that PSD inhibition may hold promise as an anti-cancer strategy.

We demonstrate here that WT C. albicans is sensitive to YU253467 and YU254403 but that this sensitivity depended on the availability of ethanolamine in the medium. The drugs’ MIC₉₀ values increased 3.3-fold in the case of YU253467 and 4-fold in the case of YU254403 in the presence of ethanolamine. Because the primary cellular source of PE in the absence of exogenous supplementation is driven by PSD conversion of endogenous PS to PE, the sensitivity of these compounds to ethanolamine supplementation (which creates an effective shunt around PSD) strongly suggests that they are acting via PSD inhibition. Interestingly, the inhibitors were not as effective at 75 μg/ml against the C. albicans psd2Δ/Δ mutant (Fig. 6A). Although the exact mechanism for psd2Δ/Δ resistance to the compounds remains to be elucidated, possible explanations include increased activity of the endogenous Psd1 in the mutant to compensate for the loss of Psd2 or increased activity in other metabolic processes or detoxification mechanisms caused by the loss of Psd2. However, ethanolamine in the host is limiting for C. albicans (29). In addition, compensation mechanisms, such as potential enhancement of Psd1, can be overcome by increasing the drug concentration to 200 μg/ml (Fig. 6A). Thus, future iterations of these proof-of-concept compounds should be able to overcome this potential resistance mechanism.

Compared with their effect in C. albicans, YU253467 and YU254403 had moderate activity against C. glabrata and moderate activity against C. parapsilosis. In C. glabrata, the compounds at 200 μg/ml inhibited growth by ~70 and ~91% in the absence of ethanolamine and by ~75 and ~55% in the presence of ethanolamine, whereas in C. parapsilosis, 200 μg/ml results in ~57 and ~44% growth inhibition in the absence of ethanolamine and ~58 and ~46% inhibition in the presence of ethanolamine. Further examination of the PSD pathway in these organisms may shed light on the level of efficacy and mode of action of these compounds.

In addition to the various species of Candida yeasts, we also tested the efficacy of YU25433 against two mold (filamentous) pathogens: A. fumigatus and F. solani (var. petroliophilum). A. fumigatus is the predominant cause of invasive aspergillosis, which carries mortality rates as high as 95% in immunocompromised patients (30). This underscores a critical need for novel antifungal modalities to combat this organism, and to this end, YU25433 demonstrated marked activity in both the presence and absence of ethanolamine. By contrast, we observed only a very weak activity of the compound against F. solani (var. petroliophilum), a significant cause of fungal keratitis (31).

Notably, none of the structures disclosed herein have been optimized for pharmaceutical properties or on-target potency. Furthermore, several of these compounds are known pan-assay interference compounds (23, 32). The goal of this study was to demonstrate tractability of the miniaturized DSB-3–based PSD assay format as a platform for medium-to-large-scale screening and to articulate a basic approach for early triage. In doing so, we identified YU253467 and its analog YU254403 as inhibitors of PSD. Although YU253467 is a recognized pan-assay interference compound and has a fluorescent interfering signal, the fact that YU253467 and its de-risked analog YU254403 cause growth inhibition of C. albicans that is recoverable by the addi-
tion of ethanolamine suggests that they may be useful for further elucidation of PSD inhibition.

The current body of work indicates a target class with significant opportunity across multiple indications. Accordingly, the emerging understanding of the role for PSDs in microbial pathogenesis and cancer have stimulated efforts to identify selective inhibitors for use in validating the enzymes as drug targets, several of which are detailed herein. Whereas the PSDs have attracted interest as drug targets based on the centrality of membrane phospholipid homeostasis to a variety of microbial pathogens as well as to the tumor microenvironment, they have also been described as undruggable (21).

To date, no druglike inhibitors have been disclosed, and despite the obvious potential of the target, the field has languished. Our previous work described the design and validation of a low-cost, automation-amenable, medium-to-high-throughput screening assay with demonstrable ability to detect micromolar inhibitors of PSD (22). The DSB-3 primary assay and screening triage described here were developed with the ultimate intent of large-scale HTS campaigns for novel PSD inhibitors of phospholipid synthesis. Here we describe in detail one such campaign and disclose the structures of a series of potential tool compounds. Although beyond the scope of the present study, which was focused on articulating a path forward for HTS campaigns versus PSD, secondary biophysical studies (SPR, thermal shift) aimed at direct confirmation of selective binding would be a logical next step.

Experimental procedures

Expression and purification of MBP-His$_6$-$\Delta$34PkPSD

Expression and purification of MBP-His$_6$-$\Delta$34PkPSD was performed as described previously (3, 22). In brief, a Rosetta DE3 strain harboring a pMAL-C2X-His$_6$-$\Delta$34PkPSD was
grown overnight in 10 ml of lysogeny broth medium supplemented with 0.2% glucose, ampicillin (100 μg/ml), and chloramphenicol (34 μg/ml) at 37 °C. The following day, cells were inoculated into 1 liter of fresh medium and then grown to an A600 of 0.5. Expression of the MBP-His$_6$-34PkPSD was initiated by the addition of 3 ml of 0.1M isopropylthiogalactoside. After incubation for 2 h at 37 °C, the cells were harvested and washed by resuspension in water and recentrifugation. The cells were resuspended in 25 ml of a column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, and 10 mM β-mercaptoethanol), stored overnight at 20 °C. The next day, the frozen cells were slowly thawed on ice water and then broken by sonication (15-s burst at 30% amplitude using a Fisher Sonic Dismembrator 500, performed eight times with 30 s on ice between intervals). Cell-free extracts were obtained by centrifugation at 20,000 × g for 20 min at 4 °C. MBP-His$_6$-Δ34PkPSD protein was purified by amylose affinity chromatography according to the manufacturer’s protocol (New England Biolabs, catalog no. E8200S). The fractions containing the MBP-His$_6$-Δ34PkPSD protein were identified by Western blot analysis using anti-His$_6$ antibody (Clontech, catalog no. 631212).

**PSD activity assay using [14C]PS as a substrate**

Purified MBP-His$_6$-Δ34PkPSD, purified MBP-Δ40PfPSD, and mitochondria prepared from *C. albicans* strains and HeLa Ohio cells were used in a radiochemical PSD enzyme assay. The assay contained 50 μM [14C]PS (4,000 cpm/nmol) as the substrate (approximately K$_m$ concentration for PkPSD) in the presence of specified concentrations of inhibitor molecules, and the reaction product was trapped as 14CO$_2$ on 2 M KOH-impregnated filter paper, as described previously (16).

**384-Well HTS assay for PSD inhibitors**

The HTS was performed using commercially available compound libraries or acquired compounds formatted as 10 mM stocks in DMSO. The screening collection for this campaign was composed of Screen-Well Food and Drug Administration–approved drugs (Enzo Life Sciences), Screen-Well kinase inhibitors (Enzo Life Sciences), MicroSource Pharmakon 1600...
Freshly prepared assay reagents and buffers were frozen at −80 °C in convenient aliquots and used within a month of preparation. The thawed MBP-His₆-Δ34Pkd was diluted in buffer A-1 (100 mM NaCl, 16 mM EDTA, 160 μM β-mercaptoethanol, 319 mM Tris-HCl, 1 mM potassium phosphate (KPO₄) buffer, pH 7.4). The assay was conducted in black-wall, flat-bottom, opaque, untreated 384-well microtiter plates (Corning, catalog no. 3575). The diluted enzyme preparation (10 μl/well) was dispensed using a small cassette Multidrop Combi dis-

Figure 7. Efficacy of YU253467 and YU254403 against nonalbicans Candida species. A–C, activity of YU253467 and YU254403 against C. albicans, C. glabrata, and C. parapsilosis at 75 or 200 μg/ml in the absence or presence of 2 mM ethanolamine. D, in vitro efficacy of combinations of YU254403 with either fluconazole, amphotericin B, or terbinafine. Compounds were evaluated at their MIC₅₀ alone or in combination against C. albicans using the 48-h growth assay. ns, not significant. Error bars, S.D. Flu 2.5: Fluconazole (2.5 μg/ml); YU403 15: YU254403 (15 μg/ml); AmpB 5: Amphotericin B (5 μg/ml); and Terb 8: Terbinafine (8 μg/ml).
penser (Thermo Fisher Scientific) for active enzyme and a multichannel pipette for heat-inactivated (negative control) enzyme. The plates were centrifuged (1,000 rpm/30 s) to ensure reagent mixing. 20-nl aliquots of 10 mM library compounds were transferred to the wells via acoustic dispensing on the Echo 550 (LabCyte), and the plates were again centrifuged and subsequently incubated for 20 min at room temperature to allow binding of test compounds to the PSD enzyme. The PSD assay was initiated by the addition of 10 ^\text{H}_9262 \text{l} of PS lipid substrate to appropriate wells using the small cassette multidrop combi reagent dispenser, followed by centrifugation. Control reactions consisting of either detergent (no substrate control) or PE were dispensed with a multichannel pipettor. Plates were then incubated at room temperature for 75 min to allow the enzymatic conversion of PS to PE to proceed in the presence of test compounds. The final assay conditions were as follows: 50 mM NaCl, 0.75 mM Triton X-100, 50 ^\text{M}_9262 \text{M} PS (or 50 ^\text{M}_9262 \text{M} PE control), 80 ^\text{M}_9262 \text{M} \beta\text{-mercaptoethanol}, 160 ^\text{M}_9262 \text{M} Tris-HCl, 1 ^\text{M}_9262 \text{M} KPO_4, pH 7.4, 30 ng of MBP-His6-34PkpPSD (or boiled enzyme control) in a volume of 20 ^\text{M}_9262 \text{M} . The enzyme reaction was arrested by the addition of 2.5 ^\text{M}_9262 \text{l} of 100 mM sodium tetraborate buffer (pH 9) and brief centrifugation. Subsequently, 2.5 ^\text{M}_9262 \text{l} of 100 ^\text{M}_9262 \text{M} DSB-3 in 1 mM KH_2PO_4, pH 7.4, was added to the plate, followed by brief centrifugation and incubation for 2 h in the dark. The PSD activity was monitored by measuring fluorescence intensities (403-nm excitation/508-nm emission, TECAN).

**HTS data analysis and normalization**

Within plates, primary HTS data were normalized as follows: low signal (positive control, analogous to total PSD1 inhibition by a test compound) was 16 wells containing screening concentrations of PS + active PSD + DSB-3. Primary data were expressed as a percentage of the positive control wells, and Z’ and S/B were calculated between the positive and negative controls. Secondary dose-response data were normalized as above. Normalized percentage inhibition data were fitted for IC_{50} determination via a four-parameter logistic equation. All offline data transformation and curve fitting was performed in ActivityBase (Abase) high-content and throughput screening software (IDBS).

**Strains**

*C. albicans* WT (strain SC5314) and mutant (psd1Δ/psd1Δ and psd2Δ/psd2Δ), *C. parapsilosis* (strain ATCC 22019), *C. glabrata* (strain CSH10 CAG-1), *A. fumigatus* (strain CEA10), and *F. solani* (var. *petroliphilum*, a fungal keratitis clinical isolate) were used in this study.

**Growth assays**

For liquid assay in a 96-well plate format, *C. albicans* cells were precultured overnight in liquid YPD medium at 30 °C, washed three times in water, and diluted to 10^3 cells/well in 100 ^\text{M}_9262 \text{M} DOB medium in the absence or presence of 2 mM ethanolamine (pH 6.5). All plates were incubated at 30 °C. A_e_{630} measurements were taken with a BioTek SynergyMx microplate reader every 8 h for a total of 50 h. The compounds YU253467, YU253454, YU224252, and YU196325 were tested at 0, 15, 30, and 60 ^\text{M}_9262 \text{g/ml}. Fluconazole was used as a positive control and tested at concentrations of 0, 3, and 10 ^\text{M}_9262 \text{g/ml}. MIC_{50} values were calculated at 48 h for YU253467, YU253454, and fluconazole, and fluconazole concentrations ranging from 5 to 100 ^\text{M}_9262 \text{g/ml} and from 1 to 10 ^\text{M}_9262 \text{g/ml}, respectively.

Figure 8. Efficacy of YU254403 against mold pathogens. Activity of YU254403 against *A. fumigatus* and *F. solani* (var. *petroliphilum*) at 9, 18, 36, and 72 ^\text{M}_9262 \text{g/ml} in the absence or presence of 2 mM ethanolamine. Error bars, S.D.
A. fumigatus growth assays

Conidia were harvested from Sabouraud’s dextrose agar plates (Difco), filtered through Miracloth (Millipore), washed twice in PBS, and inoculated to a density of 1.0 × 10⁶/mL in glucose minimal medium (containing 10 mM ammonium tartrate) or RPMI 1640 (pH 7; supplemented with 1.8% glucose), with or without 2 mM ethanolamine (pH 6.5). 100 µL of sample were transferred into wells of 96-well plates and incubated at 35°C for 48 h in the presence of 0, 9, 18, 36, and 72 µg/mL of YU254403. Absorbance was read at 530 nm in a CLARIOstar (BMG Labtech) plate reader.

F. solani (var. petrophilum) growth assays

Microconidia were collected from yeast extract peptone (YPD) liquid cultures by filtration through Miracloth and washed twice with PBS. Growth assays were otherwise performed as described above for A. fumigatus in glucose minimal medium.

HeLa and HepG2 cell toxicity assays

ATCC HeLa and HepG2 cells were dispensed into sterile black-walled, clear-bottom, tissue culture–treated, 384-well plates (Corning, catalog no. 3712) via MultiDrop (Thermo) at a density of 400 cells/well in a volume of 20 µL of complete medium. Cell plates were centrifuged at 46 × g for 10 s and incubated overnight at 37°C in a humidified 5% CO2 incubator. 24 h after cell plating, test compounds (20 nL) were transferred from the compound source plate to the cell assay plate via an Echo 550 acoustic dispenser (Labcyte). The final concentrations of test compounds and DMSO were 10 µM and 0.1%, respectively. Tamoxifen (60 µM final assay concentration) was added to columns 1–2 as a positive control (maximum cell death), and columns 23–24 received DMSO vehicle only (negative control). Assay plates were centrifuged at 46 × g for 10 s and incubated for 72 h at 37°C in a humidified 5% CO2 incubator. CellTiter-Glo (Promega) was used to measure cell viability in the assay wells according to the manufacturer’s instructions. Briefly, CellTiter-Glo reagent (20 µL/well) was added to the assay plates using the MultiDrop dispenser. The plates were shaken on a Thermomixer R (Eppendorf) at 1,100 rpm for 1 min and incubated in the dark for 10 min at room temperature. Luminescence was measured in the Synergy Neo2 plate reader (BioTek) with 0.3-s sampling time per well. Wells displaying cytotoxicity have lower luminescence signals relative to the vehicle control wells. Raw data (luminescence counts per second) were normalized to percentage effect by the formula, 100 − ((sample − positive control mean)/(negative control mean) × 100).

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