Pharmacokinetics of Posaconazole Within Epithelial Cells and Fungi: Insights Into Potential Mechanisms of Action During Treatment and Prophylaxis

Paolo Campoli,1 David S. Perlin,2 Arnold S. Kristof,3 Theodore C. White,4 Scott G. Filler,5 and Donald C. Sheppard1

1Department of Microbiology and Immunology and 2Critical Care and Respiratory Divisions and Meakins-Christie Laboratories, McGill University, Montréal, Québec, Canada; 3Public Health Research Institute and UMDNJ Medical School, Newark, New Jersey; 4Division of Cell Biology and Biochemistry, University of Missouri at Kansas City; and 5Division of Infectious Disease, Los Angeles Biomedical Research Institute Harbor—UCLA Medical Center, Torrance, California

**Background.** The antifungal posaconazole concentrates within host cells and protects against *Aspergillus fumigatus*. The specific subcellular location of posaconazole and the mechanism by which cell-associated posaconazole inhibits fungal growth remain uncharacterized.

**Methods.** Posaconazole was conjugated with the fluorophore boron-dipyrromethene (BDP-PCZ). A549 pulmonary epithelial cells and *A. fumigatus* were exposed to BDP-PCZ individually and in coculture. BDP-PCZ subcellular localization and trafficking were observed using confocal microscopy and flow cytometry.

**Results.** BDP-PCZ concentrated within A549 cell membranes, and in particular within the endoplasmic reticulum. Epithelial cell-associated BDP-PCZ rapidly transferred to and concentrated within *A. fumigatus* cell membranes on contact. BDP-PCZ transfer to conidia did not require phagocytosis and was markedly enhanced by the conidial hydrophobin RodA. Within AF, BDP-PCZ also concentrated in membranes including the endoplasmic reticulum and colocalized with the azole target enzyme CYP51a. Concentration of BDP-PCZ within host and fungal cell membranes persisted for >48 hours and could be competitively inhibited by posaconazole but not voriconazole.

**Conclusions.** Posaconazole concentrates within host cell membranes and rapidly transfers to *A. fumigatus*, where it accumulates to high concentrations and persists at the site of its target enzyme. These intracellular and intercellular pharmacokinetic properties probably contribute to the efficacy of PCZ.

**Keywords.** posaconazole; localization; pharmacokinetics; endoplasmic reticulum; epithelial cells; *Aspergillus*; postantifungal effect; hydrophobin rodA.

*Aspergillus fumigatus* infections remain an important cause of mortality in hematology patients receiving cytotoxic chemotherapy or undergoing allogeneic bone marrow transplantation [1–5]. In clinical trials, prophylactic use of theazole antifungal, posaconazole, has been found to reduce fungal infections and reduce mortality due to invasive aspergillosis [6, 7]. Interestingly, despite the efficacy of this agent in prophylaxis trials, serum levels of posaconazole reported in these patients were relatively low [6–8]. However, posaconazole is a lipophilic molecule and, as a result, the tissue levels of this agent are up to 40-fold higher than serum levels [9–11]. We and others have hypothesized that the high tissue levels of posaconazole may underlie the effectiveness of this agent in antifungal prophylaxis despite the relatively low serum levels observed in clinical trials [12–14].

In support of this hypothesis, we previously demonstrated that pulmonary epithelial cells and macrophages exposed to posaconazole were highly resistant to infection with *A. fumigatus* and other fungi even after the extracellular drug was removed [12]. The ability of cell-associated...
antifungals to protect against fungal infection was unique to posaconazole and its parent molecule itraconazole. Pharmacodynamic studies demonstrated a prolonged postantifungal effect of up to 48 hours when *A. fumigatus* was exposed to cell-associated posaconazole [12]. Consistent with the observation that posaconazole is highly lipophilic, cellular fractionation experiments revealed that the cellular distribution of posaconazole was restricted to epithelial cell membranes [12]. The subcellular location of posaconazole within epithelial cells and the mechanism by which cell-associated posaconazole inhibits fungal growth and induces a prolonged postantifungal effect remain undefined. In this study, we used a fluorophore-conjugated posaconazole [15] to determine the subcellular localization of posaconazole within host and fungal cells and investigate the unique pharmacokinetic and pharmacodynamic properties of this hydrophobic antifungal.

**METHODS**

**Cell Line**

Pulmonary epithelial cells (A549) were obtained from the American Type Cultures Collection and grown according to the suppliers recommendations using F12 Kaighns (HyClone) medium (F12K) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were grown on tissue culture–treated 100-mm dishes, sterile cover slips, 4-chamber wells, and 24-well dishes, as appropriate.

**Strains**

*Aspergillus fumigatus* strain AF293 was used for mutant construction and other studies. *Aspergillus* strains were grown on YPD agar (Gibco) at 37°C for 6 days. The ΔrodA mutant strain was described previously [16].

**Labeling of Posaconazole With Boron-Dipyrromethene (BDP)**

Labelling of posaconazole with boron-dipyrromethene (BODIPY) was performed as described elsewhere [15]. Briefly, a hydroxyl group of posaconazole was modified using succinic anhydride. This product was incubated with 4-nitrophenol in the presence of N,N’-dicyclohexylcarbodiimide. The compound was further incubated with ethylenediamine and finally with the amine-butane derivative of BDP fluorophore to yield the fluorescent posaconazole-BDP product (BDP-PCZ). Because of the difference in molecular weight between the unaltered posaconazole molecule and BDP-PCZ, concentrations of both posaconazole and BDP-PCZ were standardized based on molar concentration.

**Drug Preparation**

Posaconazole (Merck Canada), voriconazole (Pfizer), and BDP-PCZ were diluted in dimethyl sulfoxide (DMSO). Fresh dilutions were made from these stock solutions just before the experiment and diluted further in phosphate-buffered saline (PBS) or F12K. A control stock containing DMSO but without antifungals was also prepared and used in all experiments as a solvent control.

**Antifungal Susceptibility Testing**

Antifungal susceptibility testing was performed in accordance with the CLSI M38-A document for broth dilution antifungal susceptibility testing of filamentous fungi [17], as described elsewhere [12]. Stock solutions of antifungals were prepared in DMSO and then diluted in either Roswell Park Memorial Institute 1640 medium buffered with MOPS (3-[N-morpholino]propanesulfonic acid) or F12K with serum; per well, 100 µL of drug stock was added to 100 µL of a 10⁵ conidia per milliliter solution. Plates were examined after 24 and 48 hours of incubation, and the minimal inhibitory concentration (MIC) resulting in 100% growth inhibition was determined by visual and microscopic inspection.

**Cell-Associated Antifungal MIC Testing**

The ability of intracellular antifungals to inhibit fungal growth was assayed as described elsewhere [12]. Briefly, monolayers of A549 pulmonary epithelial cells were prepared by inoculating 24-well plates tissue culture treated plates with 10⁵ cells per well. Cells were grown to confluence and incubated with the appropriate antifungal in F12K medium for 2 hours. After incubation, the free drug was removed by aspirating the medium and washing the cells with Dulbecco PBS 3 times. Drug-exposed monolayers in 24-well dishes were infected with 1 mL of a 10⁵ conidia per milliliter stock of *A. fumigatus* in F12K medium and incubated for 48 hours.

**Construction of an A. fumigatus Strain Expressing Red Fluorescent Protein**

To facilitate the visualization of *A. fumigatus* during posaconazole transfer experiments, we constructed a strain of *A. fumigatus* that expressed red fluorescent protein (RFP), using pSK379 encoding the red fluorescent protein mRFP1. An mRFP1 containing fragment was amplified with polymerase chain reaction from this plasmid using primers RFP FWD and RFP REV (Supplementary Table 1). This fragment was then subcloned between the SpeI and EcoRV restriction sites of plasmid pGFP-Pheo [12] to replace green fluorescent protein with mRFP1. The resulting plasmid, pRFP-Pheo, was used to transform *A. fumigatus* strain AF293, by protoplasting [18]. Transformants were recovered by phleomycin selection, and mRFP1 expression was verified microscopically.

**Construction of AF-CYP51a-RFP**

To determine whether posaconazole colocalizes with CYP51a, its target enzyme, we constructed an *A. fumigatus* strain expressing a fusion construct of RFP-CYP51a. Briefly, the CYP51a open reading frame was cloned using polymerase chain reaction with primers CYP51a FWD and CYP51a REV (Supplementary Table 1). This fragment was then cloned in frame with mRFP1.
using the previously constructed pRFP-Phleo plasmid with EcoRV. The resulting plasmid pRFP-CYP51a-Phleo was used to transform wild-type A. fumigatus Af293, as described above [18].

**Fluorescent Cell Microscopy**

For microscopic studies, 10^5 A549 pulmonary epithelial cells were grown to confluency in 4 chamber slides or on glass cover slips in 24-well plates for approximately 24 hours in F12K medium. Alternately, 10^5 germings were added to cover slips and grown for 6-8 hours. Organisms and cells were exposed to BDP-PCZ at the indicated concentrations for 2 hours, after which the free drug was removed by washing. When the lipid dye, FM4-64 (Invitrogen), was used in conjunction with BDP-PCZ, it was added directly into the wells containing BDP-PCZ during the last 30-45 minutes of the incubation. Immunostaining for ERP57 was conducted by confocal immunofluorescence, as described elsewhere [19]. Briefly, after BDP-PCZ exposure, cells were fixed in 4% paraformaldehyde, permeabilized with 5% saponin, and blocked with 3% bovine serum albumin supplemented with 5% saponin and 0.2% sodium azide. After washing, cells were then incubated with the primary rabbit anti-ERP57 antibody (AbCam), followed by detection with a goat anti-rabbit Alexa Fluor 555 secondary antibody before confocal microscopy imaging.

**Transfer Experiments**

A549 cells were grown on glass cover slips and exposed to 5.97 mmol/L BDP-PCZ, as described above. Cells were washed to remove free BDP-PCZ and then infected with 10^5 conidia or germings of A. fumigatus strains. Plates were centrifuged at 1200 rpm for 5 minutes before being incubated at 37°C. Cover slips were removed after 0, 1, 2, and 4 hours and imaged with confocal microscopy. To determine whether phagocytosis was necessary for transfer of posaconazole, cocultures were incubated with 70 mmol/L cytochalasin D, as described elsewhere [12, 20].

**Binding BDP-PCZ by Conidia**

Conidia of Af293 and ΔrodA were exposed to 5.97 mmol/L BDP-PCZ, and fluorescence was determined at 0, 30, and 60 minutes using flow cytometry (BD LSR Fortessa). Conidia were gated based on the forward and side scatter profiles, and histograms were constructed comparing Af293 and ΔrodA fluorescence.

**Competition Assay**

A549 epithelial cells were exposed to 2.99 mmol/L BDP-PCZ for 2 hours and posaconazole, itraconazole, or voriconazole at varying ratios (ratio of BDP-PCZ to test azole, 1:1, 1:2, 1:5, 1:10, or 0:10). Free drug was removed by washing in Dulbecco PBS, and total fluorescence was detected with fluorometry (485-nm excitation and 535-nm emission). To view BDP-PCZ, exposed cells were also examined with confocal microscopy; A549 cells or germings were grown on glass cover slips in 24-well plates, exposed to antifungal agents as described above at a ratio of 1:2, and imaged with confocal microscopy. A drug-free autofluorescence control with 1% DMSO was also included.

**RESULTS**

**Antifungal Activity of BDP-PCZ**

To determine if conjugation to BDP alters the antimicrobial properties of posaconazole, the antifungal activity of unmodified PCZ was compared with BDP-PCZ. In a standard microbroth dilution assay, BDP-PCZ inhibited A. fumigatus Af293 growth, although with an approximately 4-fold decrease in potency (MIC for PCZ and BDP-PCZ, 0.4 and 1.5 mmol/L, respectively). Similarly, as we reported elsewhere with posaconazole, epithelial cells exposed to BDP-PCZ inhibited the growth of A. fumigatus, although a higher-exposure concentration of BDP-PCZ was again required (3.0 and 11.9 mmol/L for PCZ and BDP-PCZ, respectively). Thus, posaconazole conjugated with BDP retained substantial potency against A. fumigatus in free and cell-associated systems, supporting its use as a tool to study the pharmacokinetics and pharmacodynamics of posaconazole.

**BDP-PCZ Concentration Within Internal Cell Membranes of Epithelial Cells**

To determine the intracellular location of posaconazole, A549 cells were exposed to BDP-PCZ and imaged with confocal microscopy. Predominant fluorescent staining of intracellular structures was observed, suggesting that BDP-PCZ accumulated within cytoplasmic membranes (Figure 1A). To test this hypothesis, BDP-PCZ–exposed cells were costained with FM4-64, a lipophilic dye that stains all cellular membranes. Complete colocalization of intracellular membrane staining with FM4-64 and BDP-PCZ was observed. The highest level of staining for both FM4-64 and BDP-PCZ was the perinuclear region, consistent with accumulation within membranes of the endoplasmic reticulum (ER; Figure 1B). Immunostaining of BDP-PCZ exposed cells with an antibody for the ER marker, ERP57, confirmed that BDP-PCZ accumulates within the membrane rich ER (Figure 1C). Collectively, these data demonstrate that within epithelial cells, BDP-PCZ accumulates within cytoplasmic membranes, including those of the ER.

**Transfer of BDP-PCZ From Host to Fungal Membranes During In Vitro Infection**

We and others have found that posaconazole concentrates to high levels within the membranes of epithelial cells [10–12]. We hypothesized that intracellular posaconazole can serve as an important antifungal reservoir and that membrane-bound posaconazole can transfer to fungal cells resulting in growth inhibition. We therefore investigated the ability of epithelial cell–associated BDP-PCZ to transfer to A. fumigatus conidia in vitro. A549 epithelial cells exposed to BDP-PCZ were infected with conidia of A. fumigatus and examined with confocal microscopy.
microscopy. To facilitate visualization of fungi, a strain of *A. fumigatus* with constitutive expression of RFP was constructed. Within epithelial cells BDP-PCZ fluorescence was confined to the epithelial cell membranes, most notably the ER. Within minutes of infection, BDP-PCZ staining of conidia that were in contact with epithelial cells was observed (Figure 2). Epithelial cell contact was required for transfer of BDP-PCZ, as conidia cocultured with epithelial cells in a Transwell barrier culture took up trace amounts BDP-PCZ only after prolonged incubation (data not shown).

The rapidity of BPD-PCZ staining of conidia observed (Figure 2) suggests that phagocytosis was not required for

Figure 1. BDP-PCZ, a fluorophore-conjugated posaconazole, concentrates within the endoplasmic reticulum of A549 cells. A, B, A549 cells were exposed to BDP-PCZ alone (A), or BDP-PCZ and the membrane dye FM4-64 (B). C, A549 cells were exposed to BDP-PCZ and immunostained for the endoplasmic reticulum marker ERP57. All cultures were visualized under confocal microscopy. Colocalization with FM4-64 and ERP57 suggests that BDP-PCZ distributes to cellular membranes, particularly those of the endoplasmic reticulum. Abbreviation: DIC, differential interference contrast microscopy.
transfer of BDP-PCZ. To test this hypothesis, epithelial cells were pretreated with cytochalasin D to block phagocytosis of conidia. Cytochalasin D prevented phagocytosis of conidia but had no effect on the transfer of BDP-PCZ to conidia, suggesting that BDP-PCZ transfers to conidia on contact with the epithelial cell plasma membrane rather than intracellular membranes.

The small size of conidia limits the ability to study intracellular morphology by light microscopy. Therefore, to facilitate the observation of intracellular trafficking of BDP-PCZ within fungi, we infected epithelial cells with germlings of *A. fumigatus* expressing RFP. Transfer of BDP-PCZ to germlings from epithelial cells was significantly slower than with hyphae. After 1 hour of coinfection, BDP-PCZ was visible only in the plasma membrane of germlings (Figure 3). After 4 hours of coinfection, a decrease in the amount of BDP-PCZ within host cells was evident, and BDP-PCZ accumulation within fungal intracellular structures was evident (Figure 3). Interestingly, focal areas of perinuclear staining with BDP-PCZ were evident within germlings, suggesting that, as with epithelial cells, posaconazole may accumulate in the fungal ER.

**Effect of the Conidial Hydrophobin RodA on Rapid Transfer of BDP-PCZ From Host Cells to Conidia**

The transfer of BDP-PCZ from host cells was much more rapid with *A. fumigatus* conidia than with hyphae. Conidia of *A. fumigatus* are covered with an extremely hydrophobic coat comprising an organized layer of the conidial hydrophobin RodA that is shed during germination [21]. We hypothesized that this highly hydrophobic protein might facilitate the binding and transfer of lipophilic posaconazole. To test this hypothesis, we examined the kinetics of BDP-PCZ transfer between epithelial cells and a mutant strain of *A. fumigatus* deficient in the hydrophobin RodA [22]. After 1 hour of coculture, all conidia of the wild-type *A. fumigatus*-RFP strain in contact with epithelial cells were highly fluorescent, whereas conidia of the ΔrodA-RFP mutant displayed little to no uptake of BDP-PCZ (Figure 4). Similarly, when conidia of wild-type *A. fumigatus* and the ΔrodA mutant were exposed to free BDP-PCZ and their resulting fluorescence quantified with flow cytometry, the ΔrodA mutant exhibited a >10-fold reduction in binding of free BDP-PCZ (Figure 4). Collectively these results

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**Figure 2.** The fluorophore-conjugated posaconazole BDP-PCZ transfers rapidly from host epithelial cells to conidia on contact. A549 cells were exposed to BDP-PCZ, washed, and then infected with conidia of a red fluorescent protein–producing strain of *Aspergillus fumigatus* (AF-RFP) and imaged with confocal microscopy. After 5 minutes of coculture, BDP-PCZ staining of conidia in contact with epithelial cells was observed (arrows). Inhibition of A549 cell endocytosis of conidia with cytochalasin D (CD) had no effect on transfer of BDP-PCZ to conidia.
suggest that conidial hydrophobins directly bind BDP-PCZ and enhance uptake of this antifungal agent.

Accumulation of BDP-PCZ Within Fungal Intracellular Membranes and Colocalization With Its Target Enzyme CYP51a

Exposing germlings to BDP-PCZ resulted in fluorescence in the perinuclear region suggestive of accumulation of the drug within the fungal ER membranes (Figure 5A). CYP51a, the target enzyme inhibited by posaconazole and other azoles, is a membrane-bound protein found in the ER of fungi [23]. Therefore, to determine whether posaconazole accumulates specifically in the ER at the site of CYP51a, we compared the cellular distribution of BDP-PCZ and CYP51a in germlings of A. fumigatus by expression of a CYP51a-RFP fusion protein. Fluorescent microscopy demonstrated expression of CYP51a-RFP in the ER and other cytoplasmic membranes within hyphae.

Figure 3. The fluorophore-conjugated posaconazole BDP-PCZ transfers from host tissues to germlings during infection. A549 cells were exposed to BDP-PCZ, washed, infected with germlings of a red fluorescent protein–producing strain of Aspergillus fumigatus (arrows) and imaged with confocal microscopy. BDP-PCZ staining of the plasma membrane of germlings was observed after 1 hour of infection, and staining of intracellular membranes of germlings with BDP-PCZ after 4 hours.
When the CYP51a-RFP-expressing strain of *A. fumigatus* was exposed to BDP-PCZ, colocalization of BDP-PCZ and RFP-tagged CYP51a protein was observed (Figure 5B), confirming that BDP-PCZ concentrates at the location of its target enzyme CYP51a.

**Persistence of Posaconazole Within Fungal Membranes**

We demonstrated previously that posaconazole exposure was associated with prolonged postantifungal effect [12]. To determine whether this postantifungal effect might reflect a persistence of posaconazole within fungal cell membranes, we exposed...
germlings of \textit{A. fumigatus} to BDP-PCZ for 8 hours and then incubated them in drug-free medium. Consistent with the hypothesis that BDP-PCZ persists within fungi to inhibit growth, persistence of fluorescence within internal membranes of \textit{A. fumigatus} was observed for >48 hours (Figure 6).

Intracellular Membrane Accumulation of Hydrophobic Antifungals in Fungi and Epithelial Cells

To confirm that our findings with BDP-PCZ reflect that unmodified posaconazole also accumulates within host and fungal intracellular membranes, we performed competition assays to determine the ability of unlabeled posaconazole to compete with BDP-PCZ within membranes. Exposure of BDP-PCZ treated host cells or \textit{Aspergillus} to increasing concentrations of unlabeled posaconazole resulted in dose-dependent decreases in fluorescence (Figure 7A, C, and D). Microscopically, this corresponded to a marked reduction in fluorescence throughout the cell, suggesting that the labeled and unlabeled drugs are competing for the same membranes (Figure 7C and D). Importantly, this competition was specific to posaconazole and its parent compound itraconazole (Supplemental Figure 1); the addition of unlabeled voriconazole did not influence total cell fluorescence or distribution of BDP-PCZ (Figure 7B, C, and D). Collectively, these results suggest that although the lipophilic agents BDP-PCZ, posaconazole, and itraconazole compete for the same membrane compartment within host cells and fungi, voriconazole does not.

DISCUSSION

This study demonstrates that posaconazole can be conjugated with the fluorophore BODIPY, while largely retaining the antimicrobial properties of the parent molecule. We tested the activity, specificity, and localization of BDP-PCZ, using both susceptibility testing and competition assays. BDP-PCZ was
found to exhibit only a modest decrease in antifungal activity in a standard MIC assay compared with PCZ. Importantly, BDP-PCZ also retained the ability to mediate cellular protection against fungal infection that has been reported for PCZ [12]. Furthermore, in competition assays, unlabeled posaconazole, but not voriconazole, competed with BDP-PCZ with respect to accumulation in fungal and host cell membranes, suggesting that BDP-PCZ traffics to the same cellular compartments as unlabeled posaconazole. Collectively, these results suggest that BDP-PCZ is a useful tool to test the cellular pharmacokinetic properties of posaconazole.

BDP-PCZ accumulated to high levels within the ER of epithelial cells. This binding within the ER was reversible, because it decreased both over time during the transfer experiments and with the addition of unlabelled posaconazole in a dose-dependent manner. This finding distinguishes posaconazole from other intracellular antimicrobials, such as the macrolide azithromycin. Azithromycin is a weak base that is protonated on entry into the phagolysosome, becoming markedly less soluble and remaining trapped within the cells [24]. In contrast, the ER and other internal membranes are a dynamic reservoir for posaconazole and probably contribute to the long duration of antifungal protection observed in epithelial cells exposed to posaconazole [12].

In addition, our findings that conidial hydrophobins facilitate uptake of posaconazole provide a second possible explanation for the success of posaconazole in antifungal prophylaxis in clinical studies [6, 25]. Although treatment of established fungal disease requires activity against hyphae, prophylactic

**Figure 6.** The fluorophore-conjugated posaconazole BDP-PCZ persists in fungal membranes. Germlings were exposed to BDP-PCZ for 8 hours, washed, and transferred to fresh drug-free medium. Samples were then imaged using confocal microscopy after 0, 24, and 48 hours of incubation. Significant residual BDP-PCZ was detected within internal membranes of *Aspergillus fumigatus* for >48 hours after removal of free drug.
antifungal regimes target inhaled conidia. Thus, a hydrophobic antifungal with a dramatic affinity for hydrophobic conidia has an intrinsic advantage in terms of antifungal efficacy. Indeed, these data are consistent with our previous findings demonstrating a lower cell-associated posaconazole MIC for conidia compared with young hyphae of *A. fumigatus*.

Among the azole antifungals, posaconazole has the broadest spectrum of action against fungi and has been reported to have the lowest MIC90 against *Aspergillus* species [26]. This increased antifungal efficacy has been attributed to the ability of the hydrophobic side chain of posaconazole to stabilize binding to the target enzyme CYP51a [27, 28]. Accumulation of posaconazole within intracellular membranes, such as the ER of fungi, provides a second mechanism that may contribute to its broad activity against fungi. Given that CYP51a is thought to be a membrane-bound protein found in the ER [22, 23, 29], the locally increased concentration of posaconazole within this membrane compartment is highly likely to contribute to the molecule’s ability to inhibit ergosterol biosynthesis. In addition, the persistence of posaconazole within the ER membrane for up to 48 hours after drug exposure provides an explanation for the observed prolonged postantifungal effect seen with posaconazole [12].

Figure 7. Posaconazole, but not voriconazole (VCZ), competitively inhibits membrane accumulation of BDP-PCZ, a fluorophore-conjugated posaconazole, in host cells and fungi. A549 cells were exposed to BDP-PCZ and either unlabelled PCZ (A), or unlabelled VCZ (B) at the indicated ratios for 2 hours. Free drug was then removed by washing, and the total residual BDP-PCZ–associated fluorescence was then determined using a spectrofluorometer. C, A549 cells were exposed to BDP-PCZ, PCZ, or VCZ alone and combinations of BDP-PCZ and PCZ or BDP-PCZ and VCZ at a ratio of 1:2 respectively, as described previously. Cells were then washed to remove free drug and imaged using confocal microscopy. D, *Aspergillus fumigatus* germings were exposed to BDP-PCZ, PCZ or VCZ alone and combinations of BDP-PCZ and PCZ or BDP-PCZ and VCZ as in C and imaged using confocal microscopy.
Our findings suggest that distribution of posaconazole into host and fungal cell membranes, particularly the fungal ER, represents an important pharmacokinetic compartment that should be considered when analyzing the kinetics of this molecule. Although intracellular concentrations of antifungals have previously been linked to efficacy, these studies have largely focused on the efflux of azoles from fungal cells as an important cause of drug resistance [30]. Our current findings expand our understanding of intracellular pharmacokinetics to suggest that intracellular and subcellular accumulation of some antifungals may contribute to their efficacy. Little is known about the mechanisms of entry of azoles into fungal cells. A recent report suggests that fluconazole and other aqueous azoles may be actively imported into host cells [30], but the intracellular fate of azoles within fungi has received limited attention. Our results suggest that posaconazole may enter into the cell by diffusion into the plasma membrane, but the mechanisms governing subsequent transport between the plasma membrane and internal membranes remain undefined. It is possible that posaconazole is transported via the endogenous membrane recycling pathways of the cell and thus can move bidirectionally between the plasma and intracellular membrane compartments. This hypothesis would be consistent with our observations, although further study is required for confirmation.

Overall, we have used fluorescent BDP-PCZ as a tool to confirm our previous observation that posaconazole accumulates in lipid-rich membranes of host cells, including the ER. We have observed that rapid transfer of posaconazole from the host cell membranes to fungi occurs on contact of conidia with host cells, does not require invasion of host cells, and is facilitated by the conidial hydrophobin RodA. As with host-cells, BDP-PCZ accumulated within the fungal membranes, including the ER, and colocalized with CYP51a, the target enzyme inhibited by posaconazole. Furthermore, consistent with our previous observation of a prolonged postantifungal effect with posaconazole, BDP-PCZ was found to persist within fungal membranes for over 48 hours after removal of extracellular drug. Thus, host cell membrane-associated posaconazole can transfer to and concentrate within membranes of fungal cells, where it persists at its subcellular site of action, leading to prolonged inhibition of CYP51a and, ultimately, fungal death. This intracellular distribution and persistence of posaconazole may contribute to the antifungal efficacy and spectrum of action of posaconazole and provide an explanation for its observed prolonged postantifungal effect.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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**Potential conflicts of interests.** D. C. S. has served on advisory boards and been a paid consultant for Merck Canada, Pfizer Canada, and Astellas Canada. All other authors report no potential conflicts.

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