TACN (1, 4, 7-Triazacyclononane) restores the activity of β-lactam antibiotics against Metallo-β-Lactamase producing *Enterobacteriaceae*: The exploration of potential Metallo-β-Lactamase inhibitors

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Running title: TACN inhibits MBLs

Abstract:

Metallo-β-lactamase producing *Enterobacteriaceae* are of grave clinical concern particularly as there are no Metallo-β-lactamase (MBL) inhibitors approved for clinical use. The discovery and development of MBL inhibitors to restore the efficacy of available β-lactams are thus imperative. We investigated a zinc-chelating moiety, 1, 4, 7-triazacyclononane (TACN) for its inhibitory activity against clinical carbapenem-

resistant *Enterobacteriaceae*. Minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs), serum effect, fractional inhibitory concentrations index and time-kill kinetics were performed using broth microdilution techniques according to the Clinical Laboratory Standard Institute (CSLI) guidelines. Enzyme kinetic parameters and cytotoxicity effects of TACN were determined using spectrophotometric assays. The interactions of the enzyme-TACN complex were investigated by computational studies. Meropenem regained its activity against carbapenemase-producing *Enterobacteriaceae*, with the MIC decreasing to 0.03 mg/L in the presence of TACN. TACN-Meropenem combinations showed bactericidal effects with MIC/MBC ratio of ≤4, and synergistic activity was observed. Human serum effects on the MICs were insignificant, and TACN was found to be non-cytotoxic at concentrations above the MIC values. Computational studies predicted that TACN inhibits MBLs by targeting their catalytic active site pockets. This was supported by its inhibition constant K_i = 0.044 μM and inactivation constant k_{inact} = 0.0406 (min^{-1}) demonstrating that TACN inhibits MBLs efficiently and holds promise as a potential inhibitor.

Importance

Carbapenem-resistant *Enterobacteriaceae* (CRE)-mediated infections remain a significant public health concern and have been reported as critical in the World Health Organization's Priority Pathogens List for the Research and Development of New Antibiotics. CRE produce enzymes such as Metallo-β-lactamases (MBLs), which inactivate β-lactam antibiotics. Combination therapies involving a β-lactam antibiotic and a β-lactamase inhibitor remain a major treatment option for infections caused by β-lactamase-producing organisms. Currently, no MBL inhibitor-β-lactam combination therapy is clinically available for MBL-positive bacterial infections. Hence, developing efficient molecules capable of inhibiting these enzymes could be a promising way to overcome this phenomenon. TACN played a significant role in the inhibitory activity of the tested molecules against CREs by potentiating the carbapenem. This study demonstrated that TACN inhibits MBLs efficiently and holds promises as a potential MBLs inhibitor to help curb the global health threat posed by MBL-producing CREs.

48 **Keywords**: TACN, Metallo-β-lactamase inhibitor, binding affinity, β-lactams, *Enterobacteriaceae*

49 **Introduction**

50 β-lactams are broad-spectrum antibiotics with relatively minimal toxicity, making them antibiotics of
51 choice for many bacterial infections(1). However, the surge in resistance to β-lactams in Gram-negative
52 bacteria is affecting the management of infections worldwide(2). β-lactams are rendered ineffective
53 through different mechanisms, including reduced membrane permeability through porin down-regulation
54 and efflux, target site modification, and most importantly, the production of β-lactamases, enzymes that
55 hydrolyze β-lactams(3).

56 Metallo-β-lactamases (MBLs) are Ambler class B β-lactamases with zinc ions at their active sites. The
57 zinc ions orient a hydroxide nucleophile to attack the carbonyl on a β-lactam ring(4, 5). Sub-class B1
58 MBLs are the most clinically relevant among the class B MBLs. They possess a binuclear active site,
59 which contains either one or two Zn (II) ions and hydrolyze all β-lactam antibiotics in clinical use except
60 monobactams(6). MBLs such as New Delhi Metallo-beta-lactamase (NDM), Verona Integron-Borne
61 Metallo-β-Lactamase (VIM) and Imipenemase (IMP) mediate high-level resistance to antibiotics in
62 carbapenem-resistant *Enterobacteriaceae* (CRE)(7). CRE have been identified across the globe,
63 including in all African regions(8–10) and have been ranked as critical in the World Health Organization's
64 Priority Pathogens List for the Research and Development of New Antibiotics(11). It is therefore
65 imperative to invest in the discovery and development of MBL inhibitors (MBLIs) for combination therapy
66 with carbapenems.

67 Potential MBLIs investigated to date have shown substantial *in vitro* activity but cannot be used clinically
68 due to the simultaneous inhibition of human metallo-enzymes or their cytotoxicity effects(12, 13). MBLIs
69 such as aspergillomarasmine A (AMA)(13), Ca-ethylenediaminetetraacetic acid (Ca-EDTA)(12), 1,4,7-
70 triazacyclononane-1,4,7-triacetic acid (NOTA) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic
71 acid (DOTA)(14) are the few non-toxic MBLIs identified to date and act by chelating the zinc ions(15).

72 Herein, we investigated the inhibitory activity of 1, 4, 7-triazacyclononane (TACN) against well-
73 characterized sub-class B1 MBL-producing *Enterobacteriaceae*, and to the best of our knowledge, this is
74 the first time TACN has been investigated for its inhibitory activity against MBL-producing
75 *Enterobacteriaceae* to potentiate the activity of meropenem (MEM). TACN has a different chemical
76 structure with the smallest molecular weight in the family of metal chelator series reported. We believe
77 that exploring and understanding the mechanisms of biologically relevant chemical moieties is the surest
78 way to find clinically potent MBL Inhibitors that will give a lasting solution to this global menace.

79 **Results**

80 ***Minimum inhibitory concentrations (MICs), minimal bactericidal concentrations (MBCs), and***
81 ***MIC/MBC ratio***

82 TACN is a cyclic organic compound within the NOTA and DOTA series and derived from cyclononane by
83 replacing three equidistant CH_2 groups with NH groups(16) (Figure 1). It can be seen from the structures
84 that TACN is unique from that of NOTA and DOTA (family of compounds) (Figure 1). The results revealed
85 that this molecule (TACN) successfully restored the activity of MEM against characterized clinical isolates
86 of carbapenem-resistant *Enterobacteriaceae* expressing acquired sub-class B1 metallo-beta-
87 carbapenemases and reference strains with MIC values as low as 0.03 mg/L shown in table 1 and 2.
88 Also varying inhibitory concentrations of TACN considerably enhanced the efficacy of MEM against the
89 MBL-producing bacteria (varying from 2 mg/L to 64 mg/L). The lowest concentration of TACN at which
90 most of the MEM activity was restored was 8 mg/L (Table 1 and 2). Therefore, all subsequent
91 experiments were conducted with a fixed concentration of 8 mg/L (6.25%) of TACN. None of the tested
92 serine β-lactamases (SBLs) types (*K. pneumonia* OXA-48 and *E. cloacae* KPC-2) was affected by TACN,
93 substantiating the substrate spectrum of this compound (Table 1). MBCs of MEM were determined in the
94 presence of TACN at a fixed concentration, and the MBC/MIC ratio of the combination ranged from 1- to
95 4-fold against both reference and clinical CRE isolates used in this study, except two clinical isolates,

96 which had 8x the MIC values (Table 2). *K. pneumoniae* ATCC BAA 1706 and *E. coli* ATCC 25922 had
97 no changes in their MIC values when challenged with MEM alone and MEM+TACN.

98 ***Serum effect on MIC***

99 The MIC values of MEM-TACN combinations in the presence of 50% human serum varied from ±1- to 4-
100 fold dilutions compared to that of MEM-TACN in the absence of serum indicating that the inhibition
101 properties of the combination were not affected by the presence of human serum (Table 1).

102 ***Synergistic effect of TACN and MEM on CRE reference strains***

103 The synergy between TACN and MEM against CRE isolates was investigated. In most of the cases, the
104 minimum concentrations of TACN alone required to inhibit the MBL-producing *Enterobacteriaceae* were
105 ≥128 mg/L (Table 1). MEM alone exhibited inhibitory concentrations of ≥8 mg/L, indicating carbapenem-
106 resistant phenotype of these pathogens(17). Challenging these CREs with a combined regimen of MEM-
107 TACN potentiated the effectiveness of MEM. The calculated FIC index of MEM-TACN was ≤0.5,
108 indicating a synergistic effect (Table 1).

109 ***Time-kill kinetics***

110 The combination of MEM-TACN caused a considerable decrease in the CFU/mL relative to the initial
111 bacterial density of approximately 10^6 CFU/mL) over the increasing time points (0, 1, 2, 4, 6, 8 and 24 h)
112 against the tested isolates at different concentrations of the MIC values evaluated in this study (1x, 4x,
113 8x MICs) (Figure 2). A $3\log_{10}$ decrease in the CFU/ml was observed when cells were treated with MEM-
114 TACN at 1x, 4x and 8x MICs at 4 h. The time-kill kinetics results indicated that the TACN-MEM
115 combinations had bactericidal activity against the selected CRE isolates. MEM alone when challenged at
116 concentrations of 4x and 8x the MICs no significant decrease was observed during the time interval of
117 24 h.

118 ***Cytotoxicity***

119 The MTT assay was used to measure TACN cytotoxicity (0 –1024 mg/L) in HepG2 cells after 24-hour
120 exposure. A decrease in cell viability was associated with an increase in TACN concentration (Figure 3),
121 and the regression analysis yielded an IC_{50} of 56 mg/L. However, this IC_{50} of TACN was far higher than
122 the fixed MIC value of 8 mg/L used in this study.

123 ***Enzymatic assays***

124 TACN efficiently inhibited the activity of NDM-1 in a concentration- and time-dependent manner illustrated
125 by Figure 4A where the effect of increasing concentrations of TACN (0 – 50 µM) on the time-dependent
126 decrease in NDM-1 activity. The kinetic parameters were constructed by nonlinear regression of the K_{obs}
127 versus TACN concentration plot shown in figure 4B. TACN was found to be a potent time-dependent
128 inhibitor with an inhibition constant K_i and inactivation constant K_{inact} of 0.044±018 µM and 0.0406 ±0.007
129 (min^{-1}) respectively.

130 ***Computational simulations***

131 The fluctuations of enzyme-ligand complex and ligand-associated movements were analyzed to ascertain
132 their structural stability and movements during the simulation. These movements, which ensure their
133 functional reliability and working efficiency, are essential for functionality and existence inside the living
134 system. The overall systems showed appreciable stability as depicted in Figure 6. The root-mean-square
135 deviation (RMSD) of the entire system complex remained stable during the course of simulation of 100
136 ns.

137 The calculated binding free energy of TACN to NDM-1 and VIM-2 by the Molecular
138 Mechanics/Poisson−Boltzmann Surface Area *(MM/GBSA) protocol is shown in Table 3. The calculated
139 contributions favoring the inhibitors' binding include the electrostatic interactions (Δ Eele), which were -
140 9.4229 kcal/mol for NDM-1_{TACN} complex. The intermolecular van der Waals energy (Δ Evdw) for NDM-
141 1_{TACN} was -46.0898 kcal/mol, also favoring binding. The total binding free energies (Δ Gbind) for NDM-
142 1_{TACN} was -39.1603 kcal/mol. A similar trend was also observed in the complex VIM-2_{TACN} as shown in

143 table 3. The results suggest that the theoretical calculations of the binding free energies support the
144 experimental findings in term of the potentiality of TACN to inhibit the MBL enzymes.

145 To gain further understanding of the active-site residue interactions with the compounds, the simulated
146 complexes were observed with LigPlot protein-ligand interaction software. It was observed that the ligand
147 is attached with the active site residues in all complexes as shown in Figure 7. For TACN, His250, Asp124
148 residues provided H-bonding capability while His122, Asn220 and Trp93 roof the hydrophobic active-site
149 pocket. These residues are providing the hydrophobic surface area while maintaining H-bonding
150 capability.

151 **Discussion**

152 This study evaluated the TACN-MEM combination against clinical and reference CRE isolates and
153 identified TACN as a promising MBL inhibitor. The affinity of metal chelators for binding/sequestering
154 metal ions(18) such as zinc at MBLs' active sites makes them potential inhibitors that can be developed
155 for treating CRE infections. TACN is a metal chelating agent that exhibited no cytotoxic effect at effective
156 concentrations.

157 We showed that TACN significantly reduced the MIC of MEM, reinstating the carbapenem's efficacy
158 against 33 South African clinical isolates and 15 MBL-producing reference strains. MEM MICs decreased
159 by 4- to >512-fold changes in the presence of TACN against all the tested isolates, except for SBL
160 producers and susceptible ATCC strains. The SBL-producing *E. cloacae* KPC-2 and *K. pneumoniae*
161 OXA-48 only exhibited 2 or no fold decrease in MEM MICs, showing that the inhibitory effect of TACN
162 was specific for zinc-based β-lactamases. In our previous study NOTA and DPA exhibited similar activity
163 against MBLs at 4 and 8 mg/L(14, 19). Metal chelators are known for their strong affinity toward metal
164 ions(18). Class B MBLs possess zinc at their active sites, thus enabling the TACN to bind or sequester
165 the zinc ions and inhibit the enzymes' β-lactam hydrolysis activity.

166 MEM regained its pharmacological properties when combined with TACN. Most of the tested isolates
167 exhibited a MBC/MIC ratio of ≤ 4, except for two clinical isolates, *S. marcescens and E. cloacae,* where
168 the MBC/MIC ratio was 8 (Table 2). Based on the MBC/MIC ratio values, MEM demonstrated its known
169 bactericidal activity in the presence of TACN, and time-kill kinetic studies confirmed the bactericidal
170 property of MEM-TACN combinations. The MEM-TACN combination was synergistic, with an FIC index
171 ranging from ≤ 0.005 to 0.25 for all the CRE reference strains used in the study. Aspergillomarasmine A,
172 a metal chelating agent, was also found synergistic in combination with MEM toward MBLs(20).

173 The results also showed that serum had no substantial effect on the MICs of TACN-MEM combinations.
174 The minimal or no effects of serum on the activity of MEM was attributed to MEM's low protein-binding
175 properties(21). An investigation of the effects of serum on TACN or MEM's MICs may allow speculation
176 on the probable *in vivo* properties of the molecules at pre-clinical stage. Furthermore, TACN did not affect
177 the pharmacological property of MEM, suggesting that TACN could have low plasma protein-binding
178 properties either with or without MEM.

179 The MTT assay measures the ability of cells to reduce the MTT salt to a formazan product (22). This
180 reaction can only occur in living cells with healthy mitochondria, as it is reliant on NADPH-dependent
181 oxidoreductase enzymes. The cell viability obtained, therefore, reflects the number of viable cells present.
182 TACN exposure resulted in a dose-dependent decrease in cellular metabolic activity and viability in
183 HepG2 cells (Figure 3). However, the IC_{50} obtained was ≥4-fold higher than the TACN inhibitory dose (8
184 mg/L), and the cell viability was 91% at the upper limit of the inhibitory dose. This cytotoxicity analysis
185 on TACN found the compound possessing tolerable toxicity on HepG2 cell line at the applied
186 concentration, hence showing potential for development as MBLI for clinical use.

187 An experimental assay together with computational simulation demonstrated an excellent binding affinity
188 between ligand and enzyme complexes as well as potential inhibitory properties. It is known that Class
189 B β-lactamases are susceptible to inhibition by chelating agents during interactions between the chelators

190 (ligands) and the metal ions after ions are released from the proteins (MBL) active site or while still
191 associated with the enzyme complex(23). TACN possessed an apparent potential inhibition property
192 toward NDM-1 enzyme like Aspergillomarasmine A(20) as shown in figure 5. Overall, the calculated
193 variation of RMSD did not show considerable structural shifts, suggesting the stability of the enzyme
194 structure and strength of ligand attachment inside the active site pocket. The binding free energy analysis
195 showed that the intermolecular van der Waals and the electrostatic interaction were the forces driving
196 both systems. However, the total solvation energy (ΔGsol) was unfavorable for all the complexes. Even
197 though hydrogen bonds between the ligand and receptors were observed using LigPlot and chimera
198 (Figure 7), their influences could not compensate for the substantial desolvation penalties during the
199 interaction, thereby leading to the total solvation energy (ΔGsol). Similar results have been reported
200 previously where inhibition was dominated by the van der Waals interactions (24, 25).

201 In conclusion, this study showed that TACN inhibits Class B1 MBL-producing CREs with minimal
202 cytotoxic effect at effective concentrations. TACN successfully potentiated the antimicrobial properties of
203 MEM as should thus be further investigated as an MBLI for clinical development, in combination with
204 carbapenems. Molecular modeling and docking studies demonstrated the mechanistic pathway of TACN
205 to inhibit NDM-1 and VIM-2 sustaining a stable enzyme-ligand complex with the caveat that this is only
206 simulation of a prediction and not likely to represent the actual binding mode. In-depth analysis and
207 optimization of the metal-chelating agent (TACN), employing a multidisciplinary research approach
208 involving advanced computational simulations, biochemical, microbiological, bio-informatics are needed
209 in order to provide more evidence.

210 **Materials and methods:**

211 **Materials**

212 Meropenem, TACN, dimethyl sulfoxide (DMSO), human serum, cation-adjusted Mueller-Hinton broth
213 (CAMHB) and Mueller-Hinton agar (MHA), phosphate buffered saline (PBS), β-lactamase inhibitor

214 screening kit (MAK222) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were
215 purchased from Sigma Aldrich (St. Louis, MO, USA). Purified NDM-1 was obtained from RayBiotech,
216 Norcross, GA 30092, USA. Immortalized liver carcinoma (HepG2) cells and cell culture consumables
217 were procured from Highveld Biologicals (Johannesburg, South Africa) and Whitehead Scientific
218 (Johannesburg, South Africa) respectively.

219 **Bacterial isolates**

220 Seventeen carbapenem-resistant *Enterobacteriaceae* reference isolates (comprising 15 metallo-beta-
221 lactamases and two serine-beta-lactamases) obtained from France (Institut Pasteur de France) (Table
222 1)(26) and thirty-three clinical isolates of metallo-beta-lactamases obtained from Lancet laboratories
223 (private sector, South Africa) were employed in this study (Table 2)(27). *Klebsiella pneumoniae* ATCC
224 BAA 1706 and *Escherichia coli* ATCC 25922 purchased from the American Type Culture Collection
225 (ATCC) were susceptible reference strains used as quality control. All bacterial isolates were fully
226 characterized in previous studies for their phenotypic and genotypic resistance profiles(26, 27). The
227 isolates were sub-cultured twice from freezer stocks onto MHA plates and incubated at 37 °C before the
228 experiments. All subsequent liquid subcultures were derived from colonies isolated from the agar plates
229 and were grown in broth medium (CAMHB).

230 **Methods**

231 ***Minimum Inhibitory Concentrations (MICs), Minimum Bactericidal Concentrations (MBCs)***

232 MICs/MBCs determinations were performed according to CLSI guidelines and protocols described by
233 Keepers *et al.* (17, 28). Briefly, two-fold dilutions of MEM and TACN, from 0.015 to 16 mg/L and 1 to 64
234 mg/L respectively, were made with CAMHB in 96-well microtiter plates using the checkerboard
235 method(13). A 0.5 McFarland-standardized bacterial inoculum was suspended into 200 μl CAMHB in
236 each microtiter well. The plates were then incubated for 18-22 h at 37°C under aerobic conditions. The

237 MIC was determined as the lowest concentration at which there was no visible growth. Afterwards, an
238 aliquot of 100 µL was taken from the MIC assay wells in which no visible growth was observed and
239 inoculated onto MHA plates for MBC determination by incubating at 37 °C for 24 h. The MBC was
240 determined as the lowest concentration of the test compound that resulted in a ≥99.9% decrease of the
241 initial inoculum in the viable bacterial count on the agar plates. Control wells were filled with the amount
242 of solvent(s) used in dissolving drug candidates, CAMHB and bacteria. Experiments were conducted in
243 triplicate.

244 ***Serum effects on the MIC***

245 The effects of serum on the MIC of TACN-MEM combination were performed using the above-described
246 MIC method. In this assay, however, sterile-filtered serum from human male AB plasma was added to
247 the broth (CAMHB) to prepare 50% human serum in the final culture broth. Reference CRE strains
248 carrying different enzymes (NDM-1, NDM-4, VIM-1, IMP-1 and IMP-8) were used to conduct this
249 experiment.

250 ***Synergistic activity***

251 The Synergistic activity assay was used to describe the effect of compounds (TACN and MEM) working
252 together. The synergistic effect between TACN and MEM was determined by the combination assay as
253 described previously with few modifications(29). Briefly, two-fold serial dilutions of MEM were titrated with
254 a fixed concentration (8 mg/L) of TACN. This 8 mg/L TACN was the lowest concentration that did not
255 inhibit the growth of bacteria alone but considerably potentiated the activity of MEM. MEM and TACN
256 were also tested individually to determine their MICs. The fractional inhibitory concentration (FIC) index
257 was calculated as follows: FIC of MEM (MIC of MEM + TACN/MIC of MEM alone) + FIC of TACN (MIC
258 of MEM + TACN/MIC of TACN alone). The effect/activity of a combination with an FIC index of ≤ 0.5 was
259 considered synergistic while an FIC index of 1 was defined as additive and an FIC index of > 4 was
260 characterized as antagonistic.

261 ***Time-kill assays***

262 Time Kill assays were to measure the quantitative kinetic kill model of TACN for the selected organisms
263 and different time points. Time-kill kinetic studies were conducted referring to the previously reported
264 methods, including those described by CLSI (17, 28). *E. coli* producing bla_{NDM-1}, *E. coli* carrying bla_{VIM-1}
265 as well as *E. cloacae* producing bla_{IMP-1} were selected for this assay. Briefly, freshly prepared colonies
266 were re-suspended in 10 mL CAMHB and incubated in an orbital shaking incubator (37°C, 180 rpm) for
267 1 to 2 h. Cultures were then adjusted to a 0.5 McFarland standard (approximately 1.5x 10^8 CFU/mL) and
268 further diluted 1:20 in CAMHB so that the starting inoculum was approximately 1.5x 10^6 CFU/mL. MEM
269 was added to the prepared bacterial suspensions at final concentrations corresponding to 1x, 4x or 8x
270 the MIC of MEM while in combination with TACN at a fixed concentration of 8 mg/L. Also, the time-kill
271 kinetic of the MEM 4x and 8x MIC without TACN was investigated. A growth control with no antibiotic
272 was included in the assay. The initial bacterial load was determined from the growth control test tube
273 instantly after dilution step and was recorded as the CFU count at time zero. The test tubes were
274 incubated in an orbital shaking incubator at 37°C and 180 rpm, and bacterial cell viability counts were
275 performed at 1, 2, 4, 6, 8 and 24 hours by removing 100 µL of the culture, diluting as appropriate, and
276 plating on MHA. The agar plates were incubated at 37°C for at least 22 h. Colonies were counted, and
277 the results were recorded as the number of CFU/mL. A ≥3-$\log_{10}$ decrease within 24 hours in the number
278 of CFU/mL was considered bactericidal. The assays were executed in triplicate.

279 ***Cytotoxicity Assay***

280 HepG2 cells were maintained at 37°C in 10% complete culture medium (CCM; Eagles minimum essential
281 medium supplemented with fetal bovine serum, antibiotics and L-glutamine) until confluent. Following
282 trypsinization, 1.5x 10^4 cells/well were allowed to adhere to a 96-well plate overnight. The cells were
283 treated with seven dilutions of TACN (16 – 1024 mg/L) prepared in CCM. Untreated cells (CCM only)
284 served as the control. After 24 hours the treatment was replaced with 20µl MTT solution (5mg/mL MTT

285 in PBS) and 100µl CCM for 4 hours. The resulting formazan product was solubilized in 100µl DMSO (1
286 hour), and the absorbance at 570nm/690nm was determined (BioTek µQuant plate reader; BioTek
287 Instruments Inc., USA). The average absorbance values were used to calculate cell viability
288 ($\frac{Absorbance\ of\ treatment}{Absorbance\ of\ control}\ x\ 100$) and determine the IC_{50} (GraphPad Prism® v5.01). All experiments
289 were performed in triplicate.

290 ***Enzymatic assays: Enzyme inhibition and inactivation kinetic parameters***

291 The inhibitory effect of TACN was monitored by observing the rate of hydrolysis of the chromogenic
292 nitrocefin, using purified preparations of sub-class B1 MBL enzyme (NDM-1) obtained from Raybiotech
293 (Norcross, GA 30092, USA). The assays were performed using the β-lactamase inhibitor screening kit's
294 (MAK222) according to the manufacturer's instructions. Briefly, 5 nM of NDM-1 was pre-incubated with
295 various concentrations of TACN varying from 0 - 50 µM at 25°C in a final experimental volume of 100 µL
296 of β-lactamase assay buffer before adding nitrocefin. The nitrocefin hydrolysis was monitored at 25°C by
297 following the absorbance variation at 490nm using a plate reader spectrophotometer (SPECTROstar^{Nano}
298 BMG Labtech, Germany). The residual activity was determined and compared to that from the pre-
299 incubation of the enzyme with the same procedure but without TACN. The effect of TACN-mediated
300 inactivation of NDM-1 was determined by plotting the natural logarithm (LN)-linear plot of the percentage
301 of the remaining enzyme residual activity versus the pre-incubation time, and the observed initial
302 inactivation rate constant (K_{obs}) was calculated from the pseudo-first-order kinetic slope. The rate
303 constant for enzyme inactivation (k_{inact}) and the inactivation constant (K_I) were calculated (GraphPad
304 Prism5; Software Inc., San Diego, CA) according to the following hyperbolic equation as previously
305 described by Wong *et al.* (30):

306 $$Kobs = \frac{Kinact - [I]}{Ki + [I]}$$

307 Where K_{obs} is the initial rate constant for TACN inactivation and [I] is the initial inhibitor concentration.

308 The preliminary mode of inhibition of NDM-1 by TACN was also assessed using the β-lactamase inhibitor
309 screening kit reagents. Briefly, 500 nM of purified NDM-1 was pre-incubated with 20 µM of TACN at 25°C
310 for 20 min, followed by rapid dilution with the addition of 30 µM of nitrocefin; after which, changes in
311 absorbance were monitored at 490 nm for 30 min. Experiments were conducted in triplicate.

312 ***Molecular modelling***

313 ***System preparation***

314 The crystal structures (PDB ID: 3Q6X, and 5ACU) were retrieved from the RSCB Protein Data Bank
315 (https://www.rcsb.org/pdb/). The missing residues were added using a graphical user interface of
316 Chimera, a molecular modelling tool(31). A ligand interaction map was generated using the web version
317 of PoseView(32).

318 ***Molecular Docking***

319 Docking calculations were obtained using AutoDock Vina software(33). Geister partial charges were
320 assigned, and the AutoDock atom types were defined using the AutoDock graphical user interface
321 supplied by MGL tools(34). The docked conformations were generated using the Lamarckian genetic
322 algorithm (LGA)(35). The binding affinities calculated for each of the two structures (enzyme: inhibitor
323 complexes) were expressed in kcal/mol. This technique has been validated in previous studies(36). The
324 grid box was defined using Autodock Vina with the grid parameters being X= 26, Y= 18 and Z= 20 for the
325 dimensions and X= -2.502, Y= -5.255 and Z= 32.723 for the center grid box. After using Autodock Vina,
326 the ten conformations with lowest binding energy were chosen for molecular docking.

327 ***Molecular dynamics simulation***

328 Missing parameters for the ligand in the Cornell et al. force field were created (37) in the absence of
329 available parameters. Optimization of the ligands was first performed at the HF/6-31G* level with the
330 Gaussian 03 package. The restrained electrostatic potential (RESP) procedure(38) was used to calculate

331 the partial atomic charges. General amber force GAFF(39) force field parameters and RESP partial
332 charges were assigned using the ANTECHAMBER module in the Amber14 package. Hydrogen atoms
333 of the proteins were added using the Leap module in Amber12. The standard AMBER force field for
334 bioorganic systems (ff03) was used to define the enzyme parameters. Counter ions were added to
335 neutralize the enzyme charge. The system was enveloped in a box of equilibrated TIP3P water molecules
336 with 8 Å distance around the enzyme. Cubic periodic boundary conditions were imposed, and the long-
337 range electrostatic interactions were treated with the particle-mesh Ewald method (40) implemented in
338 Amber12 with a non-bonding cut-off distance of 10 Å.

339 Initial energy minimization, with a restraint potential of 2 kcal/mol $Å^2$ applied to the solute, was carried out
340 using the steepest descent method in Amber12 for 1000 iterations followed by conjugate gradient protocol
341 for 2000 steps. The entire system was then freely minimized for 1000 iterations. Harmonic restraints with
342 force constants 5 kcal/mol $Å^2$ were applied to all solute atoms during the heating phase. A canonical
343 ensemble constant number (N), volume (V), and temperature (NVT) MD was carried out for 50 ps, during
344 which the system was gradually annealed from 0 to 300 K using a Langevin thermostat with a coupling
345 coefficient of 1/ps. Subsequently, the system was equilibrated at 300 K with a 2 fs time step for 100 ps
346 while maintaining the force constants on the restrained solute. The SHAKE algorithm(41) was employed
347 on all atoms covalently bonded to a hydrogen atom during equilibration and production runs. A production
348 run was performed for 2 ns in an isothermal-isobaric (NPT) ensemble using a Berendsen barostat(42)
349 with a target pressure of 1 bar and a pressure coupling constant of 2 ps with no restraints imposed. The
350 coordinate file was saved every 1 ps, and the trajectory was analyzed every 1 ps using Ptraj module
351 implemented in Amber14.

352 **Author contributions:**

353 Co-conceptualized the study: AMS, DGA, JOS, LAB and SYE. Performed the experiments: AMS, DGA,
354 HMK and RK. Analyzed the data: AMS, DGA, HMK and RK. Vetting of the results: All. Wrote the paper:
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470 Table 1: Inhibitory activity of TACN and MEM alone and in combination against reference strains of carbapenem-resistant Enterobacteriaceae.

Bacterial species	Metallo-β-lactamase produced (Class B)	MIC (mg/L)					MBC (mg/L)	MIC/MBC (mg/L)	Serum effect (mg/L)	FIC index
		MEM alone	TACN alone	{MEM-TACN4*}	{MEM-TACN8*}	{MEM-TACN16*}	MEM-TACN8*	MEM-TACN8*	MEM-TACN8*/Serum	
Metallo-beta-lactamases										
E. coli	NDM-1	128	1024	{0.125-4}	{0.125-8}	{0.06-16}	0.5	2	0.25	0.008
E. cloacae	NDM-1	16	>1024	{{0.06-4}	{0.03-8}	-	0.125	2	0.125	≤0.005
C. freundii	NDM-1	8	1024	{0.25-4}	{0.125-8}	{0.125-16}	0.125	1	0.25	0.023
P. rettgeri	NDM-1	64	>1024	{8-4}	{2-8}	-	2	1	2	≤0.035
P. stuartii	NDM-1	16	>1024	{2-4}	{2-8}	-	2	1	4	≤0.253
E. coli	NDM-4	128	1024	-	{0.125-8}	{0.06-16}	0.125	1	1	0.008
E. coli	VIM-1	32	1024	{0.25-4}	{0.125-8}	{0.06-16}	0.125	1	0.25	0.011
K. pneumoniae	VIM-1	32	512	{2-4}	{0.125-8}	{0.06-16}	0.5	4	8	0.019
E. cloacae	VIM-1	8	128	{1-4}	{1-8}	-	1	1	2	0.187
E. coli	VIM-2	8	128	{0.06-4}	{0.03-8}	-	0.06	1	0.125	0.066
E. cloacae	IMP-1	32	512	{0.25-4}	{0.125-8}	-	0.5	2	2	0.019
K. pneumoniae	IMP-1	32	512	{8-4}	{4-8}	-	4	1	16	0.141
E. coli	IMP-1	16	512	{1-4}	{0.06-8}	{0.03-16}	0.125	2	0.25	0.019
E. cloacae	IMP-8	8	512	{0.5-4}	{0.125-8}	{0.03-16}	0.25	2	0.125	0.031
K. pneumoniae	IMP-8	16	1024	{8-4}	{4-8}	{2-16}	8	2	4	0.250
Serine-beta-lactamases										
K. pneumoniae	OXA-48	8	128	{8-8}	{8-16}	{4-64}	NA**	NA	NA	NA
E. cloaeae	KPC-2	32	256		{32-32}	{32-64}	NA	NA	NA	NA
ATCC susceptible strains										
K. pneumoniae ATCC BAA 1706	Susceptible strain	0.5	1024	NE***			NA	NA	NA	NA
E. coli ATCC 25922	Susceptible strain	0.03	1024	NE			NA	NA	NA	NA

471 *Fixed concentration of TACN (4, 8, 16 mg/L etc…) with varying concentrations of Meropenem **Not applicable *** No inhibitory effect

472 *Table 2: Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of TACN against South African clinical isolates.*

Bacterial species and isolates	MBLs produced (Class B)	Other β-lactamase (s)	MEM with 8 mg/L of TACN		
			MIC (mg/L)	MBC (mg/L)	MIC/MBC ratio (mg/L)
K. pneumoniae (N=11)					
D(UNN40_S4)	NDM-1	DHA-1, CTX-M-15, SHV-1, OXA-1	0.5	2	4
C(UNN39_S3)	NDM-1	SHV-1, CTX-M-15, OXA-1, DHA-1	0.5	2	4
I(UNN45_S9)	NDM-1	OXA-1, CTX-M-15, SHV-99, TEM-1B	4	8	2
J(UNN46_S10)	NDM-1	OXA-1, CTX-M-15, SHV-1&-28, DHA-7	0.5	2	4
53_S27	NDM-1	OXA-1, DHA-1, CTX-M-15, SHV-1&-28,	4	16	4
12_S5	NDM-1	CTX-M-15, SHV-1&-28, DHA-1, OXA-1	2	8	4
13_S6	NDM-1	CTX-M-15, SHV-1&-28, DHA-7	4	16	4
20_S11	NDM-1	CTX-M-15, TEM-1A, OXA-9, SHV-1&-28, OXA-1&-9	0.125	1	4
29_S13	NDM-1	CTX-M-15, SHV-1&-28, OXA-1&-9, TEM-1A	0.125	1	4
32_S15	NDM-1	DHA-1, CTX-M-15, SHV-1&-28, OXA-1	0.125	1	4
21_S12	NDM-1	TEM-1A, SHV-1&-28, CTX-M-15, OXA1&-9	0.5	1	2
S. marcescens (N=10)					
B(UNN38_S2)	NDM-1	OXA-10, OXA-1, CTX-M-15, SCO-1, TEM-133, TEM-198	0.125	1	4
E(UNN41_S5)	NDM-1	CTX-M-15, SCO-1, TEM-198, OXA-10, OXA - 1	1	2	2
G(UNN43_S7)	NDM-1	OXA-10, TEM-1B	1	4	4
K(UNN47_S11)	NDM-1	TEM-1B, OXA-1, OXA-10, SCO-1	2	16	8
L(UNN48_S12)	NDM-1	CTX-M-15, OXA-10, OXA -1, SCO-1, TEM-1	2	4	2
7_S3	NDM-1	TEM- 1B, OXA-10	2	8	4
56_S29	NDM-1	NONE	0.5	2	4
59_S30	NDM-1	TEM-1B, CTX-M-3	1	2	2
67_S33	NDM-1	OXA-10, CTX-M -15, OXA-1, TEM-1, SCO-1	4	16	4
71_S36	NDM-1	CTX-M-11, TEM -1B, OXA -10, OXA-1, SCO-1	2	2	1
E. cloacae (N=9)					
A(UNN37_S1)	NDM-1	CTX-M-3, ACT-3	0.5	1	2
F (UNN42_S6)	NDM-1	CTX-M-15, TEM -1B, OXA-1, ACT-7	0.5	2	4
H(UNN44_S8)	NDM-1	CTX-M-15, TEM -1B, OXA-1, ACT-14	1	1	1
16_S9	NDM-1	CTX-M-15, OXA-1, ACT-4,	0.125	2	8
43_S20	NDM-1	TEM -1B, SHV-12, MIR-1	2	4	2
49_S24	NDM-1	CTX-M-15, TEM-1B, OXA-1, ACT-3, CMY-77	0.5	1	2
51	NDM-1	CTX-M-3, TEM-1B, CMY-77	0.25	1	4
55_S28	NDM-1	TEM -1B, ACT-4, SHV-12	4	2	2

Bacterial species and isolates	MBLs produced (Class B)	Other β-lactamase (s)	MEM with 8 mg/L of TACN		
			MIC (mg/L)	MBC (mg/L)	MIC/MBC ratio (mg/L)
63_S31	NDM-1	TEM-1B, ACT-1	0.5	2	4
E.coli (N=1)					
10	NDM-5	CMY-42	2	4	2
C. freundii (N=1)					
48	NDM-1	CTX-M-3, TEM-1B, CMY-77	0.25	2	4
K.oxytoca (N=1)					
69	NDM-1	CTX-M-3, TEM-1B, OXY-14	0.25	2	4

Table 3: MM/GBSA binding free energyprofile for NMD1$_{TACN}$, and VIM-2$_{TCN}$

Binding free energy profile					
Systems	**Evdw**	**Eelec**	**ΔGsolv**	**ΔGgas**	**ΔGbind**
NDM-1$_{TACN}$ complex	-46.0898	-9.4229	16.3523	-55.5126	-39.1603
VIM-2$_{TACN}$ complex	-50.9300	-9.9143	19.6230	-63.4178	-41.2199

ΔG_{bind}- binding energy; ΔE_{ele} – electrostatic; ΔE_{vdw} – van der waals; ΔE_{gas} - gas-phase energy; ΔE_{sol} – solvation energy. All binding free energy profile are expressed in Kcal/mol.

481 **List of figures**

482 **Figure 1:** Chemical structures of [1]: 1, 4, 7-triazacyclononane (TACN), [2]: 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) and [3]: 1,4,7,10-
483 tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA).

484 **Figure 2:** Time kill kinetics at varying concentrations of MEM and fixed concentration of TACN (8 mg/mL)**.** A) *E. coli* producing NDM-1; B). *E. coli* producing VIM-
485 1; C) *E. cloacae* producing IMP-1 were challenged at 1x, 4x and 8x MIC levels with MEM-TACN combination. The surviving CFU were plated at different time
486 intervals (1, 2, 4, 6, 8, 24 hours). All data points represent the average results for 3 independent experiments.

487 **Figure 3:** TACN induced a dose-dependent decrease in HepG2 cell viability following treatment for 24-hour treatment. Higher TACN concentrations resulted in
488 increased cell death.

489 **Figure 4:** A) Time- and concentration-dependent inhibition of NDM-1 by TACN, B) The logarithmic plot of kobs of TACN against TACN concentrations.

490 **Figure 5:** Residual activity after rapid dilution of enzyme-inhibitor complex. The absorbance changes from the co-incubation of NDM-1 with no inhibitor (●) and
491 NDM-1 + TACN (□) are shown. Slope of the initial rate represents the hydrolytic activity of NDM-1.

492 **Figure 6:** Time-dependent RMSD plots of A) $NDM1_{TACN,}$ B) $VIM\text{-}2_{TACN.}$ The complexes are represented in black and the free enzymes in red.

493 **Figure 7:** Binding mechanism, illustrated by the interaction of docked ligand (TACN) at the active side of NDM-1 (A) and VIM-2 (B).