Binding of Azole Antibiotics to *Staphylococcus aureus* Flavohemoglobin Increases Intracellular Oxidative Stress

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In this work, we report that flavohemoglobin contributes to the azole susceptibility of *Staphylococcus aureus*. We first observed that deletion of the flavohemoglobin gene leads to an increase in the viability of imidazole-treated *S. aureus* cells and that reversion to the wild-type phenotype occurs upon expression of flavohemoglobin from a multicopy plasmid. Further spectroscopic analyses showed that miconazole, the most efficient azole antibiotic against *S. aureus*, ligates to heme of both oxidized and reduced flavohemoglobin. The binding of miconazole to oxidized flavohemoglobin, with an association constant of $1.7 \times 10^9$ M$^{-1}$, typical of a tight, specific binding equilibrium, results in augmentation of the superoxide production by the enzyme. These results are corroborated by in vivo studies showing that imidazole-treated *S. aureus* cells expressing flavohemoglobin contain a larger amount of reactive oxygen species. Moreover, it was observed that the survival of miconazole-treated *S. aureus* internalized by murine macrophages is higher for cells lacking flavohemoglobin. Altogether, the present data revealed that in *S. aureus*, flavohemoglobin enhances the antimicrobial activity of imidazoles via an increase of intracellular oxidative stress.

*Staphylococcus aureus* is an opportunistic pathogen responsible for a large number of human infections that cause systemic diseases from a mild to life-threatening character. The increasing incidence of methicillin-resistant *S. aureus* (MRSA) strains observed in the past few years makes *S. aureus* infections a leading threat to public health, causing more deaths in the United States and Europe than human immunodeficiency virus (AIDS) (11). Like other Gram-positive bacteria, staphylococci are sensitive to imidazoles (27). Imidazoles (such as clotrimazole, miconazole, ketoconazole, and sulconazole) (Fig. 1) represent one of the major classes of azole antifungal that are useful in the treatment of infections, including cutaneous and vaginal candidiasis (8). The activity of these antifungal drugs derives primarily from inhibition of the biosynthesis of ergosterol, an essential component of the fungal plasma membrane, at the level of lanosterol 14-alpha demethylase. Furthermore, in fungi and yeast, azole treatment leads to an increase in the endogenous production of reactive oxygen species (ROS) (12, 25). For example, in *Candida albicans* and *Saccharomyces cerevisiae*, the miconazole inhibition of cytochrome c oxidase, peroxidase, and catalase has been reported to be responsible for a high level of ROS production (3, 4). It has also been reported that clotrimazole inhibition of *Plasmodium falciparum* hemoperoxidase leads to ROS accumulation in this protozoan pathogen (26). For *S. cerevisiae*, *C. albicans*, and *Escherichia coli*, the action of imidazoles was also correlated with the inhibition of the nitric oxide (NO) scavenger activity of flavohemoglobin (7).

Flavohemoglobins (Hmp) are widespread among bacteria and yeast and contain three domains: C-terminal NAD- and flavin adenine dinucleotide (FAD)-binding domains, which together constitute a ferredoxin-NADP$^+$ oxidoreductase-like domain, and an N-terminal globin domain, which harbors a single B-type heme. The high-spin heme contains one axial histidine and binds small molecules like NO, carbon monoxide (CO), and oxygen (O$_2$). The heme can also bind bulky aromatic bases, since it is inserted in a large hydrophobic pocket (7). We observed that the binding of imidazoles to *S. aureus* flavohemoglobin results in an increase in the amount of deleterious reactive oxygen species produced by flavohemoglobin that contributes to the bactericidal effect ofazole antibiotics toward *S. aureus*.

**MATERIALS AND METHODS**

Reagents. Miconazole, sulconazole, clotrimazole, ketoconazole, and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma, and 5-tert-butoxycarbonyl 5-methyl-1-pyrroline N-oxide (BMPO) was purchased from Northwest. All of the reagents were dissolved in dimethyl sulfoxide (DMSO), except for ketoconazole and BMPO, which were prepared in methanol and water, respectively. For the UV-visible and resonance Raman (RR) spectroscopic studies, a water-saturated solution of miconazole was used, to avoid the effect of DMSO on the reduced form of flavohemoglobin.

Bacterial strains, culture conditions, and viability assays. Overnight cultures of *S. aureus* wild-type (RN4220) and *S. aureus* Δhmp (LMS800) cells (6) grown in tryptic soy broth (TSB) medium were used to inoculate, to an OD$_{600}$ of 0.1, Luria-Bertani (LB) medium supplemented with the appropriate antibiotics (2 $\mu$M miconazole, 5 $\mu$M sulconazole, 12 $\mu$M clotrimazole, and 120 $\mu$M ketoconazole) and contained in closed flasks. For control purposes, untreated cultures, in which an equal volume of the corresponding antibiotic's solvent was added, were also analyzed. *S. aureus* viability was then evaluated after a 5-h treatment of the liquid cultures with antibiotic, and the number of viable cells was determined.
by measuring the CFU per milliliter upon plating 5 μl of each dilution on agar and counting the isolated colonies formed after overnight incubation. The percentage of survival was calculated as the number of cells originated by the treated cultures divided by the number of colonies formed after plating the control cultures.

MICs of theazole antibiotics were determined on 24-well microtiter plates as previously described (19). Assays were conducted in LB medium at 37°C, performed in triplicate and repeated at least twice.

**Complementation studies.** For the complementation analysis, a vector expressing *S. aureus hmp* was constructed. To this end, a fragment containing the complete *S. aureus hmp* gene was amplified, using oligonucleotides SAHmpFw (5′-TCATATTATTAATCTATGTTATTCTTCTTAGGA-3′) and SAHmp-EcoRI (5′-CGGTCATTAAATGAGCATAAATCCCTTT-3′), and ligated to pMK4 (24). The resulting vector (pHmp) and the empty vector (pMK4) were electroporated into *S. aureus Δhmp* mutant and wild-type (RN4220) strains. For the mutant strain, the positive transformants were selected on tryptase soy agar (TSA) medium containing 10 μg/ml erythromycin plus 5 μg/ml chloramphenicol, while selection of the wild type was achieved using only chloramphenicol. Cell growth was performed in liquid medium treated with miconazole for 5 h, as described above, and analyzed by serial dilutions plated on agar.

**Spectroscopic studies:** UV-visible, resonance Raman, and EPR. *S. aureus* flavohemoglobin was cloned, expressed and purified as described previously (18). UV-visible spectra were recorded on a Shimadzu UV-1700 spectrophotometer, using 10 μM flavohemoglobin in 10 mM Tris-HCl (pH 7.6) buffer containing 9% glycerol.

Electron paramagnetic resonance (EPR) spectra were obtained on a Bruker EMX spectrometer equipped with an Oxford Instruments continuous-flow He cryostat. 5,5′-Dibutyrylcytosine 5-methyl-1-pyrroline N-oxide (BMPo) was used as a trap for the detection of reactive oxygen species, which allows us to distinguish between superoxide anion and the hydroxyl radical (30). These experiments were performed using 10 μM *S. aureus* flavohemoglobin, 200 mM NaCl, 25 mM BMPo, and 50 μM miconazole, in a quartz flat cell, at room temperature.

Resonance Raman spectra were measured using a confocal microscope coupled to a Raman spectrometer (Jobin Yvon U1000) equipped with 1,200 lines/mm grating and a liquid nitrogen-cooled back-illuminated charge-coupled device (CCD) detector. Samples of flavohemoglobin (20 μM) were placed in a quartz rotating cell and excited with the 413-nm line of a krypton ion laser (Coherent Innova 302), with a laser power of 2 to 4 mW and accumulation times of 60 s. After polynomial background subtraction, the positions and line widths of the Raman bands were determined by component analysis using in-house software.

**Enzymatic studies.** The equilibrium constant for miconazole binding was determined by titrating a fixed amount of flavohemoglobin with increasing quantities of the antibiotic and monitoring the changes in absorbance in the visible region. The amount of miconazole-protein complex was calculated using a differential absorbance at 418 – 500 nm, determined from the difference of a spectrum of a solution having excess antibiotic (thus ensuring full complex formation) and the spectrum of the oxidized, isolated protein. A value of Δε (418 – 500) = 69,565 M⁻¹ cm⁻¹ was obtained; from this value, the amount of complex at each solution could be determined, and by using the appropriate mass balance equations, the concentrations of free antibiotic and free protein were also calculated. The number of binding sites and the equilibrium constant were then determined by a Scatchard equation (17). The same procedure could not be applied with precision to the reduced protein, due to the interference of DMSO; nevertheless, a lower limit for the binding constant could be determined using a differential absorbance at 426 – 390 nm. The assays were performed with 5 μM *S. aureus* flavohemoglobin, and the miconazole concentrations varied between 2 μM and 60 μM. The percentage of DMSO used in all the assays was 0.4% (vol/vol).

**Measurement of endogenous ROS production.** Endogenous ROS production was determined by a fluorometric assay according to the method described previously (12). Cells of wild-type *S. aureus* (RN4220) and the Δemp (LMS800) mutant were grown for 5 h, in the absence or presence of azoles. Cells were then collected by centrifugation, washed, and resuspended in phosphate-buffered saline (PBS), followed by the addition of 10 μM DCFH-DA. The fluorescence intensities (Fls) were measured on a Varian Eclipse 96-well spectrofluorimeter (excitation at 485 nm and emission at 538 nm). The Fls were normalized in relation to the final OD₆₀₀ of each culture. To assess the variation of ROS, the Fl of control cultures and the Fl of azole-treated cultures were subtracted.

**Quantitative real-time RT-PCR.** For real-time reverse transcription (RT)-PCR experiments, 2.0 μg of *S. aureus* total RNA derived from samples grown in LB and treated with 2 μM miconazole for 5 h was used to synthesize cDNA, according to the Transcriptor high-fidelity cDNA synthesis kit protocol (Roche Applied Science). Real-time PCRs were performed in a LightCycler Instrument using LightCycler FastStart DNA master SYBR Green I kit according to the manufacturer’s instructions (Roche Applied Science). The amplification reactions were carried out with equal amounts of cDNA (100 ng) as the initial template, and each reaction mixture contained 0.5 μM specific primers, 2 μM MgCl₂, 1x LightCycler FastStart DNA master SYBR green I kit from Roche Applied Science. The expression ratio of the target gene was determined relatively to a reference gene, *S. aureus* 16S rRNA, whose transcription abundance remains invariant under the tested conditions. The samples were assayed in triplicate.

**Assay of intracellular *S. aureus* viability in J774A.1 macrophages.** Murine macrophages J774A.1 (LGC Promochem) were inoculated with 5 x 10⁵ cells/ml and cultured for 2 days at 37°C in a 5% CO₂-air atmosphere in 24-well plates containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 4.5 g/liter glucose, 110 mg/ml sodium pyruvate (DMEM Glutamax), 10% fetal bovine serum, 100 μM nonessential amino acids, 50 U/ml penicillin, and 50 μg/ml streptomycin (all from Gibco). Prior to infection, macrophages were activated for 5 h with 1 μg/ml gamma interferon (IFN-γ; Sigma) and 5 μg/ml lipopolysaccharides (LPS; Sigma). When required, 800 μM N⁶-monomethyl-L-arginine acetate salt (L-NMMA; Sigma) was also added to achieve inhibition of the murine macrophage inducible NO synthase (iNOS). *S. aureus* wild-type and Δemp strain cells were grown for 5 h in the presence or absence of miconazole (2 μM), washed three times with PBS, and resuspended in DMEM, to obtain for all cultures an initial bacterial concentration of 10⁷ CFU/ml. Macrophages were then infected with these bacterial suspensions, at a multiplicity of infection (MOI) of at least 16, for 30 min at 37°C. The supernatants were then collected to determine the number of bacteria not internalized. Extracellular bacteria were killed by incubation in DMEM supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin for 5 min, and the wells were washed three times with PBS. After that, macrophages were lysed with 2% saponin, and the number of intracellular bacteria was determined by CFU counting of viable bacteria.

**RESULTS**

*S. aureus* is susceptible to azole antibiotics. The susceptibility of *S. aureus* to several azole antibiotics was analyzed. For *S. aureus* RN4220, the MICs were of the same order of magnitude for miconazole (15 ± 2 μM), sulconazole (20 ± 0 μM), and clotrimazole (30 ± 3 μM), while a much higher value was determined for ketoconazole (500 ± 70 μM). In previous work, *S. aureus* viability was also reported to decrease significantly with miconazole, while essentially no effect (>200 μM) was observed with ketoconazole (23). Additionally, we observed that *S. aureus* is resistant to concentrations of the triazole antibiotics fluconazole and itraconazole (Fig. 1) up to 2 mM.

*S. aureus* susceptibility to azoles involves flavohemoglobin. We next investigated the possible role of flavohemoglobin in the sensitivity of *S. aureus* to imidazoles. To this end, we compared the viability of *S. aureus* wild-type and Δhmp mutant
cells upon treatment with several azole antibiotics. The results showed that inactivation of flavohemoglobin caused an increase in the resistance of \textit{S. aureus} to imidazoles: i.e., the mutant strain produced higher number of viable cells, a result which was observed for all imidazoles tested (Fig. 2A and B).

To confirm that imidazole resistance was a specific consequence of flavohemoglobin gene deletion, the viability of the \textit{Δhmp} strain expressing flavohemoglobin (from a multicopy plasmid) treated with miconazole was evaluated. As expected, upon complementation, reversion to the wild-type phenotype was observed (Fig. 2C). Furthermore, the overexpression of \textit{S. aureus} flavohemoglobin in the wild-type strain led to an increase in the sensitivity to imidazoles (Fig. 2C). These results show that flavohemoglobin contributes to the activity of imidazoles against \textit{S. aureus}, independently of any other stress agent.

To understand the mechanism by which flavohemoglobin affects the susceptibility of \textit{S. aureus} to imidazoles, we analyzed the binding of miconazole to the oxidized flavohemoglobin heme, yielding a low-spin ferric species, as expected for ligation through imidazole nitrogens. These results were confirmed by resonance Raman (RR) spectroscopy since the spectra of hemes include marker bands that are sensitive to the oxidation, coordination, and spin state of the heme iron (9, 22, 29), for both paramagnetic and diamagnetic species. We first investigated the ferric protein, in the presence or absence of miconazole. RR spectra of oxidized flavohemoglobin exhibit characteristic vibrational modes at 1,370 cm$^{-1}$ and 1,494 cm$^{-1}$, respectively. This form under-
goes a change of spin state upon miconazole binding, as revealed by the shift of the \( \nu_3 \) band to 1,505 cm\(^{-1} \), characteristic of a 6-coordinated, low-spin (6cLS) ferric heme (Table 1). The reduced \( S. aureus \) flavohemoglobin is in a 5cHS configuration, with \( \nu_4 \) at 1,357 cm\(^{-1} \) and \( \nu_3 \) at 1,474 cm\(^{-1} \) (Fig. 3C, spectrum I). Component analysis of the spectrum obtained upon addition of miconazole revealed the presence of two species: 5-coordinated ferrous heme, as in the pure protein, and a major component with \( \nu_4 \) upshifted to 1,364 cm\(^{-1} \) (Fig. 3C, spectrum II; Table 1), indicating the formation of a 6-coordinated, low-spin ferrous form. Moreover, since the \( \nu_4 \) band upshifts by 7 cm\(^{-1} \) (in comparison with the \( \nu_4 \) of the unbound ferrous flavohemoglobin), it is apparent that delocalization of the electron cloud from the heme to the miconazole takes place upon binding of the antibiotic (22).

We have also observed, by both RR and UV-visible spectroscopy that DMSO binds to the ferrous heme, resulting in a distinct low-spin form of flavohemoglobin.

In summary, the spectroscopic results show that miconazole coordinates to the heme moiety of \( S. aureus \) flavohemoglobin in the oxidized as well as in the reduced state.

The binding of miconazole to \( S. aureus \) flavohemoglobin was also studied by titrating the protein with different antibiotic concentrations and following the absorption change of the Soret band of the flavohemoglobin after ligation of the antibiotic. Following the procedure described in Materials and Methods, we determined an equilibrium association constant of \( 1.7 \times 10^6 \) M\(^{-1} \), indicating that one molecule of miconazole binds tightly to oxidized flavohemoglobin. A lower limit for the

**TABLE 1.** Positions of the marker bands \( \nu_4 \) and \( \nu_3 \) in the resonance Raman spectra of oxidized and reduced flavohemoglobin, unbound or bound to miconazole

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<tr>
<th>Flavohemoglobin species</th>
<th>( \nu_4 ) (cm(^{-1} ))</th>
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<tr>
<td>Oxidized Hmp(III)</td>
<td>1,370</td>
<td>1,494</td>
<td>1,372</td>
<td>1,505</td>
</tr>
<tr>
<td>Reduced Hmp(II)</td>
<td>1,357</td>
<td>1,474</td>
<td>1,364</td>
<td>1,494</td>
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<td>Reduced Hmp(II)-miconazole</td>
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More importantly, when the ROS production was evaluated for S. aureus treated with miconazole or ketoconazole (Fig. 4A).

Imidazoles increase the ROS production by flavohemoglobin. Since, as mentioned before, we detected an increase in imidazole resistance upon inactivation of S. aureus hmp (Fig. 2), we next addressed the origin of this behavior. We first observed that the level of endogenously produced reactive oxygen species augmented in cells of wild-type S. aureus treated with miconazole or ketoconazole (Fig. 4A).

More importantly, when the ROS production was evaluated in the Δhmp mutant treated with imidazoles the ROS levels were lower, indicating that the presence of flavohemoglobin contributes to the imidazole-associated ROS generation (Fig. 4A).

It has been reported that, under certain conditions, E. coli flavohemoglobin produces superoxide ions (16, 28). To investigate the effect of antibiotic binding to flavohemoglobin on radical formation, we used the spin trap BMPO in EPR experiments. In the absence of the antibiotic, when using NADH and under aerobic conditions, the BMPO-OOH adduct was observed (30) being indicative of the formation of superoxide by flavohemoglobin (Fig. 4B, upper line). In the presence of miconazole, the same EPR species was detected but at a significantly higher concentration (Fig. 4B, lower line). By comparing the intensities of the spectra of the BMPO-OOH adduct after 15 min (the time determined to yield the maximum concentration of this adduct), we determined the concentration of BMPO-OOH to be ca. 3-fold higher in samples with miconazole. We thus conclude that not only is superoxide formed by S. aureus flavohemoglobin, since its EPR spectrum is identical to that of the superoxide-BMPO adduct (30), but also that this production increases upon the binding of miconazole to flavohemoglobin. Furthermore, we detected, by mass spectrometry, that the integrity of the imidazole was maintained since no changes occurred in the mass and intensity of the peak of the antibiotic before and after incubation of miconazole with flavohemoglobin in the presence of NADH (data not shown).

Real-time RT-PCR experiments revealed that exposure of S. aureus to miconazole leads to an increase in the expression of the katA gene encoding catalase, a marker of oxidative stress, showing a 11.7 ± 1.4-fold increase in katA expression in the wild-type strain but only a 7.8 ± 0.4-fold increase in expression the hmp mutant: i.e., in the absence of flavohemoglobin, the expression of katA decreased ~30%. These results are in agreement with a lower production of ROS in the hmp mutant.

Flavohemoglobin decreases survival of miconazole-treated S. aureus in macrophages. The increased resistance of the S. aureus Δhmp mutant to imidazoles compared to the parent strain (Fig. 2A and B) led us to examine the effect of flavohemoglobin on the survival of miconazole-treated S. aureus cells phagocytized by macrophages. In the absence of the antibiotic, the Δhmp strain was killed more efficiently by activated macrophages (Fig. 5A) due to the lack of the NO detoxifying activity of flavohemoglobin. For miconazole-treated cells, we observed that while incubation in macrophages of antibiotic-treated wild-type cells resulted in a decrease of survival of approximately 50%, the Δhmp cell counts showed no statistical difference between cells unexposed or exposed to miconazole (Fig. 5A). Similar data were obtained in assays performed in the presence of L-NMMA, the mammalian inhibitor of iNOS, which shows that in the presence or absence of NO, flavohemoglobin contributes to the lower survival of azole-treated cells (Fig. 5B). The decreased viability of antibiotic-treated S. aureus in macrophage cell lines can be rationalized taking into consideration that the simultaneous presence of flavohemoglobin and miconazole leads to an increase in the level of deleterious reactive oxygen species, as previously demonstrated by fluorometric and EPR experiments.
S. aureus malian iNOS inhibitor L-NMMA (B) were infected with macrophages activated with IFN-α/H9253 0.05). cate, exhibiting the indicated standard errors (bars). After 30 min of infection, macrophages were lysed and bacterial counts determined. Two independent assays were performed in triplicate, exhibiting the indicated standard errors (*, P < 0.01; *, P < 0.05).

FIG. 5. Intracellular survival in murine macrophages of miconazole-treated S. aureus wild-type (wt) and Δhmp mutant cells. Murine macrophages activated with IFN-γ/LPS (A) and treated with the mammalian iNOS inhibitor L-NMMA (B) were infected with S. aureus wild-type cells in the absence (black bars) and in the presence (diagonal striped bars) of miconazole or incubated with Δhmp cells grown without antibiotic (white bars) or with miconazole (horizontal striped bars). After 30 min of infection, macrophages were lysed and bacterial counts determined. Two independent assays were performed in triplicate, exhibiting the indicated standard errors (*, P < 0.01; *, P < 0.05).

DISCUSSION

In this study, we show that S. aureus is susceptible to several azole antibiotics, with miconazole being, among those tested, the most effective one. The mechanism by which imidazole-treated bacteria undergo growth inhibition was assessed, and the results provide the first evidence that imidazoles induce intracellular ROS production in S. aureus after miconazole and ketoconazole treatment. Remarkably, a strain of S. aureus lacking flavohemoglobin resulted in increased cell viability, while overexpression of flavohemoglobin restored the azole-susceptibility phenotype. These results indicate that flavohemoglobin and imidazoles act together in the killing mechanism.

The binding of miconazole to S. aureus flavohemoglobin was analyzed by UV-visible, EPR, and resonance Raman spectroscopy and revealed that miconazole acts as a strong field heme ligand, since upon miconazole binding the 5-coordinated, high-spin configuration in both oxidation states is converted to a 6-coordinated, low-spin species. Interestingly, the binding of similar-size imidazoles led to similar MIC values, while the larger imidazole ketoconazole exhibits a much higher MIC value. Intriguingly, the two triazoles tested, itraconazole and fluconazole, had no activity against S. aureus. As seen in Fig. 1, this lack of activity cannot solely be related to the size of these molecules since flavohemoglobin is, in fact, the smallest system investigated. Both flavohemazoles and itraconazole are potentazole antifungals, but rather than having an imidazole side chain, they possess 1,2,4-triazoles. These triazoles do bind well to their P450 targets: since in the P450 enzymes there is an axial site, rather than an axial histidine (as in flavohemoglobin), it seems likely there may simply be large differences between imidazoles and triazoles, binding to P450 or flavohemoglobin, due to electronic effects.

Flavohemoglobinins have been reported to have several enzymatic activities, namely, NO dioxygenase (NOD) and alkyl hydroperoxide reductase, as well as production of superoxide (1, 16). We noticed that ligation of imidazoles to S. aureus flavohemoglobin leads to impairment of NOD activity (data not shown), which is in accordance with results described for fungal, yeast, and E. coli flavohemoglobinins (7). However, both the susceptibility of wild-type S. aureus to imidazoles and the increase in the resistance of S. aureus to imidazoles upon deletion of the flavohemoglobin gene occur in the absence of any source of nitric oxide. Hence, we tested if binding of imidazoles could interfere with superoxide generation by flavohemoglobin. In fact, EPR spin trap experiments showed that the binding of miconazole magnifies the superoxide production by S. aureus flavohemoglobin. Since the heme is blocked with miconazole, we concluded that the superoxide was generated at the level of the FAD center. In this mechanism, FAD receives electrons from NAD(P)H and reduces oxygen to superoxide. This hypothesis is supported by the ability of several flavin-containing proteins to generate superoxide upon one-electron oxidation by dioxygen (10). Also, it was previously shown that NAD(P)H is oxidized by flavohemoglobin with the electrons transferred via FAD to external acceptors when the ferrous heme is blocked (28).

Therefore, upon ligation of azoles to flavohemoglobin, the S. aureus cells become exposed to larger amounts of deleterious ROS, which explains the lower survival of antibiotic-treated S. aureus wild-type cells and the higher resistance of the Δhmp mutant strain.

Note that in the simultaneous presence of azoles and NO, the higher level of ROS and the inhibition of the NO scavenging activity of flavohemoglobin both will contribute to the more efficient killing of wild-type S. aureus that was indeed detected in activated macrophages (Fig. 5A). Interestingly, the present results may explain the previously observed synergistic antimicrobial action of imidazole antibiotics and NO releasers exerted on Candida species (15), which can be now rationalized considering that in the presence of the two compounds, Candida flavohemoglobin no longer can act as a fungal protective factor.

Finally, we consider future prospects and possible applications. The observation that some azole antifungals also have antibacterial activity is not new, with Janssen et al. (5) reporting as early as 1969 that miconazole had potent (10 nM) activity against Streptococcus hemolyticus (Streptococcus pyogenes). It is also of interest to note that earlier works reported that miconazole killed S. aureus (23) and that cells grown in the presence of miconazole had decreased levels of vitamin K₂ and increased levels on octaprenyl diphosphate (2). This would be consistent with targeting MenA biosynthesis, which could also potentially contribute to an increase in ROS levels, and indeed we did observe that in the hmp mutant strain there is still ROS production (Fig. 4A): i.e., even in the absence of flavohemoglobin. So, it is possible that the imidazole antibiotics have more than one target in S. aureus and, potentially, in other bacteria as well.

In S. aureus, the buildup of ROS is of particular interest since S. aureus has a protective golden carotenoid “shield” called staphyloxanthin (20) that protects the bacterium from attack by neutrophil-generated ROS (14). Inhibition of carotenoid biosynthesis (13) results in bacteria that are white (since they lack the carotenoid pigment) and are thus cleared by host defenses. Combining azoles with staphyloxanthin biosynthesis inhibitors may enhance intracellular levels of ROS and NO killing by stripping bacteria of their defenses.

In conclusion, this work revealed for the first time that the binding of azoles to S. aureus flavohemoglobin leads to an
increase of the intracellular level of reactive oxygen species, therefore enhancing the antimicrobial activity of these antibi-

tics.

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