INFLUENCE OF ALKYL CHAIN LENGTH IN *N*-ALKYLMORPHOLINE DERIVATIVES ON THEIR ANTIBACTERIAL EFFECT AGAINST METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) ISOLATES

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Abstract: A series of N-alkyl morpholines with different number alkyl chain lengths from 1 to 18 were synthesized and their antibacterial activity against Methicillin Resistant *Staphylococcus aureus* was evaluated. We observed that active compounds were those with longer chain structures (i.e. CH_3 $(CH_2)_{11}$, CH_3 $(CH_2)_{13}$, and CH_3 $(CH_2)_{15}$) with MIC and MBC values of 3.9 µg/mL, more potent than the commercial drug Linezolid. In contrast, compounds with short chains (<5C) were inactive against MRSA bacteria, finding a structure-activity relationship. Analysis suggests the possibilities of their inhibitory potential against RNA. Assays on *A. salina* showed that the inactive compounds were classified as moderately toxic, while active compounds resulted highly or extremely toxic according to the CYTED scale. The results show that the structures of these compounds could be considered to develop drugs against Methicillin Resistant *Staphylococcus aureus*.

Introduction

Antibiotic resistance is a growing threat to public health worldwide and infections caused by antibiotic-resistant pathogens are increasing substantially. In 2017, the World Health Organization (WHO) published a list of six antibiotic-resistant bacteria of international concern to help in prioritizing research and development. The list of bacteria classified as high priority includes *Enterococcus faecium* (vancomycin-resistant), *Helicobacter pylori*, (clarithromycin-resistant), *Campylobacter* (fluoroquinolone-resistant), *Salmonella* spp. (fluoroquinolone-resistant), *Neisseria gonorrhoeae* (3rd generation cephalosporin-resistant, fluoroquinolone-resistant) and *Staphylococcus aureus* (methicillin-resistant, vancomycin-intermediate, and resistant)[1]. During the last decade, *Staphylococcus aureus*, including methicillin resistant (MRSA) strains, have remained the main cause of hospital-acquired infection (HAI) in the United States and throughout the world[2]. The WHO estimates that patients with MRSA have 64% more likely to die than patients with non-resistant infections. In this sense, the need for effective therapy has led to the emerging search for new antimicrobial agents.

Morpholine is a heterocyclic structure that has physicochemical, biological, and metabolic properties used to synthesize compounds. The pharmacological effect of morpholine compounds is of great importance for research and in the pharmaceutical industry, due to the resulting improvement of the pharmacokinetic properties it can provide. The derivatives of morpholine play an important role in the treatment of various

diseases. The morpholine ring has aroused great interest in recent years due to its variety of biological activities[3-4]. It has been reported that morpholine derivatives possess a broad spectrum of antimicrobial activity such as anthelmintic, bactericidal and antifungal[5-6].

Various morpholine derivatives such as chloroacetyl, pyrimidine and pyrazoline have been evaluated as antimicrobials against microorganisms such as *E. coli*, *S. aureus*, *B. subtilis*. *P. aeruginosa*, *K. pneumonia* and others. The morpholine scaffold possesses drug properties (e.g. affinity, selectivity potency, *in vivo* stability, metabolic profile, reduced CYP3A4 liability, prolonged pharmacokinetics, solubility, blood-brain barrier permeability or bioavailability), it hast been used as a heterocyclic scaffold or substituent to synthesize antimicrobial compounds by substituting the amino or nitro position with several structures. An important property related to the drug action of morpholine-containing molecules is lipophilicity due to ionization and interactions of the ring**.**

Morpholine is a frequently used heterocycle in medicinal chemistry and a privileged structural component of bioactive molecules. This is mainly due to its contribution to a plethora of biological activities as well as to an improved pharmacokinetic profile of such bioactive molecules[7]**.** Morpholines are part of a group of pharmacophores, incorporated into various therapeutically important drugs, such as the antimicrobial drug Linezolid[8]. Considering the biological importance of the morpholinic ring, here we report the evaluation of nine morpholine compounds N-alkyl substituted which were evaluated as antibacterial drugs against five MRSA strains isolated from apparently healthy children**.**

Results and Discussion

CHEMISTRY

A total of nine morpholinic compounds were synthesized with the following chemical characteristics (table 1).

Compound M-1 [17]

4,4-dimethylmorpholin-4-ium iodine. The product was obtained as a white solid with an 82% yield. Melting point 248-249°C: 1H NMR (DMSO-d6) δ [ppm] = 3.22 (s, 6H); 3.41-3.45 (m, 4H); 3.88-3.94 (m, 4H).

13C NMR (DMSO-d6) δ [ppm] = 60.21, 59.98, 50,93.
FTIR-ATR (v, cm-1): 2965, 2873, 1231, 1105, 931, 923, 886, 626.

Compound M-2 [18]

4-ethyl-4-methylmorpholin-4-ium iodine. The product was obtained as a white solid with a 91% yield. Melting point 150-153°C: 1H NMR (DMSO-d6) δ [ppm] = 1.24 (t, J=7Hz, 3H); 3.12 (s, 3H); 3.33-3.46 (m, 4H); 3.53 (q, J= 7.36 Hz, 2H); 3.86- 3.97 (m, 4H)

13C NMR (DMSO-d6) δ [ppm] = 60.22, 59.76, 58.71, 45.89, 7.42
FTIR-ATR (v, cm-1): 2967, 2878, 1431, 1107, 952, 923, 885, 618**.**

Compound M-3 [19]

4-propyl-4-methylmorpholin-4-ium iodine. The product was obtained as a white solid with an 81% yield. Melting point 96-98°C: 1H NMR (DMSO-d6) δ [ppm] = 0.9 (t, J=7Hz, 3H); 1.64-1.75 (m, 2H); 3.14 (s, 3H); 3.39-3.44 (m,4H); 3,46-3.41 (m, 2H); 3.88-3.95 (m, 4H).

13C NMR (DMSO-d6) δ [ppm] = 64.92, 59.80, 58.95, 46.31, 14.50, 10.49.
FTIR-ATR (v, cm-1): 2963, 2873, 1460, 1107, 940, 886, 754, 618.

Compound M-4 [20, 21]

4-butyl-4-methylmorpholin-4-ium bromide. The product was obtained as a white solid with an 82 % yield. Melting point 37-38.5°C: 1H NMR (DMSO-d6) δ [ppm] = 0.92 (t, J=7Hz, 3H); 1.39 (s, 2H); 1.60-1.70 (m, 2H); 3.15 (s, 3H); 3.41-3.46 (m, 4H); 3.53-3.46 (m, 2H); 3.86-3.97 (m, 4H).

13C NMR (DMSO-d6) δ [ppm] = 13.59, 19.20, 22.78, 46.04, 58.89, 59.83, 63.43
FTIR-ATR (v, cm-1): 2964, 2873, 1459, 1110, 891, 627.

Compound M-5 [22]

4-hexyl-4-methylmorpholin-4-ium iodine. The product was obtained as a white solid with a 90% yield. Melting point 98-99°C: 1H NMR (DMSO-d6) δ [ppm] = 0.86 (t, J=7Hz, 3H), 1.22-1.34 (m, 6H), 1.61-1.72 (m, 2H), 3.14 (s, 3H), 3.39-3.43 (m 4H), 3.43-3.49 (m, 2H), 3.86-3.95 (m, 4H).

13C NMR (DMSO-d6) δ [ppm] = 63.65, 59.80, 58.91, 46.11, 30.66, 25.37, 21.88, 20.70, 13.86,
FTIR-ATR (v, cm-1): 2915, 2873, 1470, 1111, 914, 642.

Compound M-6 [23]

4-dodecyl-4-methylmorpholinium bromide. The product was obtained as a white solid with a 77% yield. Melting point 154-156°C: 1H NMR (DMSO-d6) δ [ppm] = 0.84(t, J=7.56 Hz,3H); 1,20 (s, 18H); 1.60-1.73(m, 2H); 3.17 (s, 3H); 3.41-3.47 (m, 6H); 3.87(m,4H);

13C NMR (DMSO-d6) δ [ppm] = 63.60, 63.25, 59.81, 58.84, 52.36, 45.98, 42.33, 31.31, 29.04, 29.03, 28.97, 28.86, 28.73, 28.56, 25.77, 22.11, 20.78, 13.95.
FTIR-ATR (v, cm-1): 2918, 2850, 1468, 1119, 1064, 898, 717, 649

HPLC-MS (Q TOF) mass calcd for C17H36BrNO: [M-Br] 270.28 found M/Z 270.28236

Compound M-7 [24]

4-tetradecyl-4-methylmorpholinium bromide [32]. The product was obtained as a white solid with a 78% yield. Melting point 146°C-149°C: 1H NMR (DMSO-d6) δ [ppm] = 0.85(t, J 7.5 Hz, 3H); 1.23(s, 22H); 1.59-1.70(m, 2H); 3.14(s, 3H); 3.44-3,47 (m, 6H); 3.84-3.95(m, 4H);

13C NMR (DMSO-d6) δ [ppm] = 63,66, 63.30, 59.82, 58.88, 52.43, 46.0, 42.38, 31.32, 29.09, 29.03, 28.98, 28.86, 28.74, 28.56, 25,79, 22.12, 20.76, 13.98

FTIR-ATR (v, cm-1): 2918, 2848, 1473, 1120, 1068, 908, 717, 648
HPLC-MS (Q TOF) mass calcd for C19H40BrNO: [M-Br] 298.31 found M/Z 298.3148

Compound M-8 [25]

4-hexadecyl-4-methylmorpholinium bromide. The product was obtained as a white solid with a 78% yield. Melting point 132-134°C: 1H NMR (DMSO-d6) δ [ppm] = 0.84 (t, J=7.5 Hz, 3H); 1.23 (s, 26H); 1.60-1.70 (m, 2H); 3.13 (s, 3H); 3.40-3.44 (m, 6H); 3.89-3.97 (m, 4H);

13C NMR (DMSO-d6) δ [ppm] = 63.65, 63.29, 59.81, 58.87, 52.42, 46.0, 42.3, 31.31, 29.09, 29.06, 28.99, 28.87, 28.73, 28.57, 25.79, 22.11, 20.76, 13.96.
FTIR-ATR (v, cm-1): 2918, 2850, 1473, 1120, 1064, 898, 718, 648;
HPLC-MS (Q TOF) mass calcd for C21H44BrNO: [M-Br] 326.34 found M/Z 326.34603

Compound M-9 [25]

4-methyl-4-octadecyl-morpholinium bromide. The product was obtained as a white solid with a 90% yield. Melting point 98-100°C: 1H NMR (DMSO-d6) δ [ppm] = 0.84 (t, J=7.5Hz, 3H); 1.23 (s, 30H); 1.60-1.72 (m, 2H); 3.12 (s, 3H); 3.39-3.45 (m, 6H); 3.89-3.94 (m, 4H).

13C NMR (DMSO-d6) δ [ppm] = 63.69, 63.36, 59.83, 58.91, 52, 56, 46.02, 42, 25, 31.33, 29.08, 29.04, 29.00, 28.87, 28.74, 28.57, 25.80, 22.13, 20.75, 13.99.
FTIR-ATR (v, cm-1): 2914, 2849, 1473, 1120, 1064, 900, 717, 648.
HPLC-MS (Q TOF) mass calcd for C23H48BrNO: [M-Br] 354.63 found M/Z 354.37715

An increase in the incidence of infections caused by multiresistant bacteria has been observed since the last decade. These microorganisms are usually involved in serious infections and they currently represent a major global public health problem. An increment in antimicrobial resistance and a scarce development of new antibiotics suggest that we have fewer therapeutic options for the treatment of these infectious diseases. Community-acquired infections of *Staphylococcus aureus* (CA-MRSA) are the major global challenge due to the presence of multiple drug-resistant genes. *Staphylococcus* strains pose the ability to acquire antimicrobial resistance over time and MRSA represents a problem in the future[26].

Table 1. Chemical characteristics of N-alkyl morpholine compounds

Code	IUPAC Nomenclature	R	Molecular Formula	Molecular weight	Yield	Melting point (°C)	Solubility[1]			
							CH_3OH	C_2H_5OH	H_2O	C_2H_6OS
M-1	4,4-dimethylmorpholin-4-ium iodide	CH_3	$C_6H_{14}INO$	243.09	82%	248-249	+	+	+	+
M-2	4-ethyl-4-methylmorpholin-4-ium iodide	CH_3 CH_2	$C_7H_{16}INO$	257.11	91%	150-153	+	+	+	+
M-3	4-methyl-4-propylmorpholin-4-ium iodide	$CH_3(CH_2)_2$	$C_8H_{18}INO$	271.14	81%	96-98	+	+	+	+
M-4	4-butyl-4-methylmorpholin-4-ium bromide	$CH_3(CH_2)_3$	$C_9H_{20}BrNO$	238.17	82%	37-38.5	+	+	+	+
M-5	4-hexyl-4-methylmorpholin-4-ium iodide	$CH_3(CH_2)_5$	$C_{10}H_{22}INO$	299.19	90%	98-99	+	+	+	+
M-6	4-dodecyl-4-methylmorpholin-4-ium bromide	$CH_3(CH_2)_{11}$	$C_{17}H_{36}BrNO$	350.38	77%	154-156	+	+	+	+
M-7	4-tetradecyl-4-methylmorpholin-4-ium bromide	$CH_3(CH_2)_{13}$	$C_{19}H_{40}BrNO$	378.43	78%	146-149	+	+	+	+
M-8	4-hexadecyl-4-methylmorpholin-4-ium bromide	$CH_3(CH_2)_{15}$	$C_{21}H_{44}BrNO$	406.48	78%	132-134	+	+	-	+
M-9	4-methyl-4-octadecyl-morpholin-4-ium bromide	$CH_3(CH_2)_{17}$	$C_{23}H_{48}BrNO$	434.54	90%	98-100	+	+	-	+

Concentrations: CH_3OH (32.04 g/mol) (400 µL), C_2H_50H (46.07 g/mol) (500 µL), H_20 (500 µL) (18.01528 g/mol) and C2H60S (78.13 g/mol) (900 µL). X- : Br- and I-. All compounds were observed as white solids

Several efforts are made in response to the request made by the World Health Organization in February 2017, when the list of microorganisms with the highest priority to design a drug was published, where *S. aureus* is placed in category 2 and considered as elevated [27,1]. In this sense, diverse scientific groups work in the development of drugs using different structures as scaffold-like morpholines. In this work, a series of trans alkyl morpholines were synthesized from starting materials namely morpholine and alkanes with good yield.

The antimicrobial activity of the obtained compounds was evaluated against MRSA *S. aureus* strains isolated from pharyngeal exudates of healthy children. The biochemical profile of the bacterial isolates was positive to the conventional tests performed (*i, e;* coagulase, catalase, DNase, oxidase, mannitol fermentation and hemolysis). Also, the resistance profile of the bacterial isolates was consistent with the characteristic phenotype of a resistant methicillin isolate, taking as a reference a previous association with the presence of the *mecA* gene (data not shown).

In the growing assays for the ATCC 25923 strain with the solvent, we detected a transmittance of 27.4, 20.3, 19.3, and 15.7% and CFU values of 32, 34, 57, and 64 at 10, 8, 6, and 4% of DMSO, respectively. Also, a 13.6% of transmittance and 74 CFU for the control assay without solvents. Additionally, we noted that with concentrations lower than 1% of DMSO the growing increase.

Susceptibility testing

The antimicrobial susceptibility test evaluation with the 15 drugs on the five *S. aureus* CA-MRSA strains presented different susceptibility profiles (Figure 1). The results showed that 100% of all the strains were resistant to AMP, KF, FEP, CXM, DC, P, OX and FOX and 80% were resistant to the E drug. In the analysis of resistance for each strain it is observed that strain 46-1 was resistant to 66.6% of the evaluated drugs, the strains 5-2, 52-5 and 36-2 were resistant to 60.0% while the strain 57-2 was resistant to 53.3% (Table 2).

On the other hand, 100% of the strains were susceptible to TE, SXT, GN, and LEV. The percentage of susceptibility for CA-MRSA strains were 26.6% for the 46-1, 5-2, 52-5, 36-2, and 57-2. Likewise, 100% of the isolates showed intermediate resistance to VA and 80% to CTX. The percentage of intermediate resistance for the CA-MRSA 57-2 strain was 20%, for strains 5-2, 52-5, and 36-2 was 13.3% and 6.7% for the strain 46-1.

Results showed that the ATCC 25923 presented resistance to FEP, P, OX, and DC; susceptibility to KF, FOX, E, TE, SXT, VA, GN, and LEV and intermediate resistance to AMP, CTX, and CXM, which represent 26.6%, 53.3% and 20% of the 15 antibacterial agents tested respectively against the ATCC strains used as control. In contrast, the ATCC 43300 strain was resistant to AMP, KF, CTX, FEP, CXM, DC, P, OX, FOX, and E; susceptibility to TE, SXT, VA, GN, and LEV, representing 66.6% of the resistance, 33.3% of susceptibility and the strain did not present intermediate resistance.

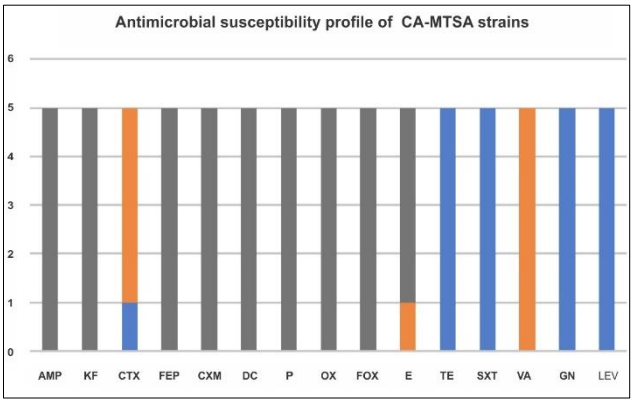

Figure 1. Antimicrobial susceptibility profile of Community-acquired Methicillin-Resistant *Staphylococcus aureus* strains isolated from oropharyngeal exudates of apparently healthy children. Blue indicate susceptibles strain, orange indicate intermediate strain and grey color indicate resistant strains.

The antimicrobial activity of the obtained compounds was evaluated against MRSA *S.* aureus strains isolated from pharyngeal exudates of healthy children. The data showed that all the strains were sensible to the tetracyclines, sulfonamides, aminoglycosides, and quinolones drugs. Several studies have reported resistance to commonly used antibiotics for CA-MRSA, in which greater resistance is evidenced for β-lactams (Penicillin) and macrolides (Erythromycin), while for aminoglycosides (Gentamicin), Tetracyclines (Tetracycline), and Sulfonamides (Sulfamethoxazole/Trimethoprim) have reported low levels of resistance. Our results are comparable to a related study in southwestern Nigeria, where 99% of isolates were resistant to penicillin and only 2% of isolates were resistant to Gentamicin [28]. Similar high resistance to Penicillin (100%,) and high sensitivity to Gentamicin (95.3%) was observed when antibiotic susceptibility tests were conducted on 169 *S. aureus* isolates among primary school children and prisoners in Jimma Town, Southwest Ethiopia[29]. Also, the MRSA isolates are resistant to eight of the nine β-lactams drugs tested and this could be related to the presence of the *mecA* gene; observed in the five strains and confirmed by the resistance to cefoxitin [26, 30, 31]. Interestingly, the profile of susceptibility to Sulfamethoxazole/Trimethoprim, Tetracycline, Levofloxacin and Gentamicin could be due to a lack or to the inhibition of protein synthesis and DNA gyrase which is the target of these drugs.

Table 2. Antimicrobial Susceptibility profile of Community-acquired Methicillin-Resistant *Staphylococcus aureus* strains isolated from oropharyngeal exudates of apparently healthy children.

Antimicrobial agent (µg)		*S. aureus* ATCC		CA-MRSA					Total (%)		
	Family	25923	43300	46-1	5-2	52-2	36-2	57-2	R	I	S
AMP (10)	B	I	R	R	R	R	R	R	5 (100)	0 (0)	0 (0)
KF (30)	B	S	R	R	R	R	R	R	5 (100)	0 (0)	0 (0)
CTX (30)	B	I	R	R	I	I	I	I	0 (0)	4 (80)	1 (20)
FEP (30)	B	R	R	R	R	R	R	R	5 (100)	0 (0)	0 (0)
CXM (30)	B	I	R	R	R	R	R	R	5 (100)	0 (0)	0(0)
DC (1)	B	R	R	R	R	R	R	R	5 (100)	0 (0)	0 (0)
P (10)	B	R	R	R	R	R	R	R	5 (100)	0 (0)	0 (0)
OX (1)	B	R	R	R	R	R	R	R	5 (100)	0 (0)	0 (0)
FOX (30)	B	S	R	R	R	R	R	R	5 (100)	0 (0)	0 (0)
E (15)	M	S	R	R	R	R	R	I	4 (80)	1 (20)	0 (0)
TE (30)	T	S	S	S	S	S	S	S	0 (0)	0 (0)	5 (100)
SXT (25)	S	S	S	S	S	S	S	S	0 (0)	0 (0)	5 (100)
VA (30)	G	S	S	I	I	I	I	I	0 (0)	5 (100)	0(0)
GN (10)	A	S	S	S	S	S	S	S	0 (0)	0 (0)	5 (100)
LEV (5)	Q	S	S	S	S	S	S	S	0 (0)	0 (0)	5 (100)
	R	26.6	66.6	66.6	60.0	60.0	60.0	53.3			
Percentages by strain	I	20	0	6.7	13.3	13.3	13.3	20			
	S	53.3	33.3	26.6	26.6	26.6	26.6	26.6			

R, resistant; **I**, intermediate resistance; **S**, susceptible. **MRSA**, methicillin-resistant *Staphylococcus aureus*. **AMP**, Ampicillin; **KF**, Cephalothin; **CTX**, Cefotaxime; **FEP**, Cefepime; **CXM**, Cefuroxime; **DC**, Dicloxacillin; **P**, Penicillin; **OX**, Oxacilline: **FOX**, Cefoxitin; **E**, Erythromycin; **TE**, Tetracycline; **SXT**, Sulfamethoxazole/Trimethoprim; **VA**, Vancomycin; **GN**, Gentamicin; **LEV**, Levofloxacin. **(B)** β-lactams, **(M)** Macrolides, **(T)** Tetracyclines, **(S)** Sulfonamides, **(G)** Glycopeptides. **(A)** Aminoglycosides, **(Q)** Quinolones.

Four N-alkyl morpholines (**M-6 to M-9**) of the nine evaluated showed antibacterial activity *in vitro* against MRSA strains with MIC and MBC ranges from 3.9 to 15.6 µg/mL. In contrast, the other five N-alkyl morpholines (**M-1 to M-5**) did not show activity up to the maximum concentration evaluated (200 µg/mL). The compound **M-6** had an MBC value of 15.6 µg/mL for 46-1, 5-2, 52-2 and 47-2 strains and 62.5 µg/mL for 36-2 strains. Compound **M-7** presented bactericidal activity against the five MRSA isolates with MBC=15.6 µg/mL. The compound **M-8** presented bactericidal activity against the five MRSA isolates with an MBC=3.9 µg/mL for 5-2, 52-2, 36-2 and 57-2 strains and 15.6 µg/mL for 46-1 strain. The morpholine **M-9** showed MBC values of 62.5 µg/mL for 5-2, 52-2, 36-2 and 57-2 MRSA strains and 15.6 for the 46-1 strain. The inhibitory activity was similar to the MIC activity except for **M-8** with a MIC value of 3.9 µg/mL (Table 3).

Bactericidal activity for both *S. aureus* ATCC reference strains with compounds **M-6**, **M-7**, **M-8**, and **M-9** showed MBC values in a range of 3.9 µg/mL to 62.5 µg/mL. The compound **M-6** presented an MBC value of 15.6 µg/mL for both strains. The compounds **M-7** and **M-8** were active at 3.9 µg/mL with the ATCC 43300 and 25923 strains respectively; compound **M-9** had an MBC at 62.5 µg/mL. The **M-1** to **M-5** N-alkyl morpholines did not show activity up to the maximum concentration evaluated (200 µg/mL) on *S. aureus* ATCC reference strains.

Regarding the *S. aureus* ATCC strains, compounds **M-6**, **M-8** and **M-9** presented MIC values of 15.6, 3.9 and 62.5 µg/mL respectively in both strains. Compound **M-7** presented a MIC= 3.9 µg/mL for *S. aureus* ATCC 25923 and a MIC = 15.6 µg/mL for *S. aureus* ATCC 43300.

In the case of Linezolid, a MIC of 4 µg/mL and an MBC >32.0 µg/mL were obtained against all the MRSA. Gentamicin showed bactericidal activity in a range of 1.0 µg/mL for 46-1 and 52-2 strains and 2 µg/mL for the rest of the MRSA strains. The inhibitory activity for Gentamicin was 1.0 µg/mL for 46-1, 5-2, and 52-2 strains and 2.0 µg/mL for 36-2 and 57-2 MRSA strains.

The Linezolid presented values of MIC=4.0 µg/mL and MBC >32.0 µg/mL against both ATCC isolates. The bactericidal activities observed for Gentamicin were 1.0 and >32.0 µg/mL for the 25923 and 43300 ATCC strains, respectively. In the case of Gentamicin, a MIC of 0.5 µg/mL was obtained for the ATCC 25923 strain, while a MIC value was not obtained until the maximum concentration tested (32.0 µg/mL) for the ATCC 43300 strain.

Research supports the use of the morpholine structure to develop drugs and they are an attractive alternative to be used as antibacterials. A chemical substance is considered active and biologically relevant when the MIC value is less than 64 µg/mL.[32]. According to our findings, the antibacterial activity of the compounds differs between morpholinic compounds tested and between the commercial morpholine-based antibacterial drugs used (Linezolid). The morpholine-derived compounds **M-6** to **M-9** were active because the MICs obtained were in concentrations from 3.9 to 62.5 µg/mL against the multiresistant isolates of *S. aureus* (Table 3). In contrast, compounds **M-1** to **M-5** were not active because MICs and MBCs were greater than 200 µg/mL. Compound **M-8** was the most active of the morpholine compounds evaluated with MIC of 3.9 µg/mL against the MRSA strains 5-2, 52-2, 36-2 and 57-2 , as well as the ATCC 25923 and 43300 strains.

Table 3. Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) (µg/mL) for N-alkyl morpholine compounds and antibiotics against Community-acquired Methicillin-Resistant *Staphylococcus aureus* strains.

Bacterial strains CA-MRSA	MIC/MBC (µg/mL)						
	M-1 - M-5	M-6	M-7	M-8	M-9	LN	GN
ATCC 25923	>200/>200	15.6/15.6	3.9/3.9	3.9/62.5	62.5/62.5	4/>32	0.5/1
ATCC 43300	>200/>200	15.6/15.6	15.6/15.6	3.9/3.9	62.5/62.5	4/>32	>32/>32
46-1	>200/>200	3.9/15.6	15.6/15.6	15.6/15.6	15.6/15.6	4/>32	1/1
5-2	>200/>200	3.9/15.6	15.6/15.6	3.9/3.9	15.6/62.5	4/>32	1/2
52-2	>200/>200	3.9/15.6	15.6/15.6	3.9/3.9	15.6/62.5	4/>32	1/1
36-2	>200/>200	3.9/62.5	15.6/15.6	3.9/3.9	15.6/62.5	4/>32	2/2
57-2	>200/>200	3.9/15.6	15.6/15.6	3.9/3.9	15.6/62.5	4/>32	2/2

Compounds M-2, M-3, M-4 and M-5 did not show activity up the maximum concentration evaluated against all the isolates in study (200µg/mL).
GN: Gentamicin, **LN**: Linezolid, **CA-MRSA**: Community-acquired Methicillin Resistant *Staphylococcus aureus*

The compound **M-6** is active against all MRSA strains but presents higher MICs and MBCs (≥3.9 µg/mL) than **M-8**. The compound **M-7**, presented a marked activity showing the same MIC and MBC values for ATTC and MRSA strains (15.6 µg/mL) and remarkable activity on the sensible ATCC 25923 (3.9 µg/mL). Interestingly, we can observe a structure-activity relationship of the compounds, that is the most active compounds were those with longer chain structure, in a range of between 11 to 15 carbons (i.e. CH_3 $(CH_2)_{11}$, CH_3 $(CH_2)_{13}$, and CH3 $(CH_2)_{15}$); compounds **M-6**, **M-7**, and **M-8** respectively. Meanwhile, the inactive compounds presented short chains (<5C) for compounds **M-1** to **M-5** (Table 1). Compounds **M-1** to **M-7** are reasonably soluble in water in contrast, the compounds **M-8** and **M-9** are less soluble in water. Hydrophobic drugs tend to be more toxic because, in general, are kept longer, have a wider distribution in the body, are somewhat less selective in their binding to molecules and finally are often extensively metabolized. Microbial cell membranes consist of a lipid bilayer interspersed with

proteins. Probably, compounds **M-6** to **M-9** can penetrate the lipid bilayer, inhibiting substrate transport and allowing the leakage of cellular components such as potassium and ribosomes. The chain size (11-17C) of the compounds may be associated with penetration into the lipid bilayer and the disruptive effect is greater.

The activity of the compound **M-8** ($CH_3(CH_2)_{15}$) can be due to its optimum hydrophilic/hydrophobic balance and good water solubility, which enhances the interaction with the bacterial membranes. Several studies showed that the number of carbon atoms present in the alkyl chain and can be modulated by the long-chain that alters its polarity could influence the antimicrobial activity [33,34]. In addition, the type of organism and its cell wall composition also influence the anti-microbial activity of a particular agent. Bacterial cell membranes are composed of mainly negatively charged phospholipids and the adsorption of alkyl group with quaternary ammonium salts like morpholine into the cell surface is facilitated by electrostatic interactions. Furthermore, their hydrophobic alkyl chains allow the alkyl group and morpholine to interact further with the hydrophobic lipid membrane and disrupt them.

We suggest that the chain short of the alkyl compounds **M-1** to **M-5**, gives rise to weak hydrophobic interaction with the lipid membranes, resulting in the absence of antimicrobial activity. On the other hand, the strongly hydrophobic character of compound **M-9** ($CH_3(CH_2)_{17}$) reduces its solubility, thereby hampering its transport through the bacterial cell membranes and reducing antimicrobial effectiveness[35]. This behavior, which is very common for cationic surfactants, is known as the cut-off effect. On the other hand, it is observed that the toxicity of compounds from **M-1** to **M-9** increases with the length of the chain. It has been reported that chemical groups such as pyridinium, imidazolium, morpholino and cholinium follow this side chain length effect up to certain alkyl chain length [36,-37]**.** Latter observations are consistent with the literature findings [38-39]. Other morpholines have been used as antimicrobials against Gram-positive bacteria such as *Bacillus.subtilis, Bacillus cereus, Micrococcus luteus* and *Salmonella typhi* with similar results which suggest the effectiveness of morpholines as broad-spectrum antibacterials [40]**.**

Compound **M-8** and Linezolid presented similar MIC activity (3.9-4 µg/mL) but MBC activity is lower for the compound **M-8** (3.9 µg/mL) than the Linezolid (32 µg/mL). In the case of Linezolid against the ATCC 29213 isolates under study, the MIC of 4 µg/mL value obtained is consistent with the ranges established by the CLSI. Another study reported a MIC of 2 µg/mL for clinical isolates of *S. aureus*; a lower concentration of inhibition than obtained in this study (4 µg/mL)[41]. Linezolid is a bacteriostatic oxazolidinone that possesses a 4-phenyl-morpholine substituent and is a commercially available antimicrobial drug with action against Gram-positive cocci. Previously, MIC values had been reported in a range of 6.25 to 200 µg/ mL of 9 morpholino pyrimidine compounds substituted at the para position of the phenyl rings attached to C-4 and C-6 carbons of pyrimidine moiety with different functional groups (CH_3, Cl, OCH_3, F, Br, NO_2 and H). Oxazolidinones are molecules with good activities against multi-drug-resistant (MDR) Gram-positive isolates, including MRSA and against vancomycin-resistant enterococci (VRE)[42]. The Linezolid action mechanism is through inhibition of the early protein synthesis by binding to the 23S site of the bacterial ribosomal RNA at the 50S subunit, blocking the formation of the 70S complex (an essential component in the protein synthesis process). However, a significant interindividual variability has been associated with exposure to linezolid, which could elevate the incidence of safety issues and compromise the effectiveness of treatment with this drug[43].

Brine shrimp bioassay

The N-alkyl morpholines analyzed presented different responses on the morphology of *Artemia salina*. No changes in morphology, structure and mobility when *A. salina* was exposed to **M-1** to **M-5** at all concentrations. In contrast, the nauplii exposed to increasing concentrations of the compounds **M-6** to **M-9** produced different morphological changes (Figure 2). For compound **M-9**, an enlarged body, and dark areas in the thoracopods and intestine were observed at concentrations of 10 and 20 ppm. At 60 ppm, the crustacean presented a process of detachment of tissue, as well as dark areas in the body, tissue and extremities. Furthermore, the crustacean assumed a shape and size twice compared to the negative control (Figure 2A) at concentrations of 100 and 80 ppm (Figures 2B and 2C respectively). Compound **M-8** against *A. salina*, exhibited tissue detachment and a smaller size compared with the negative control in all concentrations tested. When we exposed *A. salina* with compound **M-7**, we observed dark areas in the intestine, widening of the head and tissue detachment at concentrations of 100 and 80 ppm. At concentrations of 60, 20, and 10 ppm (Figures 2D, 2E and 2F respectively), the nauplii were smaller and with scattered dark areas compared with the controls. The *Artemia* exposed to compound **M-6** showed a widening of the head at the 100 ppm concentration. At the concentrations of 80, 60, 20, and 10 ppm no visible morphological changes were observed. Is important to note that no damage was observed in the tests without

compound. Furthermore, we did not observe any change in morphology, structure and mobility when *A. salina* was exposed to Gentamicin and Linezolid at 200, 100, and 10 ppm.

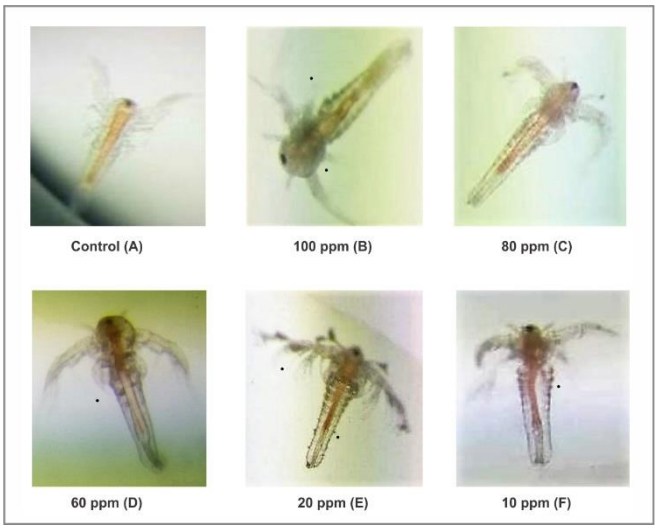

Figure. 2. Morphological variations in brine shrimp *(A. salina)* after 24 hour exposure to N-alkyl morpholine compound **M-9** (Representative) (100X).

Additionally, different mortality percentages for *A. salina* were observed at increasing concentrations of all compounds evaluated. **M-6** killed 76.6, 16.6 and 6.6% of nauplii at 100, 80 and 60 ppm respectively; all organisms were alive at 20 and 10 ppm. **M-7** presented 100% of mortality at 100, 80, 60, and 20 ppm respectively, and 6.66% at 10 ppm. Compound **M-8** was 100% mortal at 100 ppm; 90% for 80 and 60 ppm, and finally 86.6 and 76.6% at 20 and 10 ppm. Additionally, M-**9** 100% at 100, 80, and 60 ppm, 53% at 20 ppm and 20% when exposed at 10 ppm. In contrast, all *Artemia* were alive when they were exposed to 100 ppm of Gentamicin, Linezolid and the morpholines **M-1** to **M-5** (Table 4).

The lethal concentration index 50 (LC_{50}) were 441.77, 426.47, 418.45, 408.51, 408.48, 90.48, 19.07, 12.07, and 9.07 ppm for compounds **M-1**, **M-2**, **M-5**, **M-3**, **M-4**, **M-6**, **M-9**, **M-7,** and **M-8** respectively. It was observed that Gentamicin and Linezolid did not kill *Artemia salina* (Table 5). According to the results obtained and the CYTED scale; compounds; **M-1** to **M-5** are classified as moderately toxic; compounds **M-6**, **M-7**, and **M-9** highly toxic; and compound **M-8** extremely toxic (Table 5).

The results of the toxicity assay on *A. salina,* showed that the inactive compounds (**M-1** to **M-5**) were classified as moderately toxic, while the active compounds (**M-6** to **M-9**) were highly or extremely toxic according to the CYTED scale. In this sense, the antibacterial activity established for morpholines was related to their toxicity against *A. salina* with the structure of the compounds.

Indeed, it was observed that the LC_{50} is less as the length of the carbon chain increases, and this relationship is equal to the antibacterial activity so that the compounds of morpholine with the chain of longer carbon are more active as antibacterial and most toxic. In addition, morpholine ring has been reported as moderately toxic to various aquatic organisms. For *Daphnia magna*, an EC_{50} (mean effective concentration) of 100 to 119 µg/mL was found, observing an immobilization effect. In another study, with morpholines, LC_{50}´s of 180, 285, and 400 µg/mL were reported for *Oncorhynchus mykiss, Leuciscus idus* and *Odontesthes regia*, respectively [44]. However, they have also been evidenced as an extremely toxic structure against *Danio rerio*, with an LC_{50} of 1 µg/mL [45]. Regarding this, we can consider morpholines as compounds with different toxicity levels in aquatic organisms, depending on the species. The difference of toxicity order stated in other studies could be due to the different tested organisms or the effects of alkyl-chain lengths[46]. However, it is important to mention that most of the morpholine compounds reported in the literature exhibit moderate toxicity.

Table 4. The result for mortality rate of brine shrimp (*Artemia salina*) treated with N-alkyl morpholines.

	Percent of Mortality after 24 hours										
Compounds	**M-1**	**M-2**	**M-3**	**M-4**	**M-5**	**M-6**	**M-7**	**M-8**	**M-9**	**LN**	**GN**
100 ppm	0%	0%	0%	0%	0%	76.6%	100%	100%	100%	0%	0%
80 ppm	0%	0%	0%	0%	0%	16.6%	100%	90%	100%	0%	0%
60 ppm	0%	0%	0%	0%	0%	6.66%	100%	90%	100%	0%	0%
20 ppm	0%	0%	0%	0%	0%	0%	100%	86.6%	53.3%	0%	0%
10 ppm	0%	0%	0%	0%	0%	0%	6.66%	76.6%	20%	0%	0%

Mean of assay for triplicate. Initial number of *Artemia salina* in assay (30). ppm (parts per million).

Additionally, morpholines toxicity has been determined in rats for whose LC_{50} was 260 mg/L for 4h by inhalation. Besides are considered noxious with an oral LD_{50} of 1050 mg/kg, the least toxic level of the toxicity classification established by the Globally Harmonized System of Classification and Labeling of Chemical Products and the European Regulation [47]. Likewise, the EPA (Environmental Protection Agency) or equivalent to moderately toxic classifies the morpholines as harmful for the WHO. Additionally, it has been reported a LD_{50} of 1210 mg/kg in rabbits by cutaneous way, positioning also in the same category [45]. It is important to mention that there are no Linezolid toxicity studies in aquatic organisms such as *A. salina*. However, it has been reported as non-toxic in flies up to the concentration of 2000 µg/mL orally (*Drosophila melanogaster*) [43].

ADMET prediction/Pharmacokinetic parameters

The software predicted that all the alkyl morpholines and antibiotics studied resulted in category III of acute oral toxicity. All the compounds are non-carcinogenics, non-mutagenics, non-hepatotoxics, non-genotoxics, non-toxics for avian and fish aquatic and a negative value for human intestinal absorption. Likewise, all compounds can cross the blood brain barrier and can permeate Caco-2 cells. In contrast, different values were predicted for human oral bioavailability and crustacean aquatic toxicity. For example, morpholines M-**6** to **M-9** are toxic for aquatic crustraceans but do not exhibit human oral bioavailability and compounds **M-1** to **M-4** present human oral bioavailability and are non-toxic to aquatic crustaceans. **M-5** is not oral bioavailability and is not toxic to crustaceans aquatic (Table 6).

The plasma protein binding values for alkyl morpholines were (0.41, 0.49, 0.43, 0.52, 0.50, 0.70, 0.72, 0.74 and 0.75) for **M-1** to **M-9**. Linezolid can cross the blood brain barrier, is hepatotoxic, presents human oral bioavailability, can be absorbed by the human intestine, and is toxic for aquatic fish; opposite values were predicted for Gentamicin. Likewise, gentamicin is permeable in Caco-2 cells. The plasma protein binding values for Gentamicin and Linezolid were (0.52 and 0.59) respectively.

Table 5. LC_{50} of the N-alkyl morpholine compounds and antibiotics evaluated against *Artemia salina* nauplii (CYTED, 1995) [13].

Compounds /Antibiotic	*Preliminary acute toxicity*	
	LC50(ppm)	Toxicity category
M-1 [CH_3]	441.77	Moderately toxic
M-2 [CH_3 CH_2]	426.47	Moderately toxic
M-3 [$CH_3(CH_2)_2$]	408.51	Moderately toxic
M-4 [$CH_3(CH_2)_3$]	408.48	Moderately toxic
M-5 [$CH_3(CH_2)_4$]	418.45	Moderately toxic
M-6 [$CH_3(CH_2)_{11}$]	90.48	Highly toxic
M-7 [$CH_3(CH_2)_{13}$]	12.07	Highly toxic
M-8 [$CH_3(CH_2)_{15}$]	9.07	Extremely toxic
M-9 [$CH_3(CH_2)_{17}$]	19.07	Highly toxic
LN	--	Relatively innocuous
GN	--	Relatively innocuous

No mortality was presented in the nauplii of *Artemia salina*. **LN= GN=**

The prediction of the pharmacokinetic parameters of the N-alkyl morpholines are non-genotoxic, while gentamicin and linezolid are predicted to be genotoxic. Interestingly, results for aquatic toxicity of crustaceans agrees with the experiments carried out *in vitro*, that is, only the compounds (11-17) with long chains of carbons were toxic in this model. The results for the tested compounds indicate that they could be used as drug candidates. Therefore, it is essential to carry out more studies, to adequately establish the toxicity suggested in this analysis. The effectiveness of the method lies in the comparison of the LC_{50} with the LD_{50} obtained from the acute toxicity tests in rats, a methodology that precedes the initial toxicity analysis, and which is necessary to establish exact parameters of the effects of any substance depending on the dose administered.

Table 6. List of ADMET properties of the N-alkyl morpholine compounds with increasing alkylic chain length and antibiotics.

Model	M-1	M-2	M-3	M-4	M-5	M-6	M-7	M-8	M-9	LN	GN
Blood Brain Barrier	+	+	+	+	+	+	+	+	+	+	-
Ames Mutagenesis	-	-	-	-	-	-	-	-	-	+	-
Acute Oral Toxicity (c) kg/mol	III	III	III	III	III	III	III	III	III	III	III
Avian Toxicity	-	-	-	-	-	-	-	-	-	-	-
Caco-2 Permeability	+	+	+	+	+	+	+	+	+	+	+
Carcinogenecity	-	-	-	-	-	-	-	-	-	-	-
Crustacean Aquatic Toxicity	-	-	-	-	-	+	+	+	+	-	-
Hepatotoxicity	-	-	-	-	-	-	-	-	-	+	-
Genotoxicity	-	-	-	-	-	-	-	-	-	+	+
Human intestinal absorption	-	-	-	-	-	-	-	-	-	+	-
Human oral bioavailability	+	+	+	+	-	-	-	-	-	+	-
Plasma protein binding	0.41	0.49	0.43	0.52	0.50	0.70	0.72	0.74	0.75	0.59	0.52
Fish aquatic toxicity	-	-	-	-	-	-	-	-	-	+	-

***In-silico* Molecular Docking**

Autodock vina runed millions of positions analyzes between ligands and the RNA 50s subunit. Table 7 shows the affinity, interaction and type of distances of the nine morpholine N-alkyl substituted, as can be seen, the compounds M-1 to M3 and M-4 to M5 present similar values of affinity energy, while from the components M-6 to M-9 the energy varies only one little, which can be associated with the longer in the morpholine ring chain the energy decreases, which represents that there is greater binding with the receptor.

The positions of the interactions found can be observed in table 7, for the case of compounds M-1, M2 and M5 it can be observed that there is a link between the compounds and Phe 57 (Figure 3). The variations between the positions of the interactions begin to be observed from compounds M-6 to M-9 where the binding to the receptor occurs in almost the same positions as Lys 54, and Trp 61, while for the specific case of the compound M-7, two distinct types of interactions were observed, hydrophobic interaction and pi-cation interaction, and a total of four interactions were observed at positions Lys 54, Arg 58, Asn 91, and Phe 57 (Figure 4).

Table 7. Affinity, interactions and type of distances of the nine morpholine compounds N-alkyl substituted.

Compounds	Affinity	Aminoacids	Residue	Distance	Type of interaction
M1	-3.6	Arg	2	3.07	Hydrogen bond
		Phe	57	4.83	Pi-cation interaction
		Arg	53	3.73	Halogen bond
M2	-3,7	Phe	57	3.65	Hydrophobic interaction
		Lys	93	3.54	Hydrogen bond
M3	-3.7	Arg	106	3.77	Hydrophobic interaction
		Leu	110	3.77	
M4	-4.2	Leu	38	3.67	Hydrophobic interaction
		Leu	38	3.65	
M5	-4.2	Phe	57	3.62	Hydrophobic interaction
		Trp	61	3.81	
M6	-4.6	Lys	54	3.97	Hydrophobic interaction
		Arg	58	4	
		Trp	61	3.67	
		Trp	61	3.62	
M7	-4.5	Lys	54	3.71	Hydrophobic interaction
		Arg	58	3.75	
		Asn	91	3.79	
		Phe	57	5.65	Pi-cation interaction
M8	-4.3	Lys	54	3.75	Hydrophobic interaction
		Phe	57	3.77	
		Phe	57	3.64	
		Phe	57	3.7	
		Trp	61	3.77	
		Lys	93	3.89	
M9	-5.1	Thr	4	3.86	Hydrophobic interaction
		Arg	53	3.77	
		Lys	54	3.61	
		Phe	57	3.39	
		Phe	57	3.67	
		Phe	57	3.78	
		Phe	57	3.77	
		Trp	61	3.77	
		Trp	61	3.7	
		Trp	61	3.98	
		Lys	93	3.95	
Gentamicin	-6.9	Phe	2	3.67	Hydrophobic interaction
		Phe	2	3.93	
		Phe	5	3.73	
		Met	6	3.73	
		Asn	8	3.74	
		Met	1	3.1	Hydrogen bond
		Met	1	3	
		Met	1	3.27	
		Arg	2	1.92	
		Arg	2	2.7	
		Gln	3	3.35	
		Thr	4	2.93	
		Lys	13	3.34	
		Glu	97	3.28	
Lynezolid	-5.7	Lys	40	3.65	Hydrophobic interaction
		Ile	62	2.49	Hydrogen bond

The docking results of the *S.aureus* 50s unit with gentamicin have affinity energies of -6.9. Interactions with gentamicin were found with respect to amino acid residues Phe 2, Phe 5, Met 6, Asn 8, Met 1, Arg 2, Gln 3, Thr 4, Lys 13 and Glu 97 with distances between 1.92 and 3.95 (Fig.5A).

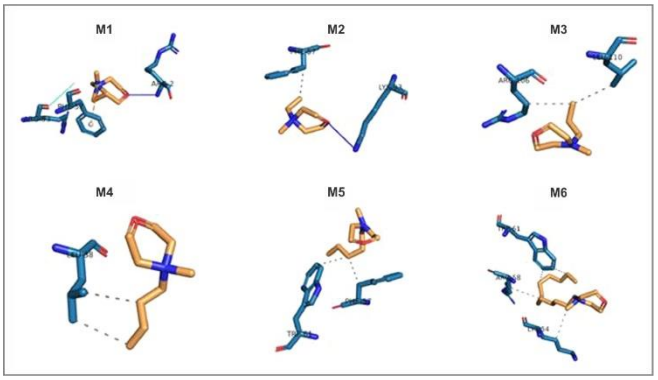

Figure 3. Molecular docking and interaction maps of the three morpholine compounds N-alkyl substituted (**M-1** to **M-6**) with the Phosphoryl Transferase Center (PTC) of the crystallized structure of the 50s ribosome subunit of *S.aureus.*

While in the case of docking with linezolid a synthetic compound belonging to the oxazolidinone class of antibiotics,gave affinity energies almost equal to gentamicin with values of -5.3 but the lowest values were -2.6. On the other hand, the positions of the interactions varied with the drug linezolid since they were found in positions Lys 40 and Ile 62 (Fig.5B).

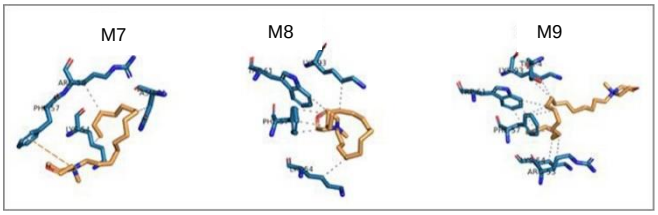

Figure 4. Molecular docking and interaction maps of the three morpholine compounds N-alkyl substituted (**M-7**, **M-8**, **M-9**) with the Phosphoryl Transferase Center (PTC) of the crystallized structure of the 50s ribosome subunit of *S.aureus*

On the other hand, the results of the coupling against the ribosomal protein 50s of *S. aureus* indicated a well conserved binding region, but with slightly different predicted binding energy values. For compounds M1 to M-5, the affinity energies are between -3.6 and -4.2 kcal/mol, while the affinity values begin to decrease for components M-6 to M-9 with energies from -5.1 to -4.3 kcal/mol. Components M-6 to M-9 presented a higher degree of antimicrobial activity.

Other studies have evaluated various structures, where the 50s ribosomal subunit appears to be a common target. The evaluation of hydroxy-3-aryl coumarins against various bacterial genera has been previously reported. Likewise, after carrying out molecular modeling, both tyrosyl-tRNA synthetase and *S. aureus* topoisomerase II DNA gyrase were studied as possible targets

and a correlation was obtained between the observed inhibitory activity and the molecular coupling scores[53]. On the other hand, the antimicrobial evaluation of N-acyl-morpholine-4-carbothioamides has also been reported and their molecular coupling was analyzed. In addition, the selected compounds were found to stack well within the active site of the major groove of the RNA complex[54].

In the case of controls like Linezolid; biochemical and structural evidence previously reviewed, indicates that Linezolid binds to the peptidyl transferase center of the large ribosomal subunit (50s), in a position that overlaps with the aminoacyl residue of an A site attached to tRNA[55,56].

Based on our study, the binding site of the nine N-alkyl substituted morpholine compounds and Linezolid should prevent the correct placement of the aminoacyl residue of the A-tRNA and inhibit the formation of peptide bonds.

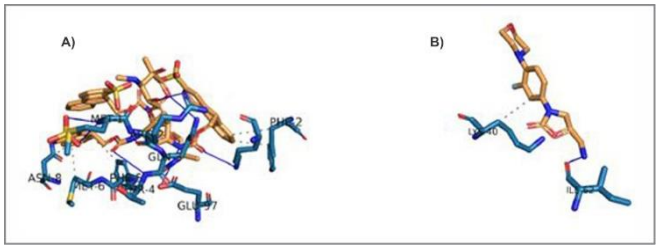

Figure 5. Molecular docking and interaction maps of controls. A) Positive control (Gentamicin), B) Structural control (Linezolid) with the crystallized structure of the 50s ribosome subunit of *S.aureus.*

The existence of commercial and antibacterial drugs with morpholinic structures (such as Linezolid), allows us to make use of all the information already generated and approved. Morpholines are chemical structures present in numerous commercial and experimental drugs. Several publications show that the morpholine ring is important for antimicrobial activity [48]. We have synthesized a novel series of hybrid molecules, which exhibited broad-spectrum antimicrobial applicability also similar to Linezolid. Structural optimization showed that the combination of morpholine and alkyl chains has a positive impact on activity. The antimicrobial screening data reveals that among the compounds only a few of them exhibit moderate to good activity. We consider modifications of the compounds to increase the antibacterial activity and reduce the toxicity.

Future research should be addressed to *in vivo* tests that demonstrate the antibacterial effect evidenced in the present study, as well as to discard the harmful effects of the chemical structures and the search for other biological activities. This finding suggests that morpholines could represent a chemical structure for the development of new antibiotics, given the fact that they are effective against multiresistant strains of *S. aureus*. The N-alkyl morpholines evaluated can be modified to improve the activity and reduce toxicity. Morpholines are an option that could be used against multidrug-resistant bacteria. More research is needed to develop new drugs to ensure successful treatment after the emerging infectious disease priority alert declared by the World Health Organization in 2017.

Conclusion

In this study, we have prepared nine N-alkyl morpholines with carbon number lengths from 11 to 17 and their antibacterial activity against MRSA was evaluated. We observed a structure-activity relationship, that active compounds were those with longer chain structures (i.e. CH_3 $(CH_2)_{11}$, CH_3 $(CH_2)_{13}$, and CH3 $(CH_2)_{15}$). In contrast, compounds with short chains (<5C) were inactive against MRSA bacteria. The compound with long chain structure $CH_3(CH_2)_{15}$ and Linezolid presented similar MIC activity (3.9-4 µg/mL) but MBC activity is lower for the compound **M-8** (3.9 µg/mL) than the Linezolid (32 µg/mL). Assays on *A. salina* showed that the inactive compounds were classified as moderately toxic, while the active compounds resulted in highly or extremely toxic according to the CYTED scale. Docking analysis allowed the elucidation of a possible mechanism of action of the nine N-alkyl substituted morpholine compounds regarding their possible antimicrobial effect with RNA inhibition. The importance of the ring in compounds **M1-M9** was corroborated by some of the aromatic-aromatic stacking interactions observed in the coupling studies.

It was observed that as the N-substituted chain of the morpholine ring grows (**M-6** to **M-9**), the interactions on the 50s ribosomal subunit vary in ***in-silico*** coupling, which may be related to increased activity antimicrobial of these components.

Experimental section

Reactives. the following reagents used throughout the study were purchased from Sigma-Aldrich and used without further purification: 1-bromododecane (97%, CAS 143-15-7), 1-bromotetradecane (97%, CAS 112-71-0), 1-bromohexadecane (97%, CAS 112-82-3), 1-bromooctadecane (97%, CAS 112-89-0), iodomethane (99.5%, CAS 74-88-4), iodoethane (99%, CAS 75-03-6), 1-iodopropane (99%, CAS 107-08-4), 1-bromobutane (99%, CAS 109-65-9), 1-iodohexane (98%, CAS 638-45-9), 4-methylmorpholine (98%, CAS 109-02-4).

Biological material

Five multi-resistant (MDR) *Staphylococcus aureus* isolates were isolated from clinical samples of apparently healthy children in childcare centers in the city of Culiacán, Sinaloa. Likewise, the *Staphylococcus aureus* strains ATCC 43300 [methicillin resistant, *mecA* (+)] and ATCC 25923 [methicillin susceptible, *mecA* (-)] were used [11]. On the other hand, *Artemia salina* cysts Instant Ocean®, Blacksburg, VA, USA were used.

Instruments

^{1}H, ^{13}C, NMR spectra were recorded using a Varian 400 spectrometer. Chemical shifts (/ppm) are reported relative to DMSO-d6.

FTIR spectra were measured on an Agilent series Cary Spectrum 600 FT-IR spectrophotometer (units are in cm^{-1}).

High-resolution mass spectra were obtained by direct infusion using an Agilent Accurate Mass QTOF 6530 and a Cole-Parmer single-syringe infusion pump.

General procedure for reactions

In a round bottom flask, 10 mmol of N-methylmorpholine, 10 mL of ethanol, and 10 mmol of the corresponding alkyl halide were placed, if the boiling point of the alkyl halide was low, it was necessary to add 5-10% excess of it, monitoring the reaction through thin layer chromatography. (CH_2Cl_2: MeOH 9:1 v/v). The solvent was removed using a vacuum evaporator. After the solvent was removed, 20 mL of hexane was added to the residue and the product precipitated as a white solid, the product was separated by vacuum filtration and washed with a small portion of hexane, and dried. The structures of all morpholine derivatives were determined by IR, NMR, and Mass spectroscopic analysis, and the results were in agreement with the proposed structures. Reactions were monitored by TLC on precoated silica gel plates (ALUGRAM SIL G/UV254) and revealed by exposure to Iodine [9,10]. A summary of the chemical characteristics of N-alkyl morpholine compounds is presented in Table 1.

Characterization of *Staphylococcus aureus* strains

The five strains of MRSA isolated from pharyngeal exudates in children were maintained in cryopreservation at -80°C in 10% of dimethyl sulfoxide (DMSO). The ATCC strains *S. aureus* 25923 [susceptible to methicillin, *mecA* (-)] and *S. aureus* 43300 [resistant to methicillin, *mecA* (+)], were used as controls to perform the antibacterial tests following the recommendations of the Clinical and Laboratory Standards Institute. The biochemical profile of the bacterial isolates was obtained by performing conventional tests, such as coagulase, catalase, DNase, oxidase, mannitol fermentation, and hemolysis. Besides, the resistance profile of the bacterial isolates was previously obtained by the Kirby-Bauer method. The methicillin resistance phenotype of *S. aureus* was previously confirmed by PCR amplification of the *mecA* gene (data are not shown), which confers resistance to β-lactams [12].

Susceptibility testing

First, the most suitable concentration of the solvent to be used was determined, in a manner that is soluble for the compound and viable for *S. aureus*, a broth macrodilution test was carried out. All compounds were soluble and evaluated in DMSO at concentrations of 4, 6, 8, and 10%. The strain *S. aureus* ATCC 25923 (characterized as susceptible to methicillin), was selected for its susceptibility profile with the aim that factors such as the resistance did not exert an effect on the interpretations [11].

The inoculum was adjusted to the same extent to be used in the determination of MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) (1×10^6 CFU/mL) at a wavelength of 530 nm, in 0.85% saline. When 100 µL of the inoculum and 1.9 mL of solvent to each tube, they were shaken and incubated at 35 °C for 18-20 h. Likewise, a positive control without solvent was used, which made it possible to observe the growth of *S. aureus* under normal conditions, to visualize the impact of the presence of DMSO. Subsequently, each culture was placed in different Petri dishes with TSA medium, adding 1 µL of each concentration and striating completely, to cover the entire surface of the agar. Finally, after incubation at 35ºC for 18-20 h, the colony forming units (CFU) were counted in each of the Petri dishes corresponding to the concentrations to be evaluated of the solvents, to determine the concentration of DMSO to be used.

The susceptibility profile was confirmed with the Kirby-Bauer method in the Mueller-Hinton culture medium with the CLSI recommendations (2012) [11,13]. First, four or five colonies of similar morphology were taken and inoculated in four mL of Mueller Hinton broth. The culture was incubated (35°C/2-5 h) to obtain turbidity equivalent to 0.5 of the McFarland scale which corresponds to a suspension containing approximately 1 to 2 × 10^8 CFU/ mL. For sow the inoculum, a sterile cotton applicator was immersed in the suspension removing the excess and sow in solid Mueller Hinton medium in three directions over the entire

surface of the agar. Subsequently, the surface of the seeded medium was allowed to dry (5-20 min) keeping the box with the lid closed. Then, the discs with antibiotics were placed on the surface of the agar with a dispenser. Finally, the inverted plates were incubated at 35°C for 16-18h. The 15 antibiotics used were Ampicillin (AMP) (10 µg), Cephalothin (KF) (30 µg), Cefotaxime (CTX) (30 µg), Cefuroxime (CXM) (30 µg), Cefepime (FEP) (30 µg), Dicloxacillin (DC) (1 µg), Erythromycin (E) (15 µg), Levofloxacin (LEV) (5 µg), Gentamicin (GN) (10 µg), Penicillin (P) (10 µg), Tetracycline (TE) (30 µg), Sulfamethoxazole/Trimethoprim (SXT) (25 µg), Vancomycin (VA) (30 µg), Oxacillin (OX) (1 µg), and Cefoxitin (FOX) (30 µg). With the help of a black surface that will not reflect the light, the diameter of the zone of inhibition was measured, including the six mm of the disc, with a Vernier ruler on the back of the petri dish without removing the lid. The strains *S. aureus* ATCC 43300 and ATCC 25923 were used as controls. Tables of the CLSI version 2021 were used as a guide for the interpretation and reporting of results.

Antibacterial activity evaluation (MIC/MBC)

To measure the antibacterial activity, we determined the minimum inhibitory (MIC) and bactericidal (MBC) concentrations according to the gold standard method established by the CLSI version 2012. Nine compounds were analyzed quantitatively in broth microdilution tests to determine the lowest concentration to inhibit bacterial growth completely in a range of concentrations of 0.06-250 µg/mL[11]. First, a series of dilutions was prepared twice at the desired concentration to be evaluated, from a stock solution at a concentration of 500 µg/mL to fill a 96-well microplate in triplicate with 50 µL of each solution of the compounds. Then, 50 µL of the bacterial solution containing 10^6 CFU/mL was added. Gentamicin (GN) (0.125-16 µg/mL) was used as a positive control, whereas negative controls were inoculum without antibiotic or compound. Besides, linezolid (LN) (0.5-32 µg/mL) was used as a structural control, because this drug carries a morpholino ring in its structure and is an antibiotic reserved for MRSA infections. The compounds were dissolved with DMSO (5%) and we did not observe any detectable effect on bacterial growth. The microplate was incubated at 35 °C for 18-20 h.

The MIC value was defined as the lowest compound concentration at which no turbidity or growth button was observed in the well. The minimum bactericidal concentration MBC was determined by subculturing the assay dilution of microtiter plate wells where no turbidity was observed from the MIC in salt and mannitol medium and incubating at 35 °C for 18 - 20 hours. The highest dilution that did not produce a bacterial colony was taken as MBC.

Brine shrimp toxicity bioassay

To evaluate the toxicity of the morpholinic compounds, a test of lethality to *Artemia salina* brine shrimp was made [14]. *Artemia salina* eggs (300 mg) were suspended in 500 mL of sea artificial medium, incubated at 25±1 ºC for 24 h with oxygenation, and provided with continuous light. Nauplii were recovered and placed in fresh saline solution, staying 24 hours more at the same conditions. Compounds were dissolved in saline solution and DMSO (10%). Each tube contained 10 nauplii, 200 µL of the compound at different concentrations, and saline solution up to 2 mL; final sample concentrations (10, 20, 60, 80, and 100 ppm), were evaluated in triplicate. Tubes were incubated at 25±1 ºC for 24 h and the number of living nauplii per tube was counted. *Artemia salina* nauplii treated with solvent or samples were observed under magnification (100x) using a light field microscope and results were photographed. The highest concentration of DMSO used was 1% without any detectable effect on *A. salina* larvae [14]. The mortality percentage (%M) was calculated with the following formula: %M= (DLT/TLT)*100, where DLT: Dead larvae by tube and TLT: Total larvae by tube.

Finally, the Probit analysis was used to determine the values of the lethal concentration 50 (LC_{50}) and this was done using the Minitab 15.1.20.0 (2007) software. Toxicity scale in the *A. salina* assay was registered as extremely toxic (1–9.9 µg/mL), highly toxic (10–99.9 µg/mL), moderately toxic (100–499.99 µg/mL), slightly toxic (500-999.99 µg/mL), practically not toxic (1000-1500 µg/mL) and relatively innocuous (>1500 µg/mL) according to the CYTED (Iberoamerican Program of Science and Technology for Development) scale[15].

ADMET prediction/Pharmacokinetic parameters

The prediction of absorption, distribution, metabolic, excretion, and toxicity (ADMET) properties plays a key role in drug design/development. Blood-brain barrier penetration, human intestinal absorption (HIA), Human oral bioavailability, Plasma protein binding, Caco-2 Permeability (Caco2+), Ames toxicity, carcinogenicity, Avian toxicity, Crustacean aquatic toxicity, Fish aquatic toxicity, hepatotoxicity, and genotoxicity were calculated using admetSAR (http://lmmd.ecust.edu.cn/ admetsar1/)[16].

***In -Silico* Molecular Docking.**

Ligand preparation.

The three-dimensional models of the synthesized compounds (M1-M9) were generated using Avogadro and saved as PDB format. These models may not accurately represent the atom's location in the actual molecules and possess high energy strain at various bonds or conformational strain between atoms. To correct the models, the sketched structures were energy-minimized using the MMF94 force field method which is an application of Avogadro. This application calculates a new position of each atom so that the cumulative potential energy for the models is minimized. PDBQT files were generated by using the Autodock tool (ADT) to add charges to the ligands which also automatically merge the non-polar hydrogen. AutoDock Vina 1.1.2.

For the analysis of the control drugs, gentamicin was used, whose 2D structure was extracted from Pubchem (CID: 3467), later Avogadro was used to determine the 3D structure necessary for docking, the minimization of the energy between the gentamicin bonds was done by means of the MMF94 method that is inside the Avogadro program[49].

Continuing with the controls we have the linezolid that, although its 3D structure is found in Pubchem (CID:441401), it is in.SDF format, so Avogadro was also used to reduce the energy stabilize the component, and convert it to .pdb format[50].

Accession of Target Protein.

In order to rationalize this significant difference in the antibacterial activity at the molecular level, the molecular docking of the nine-morpholine compounds N-alkyl substituted was performed. The three-dimensional structure of the target protein was retrieved from PDB by giving the PDB ID in the database; the target protein used is the Cryo-EM structure of 50S-RsfS complex from *Staphylococcus aureus*[51]. Before docking, the entire water molecules were removed from the protein molecule. Polar hydrogens were added as they are needed in the input structures to correctly type heavy atoms as hydrogen bond donors. The modified receptor file was then saved in the PDBQT format for docking studies.

Docking Run.

Once our ligands and the receptor were prepared, Autodock Vina was used for docking. This software uses the specification of the "search" space in the coordinate system of the receptor, within which various positions of the ligand are to be considered. The dimension of the search space was defined with Grid Center at X: 146.0, Y: 144.54 Z: 146.669 Å and the number of points in each dimension as X: 15, Y: 15, Z: 15 and Spacing (Angstrom): 0.3750. Autodock vina runs millions of position analyses where the ligand can bind the receptor and each output file has several patterns ranked in descending order in terms of binding energy[52]. The predicted binding affinity of the ligand to the target protein is plotted in kcal/mol. For the case of the nine morpholine components, the first model was selected to perform the interaction analysis.

Analysis of interactions.

For the analysis of the searches of the specific interactions between the nine components and the protein 6SJ6 of *S.aureus*, we proceeded to use bioinformatics platform PYMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC) which is a user-sponsored molecular display system on an open source basis, maintained and distributed by Schrödinger. PYMOL shows the sizes and locations of the binding sites, hydrogen bond interactions, hydrophobic interactions, and distances of the bond as interaction radii of <5 Ă from the position of the coupled ligand up to the protein. The determination of the amino acids around the binding site of the components and the receptor protein was obtained by means of the Pymol command line.

Acknowledgements: This study was funded by Consejo Nacional de Ciencia y Tecnología and scholarships were awarded to postgraduate students in biomedical sciences I am grateful to Blas Flores Perez whom we have had the pleasure to know and who was an important part to work during this and other related projects. Also, we thank all of those with who provided insight and expertise that greatly assisted the research

Keywords: MRSA, morpholine, Antibacterial, Multidrug-resistant.

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