Antibacterial Nanoassembled Calix[4]arene Exposing Choline Units Inhibits Biofilm and Motility of Gram Negative Bacteria

Grazia Maria Letizia Consoli[a,*], Giuseppe Granata[a], Giovanna Ginestra[b], Andreana Marino[b], Giovanni Toscano[b], and Antonia Nostro[b,*]

[a]Institute of Biomolecular Chemistry-C.N.R. 95126 Catania, Italy

[b] Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Italy

ABSTRACT: The high incidence of antibiotic resistance and biofilm associated infections is still a major cause of morbidity and mortality and triggers the need for new antimicrobial drugs and strategies. Nanotechnology is an emerging approach in the search for novel antimicrobial agents. The aim of this study was to investigate the inherent antibacterial effects of a self-assembling amphiphilic choline-calix[4]arene derivative (**Chol-Calix**) against Gram negative bacteria. **Chol-Calix** showed activity against *Escherichia coli* and *Pseudomonas aeruginosa* including antibiotic resistant strains and affected the bacterial biofilm and motility. The activity is likely related to the amphipathicity and cationic surface of **Chol-Calix** nanoassembly that can establish large contact interactions with the bacterial surface. **Chol-Calix** appears a promising candidate in the research of novel nanosized non-conventional antimicrobials.

KEYWORDS Antibacterial, Antibiofilm, Motility Inhibition, Choline-Calix[4]arene, Escherichia coli, Pseudomonas aeruginosa, Antibiotic Resistance

Diffusion of bacterial infections, low antibiotic turnover, and antibiotic resistance phenomena are serious problems and trigger a strong demand for novel strategies and materials that can manage with these issues. Nanotechnology is emerging as a sound approach to generate novel compounds alternative or complementary to traditional antibiotics.[1] Indeed, nano-antimicrobials might fill the gaps where traditional antibiotics fail and potentially revolutionize antimicrobial therapy. Compared with conventional antimicrobials, nano-antimicrobials offer attractive advantages deriving from their chemical but also physical properties (size, shape) that may lead to fine-tuned interactions with bacterial cells. A nano-antimicrobial may amplify the contact with the bacterial cell surface and might penetrate the bacterium with modalities different from those of small molecules.[2] Moreover, as reported for some ammonium quaternary compounds, the assembly in nanosized aggregates can result in multivalent antibacterial agents with reduced toxicity to eukaryotic cells.[3]

The applications of nanotechnology bases on both inorganic nanoparticles and organic nanoassemblies with intrinsic antibacterial properties or working as nanocarriers for the vehiculation of antibiotics.[4,5]

Calix[n]arenes are a family of macrocyclic polyphenols of great interest in supramolecular chemistry.[6] Due to the presence of a host cavity, synthetic versatility, and ability of amphiphilic derivatives to self-assembly in nanostructured systems, calix[n]arene oligomers have been proposed as molecular scaffolds in drug discovery[7] and drug delivery[8]. Calixarene derivatives have shown low cytotoxicity and immunogenicity[9] and activity as antitumoral,[10] synthetic vaccine,[11] antimicrobial agents,[12] and more. Antibacterial calixarene derivatives include polycationic,[13] glycosylated[14] and polyanionic[15] derivatives, other than calixarene-coating inorganic nanoparticles.[16,17]

The clustering and spatial organization of multiple bioactive groups on a macrocyclic calix[4]arene platform is a promising approach for developing novel antimicrobial agents that have showed higher antibacterial activity and lower toxicity to eukaryotic cells compared with monomeric analogues[18,19] and known antiseptics.[20] Previously, we demonstrated that the clustering of four *N*-methyldiethanol ammonium groups on a calix[4]arene scaffold provided a polycationic derivative that showed antibacterial activity against *Staphylococcus aureus* and *S. epidermidis*, including methicillin-resistant strains, and in combination with old antibiotics enhanced the antibiotic effect against a *P. aeruginosa* strain.[21]

Among polycationic calixarenes, a choline-calix[4]arene derivative (**Chol-Calix**), bearing choline groups at the upper rim of a calix[4]arene scaffold and dodecyl alkyl chain at its lower rim (Figure 1), is an amphiphilic compound able to spontaneously self-assemble in stable micellar nanoaggregates.[22,23]

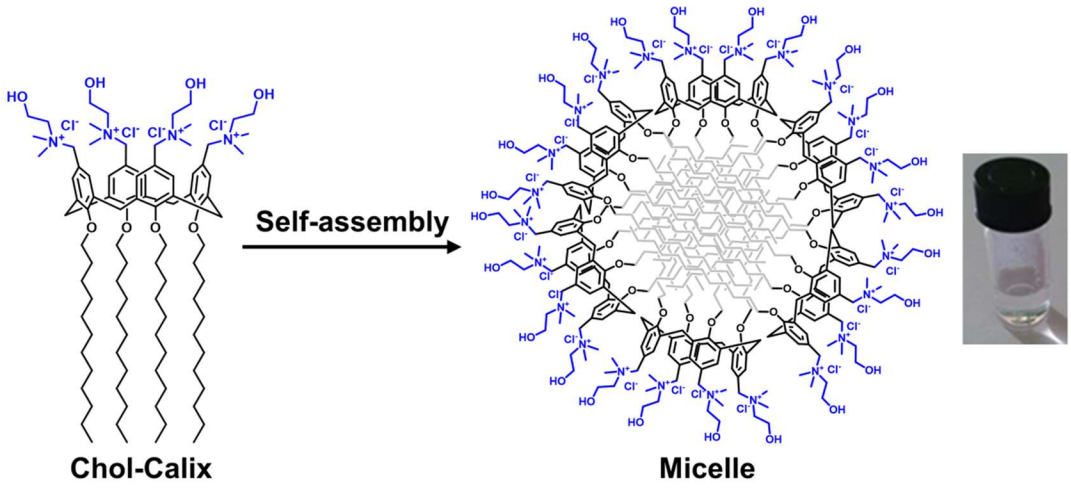

Figure 1. Molecular structure of **Chol-Calix**, schematic representation of its self-assembly in a micellar nanoaggregate and picture of the limpid nanoggregate solution. Adapted with permission from Ref. 28, Copyright 2017, *Molecular Pharmaceutics*.

We previously demonstrated that **Chol-Calix** at low concentration (c.a. 10 μM) in a biomimetic medium (10 mM PBS, pH 7.4) forms aggregates with diameter around 40 nm, polydispersity index 0.2, and zeta potential +24.7 mV.[22] The **Chol-Calix** micellar nanoassemblies showed to be a promising nanocarrier for poorly water-soluble and easily degradable drugs,[24] old antibiotics (i.e. ofloxacin, chloramphenicol and tetracycline),[25] and photo-responsive molecules eliciting photo-induced antibacterial activity due to the generation of nitric oxide and singlet oxygen radicals.[22,26,27] The potential of **Chol-Calix** as a curcumin delivery system was also successfully proved *in vivo*, in animal models of uveitis[28] and psoriasis.[29,30]

The antibacterial activity reported for polycationic calixarenes and for choline derivatives,[31] suggested that the nanosized **Chol-Calix** might also possess antibacterial properties. The quaternary ammonium and hydroxyl groups of the choline moieties exposed on the surface of the **Chol-Calix** micelle might establish electrostatic and hydrogen bond interactions with the bacterial membrane and as ligands the choline groups might bind choline-transporters expressed on the surface of *E. coli*[32] and *P. aeruginosa*.[33] Amphypathicity and nanosize of **Chol-Calix** are further advantageous features for larger interactions with the bacterial surface. A limit of cationic antibacterial agents may be the toxicity to human cells and tissues, but previously we observed no significant toxicity of **Chol-Calix** on fibroblasts[22] and corneal cells.[28]

With a view to discovering new antimicrobial compounds, here we investigated whether **Chol-Calix** possesses intrinsic antibacterial activity and affects bacterial biofilm and motility.

The susceptibility of bacteria to **Chol-Calix** was investigated on *E. coli* and *P. aeruginosa* strains by evaluation of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The values reported in Table 1 were comparable with those of other cationic antibacterial calixarene derivatives[18-21] and showed no significant differences between ATCC and carbapenemase producing *P. aeruginosa* DSM 102273 and ofloxacin-resistant *P. aeruginosa* 1 ocular isolate. The activity on resistant bacteria enhances the appeal of **Chol-Calix** in the search for novel not conventional antimicrobials.

Table 1. Antimicrobial activity of **Chol-Calix**.

Strains	MIC (μg/mL)	MBC (μg/mL)
E. coli ATCC 10536	18.8	18.8
P. aeruginosa ATCC 9027	9.4	18.8
P. aeruginosa DSM 102273	18.8	18.8
P. aeruginosa 1 ocular isolate	9.4	18.8

The outer membrane of Gram-negative bacteria provides the cell with an effective permeability barrier against external noxious agents but is itself a target for antibacterial agents. It is plausible to hypothesize that, analogously to other polycationic calixarene derivatives,[34] ammonium quaternary compounds,[35] and choline derivatives,[36] **Chol-Calix** elicits its antibacterial action by disturbance of the bacterial membrane. The initial interactions between the positively charged **Chol-Calix** and the negatively charged lipopolysaccharides of the bacterial membrane may be followed by integration of the dodecyl lipophilic tails of **Chol-Calix** into the hydrophobic membrane core with consequent weakening and alteration of the outer membrane integrity. The involvement of the dodecyl chains, that also induce the formation of stable nanoaggregates with enhanced surface contact area, is supported by the higher

antibacterial effect of **Chol-Calix** compared with analogues choline-calix[4]arenes bearing shorter propyl or octyl chains at the calix[4]arene lower rim, against *E. coli* and *P. aeruginosa* ATCC strains.[37]

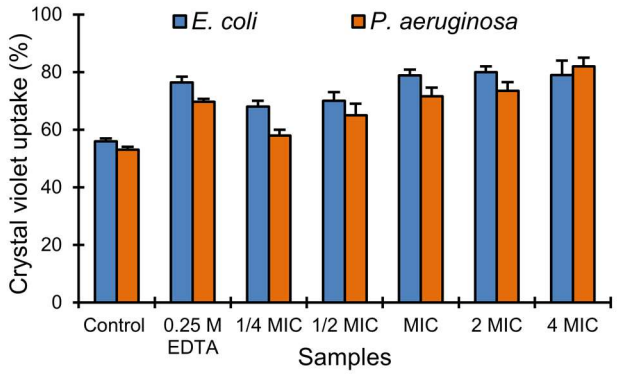

Figure 2. Crystal violet uptake assay. Percentage of crystal violet uptake by *E. coli* ATCC 10536 and *P. aeruginosa* ATCC 9027 alone (Control) and after treatment with EDTA and **Chol-Calix** at different concentrations.

To get a preliminary information about the mechanism of action, we performed the crystal violet assay,[38] notoriously used to evaluate the membrane permeabilizing effect of antibacterial agents.[39-41] Data in Figure 2 show that the uptake of crystal violet by *E. coli* and *P. aeruginosa* ATCC strains enhances from 56% and 53% (control) to 76.4% and 69.7% respectively, after treatment with EDTA, a known bacterial outer membrane permeabilizing agent (positive control). Analogously to EDTA, **Chol-Calix** enhanced the uptake of crystal violet in both *E. coli* and *P. aeruginosa* ATCC strains in dose-dependent manner (78.8% and 71.6% respectively at MIC concentration). The uptake values referred to the control were significant ($p < 0.05$) for all samples except for *P. aeruginosa* with **Chol-Calix** at ¼ MIC. The absence of released intracellular material (data not shown), as indicated by no significant increase of the absorption at 260 nm in the supernatant of the bacterial cells treated with **Chol-Calix** suggested that its activity is confined to the outer bacterial membrane without cell lysis.

Biofilms are microbial communities, held together by a self-produced extracellular matrix, very difficult to eradicate for their poor susceptibility to conventional antimicrobial agents.[42] Having antimicrobial agents that are also active against biofilm is a considerable achievement. Therefore, we investigated the effect of **Chol-Calix** on biofilm formation and preformed biofilm.

Concentrations of **Chol-Calix** lower than the MIC value poorly interfered with the planktonic growth of *E. coli* and *P. aeruginosa*, causing a slight decrease respect to the control, while caused a significant ($p < 0.05$) antibiofilm activity (Figures 3-4 and Table S1). In particular, the antibiofilm effect of **Chol-Calix** was more evident on *E. coli* than *P. aeruginosa*, being the inhibition of biofilm biomass at 1/2 MIC equal to 90.4 % and 40-55% respectively (Figures 3 and 4). Moreover, the results in Figure 3 clearly demonstrated that 1/4 MIC and 1/8 MIC of **Chol-Calix** determined a poor growth inhibition (14.5 % and 1.8 %) while caused a good biofilm inhibition (77.6 % and 51 %) of *E. coli*. These results were substantiated by observing bacterial biofilms under the light microscope (Figure 4). Direct microscopic observation showed that, after 24 h, in the absence of **Chol-Calix**, bacterial cells formed evident biofilms. In the presence of concentrations of **Chol-Calix** equal to 1/2 and 1/4 MIC, bacterial cells grew as looser colonies and the amount of formed biofilm was reduced, being almost absent at 1/2 MIC for *E. coli* (Figure 4).

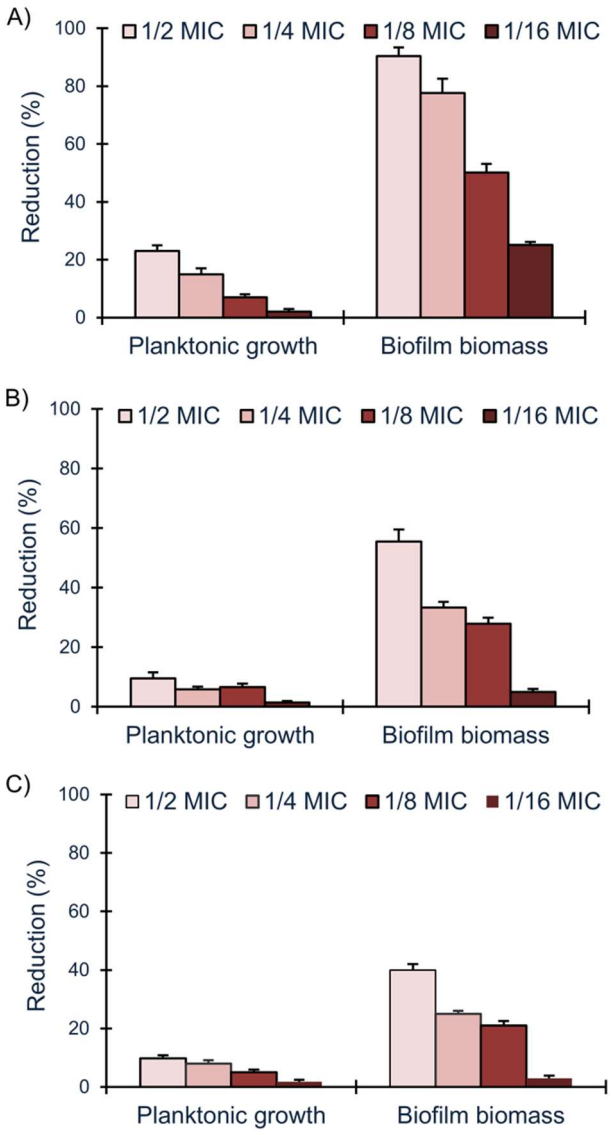

Figure 3. Reduction (%) of *E. coli* ATCC 10536 (A), *P. aeruginosa* ATCC 9027 (B) and *P. aeruginosa* 1 ocular isolate (C) planktonic growth and biofilm formation in the presence of various concentrations of **Chol-Calix**.

Cationic compounds with both antibacterial and antibiofilm activity were reported.[43-45] Mechanisms involved in bacterial biofilm formation are multiple and very complex and nowadays are not yet fully understood,[46] therefore to establish the exact antibiofilm mechanism of **Chol-Calix** is not trivial. However, the long dodecyl chains could play a

role in the anti-biofilm activity of **Chol-Calix**, indeed analogue choline-calix[4]arene derivatives, bearing shorter propyl and octyl chains, did not show antibiofilm activity against *E. coli* and *P. aeruginosa* ATCC strains.[36]

We also evaluated the effect of **Chol-Calix** on preformed biofilms in terms of influence on biofilm biomass. The results documented that **Chol-Calix** was capable to reduce preformed biofilm of both *E. coli* and *P. aeruginosa* ATTC and ofloxacin resistant *P. aeruginosa* 1 ocular isolate (Figure 5, Table S2). For *P. aeruginosa* strains a 37-50% reduction of preformed biofilm was detected at 2 MIC and 4 MIC (18.8 and 37.6 μg/mL) and 58-68% reduction at 8 MIC (75 μg/mL). For *E. coli* a 66.5% reduction was detected at 8 MIC dose corresponding to 150 μg/mL. Compounds reducing preformed biofilm at concentration > 100 μg/mL are present in literature.[47,48] Furthermore, it is remarkable that the potency of **Chol-Calix** may be enhanced by loading an additional antibiofilm agent in the micellar structure, which can entrap bioactive molecules including antibiotics.[25]

The disturbance of the bacterial cell membrane could be responsible for the activity on the biofilm as reported for a choline-based ionic liquid.[49] Moreover, it is plausible thinking that, analogously to other micelles of quaternary ammonium salts,[50] **Chol-Calix** first adsorbs onto the biofilm surface through multicharged interactions, then penetrates the extracellular polymeric matrix, diffuses throughout the biofilm, and reduces the biofilm biomass.

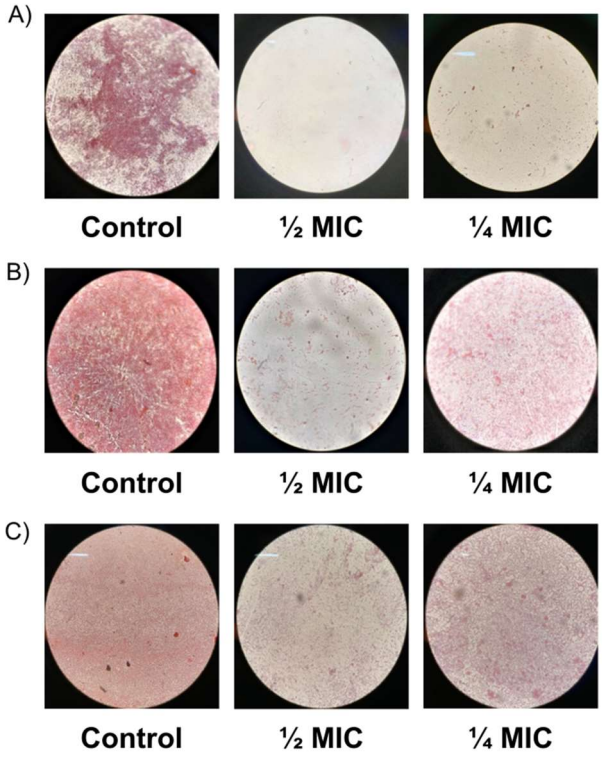

Figure 4. Microscopic images of the biofilm formation inhibition of *E. coli* (A) ATCC 10536, (B) *P. aeruginosa* ATCC 9027 and (C) *P. aeruginosa* 1 ocular isolate in the presence of various concentrations of **Chol-Calix**.

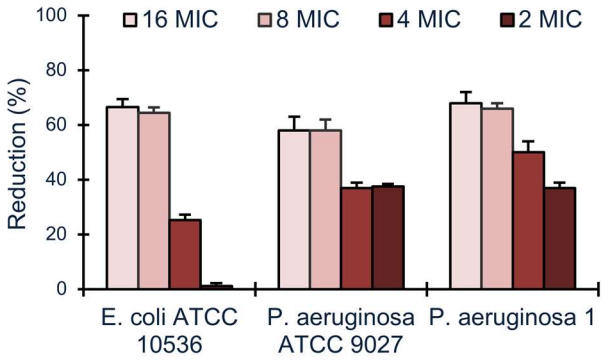

Figure 5. Reduction (%) of preformed biofilm in the presence of various concentrations of **Chol-Calix**.

Motility is important for the survival, dissemination, and virulence of bacteria. Therefore, inhibiting the motility can be a way to attack pathogens.[51] With this in mind, we decided to investigate whether **Chol-Calix** can affect swarming, swimming and twitching motility of *E. coli* and *P. aeruginosa* strains. At the best of our knowledge, this is the first work investigating the effect of an antibacterial candidate built on a calixarene scaffold on bacterial motility.

Swarming is described as a social phenomenon involving the movement of bacteria across a semisolid surface.[52] It is associated with enhanced virulence and antibiotic resistance of various human pathogens, including *P. aeruginosa*.[53] Controlling swarming is of major interest for the development of novel anti-infectives. The pretreatment with **Chol-Calix** at sub-MIC concentrations (1/2 MIC) reduced swarming and twitching of approximatively 50 - 60% on *E. coli* and *P. aeruginosa* respectively. A different effect was instead observed on swimming motility, in fact a weaker inhibition was observed for *E. coli* compared to *P. aeruginosa* (Figure 6).

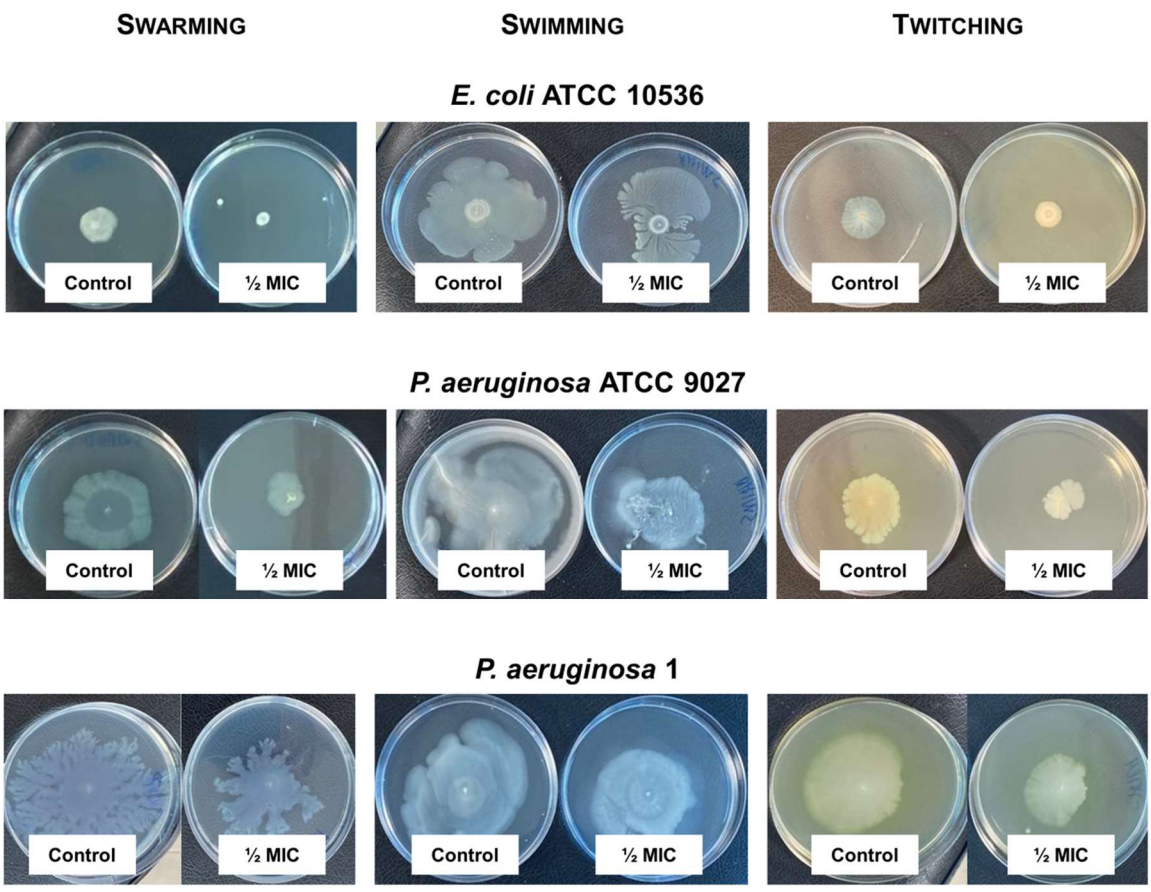

Figure 6. Microscopic images showing the swarming, swimming and twitching of *E. coli* ATCC 10536, *P. aeruginosa* ATCC 9027 and *P. aeruginosa* 1, without (control) and with pretreatment with **Chol-Calix** at different sub-MIC concentrations.

The effect of **Chol-Calix** on the biofilm formation and bacterial motility agreed with findings showing that in several Gram-negative bacteria, the motility is critical for both initial surface attachment and subsequent biofilm formation.

In conclusion, in this study we proved that the nanoassembling **Chol-Calix** possesses intrinsic antimicrobial activity against *E. coli* and *P. aeruginosa* strains and affects bacterial biofilm and motility. The effective activity also against antibiotic-resistant strains of *P. aeruginosa*, makes **Chol-Calix** an appealing potential nanosized antibacterial agent. The combination of its intrinsic antibacterial activities with the previously demonstrated advantage as a nanocarrier for drug delivery opens perspectives for application in antibacterial combined multidrug therapy.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge at……..Materials, Chol-Calix synthesis and characterization (NMR, DLS, TEM), Microbiological methods, Tables reporting the values relative to the effect of Chol-Calix on planktonic growth, biofilm formation and preformed biofilm.

AUTHOR INFORMATION

Corresponding Author

Grazia Maria Letizia Consoli – Institute of Biomolecular Chemistry C.N.R., Via P. Gaifami 18, 95126 Catania, Italy; orcid.org/0000-0002-9608-5138; Email: grazia.consoli@icb.cnr.it

Antonia Nostro - Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, viale Ferdinando Stagno d'Alcontres, 31, 98166 Messina, Italy; orcid.org/0000-0002-4280-7895; Email: anostro@unime.it

Authors

Giuseppe Granata – Institute of Biomolecular Chemistry C.N.R., 95126 Catania, Italy; https://orcid.org/0000-0001-9428-3985

Giovanna Ginestra – Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, 98168 Messina, Italy

Andreana Marino – Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, 98168 Messina, Italy; orcid.org/0000-0001-8751-8811

Giovanni Toscano– Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, 98168 Messina, Italy.

Author Contributions

All authors were involved in the preparation of this manuscript, including the conception and design of the study (GML.C., A.N.), Chol-Calix synthesis and characterization (GML.C., G.Gr.), microbiological studies (G.Gi, A.M, G.T., A.N.),

writing and editing of the manuscript (GML.C., A.N.). All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Seil, J. T.; Webster, T. J. Antimicrobial applications of nanotechnology: methods and literature. *Int. J. Nanomed.* **2012**, *7*, 2767–2781.

(2) Rizzello L.; Cingolani, R.; Pompa, P. P. Nanotechnology tools for antibacterial materials. *Nanomedicine* **2013**, *8*, 807–821.

(3) Lallemand, F.; Daull, P.; Benita, S.; Buggage, R.; Garrigue, J.-S. Successfully improving ocular drug delivery using the cationic nanoemulsion, Novasorb. *J. Drug Delivery* **2012**, *2012*, 604204, 16 p.

(4) Nurit, B.; Houri-Haddad, Y.; Domb, A.; Khan, W.; Hazan, R. Alternative antimicrobial approach: Nano-antimicrobial materials. Evid-Based Complement. *Altern. Med.* **2015**, *2015*, 246012.

(5) Abed, N.; Couvreur, P. Nanocarriers for antibiotics: A promising solution to treat intracellular bacterial infections. *Int. J. Antimicrob. Agents* **2014**, *43*, 485–496.

(6) Neri, P.; Sessler, J. L.; Wang, M.-X. Calixarenes and Beyond, Springer:Berlin-Heidelberg; 2016.

(7) Pan, Y.-C.; Hu, X.-Y.; Guo, D.-S. Biomedical applications of calixarenes: State of the art and perspectives. *Angew. Chem. Int. Ed.* **2021**, *60*, 2768–2794.

(8) Wang, J.; Ding, X.; Guo, X. Assembly behaviors of calixarene-based amphiphile and supra-amphiphile and the applications in drug delivery and protein recognition. *Adv. Colloid Interface Sci.* **2019**, *269*, 187–202.

(9) Naseer, M. M.; Ahmed, M.; Hameed, S. Functionalized calix[4]arenes as potential therapeutic agents. *Chem. Biol. Drug Des*. **2017**, *89*, 243–256.

(10) Viola, S.; Consoli, G. M. L.; Merlo, S.; Drago, F.; Sortino, M. A.; Geraci, C. Inhibition of rat glioma cell migration and proliferation by a calix[8]arene scaffold exposing multiple GlcNAc and ureido functionalities. *J. Neurochem*. **2008**, *107*, 1047–1055.

(11) Geraci, C.; Consoli, G. M. L.; Granata, G.; Galante, E.; Palmigiano, A.; Pappalardo, M.; Di Puma, S. D.; Spadaro, A. First self-adjuvant multicomponent potential vaccine candidates by tethering of four or eight muc1 antigenic immunodominant PDTRP units on a calixarene platform: Synthesis and biological evaluation. *Bioconjugate Chem*. **2013**, *24*, 1710–1720.

(12) Rodik, R. V. The antimicrobial and antiviral activity of calixarenes. *J. Org. Pharm. Chem.* **2015**, *13*, 67–78.

(13) Mourer, M.; Duval, R. E.; Constant, P.; Daffé, M.; Regnouf-de-Vains J-B. Impact of tetracationic calix[4]arene conformation-from conic structure to expanded bolaform on their antibacterial and antimycobacterial activities. *ChemBioChem* **2019**, *20*, 11–21.

(14) Granata, G.; Stracquadanio, S.; Consoli, G. M. L.; Cafiso, V.; Stefani, S.; Geraci, C. Synthesis of a calix[4]arene derivative exposing multiple units of fucose and preliminary investigation as a as potential broad-spectrum antibiofilm agent. *Carbohydr. Res*. **2019**, *476*, 60–64.

(15) Lamartine, R.; Tsukada, M.; Wilson, D.; Shirata, A. Antimicrobial activity of calixarenes. *Chimie* **2002**, *5*, 163–169.

(16) Khalid, S.; Parveen, S.; Raza Shah, M.; Rahim, S.; Ahmed, S.; Malik, M. I. Calixarene coated gold nanoparticles as a novel therapeutic agent. *Arab. J. Chem.* **2015**, *13*, 3988–3996.

(17) Tauran, Y.; Coleman, A. W.; Kim, B. Bio-Applications of calix[n]arene capped silver nanoparticles. *J. Nanosc. Nanotechnol.* **2015**, *15*, 6308–6326.

(18) Mourer, M.; Dibama, H. M.; Fontanay, S.; Grare, M.; Duval, R. E-; Finance, C.; Regnouf-de-Vains, J.-B. *p*-Guanidinoethyl calixarene and parent phenol derivatives exhibiting antibacterial activities. Synthesis and biological evaluation. *Bioorg. Med. Chem*. **2009**, *17*, 5496–5509.

(19) Boukerb, A. M.; Rousset, A.; Galanos, N.; Méar, J.-B.; Thépaut, M.; Grandjean, T.; Gillon, E.; Cecioni, S.; Abderrahmen, C.; Faure, K.; Redelberger, D.; Kipnis, E.; Dessein, R., Havet, S.; Darblade, B.; Matthews, S. E.; de Bentzmann, S.; Guéry, B.; Cournoyer, B.; Imberty, A.; Vidal, S. Antiadhesive properties of glycoclusters against *Pseudomonas aeruginosa* lung infection. *J. Med. Chem*. **2014**, *57*, 10275–10289.

(20) Grare, M.; Dibama, H. M.; Lafosse, S.; Regnouf-de-Vains J.-B.; Finance, C.; Duval, R. E. Cationic compounds with activity against multidrug-resistant bacteria: interest of a new compound compared with two older antiseptics, hexamidine and chlorhexidine. *Clin. Microbiol. Infect*. **2010**, *16*, 432–438.

(21) Consoli, G. M. L.; Granata, G.; Picciotto, R.; Blanco, A. R.; Geraci, C.; Marino, A.; Nostro, A. Design, synthesis and antibacterial evaluation of a polycationic calix[4]arene derivative alone and in combination with antibiotics. *Med. Chem. Commun*. **2018**, *9*, 160–164.

(22) Di Bari, I.; Picciotto, R.; Granata, G.; Blanco, A. R.; Consoli, G. M. L.; Sortino, S. A bactericidal calix[4]arene-based nanoconstruct with amplified NO photorelease. *Org. Biomol. Chem.* **2016**, *14*, 8047–8052.

(23) Rodik, R. V.; Anthony, A.-S.; Kalchenko, V. I.; Mély, Y.; Klymchenko, A. S. Cationic amphiphilic calixarenes to compact DNA into small nanoparticles for gene delivery. *New J. Chem*. **2015**, *39*, 1654-1664.

(24) Blanco, R.; Bondì, M. L.; Cavallaro, G.; Consoli, G. M. L.; Craparo, E. F.; Giammona, G.; Licciardi, M., Pitarresi, G.; Granata, G.; Saladino, P.; La Marca, C.; Deidda, I.; Papasergi, S.; Guarneri, P.; Cuzzocrea, S.; Esposito, E.; Viola, S. Nanostructured formulations for the delivery of silibinin and otheractive ingredients for treating ocular diseases. Patent WO/**2016**/055976, PCT/IB2015/057732

(25) Migliore, R.; Granata, G.; Rivoli, A.; Consoli, G. M. L.; Sgarlata, C. Binding affinity and driving forces for the interaction of calixarene-based micellar aggregates with model antibiotics in neutral aqueous solution. *Front. Chem*. **2021**, *8*, 626467.

(26) Di Bari, I.; Fraix, A.; Picciotto, R.; Blanco, A. R.; Petralia, S.; Conoci, S.; Granata, G.; Consoli, G. M. L.; Sortino, S. Supramolecular activation of the photodynamic properties of porphyrinoid photosensitizers by calix[4]arene nanoassemblies. *RSC Adv*. **2016**, *6*, 105573-105577.

(27) Di Bari, I.; Granata, G.; Consoli, G. M. L.; Sortino, S. Simultaneous supramolecular activation of NO photodonor/photosensitizer ensembles by a calix[4]arene nanoreactor. *New J. Chem*. **2018**, *42*, 18096–18101.

(28) Granata, G.; Paterniti, I.; Geraci, C.; Cunsolo, F.; Esposito, E.; Cordaro, M.; Blanco, A. R.; Cuzzocrea, S.; Consoli, G. M. L. Potential eye drop based on a calix[4]arene nanoassembly for curcumin delivery: Enhanced drug solubility, stability, and anti-inflammatory effect. *Mol*. *Pharm*. **2017**, *14*, 1610–1622.

(29) Granata, G.; Petralia, S.; Forte, G.; Conoci, S.; Consoli, G. M. L. Injectable supramolecular nanohydrogel from a micellar self-assembling calix [4] arene derivative and curcumin for a sustained drug release. *Mater. Sci. Eng. C* **2020**, *111*, 110842.

(30) Filippone, A.; Consoli, G. M. L.; Granata, G.; Casili, G-; Lanza, M.; Ardizzone, A.; Cuzzocrea, S.; Esposito, E.; Paterniti, I. Topical delivery of curcumin by choline-calix[4]arene-based nanohydrogel improves its therapeutic effect on a psoriasis mouse model. *Int. J. Mol. Sci*. **2020**, *21*, 5053.

(31) Siopa, F.; Figueiredo, T.; Frade, R. F. M.; Neto, I.; Meirinhos, A.; Reis, C. P.; Sobral, R. G.; Afonso, C. A. M.; Rijo, P. Choline-based ionic liquids: Improvement of antimicrobial activity. *ChemistrySelect* **2016**, *1*, 5909–5916.

(32) Ly, A.; Henderson, J.; Lu, A.; Culham, D. E.; Wood, J. M. Osmoregulatory systems of *Escherichia coli*: Identification of betaine-carnitine-choline transporter family member BetU and distributions of betU and trkG among pathogenic and nonpathogenic isolates. *J. Bacteriol.* **2004**, *186*, 296–306.

(33) Malek, A. A.; Chen, C.; Wargo, M. J.; Beattie, J. A.; Hogan, D. A. Roles of three transporters, CbcXWV, BetT1, and BetT3, in *Pseudomonas aeruginosa* choline uptake for catabolism. *J. Bacteriol*. **2011**, *193*, 3033–3041.

(34) Formosa, C.; Grare, M.; Jauvert, E.; Coutable, A.; Regnouf-de-Vains, J.-B.; Mourer, M.; Duval, R. E.; Dague, E. Nanoscale analysis of the effects of antibiotics and CX1 on a *Pseudomonas aeruginosa* multidrug-resistant strains. *Sci. Rep.* **2012**, *2*, 575.

(35) Alkhalifa, S.; Jennings, M. C.; Granata, D.; Klein, M.; Wuest, W. M.; Minbiole, K. P. C.; Carnevale, V. Analysis of the destabilization of bacterial membranes by quaternary ammonium compounds: a combined experimental and computational study. *ChemBiochem* **2020**, *21*, 1510–1516.

(36) Pernak, J.; Chwala, P. Synthesis and anti-microbial activities of choline-like quaternary ammonium chlorides. *Eur. J. Med. Chem*. **2003**, *38*, 1035–1042.

(37) Melezhyk, I. O.; Rodik, R. V.; Iavorska, N. V.; Klymchenko, A. S.; Mely, Y.; Shepelevych, V. V.; Skivka, L. M.; Kalchenko, V. I. Antibacterial properties of tetraalkylammonium and imidazolium tetraalkoxycalix[4]arene derivatives. *Anti-Infect. Agents* **2015**, *13*, 87–94.

(38) Devi, K. P.; Nisha, S. A.; Sakthivel, R.; Pandian, S. K. Eugenol (an essential oil of clove) acts as an antibacterial agent against *Salmonella typhi* by disrupting the cellular membrane. *J. Ethnopharmacol.* **2010**, *130*, 107–15.

(39) Halder, S.; Yadav, K. K.; Sarkar, R.; Mukherjee, S.; Saha, P.; Haldar, S.; Karmakar, S.; Sen, T. Alteration of Zeta potential and membrane permeability in bacteria: a study with cationic agents. *SpringerPlus* **2015**, *4*, 672.

(40) Li, N.; Tan, S.; Cui, J.; Guo, N.; Wang, W.; Zu, Y-g.; Jin, S.; Xu, X.-x.; Liu, Q.; Fuet, Y.-j. PA-1, a novel synthesized pyrrolizidine alkaloid, inhibits the growth of *Escherichia coli* and *Staphylococcus aureus* by damaging the cell membrane. *The Journal of Antibiotics* **2014**, *67*, 689–696.

(41) Sana, S.; Datta, S.; Biswas, D.; Sengupta, D. Assessment of synergistic antibacterial activity of combined biosurfactants revealed by bacterial cell envelop damage. *Biochim. Biophys. Acta (BBA) – Biomembranes* **2018**, *1860*, 2, 579–585.

(42) Olsen, I. Biofilm-specific antibiotic tolerance and resistance. *Eur. J. Clin. Microbiol. Infect. Dis*. **2015**, *34*, 877–886.

(43) Hou, S.; Liu, Z.; Young, A. W.; Mark, S. L.; Kallen-bach, N. R.; Ren, D. Effects of Trp- and Arg-Containing Antimicrobial-Peptide Structure on Inhibition of Esche-richia coli Planktonic Growth and Biofilm Formation. *Appl. Environ. Microbiol*. **2010**, *76*, 1967–1974.

(44) Harrison, J. J.; Turner, R. J.; Joo, D. A.; Stan, M. A ; Chan, C. S.; Allan, N. D.; Vrionis, H. A.; Olson, M. E.; Ceri, H. Copper and quaternary ammonium cations exert synergistic bactericidal and antibiofilm activity against Pseudomonas aeruginosa. *Antimicrob. Agents Chemother*. **2008**, *52*, 2870-2881.

(45) Kwaśniewska, D.; Chen, Y. L.; Wieczorek, D. Biological Activity of Quaternary Ammonium Salts and Their Derivatives. *Pathogens* **2020**, 9, 459.

(46) Melezhyk, I. O.; Rodik, R. V.; Iavorska, N. V.; Klymchenko, A. S.; Roy R, Tiwari M, Donelli G, Tiwari V. Strategies for combating bacterial biofilms: A focus on anti-biofilm agents and their mechanisms of action. *Virulence* **2018**, *9*, 22–54.

(47) Farha, A. K.; Yang, Q.-Q.; Kim, G.; Zhang, D.; Mavumengwana, V.; Habimana, O.; Li, H.-B.; Corke, H.; Gan, R.-Y. Inhibition of multidrug-resistant foodborne Staphylococcus aureus biofilms by a natural terpenoid (+)-nootkatone and related molecular mechanism. *Food control* **2020**, *112*, 107112.

(48) Harrison, Z. L.; Awais, R.; Harris, M.; Raji, B.; Hoffman, B. C.; Baker, D. L.; Jennings, J. A. 2-Heptylcyclopropane-1-Carboxylic Acid Disperses and Inhibits Bacterial Biofilms. *Front. Microbiol*. **2021**, *12*, 645180.

(49) Zakrewsky, M.; Lovejoy, K. S.; Kern, T. L.; Miller, T. E.; Le, V.; Nagy, A.; Goumas, A. M.; Iyer. R. S.; Del Sesto, R. E.; Koppisch, A. T.; Fox, D. T.; Mitragotri, S. Ionic liquids as a class of materials for transdermal delivery and pathogen neutralization. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 13313–13318.

(50) Liu, F.; He, D.; Yu, Y.; Cheng, L.; Zhang, S. Quaternary ammonium salt-based cross-linked micelles to combat biofilm. *Bioconjugate Chem.* **2019**, *30*, 541–546.

(51) Josenhans, C.; Suerbaum, S. The role of motility as a virulence factor in bacteria. *Int. J. Med. Microbiol.* **2002**, *291*, 605–614.

(52) Jindai, K.; Nakade, K.; Masuda, K.; Sagawa, T.; Kojima, H.; Shimizu, T.; Shingubara, S. Adhesion and bactericidal properties of nanostructured surfaces dependent on bacterial motility. *RSC Adv*. **2020**, *10*, 5673–5680.

(53) Overhage, J.; Bains, M.; Brazas, M. D.; Hancock, R. E. Swarming of *Pseudomonas aeruginosa* is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. *J. Bacteriol.* **2008**, *190*, 2671–2679.

"For Table of Contents Use Only"

Antibacterial Nanoassembled Calix[4]arene Exposing Choline Units Inhibits Biofilm and Motility of Gram Negative Bacteria

Grazia Maria Letizia Consoli*, Giuseppe Granata, Giovanna Ginestra, Andreana Marino, Giovanni Toscano, and Antonia Nostro*