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New antibacterial agents: Hybrid bioisoster derivatives as potential *E. coli* FabH inhibitors

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ABSTRACT

The development of resistance to antibiotics by microorganisms is a major problem for the treatment of bacterial infections worldwide, and therefore, it is imperative to study new scaffolds that are potentially useful in the development of new antibiotics. In this regard, we propose the design, synthesis and biological evaluation of hybrid sulfonylhydrazone bioisosters/furoxans with potential antibacterial (*Escherichia coli*) activity. The most active compound of the series, (*E*)-3-methyl-4-((2-tosylhydrazono)methyl)-1,2,5-oxadiazole 2-oxide, with a MIC = 0.36 μM, was not cytotoxic when tested on Vero cells (IC_{50} >100 μM). To complement the in vitro screening, we also studied the interaction of the test compounds with β-ketoacyl acyl carrier protein synthase (FabH), the target for the parent compounds, and we observed three important hydrogen-bonding interactions with two important active site residues in the catalytic site of the enzyme, providing complementary evidence to support the target of the new hybrid molecules.

Bacterial infections are the subject of recent concerns in clinic and represent a potential life-threatening problem worldwide, mainly because of increasing resistance to existing antibiotics.[1] Microbial resistance to antibacterial and antifungal drugs has reached critical levels, to the extent that it actually interferes with the potency and efficacy of antibiotics.[2] Consequently, the increasing threat to global public health requires extensive research efforts to develop new antimicrobial compounds.[3] To circumvent drug resistance, new antimicrobials based on new targets (different mechanisms of action) are urgently needed. In this regard, an attractive target for drug discovery is the fatty acid synthesis (FAS) because of its critical role in bacterial metabolism and survival. Additionally, the constitutive differences between human and bacterial FAS[4,5] would allow significant drug selectivity.

β-Ketoacyl acyl carrier protein synthase (FabH) is one of the key enzymes in bacterial type II FAS. This enzyme catalyzes the first step of fatty acid synthesis by condensing malonyl-ACP with either 2-methylbutyryl-CoA, for branched-chain *anteiso*-fatty acid synthesis, or acetyl-CoA, for straight-chain fatty acid synthesis, resulting in β-ketoacyl-ACP (Fig. 1). Considering that FabH is essential for bacterial viability,[6,7] FabH small molecule inhibitors are interesting and promising candidates for the development of more selective and less toxic antibacterial agents.

Many different compounds with good antibacterial profiles and capable of selectively inhibiting FabH have been described in the literature.[8–13] These compounds have been obtained through structure-based drug design (SBDD) or by high-throughput screening (HTS) of natural products and small libraries of molecules (Fig. 2). It is worth mentioning that structures bearing a Schiff base or a hydrazone moiety have been reported as FabH inhibitors, with activity against different types of bacteria; nevertheless, some of the functional groups present in these molecules are metabolically unstable, hindering their potential as drug candidates.[14] Wang and collaborators[11] reported the synthesis and antimicrobial activity of vanillic acylhydrazone derivatives on Gram-negative strains, including *Escherichia coli*. The most active compound of their series exhibited potent *E. coli* activity by inhibiting the catalytic activity of FabH. Additionally, Song et al.[12] also described cinnamaldehyde acylhydrazone derivatives as a novel class of effective *E. coli* FabH inhibitors, suggesting the relevance of this group to exert interactions in the receptor binding site (Fig. 2E).

The description of crystal structures of the FabH enzyme, complexed with different substrates and inhibitors, has provided

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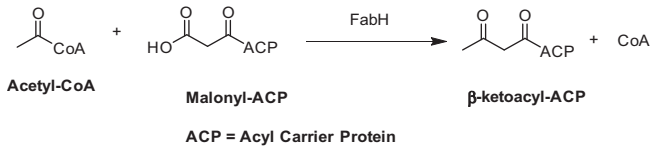


Figure 1. Condensation step in bacterial type II FAS system catalyzed by FabH.

support for the optimization of new lead compounds. Daines and co-workers[13] described the essential elements for the recognition of *E. coli* FabH at the molecular level, through the co-crystallization of FabH with small molecule inhibitors. They highlighted two important features: (I) a hydrophobic bulky group that fits in a complementary hydrophobic region within the active site; and (II) the presence of acidic groups to form an electrostatic interaction with arginine residues in the upper side of the FabH active site.

Based on the structural features described above, and our previous experience designing hybrid bioisosters of sulfonylhydrazone/-furoxan groups[15] (Fig. 3), we report the addition of a complementary binding component to improve the potency of FabH inhibitors by increasing ionic interactions as follows. Sulfonylhydrazones have been reported to improve the biological activity of new antibiotics.[16] We propose a non-classical carbonyl-sulfone bioisosteric replacement in the lead molecule (compound **B**), expecting increased potency and lower toxicity. With this bioisosteric replacement, the number of available hydrogen bonding interactions is likely to increase.

Previously, Reynolds et al.[17] successfully designed new sulfonyl-naphthalene-1,4-diol analogues with potent *E. coli* FabH inhibitory activity. They suggested that the sulfone group is an important group to exert binding interactions directly correlated to the antimicrobial effect. Furthermore, the furoxan group (1,2,5-oxadiazole *N*-oxide) is a useful group in Medicinal Chemistry (due to its ionic nature), and this group has been reported in many active compounds.[18] The furoxan ring has a negative charge (it is an electron rich system), capable of producing ionic interactions with receptors and enzyme binding sites.

Based on the foregoing considerations, in this work we describe the synthesis, antibacterial evaluation, cytotoxicity assay and molecular modeling of a new series of potential FabH inhibitors. It is worth mentioning that although non-resistant, the strains used herein are very pathogenic and have been employed as a preliminary test. In addition, the test, which has been performed,

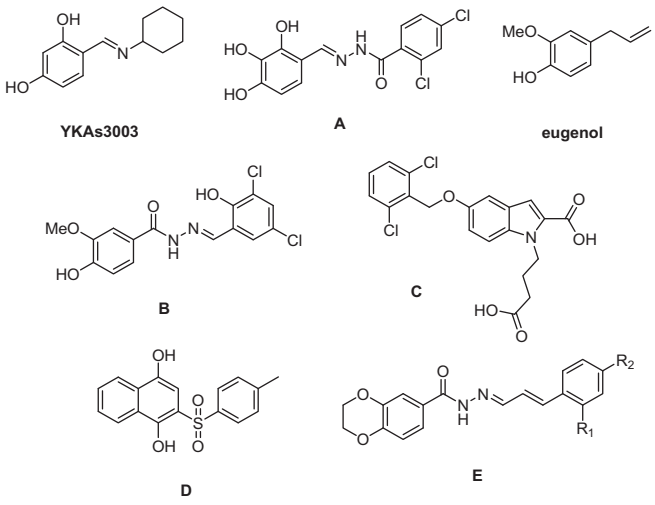


Figure 2. Potent FabH inhibitors and antibacterial compounds described in the literature.[8–13]

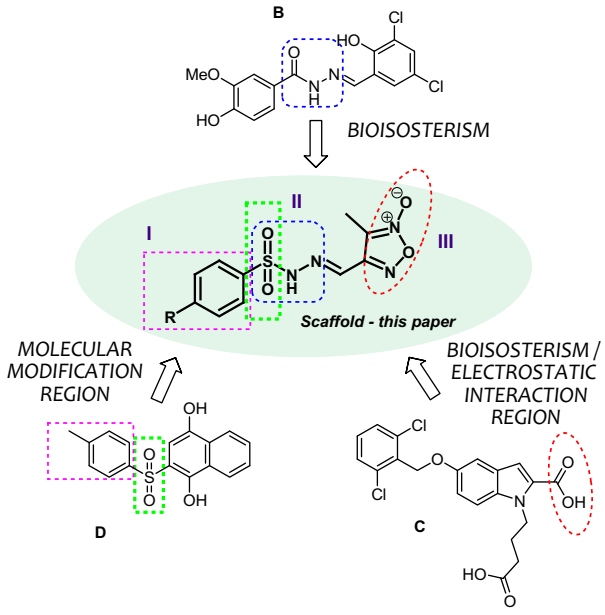


Figure 3. Strategy used in the design of FabH inhibitors.

points out the selectivity in *E. coli* and the low cytotoxicity of the compounds evaluated.

The test compounds were divided using three structural regions, namely region I, II and III, based on the prototype structures **B**, **D** and **C** (Fig. 3). The most active compounds in the series showed: (I) a substituted aromatic ring, (II) a sulfonylhydrazone group and (III) a furoxan group (electrostatic interaction region). Additionally, the applied strategy was based on the alignment of prototype B and the proposed scaffold, obtained through Computer Aided Drug Design (CADD) (Supplementary information).

Five (**1**–**5**) furoxan-containing compounds were synthesized in a simple two step-route (Fig. 4): (a) cyclization of crotonaldehyde to generate the furoxan ring (**VII**); and (b) synthesis of the final compounds from Schiffs bases (**1**–**5**). Yields of the last step were considered good, ranging from 72% to 87%. All final compounds were characterized by melting point, TLC, IR, ^{1}H NMR, ^{13}C NMR, HRMS and elemental analysis (spectra are available in the Supplementary information).

Derivatives **1**–**5** were tested against *E. coli* (ATCC 25922) by broth microdilution method.[19–22] The minimum inhibitory concentration (MIC) values for all compounds are presented in Table 1. Ampicillin and gentamicin were used as standard drugs in microbiological assays, and rifampicin was used as standard for cytotoxicity study.

The furoxan/sulfonylhydrazone hybrid seems to be an interesting scaffold to increase antibacterial activity against *E. coli*. For example, compound **1** showed a MIC around 10 μM, close to that of ampicillin. The introduction of a methyl group in the *para* position (compound **5**) yielded the most potent derivative with a MIC value of 0.36 μM, lower than that observed for the reference drug ampicillin (MIC = 9.59). It is noteworthy that methyl groups can drastically change the potency and pharmacological effect of molecules, modifying properties related to solubility, conformation, electronic factors, bioavailability and phamacokinetics.[23]

However, groups other than methyl in the benzene ring *para* position strongly reduced the activity (Table 1). The presence of a nitro group (compound **3**) decreased potency leading to a loss of biological activity. This is not consistent with previous reports where this group is able to interfere with redox systems of different microorganisms,[24] and it is considered essential for the

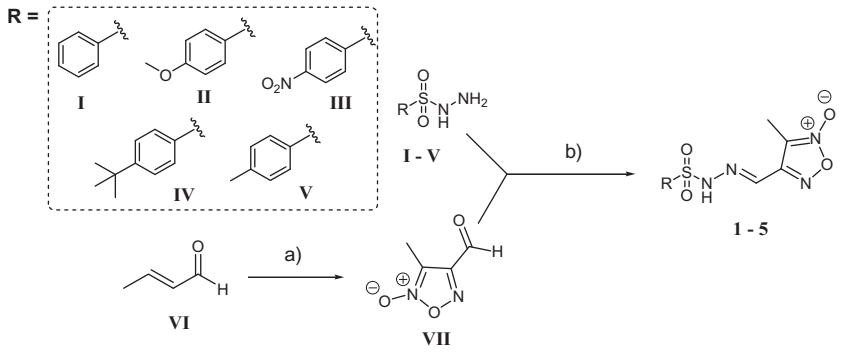
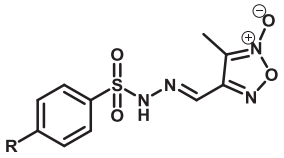


Figure 4. Synthetic route for the synthesis of furoxan-containing inhibitors. Reaction conditions: (a) $NaNO_2$(aq), AcOH/14 °C, 1 h; (b) CH_3OH, H^+/reflux.

Table 1
In vitro antibacterial activity and cytotoxicity of furoxan/sulfonylhydrazone derivatives



Compound	R	MW[d]	*c*Log *D*[d]	*E. coli* MIC[a] (μM)	Cytotoxicity IC_{50}[b] (μM)	Inhibition at highest conc[c] (%)
1	H	282.27	0.23	10.72 ± 0.9	>100	63.44
2	OCH_3	312.30	-0.02	77.83 ± 1.7	>100	0.00
3	NO_2	327.27	0.18	>300 ± 0.0	>100	0.00
4	*t*-Butyl	338.38	1.86	286.91 ± 0.4	>100	74.78
5	CH_3	296.30	0.69	0.36 ± 0.3	>100	8.38
Ampicillin	—	—	—	9.59 ± 0.09	—	—
Gentamicin	—	—	—	<0.02 ± 0.01	—	—
Rifampicin	—	—	—	—	>100	44.78

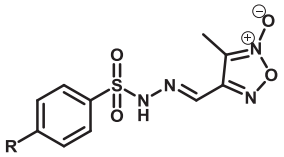
[a] Results were determined in duplicate by the broth microdilution method described in the Supplementary data.
[b] IC_{50} values were determined by the MTS assay using Vero cells as described in the Supplementary data.
[c] Percentage of Vero cells growth inhibition at the highest concentration of the compound.
[d] *c*Log *D* and molecular weight values were calculated using the MarvinSketch software.

antimicrobial activity in some cases.[15,25] Notwithstanding, methoxyl and *t*-butyl substitutions on the aromatic ring also led to a drastic decrease in antibacterial activity of compounds **2** and **4**. This suggests that the volume of the test molecules is an essential factor to exert binding interactions, and the steric effects influence more the biological activity of the compounds than the electronic effect.

A major concern regarding the design of antimicrobial drugs is the lack of selectivity, where some antibiotics cause toxicity to human cells.[26] Thus, we examined the potential cytotoxicity exerted by the synthesized compounds on Vero cells, using the MTS assay (Table 1).[20,27] We observed that all the test compounds were devoid of significant cytotoxicity (IC_{50} values higher than 100 μM). Besides, the percent inhibition of Vero cell proliferation at the highest test compound concentration (drug **5**) was about five times lower than that exerted by rifampicin (the reference drug), with values = 8.3% and 44.8%, respectively. The effective antibacterial activity and the low toxicity of this compound suggest that it may be a promising antibacterial compound for further modification.

To evaluate the selectivity of the compounds against *E. coli*, we carried out an additional screening assay against another representative Gram-positive bacteria, namely *Staphylococcus aureus*, and a representative yeast, *Candida albicans* (Table 2), as performed previously.[28] As expected, the test compounds displayed no activity at 100 μM against these microorganisms.

Table 2
In vitro activity of furoxan/sulfonylhydrazone derivatives against *S. aureus* and *Candida albicans*



Compound	R	*S. aureus* MIC[a] (μM)	*Candida albicans* MIC[a] (μM)
1	H	>100	>100
2	OCH_3	>100	>100
3	NO_2	>100	>100
4	*t*-Butyl	>100	>100
5	CH_3	>100	>100
Ampicillin	—	1.10	nd[b]
Gentamicin	—	0.076	nd[b]
Amphotericin B	—	nd[b]	0.027

[a] Results were determined by the broth microdilution method described in the Supplementary data.
[b] No MIC values were determined.

Since furoxan rings can decompose in biological media releasing nitric oxide, we evaluated formation of the nitric oxide product nitrite from the synthesized compounds.[29] The compounds

(100 μM) were incubated with L-cysteine (5 mM) in a mixture of 50 mM phosphate buffer, pH 7.4 and methanol (1:1) for 1 h at 37 °C. Aliquots were removed and nitrite formation was measured by the Griess assay. Despite positive control experiments showing nitrite production, no nitrite was detectable (⩽1 μM) from the synthesized compounds (Supporting information). Although this does not eliminate nitric oxide involvement in the anti *E. coli* action, it suggests that other targets and mechanisms can be envisioned.

The search for biological targets with experimentally validated bioactivities is a challenge because in vivo effects may result from multiple aspects. The availability of a wide number of 3D protein structures has simplified the search of drug targets.[30] Therefore, based on a previous study in which Schiff bases and acylhydrazones were identified as *E. coli* FabH inhibitors, through pharmacophore-based in silico approaches and bioisosterism,[6] we suggest that the FabH enzyme is likely to be the target of our test molecules. Thus, we conducted a series of molecular docking experiments to obtain detailed information at the molecular level, related to the binding mode exerted by compounds **1**–**5** in the FabH active site.

To increase the accuracy of the docking protocol for this particular system, we also carried out a redocking validation test using the AutoDock Vina program, using four complexes selected from the PDB, namely 1HNJ, 1HND, 1NHN and 1MZS, after a BLAST alignment sequence search. This set of complexes was used with the purpose of exploring, as much as possible, all characteristics defined in the literature for substrate binding to the FabH active site. Once validated (Supplementary information), the protocol with AutoDock Vina provided insights about the conformation and possible binding orientations for all ligands, complexed with the choosing 1HNJ crystal structure.

Nevertheless, it was difficult to choose the best docking solutions (conformations) because the results are broadly sensitive to the score function. The limitations of scoring functions producing data that correlate with activity are well-known.[31] Therefore, the docking solutions of the synthesized compounds to FabH were carefully analyzed. As the main inclusion criteria, we selected the lowest docking energy considering the fitting conformation of the ligands in the surface area within the receptor as well as the interaction between the ligands and FabH active site. The docking simulation results with the tested compounds in the binding site of FabH are summarized in Table 3.

All compounds showed comparable values of energy and good interaction profile with *E. coli* FabH. The most active compound, compound **5** (Table 3), showed the lowest interaction energy value of the series, indicating a better ligand-binding mode with FabH (Fig. 5A).

Interestingly, the three less potent sulfonylhydrazone derivatives presented similar interaction profiles at the FabH active site, which are different than that for compounds **1** and **5** the most active compounds (Fig. 5B). This observation suggests that the active conformation of the drug is important to the ligand–protein binding. However, in all cases, the aromatic group is filling the complementary hydrophobic region within the active site tunnel, and the furoxan group is facing the arginine residues at the protein surface surrounding this tunnel. These data are in agreement with the initial drug design approach proposed at the beginning of this work, that was based on the key binding features of *E. coli* FabH. Additionally, these results suggest that all compounds bind in the active site of FabH to different extents and differently, which may explain the differences in their antimicrobial potency.[13]

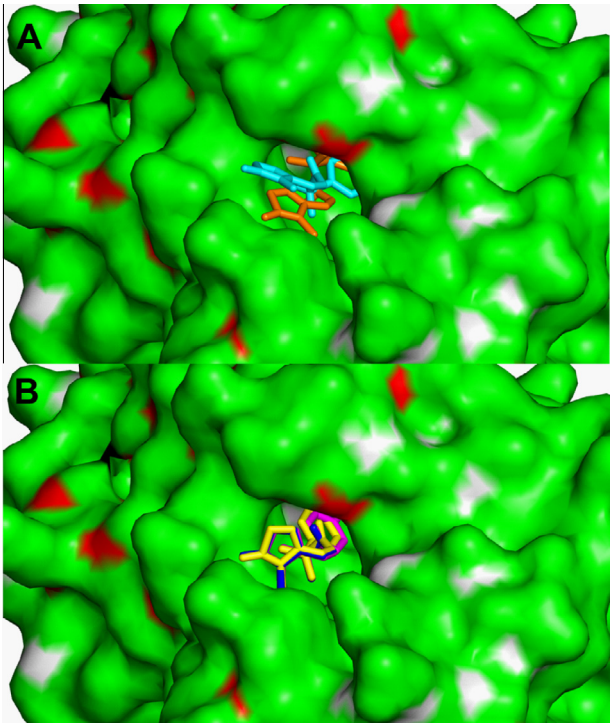


Figure 5. Representation of the compound binding modes in 1HNJ structure. (A) Orientation profile of the best solutions obtained by AutoDock Vina software for compound **1** (Orange) and compound **5** (Cyan). (B) Orientation profile of the best solutions for compound **2** (Blue), compound **3** (Yellow) and compound **4** (Magenta).

To achieve a better understanding of the interactions at molecular level, we selected 10 residues located in the FabH binding site, including the catalytic triad, and evaluated the interatomic distances using the LPC/CSU server (Supplementary information).[11–13] The results showed several putative interactions between all compounds and the enzyme-binding site. Those interactions include hydrogen bonding, π–π stacking (aromatic interactions), hydrophobic and van der Waals bonding, according to the classification of ligand–protein contacts by the server.[32] In general, all ligands interact with at least one arginine residue at protein surface and the active site tunnel without affecting the catalytic triad (Cys112, His244 and Asn274).

Analyzing the results obtained for compound **5**/*E. coli* FabH complex it was possible to identify three important hydrogen-bonding interactions with two important active site residues. (Fig. 6A): compound **5**-O and Asn247-NH_2 (O–N: d = 3.3 Å); compound **5**-N and Gly209-O (N–O: d = 3.5 Å), and compound **5**-N and Gly209-O (N–O: d = 3.4 Å). Comparatively, compound **3** interact nicely to FabH through just two hydrogen bonds. (Fig. 6B): compound **3**-O and Asn247-NH_2 (O–N: d = 3.3 Å) and compound **3**-O and Arg36-NH_2 (O–N: d = 3.3 Å). These differences are likely to contribute to the higher binding energy found for compound **3** as compared with compound **5**. Additionally, they may reproduce the poor biological activity since the nitro group is facing the

Table 3
Molecular docking results obtained for each simulation performed using AutoDock Vina between all compounds and 1HNJ, number of solution or positions, lowest energy value of the best solutions and individual molecular properties

Compound	N° of solutions	Lowest energy solution (kcal/mol)	PSA	VDW	*c*Log*D*
1	9	−6.5	111.50	359.0	0.23
2	9	−6.4	120.73	406.9	−0.02
3	9	−6.6	157.32	399.7	0.18
4	9	−6.6	111.50	487.0	1.86
5	9	−6.7	111.50	391.2	0.69

N° of solution: Number of solutions obtained using AutoDock Vina. PSA: polar surface area. VDW: Van der Waals area. *c*Log*D*: Log*D* calculated using MarvinSketch software.

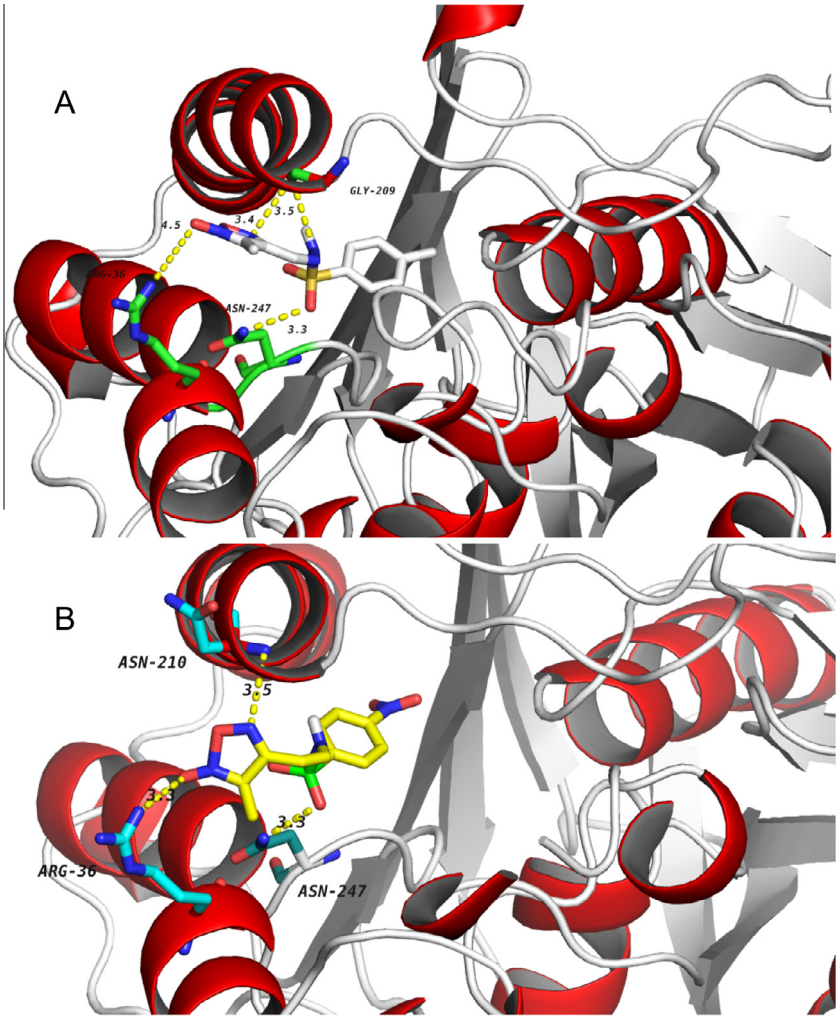


Figure 6. Representations of hydrogen-bonding and electrostatic interaction distances of compound **5** (A) and compound **3** (B) in complex with 1HNJ. Noteworthy, hydrogen bonding interactions have been considered less than 3.5 Å and electrostatic interactions, less than 5 Å.

hydrophobic pocket. Although there was no hydrogen bond between compound **5** and Arg36, electrostatic interactions of the furoxan group with this residue are likely to occur efficiently (Fig. 6A).[33]

On the other hand, compound **4**, which displayed low antibacterial activity in the in vitro assay, showed a good result in molecular docking with a binding energy of −6.6 kcal/mol. This suggests a permeability issue, precluding compound penetration into the bacterial cell. To estimate the cell permeability of the compounds, *c*Log*D* and polar surface (PSA) were calculated (Table 3). These two molecular properties are frequently used to estimate the transport of ligands across membranes. Although PSA values of compounds **4** and **5** are very similar, *c*Log*D* is higher for compound **4** (*c*Log*D* = 1.86). Additionally, Van der Waals area (VDW) is slightly greater for compound **4** than for all other compounds. Therefore, we advanced the hypothesis that the bulky group of compound **4** hampers the interaction with the target active site of *E. coli* FabH.

In conclusion, we have synthesized a small series of hybrid bioisosters of sulfonylhydrazones and furoxan groups as part of a preliminary study to develop new antibacterial compounds targeting FabH. The design proposed in this work was successful with compound **5**, which displayed activity in nM range. All the compounds are devoid of toxicity in Vero cell assays at the drug concentration tested (compared with a standard drug), indicating the advantages of using the scaffold designed. To optimize the antibacterial activity further efforts to design structures exploring other regions of the aromatic ring and the furoxan groups are being made. To prove the hypothesis here advanced, inhibitory enzymatic studies with FabH will be necessary.

This study is a starting point to further work on resistant strains, which have been a challenge for the chemotherapy, as mentioned before.

Author contributions: N.D.S. and R.A.M.S. contributed equally to this work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Supplementary data

Supplementary data (synthetic procedure, NMR spectra of all final compounds, description of the method for nitrite detection, biological assay and molecular modeling procedure) associated

with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.06.089.

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