Synergistic antibacterial effect of statins with the complex \{1-(4-bromophenyl)-3-phenyltriazene N₃-oxide-κ² N¹,O⁴\} (dimethylbenzylamine-κ² C¹,N⁴)palladium(II) \(\text{Pd(DMBA)LBr}\)

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The treatment of infections caused by resistant microorganisms represents a big challenge in healthcare due to limited treatment options. For this reason, the discovery of new active substances which are able to perform innovative and selective actions is of great impact nowadays. Statins and triazenes (TZC) have consolidated as a promising class of compounds, characterized by the expressive biological activity, especially antimicrobial activities. The aim of this study was to assess the in vitro synergistic antibacterial effect of the association of statins and a new TZC complex \{1-(4-bromophenyl)-3-phenyltriazene N₃-oxide-κ² N¹,O⁴\} (dimethylbenzylamine-κ² C¹,N⁴)palladium(II) \(\text{Pd(DMBA)LBr}\) against American Type Culture Collection (ATCC) strains and clinical isolates. The complex and the statins showed bacterial activity of all tested strains and clinical isolates, evidencing that TZC complexion with metals can be promising. Simvastatin showed synergy when associated to the complex (FICI≤0.5), being the minimum inhibitory concentration (MIC) of 16 µg mL⁻¹ found in 6 samples. Thus, it is possible to infer that the association between \text{Pd(DMBA)LBr}\ and simvastatin consists of an alternative to increase the potential of these compounds, since statins have low toxicity.

Keywords: Statins/antimicrobial activity. Triazenide. Atorvastatin. Simvastatin.

INTRODUCTION

Antimicrobial resistance is a serious public health issue worldwide. Multi-drug resistant microorganisms (MDR) are an increasing concern since they show low susceptibility to different classes of antimicrobials that are commonly prescribed in hospitals (WHO, 2014; Tzialla et al., 2015). It has also become a challenge for healthcare professionals because therapy options for the treatment of some infections caused by MDR are more and more restrict due to the fast emergence and dissemination of these microorganisms (Azevedo, Silva, 2012; Thangamani et al., 2015; Karam et al., 2016).

This resistance occurs due to several reasons, mainly the indiscriminate use of these agents, thus decreasing the amount of drugs available for the treatment of such infections (Azevedo, Silva, 2012; Karam et al., 2016). Therefore, there is the urge to discover new drugs with antibacterial properties, in addition to associations in the search of synergistic effects (Kalaivani, 2012; Thangamani et al., 2015; Tizotti et al., 2016).

Statins are a class of drugs which have shown a promising antibacterial activity against several bacterial species, being used for the reduction of lipids in patients with high cholesterol levels as well as showing anti-inflammatory activity (Almog et al., 2004; Lopez-
Cortes et al., 2013; Kozarov, Padro, Badimon, 2014; Thangamani et al., 2015). Also, other substances worth noticing are triazenes (TZC), which contain an aliphatic chain composed by three nitrogen atoms interconnected in sequence (N=N-N), responsible for their biological properties (Moore, Robinson, 1986). These substances show wide pharmacological versatility such as antifungal, antileukemia and antibacterial activity, making them the focus of several studies (Hörner et al., 2008; Domingues et al., 2010; Mohammadi, 2014; Tizotti et al., 2016).

Also, in order to increase the biological activity and stability of TZC in medicines, there is a growing interest in associating these compounds with metals (Sreedhara, Cowan, 2001; Karami et al., 2017). Compounds that contain palladium (Pd(II)) are worth highlighting, mainly regarding antitumor activity, since they have more stability and less toxicity when compared to platin-based anticancer compounds, due to their similar structural behaviour (Dupont, Consorti, Spencer, 2005; Massai et al., 2016; Karami et al., 2017). Also, their potential antibacterial activity can have their action significantly increased up to 16 times when associated to antimicrobials than the free drug (Guerra et al., 2005).

The aim of this study was to assess the in vitro synergistic antibacterial effect of the association between statins and a new TZC complex \([1-(4\text{-}bromophenyl)-3\text{-}phenyltriazene\ N^3\text{-}oxide\ \kappa^2\ N^1,\ O^4](\text{dimethylbenzylamine}\ \kappa^2\ C^1,N^4)\text{palladium(II)}\) (Pd(DMBA)LBr)

\[
\text{Pd(DMBA)LBr} \rightarrow \text{Pd(DMBA)} + \text{LBr}.
\]

MATERIAL AND METHODS

Chemical compounds

The TZC were previously synthesized and chemically characterized in the Núcleo de Investigação de Triazenos e Complexos (NiTriCo) of Universidade Federal de Santa Maria (UFSM). Statins were purchased commercially in the form of their active principle (atorvastatin, formula: \(C_{66}H_{68}CaF_2N_4O_{10}.3H_2O\), PM = 1209.4; simvastatin, formula: \(C_{25}H_{38}O_5\), PM = 418.57).

Experimental

The synthesis of the ligand HLBBr was realized according the literature and based on Scheme 1, while the synthesis of the complex Pd(DMBA)LBr from the ligand HLBBr and the precursor complex \([\text{Pd(DMBA)Cl}_2]\) followed according Scheme 2 (Martins et al., 2017).

Synthesis of \([1-(4\text{-}bromophenyl)-3\text{-}phenyltriazene\ N^3\text{-}oxide\ \kappa^2\ N^1,\ O^4](\text{dimethylbenzylamine}\ \kappa^2\ C^1,N^4)\text{palladium(II)}\) (Pd(DMBA)LBr)

To obtain the complex Pd(DMBA)LBr a solution of the protonated ligand HLBBr (0.05 g; 17.12 mmol) in 20 mL of tetrahydrofuran was prepared. To this transparent pale-yellow solution five drops of a concentrate solution of KOH in methanol were added under continuous stirring at room temperature. The reaction mixture changes to intense yellow indicating the presence of the deprotonated free...
Synergistic antibacterial effect of statins with the complex (Pd(DMBA)LBr)

**In vitro antibacterial activity**

The **in vitro** antibacterial activity was evaluated against different strains ATCC, including Bacillus cereus ATCC 14579, Enterobacter hormaechei ATCC 700323, Enterococcus casseliflavus ATCC 700327, Enterococcus faecalis ATCC 29212, Enterococcus faecalis ATCC 51299, Escherichia coli ATCC 25922, Escherichia coli ATCC 35218, Klebsiella pneumoniae ATCC 700603, Micrococcus luteus ATCC 7468, Pseudomonas aeruginosa ATCC 27853, Salmonella typhimurium ATCC 14028, Salmonella spp. ATCC 52117, Staphylococcus aureus ATCC 25923, Staphylococcus aureus ATCC 29213, Staphylococcus aureus BAA 1026, Staphylococcus aureus BAA 976, Staphylococcus aureus BAA 977, Staphylococcus epidermidis ATCC 12228 and against ten coagulase-negative staphylococci isolates in newborn blood cultures in 2014. Clinical isolates were identified through by automated system Vitek® 2 (bioMérieux, France).

**Determination of the Minimum Inhibitory Concentration (MIC)**

Bacterial isolates and ATCC strains, stored in 15% glycerol at -80 °C, were pre-activated using the agar tryptcase soy medium (TSA) for 24 h at 35 ± 2 °C. Evaluation of the antibacterial activity of the compounds was performed using the conventional method of broth microdilution for Minimum Inhibitory Concentration (MIC) based on the Clinical and Laboratory Standards Institute guidelines (CLSI, 2012a). The test compounds was diluted in ethanol at a concentration of 20.480 µg mL⁻¹ and then successive dilutions were made with concentrations from 1.024 to 1 µg mL⁻¹, with ethanol concentration 5% to 0.0048%. The bacterial inoculum was prepared using a 0.5 McFarland scale, so that each well contained 5 x 10⁵ CFU mL⁻¹. Plates were incubated for 24 h at 35 ± 2 °C and after this period, the MIC
was determined visually as the lowest concentration that completely inhibited growth of microorganisms in dilution wells. For control and comparison MIC was determined as a broad spectrum antibacterial drug used in therapeutics: Tigecycline (Tygacil® - Wyeth). The drug was dissolved in physiological solution in the same manner as the compounds, but at a lower concentration (5.120 µg mL⁻¹). After successive dilutions were performed at concentrations of 256 to 0.25 µg mL⁻¹. Tests were also conducted using only ethanol to demonstrate that it did not interfere with the activity.

Statistical analysis

The analysis of the combination of TZC and statins was obtained by calculating the Fractional Inhibitory Concentration Index (FICI). The FICI was interpreted as “synergetic” (FICI≤0.5); “no interaction” (FICI>0.5 and ≤4.0) and “antagonism” (FICI>4.0) (Odds, 2003; Konaté et al., 2012).

Ethical considerations

This study was approved by the Research Ethics Committee (CEP) of UFSM, under the certificate number of presentation for ethical consideration (CAAE) 38850614.4.0000.5346.

RESULTS

All compounds showed antibacterial activity against the strains tested. The MIC of ATCC strains against ligands, precursor and complex, associated or not to statins, free palladium(II), tigecycline and fici values, are shown in Table I; and it is possible to observe the same parameters in Table II, however against the 10 clinical isolates. Simvastatin showed an activity similar or better than atorvastatin in all ATCC strains and clinical isolates analyzed, with the lowest MIC (=16 µg mL⁻¹) found in the ATCC strain of S. aureus BAA 976. Also, S. aureus BAA 977, Micrococcus luteus 7468 and the isolates 8 showed MIC=32 µg mL⁻¹. It has also been possible to observe that the best activity occurred in Gram-positive bacteria.

Regarding TZC, the DMBA ligand and the precursor ([Pd(DMBA)(μ-Cl)]₂) showed MIC≥128 µg mL⁻¹ for all the microorganisms analyzed. The ligand HLBr showed good activity in ATCC strains, with MIC=64 µg mL⁻¹ for E. faecalis ATCC 51299 and S. aureus ATCC 25923, and for the clinical isolates the lowest MIC was 128 µg mL⁻¹. As for the palladium(II) complex, (Pd(DMBA)HLBr) showed greater antibacterial potential in nearly all microorganisms when compared to the ligand and the precursor, resulting in MIC=64 µg mL⁻¹ for some clinical isolates and MIC=32 µg mL⁻¹ for the ATCC strain of E. faecalis 51299.

When TZC was associated with simvastatin, it was possible to observe a decrease of MIC values in both ATCC strains and clinical isolates. The DMBA associated with simvastatin showed synergy (FICI≤0.5) against strains of B. cereus ATCC 14579, E. faecalis ATCC 51299, S. aureus ATCC 25923, S. aureus 29213, and S. aureus BAA 1026. The precursor ([Pd(DMBA)(μ-Cl)]₂) showed synergy against these strains as well as Salmonella ATCC 52117 and isolate 2.

The ligand 2 (HLBr), when associated to simvastatin, showed MIC=64 µg mL⁻¹ for Salmonella ATCC 52117; MIC=32 µg mL⁻¹ for B. cereus, S. aureus 29223, S. aureus BAA 1026, isolates 4 and 6; MIC=16 µg mL⁻¹ for E. faecalis ATCC 51299 and S. aureus 25923, with FICI<0.5 against these samples. The complex (Pd(DMBA)HLBr) was twice more active than its free ligands and precursor when associated with simvastatin, showing synergy against the strains E. casseliflavus ATCC 700327, E. coli ATCC 25922, P. aeruginosa ATCC 27853 and S. aureus 29213, S. aureus BAA 1026, isolates 1, 2, 3, 4, 5, 6 and 7, with FICI≤0.5. As for the association of TZC with atorvastatin, no synergy was evidenced, but MIC values were similar to the ones obtained when the compounds were separately tested, except for a few strains.

DISCUSSION

The emergence of antimicrobial resistance shows the need for searching new drugs, as well as their association (Masadeh et al., 2012). Some studies have evidenced a possible antibacterial effect of this class of drugs, associated to antimicrobials or not in order to reduce morbidity and mortality of several infectious diseases (Masadeh et al., 2012; Ajrouche et al., 2013; López-Cortés et al., 2013; Kozarov, Padro, Badimon, 2014; Graziano et al., 2015; Thangamani et al., 2015).

In this study, it was possible to observe that statins are able to induce variable degrees of antibacterial activity, with simvastatin being more potent than atorvastatin. A study developed in 2012 comparing the antibacterial activity of atorvastatin, simvastatin and rosuvastatin showed that the two first statins were the most potent in Gram-positive microorganisms, with MIC=166.67±72.16 µg mL⁻¹ for atorvastatin and MIC=104.17±36.08 µg mL⁻¹ for simvastatin in E. faecalis 51299 strains, a result similar to the one found in our study. However, below-average results were found for E. coli 35218 (MIC=26.04±9.02 µg mL⁻¹ and
### TABLE I - Minimum Inhibitory Concentrations (MIC) of ligands, precursor and complex, associated or not with statins against standard bacteria

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<td><strong>Staphylococcus epidermidis ATCC 12228</strong></td>
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<td>512</td>
<td>128</td>
<td>0.8</td>
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<td>256</td>
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<td>128</td>
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<td>1.5</td>
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<td>256</td>
<td>&lt;0.25</td>
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</table>

A = Atorvastatin; S = Simvastatin; L₁ = Ligand 1 N,N'-dimethylbenzylamine (DMBA); FICI = Fractional Inhibitory Concentration Index; L₂ = Ligand 2 1-Phenyl-3-(4-bromophenyl)triazene N₂-hydroxide (HLBr); P = Precursor [Pd(DMBA)(µ-Cl)]; C = Complex [1-(4-bromophenyl)-3-phenyltriazene N₂-oxide·κ²N₂', O²][dimethylbenzylamine·κ²C³, N⁶]palladium(II); (Pd(DMBA)LBr); Pd = palladium(II); Tig = Tigecycline; * = Test not realized.
**TABLE II - Minimum Inhibitory Concentrations (MIC) of ligands, precursor and complex, associated or not with statins against CoNS**

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>A</th>
<th>S</th>
<th>L₃</th>
<th>L₃+A</th>
<th>FICI</th>
<th>L₃+S</th>
<th>FICI</th>
<th>L₃+A</th>
<th>FICI</th>
<th>L₃+S</th>
<th>FICI</th>
<th>P</th>
<th>P+A</th>
<th>FICI</th>
<th>P+S</th>
<th>FICI</th>
<th>C</th>
<th>C+A</th>
<th>FICI</th>
<th>C+S</th>
<th>FICI</th>
<th>Pd</th>
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</tbody>
</table>

A = Atorvastatin; S = Simvastatin; L₃ = Ligand 1 N,N'-dimehtylbenzylamine (DMBA); FICI = Fractional Inhibitory Concentration Index; L₃ = Ligand 2 1-Phenyl-3-(4-bromophenyl)triazene N₂-hydroxide (HLBr); P = Precursor [Pd(DMBA)(Cl)]; C = Complex [1-(4-bromophenyl)-3-phenyltriazane N₂-oxide-xe<sup>2</sup> N<sup>1</sup>, O<sup>2</sup>][dimethylbenzylamine-xe² C<sup>5</sup>, N<sup>2</sup>][palladium(II)] (Pd(DMBA)Br); Pd = Palladium; Tig = Tigecycline; * = Test not realized. Isolates 1, 10 = *Staphylococcus hominis*; Isolates 2, 3, 4, 5, 7, 9 = *Staphylococcus epidermidis*; Isolate 6 = *Staphylococcus saprophyticus*; Isolate 8 = *Staphylococcus capitis*.

MIC=58.08±18.04 μg mL⁻¹, and *S. epidermidis* 12228 (MIC=26.04±9.02 μg mL⁻¹), respectively (Masadeh et al., 2012).

Graziano et al. has detected that simvastatin was the only statin with antibacterial activity against clinical isolates and ATCC strains of *S. aureus* susceptible (MSSA) and resistant to methicillin (MRSA), with *S. aureus* 29213 showing MIC=15.65 μg mL⁻¹ (Graziano et al., 2015). The difference of MIC between these studies can be attributed to the different design of the study performed (Ting, Whitaker, Albandar, 2016). We suppose that it could have been due to the different methodology used in the solubility of statins, since the active principles used in our study were diluted in ethanol, whereas other researchers used dimethyl sulfoxide (DMSO). Thus, we performed the MIC of these strains by using the same methodology used in other studies, and results remained the same as when principles were diluted in ethanol. However, it is known that according to the document M100-S22 from CLSI, DMSO can inactivate DNA of microorganisms when used in doses higher than 1%, interfering with the antibacterial activity (CLSI, 2012b).

All statins induce their antihyperlipidemic activity through the same mechanism of action in eukaryotic cells, completely inhibiting the Class 1 3-hydroxy-3-methylglutaryl-coenzyme to reductase (HMG-CoA), hindering the formation of mevalonate of HMG-CoA, and leading to a decrease in biosynthesis of cholesterol and an increase in the removal of circulation of low density lipoproteins (LDL) (Shitara, Sugiyama, 2006; Masadeh et al., 2012; Graziano et al., 2015). However, studies have shown that it is unlikely that the antibacterial activity shown by this class of drugs is related to this action mechanism, since mechanisms of antimicrobial effects of statins are yet to be elucidated (Masadeh et al., 2012; Ting, Whitaker, Albandar, 2016). A possible mechanism which can be related would be due to promoting apoptosis in microbial cells or the hydrofobic nature of statins, leading to the rupture of the bacterial membrane, resulting in cell death (Bergman et al., 2011; Tapia-Perez et al., 2011; Masadeh et al., 2012).

Other researchers have revealed that simvastatin inhibits the multiple biosynthetic pathways and the cellular processes in bacteria, including the selective interference of bacterial proteic synthesis, aiding the ability of this drug to suppress the production of some bacterial toxins such as α-hemolysin and Panton-Valentine leucocidin (Thangamani et al., 2015). Furthermore, Thangamani et al. (2015) have shown that simvastatin has an excellent activity against biofilm-forming bacteria in *Staphylococcus*.

In terms of the difference of MIC in statins, it can be due to the difference in their chemical structure as well as their production. Simvastatin is the semisynthetic form which derives from lovastatin, a product of *Penicillium citrinum*, with higher intrinsic antibacterial activity. Atorvastatin, on the other hand, is the pure synthetic form (Mason et al., 2005; Jerwood, Cohen, 2008; Ting,
Synergistic antibacterial effect of statins with the complex (Pd(DMBA)LBr)

Whitaker, Albandar, 2016). These two are lipophilic statins, thus simvastatin probably goes through the cell membrane more easily, causing the inhibition of bacteria, depending on the dose. Atorvastatin has not shown significant antimicrobial activity, although it is lipophilic, and it is justified because this statin is not derived from fungi (Mason et al., 2005; Graziano et al., 2015). However, more studies regarding statins structure are needed in order to elucidate their effect against bacteria (Graziano et al., 2015).

Regarding TZC, several studies using these compounds have shown that they have antimicrobial activity (Hörner et al., 2008; Domingues et al., 2010; Ombaka, Muguna, Gichumbi, 2012; Mohammadi, 2014; Paraginski et al., 2014; Tizotti et al., 2016), being proposed that their action mechanism occurs due to the chelating activity of metallic ions from the bacterial cell wall, inhibiting stages of bacterial synthesis, leading to cell death (Hörner et al., 2008; Ombaka, Muguna, Gichumbi, 2012; Yeo et al., 2013).

The highest antibacterial activity was detected in Gram-positive microorganisms, and could be justified by the difference in the cell wall structure of these bacteria, which is less complex and has a thick layer of peptidoglycan. Gram-positive bacteria need this layer for their protection and the maintenance of osmotic pressure, thus, antibacterial activity may be related to the inhibition of the synthesis of the peptidoglycan, leading to cell death (Yeo et al., 2013).

The DMBA ligands and the precursor [Pd(DMBA)(µ-Cl)]₂ showed MIC≥128 µg mL⁻¹. TZC complexed with palladium(II) showed better activity against the strains tested when compared to free ligands and precursor, proving to be an alternative for a new class of drugs with antibacterial activity. This activity can be justified since TZC complexed with transition metals such as Pd(II) can interact and cause damage to the DNA, providing a potent antibacterial activity, in addition to blocking cancer cells division leading to cell death (Hecht, 2000; Song et al., 2006; Paraginski et al., 2014). Therefore, complementary studies must be performed for the assessment of other biological parameters, their toxicity and therapeutic efficacy (Nunes et al., 2014).

When associating TZC with atorvastatin or simvastatin, simvastatin has shown synergy. It is estimated that a third of American adults over 45 years old make use of these drugs routinely (Wang et al., 2016). These drugs show a good safety profile with limited secondary effects, with low toxicity, allowing a frequent use in patients with high cholesterol levels (Thangamani et al., 2015; Ting, Whitaker, Albandar, 2016). Also, studies show that patients who make use of this class of drugs have a lower risk of acquiring bacterial infections, proving the correlation of the use of statins and a lower incidence of sepsis and mortality related to these infections (Almog et al., 2004; Ajrouche et al., 2013; López-Cortés et al., 2013).

Thus, according to MIC and FICI values obtained, we can infer that TZC complexed with palladium(II) significantly increases the antibacterial activity, and worked even better when associated with simvastatin. The documentation of this synergistic effect is of great impact for the treatment of MDR bacteria together with the low toxicity performance of simvastatin.

CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

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Synergistic antibacterial effect of statins with the complex (Pd(DMBA)LBr)


Yeo CY, Sim JH, Khoo CH, Goh ZJ, Ang KP, Cheah YK, et al. Pathogenic Gram-positive bacteria are highly sensitive to triphenylphosphane gold(O-alkylthiocarbamates), Ph3PAu[SC(OR)=N(p-tolyl)] (R = Me, Et and iPr). Gold Bull. 2013;46(3):145-52.

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