Phenotypic and *in silico* studies for a series of synthetic thiosemicarbazones as New Delhi metallo-beta-lactamase carbapenemase inhibitors

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**ABSTRACT**

The past two decades have been marked by a global spread of bacterial resistance to β-lactam drugs and carbapenems derivatives are the ultimate treatment against multidrug-resistant bacteria. β-lactamase expression is related to resistance which demands the development of bacterial resistance blockers. Drug inhibitor combinations of serine-β-lactamase and β-lactam were successful employed in therapy despite their inactivity against New Delhi metallo-beta-lactamase (NDM). Until now, few compounds are active against NDM-producing bacteria and no specific inhibitors are available yet. The rational strategy for NDM inhibitors development starts with *in vitro* assays aiming to seek compounds that could act synergistically with β-lactam antibiotics. Thus, eight thiosemicarbazone derivatives were synthesized and investigated for their ability to reverse the resistant phenotype in NDM in *Enterobacter cloacae*. Phenotypic screening indicated that four isatin-beta-thiosemicarbazones showed Fractional Inhibitory Concentration (FIC) ≤ 250 μM in the presence of meropenem (4 μg/mL). The most promising compound (FIC= 31.25 μM) also presented synergistic effect (FICI = 0.34). Docking and molecular dynamics studies on NDM-thiosemicarbazone complex suggested that 2,3-dihydro-1H-indol-2-one subunit interacts with catalytic zinc and interacted through hydrogen bonds with Asp124 acting like a carboxylic acid bioisostere. Additionally, thiosemicarbazone tautomer with oxidized sulfur (thione) seems to act as a spacer rather than zinc chelator, and the aromatic moieties are stabilized by π–π and cation–π interactions with His189 and Lys221 residues. Our results addressed some thiosemicarbazone structural changes to increase its biological activity against NDM and highlight its scaffold as promising alternatives to treat bacterial resistance.

**1. Introduction**

The research and development of antibiotics were one of the greatest challenges of the twentieth century (Hutchings et al., 2019). However, the emergence of multidrug-resistant (MDR) or extensively resistant (XDR) pathogens over the past two decades due to antibiotics overuse both in the clinic and for animal production has increasingly hindered treatment (Mendelson & Matsoso, 2015; Talebi Bezmin Abadi et al., 2019). The true magnitude of antimicrobial resistance in humans is not fully known, but globally about 700,000 deaths/year can be related to antibiotic-resistant superbugs, and it will be the leading cause of death in 2050, overcoming traffic accidents, myocardial infarction and cancer, reaching more than 10 million people a year (Cecchini et al., 2015; World Health Organization, 2019). The economic impact of uncontrolled antimicrobial resistance would also be catastrophic, the superbugs spread would increase health care expenditures, and put at risk sustainable food and feed production worldwide (IACG, 2019). As a result, by 2030, antimicrobial resistance could force up to 24 million people into extreme poverty (Jonas et al., 2017). This scenario is alarming as several resistance mechanisms are readily being developed and shared among many bacteria species (Mulani et al., 2019). Some nosocomial pathogens were classified as highly critical due to rising multidrug resistance and virulence, for example, the ones summarized by the acronym ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) (Wolff et al., 2021).

Although β-lactam drugs are currently the main chemical class of antibiotics used clinically, the usefulness of these agents has been drastically reduced due to the emergence and dissemination of β-lactamases, which catalyze the cleavage of β-lactam rings of the antibiotics (Parkova et al., 2020; Zhao et al., 2021). Continuous structural changes in β-lactams scaffolds have become an obsolete strategy, thus, encouraging the search for new classes of antibiotics or...
inhibitors of resistance and virulence factors (van den Akker & Bonomo, 2018). The discovery of serine β-lactamase inhibitors, such as clavulanic acid, tazobactam, avibactam and sulbactam allowed the clinical reuse of β-lactam drugs, however, these adjuvants are not active against Metallo-β-Lactamases (MBL), especially New Delhi metallo-β-lactamase (NDM) (Celenza et al., 2018).

NDM is a type 1 MBL (B1) that has spread faster and more widely than other MBLs and bacterial infections that harbor plasmid-encoded NDM have emerged rapidly worldwide (Khan et al., 2017; Wu et al., 2019). Currently, 24 variants of NDM have been identified in more than 60 species from 11 different bacterial families (Halat & Moubareck, 2020). In fact, NDMs exhibit a broad substrate profile for hydrolysis of the penicillin, cephalosporin and ‘last resort’ carbapenems, and thus, confers bacterial resistance to nearly all β-lactam antibiotics (Sun et al., 2018). It makes the search for therapeutic alternatives a daunting challenge (Li et al., 2020). Although many NDM inhibitors have been reported, for example, thiols (Büttner et al., 2018; Song et al., 2018), carboxylic acids (King et al., 2014; Yoshizumi et al., 2013), and azoles (Jackson et al., 2021), there is no NDM inhibitor for clinical purposes to date.

Thiosemicarbazones have been reported to have a wide pharmacological activity, including antibacterial, antiviral, antiparasitic, antifungal, antimalarial and anticancer (Heffeter et al., 2019; Nishida & Ortiz De Montellano, 2011; Pape et al., 2016; Sarkanj et al., 2013; Summers, 2019). Recently, thiosemicarbazones have been reported as MBL inhibitors (Song et al., 2018; Zhao et al., 2021). The promising thiosemicarbazone scaffold shed light to investigate, by phenotypic (in vitro) and computer-aided approaches, a series of synthetic thiosemicarbazone derivatives as NDM inhibitors. Our goals were to identify MBL inhibitors that synergistically combat resistant bacterial infections in combination with β-lactam antibiotics and understand the binding mode of the NDM inhibitor through molecular modeling assays.

2. Materials and methods

2.1. Chemistry

The compounds 1a-d and 2a-d were synthesized according to (Cunha & Silva, 2009) and (Omar et al., 1984), respectively, as outlined in Scheme 1. All compounds have >85% purity. Infrared spectra were recorded as KBr discs on the Shimadzu IR Affinity-1 instrument. NMR spectra were obtained using a Varian Gemini 300 spectrometer or a Bruker Avance Ill 500 spectrometer, with tetramethylsilane as an internal standard for 1H and 13C. For 1H NMR spectra, chemical shifts were reported in ppm (δ), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet) and coupling constants (Hz). Additional IV and RMN data are described below.

2.1.1. Compound 1d

I.R. (KBr): 3311 (N-H), 3184 (N-H), 1525 (C = N), 1492 (C = N), 1463 (N-CS-N), 1168 (C = S), 1089 (C = S) cm⁻¹. 1H NMR (300 MHz, DMSO-d6): 12.04 (1H, s); 11.33 (1H, s); 10.83 (1H, s); 7.76 (1H, d, J = 7.8 Hz); 7.66 (2H, d, J = 9 Hz); 7.48 (2H, d, J = 9 Hz); 7.37 (1H, t, J = 7.5 Hz); 7.11 (1H, t, J = 7.5 Hz); 6.94 (1H, d, J = 8.1 Hz). 13C NMR (78 MHz) δ: 176.3; 162.6; 142.5; 137.4; 132.5; 131.5; 130.0; 128.2; 127.2; 122.3; 121.4; 119.8; 111.1.

2.1.2. Compound 2a

I.R. (KBr): 3348 (N-H), 3190 (N-H), 2958, 2931, 1535 (C = N), 1408 (N-CS-N), 1080 (C = S) cm⁻¹. 1H NMR (500 MHz, CD₂J₂CO) δ: 10.69 (1H, s); 10.05 (1H, s); 9.61 (1H, s); 7.59 (1H, d, J = 7.5 Hz); 7.36 (1H, t, J = 7.5 Hz); 7.08 (1H, t, J = 7.5 Hz); 7.02 (1H, d, J = 7.5 Hz); 3.74 (2H, dt, J = 7.5 Hz); 1.70 (2H, qt, J = 7.5 Hz); 1.42 (2H, st, J = 7.5 Hz); 0.96 (3H, t, J = 7.5 Hz). 13C NMR (125 MHz; CD₂J₂CO) δ: 178.2, 142.2, 139.1, 134.1, 122.5, 120.6, 110.2, 44.1, 30.8, 19.8, 13.2.

2.1.3. Compound 2b

I.R. (KBr): 3410 (N-H), 3236 (N-H), 2958, 2931, 1531 (C = N), 1446 (N-CS-N), 1184 (C = S), 1080 (C = S) cm⁻¹. 1H NMR (500 MHz, CD₂J₂CO) δ: 12.62 (1H, s); 10.14 (1H, s); 9.73 (1H, s); 7.36 (1H, m); 7.18 (1H, dd, J = 2,5 Hz); 6.95 (1H, d, J = 8.5 Hz); 3.74 (2H, dt, J = 7.5 Hz); 1.70 (2H, qt, J = 7.5 Hz); 1.42 (2H, st, J = 7.5 Hz); 0.96 (3H, t, J = 7.5 Hz). 13C NMR (125 MHz; CD₂J₂CO) δ: 178.1, 162.9, 137.5, 129.7, 128.7, 127.1, 126.7, 117.5, 111.3, 44.2, 30.8, 19.8, 13.2.

2.1.4. Compound 2c

I.R. (KBr): IV (KBr): 3340 (N-H); 3197 (N-H); 2958; 2931; 1593 (C = N); 1462 (N-CS-N); 1184 (C = S); 1080 (C = S) cm⁻¹. 1H NMR (500 MHz, CD₂J₂CO) δ: 12.57 (1H, s); 10.61 (1H, s); 9.15 (1H, s); 8.38 (1H, d, J = 2 Hz); 8.29 (1H, dd, J = 8 Hz, J = 2 Hz); 7.24 (1H, d, J = 8 Hz); 3.75 (2H, dt, J = 7.5 Hz); 1.71 (2H, qt, J = 7.5 Hz); 1.42 (2H, st, J = 7.5 Hz); 0.96 (3H, t, J = 7.5 Hz). 13C NMR (125 MHz; CD₂J₂CO) δ: 178.0, 162.8, 146.9, 143.6, 126.5, 115.8, 111.2, 44.3, 30.7, 19.8, 13.2.

2.1.5. Compound 2d

I.R. (KBr): IV (KBr): 3228 (N-H); 3147 (N-H); 2958; 2931; 1546 (C = N); 1458 (N-CS-N); 1188 (C = S); 1080 (C = S) cm⁻¹. 1H NMR (500 MHz, CD₂J₂CO) δ: 10.09 (1H, s); 9.76 (1H, s); 9.36 (1H, s); 7.57 (1H, d, J = 2 Hz); 7.45 (1H, d, J = 2 Hz); 3.75 (2H, dt, J = 7.5 Hz); 1.71 (2H, qt, J = 7.5 Hz); 1.42 (2H, st, J = 7.5 Hz); 0.96 (3H, t, J = 7.5 Hz). 13C NMR (125 MHz; CD₂J₂CO) δ: 178.0, 162.9, 149.1, 137.5, 134.8, 127.0, 126.6, 124.4, 115.4, 44.2, 30.7, 19.8, 13.2.

2.2. In vitro assays

2.2.1. Bacterial strain and culture condition

All phenotypic assays were conducted against a carbapenem-resistant strain (Enterobacter cloacae CC810892) (Barberino et al., 2018). Before each in vitro experiment, the
E. cloacae CCBH10892 have grown in Mueller-Hinton (MH) agar (Difco) overnight at 37°C and the bacterial suspension was prepared in 0.9% NaCl sterile solution to $3 \times 10^8$ UFC/mL concentration using densitometer model DEN-1 (Biosan, Latvia). Meropenem (ABL, Brazil) were diluted in MH Broth to 5 mg/mL fresh stock concentration. Compounds stock solutions (25 mM) were prepared in Dimethyl Sulfoxide (DMSO, Merck - Germany) and stored at -20°C.

2.2.2. Minimal inhibitory concentration (MIC)

The meropenem sensibility against E. cloacae CCBH10892 strain was assessed using the broth microdilution method described on Clinical and Laboratory Standards Institute M7-A6 protocol (CLSI, 2018). Serial dilutions of meropenem (0.5 – 128 µg/mL) were performed in a 96-well microplate (Costar, NY/USA). The final suspension density in each well was $1.5 \times 10^8$ cells/mL (0.5 McFarland). The microplates were incubated at 35±2°C for 24 h and the MIC value was reported as the lowest antibiotic concentration without residual bacterial growth after 24 h incubation. Only the broth and broth plus bacterial suspension were used as a negative (sterility) and positive (bacterial growth) control, respectively. All tests were performed in triplicate and the MIC values were described as mean and standard deviations.

### 2.2.3. Fractional inhibitory concentration

The fractional inhibitory concentration (FIC) is an adaptation of the MIC, as described in the M7-A6 protocol (CLSI, 2018). Briefly, serial dilutions of the compounds (1.95 – 250 µM) were performed at the presence of meropenem (4 µg/mL) as previously defined through the MIC test. Wells with MH Broth were used as a negative control and the bacterial growth controls were evaluated in the following conditions: wells containing MH broth, bacterial suspension and 4 µg/mL meropenem; MH broth, bacterial suspension, 1% DMSO and 4 µg/mL meropenem. Wells containing MH broth, bacterial suspension, 250 µM EDTA and 4 µg/mL meropenem were used as a control of carbapenemase inhibition (NDM).

All experiments were done in triplicate for each compound and the FIC values were described as mean and standard deviation. The maximum percentage of DMSO in the wells was 1% (v/v). After static incubation (~20 h at 35±2°C) in a bacteriological incubator (Fanem, Brazil), the lowest concentration of compounds in combination with meropenem at which no bacterial growth was observed.

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**Scheme 1.** Synthetic route for the series of thiosemicarbazone derivatives.
tests were performed in triplicate and the MIC and FIC values were described as mean and standard deviations.

### 2.2.4. Fractional inhibitory concentration index

For compounds with FIC/250 µM (highest compound concentration) < 0.5, the Fractional inhibitory concentration index (FICI) (Pillai & Moellering, 2005) were determined. Briefly, in a 96-well microplate, twofold dilutions of meropenem (0.5 – 32 µg/mL) were added with twofold dilutions of selected compounds (0.24 – 250 µM) to determine the FIC values at each combination. Then, the MH broth containing the bacterial suspension (Final concentration: 1.5 × 10^8 cells/mL) was added to each well. Column 12 contains a serial dilution of meropenem alone, while row H contains a serial dilution of compound alone. These controls are used to determine the MIC value for meropenem (MICm) and each test compound (MICc). The ‘H12’ well containing only the MH broth was used as sterility control. The maximum percentage of DMSO in the wells was 1% (v/v). After incubation (~20 h at 35 ± 2 °C), MICm and MICc were determined along with the FIC values for compounds (FICc) and meropenem (FICm) at the combinations that inhibited bacterial growth. Then, to quantify the interactions between the antibiotic and compound, the FICI value were calculated through Equation (1): 

\[
\text{FICI} = \frac{\text{FICm}}{\text{MICm}} + \frac{\text{FICc}}{\text{MICc}}
\]

The effect of the compounds on the meropenem activity was classified according to (Kumar et al., 2012) into synergic (FICI < 0.5), additive (0.5 ≤ FICI < 1), indifferent (1 ≤ FICI < 4) and antagonist (FICI ≥ 4.0). All tests were performed in triplicate and the MIC, FIC and FICI values were described as mean and standard deviations.

### 2.2.5. Time-kill assay

Time-kill assay as described in M26-A protocol (CLSI, 1999) with adaptations was used to evaluate the effect of DMSO on bacterial growth. Then, different DMSO percentages (0.5, 1, 2, 5 and 10% v/v) were added to the MH broth plus bacterial suspension (Final concentration: 1.5 × 10^8 cells/mL) and distributed in a 96-well plate. After incubation (24 h at 35 ± 2 °C), the bacterial density (scale = 10^8 cells/mL) was estimated spectrophotometrically at 600 nm through NanoDrop One (Thermo Scientific, USA). Wells containing MH broth alone or MH broth plus suspension were used as a negative (sterility) and positive (bacterial growth) controls. The time-kill assay as described above was also used to investigate the effect of combinations of meropenem plus compounds, previously determined in the FICI study, on bacterial growth. All tests were performed in triplicate and the bacterial density values were described as mean ± SD.

### 2.2.6. Statistical analysis

The results were expressed as mean ± SD. Statistical significance between the two groups was determined using Student’s t-test and p-values < .05 were considered statistically significant. All analyzes were performed using the GraphPad Prisma software v. 8.0.2 (California, USA).

### 2.3. Computational modeling

#### 2.3.1. Ligand and NDM-1 structures preparation

The 3D crystallographic structures of NDM-1 enzyme available on the Protein Data Bank (http://www.rcsb.org/) were selected from the following criteria: (1) Structures co-crystallized with a noncovalent ligand at the active site; (2) Enzymatic classification number: EC 3.5.2.6; (3) monomeric form; (4) resolution < 2 Å. Following, the 3D structures were prepared for docking assays using the Biopolymer module, which is available on the Sybyl-X 2.1.1 platform (Tripos Associates, 2012). First, co-crystallized ligands, waters and crystallization adjuvants were excluded. Hydrogen atoms were added and optimized to prioritize H-bond interactions. Histidine, glutamate and aspartate residues were manually checked for orientation, protonation and tautomeric states. The protonation state was determined at pH = 7.5 (Sharma et al., 2020) using Propka 3.1 server (https://server.poissonboltzmann.org/pdb2pqr) (Rostkowski et al., 2011; Søndergaard et al., 2011). Finally, Gasteiger–Hückel partial charges (Gasteiger & Marsili, 1980) were calculated at the Tripos force field (Clark et al., 1989).

The thiosemicarbazone derivative on prevalent tautomeric state (Kohli et al., 2014) and co-crystallized ligands had their 2D structure drawn on MarvinSketch v. 19.1 (ChemAxon, 2019) at pH = 7.4 and were later converted to the 3D format with Concord module (standard parameters) available on Sybyl®-X 2.1.1 (Tripos Associates, 2012). Afterward, the 3D structures were energy minimized using the Tripos force field (parameters: 50,000 steps, conjugate gradient algorithm, convergence criterion of 0.001 Kcal/mol) (Clark et al., 1989) on implicit solvent at dielectric constant = 80.4. Gasteiger–Marsili charges (Gasteiger & Marsili, 1980; Xu et al., 2013) were assigned at the Tripos force field. The final optimized 3D structure was applied to docking assay.

#### 2.3.2. Docking

The docking calculations were carried out on the GOLD 5.5 software (Jones et al., 1997; Verdonk et al., 2003). The search space was defined as a sphere with a 10 Å radius positioned on the center of mass of the co-crystallized ligand for each NDM-1 structure. Docking simulations were performed using a Lamarckian genetic algorithm (LGA) (Morris et al., 1998), a hybrid of the genetic algorithm and local search algorithm available on the GOLD software (Jones et al., 1997). The docking parameters were previously described (Cassiano et al., 2019) and other parameters were kept as default. Each docking simulation produced 2.5 × 10^4 different docked conformations and the ligands were scored using the Piecewise Linear Potential function (ChemPLP) implemented on the GOLD software (Korb et al., 2009; Liebeschuetz et al., 2012).

The three poses that showed better steric and electronic complementarity with the protein, according to ChemPLP function, were submitted to the PLIP server (Salentin et al., 2015) for interaction analysis. The final poses were selected using Pymol 1.3 (Schrödinger L. L. C, 2009) for visual analysis on interaction patterns described by the PLIP server.
For docking validation, all ligands from X-ray structures were mutually docked and the Root Mean Square deviation (RMSD) of the atomic positions of docked ligands regarding co-crystallized position was calculated with the ‘Align compounds, Match’ command available on the SYBYL-X 2.1.1 platform. The docked and its crystallography pose (redock) was satisfactory for RMSD < 2 Å (Sargsyan et al., 2017; Westermaier et al., 2015) and used to estimate the quality and reliability of docking parameters (Kumar & Zhang, 2018). Conversely, RMSD of ligands docked on related NDM-1 structures (cross-docking) was used to select the best protein for compounds docking by the lowest RMSD sum values (ΣRMSD).

2.3.3. Molecular dynamic simulations

The topology file of compound 1d was built using the Automated Topology Builder (ATB) server (http://compbio.biosci.uq.edu.au/ATB/) (Koziara et al., 2014; Malde et al., 2011; Stroet et al., 2018). Subsequently, NDM-1 (Apo) and its complex (NDM/1d) were submitted to Molecular Dynamic (MD) simulations on GROMACS 2019-1 (Abraham et al., 2015; Berendsen et al., 1995; Pronk et al., 2013; Van der Spoel et al., 2005). The protonation state of acid and basic residues of both systems were adjusted at pH = 7.5 by pdb2gmx module available on GROMACS 2019-1 using microenvironment pKa values calculated on PROPKA 3.1 version (Olsson et al., 2011; Sondergaard et al., 2011). The MD parameters were set to 1 bar, 310K, GROMOS54A7 force field (Schmid et al., 2011), PME (Darden et al., 1993) for electrostatic treatment with cut-off = 0.9 nm in a dodecahedral box solvated with SPC/E water model (Berendsen et al., 1987) at periodic boundary conditions (PBC). Next, one Na+ ion was added to neutralize the residual system charge.

The systems were submitted to two sequential energy minimization steps (steepest descent and conjugate gradient) of 1000 cycle/step followed by a pre-equilibrium simulation step (nonhydrogen atoms were position restrained for 1 ns). Finally, 80 ns of unrestrained simulation was performed for both Apo and NDM/1d systems where the atomic coordinates were recorded every 100 ps for further analyses on an NPT ensemble with V-rescale thermostat (Bussi et al., 2007) and Berendsen barostat (Berendsen et al., 1984) with the SETTLE (Miyamoto & Kollman, 1992) algorithm for solvent bonds and LINCS (Hess et al., 2008) for other bonds.

2.3.4. Analysis of MD simulation trajectories

Root Mean Square Deviation (RMSD) was calculated for both systems to provides information on the variation of the atomic position during MD. The crystallographic structure (Verli, 2014) was employed as a reference. Afterward, Radius of gyration (Rg) and Root Mean Square Fluctuation (RMSF) were computed for both systems to analyze the protein’s compactness (Lobanov et al., 2008) and residues’ movement over time. All these analyses were computed through g_rms, g_gyrate and g_rmsf modules from GROMACS 2019-1, respectively.

The hydrogen bonds (H-bonds) between compound 1d and NDM-1 residues were computed using the g_hbond module (Abraham et al., 2015) from GROMACS 2019-1. All possible interactions between donors and acceptors at donor–acceptor distance ≤ 0.35 nm and hydrogen-donor–acceptor angle ≤ 60° were considered. The H-bond permanence was calculated through hmap2grace program (http://lmdm.biof.ufrj.br/software.html) (Gomes et al., 2009) and used as a stability measure over the MD. H-bonds with <10% permanence were discarded. Surface contact area (Å²) between NDM-1 residues and 1d was calculated with rsurf and surf modules from SurfMD program (http://lmdm.biof.ufrj.br/software.html) (Gomes et al., 2009) to investigate hydrophobic and π interactions at a default probe radius (1.4 Å) and probe density (1.0 Å²).

The averaged structure (time = 13.6 ns) from the productive phase of each MD simulation was extracted and analyzed. The main interactions between derivative 1d and NDM-1 residues were assigned by protein–ligand interaction profiler (PLIP) (Salentin et al., 2015). All interactions, as well as coordination/bonding between divalent ions and water molecules, were visually analyzed with the PyMOL™ 1.3 software (Schrödinger L. L. C, 2009).

3. Results

3.1. Phenotypic assay for NDM inhibitor screening

To screen inhibitors of NDM using broth microdilution was determined the minimum inhibitory concentration (MIC) of meropenem against a carbapenem-resistant E. cloacae CCBH10982 strain. The test showed a MIC of 16 µg/mL that was interpreted as a resistant phenotype according to CLSI standard M7-A6 protocol. Based on MIC value, we used meropenem at 4 µg/mL (1/4 MIC) for FIC assay aiming to quantitatively determine the effect of thiosemicarbazone derivatives on resistant phenotype. However, as these compounds were solubilized in DMSO, we first investigated the effect of solvent on bacterial growth (Figure 1S, Supporting Information). The time-kill assay showed that up to 2% DMSO does not significantly reduce the bacterial growth compared to control. In contrast, 5% DMSO blocks 65% bacterial growth (p < 0.05) and 10% DMSO completely inhibits
Table 1. Fractional inhibitory concentration of thiosemicarbazones in combination with meropenem (4 μg/mL).

| Compound | FIC (μM)* | Compound | FIC (μM)* |
|----------|-----------|----------|-----------|
| 1a       | >250      | 2a       | 125       |
| 1b       | >250      | 2b       | 125       |
| 1c       | >250      | 2c       | 250       |
| 1d       | 31.25     | 2d       | >250      |

*Standard deviation was not calculated for identical FIC repetitions.

the bacterial growth (p < .01). Therefore, 1% DMSO was the maximum percentage used in the following phenotype tests.

Next, FIC assays were performed using different concentrations of thiosemicarbazone compounds (Table 1, see structures in Scheme 1), however, only compounds 1d (31.25 μM), 2a (125 μM), 2b (125 μM) and 2c (250 μM) promoted a reduction in bacterial growth in combination with meropenem.

The results of the phenotypic screening indicate that the thiosemicarbazone group does not seem essential to biological activity, however, the presence of the isatin-β group (R1/R2 substituents) may be important to block bacterial resistance since only derivatives containing this group (1d, 2a, 2b and 2c) showed some activity in combination with meropenem. The replacement of isatin-beta moiety by other aromatic systems, such as phenyl (1a), p-nitrophenyl (1b) and benzodioxole (1c) eliminates the activity. Although the small number of isatin-beta-containing derivatives limits a robust structure-activity relationship interpretation of these derivatives, a preliminary view allows supposing that substitutions in the isatin-beta ring can drop the activity, for example, compound 2a versus 2c. Conversely, the coexistence of an aromatic group linked to the R3 substituents of thiosemicarbazone, for example, p-chlorophenyl of compound 1d, promotes greater inhibition of NDM than aliphatic groups, such as the n-butyl group of compound 2a.

The FIC assays indicate which compounds inhibit bacterial growth in combination with meropenem, but it was not possible to determine whether the effect is synergistic or not and if it was caused by direct inhibition of Metallo-β-lactamase or by an effect on other proteins of the microorganism. To investigate these hypotheses, several combinations of thiosemicarbazones and meropenem were tested in order to calculate the fractional inhibitory concentration index (FICI) and determine the MIC of compounds. However, only compounds with the ratio of FIC/250 < 0.5 were investigated. Thus, compound 1d (FIC/250 = 0.125) proceeded for the FICI assay while other less active compounds (2a, 2b and 2c) were discarded from further analysis because they would tend to be classified as additive, indifferent or antagonistic in any combination.

Initially, the MICs of meropenem (MICm > 32 μg/mL) and compound 1d (MICc > 250 μM) were determined in the FICI assays. These results suggest that bacterium kept the expected resistance profile and the compound does not show activity or toxicity at the highest concentrations used. We then used the highest values of MICm and MICc to calculate the FICI. The combined concentrations of the compound (FICc) and antibiotic (FICm; Figure 1) show the effect of increasing concentrations of compound 1d on the meropenem concentration needed to inhibit bacterial growth. The change from resistant to sensitive phenotype was achieved at 62.5 μM of thiosemicarbazone 1d together with 3 ± 1.3 μg/mL of meropenem. At these concentrations, the FICI was 0.34 ± 0.04 suggesting a synergic effect (FICI < 0.5) as a result of direct action on the bacterial resistance mechanism. Another combination of compound 1d (FICc = 125 μM) and meropenem (FICm = 1.16 ± 0.6 μg/mL) showed only an additive effect (FICI = 0.54 ± 0.02).

It is noted from the FICm standard deviation that more than one meropenem concentration (between 1.7 and 4.3 μg/mL) may be useful as a synergistic concentration in combination with 62.5 μM of compound 1d. Although the difference in FICm does not alter the FICI classification, many compounds classified as synergistic may have a bacteriostatic profile instead of bactericide in some combinations with the antibiotic. Based on this assumption, time-kill assays were performed to quantitatively evaluate the effect of meropenem (2 and 4 μg/mL) together with 62.5 μM compound 1d to the bacterial growth (Figure 2). The combination of 2 μg/mL meropenem plus 62.5 μM compound 1d reduced the bacterial growth not significantly by 45.7% compared to the control. In contrast, 4 μg/mL meropenem in combination with compound 1d showed statistically significant bactericidal potential (p = .0034).

3.2. Docking of thiosemicarbazone derivative 1d on NDM-1

For docking of thiosemicarbazone 1d into active site of crystallographic NDM-1 structure, four 3D complexed NDM-1 structures (PDBs: 4EYF, 4U4L, 5YPL and 5ZQG) were first selected (See docking methods). After, two validation assays (redocking and cross-docking) were done to determine the search parameter and select a suitable structure for thiosemicarbazone dockings.

Redocking seeks to reproduce the co-crystallized geometry and orientation. Cross-docking reproduces the crystallographic positioning in several complexes. In both cases, lower root-mean-square deviation (RMSD) values between the docked and crystallographic conformation indicate success on docking poses. After redocking and cross-docking studies, RMSD values were calculated and analyzed (Table 2).
Table 2. Root-mean-square deviation (RMSD, Å) of docked and crystallographic conformation.

| Ligands         | Crystallographic protein structure |
|-----------------|-----------------------------------|
|                 | 4EYF | 4U4L | 5GZQ | 5YPL |
| Hydrolyzed benzylpenicillin | 1.98  | 2.36  | 2.49  | 2.52  |
| Bis-thiazolidine | 0.13  | 0.18  | 0.13  | 0.00  |
| Hydrolyzed imipenem | 1.05  | 2.75  | 2.08  | 2.51  |
| Hydrolyzed ampicillin | 0.80  | 1.34  | 1.46  | 1.84  |
| ΣRMSD<sup>a</sup> | 3.96  | 6.63  | 6.16  | 6.88  |

<sup>a</sup>Calculated for docking evaluation.

Redocking results (diagonal values) reveal that bis-thiazolidine had the lowest RMSD (0.18 Å), whereas hydrolyzed imipenem presents the largest deviation (2.08 Å). The 5GZQ structure was excluded from further analysis, as its redocking RMSD value (>2 Å) is unreliable. Next, ΣRMSD values were calculated from the cross-docking assays to assess the ability of NDM-1 protein to accommodate different ligands. The results showed that 4EYF had the lowest ΣRMSD (3.96 Å) and additionally, all ligands presented lower RMSD (Figure 25, Supporting Information). Therefore, this structure was employed on our docking assays.

Subsequently, docking calculations in the NDM active site for thiosemicarbazone derivative (1d) show that the 2,3-dihydro-1H-indol-2-one from isatin-β ring and thiosemicarbazone nitrogen made several electrostatic interactions with polar residues (Asp124, Lys211 and Asn220) and zinc ions (Figure 3S, Supporting Information). Hydrophobic and π-stacking interactions between aromatic rings of 1d and residues Ile35, Trp93, His122 and Gln123 were also performed. Their interaction suggests that thiosemicarbazone moiety may act as a spacer (not a zinc chelator), which corroborates with our previous in vitro results. Aiming to evaluate the ligand-protein movements, MD assays were performed.

3.3. MD simulations

The Apo and the NDM/1d systems were analyzed through RMSD and Radius of gyration (Rg) over the 80 ns trajectory (Figure 3(A)) in order to evaluate the systems stability.

The RMSD evolution (Figure 3(A)) shows that both systems stabilize after 30 ns (productive phase) and these data were employed on later analysis. In addition, compound 1d greatly stabilise the NDM enzyme (RMSD<sub>30–80 ns</sub> = 0.282 ± 0.012 nm) when compared to Apo form (RMSD<sub>30–80 ns</sub> = 0.345 ± 0.018 nm). However, the RMSD plot alone is not sufficient to fully demonstrate the complex stability, since NDM-1 can undergo significant changes during simulation. Thus, Rg values of NDM/1d were calculated on productive phase (Figure 3(B)). The Rg evaluates the protein geometry through their decomposed values (RgX, RgY and, RgZ). Since Rg values remain stable over the simulation (Rg = 1.69 ± 0.01 nm), the compactness of the protein structure was conserved. Therefore, RMSD and Rg assure that MD simulations were reliable and useful for further analysis.

The root-mean-square fluctuations (RMSF) were calculated on these two systems aiming to determine the thiosemicarbazone 1d influence on NDM subdomains (Figure 4).

The smaller fluctuation, when compared with Apo protein, was located on residues 122–136, 187–190 and 217–220. These previous analyses overlooked the hydrophobic content of the NDM-1/1d interactions that are equally important for ligand arrangement in the binding site. Thus, the contact surfaces (cut-off area > 10 Å² and distance < 4 Å) between 1d and protein residues were calculated (Figure 5(B)). The results revealed that 1d made some stable interactions:
hydrophobic contacts with Tyr229, Leu218, Cys208 and Thr34, π stacking with His120 and 189 and cation-π interactions with Lys211, despite its electrostatic contributions (Figure 6).

4. Discussion

Enzymatic (kinetic) assays containing the expressed and purified target protein have been nowadays the most used strategy for the NDM inhibitors screening, however, several promising NDM inhibitors are not effective when evaluated in cell models. Consequently, the discovery of adjuvant molecules that act synergistically with β-lactam drugs in cell models is essential at some step of the development of new β-lactamase inhibitors. Given this, we prioritized the phenotypic activity assays as the initial stage of β-lactamase inhibitors screening and demonstrated the property of isatin-β-thiosemicarbazone to synergically revert the resistance of NDM-producing Enterobacteria in combination to low concentration of meropenem.

Thus, it was used a bacterial model *E. cloacae* CCBH 10892, whose only resistance mechanism is the expression of New Delhi Metallo-β-lactamase (NDM) carbapenemase. Indeed, PCR studies for detecting genes encoding β-lactamases (KPC, NDM, VIM, etc.) in this strain demonstrated only the presence of the blaNDM gene (Barberino et al., 2018). Then, a set of eight thiosemicarbazone derivatives was evaluated in combination with meropenem using the broth microdilution methodology (MIC, FIC and FICI) to investigate the effect of these compounds on the bacterial model phenotype. In the FIC assays, the only compounds derived from isatin-β-thiosemicarbazones (1d, 2a, 2b and 2c) showed any activity (FIC ≤ 250 μM). Although thiosemicarbazones and semicarbazones have a broad pharmacological profile and constitute an important class for their metal chelating and coordinating properties (Barcelos et al., 2012; Kowol et al., 2009; Yu et al., 2009), the in vitro screening suggests
that the thiosemicarbazone group, a common scaffold to
tested compounds, does not seem essential or preponderant
for the inhibition of NDM. In contrast, the presence of the
isatin-β-moiety together with thiosemicarbazone seems to be
important for the anti-β-lactamase activity. These results cor-
roborated with drug repositioning studies employing the
methisazone, an old isatin-β-thiosemicarbazone drug used to
treat smallpox infections (Bauer, 1965) and a weak inhibitor
of NDM-1 (IC₅₀ = 297 μM) (Song et al., 2018).

Among isatin-β-thiosemicarbazones, only derivative 2d
did not show activity within the maximum concentration
limit used (250 μM), in contrast, compound 1d was more
promising (FIC = 31.25 μM). Although 1d has shown medium
to low activity, it is the first time that activity of the isatin-
β-thiosemicarbazone derivatives is observed at the cellular
level to NDM-producing bacteria, that is, with any clinical
relevance, thus, justifying more studies with analogous mole-
cules for further optimization structural. For example, a pre-
liminary analysis of the structure-activity relationship, based
on FIC data, indicates that the electron-withdrawing or bulky
substitutes attached in the isatin-β ring, such as nitro (com-
pound 2c) or chlorine groups (2b and 2d), seem to impair
biological activity. However, enzymatic-kinetic assays against
NDM suggest that the inclusion of halogen groups does not
drastically alter the activity as compared to unsubstituted
derivatives. Thus, these changes may have a greater impact
on the pharmacokinetic profile, for example, membrane pas-
sage, than the pharmacodynamic profile (interaction with the
active site) of the compounds. Also, the replacement of the
p-chlorophenyl ring by n-butyl chain (compare 1d with 2a)
seems crucial to reduce activity. Maybe the interaction of
this group, as well as its volume, shed light on why its
replacement drops the activity of other isatin-β derivatives.
These aspects were clarified in the in silico part.

Conceptually, synergism occurs when two molecules in
combination act significantly better than the response of
each used separately (Odds, 2003). Among the compounds
evaluated, only derivative 1d was investigated in subsequent
steps and showed ability at 62.5 μM concentration to reduce
the tenfold MIC of meropenem (32 to 3 μg/mL), suggesting
that it should act synergistically on the evaluated mechanism
of resistance (FICI = 0.34). Similarly, 64 μM of some diaryl-thi-
osemicarbazone derivatives promoted a tenfold reduction in
the MIC of meropenem in NDM-1-producing E. coli (Li et al.,
The effect of compound 1d also does not seem to block essential bacteria proteins because there was no decrease in cell growth at the highest concentration of compound alone. These results then support the hypothesis that compound 1d blocks bacterial resistance through inhibition of NDM. This evidence is corroborated by studies by Song et al. (2018) in which β-isatin thiosemicarbazone derivatives similar to 1d (Tanimoto index > 80%) showed activity against the enzyme NDM-1 in low concentrations and act competitively.

Therefore, in silico approaches were employed to understand the steric and electronic characteristics that govern the interaction between compound 1d and the NDM. After preceding validation by redocking and cross-docking, derivative 1d was prepared for docking. The staurometnic state of thiosemicarbazone was considered because some studies often state (Spyrakis et al., 2020) that thiosemicarbazones play a zinc chelating action due to its possible tautomeric presented a sulfhydryl group. Since thiols are soft Lewis bases that can bind to metal ions (Soldatović, 2020), they could coordinate with the catalytic zinc on NDM leading to enzyme inhibition. Nevertheless, theoretical studies of quantum mechanics with isatin-β-thiosemicarbazones (Kohli et al., 2014) suggest that the dominant tautomer has sulfur in its oxidized form (91% of possible states). Thus, we decided to employ the prevalent tautomer of compound 1d on docking studies at the active site of NDM-1.

After, MD simulations were performed in the systems Apo and NDM/1d complex to describe these intermolecular relationships as a function of time (secondary structure contents, side chains orientation, loop conformations and interaction energy between ligand and protein) (Verli, 2014).

The RMSF analysis indicates that NDM/1d complex reduces the fluctuations of some protein residues: 122–136, 187–190 and 217–220 (Figure 4). They belong to the zinc complexation core (His122, Asp124 and His189) and the L10 loop, especially Lys211 and Asn220 which stabilizes both substrate and cleaved β-lactams (King & Strynadka, 2011; Zhang & Hao, 2011). Similarly, MD simulations with mercapto-carboxylate derivatives found important fluctuation of the same region suggesting that they act on the same active site as 1d (Khan et al., 2017). The interaction map analysis revealed that isatin-β ring interacted both with catalytic Asp124 and zinc (Zn1). This residue and ion contribute to the stabilization of carbapenem carboxylate group after cleavage (Wu et al., 2019). In addition, they made interactions similar to other NDM inhibitor classes such as rhodamine derivatives carboxylates (Brem et al., 2014) and boronic acid derivatives (Santucci et al., 2017). Therefore, isatin-β group maybe act as a nonclassical bioisostere of acid groups into this enzyme.

The interaction between thiosemicarbazone sulfur and cysteine thiol (Cys208) suggests that the oxidized sulfur should act as an electron acceptor for H-bond rather than a zincons chelator. Indeed, theoretical studies on isatin-β-thiosemicarbazone sulfur reinforce this hypothesis (Kohli et al., 2014). This study corroborates with our data that thiosemicarbazone may act as a spacer and less influent on biological activity and explained the inactivity of the other tested thiosemicarbazones. Nevertheless, the electronic characteristics and spatial restraints generated by the thiosemicarbazone group maybe are useful for suitable fit and interaction of other moieties of derivative 1d within the enzyme activity site.

Last but not the least, compound 1d made hydrophobic contacts (Tyr229, Leu218, Thr34), π-π (His189) and a cation–π interaction (Lys211) with p-chlorophenyl moiety. This last interaction may explain the activity of 1d as compared with other thiosemicarbazones. The highlighted residues belong to the L10 loop, which has an important role in guiding and stabilizing β-lactam drugs for hydrolysis (Linciano et al., 2019).

5. Conclusion

The present work describes for the first time the synergic activity of isatin-β-thiosemicarbazone derivatives in combination with a carbapenem (meropenem) against NDM-producing enterobacteria. Phenotype assays showed that the most promising compound (1d) is bactericide at micromolar concentration and inhibits New Delhi Metallo-β-lactamase as the unique mechanism. The in silico data indicates that the isatin-β ring is essential for β-lactamase inhibition.

Disclosure statement
All authors have declared no conflict of interest.

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Silvio Cunha and Danilo Santana synthesized the entire series of derivatives with structure elucidation. Humberto F. Freitas and Joice Reis initiated the idea and design of the biology and computational parts. Jonatham Moreira and Carolina Xavier carrying out in vitro phenotypic screening and molecular docking. Samuel R. Pita performs the Molecular Dynamic simulations. All authors contributed to data analysis and manuscript writing in their corresponding parts.

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