Article

Cyclic and Acyclic Amine Oxide Alkyl Derivatives as Potential Adjuvants in Antimicrobial Chemotherapy against Methicillin-Resistant *Staphylococcus aureus* with an MDR Profile

Lorenza Fagnani [1], Lisaurora Nazzicone [1], Fabrizia Brisdelli [1], Luisa Giansanti [2], Sara Battista [2], Roberto Iorio [1], Sabrina Petricca [1], Gianfranco Amicosante [1], Mariagrazia Perilli [1], Giuseppe Celenza [1,*] and Pierangelo Bellio [1]

[1]   Department of Biotechnological and Applied Clinical Sciences, University of L'Aquila, Via Vetoio 1, 67100 L'Aquila, Italy; lorenza.fagnani@graduate.univaq.it (L.F.); lisaurora.nazzicone@student.univaq.it (L.N.); fabrizia.brisdelli@univaq.it (F.B.); roberto.iorio@univaq.it (R.I.); sabrina.petricca@univaq.it (S.P.); gianfranco.amicosante@univaq.it (G.A.); mariagrazia.perilli@univaq.it (M.P.); pierangelo.bellio@univaq.it (P.B.)
[2]   Department of Physical and Chemical Sciences, University of L'Aquila, Via Vetoio 1, 67100 L'Aquila, Italy; luisa.giansanti@univaq.it (L.G.); sara.battista@univaq.it (S.B.)
*   Correspondence: giuseppe.celenza@univaq.it

Abstract: The dramatic intensification of antimicrobial resistance occurrence in pathogenic bacteria concerns the global community. The revitalisation of inactive antibiotics is, at present, the only way to go through this health system crisis and the use of antimicrobial adjuvants is turning out the most promising approach. Due to their low toxicity, eco-friendly characteristics and antimicrobial activity, amphoteric surfactants are good candidates. This study investigated the adjuvant potentialities of commercial acyclic and newly cyclic *N*-oxide surfactants combined with therapeutically available antibiotics against MDR methicillin-resistant *Staphylococcus aureus* (MRSA). The safety profile of the new cyclic compounds, compared to commercial surfactants, was preliminarily assessed, evaluating the cytotoxicity on human peripheral mononuclear blood cells and the haemolysis in human red blood cells. The compounds show an efficacious antimicrobial activity strongly related to the length of the carbon atom chain. In drug–drug interaction assays, all surfactants act synergistically, restoring sensitivity to oxacillin in MRSA, with dodecyl acyclic and cyclic derivatives being the most effective. After evaluating the cytotoxicity and considering the antimicrobial action, the most promising compound is the L-prolinol amine-oxide C12NOX. These findings suggest that the combination of antibiotics with amphoteric surfactants is a valuable therapeutic option for topical infections sustained by multidrug-resistant *S. aureus*.

Keywords: amphoteric surfactants; amine oxides; adjuvants; antibiotics; multidrug resistance; MRSA; cytotoxicity; checkerboard assay

1. Introduction

In the last decade, the problem of antimicrobial resistance (AMR) has assumed the shape of a global emergency. As reported by the Centre for Disease Control and Prevention (CDC), the mortality rate per year in industrialised countries increases at an average of 23,000 deaths [1]. Six pathogens are mainly related to multi-resistance: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. The acronym "ESKAPE", from the first letter of the above-mentioned organisms, summarises their propensity to "escape" the biocidal action of antimicrobial agents [2,3]. *S. aureus* is one of the most important pathogenic bacteria, usually associated with nosocomial infections, expressing a multidrug resistance (MDR) profile, showing

resistance to more than three classes of approved antibiotics. The increasing incidence of methicillin-resistant (MRSA), vancomycin-intermediate (VISA) and vancomycin-resistant (VRSA) *S. aureus* is a crucial issue, especially for community-acquired and hospital-acquired infections [4–7].

Although the 20th century is considered the "golden age" of antimicrobials, their misuse and sometimes abuse in clinical practice and veterinary medicine has also led to the selection of resistant bacterial strains [8,9], favouring the emergence of MDR strains. Despite the global antibiotic consumption growth of more than 65% between 2000 and 2015 worldwide, the number of deaths caused by infectious diseases [10,11] increased. In some cases, there is a total failure of antimicrobial therapies, and often effective molecules are no longer clinically available. From this point of view, the development of new antimicrobial drugs has also stalled. Hence the idea to develop molecules that can restore "old" antibiotic molecules or exert their action with mechanisms unrelated to the classic ones is attractive. The use of synthetic or natural drug combinations or adjuvants is beginning to open a new way to develop new antimicrobial solutions [7,12–17].

Surfactants play a key role in nanomedicine as building blocks of functional aggregates, as nanoparticle stabilisers and drug delivery systems, besides their great variety of properties (e.g., solubilisation, detergency, wetting and foaming ability) [18–20]. These aspects are strongly related to their molecular structure and composition. The charge of the polar headgroup and/or the length of the alkyl chain are essential to determine the surfactants' behaviour in a solution; variations of the subtle balance between the hydrophobic and the hydrophilic portions of the molecules can affect the properties of both the monomers and the aggregates they form [21–28]. In particular, a pyrrolidine ring in the polar headgroup confers a lower degree of conformational freedom to the structure compared to the acyclic analogue [29]. This class of "soft" and environmentally friendly surfactants is very attractive in many fields, thanks to the possibility of modifying the length of the alkyl chain and the pyrrolidine skeleton [30–32]. *N*-oxides are very interesting among all the categories of surfactants because of their low or absent toxicity and irritative action, biodegradability, pH-sensitive aggregative behaviour and performances [33–36]. Generally, *N*-oxide surfactants can be easily prepared by the oxidation of the corresponding tertiary amine in the presence of hydrogen peroxide in an alcoholic medium. They are zwitterionic amphiphilic compounds at physiological pH, bearing a strong polar N–O bond with a high electron density on the oxygen atom [37]. In particular, these surfactants, coming from selective modification of their amphiphilic molecular structure, have opened the scenario to a plethora of systems [38–45], providing different superstructures by their self-assembly and co-assembly with other materials. They show multifunctional capabilities and are used in several industrial applications, such as washing-up products and laundry detergents, foaming and wetting agents, softeners and thickeners, besides being components of hair and body care products [46–48]. *N*-oxide surfactants can be an attractive solution as adjuvants in antimicrobial chemotherapy, to improve the activity of old antimicrobials, especially in drug formulations for external use, along with their propensity to be poorly or non-toxic.

In this study, we aimed to explore the potentialities of *N*-oxide-derived surfactants (Figure 1) as antibiotic adjuvants against MDR methicillin-resistant *S. aureus*. The antimicrobial activity of commercial acyclic *N*-oxide derivatives LDAO and TDAO, commonly found in many cleaning and personal care products, was compared with recently synthesised cyclic L-prolinol *N*-oxide analogues. The antimicrobial efficacy of the *N*-oxides was ascertained alone and in combination with therapeutically available antibiotics to determine the potential adjuvant properties. Moreover, a preliminary in vitro safety profile assessment of the cyclic L-prolinol *N*-oxide alkyl derivatives was determined and compared with commercial amphoteric surfactants.
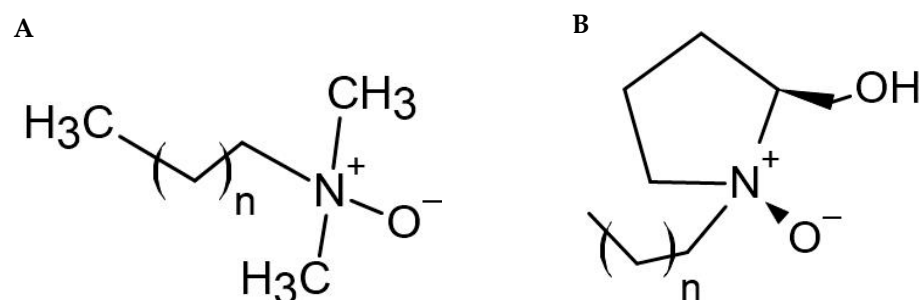
Figure 1. Structure of *N*-oxides. (**A**) Structures of commercial *N*-oxide alkyl derivatives: *n* = 10, LDAO; *n* = 12, TDAO. (**B**) Structures of L-prolinol *N*-oxide alkyl derivatives: *n* = 10, C12NOX; *n* = 12, C14NOX; *n* = 14, C16NOX.

2. Results

2.1. In Vitro Susceptibility Test

The compounds under examination were tested against 21 strains of methicillin-resistant *S. aureus* with an MDR profile (MIC for OXA ranging from 2 µg/mL to 256 µg/mL) to evaluate their antimicrobial activity. The MIC_{50} and MIC_{90} values for all compounds are reported in Table 1. The highest MIC value is related to LDAO (312.5 µM), while the lowest values are related to C14NOX and C16NOX, 4.88 µM and 2.44 µM, respectively.

Table 1. MIC_{50} and MIC_{90} calculated for 21 *S. aureus* clinical isolate strains.

Compounds (Carbon Chain Length)	MIC_{50} (µM) (Range)	MIC_{90} (µM)	CMC * (M)
LDAO (12)	312.50 (39.06–625.00)	625.00	[a] 1.7×10^{-3}
TDAO (14)	19.53 (19.53–39.06)	39.06	[a] 2.7×10^{-4}
C12NOX (12)	156.25 (9.77–156.25)	156.25	[b] 1.4×10^{-5}
C14NOX (14)	4.88 (1.22–19.53)	9.77	[b] 5.8×10^{-6}
C16NOX (16)	2.44 (0.61–19.53)	4.88	[b] 1.5×10^{-6}

* Critical micelle concentration; [a] Reference [43]; [b] Reference [49].

2.2. Checkerboard Microdilution Assay

A two-dimensional checkerboard microdilution assay was used to evaluate the adjuvant effect of the detergents when combined with antibiotics against the reference strain *S. aureus* ATCC43300, which showed, in our experimental conditions, resistance to oxacillin (MIC = 32 µg/mL), clindamycin (MIC > 16,384 µg/mL), erythromycin (MIC > 2048 µg/mL) and gentamicin (MIC = 128 µg/mL). In addition, the antimicrobial susceptibility to detergents of the reference strain was also determined to arrange the conditions for the checkerboard assay. As shown in Table 2, the MIC values for the detergents were comparable to the MIC_{50} and MIC_{90} values previously determined for the clinical strains.

Table 2. MIC values of the detergents calculated for *S. aureus* ATCC43300.

Compounds (Carbon Chain Length)	Median MIC (µM) (Range)
LDAO (12)	312.50
TDAO (14)	39.06
C12NOX (12)	78.13 (78.13–156.25)
C14NOX (14)	19.53 (9.77–19.53)
C16NOX (16)	19.53 (9.77–19.53)

The data obtained from the checkerboard assays were analysed with a non-parametric interpretative model, the Loewe additivity-based model (Fractional Inhibitory Concentration Index, FICI), as reported in Table 3. Among the four antibiotics, no synergistic

interaction was observed, except for the combination clindamycin-LDAO and oxacillin. On the other hand, the β-lactam oxacillin showed a synergistic effect with all detergents with an FICI ≤ 2. However, the most interesting results were observed when oxacillin is combined with LDAO and C12NOX, where the effective antibiotic concentration drops to 0.0625 µg/mL and 0.125 µg/mL, respectively.

Table 3. Results from the in vitro interaction experiments between detergents and antibiotics against *S. aureus* ATCC43300 determined by non-parametric FICI.

Compounds (Carbon Chain Length)	Antibiotic	FICI$_{min}$	INT [a]	Effective Combination	
				Antibiotic (µg/mL)	Detergents (µM)
LDAO (12)	clindamycin	0.5	SYN	16	156.25
	erythromycin	0.625	IND		
	gentamicin	1	IND		
	oxacillin	0.252	SYN	0.0625	78.13
TDAO (14)	clindamycin	1	IND		
	erythromycin	1	IND		
	gentamicin	0.531	IND		
	oxacillin	0.313	SYN	2	9.77
C12NOX (12)	clindamycin	1	IND		
	erythromycin	1	IND		
	gentamicin	1	IND		
	oxacillin	0.254	SYN	0.125	19.53
C14NOX (14)	clindamycin	1	IND		
	erythromycin	1	IND		
	gentamicin	1	IND		
	oxacillin	0.5	SYN	2	4.88
C16NOX (16)	clindamycin	1	IND		
	erythromycin	1	IND		
	gentamicin	1	IND		
	oxacillin	0.5	SYN	2	4.88

[a] INT, interpretation; IND, indifference; SYN, synergism; ANT, antagonism. Synergism was defined as an FICI of ≤0.5, antagonism was defined as an FICI of >4, and indifference was defined as an FICI >0.5 and ≤4.

2.3. Red Blood Cell Haemolysis and PBMC Cytotoxicity

A preliminary assessment of the safety profile of the L-prolinol *N*-oxide surfactants was accomplished by evaluating the haemolytic effect in red blood cells and the cytotoxicity in peripheral blood mononuclear cells. As described in Table 4, haemolysis occurred in a range between 19.5 µM for C16NOX and 625 µM for the dodecyl derivatives LDAO and C12NOX.

To verify the effect of the detergents on growth and viability in human cells, PBMCs were exposed to increasing concentrations of compounds for 48 h. The effects on cell growth were evaluated by estimating the IC_{20}, IC_{50} and IC_{80} values, as reported in Table 4. Minor cytotoxicity was observed in the dodecyl derivatives LDAO and C12NOX with estimated IC_{80} values above 200 µM. For instance, LDAO showed inhibitory concentration values 2-fold higher than the equivalent tetradecyl derivative TDAO, and the C12NOX about 5–12-fold higher than the related derivatives with longer alkyl chains.

Table 4. Preliminary assessment of the safety profile of L-prolinol *N*-oxide compounds compared to *N*-oxide detergents. It includes the calculated values of IC_{20}, IC_{50} and IC_{80} from dose–response experiments in human peripheral blood mononuclear cells (PBMCs) and the concentration of the detergents causing 100% of human red blood cell (RBC) haemolysis.

Compounds (Carbon Chain Length)	$IC_{20} \pm$ SEM [a] (μM)	$IC_{50} \pm$ SEM [a] (μM)	$IC_{80} \pm$ SEM [a] (μM)	Haemolysis * (μM)
LDAO (12)	192.9 ± 8.6	220.1 ± 6.6	250.0 ± 11.1	625.0
TDAO (14)	100.3 ± 2.3	113.6 ± 1.6	128.7 ± 2.9	78.2
C12NOX (12)	154.3 ± 4.1	177.3 ± 4.7	203.6 ± 5.4	625.0
C14NOX (14)	12.5 ± 1.9	20.8 ± 1.4	34.7 ± 3.3	78.2
C16NOX (16)	18.3 ± 2.8	27.6 ± 2.3	41.7 ± 5.1	19.5

[a] Error is expressed as ± standard error (SEM) of the mean of three independent experiments. * Intended as the median concentration value, obtained from three independent experiments, causing 100% of human red blood cell haemolysis in a double-serial dilution assay.

In red blood cell haemolysis experiments, the same trend can be observed for the amine oxide derivatives with a 12-atom carbon chain (Table 4). Indeed, both LDAO and C12NOX exhibit haemolytic activity at a concentration of 625 μM.

3. Discussion

N-alkyl-*N,N*-dimethylamine oxides are zwitterionic surfactants frequently used in many household cleaning products and personal care products. Amine oxides are generally considered environmentally eco-safe because they are fully biodegradable in aerobic and anaerobic conditions. Furthermore, from a human health safety profile point of view, it is known that amine oxides do not cause skin sensitisation, as well as no evidence of carcinogenic activity or reproductive and developmental toxicity has been reported [50–52].

This study aimed to investigate the potential antimicrobial activity and adjuvant antibiotic properties of L-prolinol *N*-oxide alkyl derivatives compared with the commercial *N*-oxide derivatives LDAO and TDAO, alone and in combination with therapeutically available antibiotics. Moreover, a preliminary safety profile was assessed by evaluating the induced haemolysis in red blood cells and cytotoxicity in peripheral blood mononuclear cells. The newly L-prolinol *N*-oxide derivatives differ from the "classical" alkyl dimethylamine oxide amphoteric surfactants for their peculiar headgroup, which originates by reduction of the carboxy group of the amino acid L-prolinol and oxidation of the tertiary pyrrolidinic amine [43]. Furthermore, the commercial surfactants LDAO and TDAO differ in the carbon chain length, C12 and C14, while the alkylic length of the newly synthesised L-prolinol *N*-oxide derivatives extends from 12 to 16 carbon atoms.

The compounds under examination were tested for their antimicrobial properties against twenty-one methicillin-resistant *S. aureus* clinical isolates with a multidrug resistant profile to determine their respective MIC values. Among the five formulations, the C14 and C16 alkyl L-prolinol derivatives show the greatest ability to inhibit microbial growth against all *S. aureus* strains (Table 1). This result can be elucidated by looking at the chemical features of the detergents and their composition. Subik J. et al. and further studies reported that the *N*-oxides' antimicrobial activity is influenced by the carbon chain length [53–55]. Specifically, an alkyl chain length of approximately 15 carbon atoms was associated with maximum antimicrobial activity in dimethyl alkyl *N*-oxide surfactants [55]. Therefore, as the carbon chain length increases, the antimicrobial activity becomes more efficacious among surfactants bearing the same functional group, as can be observed for C16NOX, where the MIC_{50} and MIC_{90} are the lowest recorded, even though a plateauing effect is observed as the alkyl length increases (Tables 1 and 2). One plausible explanation can be retrieved by looking at the critical micellar concentration (CMC) of these compounds. CMC describes the tendency of the surfactant monomer to form aggregates. For instance, it is reasonable to hypothesise that the low CMC values of the surfactant with the longest chain length might play a major role in determining its antimicrobial activity [43].

Since it is assumed that such compounds do not have specific targets, it is reasonable to suppose that they might be used as potential adjuvants to enhance the activity of the therapeutically available antibiotics against MDR organisms.

For this reason, *S. aureus* ATCC43300 was used as a model organism to verify a potential adjuvant effect in combination with those antibiotics whose efficacy is compromised by the presence of resistance. The MDR phenotype of *S. aureus* ATCC43300 confers resistance to various molecules belonging to four different classes of antibiotics, such as clindamycin (lincosamides), erythromycin (macrolides), gentamicin (aminoglycosides) and oxacillin (β-lactams). The potential synergistic effects of the surfactant–antibiotic combinations were assessed by checkerboard assay, and the data were analysed using the FICI model based on the Loewe additivity theory.

Among all tested antibiotics, clindamycin, erythromycin and gentamicin manifested no synergistic interaction, except for clindamycin in combination with LDAO, where a borderline value of FICI (0.5) was observed. On the contrary, both the commercial and new detergents exert a synergistic action, capable of enhancing the activity of the β-lactam oxacillin, verified by FICI values lower than 0.5. These data are worthy of attention since all surfactants reduced the MIC value of oxacillin below the breakpoint value (≤ 2 μg/mL), thus inducing a phenotypic reversion of resistance to oxacillin.

Unlike the other tested antibiotics, whose targets are located in the cytoplasmatic space of the bacterial cell, the targets of the β-lactam antibiotics are the penicillin-binding proteins (PBPs), enzymes with transpeptidase activity involved in the synthesis of peptidoglycan of the bacterial cell wall. PBPs are located outside the bacterial cell and are anchored to the external face of the bacterial plasma membrane through a 23-residue N-terminal transmembrane domain [56,57]. Resistance to methicillin in *Staphylococcus aureus* is prevalently mediated by the presence of a specific PBP, named PBP2′ or PBP2a, which is acquired by a mobile genetic element through horizontal gene transfer (SCC*mec*) [56,58]. PBP2a belongs to the subclass B1 of high-molecular-weight PBPs, characterised by a low β-lactam affinity [59]. However, bacteria gain resistance to lactam antibiotics by modifying their PBPs, thus preventing drug binding and, consequently, conferring resistance [56].

We can easily hypothesise that the tested zwitterionic surfactants most likely act at the level of the external structures of the cell, inducing a perturbation of the structure of the plasma membrane, which might affect the functionality of PBP2a.

The most effective interactions are between oxacillin and detergents with a 12-carbon atom chain length, regardless of the nature of the head. Indeed, thanks to LDAO and C12NOX, the MIC value for oxacillin drops below the breakpoint value, implying a 512-fold dose reduction in the antibiotic when combined with LDAO and a 256-fold dose reduction with C12NOX. Generally, the increase in the number of carbon atoms causes an increment in the antimicrobial activity when the compound acts alone [49,55], as demonstrated experimentally through the in vitro susceptibility test (Tables 1 and 2). In reverse, we observe that the opposite happens with the combinations between the detergents and antibiotics; it is clear, in fact, that the best ability to hinder microbial growth is linked to detergents with a lower content of carbon atoms when combined with oxacillin. This result suggests that the surfactants support the action of the combined antibiotic with more efficacy in their monomeric form, also considering the previously estimated CMC values of cyclic amine *N*-oxides [43].

The idea to combine surfactants to enhance their broad-spectrum antimicrobial activity has been exploited in C31G, an equimolar combination of two synthetic C12 and C16 alkyl amine oxide and alkyl betaine [60,61], with antimicrobial and viricidal activity. Previous studies have demonstrated that C31G is effective against both Gram-positive and Gram-negative bacteria [60,61], fungi [62] and viruses [63]. Moreover, C31G possesses wound-healing and anti-inflammatory-related properties [64], and efficacious spermicidal activity [65]. Moreover, C31G was enrolled in a pivotal Phase III clinical trial to reduce the sexual transmission of the human immunodeficiency virus (a Phase II/III multicentre, ran-

domized, double-masked study of the safety and contraceptive efficacy of C31G compared to Conceptrol®).

As stated above, global and national environmental agencies consider the safety profile of the *N*-alkyl-*N*,*N*-dimethylamine oxides sufficiently harmless from a human and environmental standpoint. In this respect, we intended to preliminary verify the safety profile of the L-prolinol *N*-oxide derivatives compared to the acyclic commercial surfactants. It was ascertained by evaluating the effect of the detergents on human peripheral blood mononuclear cell growth and viability. PBMCs were exposed to increasing concentrations of surfactants, and the cytotoxicity was evaluated by calculating the concentration inhibiting 20% (IC_{20}), 50% (IC_{50}) and 80% (IC_{80}) of cell growth.

As reported in Table 4, commercially available surfactants are more tolerated than cyclic amine-oxides, although C12NOX shows growth-inhibitory concentrations comparable to those of LDAO and about two-fold above TDAO. If we compare the cytotoxicity inside the L-prolinol *N*-oxide series, we can observe a remarkable increase in toxicity as the alkyl moiety increases in carbon atoms. Indeed, the relationship between the carbon chain length and cytotoxicity has been observed by Catalone et al. in HeLa cells, where myristyl dimethyl amine oxide (16 atom carbon length) showed higher toxicity than TDAO [61]. Since this phenomenon is also observable between LDAO and TDAO, it is reasonable to affirm that the increase in the carbon chain length leads to higher toxicity. The same trend is observable in the cyclic *N*-oxide series, where the IC_{80} values for C14NOX and C16NOX are between 5- and 6-fold lower than the C12NOX.

However, it is noteworthy that the MIC_{90} values for *N*-oxide compounds with an alkyl chain longer than 12 carbon atoms, independently of the headgroup, are lower than the IC_{50} and IC_{80} values. It means that the efficacious antimicrobial dose is far from the cytotoxic dose. For instance, the MIC_{90} value for C16NOX is about 6-fold and 9-fold lower than the IC_{50} and IC_{80} values, while the MIC_{90} for LDAO is about 3-fold higher than the cytotoxic dose.

However, the most intriguing data are those obtained by the drug–drug combination assays. All detergents showed cytotoxicity values well above their effective concentrations when combined with oxacillin. It emerges that when C12NOX is combined with oxacillin, the detergent concentration is 9-fold lower than the IC_{50} value.

We must also consider that the C12NOX MIC_{90} value is 4-fold lower than the concentration causing the complete haemolysis of human red blood cells. Moreover, the observed synergistic effect with oxacillin leads to a C12NOX dose reduction of about 32-fold lower than the haemolysis concentration. Nevertheless, LDAO and C12NOX share the same haemolytic concentration value. Looking at the evaluated safety profile, these data are encouraging, especially if we consider that LDAO is widely used in many personal care products [27]. Another remarkable aspect is that the value of the concentration causing extensive red blood cell haemolysis for C12NOX is up to 32 times higher than the same L-prolinol *N*-oxides with a longer carbon chain. In this regard, it is plausible to assert that the length of the carbon chain, as in PBMCs, influences red blood cell haemolysis, probably because of its effect on CMC values. In general, the longer the alkyl chain the lower the CMC. Furthermore, it is clear that when the surfactants are organised in micelles, they exert their detergent effect (thus causing haemolysis in this case) with greater efficacy with respect to the same amphiphiles in their monomeric form. Therefore, the chain with 12 carbon atoms would seem to guarantee greater safety of the compound. Therefore, limited to the data obtained highlighting the absence of toxicity, we could assert, along with the other detergents already on the market, that C12NOX is a good candidate from a safety point of view, deserving further investigations.

Due to their low toxicity, low environmental impact [27] and high versatility, *N*-oxide-based surfactants are compounds of great interest. For instance, it is not by chance that they are employed in a wide variety of industrial applications and daily life products [43]. Of great significance is the ability of amphoteric surfactants to hinder cell growth, acting in a non-specific way. The lack of a specific target is of extreme importance since it would

avoid the development of resistant phenotypes, opening the possibility of improving the activity of antimicrobials to which pharmacological resistance mechanisms may exist, such as oxacillin. Resistance to available antibiotics in pathogenic bacteria is currently a global challenge since the number of resistant strains to multiple classes of antibiotics has dramatically increased, spreading worldwide [66]. Nowadays, the new frontier of antimicrobial therapy is trying to revert antibacterial resistance using efficacious combinations of drugs to restore the activity of old and inactive molecules [14,15]. Therefore, antibiotic adjuvants in combination with available antibiotics are now being exploited to overcome the phenomenon of antimicrobial resistance. Antibiotic adjuvants, also known as 'resistance breakers' or 'antibiotic potentiators' [67–72], have no or poor antimicrobial activity, but when combined with antibiotics, they enhance the antimicrobial action of the drug [66]. Nowadays, resistance to amphoteric surfactants in pathogenic microorganisms has not been reported, and the use of these molecules can be exploited as adjuvants in antimicrobial therapy, specifically for topical use.

4. Materials and Methods

4.1. Organisms

Twenty methicillin-resistant *S. aureus* clinical strains with a multidrug-resistant profile (MDR), collected at the "San Salvatore" Hospital of L'Aquila, were used for the antimicrobial susceptibility test. In addition, a reference strain with a methicillin-resistant profile from the American Type Culture Collection *S. aureus* ATCC43300 was used to investigate the drug–drug interaction between the compounds and antibiotics. In addition to oxacillin, all these strains were resistant to clindamycin, erythromycin and gentamicin.

4.2. Antibiotics and Compounds

All the tested antibiotics, oxacillin, clindamycin, erythromycin and gentamicin, were from Sigma–Aldrich (Milan, Italy). *N,N*-Dimethyldodecan-1-amine *N*-oxide, also known as lauryldimethylamine *N*-oxide (LDAO) and *N,N*-Dimethyltetradecan-1-amine *N*-oxide, also known as myristyldimethylamine *N*-oxide (TDAO), were from Sigma-Aldrich (Milan, Italy). The L-prolinol amine oxide derivatives [(1*S*,2*S*)-1-dodecyl-1-oxo-1λ^5-pyrrolidin-2-yl]methanol (C12NOX), [(1*S*,2*S*)-1-tetradecyl-1-oxo-1λ^5-pyrrolidin-2-yl]methanol (C14NOX) and [(1*S*,2*S*)-1-hexadecyl-1-oxo-1λ^5-pyrrolidin-2-yl]methanol (C16NOX) were synthesised as previously described [43].

4.3. In Vitro Susceptibility Test

The in vitro antimicrobial effect of the detergents against the organisms used in this study was ascertained by the microdilution method in 96-well microplates. The determination of the MIC values was done following the CLSI guidelines (CLSI, 2018). The microtiter plates were incubated for 18 h at 37 °C before MIC determination. A microplate reader iMark, BioRad (Milan, Italy), quantified at 595 nm the growth in each well. The minimum inhibitory concentration (MIC) for drugs and detergents was defined as the concentration of drug that reduced growth by 80% compared to that of organisms grown in the absence of a drug. The MIC value was determined as the median of at least three independent experiments. In addition, the MIC_{50} and MIC_{90} were also calculated as the lowest concentration of the compound at which the growth of 50% and 90% of the organisms were inhibited.

4.4. Checkerboard Microdilution Assay

The two-dimensional checkerboard microdilution assay using a 96-well microtitration plate was used to investigate the in vitro interactions between the antibiotics and detergents, as previously described by Segatore et al. [73,74]. The microtiter plates were incubated at

37 °C for 18 h. The growth in each well was quantified spectrophotometrically at 595 nm by a microplate reader. The percentage of growth in each well was calculated as follows:

$$\frac{OD_{drug\ combination\ well} - OD_{background}}{OD_{drug\ free\ well} - OD_{background}} \times 100$$

where the background is derived from the microorganism-free plates. All experiments were performed in triplicate.

4.5. Drug–Drug Interaction Model Analysis

The data acquired from the checkerboard assay were examined by a non-parametric interpretative model based on the Loewe additivity model (Fractional Inhibitory Concentration Index, FICI), as previously described [74,75].

4.6. Human Peripheral Blood Red Cells and Mononuclear Cells (PBMCs) Isolation

Peripheral blood mononuclear cells (PBMCs) obtained from heparinised whole blood of healthy adults were isolated by density gradient centrifugation using Histopaque-1077 (Sigma–Aldrich, St. Louis, MO, USA). The PBMCs were collected, washed twice and resuspended in RPMI 1640 medium supplemented with 10% foetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin.

4.7. Human Red Blood Cell Haemolytic Assay

Haemolytic activity of detergents on human erythrocytes was determined as previously described [73]. Briefly, after washing three times with phosphate-buffered saline (PBS 5 mM phosphate buffer containing 150 mM NaCl, pH 7.4), red blood cells were isolated from heparinised human blood by centrifugation. Erythrocytes were diluted in PBS saline to reach an absorbance of 1.0 at 655 nm. In a sterile 96-well microtiter plate, already containing 100 μL of the diluted compound, an aliquot of 100 μL of erythrocytes was added to reach a final volume of 200 μL. Complete haemolysis (100% haemolysis) was ascertained by preparing wells with PBS saline containing 0.2% Triton X-100 and red blood cells. The absence of haemolysis (0% haemolysis) was verified by preparing wells with PBS saline and erythrocytes. The microtitration plate was incubated at 37 °C with gentle mixing for 20 min. The haemolysis was quantified spectrophotometrically at 655 nm by a microplate reader (iMark, BioRad, Milan, Italy). The haemolysis percentage was obtained using the following equation:

$$hemolysis\ (\%) = 100 - \frac{OD_{655\ nm\ well\ containing\ detergent} - OD_{655\ nm\ 0\%\ hemolysis}}{OD_{655\ nm\ 100\%\ hemolysis} - OD_{655\ nm\ 0\%\ hemolysis}} \times 100$$

All experiments were done in triplicate.

4.8. PBMC Cytotoxicity Assay

The effects of the detergents on peripheral blood mononuclear cells (PBMCs) viability were evaluated by the MTT colourimetric method that quantifies the cellular metabolic activity [75]. PBMCs, seeded in 96-well plates at a density of 1×10^6 cells/mL (1×10^5 cells/well), were treated with increasing concentrations of compounds (LDAO and C12NOX from 75 to 300 μM; TDAO from 25 to 150 μM; C14NOX and C16NOX from 5 to 50 μM) for 48 h, at 37 °C in a humidified atmosphere of 5% CO_2.

At the end of treatment, the MTT solution was added to each well at a concentration of 0.5 mg/mL and cells were incubated at 37 °C for a further 4 h. Then, the MTT-formazan crystals were dissolved in acidified isopropanol (0.04 M HCl in isopropanol), and the absorbance at 570 nm was determined using a microplate reader (Biorad, Model 550).

The percentage of cell survival was obtained by comparing the absorbance of the treated groups with that of the untreated cells, the viability of which is taken as 100%.

Experimental data for the calculation of the IC_{20} and IC_{50} were analysed using OriginPro 2018 software.

Author Contributions: Conceptualisation, G.C. and P.B.; investigation, L.F., L.N., F.B., R.I., S.B., L.G. and S.P.; methodology, G.C., P.B., L.N. and L.F.; resources, G.C.; data curation, L.F., L.N. and P.B.; writing and original draft preparation, L.F. and L.N.; review and editing, G.A. and M.P.; supervision, P.B.; compound synthesis L.G. and S.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Data is contained within this article.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. World Health Organization (WHO). *WHO Report on Surveillance of Antibiotic Consumption*; WHO: Geneva, Switzerland, 2019.
2. Mulani, M.S.; Kamble, E.E.; Kumkar, S.N.; Tawre, M.S.; Pardesi, K.R. Emerging strategies to combat ESKAPE pathogens in the era of antimicrobial resistance: A review. *Front. Microbiol.* **2019**, *10*, 539. [CrossRef] [PubMed]
3. León-Buitimea, A.; Garza-Cárdenas, C.R.; Garza-Cervantes, J.A.; Lerma-Escalera, J.A.; Morones-Ramírez, J.R. The Demand for New Antibiotics: Antimicrobial Peptides, Nanoparticles, and Combinatorial Therapies as Future Strategies in Antibacterial Agent Design. *Front. Microbiol.* **2020**, *11*, 1669. [CrossRef] [PubMed]
4. Magiorakos, A.P.; Srinivasan, A.; Carey, R.B.; Carmeli, Y.; Falagas, M.E.; Giske, C.G.; Harbarth, S.; Hindler, J.F.; Kahlmeter, G.; Olsson-Liljequist, B.; et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* **2012**, *18*, 268–281. [CrossRef]
5. Basak, S.; Singh, P.; Rajurkar, M. Multidrug Resistant and Extensively Drug Resistant Bacteria: A Study. *J. Pathog.* **2016**, *2016*, 4065603. [CrossRef]
6. The World Bank. Drug-Resistant Infections: A Threat to Our Economic Future (Vol. 2): Final Report. Available online: https://documents.worldbank.org/en/publication/documents-reports/documentdetail/323311493396993758/final-report (accessed on 21 April 2021).
7. Vallavan, V.; Krishnasamy, G.; Zin, N.M.; Abdul Latif, M. A Review on Antistaphylococcal Secondary Metabolites from Basidiomycetes. *Molecules* **2020**, *25*, 5848. [CrossRef] [PubMed]
8. Aminov, R. History of antimicrobial drug discovery: Major classes and health impact. *Biochem. Pharmacol.* **2017**, *133*, 4–19. [CrossRef] [PubMed]
9. Lewis, K. Platforms for antibiotic discovery. *Nat. Rev. Drug Discov.* **2013**, *12*, 371–387. [CrossRef] [PubMed]
10. Laxminarayan, R.; Matsoso, P.; Pant, S.; Brower, C.; Røttingen, J.A.; Klugman, K.; Davies, S. Access to effective antimicrobials: A worldwide challenge. *Lancet* **2016**, *387*, 168–175. [CrossRef]
11. Abat, C.; Gautret, P.; Raoult, D. Benefits of antibiotics burden in low-income countries. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E8109–E8110. [CrossRef]
12. Wright, G.D.; Sutherland, A.D. New strategies for combating multidrug-resistant bacteria. *Trends Mol. Med.* **2007**, *13*, 260–267. [CrossRef] [PubMed]
13. Cottarel, G.; Wierzbowski, J. Combination drugs, an emerging option for antibacterial therapy. *Trends Biotechnol.* **2007**, *25*, 547–555. [CrossRef]
14. Phougat, N.; Khatri, S.; Singh, A.; Dangi, M.; Kumar, M.; Dabur, R.; Chhillar, A. Combination Therapy: The Propitious Rationale for Drug Development. *Comb. Chem. High. Throughput Screen.* **2014**, *17*, 53–67. [CrossRef] [PubMed]
15. Bellio, P.; Luzi, C.; Mancini, A.; Cracchiolo, S.; Passacantando, M.; Di Pietro, L.; Perilli, M.; Amicosante, G.; Santucci, S.; Celenza, G. Cerium oxide nanoparticles as potential antibiotic adjuvant. Effects of CeO_2 nanoparticles on bacterial outer membrane permeability. *Biochim. Biophys. Acta Biomembr.* **2018**, *1860*, 2428–2435. [CrossRef] [PubMed]
16. Bellio, P.; Brisdelli, F.; Perilli, M.; Sabatini, A.; Bottoni, C.; Segatore, B.; Setacci, D.; Amicosante, G.; Celenza, G. Curcumin inhibits the SOS response induced by levofloxacin in Escherichia coli. *Phytomedicine* **2014**, *21*, 430–434. [CrossRef] [PubMed]
17. Celenza, G.; Segatore, B.; Setacci, D.; Perilli, M.; Brisdelli, F.; Bellio, P.; Piovano, M.; Garbarino, J.A.; Amicosante, G.; Nicoletti, M. Antibacterial activity of selected metabolites from Chilean lichen species against methicillin-resistant staphylococci. *Nat. Prod. Res.* **2013**, *27*, 1528–1531. [CrossRef] [PubMed]
18. Azira, H.; Tazerouti, A.; Canselier, J.P. Study of Foaming Properties and Effect of the Isomeric Distribution of Some Anionic Surfactants. *J. Surfactants Deterg.* **2008**, *11*, 279–286. [CrossRef]
19. Sehgal, P.; Doe, H.; Bakshi, M.S. Solubilisation of phospholipid vesicular structures into mixed micelles of zwitterionic surfactants. *J. Surfactants Deterg.* **2003**, *6*, 31–37. [CrossRef]
20. Lichtenberg, D.; Robson, R.J.; Dennis, E.A. Solubilization of phospholipids by detergents structural and kinetic aspects. *BBA Rev. Biomembr.* **1983**, *737*, 285–304. [CrossRef]

21. Ceccacci, F.; Giansanti, L.; Mancini, G.; Mauceri, A.; Scipioni, A.; Sperduto, C. Transcription of chirality from molecules to complex systems: The role of hydrophobic interactions. *Supramol. Chem.* **2013**, *25*, 741–747. [CrossRef]

22. Costas-Costas, U.; Bravo-Diaz, C.; Gonzalez-Romero, E. Kinetics and mechanism of the reaction between ascorbic acid derivatives and an arenediazonium salt: Cationic micellar effects. *Langmuir* **2005**, *21*, 10983–10991. [CrossRef] [PubMed]

23. Wang, X.; Gao, Y. Effects of length and unsaturation of the alkyl chain on the hydrophobic binding of curcumin with Tween micelles. *Food Chem.* **2018**, *246*, 242–248. [CrossRef]

24. Ceccacci, F.; Giansanti, L.; Mortera, S.L.; Mancini, G.; Sorrenti, A.; Villani, C. Enantiodiscrimination of bilirubin-IXα enantiomers in biomembrane models: Has chirality a role in bilirubin toxicity? *Bioorganic Chem.* **2008**, *36*, 252–254. [CrossRef] [PubMed]

25. Fuangswasdi, A.; Charoensaeng, A.; Sabatini, D.A.; Scamehorn, J.F.; Acosta, E.J.; Osathaphan, K.; Khaodhiar, S. Mixtures of anionic and cationic surfactants with single and twin head groups: Solubilisation and adsolubilization of styrene and ethylcyclohexane. *J. Surfactants Deterg.* **2006**, *9*, 29–37. [CrossRef]

26. Demissie, H.; Duraisamy, R. Effects of electrolytes on the surface and micellar characteristics of Sodium dodecyl sulphate surfactant solution. *J. Sci. Innov. Res.* **2016**, *5*, 208–214.

27. Api, A.M.; Belmonte, F.; Belsito, D.; Biserta, S.; Botelho, D.; Bruze, M.; Burton, G.A.; Buschmann, J.; Cancellieri, M.A.; Dagli, M.L.; et al. RIFM fragrance ingredient safety assessment, dodecyldimethylamine oxide, CAS Registry Number 1643-20-5. *Food Chem. Toxicol.* **2020**, *141*, 111424. [CrossRef] [PubMed]

28. Vlachy, N.; Drechsler, M.; Verbavatz, J.M.; Touraud, D.; Kunz, W. Role of the surfactant headgroup on the counterion specificity in the micelle-to-vesicle transition through salt addition. *J. Colloid Interface Sci.* **2008**, *319*, 542–548. [CrossRef] [PubMed]

29. Karukstis, K.K.; McDonough, J.R. Characterization of the aggregates of N-Alkyl-N-methylpyrrolidinium bromide surfactants in aqueous solution. *Langmuir* **2005**, *21*, 5716–5721. [CrossRef]

30. Zhao, M.; Zheng, L. Micelle formation by N-alkyl-N-methylpyrrolidinium bromide in aqueous solution. *Phys. Chem. Chem. Phys.* **2011**, *13*, 1332–1337. [CrossRef]

31. Tian, Y.; Wei, R.; Cai, B.; Dong, J.; Deng, B.; Xiao, Y. Cationic gemini pyrrolidinium surfactants based sweeping-micellar electrokinetic chromatography for simultaneous detection of nine organic pollutants in environmental water. *J. Chromatogr. A* **2016**, *1475*, 95–101. [CrossRef]

32. Bombelli, C.; Bordi, F.; Ferro, S.; Giansanti, L.; Jori, G.; Mancini, G.; Mazzuca, C.; Monti, D.; Ricchelli, F.; Sennato, S.; et al. New cationic liposomes as vehicles of m-tetrahydroxyphenylchlorin in photodynamic therapy of infectious diseases. *Mol. Pharm.* **2008**, *5*, 672–679. [CrossRef]

33. Goracci, L.; Germani, R.; Rathman, J.F.; Savelli, G. Anomalous behavior of amine oxide surfactants at the air/water interface. *Langmuir* **2007**, *23*, 10525–10532. [CrossRef] [PubMed]

34. Brinchi, L.; Germani, R.; Di Profio, P.; Marte, L.; Savelli, G.; Oda, R.; Berti, D. Viscoelastic solutions formed by worm-like micelles of amine oxide surfactant. *J. Colloid Interface Sci.* **2010**, *346*, 100–106. [CrossRef] [PubMed]

35. García, M.T.; Campos, E.; Ribosa, I. Biodegradability and ecotoxicity of amine oxide based surfactants. *Chemosphere* **2007**, *69*, 1574–1578. [CrossRef] [PubMed]

36. Goldsipe, A.; Blankschtein, D. Molecular-thermodynamic theory of micellisation of multicomponent surfactant mixtures: 2. pH-sensitive surfactants. *Langmuir* **2007**, *23*, 5953–5962. [CrossRef]

37. Łukomska, M.; Rybarczyk-Pirek, A.J.; Jabłoński, M.; Palusiak, M. The nature of NO-bonding in N-oxide group. *Phys. Chem. Chem. Phys.* **2015**, *17*, 16375–16387. [CrossRef] [PubMed]

38. Piasecki, A.; Wójcik, B.; Łuczyński, J.; Piłakowska-Pietras, D.; Witek, S.; Krasowska, A. Bifunctional N-Oxides of Alkyldiamidoamines. *J. Surfactants Deterg.* **2009**, *12*, 201–207. [CrossRef]

39. Bordi, F.; Cerichelli, G.; De Berardinis, N.; Diociaiuti, M.; Giansanti, L.; Mancini, G.; Sennato, S. Synthesis and physicochemical characterisation of new twin-tailed N-oxide based gemini surfactants. *Langmuir* **2010**, *26*, 6177–6183. [CrossRef] [PubMed]

40. Katritzky, A.R.; Duell, B.L.; Seiders, R.P.; Katritzky, A.R. Synthesis and Catalytic Activity of Surfactant Analogues of 4-(Dimethylamino) pyridine. *Langmuir* **1987**, *3*, 976–982. [CrossRef]

41. Karlovská, J.; Uhríková, D.; Kučerka, N.; Teixeira, J.; Devínsky, F.; Lacko, I.; Balgavý, P. Influence of *N*-dodecyl-*N*,*N*-dimethylamine *N*-oxide on the activity of sarcoplasmic reticulum Ca^{2+}-transporting ATPase reconstituted into diacylphosphatidylcholine vesicles: Effects of bilayer physical parameters. *Biophys. Chem.* **2006**, *119*, 69–77. [CrossRef] [PubMed]

42. Niedziółka, K.; Szymula, M.; Lewińska, A.; Wilk, K.A.; Narkiewicz-Michałek, J. Studies of vitamin C antioxidative activity in the N-oxide surfactant solutions. *Colloids Surf. A Physicochem. Eng. Asp.* **2012**, *413*, 33–37. [CrossRef]

43. Battista, S.; Campitelli, P.; Carlone, A.; Giansanti, L. Influence of structurally related micelle forming surfactants on the antioxidant activity of natural substances. *Chem. Phys. Lipids* **2019**, *225*, 104818. [CrossRef] [PubMed]

44. Battista, S.; Bellio, P.; Celenza, G.; Galantini, L.; Franceschini, I.; Mancini, G.; Giansanti, L. Correlation of Physicochemical and Antimicrobial Properties of Liposomes Loaded with (+)-Usnic Acid. *ChemPlusChem* **2020**, *85*, 1014–1021. [CrossRef] [PubMed]

45. Battista, S.; Campitelli, P.; Galantini, L.; Köber, M.; Vargas-Nadal, G.; Ventosa, N.; Giansanti, L. Use of N-oxide and cationic surfactants to enhance antioxidant properties of (+)-usnic acid loaded liposomes. *Colloids Surf. A Physicochem. Eng. Asp.* **2020**, *585*, 124154. [CrossRef]

46. Richmon, J. (Ed.) *Cationic Surfactants: Organic Chemistry*, 1st ed.; CRC Press: Boca Raton, FL, USA, 1990; Volume 34. Available online: https://www.routledge.com/Cationic-Surfactants-Organic-Chemistry/Richmond/p/book/9780824783815 (accessed on 21 April 2021).

47. Prabhu, V.S. Synthesis of Tertiary Amine Oxides. U.S. Patent US5866718A, 20 March 1997.

48. Gunstone, F.D.; Padley, F.B. (Eds.) *Lipid Technologies and Applications*, 1st ed.; CRC Press: Boca Raton, FL, USA, 1997; 848p. Available online: https://www.routledge.com/Lipid-Technologies-and-Applications/Gunstone-Padley/p/book/9780824798383 (accessed on 21 April 2021).

49. Birnie, C.R.; Malamud, D.; Schnaare, R.L. Antimicrobial evaluation of *N*-Alkyl betaines and *N*-Alkyl-*N*,*N*-dimethylamine oxides with variations in chain length. *Antimicrob. Agents Chemother.* **2000**, *44*, 2514–2517. [CrossRef]

50. European Commission. Environment, Eco-Label, Product Groups. 2007. Available online: https://ec.europa.eu/environment/archives/ecolabel/product/pg_did_list_en.htm (accessed on 21 April 2021).

51. U.S. Environmental Protection Agency. Safer Chemical Ingredients List. Available online: https://www.epa.gov/saferchoice/safer-ingredients (accessed on 21 April 2021).

52. Organisation for Economic Co-Operation and Development. SIAM 22. 18–21 April 2006. SIDS Initial Assessment Profile. Available online: https://www.aciscience.org/docs/Amine_Oxides_SIAP_April_2006.pdf (accessed on 21 April 2021).

53. Subik, J.; Takacsova, G.; Psenak, M.; Devinsky, F. Antimicrobial activity of amine oxides: Mode of action and structure-activity correlation. *Antimicrob. Agents Chemother.* **1977**, *12*, 139–146. [CrossRef] [PubMed]

54. Devínsky, F.; Kopecka-Leitmanová, A.; Šeršeň, F.; Balgavý, P. Cut-off Effect in Antimicrobial Activity and in Membrane Perturbation Efficiency of the Homologous Series of *N*,*N*-Dimethylalkylamine Oxides†. *J. Pharm. Pharmacol.* **2011**, *42*, 790–794. [CrossRef] [PubMed]

55. Berger-Bächi, B. Resistance mechanisms of gram-positive bacteria. *Int. J. Med. Microbiol.* **2002**, *292*, 27–35. [CrossRef] [PubMed]

56. Fuda, C.; Suvorov, M.; Vakulenko, S.B.; Mobashery, S. The Basis for Resistance to β-Lactam Antibiotics by Penicillin-binding Protein 2a of Methicillin-resistant Staphylococcus aureus. *J. Biol. Chem.* **2004**, *279*, 40802–40806. [CrossRef] [PubMed]

57. Ito, T.; Katayama, Y.; Asada, K.; Mori, N.; Tsutsumimoto, K.; Tiensasitorn, C.; Hiramatsu, K. Structural comparison of three types of staphylococcal cassette chromosome mec integrated in the chromosome in methicillin-resistant Staphylococcus aureus. *Antimicrob. Agents Chemother.* **2001**, *45*, 1323–1336. [CrossRef] [PubMed]

58. Goffin, C.; Ghuysen, J.-M. Multimodular Penicillin-Binding Proteins: An Enigmatic Family of Orthologs and Paralogs. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 1079–1093. [CrossRef]

59. Corner, A.M.; Dolan, M.M.; Yankell, S.L.; Malamud, D. C31G, a new agent for oral use with potent antimicrobial and antiadherence properties. *Antimicrob. Agents Chemother.* **1988**, *32*, 350. [CrossRef] [PubMed]

60. Catalone, B.J.; Ferguson, M.L.; Miller, S.R.; Malamud, D.; Kish-Catalone, T.; Thakkar, N.J.; Krebs, F.C.; Howett, M.K.; Wigdahl, B.; Malamud, D. Prolonged exposure to the candidate microbicide C31G differentially reduces cellular sensitivity to agent re-exposure. *Biomed. Pharmacother. Biomed. Pharmacother.* **2005**, *59*, 460–468. [CrossRef] [PubMed]

61. Calis, S.; Yulug, N.; Sumnu, M.; Ayhan, A.; Hincal, A.A. A non-antibiotic antimicrobial mixture (C31G): Evaluation of the antimicrobial efficiency of C31G on vaginal cultures. *Boll. Chim. Farm.* **1992**, *131*, 335–338. [PubMed]

62. Krebs, F.C.; Miller, S.R.; Malamud, D.; Howett, M.K.; Wigdahl, B. Inactivation of human immunodeficiency virus type 1 by nonoxynol-9, C31G, or an alkyl sulfate, sodium dodecyl sulfate. *Antivir. Res.* **1999**, *43*, 157–173. [CrossRef]

63. Michaels, E.B.; Hahn, E.C.; Kenyon, A.J. Effect of C31G, an antimicrobial surfactant, on healing of incised guinea pig wounds. *Am. J. Vet. Res.* **1983**, *44*, 1378–1381. [PubMed]

64. Thompson, K.A.; Malamud, D.; Storey, B.T. Assessment of the antimicrobial agent C31G as a spermicide: Comparison with nonoxynol-9. *Contraception* **1996**, *53*, 313–318. [CrossRef]

65. González-Bello, C. Antibiotic adjuvants—A strategy to unlock bacterial resistance to antibiotics. *Bioorg. Med. Chem. Lett.* **2017**, *27*, 4221–4228. [CrossRef] [PubMed]

66. Chakradhar, S. What's old is new: Reconfiguring known antibiotics to fight drug resistance. *Nat. Med.* **2016**, *22*, 1197–1199. [CrossRef] [PubMed]

67. Gill, E.E.; Franco, O.L.; Hancock, R.E.W. Antibiotic adjuvants: Diverse strategies for controlling drug-resistant pathogens. *Chem. Biol. Drug Des.* **2015**, *85*, 56–78. [CrossRef] [PubMed]

68. Bernal, P.; Molina-Santiago, C.; Daddaoua, A.; Llamas, M.A. Antibiotic adjuvants: Identification and clinical use. *Microb. Biotechnol.* **2013**, *6*, 445–449. [CrossRef] [PubMed]

69. Kalan, L.; Wright, G.D. Antibiotic adjuvants: Multicomponent anti-infective strategies. *Expert Rev. Mol. Med.* **2011**, 13. [CrossRef] [PubMed]

70. Farha, M.A.; Brown, E.D. Discovery of antibiotic adjuvants. *Nat. Biotechnol.* **2013**, *31*, 120–122. [CrossRef] [PubMed]

71. Melander, R.J.; Melander, C. The Challenge of Overcoming Antibiotic Resistance: An Adjuvant Approach? *ACS Infect. Dis.* **2017**, *3*, 559–563. [CrossRef] [PubMed]

72. Celenza, G.; Segatore, B.; Setacci, D.; Bellio, P.; Brisdelli, F.; Piovano, M.; Garbarino, J.A.; Nicoletti, M.; Perilli, M.; Amicosante, G. In vitro antimicrobial activity of pannarin alone and in combination with antibiotics against methicillin-resistant Staphylococcus aureus clinical isolates. *Phytomedicine* **2012**, *19*, 596–602. [CrossRef] [PubMed]

73. Bellio, P.; Segatore, B.; Mancini, A.; Di Pietro, L.; Bottoni, C.; Sabatini, A.; Brisdelli, F.; Piovano, M.; Nicoletti, M.; Amicosante, G.; et al. Interaction between lichen secondary metabolites and antibiotics against clinical isolates methicillin-resistant Staphylococcus aureus strains. *Phytomedicine* **2015**, *22*, 223–230. [CrossRef] [PubMed]

74. Linciano, P.; Vicario, M.; Kekez, I.; Bellio, P.; Celenza, G.; Martín-Blecua, I.; Blázquez, J.; Cendron, L.; Tondi, D. Phenylboronic acids probing molecular recognition against class A and class C β-lactamases. *Antibiotics* **2019**, *8*, 171. [CrossRef] [PubMed]
75. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55–63. [CrossRef]