

1 **Manganese complex [Mn(CO)<sub>3</sub>(tpa-κ<sup>3</sup>N)]Br increases antibiotic sensitivity in multidrug**  
2 **resistant *Streptococcus pneumoniae***

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14 **Running title:** Re-sensitizing multidrug resistant *S. pneumoniae*

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17

18 **Abstract**

19 The emergence of multidrug-resistance (MDR) in *Streptococcus pneumoniae* clones and non-  
20 vaccine serotypes is of increasing concern, necessitating the development of novel treatment  
21 strategies. Here, we determined the efficacy of the Mn complex  $[\text{Mn}(\text{CO})_3(\text{tpa-}\kappa^3\text{N})]\text{Br}$   
22 against MDR *S. pneumoniae* strains. Our data showed that  $[\text{Mn}(\text{CO})_3(\text{tpa-}\kappa^3\text{N})]\text{Br}$  has *in vitro*  
23 and *in vivo* antibacterial activity and has the potential to be used in combination with currently  
24 available antibiotics to increase their effectiveness against MDR *S. pneumoniae*.

## 25 **Introduction**

26 *Streptococcus pneumoniae* is the main cause of community-acquired pneumonia, meningitis  
27 and bacteremia in children and adults (1), with pneumonia remaining the leading cause of  
28 death in children under 5 years of age worldwide (2). In addition, pneumococcal disease  
29 causes the most deaths among vaccine-preventable diseases, according to the World Health  
30 Organization (WHO) (3). Although the available pneumococcal vaccines have reduced  
31 invasive pneumococcal disease (IPD), current vaccines only protect only against a fraction of  
32 the more than 97 circulating serotypes. Eradication of the vaccine-included serotypes has  
33 caused rapid serotype replacement, followed by an increase in the carriage, prevalence and  
34 disease caused by non-vaccine serotypes (4). As a consequence of the incomplete protection  
35 against circulating serotypes, antibiotic therapy remains a mainstay of IPD treatment (5).

36 The emergence of multidrug-resistant *S. pneumoniae* strains worldwide compromises  
37 the available treatment options for IPD (6-11) and imposes the need for alternatives to  
38 traditional anti-pneumococcal agents. Manganese-carbonyl complexes, such  $[\text{Mn}(\text{CO})_3(\text{tpa}-$   
39  $\kappa^3\text{N})]\text{Br}$ , have been proposed as antibacterials against Gram-negative bacteria (12, 13),  
40 especially in combination with membrane permeabilisers like colistin (14). Although their  
41 mechanism of action is still elusive, a combination of membrane disruption, interference of  
42 metal ion uptake and inhibition of respiration has been proposed (15). Previous data suggests  
43 that the manganese-cologand core of the title compound does not reach the intracellular  
44 environment in Gram-negative bacteria, possibly due to the inability of the compound to cross  
45 the outer bacterial membrane (16), but their antibacterial activity against Gram-positive  
46 bacteria is yet to be explored.

47 The aim of this study was to evaluate the *in vitro* and *in vivo* activity of manganese  
48 complex  $[\text{Mn}(\text{CO})_3(\text{tpa}-\kappa^3\text{N})]\text{Br}$  alone or in combination with commonly used antibiotics  
49 against multidrug-resistance strains of *S. pneumoniae*.

50

## 51 **Material and Methods**

52

### 53 **Bacterial Strains, growth conditions and media**

54 A total of 20 human-derived clinical non-duplicate invasive and multidrug resistant *S.*  
55 *pneumoniae* strains were provided by the CDC *Streptococcus* Laboratory and were included  
56 in the study. Their relevant characteristics are indicated in Table S1. *S. pneumoniae* strains  
57 were grown in cation-adjusted Mueller-Hinton broth (BD, New Jersey, USA) supplemented  
58 with 100 U of catalase (Worthington Biochemical Corporation, New Jersey, USA) and 20  
59 mg/L  $\beta$ -NAD (Sigma-Aldrich, St. Louis, USA) at 37°C under 5% CO<sub>2</sub> for 18-24 h. Blood  
60 agar plates were made from Tryptic soy agar (BD, New Jersey, USA) with the addition of  
61 0.5% yeast extract (BD, New Jersey, USA) and 5% defibrinated horse blood (Sanbio, Uden,  
62 The Netherlands). [Mn(CO)<sub>3</sub>(tpa- $\kappa^3$ N)]Br (USC-CN028) was synthesised according to a  
63 previously published procedure (13).

64

### 65 **MIC and MBC determination**

66 Minimum inhibitory concentrations (MICs) were determined in triplicate by broth  
67 microdilution according to European Committee on Antimicrobial Susceptibility Testing  
68 (EUCAST; <http://www.eucast.org>) and ISO 20776-1:2006 guidelines with the exception that  
69 cation-adjusted Mueller-Hinton broth (BD, New Jersey, USA) was supplemented with 100 U  
70 of catalase (Worthington Biochemical Corporation, New Jersey, USA) instead of 5% lysed  
71 horse blood. The lowest concentration of compound where no turbidity was observed was  
72 noted as the MIC. *S. pneumoniae* ATCC 49619 was used as quality control.

73 Minimum bactericidal concentration (MBC) of [Mn(CO)<sub>3</sub>(tpa- $\kappa^3$ N)]Br was determined  
74 using a resazurin-based microtiter plate assay as previously described (17). After adding 20  
75  $\mu$ L of 0.15 mg/mL resazurin (Cayman Chemical Company, Michigan, USA) solution in PBS  
76 to each well, plates were incubated at 37°C and the color conversion of all wells was

77 recorded. The lowest well concentration of  $[\text{Mn}(\text{CO})_3(\text{tpa-}\kappa^3\text{N})]\text{Br}$  to remain blue was  
78 considered the MBC. All assays were performed in triplicate.

79

### 80 **Disc diffusion synergy test and checkerboard assays**

81 Synergy between  $[\text{Mn}(\text{CO})_3(\text{tpa-}\kappa^3\text{N})]\text{Br}$  and 11 anti-pneumococcal agents was assessed by a  
82 modified disk diffusion test of the EUCAST method, in that a disk of each of the anti-  
83 pneumococcal agents was tested with and without the addition of 64  $\mu\text{g}$  of  $[\text{Mn}(\text{CO})_3(\text{tpa-}$   
84  $\kappa^3\text{N})]\text{Br}$ . A decrease in the inhibition zone diameter for the combination discs versus the discs  
85 alone was considered suggestive of synergy.

86 To confirm the observed synergies and determine their magnitude, checkerboard  
87 assays were performed for three randomly selected strains (SP25, SP96 and SP30) using an  
88 inoculum of approximately  $10^5$  CFU/ml onto each well and a 2-fold dilution scheme. The  
89 fractional inhibitory concentration (FIC) for each well and the FIC index were calculated as  
90 previously described (18). All assays were performed in triplicate.

91

### 92 **Time-kill assays**

93 Time-kill assays were performed in triplicate using approximately  $10^5$  CFU/mL as the starting  
94 inoculum for each strain and antimicrobials were added at the following final concentrations:  
95  $[\text{Mn}(\text{CO})_3(\text{tpa-}\kappa^3\text{N})]\text{Br}$  (1 x MIC), tetracycline (1 x and 2 x MIC) and the Mn complex-  
96 tetracycline combination (0.5 x MIC – 1 x MIC). Cultures were incubated at 37°C under 5%  
97  $\text{CO}_2$  continuous agitation (225 rpm) for 24 h. At set time points of 0, 30 min, 1, 2, 4 and 24 h  
98 post inoculation, 100  $\mu\text{L}$  samples were collected, serially diluted and cultured onto blood agar  
99 plates for viable cell titer determination. Time–kill curves (CFU/ml vs time) were plotted  
100 using GraphPad Prism 8.2.1 software. Synergy was defined as bactericidal activity ( $\geq 2 \log_{10}$   
101 difference in CFU/mL) of the combination compared with either agent alone, after 24 h  
102 incubation. Unpaired student t-tests were performed to check for significant differences.

103

## 104 ***Galleria mellonella* treatment assays**

105 *S. pneumoniae* inocula of approximately 0.3 OD<sub>600</sub> (equating to ~10<sup>8</sup> CFU/mL) in phosphate  
106 buffered saline (PBS) were serially diluted in PBS and colony forming units were determined  
107 by plating the dilutions on blood agar and incubating for 24 h. Sixteen *Galleria mellonella*  
108 larvae (TruLarv™, Biosystems Technology, Exeter, U.K) were infected with 10<sup>5</sup> CFU/larvae  
109 of each *S. pneumoniae* strain (SP25, SP30 and SP96) via a 10 µL injection in a left proleg as  
110 previously described (19). Within 30 min of infection, a second injection into a right proleg  
111 was performed to administer the Mn complex (2.56 mg/kg in PBS), tetracycline (0.64 mg/kg),  
112 a combination of Mn complex and tetracycline (2.56 + 0.64 mg/kg) or PBS, respectively.  
113 Larvae were incubated at 37°C and scored for survival (live/dead) at 0, 24, 48, 72 and 96 h  
114 post inoculation.

115 Melanisation scores for larvae were recorded over 96 h as an indicator of morbidity,  
116 based on a reversed scoring method previously published (20), whereby a score of 4 indicated  
117 total melanisation of the larvae, 2 indicated melanin spots over the larvae, 1 indicated  
118 discoloration of the tail and a score of 0 indicated no melanisation.

119 All assays were performed in triplicate and the data was plotted using GraphPad Prism  
120 8.2.1 software (San Diego, CA, USA). Analysis of survival curves was performed using the  
121 log rank test, with a *p* value of ≤ 0.05 indicating statistical significance (21). Unpaired student  
122 t-tests were performed to check for differences in bacterial counts at 24 h.

123

## 124 **Results and discussion**

125 The antibacterial activity of [Mn(CO)<sub>3</sub>(tpa-κ<sup>3</sup>N)]Br was studied on 20 *S. pneumoniae* clinical  
126 isolates exhibiting genotypically-confirmed multidrug-resistance phenotypes (Table S1).  
127 [Mn(CO)<sub>3</sub>(tpa-κ<sup>3</sup>N)]Br was weak against *S. pneumoniae*, with MICs ranging from 64 mg/L (*n*  
128 = 11; 55%) to 128 mg/L (*n* = 9; 45%) (Table S1). However, this is 8- to 16-fold more active

129 than previously shown against multidrug-resistant *E. coli* (14). This enhanced activity is  
130 potentially due to the absence of the Gram-negative outer lipopolysaccharide membrane  
131 known to reduce the permeability of many antimicrobials (22). The MBCs for all tested  
132 strains were equal to the MICs, suggesting bactericidal activity of  $[\text{Mn}(\text{CO})_3(\text{tpa-}\kappa^3\text{N})]\text{Br}$ .  
133 Time-kill assays for three randomly selected strains, SP25, SP30 and SP96, confirmed its  
134 bactericidal activity with total bacterial death observed within 2 h (SP25 and SP96) or 24 h  
135 (SP30) at 1x MIC (Figure 1a).

136 The potential synergistic effect of the Mn complex with 11 other anti-pneumococcal  
137 agents against SP25, SP30 and SP96 was assessed by a combination disc diffusion test. All  
138 three strains showed a decreased diameter of inhibition zone only for the combinations of  
139 tetracycline, erythromycin and co-trimoxazole with the Mn complex versus these agents  
140 alone, suggesting synergy between these antibiotics and  $[\text{Mn}(\text{CO})_3(\text{tpa-}\kappa^3\text{N})]\text{Br}$  (Table S2).  
141 To examine strain-specific effects, we analyzed these same synergistic combinations for the  
142 remaining 17 multidrug-resistant *S. pneumoniae* strains by a combination disc diffusion test.  
143 Among them, eight (47.0%) exhibited decreased diameter of inhibition zone for tetracycline  
144 (ranging from 1 to 2 mm), 10 (58.8%) for erythromycin (ranging from 1 to 7.5 mm) and 13  
145 (76.5%) for co-trimoxazole (ranging from 1 to 2 mm) (Table S2).

146 Checkerboard assays for SP25, SP30 and SP96 indicated that the Mn complex was  
147 able to increase susceptibility of tetracycline even against tetracycline-resistant strains of *S.*  
148 *pneumoniae*, with tetracycline MICs falling below the susceptibility breakpoint of 1 mg/L.  
149 Similar results showed that resistant strains were resensitized to erythromycin- and the co-  
150 trimoxazole-Mn complex combination (Table 1). Fractional inhibitory concentrate indexes  
151 were calculated and indicated that synergy was observed between co-trimoxazole and  
152  $[\text{Mn}(\text{CO})_3(\text{tpa-}\kappa^3\text{N})]\text{Br}$  against all strains (FICI = 0.002-0.26) and against 2 out of 3 strains  
153 with combinations of  $[\text{Mn}(\text{CO})_3(\text{tpa-}\kappa^3\text{N})]\text{Br}$  with tetracycline (FICI = 0.123-0.28) and

154 erythromycin (FICI = 0.28-0.31), with intermediate/additive activity observed with the  
155 remaining strains (0.75-2).

156 Synergy between  $[\text{Mn}(\text{CO})_3(\text{tpa-}\kappa^3\text{N})]\text{Br}$  and tetracycline was confirmed using time-  
157 kill assays for the same strains, where a subinhibitory concentration of the Mn complex not  
158 only restored the activity of tetracycline, but was bactericidal. Bacteria were completely  
159 eradicated at 4 h (SP30 and SP96) and 24 h (SP25) with the combination, versus tetracycline  
160 alone, where an increase in bacterial numbers ( $10^7 - 10^8$  CFU/mL) was observed at 24 h  
161 (Figure 1a). Previous studies have also highlighted synergy between doxycycline and  
162  $[\text{Mn}(\text{CO})_3(\text{tpa-}\kappa^3\text{N})]\text{Br}$  against *E. coli* by reducing the expression of *tet(A)* (16). Therefore it is  
163 logical to postulate that in *tet(M)*-encoding *S. pneumoniae*,  $[\text{Mn}(\text{CO})_3(\text{tpa-}\kappa^3\text{N})]\text{Br}$  may  
164 reduce the expression of *tet(M)*, increasing susceptibility to tetracycline. Further studies are  
165 needed to confirm the mechanism of synergy in *S. pneumoniae*.

166 To evaluate the efficacy of the tetracycline-Mn complex combination *in vivo*, *G.*  
167 *mellonella* larvae were infected with *S. pneumoniae* strains SP25, SP30 and SP96. Overall,  
168 data from *in vivo* experiments show a significant difference between tetracycline monotherapy  
169 and the tetracycline-Mn complex combination ( $p = <0.049$ ). With only PBS therapy,  
170 infections with SP25, SP30 and SP96 resulted in mortality rates of 75%, 62.5% and 87.5%,  
171 respectively (Fig 1b), reflecting intrinsic differences in strain virulence. Treatment with the  
172 tetracycline-Mn complex combination was superior to monotherapy with either tetracycline or  
173 the Mn complex, resulting in significantly lower mortality in infected larvae (20.8% vs 47.9%  
174 and 45.8% respectively). Consistent with mortality data, high melanisation scores indicated a  
175 strong immune response in *G. mellonella* infected with strains SP25, SP30 and SP96 (Fig.  
176 1c), with mean scores of 47 (+/- 2.3), 63 (+/- 2.7) and 63 (+/- 1.3) out of a maximum of 64 for  
177 each strain, respectively. Melanisation was reduced in larvae treated with the tetracycline-Mn  
178 complex combination, compared with tetracycline and Mn complex monotherapy, with mean



179 scores of 18.6 (+/- 7.2), 34.4 (+/- 9.1) and 33.7 (+/- 6.4) respectively. Doses of  $[\text{Mn}(\text{CO})_3(\text{tpa}-$   
180  $\kappa^3\text{N})]\text{Br}$  used in this study for treatment of *S. pneumoniae* infections, were more than 70 times  
181 lower than the concentration previously shown to be toxic 24 h post-administration in *G.*  
182 *mellonella* (14).

183 In conclusion, our results show that  $[\text{Mn}(\text{CO})_3(\text{tpa}-\kappa^3\text{N})]\text{Br}$  used in combination with  
184 traditional antibiotics like tetracycline, erythromycin and co-trimoxazole, may have potential  
185 as antimicrobial and resistance breaker against multidrug-resistant *S. pneumoniae*.

186 **Figure 1. (a)** Time-kill curves of  $[\text{Mn}(\text{CO})_3(\text{tpa-}\kappa^3\text{N})]\text{Br}$ , tetracycline and combination of  
187 both agents ( $\times 1 \text{ MIC} + 0.5 \text{ MIC}$ ) versus *S. pneumoniae* strains SP25, SP30 and SP96 over 24  
188 h. **(b)** Survival curves (live/dead) of *Galleria mellonella* over 96 h after infection with  $10^5$   
189 CFU/larvae of strains SP25, SP30 and SP96 and treatment with phosphate buffered saline  
190 (PBS), 2.56 mg/kg of  $[\text{Mn}(\text{CO})_3(\text{tpa-}\kappa^3\text{N})]\text{Br}$ , 0.64 mg/kg of tetracycline, and combination of  
191 both agents. **(c)** Melanisation assays in *G. mellonella* under the same conditions for strains  
192 SP25, SP30 and SP96.

193 **Table 1.** Minimum inhibitory concentrations (MICs) and fractional inhibitory concentration index (FICI) of the antibiotics tetracycline (TET),  
 194 erythromycin (ERY) and co-trimoxazole (SXT) alone and in combination with the Mn complex  $[\text{Mn}(\text{CO})_3(\text{tpa}-\kappa^3\text{N})]\text{Br}$  against multidrug-  
 195 resistant *S. pneumoniae* strains included in this study.

Sample ID	Mn (mg/L)	TET + Mn (mg/L)	TET (mg/L)	FICI	ERY + Mn (mg/L)	ERY (mg/L)	FICI	SXT + Mn (mg/L)	SXT (mg/L)	FICI
SP25	64	0.03	0.5	0.123	0.5	64	0.31	0.125	8	0.14
SP30	128	4	8	0.75	64	64	2	0.00025	0.25	0.002
SP96	128	0.25	8	0.28	0.5	64	0.28	0.015	2	0.26

196

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## 205 206 **Transparency declarations**

207 None to declare.

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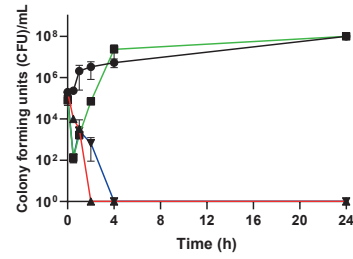
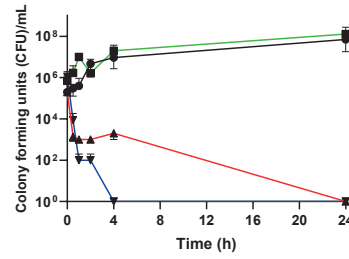
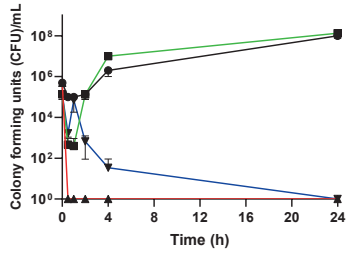
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## SP25

## SP30

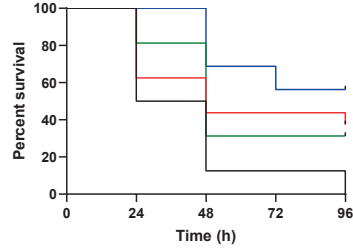
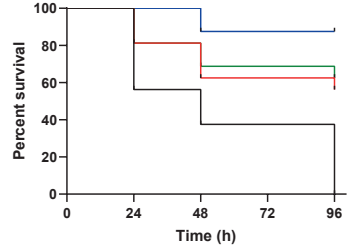
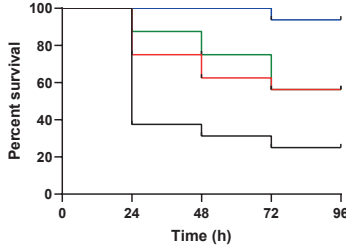
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**a**



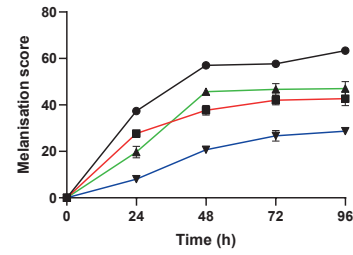
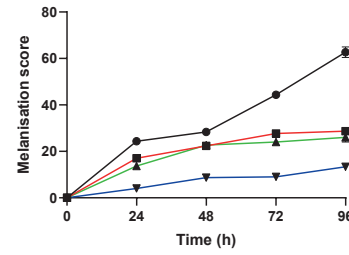
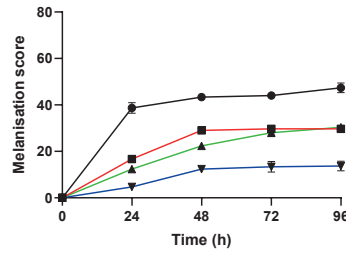
● MH2 broth control  
▲ Mn complex (x1 MIC)  
■ TET (x 1 MIC)  
▼ TET + Mn complex (x1 MIC + 0.5 MIC)

**b**



— PBS control  
— Mn complex (2.56 mg/kg)  
— TET (0.64 mg/kg)  
— TET + Mn complex (0.64 mg/kg + 2.56 mg/kg)

**c**



● PBS control  
■ Mn complex (2.56 mg/kg)  
▲ TET (0.64 mg/kg)  
▼ TET + Mn complex (0.64 mg/kg + 2.56 mg/kg)