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Antibacterial, kinetics and bacteriolytic properties of silver(I) pyridinedicarboxylate compounds

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ABSTRACT

Antibacterial properties of silver(I)-pyridinedicarboxylate compounds (with Pyridine-2,3-dicarboxylic(Lutidinic acid), pyridine-2,4-dicarboxylic (Quinolinic acid) and pyridine-2,5-dicarboxylic (Isocinchomeronic acid)) were studied against *Escherichia coli*, *Listeria monocytogenes* (ISP-65-08), *Salmonella typhi* and *Staphylococcus aureus* (ATCC 25923) using kinetics of grown inhibition, viability assays, minimum inhibitory concentration and optical microscopy. The 3 silver compounds were tested toward UV-radiation in order to characterize their light insensitivity for potential medical devices: UV-radiation curable polymers. Photophysical measurements show remarkable differences toward UV-radiation, which were explained based on their polymeric structures with multiple nature bonds between pyridinedicarboxylic ligands and Ag(I) centers. We found a bacteriolytic effect and differences in the antibacterial efficiency depending on the structure of the complexes and the nature of Ag – X (X = oxygen and nitrogen) bonds: AgQuinol > AgLutidin > AgIsocinchom.

1. Introduction

In the last decades silver complexes have been extensively studied for their excellent antibacterial properties, which have been proven to be even more effective than silver salts. On the other hand this metal is active at low concentrations, has a low toxicity and has displayed anti-carcinogenic and antiviral activity [1–3]. Currently there is a revival of silver in the medical practice, principally in the addition of silver to medical instruments to avoid infections and due to the development of antibiotic resistance [4,5].

Despite numerous investigations related to the topic, the underlying mechanisms of the silver complexes and biological activity are not well understood [6–8]. Nonetheless, the type of atom coordinated to Ag(I) and the properties of the bond are both important factors for the effectiveness of the silver complexes. For example, MIC (minimum inhibitory concentration) experiments have shown broader antimicrobial activity spectra for silver complexes with Ag – O and Ag – N bonds than with Ag – P and Ag – S bonds [1,9].

Investigations on silver complexes to date have attributed their enhanced antimicrobial properties to distinctive weak Ag – O and Ag – N bonds in their structure. Moreover, several authors have suggested that the antibacterial properties of silver complexes are more associated to the Ag–ligand bond than to solubility, chirality, or the degree of polymerization of these complexes [10,11].

Although there is consensus that the structure–activity relationship is a key factor in the display of antimicrobial properties, investigations thus far have not been able to provide a general conclusion since the antibacterial activity of the complexes studied depends on the type of bacteria tested [2,12].

Using IR of carboxylic groups, Sawyer and McKinnie [13] reported interaction of several metal ions with EDTA as a ligand. The IR data on the carboxylic groups supported the conclusion that bonding Metal–OOC could be primarily ionic for some complexes and primarily covalent for others. Koczon et al. [14] were also able to relate pyridinecarboxylate chemical structure to antimicrobial activity. Their results demonstrated that although the ionic character of the interaction is important for the display of antibacterial activity, the degree of interaction is more important and determines the effectiveness of the complexes. Therefore spectroscopic studies on the carboxylic group structure have been shown to reflect the metal–oxygen character of the bond. Consequently, this technique has been proposed as a tool for the determination of biological properties in these compounds [15–17].

In order to elucidate the differences in the behavior of various types of antibacterial silver(I) complexes (Fig. 1), we studied the effect of the structure of three silver complexes on the kinetics of antimicrobial action and the efficiency of these compounds as antimicrobial agent.

Our results show differences in the bacteriolytic mechanism, stability in the presence of light and antibacterial efficiency depending on the structure of the complexes and their Ag–ligand bonds.

2. Materials and methods

2.1. Synthesis of complexes

Silver(I) complexes were synthesized following the same methods as previously reported [15,18].

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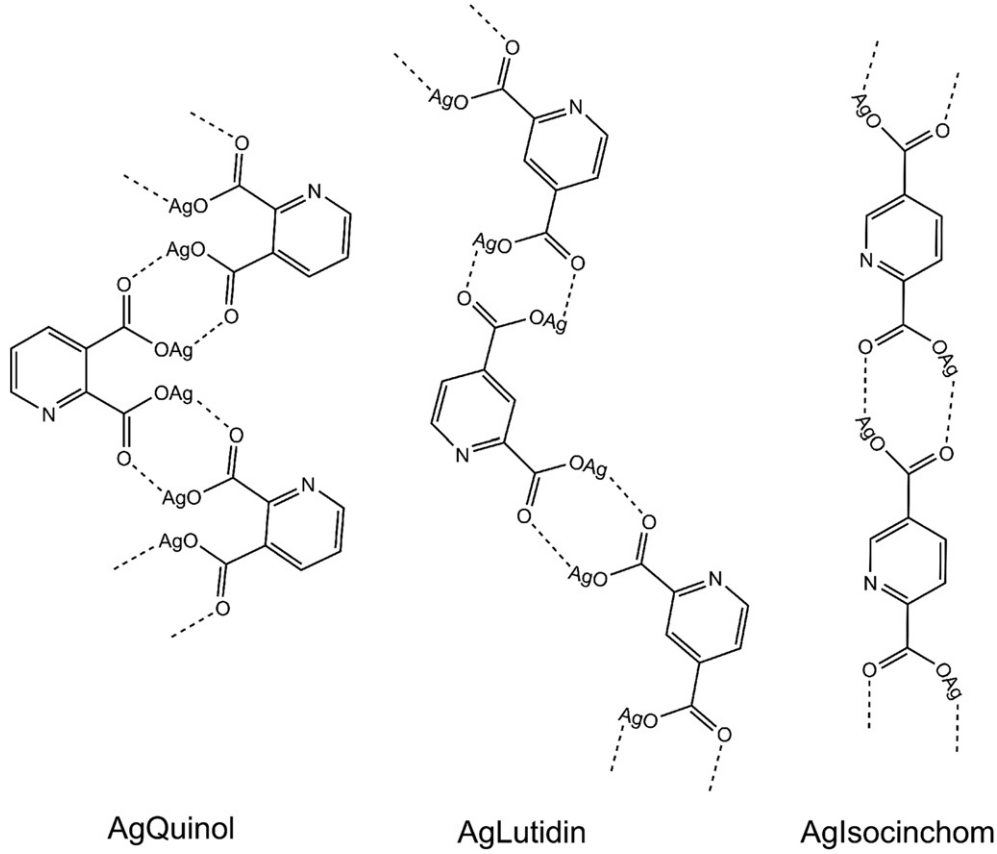

Fig. 1. Scheme of the silver(I) polymeric structures of the compounds. AgQuinol: pyridine-2,3-dicarboxylatosilver(I), AgLutidin: pyridine-2,4-dicarboxylatosilver(I), Ag-Isocinchom: pyridine-2,5-dicarboxylatosilver(I).

2.2. Photophysical measures

Light sensibility of Ag(I) compounds was studied at 293 K using a low-pressure Hg lamp: 258 nm, 30 W. The radiation source was positioned 10 cm from the sample. Fresh AgCl was used as a control substance. Digital pictures of irradiated samples were taken every 30 min using a digital camera (Canon 60D, 18 Mpx) and images were cropped to square shapes without editing.

2.3. Antimicrobial activity (MICs)

Antimicrobial activities of complexes were determined according to the recommendations of NCCLS (1999), National Committee for Clinical Laboratory Standards, by the use of a broth microdilution method. Minimum inhibitory concentrations (MICs) for the tested compound were prepared using four bacterial strains, *Escherichia coli*, *Listeria monocytogenes* (ISP-65-08), *Salmonella typhi* and *Staphylococcus aureus* (ATCC 25923). Silver complex stock solutions were diluted in broth solution to the final concentrations of 5, 10, 15, 20, 30 and 40 μg/mL. Free ligands were also tested from 20 to 200 μg/mL, although no antibacterial activity was observed, even with the higher concentrations. MIC determinations for each strain were performed after 24 h incubation at 37 °C of 10^5 CFU/mL in the respective complex concentration. The inocula were prepared from a 10^6 CFU/mL starting inoculum grown at 37 °C overnight.

2.4. Cell viability assays

Silver complexes were tested according to MIC experiment results (see Table 1). 10^5 CFU/mL of the respective bacteria was cultured in sterile MHB at 37 °C in the presence of silver compounds. Samples were taken every 0, 1, 2, 3, 4, 5, 6, 7 and 8 h and diluted to a concentration of 10^2 CFU/mL. 100 μL of these samples was plated on Mueller Hinton agar and incubated at 37 °C for 24 h. Colony counts were performed using a spiral platter. All experiments were performed in quintuplicate and values were obtained from the average of each experiment. Results were expressed in log [(% of viability) × 10^5], where % = ((N° CFUSample / N° CFUControl) × 100) and results plotted as a function of time in hours (h).

2.5. Kinetics of inhibition grown

Stock solutions (25, 50, 75, 100 and 125 μg/mL) of silver compounds were sterilized and then aseptically diluted in sterilized MH medium. 1 mL of (10^6 CFU/mL) of fresh cultures was transferred into 7 mL of MH medium containing silver compounds (2 mL) at different concentrations. Samples of 300 μL were transferred (in sextuple) in 96 well immunoplates (ELISA Plates) and incubated for 24 h at 37 °C under shaking condition. The growth inhibiting effect was measured using

Table 1
MIC[a] (μg/mL) data for the complex Ag-pyridinedicarboxylic.

N = 6 (μg/mL)	*E. coli*	*L. monocytogenes*	*S. typhi*	*S. aureus*
AgQuinol	7.5 (10: 4.6)[b]	7.5 (10: 4.6)	12.5 (15: 7.66)	12.5 (15: 7.66)
AgLutidin	7.5 (10: 5.6)	7.5 (10: 5.6)	12.5 (15: 9.33)	12.5 (15: 9.33)
AgIsocinchom	7.5 (10: 4.38)	7.5 (10: 4.38)	12.5 (15: 7.30)	12.5 (15: 7.30)

[a] Free ligand did not show antibacterial activity. MICs > 200 μg/mL.
[b] Values in parenthesis correspond to concentrations used in killing–time curves and Ag(I) of each complex in μg/mL, respectively.

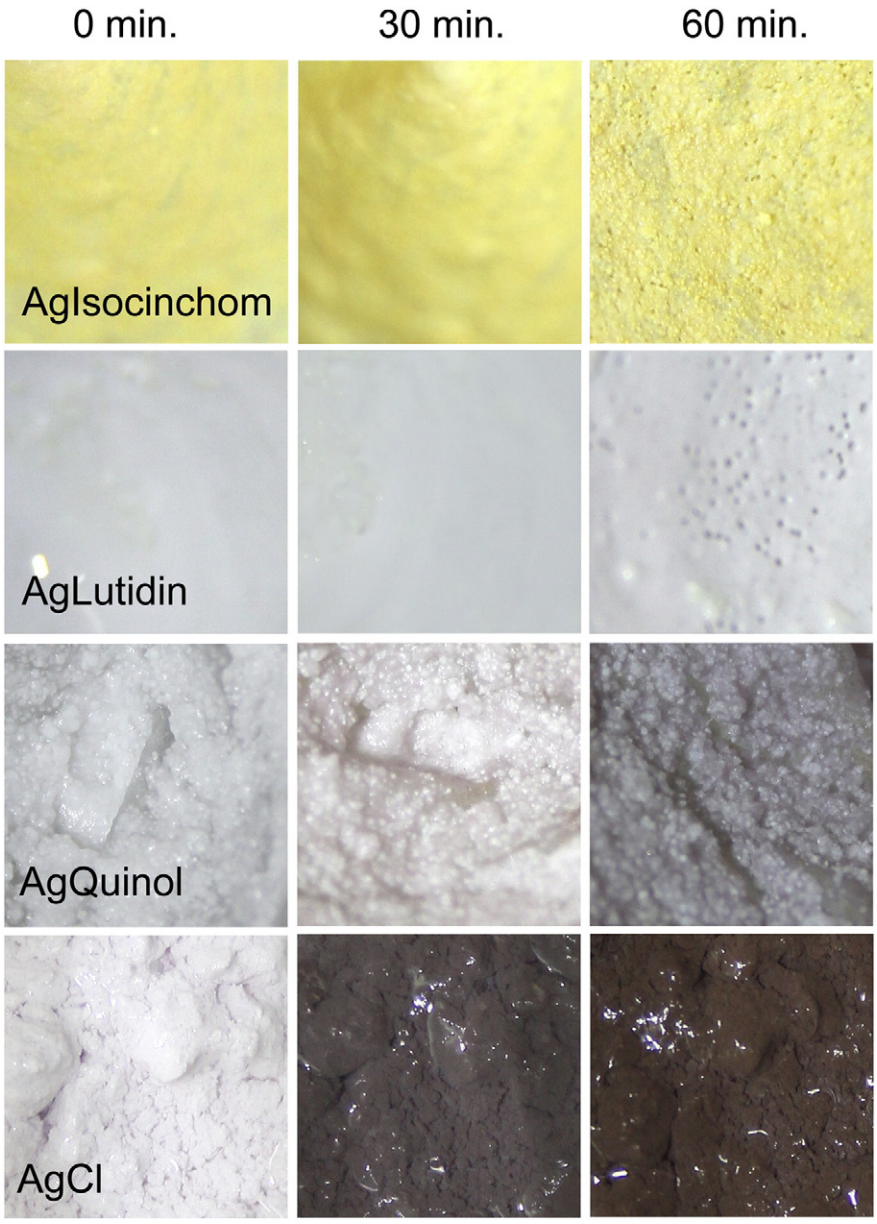

Fig. 2. Reduction of synthesized silver(I) compounds in the presence of high-energy UV radiation at 258 nm and 30 W.

optical density at 629 nm (Labsystem Multiskan, 352 and Ascent v. 2.6 Software) as a function of time for each bacterium under the presence of silver compounds (AgQuinol, AgLutidin and AgIsocinchom) and recorded every 1 h up to 24 h.

2.6. Bacteriolytic effects by optical microscopy

Bacteriolytic behavior was tested by optical microscopy and was acquired using a Leica DMLS-2 microscope coupled to a digital video camera (3.2 Mpx) with 100× magnification. The images were acquired after 24 h of incubation of the strains in the presence of silver compounds.

2.7. Statistical analysis

Viability was calculated as a percentage of solvent-treated control cells and expressed as a percent (%) of the control. The significance of any reduction in cellular viability was determined using one-way ANOVA (analysis of variance). A probability of 0.05 or less was deemed statistically significant.

3. Results and discussion

The complexes AgQuinol: pyridine-2,3-dicarboxylatosilver(I), AgLutidin: pyridine-2,4-dicarboxylatosilver(I) and Ag-Isocinchom: pyridine-2,5-dicarboxylatosilver(I) shown in Fig. 1 were available from a previous work and tested against *E. coli* and *Streptococcus agalactiae* (ISP 329-09) to ensure high antibacterial activity [15]. All studied silver complexes are sparingly soluble in water and are thermally stable up to 200 °C. The stability of Ag(I) compounds toward reduction to silver(0) by short wavelength UV-radiation is shown in Fig. 2. AgIsocinchom shows great stability for a prolonged period of time in comparison with AgQuinol and the control, ionic AgCl, which is completely darkened after 10 min and was used as a control photosensitive substance to compare the light stability of silver(I) complexes.

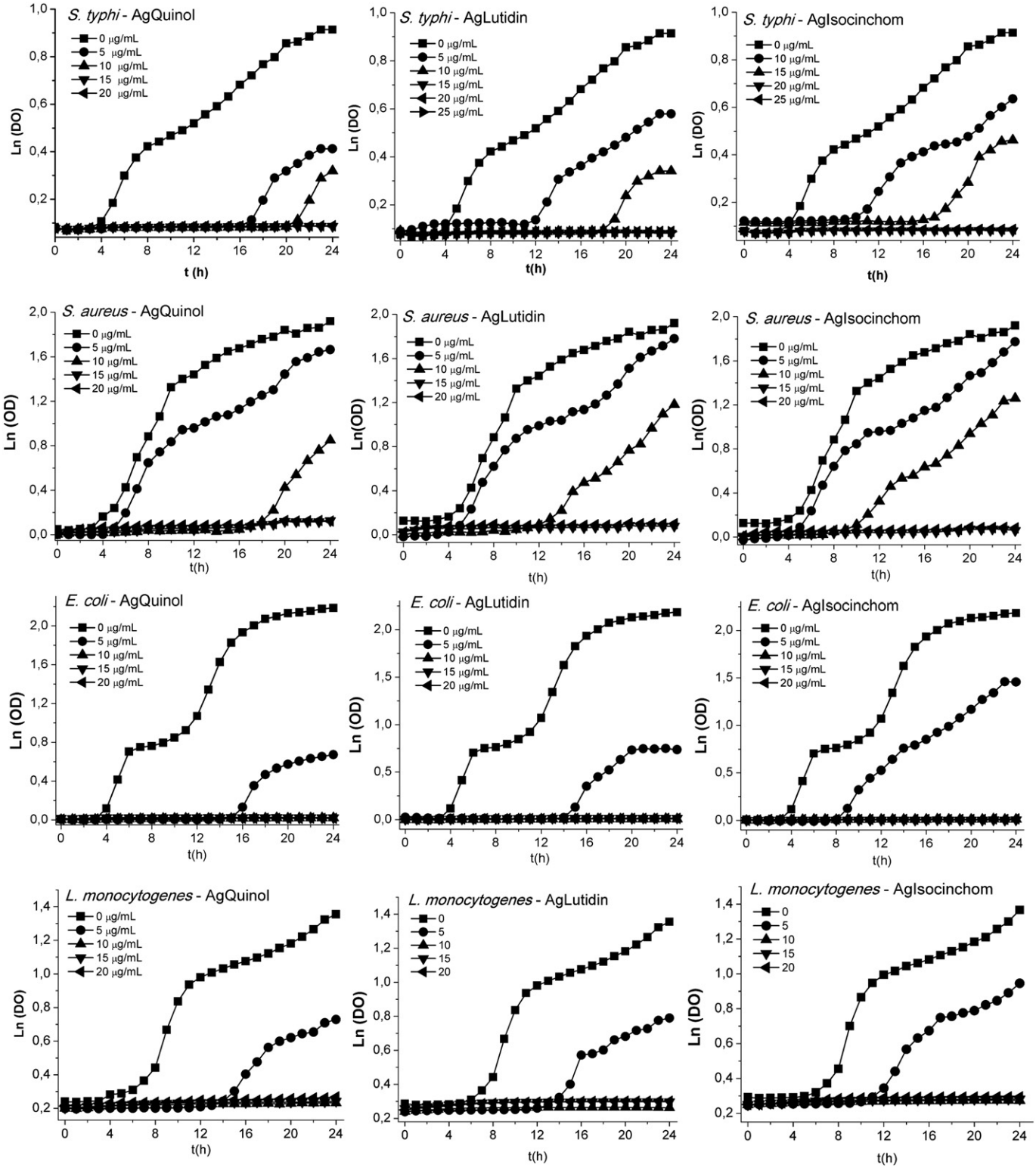

Fig. 3. Kinetics of inhibition grown at 24 h in MH broth at 37 °C.

The more light-insensitive compound, AgIsocinchom shows anisobidentate binding ($\Delta\nu$as: 226 cm^{-1}) in comparison with AgQuinol ($\Delta\nu$as: 214 cm^{-1}) because when the differences in frequency increase, the bonding between the metal and the carboxylate groups becomes more covalent [13–15].

The light-insensitive characteristics of silver(I) compounds are important for their potential applications in medical devices and also their thermal stability, antibacterial activity, poor solubility and the resistance toward intense UV radiation. These properties are important for potential candidates as additives to curable photopolymers in dental implants [19].

AgLutidin does not show differences after 1 h, but a color change very slight from white to purple light after 1 week in the presence of visible light. These differences and the origin of the resistance toward

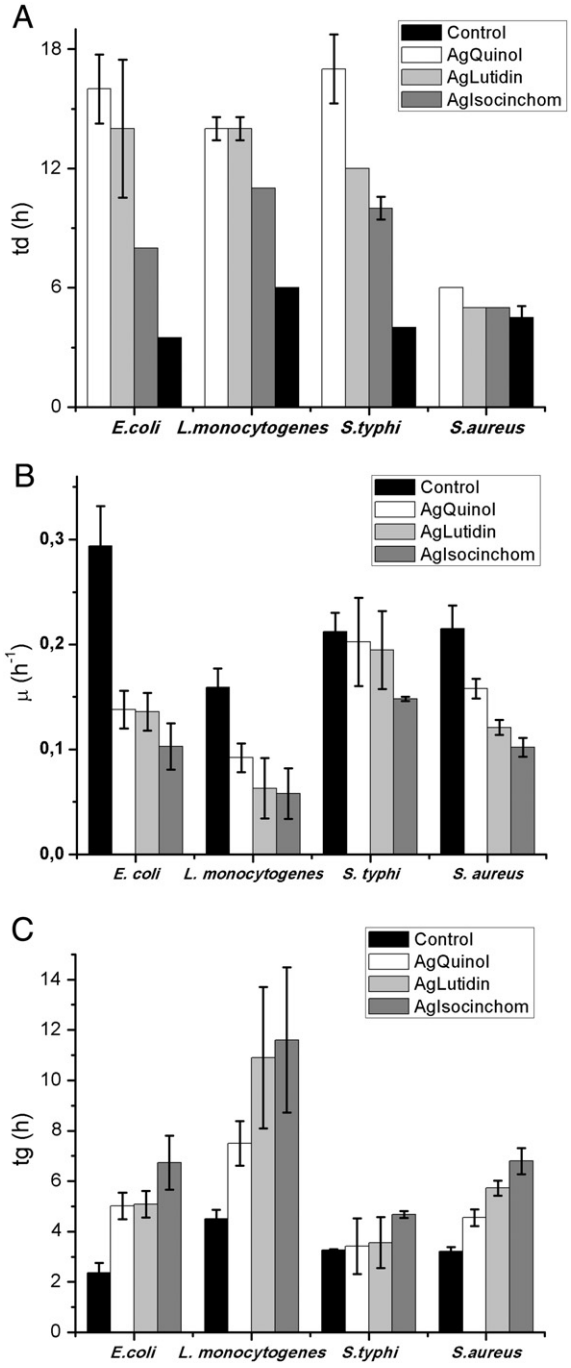

Fig. 4. (A). Detection time: Td with a dose of 5 μg/mL. (B). Maximum specific growth rate (μ) with a dose of 5 μg/mL. (C). Generation time (Tg).

visible and UV-light could be related to the different Ag – O bond energies established by FT-IR [15], short covalent bonds between silver(I) and nitrogen atoms of the ligand [15] and polymeric structure in every compound [19,20].

3.1. Antimicrobial activity (MICs)

The antibacterial activities of the silver(I) compounds over *E. coli*, *L. monocytogenes* (ISP-65-08), *S. typhi* and *S. aureus* (ATCC 25923) are presented in Table 1. The observed data show the activity of the compounds over the considered Gram-positive and Gram-negative bacterial strains. In the case of *E. coli*, this bacterium has been more sensible than others in the presence of silver complexes [21,22]. Free ligands (acids) do not show antibacterial activity even with 200 μg/mL, therefore antibacterial activity depends only on the silver ion.

In recent decades most of the works have been focused in showing the differences in the antibacterial properties of a wide variety of silver complexes. In all the cases biological activity was dependent on the microorganism tested and not on the nature of the metallic complexes [2,12], therefore it has not been able to establish the parameters to distinguish the effectiveness of a silver compound in comparison to other similar.

3.2. Kinetics of inhibition grown

Growth inhibition effects of silver complexes were tested in MHB by 24 h at 37 °C (Fig. 3). The increase of silver complex concentration resulted in an increase of lag phase (detection time: Td) and a total reduction of the bacterial population within 24 h according to the MIC results. In all the cases, even at lower concentration, the silver complexes were able to reduce the final population, but in the presence of *S. aureus* the effect was lower because after 24 h the bacterial population reaches a higher growth. This behavior may be showing greater resistance of *S. aureus* against silver complexes in concordance with MIC results.

For a better comparison between the silver compounds, Fig. 4 shows the differences in the detection times (Td or time in lag phase) for each compound with a dose of 5 μg/mL (the lowest dose used) in the presence of each bacterium. It is clear that the compounds have a general trend where Td follows a decreasing order: AgQuinol > AgLutidin > AgIsocinchom > Control.

The length of the *lag phase*(Td) depends on the intrinsic characteristics of the bacteria, the environmental conditions and the physiological history of the bacterium (Fig. 4A). Cells that are damaged may require more time to synthesize macromolecules and repair damage before they can divide [23]. This increased of Td values indicate that the compounds act at different times, being faster AgQuinol. In the case of AgIsocinchom, this compound could be reacting more slowly (releasing silver ions) and allowing bacteria to reach an exponential phase in less time.

The slope of the curve in the exponential phase represents the maximum specific growth rate (μ) as shown in Fig. 4B and it is considered an intrinsic characteristic of the organism dependent on the current environment. Silver compounds have an influence on the specific growth rate, showing a decreasing trend: Control > AgQuinol > AgLutidin > AgIsocinchom.

Specific growth rate constant is a way of measuring how fast the cells are dividing in the culture broth and it is defined on the basis of doubling rate. This growth parameter is an important way of expressing the relative success of a species to adapt to its natural environment or the experimental environment that was imposed [24]. Therefore silver compounds can modify growth parameters, independent of the type of bacteria.

A third parameter to consider is "Generation time" (Tg) or the time required for a bacterial population to double in cell number. This value varies among bacterial species and ranges from about 10 min to more than 24 h. In Fig. 4C, silver complexes also show an increased trend: Control < AgQuinol < AgLutidin < AgIsocinchom. In MH broth, tested bacteria show values between 2 and 5 h, but in the presence of the metal-compounds this parameter varies from 3 to 12 h. This is because bacterial growth is dependent upon number of factors, both nutritional, genetics and also dependent upon the initial time of cells [24].

In the case of *E. coli* and *L. monocytogenes*, the effects are more noticeable, even tripling the generation time of bacteria compared to the control. This behavior would indicate that "AgIsocinchom" acts more slowly over time, and therefore affects the rate of bacterial growth.

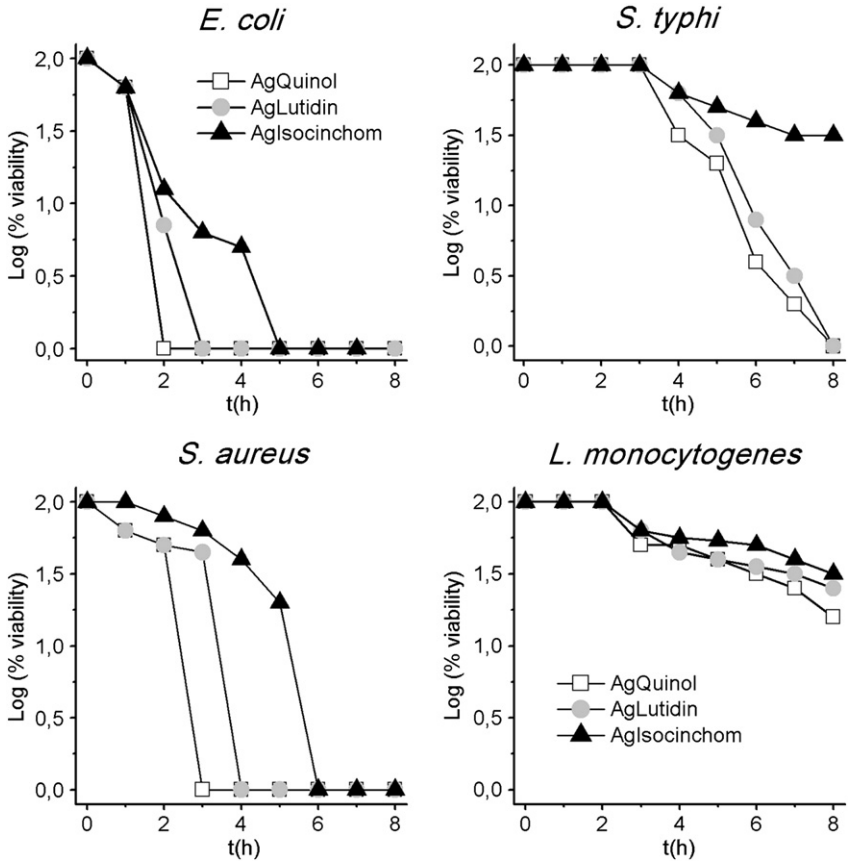

Fig. 5. Bactericidal activity (cell viability curves). A starting bacterial concentration of 1×10^5 CFU/mL was used.

These parameters (Td, μ and Tg), show for the first time the kinetic effects of the silver complex depending on their structure and associated with the nature of the metal–ligand bond.

3.3. Cell viability assays

Viability tests were conducted to correlate the antibacterial behavior and its action over time. The results of silver complexes reveal different antibacterial efficiencies as shown in Fig. 5. *E. coli* and *S. aureus* show a remarkably rapid response in the presence of AgQuinol: 3 h before AgIsocinchom and 1 h before AgLutidin demonstrating more efficiency than AgLutidin and AgIsocinchom. As previously reported, AgQuinol has primary ionic bond with $\Delta\nu$as value of 214 cm^{-1} [15] and the Ag–O bond has been reported to display distances of: 2.705 Å and 2.706 Å, which are significantly larger than those found in other similar chelates [18], would therefore more easily release silver ions, and this could explain the most efficiency in time.

In this context, it is reasonable to assume that exchange between the Ag–O and Ag–X states, where X represents a biological fragment, would be easier considering the weak Ag–O bond nature, or the stronger ionic character of the interaction.

The compounds of Ag(I) tested against *S. typhi* and *L. monocytogenes* show a similar but less evident trend in their antimicrobial efficiency. However, AgIsocinchom is always slower than the other compounds and AgQuinol could be considered more efficient in time. Even a gradual decrease of viability was observed in both of the cases, but it is evident that the efficiency could be considered in this order: AgQuinol >AgLutidin > AgIsocinchom.

This trend in cell viability curves could be directly related to the early work of Fox and Modak [25]; they suggested that the efficiency of silver sulfadiazine in vivo is the result of the sustained and slow release of silver ions into the wound environs.

3.4. Bacteriolytic effects by optical microscopy

To understand the mechanism of its antimicrobial effects, silver complex was studied by optical microscopy in the presence of the treated bacteria and compared with untreated cells (Fig. 6).

The complexes treated in the presence of bacteria demonstrated disruption of the bacterial cell morphology, therefore were observed a bacteriolytic mechanism for Gram-positive and Gram-negative strains. This behavior has been reported after treatment of *E. coli* with silver nanoparticles [26,27].

None of the untreated cells exhibited these changes of morphology and AgQuinol and AgLutidin show a total destruction of the cells after 24 h, but following the tendency in the kinetic experiments, AgIsocinchom showed in all the cases some bacteria without disruption of the cell wall, as expected for a covalent Ag–O bond and a slower action. With this background, we can assume the kinetic effects of the silver complexes and their impact on the antibacterial properties.

In summary, the effect of carbonyl group and the nature of the interaction Ag–O bonding, have been proven useful to understand differences in light stability and antibacterial effectiveness of the silver complexes. Our work supports the possibility of controlling the delivery of silver ions into varied environments, great stability to visible light, UV radiation and high capacity antibacterial against Gram-positive and Gram-negative strains. Thus, silver(I)-pyridinedicarboxylate and/or silver(I)-R-carboxylates could play an important role in the design of new antimicrobial agents with different degrees of efficiency. Finally,

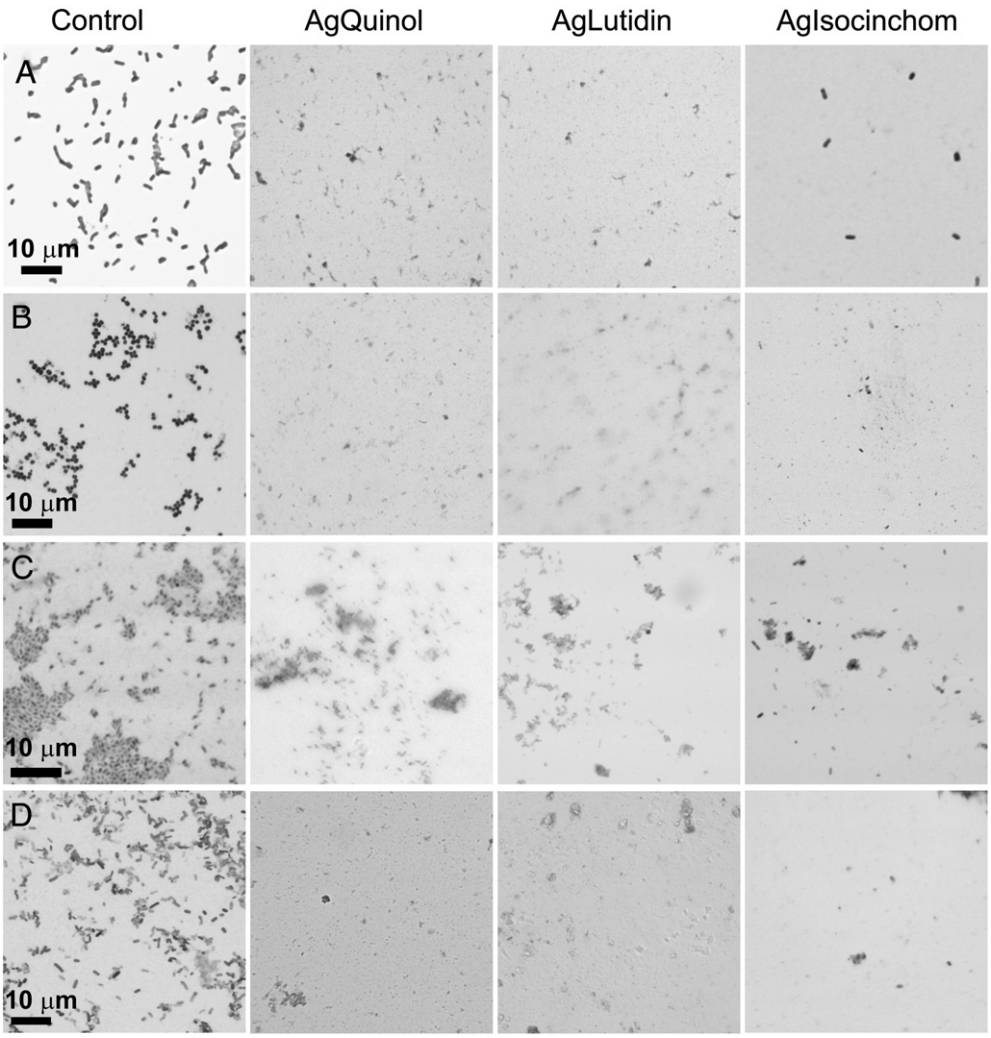

Fig. 6. (A). *E. coli*, (B). *S. aureus*, (C). *S. typhi* and (D). *L. monocytogenes* after 24 h at 37 °C in the presence of the complex Ag-pyridinedicarboxylic.

the activity–structure relationship investigated here could set the ground for the prediction and assessment of light stability, microbiological properties and efficiency of new silver compounds through the modulation of the interaction of Ag–ligands.

Acknowledgments

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