High Intracellular Concentrations of Posaconazole Do Not Impact on Functional Capacities of Human Polymorphonuclear Neutrophils and Monocyte-Derived Macrophages In Vitro

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Posaconazole is a commonly used antifungal for the prophylaxis and treatment of opportunistic invasive fungal infections (IFI) in immunocompromised patients. The majority of all IFI (probably more than 95%) are attributable to Aspergillus spp. and Candida spp. (1, 2). Aspergillus spp. are ubiquitous soil-dwelling fungi, often found in walls and dust. Due to their small size (2- to 4-μm diameter), Aspergillus conidia (spores) tend to remain airborne. It is estimated that all humans inhale at least several hundred Aspergillus fumigatus conidia per day (3). Upon inhalation, these conidia may germinate at body temperature in the terminal airways and alveoli, causing invasive pulmonary aspergillosis (3). Alveolar macrophages constitute the first line of defense against invading conidia. They immediately phagocyte conidia, thereby restricting their initial spread into the alveoli. The process of phagocytosis is accompanied by secretion of proinflammatory cytokines, leading to autocrine activation of the macrophage and attraction of other immune cells to the site of infection (4). It has been shown that recruitment of neutrophils is essential for efficient clearing of Aspergillus fumigatus conidia (5). Moreover, one of the most disposing risk factors for invasive aspergillosis is a depletion and/or functional impairment of neutrophils (6–8). Killing of Aspergillus fumigatus by neutrophils is mediated by the release of reactive oxygen species and neutrophil granular content (9–12).

Therapeutic substances are differentially distributed to various compartments of the human body, which impacts their pharmacokinetics and pharmacodynamics. With regard to the question of whether cells of the peripheral blood constitute a relevant compartment for the pharmacokinetics of triazoles, we previously measured the intracellular concentrations of posaconazole. We found that the intracellular concentration of posaconazole in peripheral blood mononuclear cells (PBMCs) and polymorphonuclear neutrophils (PMNs) was greatly increased compared to the plasma concentration. As these professional phagocytes are crucial to combat fungal infections, we set out to investigate if and how, beneficial or deleterious, this high loading of intracellular posaconazole impacts the functional capacities of these cells. Here, we show that high intracellular concentrations of posaconazole do not significantly impact PMN and monocyte-derived macrophage function in vitro. In particular, killing capacity and cytoskeletal features of PMN, such as migration, are not affected, indicating that these cells serve as vehicles for posaconazole to the site of infection. Moreover, since posaconazole as such slowed the germination of Aspergillus fumigatus conidia, infected neutrophils released less reactive oxygen species (ROS). Based on these findings, we propose that the delivery of posaconazole by neutrophils to the site of Aspergillus species infection warrants control of the pathogen and preservation of tissue integrity at the same time.

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various functional capacities of neutrophils and monocyte-de
derived macrophages upon challenge with Aspergillus fumigatus
conidia.

MATERIALS AND METHODS
Donors. Whole blood from healthy donors was collected as part of the
Cologne biobank protocol (University of Cologne Ethics Committee, no.
08-160) (15). Buffy coats were obtained from healthy blood donors at the
Department of Transfusion Medicine of Cologne University Hospital
(University of Cologne Ethics Committee, no. 14-144).

Preparation of conidial suspensions. Aspergillus fumigatus conidia
(ATCC 46645) were plated on Sabouraud agar plates (Becton Dickinson,
Heidelberg, Germany) and cultivated for 3 days at 37°C. Conidia were
then harvested, filtered through a 40-μm nylon cell strainer (BD Biosciences,
San Jose, CA), and resuspended in phosphate-buffered saline (PBS)
to a final concentration of 1.5 × 10⁵ conidia/ml.

Preparation of fungal lysates. Aspergillus fumigatus conidia were in-
oculated at a final concentration of 2 × 10⁶ conidia/ml in yeast extract,
peptone-dextrose medium (Becton Dickinson, Heidelberg, Germany)
and shaken at 37°C with 200 rpm overnight. Mycelia were recovered by
filtration and disrupted in a Micro-Dismembrator S (Sartorius Stedim
Biotech GmbH, Göttingen, Germany) at 200 rpm for 10 min using
0.5-mm glass beads.

Preparation of Aspergillus-activated serum (AAS). Aspergillus fu-
migatus conidia (1 × 10⁶/ml) were incubated with serum for 30 min at
37°C. Afterwards, the reaction mixture was centrifuged to remove conidia
and incubated at 56°C for 30 min in order to inactivate residual comple-
ment.

Labeling of Aspergillus conidia with C₁₂FDG. Aspergillus fumigatus
conidia (7.5 × 10⁷/ml) were incubated at 37°C for 1 h with intermittent
sonication (10 s every 10 min) in 0.1 M bicarbonate buffer containing 2.0
mM 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C₁₂FDG;
Molecular Probes, Invitrogen, Karlsruhe, Germany). After labeling,
conidia were washed twice with bicarbonate buffer (1,500 × g, 10 min,
4°C), resuspended in assay buffer, and used directly.

Purification of PMNs. Polymorphonuclear neutrophils (PMNs) were
isolated from 20 ml of venous blood. Blood was anticoagulated with 2 ml
acidified citrate and mixed with 18 ml dextan (7% dextan 70 in 0.9%
NaCl), and erythrocytes were allowed to sediment for 60 min at room
temperature. The leukocyte-rich supernatant was underlayered with an
equal volume of LymphoPrep (Axis-Shield PoC AS, Oslo, Norway)
and centrifuged for 20 min at 800 × g. The PMN fractions were recovered,
and contaminating erythrocytes were lysed with hypotonic saline. Afterwards,
the suspension was immediately adjusted to a volume of 40 ml with Hanks balanced salt solution (without Mg²⁺ and Ca²⁺, i.e., HBSS⁻⁻)
containing 0.1% chicken egg albumin. The suspension then was centrifuged for
5 min at 300 × g and 4°C. PMNs were resuspended and washed again using
HBSS⁻⁻ plus 0.1% chicken egg albumin, and cell counts were deter-
mined using a hemocytometer.

Generation of human monocyte-derived macrophages (hMDMs). Blood
from buffy coats (50 ml) was mixed with phosphate-buffered saline
(PBS; 50 ml). Four 25-ml aliquots were layered over 15 ml LymphoPrep
solution, PBMCs were separated by gradient centrifugation (20 min,
800 × g, 21°C) and washed twice (300 × g, 10 min) with PBS. The pellet
was resuspended in 30 ml RPMI 1640 plus 5% fetal bovine serum (FBS),
and cell counts were determined using a hemocytometer. Assuming that
5% of total PBMCs are monocytes, 4 × 10⁷ PBMCs were seeded in each
well of 24-well plates precoated with human serum in order to yield ap-
proximately 2 × 10⁵ monocytes/well. For 12-well plates, 1 × 10⁷ PBMCs,
i.e., 5 × 10⁵ monocytes/well, were used. Monocytes were allowed to attach
for 24 h. Floating lymphocytes then were removed upon washing with
warm PBS, and adherent monocytes were replenished with RPMI plus
10% FBS. Cells were maintained (washed and replenished every 48 h) for
6 to 8 days until macrophages were well differentiated and confluent in
each well.

Chemotaxis of PMNs. A stock solution of posaconazole (312 mg/liter;
Schering-Plough Research Institute, Kenilworth, NJ) was prepared in
methanol-water (50:50, vol/vol) and further diluted using HBSS⁻⁻. Cells
were prepared as described above and incubated with posaconazole at
different concentrations (0, 600, and 1,200 ng/ml). For chemotaxis assays,
calcine acetyoxymethyl ester (calcine-AM; Molecular Probes, Invitrogen,
Karlsruhe, Germany)-loaded PMNs were utilized. PMNs were incubated
with calcine-AM (5 μM) in HBSS⁻⁻ at 37°C and 5% CO₂ for 20 min.
After incubation, free calcine-AM was removed by dilution and a subse-
quent washing of the cells. PMN were resuspended to 2 × 10⁶ cells/ml in
HBSS with Ca²⁺ and Mg²⁺ (HBSS⁺⁺) and 0.1% chicken egg albumin.
Chemotaxis of calcine-AM-loaded PMN was assessed using a modified
96-well Boyden chamber with a polycarbonate polypyrrolidone (PVP)-free filter with 3-μm pores (ChemoTx 101-3; Neo Probe, Gaith-
ersburg, MD). Bottom plate wells were loaded with chemotactic trac-
tants, i.e., zymosan-activated serum (ZAS; 0.1 mg/ml), AAS (1:10), N-formyl-
leuyl-phenylalanine (fMLP; 1 × 10⁻⁷ M), and Aspergillus fu-
migatus conidia (1.5 × 10⁵/ml). Random migration of PMNs was deter-
mined using a negative control, i.e., HBSS⁺⁺ plus 0.1% chicken egg
albumin. Corresponding filter membrane wells were loaded with 25 μl of
the pretreated PMN suspensions. After a 90-min incubation period (37°C
and 5% CO₂), free cells on the top of the filter were wiped off and the
relative fluorescent intensity of cells that had migrated to the underside
of the filter was quantified using a fluorescence plate reader (Cytocount
4000; Applied Biosystems, Foster City, CA) using excitation at 485 nm
and emission at 530 nm. Each chemotactic condition was analyzed in quadru-
licate. The chemotactic index was calculated in relation to the negative
control.

ROS production of PMNs. Cells were prepared as described above,
using RPMI 1640 (without phenol red) plus 10% FBS and adjusted to 2 × 10⁶
cells/ml. Reactive oxygen species (ROS) production was determined by
chemiluminescence in the presence of luminol (4-amino-2,3-dihydro-
1,4-phenathalinedione; 4 × 10⁻⁴ M). Briefly, white 96-well microplates
were loaded with cell suspension (50 μl; 1 × 10⁶ cells/well) and different
concentrations of posaconazole (0, 300, 600, and 1,200 ng/ml), luminol
(4 × 10⁻⁴ M), and horseradish peroxidase (HRP). Afterwards, the gen-
eration of ROS was stimulated with Aspergillus fumigatus conidia (multi-
licity of infection [MOI], 1:5), lyophilized Escherichia coli (positive con-
trol) (2.5 ng/ml), and medium (negative control). The luminescence was
measured every 2 min for 6 h at 37°C using a microplate reader (Infinite
M1000 Pro; Tecan, Switzerland).

Phagosomal processing of A. fumigatus conidia by PMNs. Cells were prepared
as described above and adjusted to 5.0 × 10⁷ cells/ml using HBSS⁻⁻
plus 20 mM HEPES (2-(4-(2-hydroxyethyl) piperazin-1-yl) ethanesulfonic acid) containing different concentrations of posaconazole
and incubated (37°C) with C₁₂FDG-labeled A. fumigatus conidia (MOI,
1:2). After defined intervals (0, 30, 60, and 120 min), a fixing solution (460
μl; 5% formaldehyde in PBS plus 2% fetal calf serum [FCS]) was added
and the samples were analyzed by flow cytometry. The enzymatic cleavage
of C₁₂FDG by β-galactosidase within phagolysosomes produces a green
fluorescent product and indicates phagosomal processing of the labeled
conidia. Hence, phagosome maturation is considered to be directly pro-
portional to the increase in mean fluorescence intensity (MFI).

Killing of A. fumigatus conidia by hPMNs. For the killing of A. fu-
migatus conidia by human PMNs (hPMNs), cells were prepared as
described above, adjusted to 1.0 × 10⁷ cells/ml using HBSS⁻⁻ plus HEPES
(20 mM) containing different concentrations of posaconazole, and
incubated (37°C) with serum-opsonized A. fumigatus conidia (MOI, 1:4).
After 3 h, the neutrophils were lysed by adding cold Tween 20 (460 μl; 0.1%
Tween 20 in sterile water; 4°C) and stored at −80°C. The amount of viable
conidia/CFU was determined by serial dilution plating. In brief, samples
were thawed, serially diluted (1:10 with 0.1% Tween 20), and plated on
Sabouraud agar plates containing gentamicin and chloramphenicol (Bec-
ton Dickinson, Heidelberg, Germany). The plates were incubated for 20 h
at 37°C, and CFU were counted afterwards. A negative control of the
inoculum without neutrophils was kept under the same conditions as those applied in the infection experiment. The killing index was then calculated in relation to the CFU of that negative control.

Metabolic activity of \textit{A. fumigatus} conidia challenged by hPMNs. Cells were prepared as described above, adjusted to \(2.0 \times 10^8\) cells/ml using HBSS\(^{1/2}\) plus 20 mM HEPES containing different concentrations of \textit{A. fumigatus}, and incubated (37°C, 3 h) with serum-opsonized \textit{A. fumigatus} conidia (MOI, 1:5). Neutrophils then were centrifuged (1,500 \(\times\) g, 4°C, 5 min), lysed by adding a cold Tw3020 solution (0.1% Tween 20 in sterile water, 4°C), and stored at \(-80^\circ\)C. The metabolic activity of conidia was assessed colorimetrically based on the reduction of the tetrazolium salt 2,3-bis-(2-methoxy-4-nitro-5-[(sulfenylamino)carbonyl]-2H-tetrazolium-hydroxide (XTT) in the presence of menadione as published elsewhere, with some modifications (16). Briefly, the samples were thawed, supplemented with RPMI (without phenol red), transferred to 96-well microplates (100 \(\mu\)l/well), and incubated at 37°C. After 16 h, XTT (167 mg/ml) and menadione (167 \(\mu\)M) were added and the microplates were further incubated for 3 h at 37°C before the optical density at 450 nm (OD\(_{450}\)) was measured using a microplate reader (Infinite M1000 Pro; Tecan, Switzerland).

\textbf{IL-8 production of hPMNs stimulated by \textit{A. fumigatus} conidia.} Neutrophils were isolated from whole blood using the MACSplex neutrophil isolation kit (Miltenyi Biotec, Germany) according to the manufacturer’s instructions. Cells were counted using a hemocytometer, centrifuged (350 \(\times\) g, 10 min), and adjusted to 4.0 \(\times\) 10^6 cells/ml using RPMI 1640 (without phenol red) containing different concentrations of \textit{A. fumigatus} conidia. Equal volumes of cell suspension (250 \(\mu\)l) and \textit{A. fumigatus} conidia (250 \(\mu\)l; 2.0 \(\times\) 10^8 conidia/ml; MOI, 1:5) were admixed and incubated at 37°C. After 6 h, the infected neutrophils were centrifuged (350 \(\times\) g, 4°C, 5 min), and then the supernatant was centrifuged again (14,000 \(\times\) g, 4°C, 5 min), aliquoted, and stored at \(-80^\circ\)C until use. The supernatants were thawed and analyzed using the human CXCL8/interleukin-8 (IL-8) DuoSet kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

\textbf{ROS production by MDMs.} 6-Carboxy-2’,7’-dichlorodihydrofluorescein diacetate, di(acetoxymethyl) ester (DCF; Molecular Probes, Invitrogen, Karlsruhe, Germany) was used as an indicator for ROS production within human MDMs. Briefly, MDMs were grown in RPMI plus 10% FBS for 6 to 8 days in 24-well plates (as described above), washed (PBS), and incubated with 250 \(\mu\)l 16 \(\mu\)M DCF for 15 min at 37°C. Plates were washed twice (RPMI plus 10% FBS) and treated with posaconazole at different concentrations (0, 200, 600, and 1,200 ng/ml). ROS production was stimulated with \textit{Aspergillus fumigatus} conidia (2.0 \(\times\) 10^8 conidia/well; MOI, 1:10) and phorbol myristate acetate (4 \(\mu\)M PMA; P8139; Sigma-Aldrich, Munich, Germany) (positive control), and medium (negative control). After 3 h of incubation at 37°C, MDMs were carefully scraped off and analyzed by flow cytometry. Macrophages were identified and gated based on their appearance within the cellular forward and sideward scatter (FSC/SSC). The purity of cells within this gate was confirmed by CD45/CD14 staining. At least 98% of the cells within the respective gate were CD45^-/CD14^+ cells, i.e., evidently human MDMs. Only cells within the MDM gate were analyzed. ROS production was assessed as relative change of the MFI, calculated in relation to the negative control.

\textbf{Phagosomal processing of \textit{A. fumigatus} conidia by hMDMs.} The phagosomal processing of \textit{A. fumigatus} conidia by posaconazole-loaded MDMs was studied by flow cytometry. MDMs were incubated (37°C) with C\(_{12}\)-FDG-labeled \textit{A. fumigatus} conidia (2.0 \(\times\) 10^8 conidia/ml; MOI, 1:10) at different concentrations of posaconazole (0, 200, 600, and 1,200 ng/ml). After defined intervals (0, 30, 60, and 120 min), the MDMs were washed twice (in PBS at 37°C), resuspended in cold PBS (4°C), and immediately analyzed by flow cytometry. The increase of green (FL1) fluorescence caused by the enzymatic cleavage of C\(_{12}\)FDG by β-galactosidase within phagolysosomes is considered to be directly proportional to phagosomal processing of the labeled conidia.

\textbf{Killing of \textit{A. fumigatus} conidia by hMDMs.} MDMs were grown in RPMI plus 10% FBS for 6 to 8 days in 12-well plates (as described above), washed with PBS, and incubated (37°C) with 500 \(\mu\)l RPMI plus 10% FBS containing serum-opsonized \textit{A. fumigatus} conidia (5 \(\times\) 10^6 conidia/ml; MOI, 1:5). After 1 h, unbound conidia were washed away with PBS (37°C). Macrophages of four wells were lysed and processed (as described below), and the macrophages within the remaining wells were incubated for an additional 6 h in 500 \(\mu\)l RPMI plus 10% FBS containing different concentrations of posaconazole (0, 200, 600, and 1,200 ng/ml). Afterwards, the MDMs were washed twice with PBS (37°C), lysed by adding cold Tw3020 (500 \(\mu\)l; 0.1% Tween 20 in sterile water; 4°C), and stored at \(-80^\circ\)C until use. The amount of viable conidia/CFU was determined by serial dilution plating. In brief, samples were thawed, serially diluted (1:10 with 0.1% Tween 20), and plated on Sabouraud agar plates containing gentamicin and chloramphenicol (Becton Dickinson, Heidelberg, Germany). CFU were counted after 20 h of incubation at 37°C. The killing index, i.e., the percentage of \textit{Aspergillus fumigatus} conidia killed after 6 h in relation to the amount of phagocytized conidia (after 1 h), was calculated.

\textbf{Statistical analysis.} Statistical analysis was performed using GraphPad Prism 5.02 (GraphPad Software Inc., CA, USA). Values were compared using analysis of variance (ANOVA); intergroup differences were confirmed by a Bonferroni post hoc test with multiple-test corrections. P values lower than 0.05 were considered statistically significant. Figures were computed with GraphPad Prism 5.02 (GraphPad Software Inc., CA, USA).

\textbf{RESULTS} PMNs do not migrate toward \textit{Aspergillus fumigatus} conidia. The migration capacity of unloaded neutrophils toward \textit{Aspergillus fumigatus} conidia, as well as \textit{Aspergillus fumigatus} lysate, was studied first. However, conidia in concentrations up to 1.5 \(\times\) 10^8 did not induce PMN migration. In fact, the level of migration toward conidia was equal to that of random migration (which is the negative control) (data not shown), as previously described by Waldorf et al. (17).

Intracellular loading of posaconazole does not impact neutrophil migration capacities. Migration to the site of infection is crucial for PMNs in order to combat fungal pathogens. Thus, we investigated whether intracellular loading of posaconazole has an influence on neutrophil chemotaxis toward physiological chemotacticants. To this end we used serum which had been activated by \textit{Aspergillus fumigatus} (AAS) in our assay. We applied N-formylmethionyl-leucyl-phenylalanine (FMLP) as an additional positive control. Whenever cells remained that were not needed for the actual assay, intracellular concentrations were determined as previously described (18); the ratio between the cellular and the applied concentration was well within the expected range (4.95 \(\pm\) 1.90 versus 7.66 \(\pm\) 6.50) (13). The results of the chemotaxis assay are shown in Fig. 1; there was no significant effect of posaconazole on the migration capacity of neutrophils toward AAS or FMLP.

Phagosomal maturation is not affected by intracellular concentrations of posaconazole. We evaluated the effect of posaconazole on the phagosomal-lysosomal fusion with C\(_{12}\)-FDG-coated \textit{A. fumigatus} conidia. C\(_{12}\)-FDG is a self-quenched nonfluorescent substrate that is hydrolyzed upon exposure to β-galactosidase found in phagolysosomes, producing highly fluorescent fluorescein. Therefore, C\(_{12}\)-FDG-coated conidia become fluorescent only as they are processed within phagolysosomes (19). The flow-cytometric profiles of human neutrophils 2 h after internalization of C\(_{12}\)-FDG-coated \textit{A. fumigatus} are shown in Fig. 2A (one representative of three
individual experiments). The mean fluorescence intensity (MFI) increased with phagosomal maturation following uptake of conidia; this effect was partially reversed by bafilomycin A1, which inhibits the phagosomal-lysosomal fusion.

Posaconazole inhibits germination of conidia, thereby impacting release of ROS from PMNs. Release of ROS from PMNs is crucial for extracellular killing of pathogens as well as for the initiation of several intracellular signaling processes. However, ROS also drives inflammation in the microenvironment at the site of infection, which may be harmful to the host. Figure 3 shows the relative change in ROS production by human neutrophils upon stimulation with conidia of different Aspergillus fumigatus strains after 3 h of incubation. While posaconazole did not affect the production of ROS upon stimulation with a highly azole-resistant strain, M-491 (MIC of ≥8 mg/ml), there was a significant reduction of ROS produced by human neutrophils upon stimulation with conidia of a posaconazole-susceptible strain (ATCC 46645) (Fig. 4). Upon stimulation with another azole-resistant strain, the M-850 strain (MIC of ≥2 mg/ml), only the highest posaconazole concentration (1.2 mg/ml) showed a significant reduction in ROS production. Both azole-resistant strains (M-491 and M-850) have been described elsewhere (20). The production of ROS upon stimulation with lyophilized E. coli (2.5 ng/ml) was not affected by posaconazole (see Fig. S1 in the supplemental material). The amount of IL-8 released by human neutrophils was not affected by posaconazole, as shown in Fig. S2 in the supplemental material. In addition, the decline in ROS production does not seem to be attributable to a decrease in A. fumigatus conidia due to the antifun-
gal efficacy of posaconazole (and hence elimination of the stimulus). In fact, posaconazole did not affect killing of *A. fumigatus* conidia by human neutrophils, as shown in Fig. 5A. However, it is known from previous studies that posaconazole inhibits the germination of *Aspergillus fumigatus* conidia (21), and ROS release from neutrophils is significantly lower upon stimulation with resting conidia than with swollen conidia or hyphae (12, 22). In order to confirm inhibition of germination of *A. fumigatus* conidia by posaconazole in our experimental setting, we assessed the metabolic activity of *A. fumigatus* conidia in the presence of posaconazole and/or neutrophils.

As shown in Fig. 6, posaconazole at 0.2, 0.6, and 1.2 μg/ml led to reduced metabolic activity of *A. fumigatus* conidia by 12.7% ± 2.9%, 13.9% ± 1.9%, and 11.1% ± 2.3%, respectively. However, we could not show a dose-dependent effect on metabolic activity. In fact, since all studied concentrations of posaconazole were well above the MIC of the strain (MIC of 0.063 mg/liter), we did not expect to see any difference between those concentrations.

We also studied the functional capacities of human monocyte-derived macrophages in the presence of posaconazole.

**Release of ROS from hMDMs is not affected by posaconazole.** Since *A. fumigatus* conidia are rapidly phagocytized by macrophages, we measured intracellular ROS production of macrophages using DCF (Fig. 4) instead of luminol, which is used to quantify intra- and extracellular ROS. Although there seemed to be a slight tendency toward smaller amounts of ROS with increasing concentrations of posaconazole, we did not see any significant effect of posaconazole on the ROS production by macrophages. Therefore, we studied the phagosomal processing of *C. albicans*FDG-coated conidia after 2 h of incubation. As shown in Fig. 2B, posaconazole did not affect the phagosomal processing.

**Killing is equally effective in the presence or absence of posaconazole.** After 6 h of incubation, 90.3% ± 8.0% of the phagocytized conidia were killed by macrophages. As shown in Fig. 5B, posaconazole did not affect the killing of *A. fumigatus* conidia by human macrophages; however, a two-way analysis of variance (ANOVA) suggested that the killing was donor dependent (P < 0.0001) or at least depended on the quality of theuffy coat and hence the resulting MDMs. A mere 4.2% of the variance might have been caused by the treatment, i.e., posaconazole (P = 0.0111).

**DISCUSSION**
In this study, we assessed key functional capacities of human neutrophils and human monocyte-derived macrophages in the presence of posaconazole at various concentrations *in vitro*. We hypothesized that high intracellular loading of posaconazole either supports the professional phagocytes in their mission of pathogen killing or has a toxic effect and thereby hampers neutrophil and MDM function. Despite high intracellular concentrations of posaconazole, we did not see any significant effect, neither beneficial nor deleterious, on the functional capacities of these cells, except for ROS release. The total amount of ROS released by human neutrophils was decreased in the presence of posaconazole. We attributed this effect to reduced metabolic activity, i.e., germination of the *A. fumigatus* conidia in response to the drug than to an immunomodulatory effect of posaconazole on the neutrophil. This conclusion is supported by the fact that, when stimulated with conidia of azole-resistant *Aspergillus fumigatus* strains or *E. coli*, neutrophils showed equal ROS release in the presence and absence of posaconazole. However, the observation of decreased
ROS release in the presence of posaconazole indicates that patients who experience control of fungal germination by sufficient drug levels suffer less collateral tissue damage at the site of infection, as it is associated with excessive ROS release. Based on the available literature, overall the data on ROS release by neutrophils in the presence of azoles are inconsistent. The effect of fluconazole on the phagocytic response of polymorphonuclear leukocytes was studied in a rat model of acute bacterial sepsis. In these experiments a significant and dose-dependent inhibition of the ROS-mediated proinflammatory cascade was observed (23). In contrast, Velert et al. measured the generation of free radicals by human PMNs in vitro upon PMA stimulation with and without fluconazole (at 0.1, 1, 5, and 50 mg/ml) and itraconazole (at 0.05, 0.5, and 5 mg/ml); neither compound had a significant effect on ROS release (24).

Neutrophils are attracted to the site of Aspergillus infection, e.g., the alveoli, by the release of distinct cytokines. This scenario happens early in the infection process and is crucial for efficient clearing of Aspergillus fumigatus conidia (5). We assessed directed and random migration of human neutrophils in a modified 96-well Boyden chamber system. PMNs exhibited only random migratory activity toward Aspergillus fumigatus conidia as such. Applying FMLP, Aspergillus fumigatus-activated serum (AAS), as well as zymosan-activated serum (ZAS) as chemoattractants, we observed equal directed migration of neutrophils independent of the presence of posaconazole. In contrast, Vuddhakul et al. described a significant suppression of neutrophil chemotaxis for human neutrophils in vitro in the presence of itraconazole, which is closely related to posaconazole (25). However, these examinations were carried out in protein-free media, and it was shown by Perfect et al. that the ratio of intracellular to extracellular concentrations (C/E) of itraconazole in alveolar macrophages (AC) depends on the composition of the surrounding medium. The C/E ratio of itraconazole within protein-free medium was significantly higher than that in media which contained serum, with C/E ratios of 87.9, 18, and 3 in 0%, 5%, and 100% serum, respectively (26). Hence, the intracellular concentration of itraconazole used in the experiments by Vuddhakul et al. (25) were much higher than those under physiological conditions. This might have contributed to the observation that itraconazole suppressed neutrophil functions to a greater extent than fluconazole.

Our in vitro study used protein-supplemented medium, and neutrophil migration was not affected by high concentrations of posaconazole. Given the fact that under physiological conditions protein concentrations are even higher, we propose that neutrophils are the perfect vehicles to deliver the drug to the site of infection. The inevitable death of neutrophils would subsequently lead to the release of posaconazole, thus increasing drug concentrations directly at the infection site. This then would contribute to slowing the germination of Aspergillus spp., which leads to diminished release of ROS from further recruited neutrophils. If this hypothesis holds true, the delivery of posaconazole by neutrophils to the site of Aspergillus infection would warrant control of the pathogen and preservation of tissue integrity at the same time. Since infections with Aspergillus spp. pose a life-threatening event to patients with neutropenia, e.g., after chemotherapy, neutrophil transfusions that are preloaded with posaconazole might provide a new perspective on the treatment of fungal infections.

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