Tomatidine acts in synergy with aminoglycoside antibiotics against multiresistant Staphylococcus aureus and prevents virulence gene expression

Gabriel Mitchell1, Myriame Lafrance1, Simon Boulanger1, David Lalonde Séguin1, Isabelle Guay1, Mariza Gattuso1, Éric Marsault2, Kamal Bouarab1 and François Malouin1*

1Centre d’Étude et de Valorisation de la Diversité Microbienne (CEVDM), Département de biologie, Faculté des sciences, Université de Sherbrooke, Sherbrooke, QC, Canada, J1K 2R1; 2Département de pharmacologie, Faculté de médecine et des sciences de la santé, Université de Sherbrooke, QC, Canada, J1H 5N4

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Objectives: This study characterized the multiple biological activities of the natural compound tomatidine against Staphylococcus aureus. Notably, this work examined the antibacterial activity of tomatidine in combination with other antibiotics and the influence of this compound on the expression of virulence factors in S. aureus.

Methods: The effect of tomatidine on the susceptibility of S. aureus to several antibiotic classes was determined by a broth microdilution procedure and a chequerboard protocol to measure fractional inhibitory concentration indices and to reveal drug interactions. Time–kill experiments for aminoglycoside/tomatidine combinations were also performed. The haemolytic ability of several strains in the presence of tomatidine was measured on blood agar plates and the expression of virulence-associated genes in strain ATCC 29213 treated with tomatidine was monitored by quantitative PCR.

Results: Tomatidine specifically potentiated the inhibitory effect of aminoglycosides but not of other classes of drugs. This potentiating effect was observed against strains of different clinical origins (human blood, cystic fibrosis airways, osteomyelitis, skin tissues and bovine mastitis), including aminoglycoside-resistant bacteria possessing the aac(6′)-aph(2′′), ant(4′)-Ia and aph(3′)-IIIa genes. The killing kinetics for the combination of aminoglycosides with tomatidine revealed strong bactericidal activity. Although tomatidine did not possess growth-inhibitory activity of its own against prototypical S. aureus, it inhibited the haemolytic activity of several strains and, more specifically, blocked the expression of several genes normally influenced by the agr system.

Conclusions: These results show that tomatidine is an aminoglycoside potentiator that also acts as an anti-virulence agent targeting both antibiotic-susceptible and antibiotic-resistant S. aureus.

Keywords: aminoglycoside-resistant staphylococci, MRSA, cystic fibrosis, steroidal alkaloids, antibiotic synergy

Introduction

Antibiotic resistance now represents an overwhelming problem in human and veterinary medicine since pathogens have developed mechanisms to circumvent almost all antibiotics of the current arsenal.1,2 Staphylococcal infections are often refractory to antibiotic treatments3 and cause both life-threatening and chronic diseases affecting several host species and body sites.4–6 Staphylococcus aureus is a significant pathogen causing difficult-to-treat infections7 and is commonly recovered from cystic fibrosis (CF) patients.7–9 Antibiotic therapy usually leads to better clinical prognostics in CF.10,11 However, the emergence of antibiotic-resistant bacteria is a major problem for patients afflicted by this disease and the prevalence of methicillin-resistant S. aureus (MRSA) is currently increasing among CF patients.11 This is alarming considering that MRSA are most often resistant to multiple antibiotic classes12 and are associated with declines of lung function and worse survival in CF patients.13,14 MRSA are associated with hospital- and community-acquired infections (HA- and CA-MRSA)15,16 but are also found in livestock (LA-MRSA) and companion animals, and transmission from colonized animals to humans has been reported.15,16 The ability of S. aureus to cause multiple diseases is related to its collection of virulence factors, the expression of which is...
controlled by a variety of global regulators that allow diverse strategies for pathogenesis. Such strategies include bacterial adhesion to extracellular matrix components, the formation of biofilms, immunomodulation, the destruction of host tissues and the invasion of host cells. The quorum-sensing-dependent agr system is known to be a major regulator of staphylococcal virulence. Once activated, the agr system leads to down-regulation of several cell-surface proteins such as adhesins and to up-regulation of several exotoxins and hydrolytic enzymes. It has been shown that the agr system greatly influences the outcome of S. aureus infection in several experimental models. Targeting virulence would thus be an interesting new avenue for drug development.

Plant products providing antibiotic-like activities against a wide variety of pathogenic bacteria may represent new chemical scaffolds for the development of antimicrobials. These plant products may directly inhibit or kill pathogenic bacteria, but some may alternatively increase the antimicrobial activity of already known antibiotics ('antibiotic potentiators') or alter the virulence and/or the adaptation of the pathogen towards the host ('virulence attenuators'). The plant product tomatidine and other closely related steroidal alkaloids do not have any clinically significant growth-inhibitory activity against prototypical S. aureus. We recently discovered serendipitously that tomatidine possesses an important and specific bacteriostatic activity against respiratory-deficient S. aureus small-colony variants (SCVs). The clinical importance of SCVs is increasingly recognized as their ability to persist in non-professional phagocytes and their association with chronic infections is now well established. The development of antibiotics able to target both extra- and intracellular SCVs, such as tomatidine, could represent interesting alternatives for clinicians. However, SCVs are rarely found alone during infections and are often co-isolated with strains harbouring the normal phenotype. In fact, it has been proposed that phenotypic switching is intrinsic to S. aureus pathogenesis. Consequently, this study examined the interaction of tomatidine with antibiotics of several classes in order to reveal useful combination therapies targeting both the normal and SCV phenotypes of S. aureus.

Materials and methods

Bacterial strains and growth conditions

A variety of S. aureus clinical strains of human and veterinary origins were used in this study. MRSA Sa220c and Sa228c were isolated from human skin and soft tissue infections at the Centre Hospitalier Universitaire de Sherbrooke (CHUS). Several S. aureus isolates (CF07-L, CF1A-L, CF2A-L, CF4B-L, CF6B-L, CF7A-L, CF8E-L, CF9A-L and CF35A-L) were isolated from the airways of CF patients by the clinic of the CHUS. The strains were collected following a consent protocol approved by the ethics review board of the CHUS (protocol 06-158-R4). MA078038 is a human blood isolate (CMRSA-10/USA300) from the Laboratoire de Sante´ Publique du Québec (LSQP). Strain SHY97-3906 is a bovine mastitis isolate. New- bouldAsigB was generated from Newbould (ATCC 29740, another bovine mastitis isolate) by disrupting the sigB gene with the ermA cassette by homologous recombination. Additional S. aureus reference strains were also used, such as ATCC 29213, Newman, MRSA COL, MRSA ATCC 43300, MRSA ATCC BAA-41, MRSA N315 and MRSA Mu50, which is also a vancomycin-intermediate resistant S. aureus (VISA). Strain 8325-4 is characterized by lower SigB activity resulting from a ‘natural’ deletion in the rsbU locus. SH1000 is isogenic to strain 8325-4, but with a functional rsbU allele. Except where otherwise stated, bacteria were grown in cation-adjusted Mueller–Hinton broth (CAMHB; BD, Mississauga, ON, Canada).

Chemical reagents and antibiotics

Tomatidine hydrochloride, oxacillin, erythromycin, ciprofloxacin, tetracycline, vancomycin, gentamicin, kanamycin, tobramycin, amikacin and streptomycin were from Sigma (Oakville, ON, Canada). Tomatidine and tomatine were solubilized at 2 g/L in DMSO. Tomatidine was warmed at 70°C during the solubilization process. Oxacillin, ciprofloxacin, tetracycline, vancomycin, gentamicin, kanamycin, tobramycin, amikacin and streptomycin were solubilized in water at 10 g/L. NaOH 2.5 N was used during the solubilization of ciprofloxacin. Erythromycin was solubilized at 10 g/L in 1:1 water/ethanol. The chemical structures of tomatidine and tomatine are shown in Figure 1.

Antibiotic susceptibility testing

MICs were determined by a broth microdilution technique, following the recommendations of the CLSI. S. aureus ATCC 29213 was used as a comparator strain in all MIC tests. Resistance phenotypes were attributed using the following susceptibility breakpoints: oxacillin 4 mg/L, erythromycin 8 mg/L, ciprofloxacin 4 mg/L, tetracycline 16 mg/L, vancomycin 16 mg/L, gentamicin 16 mg/L, kanamycin 64 mg/L, and tobramycin 16 mg/L. Intermediate resistance to gentamicin and vancomycin was defined by concentrations of 8 and 4–8 mg/L, respectively. The concentration of tomatidine chosen for antibiotic combinations in MIC assays was 4–8 mg/L, which was well above the minimal tomatidine concentration showing a potentiating effect in synergy studies (see the Results section).

Synergy studies

A chequerboard protocol was conducted by a broth microdilution method similar to that used for standard MIC determinations in order to evaluate the effect of varying concentrations of tomatidine on the activity of aminoglycoside antibiotics against S. aureus strain ATCC 29213. The fractional inhibitory concentration (FIC) indices were calculated as follows: FIC index = FICA + FICB = A/MICA + B/MICB, where A and B are the MICs of compounds A and B in combination, MICA and MICB are the MICs of compound A and compound B alone and FICA and FICB are the FICs of compound A and of compound B. Indifference for drug interactions or an additive effect is demonstrated if the FIC index is >0.5–4 and synergy if the FIC index is <0.5, whereas an antagonistic effect is represented by an FIC index of >4.

Time–kill experiments

Time–kill kinetics were followed in order to determine whether the effect of compounds alone or in combination was bacteriostatic or bactericidal. Bacteria were inoculated at ~10^5–10^6 cfu/mL in 100 mL CAMHB and grown at 35°C with shaking in the absence or presence of antibiotics at the following concentrations (as specified in the figure legends): tomatidine and tomatine, 8 mg/L; ciprofloxacin and erythromycin, 1 mg/L; gentamicin, 0.12–4 mg/L. At several timepoints, bacteria were sampled, serially diluted and plated on tryptic soy agar for cfu determinations.
Aminoglycoside resistance determinants

The aminoglycoside-resistant strains used in this study were screened by PCR for the presence of genes encoding aminoglycoside-modifying enzymes. PCR detection of the aac(6′)-aph(2″) (351 bp), ant(4′)-Ia (172 bp) and aph(3′)-IIIa (268 bp) amplicons was done by using the following primer sequences:

- forward 5′-ACAGAGCCTTGGGAAGATGAAGT-3′ and reverse 5′-GCCCTCGTGTAATTCATGTTCTGGC-3′; for the amplification of aac(6′)-aph(2″);
- forward 5′-CTGCTAATCGGTAGAAGC-3′ and reverse 5′-CAGACCAGCTAACACATGGCACC-3′; for the amplification of ant(4′)-Ia;
- forward 5′-CTGATCGAAAAATACCGCTGC-3′ and reverse 5′-TCATACTCTTCCGAGCAAAGG-3′; for the amplification of aph(3′)-IIIa.

Primers for the amplification of aac(6′)-aph(2″) were designed using the aac(6′)-aph(2″) DNA sequence of strain Mu50. Primers for the amplification of ant(4′)-Ia and aph(3′)-IIIa were from Schmitz et al.31 Samples were denatured at 94°C for 10 min followed by 35 amplification cycles using the following parameters: 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. A final extension cycle of 72°C for 10 min was used. After amplification, PCR products were resolved using 1.5% (w/v) agarose gels.

Quantitative PCR (qPCR)

In order to evaluate the effect of tomatidine or tomatine on S. aureus gene expression, overnight cultures were used to inoculate CAMHB at an A600 of 0.1. Bacteria were then grown until an A600 of 0.6 and exposed to tomatidine or tomatine for 3 h. RNA extraction, cDNA synthesis and qPCR were performed as previously described26,32 using the primers described in Table 1. The relative expression ratios were calculated by using the cycle threshold (Ct) of the untreated culture (no-drug control) and that of the housekeeping gyrA gene: 

\[ n\text{-fold expression} = 2^{-\Delta\Delta Ct}, \]

where \( \Delta\Delta Ct = \Delta Ct \) (treated culture)/\( \Delta Ct \) (untreated culture) and \( \Delta Ct \) represents the difference between the Ct of the gene studied and the Ct of gyrA.

Haemolysis

Bacterial suspensions of 0.5 McFarland were prepared for each strain and were spotted (2 μL) on Mueller–Hinton agar (BD, Mississauga, ON, Canada) with 5% horse blood supplemented or not with 8 mg/L tomatidine or tomamine. Haemolysis was assessed after 24 h of incubation at 35°C followed by an overnight incubation at 4°C.

Statistical analysis

Statistical analyses were carried out with the GraphPad Prism Software (v.5.00). For qPCR results, analyses were performed on \( \Delta\Delta Ct \). Statistical
tests used for the analysis of each experiment are specified in the Results section and the figure legends.

Results
Tomatidine potentiates the action of aminoglycoside antibiotics against S. aureus

We showed previously that tomatidine had potent inhibitory activity against S. aureus SCVs but that this natural product had no clinically significant MIC against normal strains of S. aureus.10 In order to further characterize the effect of tomatidine on S. aureus and to better understand its therapeutic potential, we evaluated the activity of tomatidine in combination with several classes of antibiotics. We found that tomatidine increased the susceptibility (i.e. decreased the MIC) of ATCC 29213 to the aminoglycoside antibiotics gentamicin (8–32-fold), kanamycin (4–8-fold), tobramycin (4–8-fold), amikacin (4-fold) and streptomycin (4–8-fold), whereas it did not alter that of other classes of antibiotics (oxacillin (B-lactams), erythromycin (macrolides), ciprofloxacin (fluoroquinolones), tetracycline (tetracyclines) and vancomycin (glycopeptides)) (Table 2). The specificity of tomatidine as an aminoglycoside potentiator was also demonstrated by the use of the structurally related tomatine (Figure 1), which did not influence the susceptibility of ATCC 29213 to aminoglycosides or any other classes of antibiotics (data not shown).

The activity of tomatidine in combination with gentamicin and/or tobramycin was also studied against other Gram-positive bacteria. Tomatidine potentiated the activity of gentamicin and tobramycin against Staphylococcus epidermidis (4–8-fold), Staphylococcus haemolyticus (4–16-fold), Staphylococcus saprophyticus (1–8-fold) and Staphylococcus hominis (2–4-fold), but not against Enterococcus faecalis and Enterococcus faecium (data not shown). For evaluation against Gram-negative bacteria, the activity of tomatidine in combination with tobramycin was tested against Pseudomonas aeruginosa ATCC 27853 and Escherichia coli ATCC 25922 with no effect. Other E. coli strains tested that also showed no increase in susceptibility to the tomatidine/aminoglycoside combination included a strain highly permeable to large antibiotic molecules like erythromycin and vancomycin (Imp+213),33 a strain that lacks one of the major efflux pump systems (acrAB-aph(3′)β)34 and a rough strain (E. coli J5) lacking the long lipopolysaccharide (LPS) chains that prevent lipophilic molecules from interacting with bacterial membranes.35

The effect of tomatidine on the susceptibility of ATCC 29213 to several aminoglycoside antibiotics (gentamicin, kanamycin, tobramycin, amikacin and streptomycin) was confirmed using the chequerboard method. Results showed that tomatidine markedly increased the susceptibility of ATCC 29213 to all aminoglycoside antibiotics tested at a concentration as low as 0.12 mg/L (data not shown). At this concentration and above (tested up to 8 mg/L), a reduction of 4–32-fold in the MICs of aminoglycosides was observed. To approximate the FIC indices, we considered an MIC of 32 mg/L for tomatidine against ATCC 29213. This MIC was knowingly overestimated because there is still no complete growth inhibition of normal S. aureus strains in the presence of tomatidine at concentrations as high as 128 mg/L.19 The approximate FIC values characterizing the interaction of tomatidine with aminoglycoside antibiotics are reported in Table 2 and demonstrate strong synergy between the antimicrobial activities of these molecules (FIC <0.5).

Tomatidine specifically potentiates the action of aminoglycosides against S. aureus strains of various clinical origins and antibiotic susceptibility profiles

The antibacterial effect of tomatidine in combination with aminoglycosides was next evaluated against a group of S. aureus strains of diverse origins. These strains were isolated from a variety of diseases and body sites, such as bovine mastitis, human osteomyelitis, blood, skin and soft tissue infections and from the airways of CF patients, and are shown in Table 3. Table 3 also presents the MICs of oxacillin, erythromycin, ciprofloxacin, tetracycline, vancomycin and three aminoglycosides (gentamicin, kanamycin and tobramycin) against these S. aureus strains. It is noteworthy that several of these strains were resistant to multiple classes of antibiotics and/or were MRSA. Importantly, Table 3 shows that tomatidine increased the susceptibility of all the studied strains to aminoglycoside antibiotics. More precisely, the presence of tomatidine could increase the susceptibility of aminoglycoside-susceptible S. aureus strains to gentamicin, kanamycin or tobramycin by 2–16-fold.

Interestingly, the potentiating effect of tomatidine on the activity of aminoglycoside antibiotics was also detectable against aminoglycoside-resistant S. aureus strains (Table 3). Strains resistant to gentamicin, kanamycin and/or tobramycin were screened for genetic determinants encoding aminoglycoside-modifying enzymes (AMEs), which represent the main mechanism of aminoglycoside resistance.11,26,31 The detected AME genes matched the expected aminoglycoside susceptibility pattern for each strain. The presence of aac(6′)-aph(2′) correlated with resistance to gentamicin, kanamycin and tobramycin, that of ant(4′)-Ia with resistance to kanamycin and tobramycin and that of aph(3′)-IIIa with resistance to kanamycin (Table 3). Interestingly, as for the aminoglycoside-susceptible strains, the activity
Table 3. Effect of tomatidine on the susceptibility of S. aureus strains of diverse origins and antibiotic susceptibility profiles to aminoglycosides

<table>
<thead>
<tr>
<th>Strains</th>
<th>Details</th>
<th>Aminoglycoside modifying enzyme-encoding genes</th>
<th>MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OXA</td>
<td>ERY</td>
</tr>
<tr>
<td>B3254</td>
<td></td>
<td>0.12</td>
<td>0.25–0.5</td>
</tr>
<tr>
<td>COL</td>
<td>MRSA</td>
<td>&gt;64 (R)</td>
<td>0.5</td>
</tr>
<tr>
<td>Newman</td>
<td>OS</td>
<td>0.5–1</td>
<td>0.5</td>
</tr>
<tr>
<td>Newbould</td>
<td>BM</td>
<td>0.12</td>
<td>0.5</td>
</tr>
<tr>
<td>SHY97-3906</td>
<td>BM</td>
<td>0.12–0.25</td>
<td>0.12–0.25</td>
</tr>
<tr>
<td>Sa220c</td>
<td>SSTI, MRSA</td>
<td>16–32 (R)</td>
<td>0.5–1</td>
</tr>
<tr>
<td>CF07-L</td>
<td>CF</td>
<td>0.12–0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>CF1A-L</td>
<td>CF</td>
<td>0.25–0.5</td>
<td>0.25–0.5</td>
</tr>
<tr>
<td>CF2A-L</td>
<td>CF</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>CF4B-L</td>
<td>CF</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>CF6E-L</td>
<td>CF</td>
<td>0.125</td>
<td>0.5</td>
</tr>
<tr>
<td>CF35A-L</td>
<td>CF, MRSA</td>
<td>&gt;64 (R)</td>
<td>&gt;64 (R)</td>
</tr>
<tr>
<td>ATCC 43300</td>
<td>MRSA</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>ATCC BAA-41</td>
<td>MRSA</td>
<td>ant(4′)-Ia</td>
<td>&gt;64 (R)</td>
</tr>
<tr>
<td>N315</td>
<td>MRSA</td>
<td>ant(4′)-Ia</td>
<td>8</td>
</tr>
<tr>
<td>Mu50</td>
<td>MRSA, VISA</td>
<td>aac(6′)-aph(2′)-Ia</td>
<td>&gt;64 (R)</td>
</tr>
<tr>
<td>MAD78038</td>
<td>BI, MRSA</td>
<td>aph(3′)-IIIa</td>
<td>64 (R)</td>
</tr>
<tr>
<td>Sa228c</td>
<td>SSTI, MRSA</td>
<td>aac(6′)-aph(2′)-Ia</td>
<td>&gt;64 (R)</td>
</tr>
</tbody>
</table>

Strains susceptible to GEN, KAN and TOB

Strains resistant to GEN, KAN and/or TOB

Bl, blood; BM, bovine mastitis; CF, CF airways; OS, osteomyelitis; SSTI, skin and soft tissue infection; OXA, oxacillin; ERY, erythromycin; CIP, ciprofloxacin; TET, tetracycline; VAN, vancomycin; GEN, gentamicin; TOB, tobramycin; KAN, kanamycin; TO, tomatidine; ND, not determined.

Intermediate resistance (I) and resistance (R) are indicated.

$^a$Ratios expressed as fold differences between the aminoglycoside MICs without and with the presence of tomatidine were determined for each independent experiment and show the increase in S. aureus susceptibility.

Tomatidine is an antibiotic potentiator and an anti-virulence agent.
of aminoglycosides against strains carrying AME genes was also generally increased by 2–16-fold in the presence of tomatidine, although in this case MICs did not drop below the resistance breakpoints (Table 3). Notably, although we have previously shown that tomatidine possesses a strong antibacterial effect of its own against *S. aureus* SCVs (MIC of 0.12 mg/L), we did not observe any synergy with aminoglycosides against such variants, which are already highly susceptible to tomatidine but less susceptible to aminoglycosides with gentamicin MICs of 4–8 mg/L (data not shown).

The combination of tomatidine with aminoglycoside antibiotics is bactericidal

Time–kill experiments were performed in order to determine whether the combination of tomatidine with aminoglycoside antibiotics is bacteriostatic or bactericidal against *S. aureus*. Figure 2(a) shows the effect of tomatidine and control antibiotics (erythromycin and ciprofloxacin at 2×MIC) on the viability of *S. aureus* ATCC 29213 as a function of time. As expected, tomatidine did not affect the growth or viability of *S. aureus* (MIC >128 mg/L), while erythromycin was bacteriostatic and ciprofloxacin was bactericidal (>3 log reduction in cfu). Figure 2(b) shows that gentamicin alone at ~1–2×MIC (gentamicin MIC was 0.5–1 mg/L, Table 3) was also bactericidal against *S. aureus*, although, as anticipated for aminoglycosides, regrowth was observed within 24 h. However, Figure 2(b) also shows that regrowth of bacteria was markedly reduced when tomatidine was combined with gentamicin at 1 mg/L and shows that this combination produced significant synergy and a bactericidal effect that was sustained for 24 h. Interestingly, the combination of a subinhibitory concentration of gentamicin (0.12 mg/L, i.e. about 1/4 to 1/8 of the MIC) with tomatidine resulted in a bactericidal effect (Figure 2c) that was...
quicker than that seen with ciprofloxacin at 2×MIC (Figure 2a), although, as for the aminoglycoside alone at ~1–2×MIC, regrowth was observed within 24 h. Again, this potentiating effect was not observed with tomatine, confirming the specificity of tomatidine. Note that similar results were obtained for tobramycin at 0.12 and 1 mg/L in combination with tomatidine (data not shown).

In order to examine the possibility that tomatidine can actually prevent the emergence of bacteria with decreased susceptibility to aminoglycosides, we followed the regrowth of bacteria in cultures exposed to various concentrations of gentamicin alone or in combination with tomatidine at 8 mg/L. Figure 2(d) further demonstrates that the presence of tomatidine could significantly decrease the number of cfu recovered from cultures exposed to concentrations of gentamicin ranging from 0.5 to 2 mg/L for 24 h (unpaired t-test between control and tomatidine-exposed conditions for each gentamicin concentration). From these time–kill experiments, isolated colonies obtained from cultures exposed to gentamicin combined or not with 8 mg/L tomatidine were analysed for their susceptibility to gentamicin. When ATCC 29213 was exposed to gentamicin alone, the emergence of numerous normal-growing isolates showing decreased susceptibility to gentamicin (MIC ranging from 1 to 4 mg/L) was easily detected. Such an exposure to gentamicin also yielded numerous isolates with the SCV phenotype showing the expected reduced susceptibility to gentamicin (MIC <0.5 mg/L). It is noteworthy that these SCVs maintained their high susceptibility to tomatidine (MIC, 0.5 mg/L). Besides, although many fewer colonies were recovered from cultures exposed to the various combinations of gentamicin and tomatidine (Figure 2d), normal-growing isolates with decreased susceptibility to gentamicin were found, as seen with gentamicin alone in cultures. Interestingly, small pinpoint colonies recovered from the combination cultures were particularly

Figure 3. Tomatidine (TO) alters the expression of agr-regulated virulence factors in S. aureus. (a) Haemolytic ability of several strains in the absence or presence of 8 mg/L TO or tomatine (TN). (b) Concentration-dependent effect of TO on the expression of hld (encoding the θ-haemolysin and a fragment of RNAIII, the effector of the agr system). Data are presented as means with standard deviations from at least three independent experiments. (c) Effect of 12.8 mg/L TO or TN on the expression of the agr-regulated genes hla, hld, geh, nuc, plc and splC. Significant differences between the TO and TN conditions for the expression of each gene are shown (**P<0.01 and *P<0.05; one-tailed unpaired t-test). Data are presented as means with standard deviations from at least two independent experiments. Ctrl, control.
unstable and easily reverted to the normal-growing phenotype when sub-cultured. It was therefore not possible to adequately measure the susceptibility of these SCVs to gentamicin or tomatidine and see if resistance of SCVs to tomatidine could develop from exposure to the combination. Finally, it is important to mention that the normal-growing isolates having reduced susceptibility to gentamicin (recovered from either the gentamicin or the gentamicin/tomatidine combination cultures) were still subject to the potentiating effect of tomatidine, although it required about two times more gentamicin to attain the level of growth inhibition observed with the parental strain ATCC 29213, i.e. a concentration of gentamicin related to the new MIC.

Overall, the results in this section clearly demonstrate that tomatidine potentiates the bactericidal action of aminoglycoside antibiotics against S. aureus and suggest that tomatidine can also reduce the emergence of bacteria with decreased susceptibility to aminoglycosides.

**Tomatidine affects the haemolytic ability of S. aureus and represses several agr-regulated virulence factors**

The quorum-sensing-dependent agr system is known to repress the expression of many cell-surface proteins while allowing expression of several exoproteins. Importantly, the ability of S. aureus to lyse red blood cells is regulated by the agr system. This association between haemolysis and the activity of agr was used to easily detect if tomatidine could have an impact on the virulence of S. aureus. Figure 3(a) shows that inclusion of tomatidine in blood agar plates strikingly reduced the haemolytic activity of all S. aureus strains tested, whereas tomatidine did not. It is noteworthy that Newbould sigB and 8325-4 strains with dysfunctional SigB activity, resulting in increased agr activity and, consequently, in an increase in haemolytic ability. Figure 3(a) shows that both the increased haemolytic activity of these strains and the haemolytic activity of their counterpart strains having a functional sigB operon (Newbould and SH1000, respectively) were inhibited by tomatidine.

qPCR was then used to measure the expression of hld (encoding the α-toxin and a fragment of RNAIII, the effector of the agr system) in ATCC 29213 treated with different concentrations of tomatidine. As expected, the expression of hld was decreased as a function of tomatidine concentration (Figure 3b). Figure 3(c) shows the effects of tomatidine and tomatine at 12.8 mg/L on the expression of some of the agr-upregulated genes (hla, hld, geh, nuc, plc and splC). As expected, tomatidine significantly repressed the expression of the agr-upregulated genes whereas tomatine did not (one-tailed unpaired t-test between tomatidine- and tomatine-exposed conditions for each gene). These results demonstrate that exposure to tomatidine represses several virulence factors in S. aureus.

**Discussion**

This study demonstrates that the plant product tomatidine specifically potentiates the bactericidal activity of aminoglycoside antibiotics against S. aureus and also more broadly against staphylococci. This effect was documented for several strains of diverse clinical origins and included multiresistant S. aureus strains. Surprisingly, tomatidine also potentiates the activity of aminoglycoside antibiotics against aminoglycoside-resistant bacteria carrying AMEs, although the MICs against such strains did not drop below the resistance breakpoints. Although we could not demonstrate a potentiating effect of tomatidine on the inhibitory activity of aminoglycosides against P. aeruginosa, E. coli or even Enterococcus spp., it remains to be seen whether such a biological activity can be observed in other clinically relevant bacterial genera.

A major challenge in the clinical use of aminoglycosides is to empirically evaluate the dose and administration schedule needed to obtain a non-toxic, but still successful, therapy. If the development of tomatidine as an adjunct therapy is successful eventually, the use of tomatidine in combination with aminoglycoside antibiotics could allow new efficient treatment regimens with reduced aminoglycoside amounts and a lower risk of drug toxicity. Moreover, the emergence of bacteria that are less susceptible after exposure to aminoglycosides has been fully documented by others and the results from this study also suggest that the presence of tomatidine could reduce the incidence of bacteria that are less susceptible to aminoglycosides. Thus, an aminoglycoside potentiator such as tomatidine could possibly extend the clinical applications and safe usage of aminoglycosides.

In addition to the potentiating effect of tomatidine demonstrated here on the inhibitory activity of aminoglycosides against prototypical S. aureus, we recently reported that tomatidine also has a specific bacteriostatic activity against S. aureus SCVs. More importantly, we showed that tomatidine has the ability to inhibit the replication of SCVs internalized in CF-like human airway epithelial cells. Because S. aureus of the normal and SCV phenotypes are often recovered simultaneously from CF patients and because aminoglycoside antibiotics are already used in the management of bacterial infections in CF, this suggests that a combination therapy of tomatidine and aminoglycosides could be especially effective in the context where S. aureus and S. aureus SCVs co-infect a CF patient. Aminoglycoside antibiotics have a wide spectrum of antimicrobial activity that affects both Gram-positive and Gram-negative pathogens, and in this new era plagued by multiresistant organisms, the revitalization of a well-known class of antibiotics by potentiators such as tomatidine would certainly have its merit.

The mechanism by which tomatidine increases the activity of aminoglycoside antibiotics against prototypical S. aureus is not yet understood. It is possible that tomatidine increases the uptake of aminoglycoside antibiotics by increasing the permeability of S. aureus. This would explain the improved bactericidal activity of the combination of tomatidine with subinhibitory concentrations of aminoglycosides since the activity of such antibiotics is intimately associated with their uptake into bacteria. Inversely, it is also possible that subinhibitory concentrations of aminoglycosides allow tomatidine to reveal its own antimicrobial activity against S. aureus. As an example, it is known that the action of aminoglycosides causes channel formation in bacterial membranes that support influx of antibiotics, and this may allow the uptake of tomatidine to occur. Tomatidine could then act on protein synthesis, as previously anticipated. We currently tend to rule out that the action of tomatidine on S. aureus is through membrane permeabilization because of the lack of activity of the structurally related tomatine, the lack of potentiating activity of tomatidine on other antibiotic classes against...
Tomatidine is an antibiotic potentiator and an anti-virulence agent

S. aureus and the inability of tomatidine to potentiate aminoglycoside activity against both hyperpermeable, LPS-rough and efflux-pump-deficient E. coli strains.

The inhibition of S. aureus haemolytic activity we noticed in the presence of tomatidine was associated with the inability of the bacterium to activate the quorum-sensing agr system, which normally upregulates the expression of several virulence factors, such as α-haemolysin and other exoproteins. Accordingly, we demonstrated that tomatidine inhibits the expression of several genes known to be upregulated by the agr system. Our results thus suggest that tomatidine could alter the virulence of S. aureus by repressing the expression of several agr-upregulated genes. Here again, the mechanism by which tomatidine acts on the agr system is not known, but it is possible that tomatidine influences the production of virulence factors by interacting with the bacterial surface and/or interfering with signal transduction, as proposed for other virulence inhibitors in staphylococci.

Overall, this study demonstrates that tomatidine exerts different biological effects on S. aureus that are likely to be useful once translated into therapeutic applications. We have shown here that the synergy between tomatidine and aminoglycosides could help reduce the amounts of these drugs required for a bactericidal effect against prototypical S. aureus while also reducing the incidence of resistance. This synergy, in addition to the standalone inhibitory activity of tomatidine previously reported against extracellular and intracellular S. aureus SCVs, remains to be confirmed in vivo. However, in the meantime, tomatidine now represents an interesting new structural scaffold on which to base future investigations aiming at the development of anti-staphylococcal agents efficient against strains causing chronic and difficult-to-treat infections.

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Transparency declarations

None to declare.

References

Staphylococcus aureus is a common pathogen in infections, particularly in cystic fibrosis patients. Small colony variants isolated from the airways of cystic fibrosis patients display unusual resistance to aminoglycosides. Aminoglycoside resistance genes and their expression are influenced by various factors, including SigB expression and stress resistance. The prevalence of aminoglycoside resistance genes in clinical isolates is a concern for effective treatment. Understanding the mechanisms of resistance and developing strategies to combat this problem are crucial.

References: