Azoles containing naphthalene with activity against Gram-positive bacteria: in vitro studies and in silico predictions for flavohemoglobin inhibition

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ABSTRACT

Azoles are first-line drugs used in fungal infections. Topical antifungals, such as miconazole and econazole, are known to be active against Gram-positive bacteria, which was reported to result from bacterial flavohemoglobin (flavoHb) inhibition. Dual antibacterial/antifungal action is believed to have benefits for antimicrobial chemotherapy. In this study, we tested antibacterial effects of an in-house library of naphthalene-bearing azoles, some of which were reported as potent antifungals, in an attempt to find dual-acting hits. Several potent derivatives were obtained against the Gram-positive bacteria, Enterococcus faecalis and Staphylococcus aureus. 9 was active at a minimum inhibitor concentration (MIC) less than 1 µg/ml against E. faecalis and S. aureus, and 10 against S. aureus. 16 was also potent against E. faecalis and S. aureus (MIC = 1 and 2 µg/ml, respectively). Six more were active against S. aureus with MIC < 4 µg/ml. In vitro cytotoxicity studies showed that the active compounds were safe for healthy cells within their MIC ranges. According to the calculated descriptors, the library was found within the drug-like chemical space and free of pan-assay interference compounds (PAINS). Molecular docking studies suggested that the compounds might be bacterial flavohemoglobin (flavoHb) inhibitors and theazole and naphthalene rings were important pharmacophores, which was further supported by pharmacophore modeling study. As a result, the current study presents several non-toxic azole derivatives with antibacterial effects. In addition to their previously reported antifungal properties, they could set a promising starting point for the future design of dual acting antimicrobials.

Abbreviations: MRSA: methicillin-resistant Staphylococcus aureus; FRSA: fusidic acid-resistant Staphylococcus aureus; flavoHb: flavohemoglobin; CYP51: lanosterol 14α demethylase; PAINS: pan-assay interference compounds; ATCC: American Type Culture Collection; CLSI: Clinical and Laboratory Standards Institute; DMSO: dimethyl sulfoxide; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MIC: minimum inhibitor concentration; hERG: Human ether-à-go-go related gene

1. Introduction

Azoles play a critical role in antifungal chemotherapy in various fungal infections ranging from topical mycoses to drug resistant invasive candidiasis. Azoles have a wide spectrum of efficacy, good tolerability and side effect profile, and oral availability, which make them first line antifungal class (Mourad & Perfect, 2018). Azole antifungals have recently attracted interest with the discovery of their antibacterial effects. Especially, miconazole, econazole, and ketoconazole, which are imidazole derivatives used topically, were reported to display potent activity against a number of Gram-positive bacteria (Boyen et al., 2012; Sud & Feingold, 1982). Miconazole was shown to inhibit methicillin- and fusidic acid-resistant Staphylococcus aureus (MRSA and FRSA) growth (Nenoff et al., 2017). MRSA is a notorious strain accounting for the majority of nosocomial infections in the US with high mortality rates (Dukic et al., 2013). Bringing antifungal and antibacterial effects together may have crucial benefits in certain conditions. Fluconazole was reported to be 100-times more potent against azole-resistant Candida albicans when combined with tetracycline or doxycycline (Azevedo et al., 2015). As a result, antibacterial actions of azoles were investigated extensively and antibacterial azoles were designed in recent studies (Bozbey et al., 2020; Dogan et al., 2017; Karakurt et al., 2001; Khabnadideh et al., 2012).

Topical azoles are known to show antibacterial properties through inhibition of a bacterial heme-containing enzyme called flavohemoglobin (flavoHb) (Helmick et al., 2005). FlavoHb is a nitric oxide dioxygenase that oxidizes nitric oxide (NO•) to nitrate (NO3–) in the presence of oxygen (O2) and NAD(P)H, which is mediated by a heme cofactor. The function of flavohb is essential for bacterial survival by protecting bacteria from nitrosative stress (Frey et al., 2002). In...
vitro and crystallographic studies show that miconazole, econazole, and ketoconazole inhibit microbial flavoHbs through coordinating with the heme co-factor (El Hammi et al., 2011; Frey et al., 2002), a similar mechanism for their antifungal activity: inhibition of lanosterol 14α demethylase (CYP51), which is also a heme-containing oxidase of cytochrome P450 family (Figure 1) (Raucy et al., 1991). The azole ring of azole antifungals is responsible for this coordination, while the family (Figure 1) (Raucy et al., 1991). The azole ring of azole antifungals such as miconazole, econazole, and ketoconazole (Figure 2), which was designed in pursuit of new antifungal and anticonvulsant compounds (Acar et al., 2019; Karakurt et al., 2012; Ozdemir et al., 2019; Sari et al., 2016). The library compounds incorporate a naphthalene and an azole ring (imidazole and 1,2,4-triazole), which are connected via an ethylene linker to which various alky1, aryl, and aroylalkyl substituents are attached through alcohol ester, oxime ester, and oxime ether functionalities. Some of these derivatives were previously reported to possess highly potent anti- Candida properties (Sari et al., 2021). Therefore, finding dual-acting compounds with both antifungal and antibacterial effects was aimed. In order to evaluate safety of the active compounds, in vitro cytotoxicity was performed with healthy cell line. A number of molecular descriptors were calculated to assess druglikeness and the presence of pan-assay interference compounds (PAINS) was checked. The proposed mechanism for miconazole, econazole and ketoconazole, inhibition of bacterial flavoHb, was also tested for our active derivatives in silico through molecular docking and pharmacophore modeling approaches.

2. Methods

2.1. Microdilution test for antibacterial activity

Minimum inhibitor concentration (MIC) values of the compounds were determined against the American Type Culture Collection® (ATCC) strains of Enterococcus faecalis (ATCC 29212), Staphylococcus aureus (ATCC 29213), Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 27853), and MRSA using broth microdilution method in compliance with the Clinical and Laboratory Standards Institute (CLSI) reference documents (CLSI M07-A8; Clinical and Laboratory Standards Institute (CLSI), 2009). Gentamicin, ciprofloxacin, and piperacillin/tazobactam (64–0.0625 μg/ml) were used as positive control. The bacterial strains, which were stored in glycerol at –80 °C, were thawed, solubilized, and subcultured twice to Mueller Hinton agar. Microdilution test was conducted using Mueller Hinton broth (MHB, Difco Laboratories, USA) buffered to pH 7.0 with 3-N-morpholino propane sulfonic acid (MOPS, Sigma, USA). The inoculum densities were prepared from subcultures for 24 h. The final test concentration of the bacteria was 5 x 10^5 cfu/ml. The test compounds were dissolved in dimethyl sulfoxide (DMSO, Sigma, USA) and their final twofold concentrations (1024 to 1 μg/ml) were prepared in microtiter plate wells. The plates were incubated at 35 °C for 18–24 h and the MIC values were read as the lowest concentration (μg/ml) of the test compound in the wells which completely inhibited visual microorganism growth.

2.2. Cytotoxicity test

T3T (mouse fibroblast) cell line purchased from ATCC (Manassas, VA, USA) was preserved in DMEM (Dulbecco’s Modified Eagle’s medium) with 10% fetal calf serum and 0.5% penicillin-streptomycin. The cell line was kept in an incubator under humidified atmosphere of 95% air and 5% CO2 at 37 ± 1 °C. The culture medium was renewed twice a week. T3T cells in the test were between the second and third culture passage after thawing.

Cytotoxicity test was performed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay described by Ohguro et al. (1999) with slight changes (Ohguro et al., 1999). The cell line was grown for two weeks, then seeded at 10,000 cells/well density in 96-well plates and allowed to attach to the surface and grow for 24 h prior to the treatment. The cells were treated with each compound at varying concentrations in the 0.156–5 μg/ml range for 48 h. At the end of the incubation period, the medium in each well was replaced with MTT solution (1 mg/ml) in 100 μl culture medium and the cells were incubated at 37°C for an additional 3 h. Later, 100 μl DMSO was added to the cells to dissolve the formazan crystals and the optic density was measured at 570 nm using a microplate reader (SpectraMax M2, Molecular Devices Limited, Berkshire, UK). Cytotoxicity was determined by calculating the percentage viability of the cells treated with compound with respect to untreated (control) cells. Each experiment was repeated twice.

2.3. Molecular modeling

Molecular docking was performed using three different programs: Glide (2020-2, Schrödinger, LLC, New York, NY) (Friesner et al., 2004; 2006; Halgren et al., 2004), AutoDock (v4.2, The Scripps Research Institute, San Diego, CA) (Morris et al., 2009), and FRED (v3.5.0.4, Open Eye Scientific Software, Santa Fe, NM) (McGann, 2012). The compounds were modeled using LigPrep (2020-2, Schrödinger, LLC, New York, NY) by generating possible enantiomers, ionization and tautomeric states (pH = 7.0 ± 2). The ligands were then minimized using MacroModel (2020-2, Schrödinger, LLC, New York, NY) according to OPLS3e forcefield parameters (2020-2, Schrödinger, LLC, New York, NY) (Harder et al., 2016) and conjugate gradients method. Molecular descriptors were calculated using QikProp (2020-2, Schrödinger, LLC, New York, NY) and the compounds were checked for PAINS via SwissADME web server (www.swissadme.ch) (Daina et al., 2017). For AutoDock and FRED, the ligands were converted to mol2 and sdf file format, respectively. For AutoDock, Gasteiger charges were assigned and the ligands were converted to pdbqt format using AutoDockTools (v1.5.7, The Scripps Research Institute, San Diego, CA). The homology
model of S. aureus flavHb (SAFH) was used as receptor, modeling of which was defined in detail in our previous study (Bozbey et al., 2020). SAFH was prepared for Glide docking using the Protein Preparation Wizard panel of Maestro (2020-2, Schrödinger, LLC, New York, NY) (Sastry et al., 2013). In this process hydrogen atoms, bond orders, and partial charges were assigned, ionization and tautomeric states of the residues were set and H bonds were assigned. This process was performed using AutoDockTools for AutoDock and Make Receptor (v3.5.0.4, Open Eye Scientific Software, Santa Fe, NM) for FRED. Grid maps of the receptor active site (centroid: 11.15, 28.44, 7.41; volume: 13,000 Å³) were prepared using Maestro, AutoGrid, and Make Receptor for Glide, AutoDock, and FRED, respectively. Each ligand was docked 50 times to the receptor using standard precision for Glide, Lamarckian genetic algorithm for AutoDock, and default parameters for FRED. The results were ranked according to docking score, free energy of binding, and FRED Chemgauss4 score from Glide, AutoDock, and FRED, respectively, and each ligand was assigned the score of the best docking pose selected through visual evaluation from each program. To validate the docking methodology, miconazole in the SAFH homology model catalytic site was redocked and the results from each program was compared with its original pose by calculating the root-mean-square deviation (RMSD) values found as 0.88, 0.96, and 0.75 Å for Glide, AutoDock, and FRED, respectively. Six other compounds (2, 25, 27, 28, 30, and 33) had MIC values in 2–4 μg/ml range against S. aureus. On the other hand, 5, 6, and 11 showed promising activity against the Gram-negative strain P. aeruginosa (MIC = 4–8 μg/ml). None of the derivatives showed considerable activity against E. coli. As the general activity profile of the library was promising against S. aureus, we tested the most active three compounds (9, 10, and 16) against MRSA, however they did not obtain noteworthy MIC values (Table 1).

3. Results

3.1. Biological studies

3.1.1. Some of the compounds have significant activity against the Gram-positive bacteria

According to the results presented in Table 1, some of the derivatives obtained low MIC values against the Gram-positive bacteria. Among them, the MIC of 9 was less than 1 μg/ml against E. faecalis and S. aureus, and of 10 against S. aureus. 16 was also potent against E. faecalis and S. aureus (MIC = 1 and 2 μg/ml, respectively). Six other compounds (2, 25, 27, 28, 30, and 33) had MIC values in 2–4 μg/ml range against S. aureus. On the other hand, 5, 6, and 11 showed promising activity against the Gram-negative strain P. aeruginosa (MIC = 4–8 μg/ml). None of the derivatives showed considerable activity against E. coli. As the general activity profile of the library was promising against S. aureus, we tested the most active three compounds (9, 10, and 16) against MRSA, however they did not obtain noteworthy MIC values (Table 1).

3.1.2. The compounds show no significant cytotoxicity

In vitro cytotoxicity tests were performed to evaluate the cytotoxic effects of the most active compounds (9, 10, 16, 25, 27, 28, and 30) on the viability of mouse fibroblast cells at 0.15625 – 5 μg/ml at the end of a 48 h treatment. The fibroblast cells preserved at least 60% viability in the presence of the compounds at up to 5 μg/ml in 48h (Figure 3) (see Supporting information for details).

3.2. Molecular modeling

3.2.1. The library mostly belongs to the druglike chemical space

The compounds were evaluated for druglikeness and ADMET (Absorption, Distribution, Metabolism, Excretion, Toxicity) properties using a set of descriptors. Molecular weight (MW),
number of rotatable bonds (RB), H bond donor and acceptor counts (HD and HA), LogP (octanol/water), and polar surface area (PSA) are considered as relevant molecular descriptors for identifying drug-like chemical space (Lipinski et al., 2001; Veber et al., 2002) and reliability of QikProp for these descriptors was well-established (Ioakimidis et al., 2008). All the active compounds were found within the ideal ranges of drug-like chemical space of all descriptors according to Figure 2.
QikProp, except 30, LogP of which was higher than the upper limit (Table 2). For other descriptors, the compounds were also calculated to have ideal values except predicted hERG channel affinity and skin permeability. Also, 27 and 30 were predicted to have low aqueous solubility. The library was free of PAINS.

### Table 1. MIC values (µg/ml) of the compounds and the reference drugs against the tested bacteria.

| Comp. | E. faecalis | S. aureus | P. aeruginosa | E. coli | MRSA | Comp. | E. faecalis | S. aureus | P. aeruginosa | E. coli | MRSA |
|-------|-------------|-----------|---------------|--------|------|-------|-------------|-----------|---------------|--------|------|
| 1     | 128         | 64        | 128           | 512    | 31   | >1024 | 64          | >1024     | >1024         | >1024  |       |
| 2     | 64          | 4         | 512           | 512    | 32   | 256   | 512         | 1024      | 1024          | 1024   |       |
| 3     | 128         | 32        | 64            | 512    | 33   | 64    | 4           | 512       | 512           | 512    |       |
| 4     | 128         | 256       | 64            | 512    | 34   | 35    | 256         | 256       | 512           | 512    |       |
| 5     | 16          | 8         | 4             | 512    | 35   | 35    | 256         | 256       | 512           | 512    |       |
| 6     | 32          | 64        | 8             | 256    | 36   | 128   | 32          | 1024      | 1024          | 1024   |       |
| 7     | 32          | 32        | 16            | 64     | 37   | 64    | 64          | 256       | 256           | 512    |       |
| 8     | 8           | 32        | 64            | 512    | 38   | 32    | 16          | 256       | 256           | 512    |       |
| 9     | <1          | <1        | 512           | 1024   | 39   | 32    | 32          | 256       | 256           | 512    |       |
| 10    | <1          | 2         | 512           | 32      | 40   | 256   | 8           | 512       | 512           | 512    |       |
| 11    | 8           | 8         | 8             | 512    | 41   | 64    | 8           | 256       | 256           | 512    |       |
| 12    | 256         | 512       | 128           | 512    | 42   | 64    | 256         | 256       | >1024         |       |       |
| 13    | 64          | 32        | 64            | 512    | 43   | 128   | 32          | 512       | 512           | 512    |       |
| 14    | 128         | 512       | 512           | 512    | 44   | 512   | 512         | 512       | 512           | 512    |       |
| 15    | 1024        | 512       | 1024          | 512    | 45   | 256   | 512         | 256       | 512           | 512    |       |
| 16    | 1           | 2         | 512           | 512    | 46   | 512   | 512         | 512       | 512           | 512    |       |
| 17    | 1024        | 512       | 1024          | >1024   | 47   | 512   | 256         | 256       | 256           | 512    |       |
| 18    | >1024       | 16        | >1024         | >1024   | 48   | 1024  | 128         | 256       | 256           | 512    |       |
| 19    | 128         | 32        | >1024         | >1024   | 49   | 512   | 256         | 512       | 512           | 512    |       |
| 20    | >1024       | 8         | >1024         | >1024   | 50   | 512   | 512         | 256       | 256           | 512    |       |
| 21    | 128         | 32        | >1024         | >1024   | 51   | 256   | 256         | 256       | 512           | 512    |       |
| 22    | 128         | 32        | >1024         | >1024   | 52   | 256   | 1024        | 256       | 256           | 512    |       |
| 23    | 128         | 16        | >1024         | >1024   | 53   | 256   | 256         | 256       | 512           | 512    |       |
| 24    | 1024        | 512       | 1024          | >1024   | 54   | 512   | 512         | 1024      | 512           | 512    |       |
| 25    | 64          | 4         | >1024         | >1024   | 55   | 512   | 512         | 256       | 256           | 512    |       |
| 26    | 1024        | 8         | >1024         | >1024   | 56   | 1024  | 512         | >1024     | 256           |       |       |
| 27    | >1024       | 2         | >1024         | >1024   | 57   | 16    | 0.125       | 0.5       | 0.5           |       |       |
| 28    | >1024       | 4         | >1024         | >1024   | 58   | 0.125 | 0.125       | 0.5       | 0.0625        |       |       |
| 29    | 1024        | 16        | >1024         | >1024   | 59   | 0.625 | 0.312       | 0.625     | –             |       |       |
| 30    | 1024        | 2         | >1024         | 1024    | 60   |       |             |           |               |       |       |

Figure 3. % viability of mouse fibroblast cells in the presence of 9, 10, 16, 25, 27, 28, and 30 in 48 h.

3.2.2. The active compounds show high affinity to flavoHb with the imidazole interacting with the heme

The flavoHb catalytic site is comprised of three regions: a heme co-factor site, a deep lipophilic pocket, and a gorge that opens to the heme site and deep pocket. Miconazole interacts with the heme cofactor through imidazole. The (2,4-
Table 2. Calculated molecular descriptors of 9, 10, 16, 25, 27, 28, and 30.

| Descriptors* | 9     | 10    | 16    | 25    | 27    | 28    | 30    |
|--------------|-------|-------|-------|-------|-------|-------|-------|
| MW (130–725 Da) | 321.4 | 335.4 | 333.4 | 348.4 | 384.5 | 368.4 | 432.5 |
| RB (0–15)    | 8     | 10    | 6     | 5     | 8     | 7     | 7     |
| HD (0–6)     | 0     | 0     | 0     | 0     | 0     | 0     | 0     |
| HA (2–20)    | 4.7   | 4.7   | 4.7   | 4.7   | 4.7   | 4.7   | 4.7   |
| LogP for octanol/water (–2 to 6.5) | 4.6   | 5.1   | 4.6   | 4.6   | 5.1   | 6.0   | 5.6   |
| PSA (7–20 Å²) | 33.6  | 35.5  | 33.7  | 46.6  | 48.8  | 50.0  | 45.0  |
| Dipole moment (1.0–12.5) | 4.8   | 4.8   | 4.6   | 5.1   | 5.1   | 2.5   | 7.2   |
| Total SASA (300.0–1000.0 Å²) | 657.2 | 696.4 | 673.6 | 660.5 | 723.5 | 697.2 | 745.8 |
| Hydrophobic SASA (0.0–750.0 Å²) | 262.6 | 302.0 | 286.3 | 243.2 | 137.6 | 68.5  | 38.4  |
| Hydrophilic SASA (2.0–330.0 Å²) | 44.2  | 43.9  | 45.5  | 65.2  | 69.8  | 71.5  | 35.6  |
| Carbon n SASA (0.0–450.0 Å²) | 350.4 | 350.5 | 343.8 | 352.0 | 516.0 | 557.2 | 671.9 |
| Weakly polar SASA (0.0–175.0 Å²) | 0     | 0     | 0     | 0     | 0     | 0     | 0     |
| Molecular volume (500.0–2000.0 Å³) | 1151.4| 1217.9| 1201.0| 1191.4| 1309.3| 1237.3| 1386.4|
| Globalizability (0.75–0.95) | 0.81  | 0.79  | 0.81  | 0.82  | 0.80  | 0.80  | 0.81  |
| Polarizability (13.0–70.0 Å³) | 38.1  | 39.5  | 40.7  | 41.7  | 46.0  | 44.2  | 51.3  |
| LogP for hexadecane/gas (4.0–18.0) | 11.7  | 12.4  | 12.1  | 12.2  | 14.6  | 14.2  | 16.1  |
| LogP for octanol/gas (8.0–35.0) | 14.9  | 15.2  | 15.8  | 16.1  | 17.5  | 17.4  | 19.8  |
| LogP for water/gas (4.0–45.0) | 6.8   | 6.5   | 7.1   | 7.0   | 7.6   | 8.1   | 8.7   |
| LogS (–6.5–0.5 mol dm⁻³) | –4.9  | –5.3  | –5.4  | –5.8  | –6.5  | –6.2  | –7.1  |
| LogS conformation independent (–6.5–0.5 mol dm⁻³) | –4.6  | –4.9  | –5.1  | –5.5  | –6.6  | –6.3  | –8.0  |
| LoghERG<sup>+</sup> (–5) | –6.3  | –6.6  | –6.2  | –6.1  | –7.2  | –7.4  | –7.9  |
| PCaco<sup>+</sup> (–25 mm/sec) | 3777.9| 3797.5| 3834.4| 2383.7| 2156.2| 2081.3| 4553.1|
| LogBBf (–3.0 to 1.2) | –0.37 | –0.51 | –0.30 | –0.38 | –0.65 | –0.60 | –0.22 |
| PMDCK<sup>+</sup> (–25 mm/sec) | 2078.7| 2092.8| 2114.7| 1265.1| 1135.1| 1092.5| 2546.3|
| LogK<sub>H</sub> (–8.0 to –1.0) | –0.3  | –0.1  | –0.4  | –1.0  | –0.2  | –0.2  | 0.9   |
| IP<sup>+</sup> (7.9–10.5 ev) | 8.8   | 8.8   | 8.8   | 8.9   | 8.8   | 8.8   | 8.9   |
| EA<sup>+</sup> (–0.9–1.7 ev) | 1.0   | 1.0   | 1.0   | 0.9   | 0.9   | 1.0   | 1.0   |
| #metabol<sup>+</sup> (1–8) | 1     | 1     | 1     | 2     | 3     | 4     | 4     |
| LogK<sub>inh</sub> (–1.5 to 1.5) | 0.4   | 0.5   | 0.6   | 0.8   | 1.0   | 0.8   | 1.3   |
| Oral absorption (>25%) | 100   | 100   | 100   | 100   | 100   | 100   | 100   |
| Rule of five<sup>+</sup> | 0     | 1     | 0     | 1     | 1     | 1     | 1     |
| Rule of three<sup>+</sup> | 0     | 0     | 0     | 0     | 0     | 0     | 0     |

<sup>+</sup> Ideal values are provided in parentheses, outliers are highlighted as bold.

<sup>*</sup>Solvent accessible surface area.

<sup>1</sup>Predicted aqueous solubility.

<sup>2</sup>Predicted IC<sub>50</sub> value for blockage of hERG K<sup>+</sup> channels.

<sup>3</sup>Predicted apparent Caco-2 cell permeability.

<sup>4</sup>Predicted brain/blood partition coefficient.

<sup>5</sup>Predicted apparent MDCK cell permeability.

<sup>6</sup>Predicted skin permeability.

<sup>7</sup>Calculated ionization potential.

<sup>8</sup>Calculated electron affinity.

<sup>9</sup>Number of likely metabolic reactions.

<sup>10</sup>Prediction of binding to human serum albumin.

<sup>11</sup>Number of violations of Lipinski’s rule of five (Lipinski et al., 2001).

<sup>12</sup>Number of violations of Jorgensen’s rule of three.

dichlorophenyl)methoxy group engages with the deep lipophilic pocket including residues Leu17, Val61, Leu102, Ile106, Trp122, Ala125, Tyr126, and Ile129. The 2,4-dichlorophenyl of miconazole interacts with the entrance gorge residues (Thr25, Phe28, Tyr29, Ala56, Leu57, and Ala60) as well as heme. Compounds with low MIC values against S. aureus were predicted to bind to the active site similar as miconazole in the homology model: the azole ring engaged with the heme, the naphthalene fit in the lipophilic pocket, and the tail group shifted places. However, there were few exceptions: in the binding mode of 10 and 27 from FRED and 30 from Glide and AutoDock, the naphthalene and the tail groups shifted places.

Accordingly, the residues included in the binding of the potent derivatives were similar (Figure 5). The imidazole interacted with heme while the nitrogen at the 3<sup>rd</sup> position was in close contact with the heme iron. Naphthalene was also observed to be in strong interactions with heme in the case of 27 and 30, which showed exceptional binding as discussed above. Ala60, Val61, Ala64, Trp122, Ala125, Tyr126, and Ile129 were the most common residues of the lipophilic pocket making hydrophobic contacts mostly with naphthalene. Ile24, Phe28, Tyr29, Phe43, and Ala56 were the residues lining the entry gorge and generally made hydrophobic contacts with tail part of the compounds. Docking scores of the compounds were close to those of miconazole showing that these compounds may bind to SAFH catalytic site with good affinity (Table 3).

### 3.2.3. The pharmacophore model of the active compounds supports molecular docking results

Out of the active derivatives, a pharmacophore hypothesis was created, which comprised of an acceptor (A), a hydrophobic (H), and three ring (R) pharmacophores (Figure 6(A)). Two of the rings correspond to the naphthalene, the third ring to the imidazole, the acceptor to the oxygen of the ether/carboxylic acid that attaches the tail to the ethylene linker, which corresponds to the hydrophobic feature (Figure 6(B)–(F)). The azole and naphthalene rings were found to engage in key interactions with the receptor in docking.
studies (Figures 4 and 5). The BEDROCK and Phase Hypo score of the model was 1.00 and 1.30, respectively.

4. Discussion

Incorporating antibacterial and antifungal effects in a single entity has been suggested highly beneficial in antimicrobial chemotherapy, thus, in an attempt to achieve antibacterial hits out of a library with antifungal properties, we screened an in-house library of naphthalene analogues of azoles. We found several derivatives with low MIC values, especially against the Gram-positive bacteria just like the topical anti-fungals like miconazole, econazole, and ketoconazole. *S. aureus* was more susceptible to our compounds than the other

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**Figure 4.** Binding mode of 9 (gray), 10 (orange), 16 (yellow), 27 (magenta), and 30 (teal) in SAFH catalytic site obtained from Glide (A–E), AutoDock (F–J), and FRED (K–O). Ligands are represented as color stick and balls, heme as green sticks, iron as orange sphere, and the amino acids as molecular surface rendered in color according to electrostatic potentials.

**Figure 5.** Binding interactions of 9 (A, F, K), 10 (B, G, L), 16 (C, H, M), 27 (D, I, N), and 30 (E, J, O) with SAFH catalytic site residues obtained from Glide (A–E), AutoDock (F–J), and FRED (K–O).
species. 9, 10, 16, 2, 25, 27, 28, 30, and 33 were the compounds noteworthy for their antibacterial activities. Interestingly, 5 was an exception and a rare case with a MIC of 4 \( \mu \)g/ml against \( P. \) aeruginosa, a gram-negative bacterium, which bears hope for azole design against gram-negative bacteria, as well. Some of these compounds were previously reported for their antifungal effects against \( C. \) parapsilosis and \( C. \) krusei and the MIC of 28 was 1 \( \mu \)g/ml against \( C. \) parapsilosis, reportedly (Sari et al., 2021), which is promising for design of dual acting antifungal/antibacterial agents.

The library compounds included imidazole and 1,2,4-triazole as azole ring, naphthalene as aromatic group and various lipophilic structures in the tail attached to the ethylene linker between the azole ring and naphthalene via oxime ether, alcohol ester, or oxime ester functionalities. The active compounds were all imidazole derivatives. This finding is in line with the fact that all the reported azole drugs with antibacterial activity are imidazole derivatives. Those with aliphatic and alicyclic groups in the tail were more potent than those with aromatic groups. Derivatives with oxime ether and alcohol ester functional groups were markedly more potent than those with oxime ester.

In addition to antimicrobial efficacy, ideal antimicrobial chemotherapeutics are expected to be tolerable for the host by showing selective action towards pathogens over host cells. In cytotoxicity studies no significant dose-dependent change was observed in % viability of the compound-treated cells. The best MIC values of the tested compounds were within 1–4 \( \mu \)g/ml range; thus, these results suggest that the compounds are expected to be safe for host tissues and act selectively against pathogens at their active concentrations.

Toxicity and poor pharmacokinetic properties play critical role in high attrition rates in late-stage drug development. Also, compounds with nonspecifically reactive functional groups, i.e. PAINS, hamper late drug discovery efforts with false positives in biological assays (Baell & Walters, 2014). To reduce the loss of time and resources due to attritions in clinical trials, ADMET issues are addressed in early stages by various measures including \textit{in silico} predictions (Wang, 2009). Thus, the library was evaluated for druglikeness and PAINS, found free of PAINS and mostly complied with the druglikeness criteria except for a couple derivatives with high LogP values, high hERG affinity, low skin permeability, and low aqueous solubility. LogP value indicates lipophilicity and high lipophilicity, among a number of pharmacokinetic problems, may result in poor aqueous solubility (Freeman-Cook et al., 2013).

In order to evaluate the hypothesis that our compounds could act through inhibition of bacterial flavoHb, we performed molecular docking with \( S. \) aureus flavoHb (SAFH) homology model using three different programs. The active compounds interacted with SAFH similar as miconazole with high affinity (see Supporting information). The binding mode was also similar as that of antifungal azoles with fungal CYP51s. The azole and naphthalene rings played key roles in this binding mode, which was further supported by the pharmacophore modeling analyses. These results suggest that flavoHb inhibition could be a possible mechanism for their antibacterial effects.

**Table 3.** Docking scores (kcal/mol) of 9, 10, 16, 27, 30, and miconazole.

| Software    | 9   | 10  | 16  | 27  | 30  | Miconazole |
|-------------|-----|-----|-----|-----|-----|------------|
| Glide       | -6.0| -6.3| -6.3| -7.0| -8.2| -6.4       |
| AutoDock    | -8.4| -8.3| -9.5| -6.8| -9.1| -8.2       |
| FRED        | -11.7| -9.4| -14.2| -12.3| -9.1| -14.0     |

**Figure 6.** The features of the pharmacophore model (A) and alignment of 9 (B), 10 (C), 16 (D), 27 (E), and 30 (F) with the model itself.
5. Conclusions

Aiming to find dual-acting antimicrobial hits, we tested antibacterial effects of an in-house library of naphthalene-bearing azoles, some of which were reported as potent antifungals. Some of the title compounds were potent against the Gram-positive bacteria, E. faecalis and S. aureus. 9 was active at a MIC less than 1 µg/ml against E. faecalis and S. aureus, and 10 against S. aureus. 16 was also potent against E. faecalis and S. aureus (MIC = 1 and 2 µg/ml, respectively). The active compounds were found to have minor cytotoxicity against healthy cells within their MIC ranges. Molecular modeling studies showed that our library was within the drug-like chemical space and free of PAINS. The active compounds might be bacterial flavoHb inhibitors owing to their azole and naphthalene rings, according to the molecular docking and pharmacophore modeling study. As a result, our study presentsazole derivatives with naphthalene could be a promising field to design and develop new entities with both antifungal and antibacterial effects, which could have a major impact on applications of antimicrobial chemotherapy and molecular modeling may act as a powerful tool for this purpose. The future studies will focus on antibacterial tests against different bacterial species to establish the antibacterial spectrum of the compounds, as well as to identify their bacterial flavoHb inhibitory and pharmacokinetic profiles.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability

Additional data is provided in the Supporting information file.

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