

Antibacterial activity and cytotoxic effect of SIAB-GV3

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SUMMARY

The aim of this study was to evaluate the antibacterial activity and cytotoxic effect of the compound SIAB-GV3, a new formulation presenting as an aqueous suspension of silicon dioxide (SiO₂) functionalized with silver (Ag⁺) nanoparticles and benzalkonium. The product is formulated as an adjuvant for the treatment of inflammatory diseases of the oral cavity. This study demonstrates that SIAB-GV3 possesses strong antimicrobial activity against most of the common oral pathogens, in particular against *Streptococcus pyogenes*, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*. The cytotoxic effect against human embryo lung fibroblasts (HELF cells) was evaluated at different times, from 1 h to 168 h, using concentrations of SIAB-GV3 ranging from 50 mg/ml to 0.0008 mg/ml. At the concentration of 10 mg/ml, commonly used in clinical practice, the compound results cytotoxic after about 2 hours, this time being much longer than the typical time of local application, which is no more than 10 minutes. In conclusion, our findings suggest that SIAB-GV3 has a good antibacterial activity against the most common oral pathogens even at very low concentrations and a low cytotoxic activity, thanks to the synergistic effects of Ag nanoparticles, silicon dioxide and benzalkonium.

KEY WORDS: Silver nanoparticles, SIAB-GV3, Antibacterial activity, Cytotoxic effect.

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INTRODUCTION

The widescale use of antibiotics in the prevention and treatment of bacterial infections has led to the emergence and spread of resistant microorganisms requiring the constant discovery and development of new active molecules against bacteria. The topical use of antimicrobial substances not belonging to the group of traditional antibiotics can help overcome this emerging problem (Weber *et al.*, 2013).

Nanotechnology is an emerging branch of science that deals with designing tools and devices of size 1-100 nm with unique functions at the cellular, atomic and molecular levels (Rawat *et*

al., 2006). Nanoparticles <100 nm have a variety of optical, electronic and structural properties not possessed by macromolecules (Sinha *et al.*, 2006). Nanoparticles are smaller than human cells but similar in size to biological macromolecules like enzymes and receptors (Rawat *et al.*, 2006). The development of novel nanotherapy strategies represents a great opportunity to enhance currently available medical treatments, improving standard care and prognosis for challenging healthcare issues, such as impaired wound healing (Sandhiya *et al.*, 2009; Tocco *et al.*, 2012).

Recent literature reports encouraging results on the bactericidal activity of silver nanoparticles both of simple and composite nature (Ip *et al.*, 2006; Rai *et al.*, 2009).

Nanoparticles coated with silver represent an important tool in this field, due to their strong bactericidal activity. The antimicrobial activity of nanoparticles is known to be a function of the

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surface area in contact with microorganisms. Due to their small size, nanoparticles present a relatively large surface area, a property that enhances their interaction with microbes, allowing a broad range of possible antimicrobial activities. For example the double high affinity of silver ions to sulfur and phosphorus is the key element of his antimicrobial effect. The bacterial membrane is rich in sulfur proteins that can be bound to Ag⁺ ions, determining an increased membrane permeability (Morones *et al.*, 2005). The Ag⁺ ions released from the nanoparticles can also interact with the phosphate groups present in the DNA, blocking its replication while at the same time inhibiting the function of many enzymes through interaction with the sulfur proteins. Moreover at micromolecular levels Ag⁺ ions have been reported to uncouple respiratory electron transport from oxidative phosphorylation, inhibit respiratory chain enzymes, and interfere with membrane permeability to protons and phosphate (Bard and Holt, 2005).

Metal nanoparticles with antimicrobial activity when embedded and coated onto surfaces can be used for various applications in water treatment, synthetic textiles, biomedical and surgical devices, food processing and packaging (Gutierrez *et al.*, 2010; Jafry *et al.*, 2011). Moreover, the composites prepared using metal nanoparticles and polymers can be more effective due to their enhanced antimicrobial activity.

The goal of this study is to evaluate the antibacterial activity of a nanomolecular silicon dioxide (SiO₂) named SIAB-GV3, synthesized with a nanotechnological method. SIAB-GV3 consists of stable silica dioxide nanoparticles functionalized with ionic Ag and linked to benzalkonium molecules that exhibit high bactericidal and fungicidal action.

MATERIALS AND METHODS

SIAB-GV3 description

SIAB-GV3 is a compound developed by NM Tech S.R.L. (Italy), corresponding to the following composition: SIAB 1% w/V; gelling agent 1.8% w/V; sweetener aroma 19.4% v/V, water to 100 ml. The substance is a new formulation consisting of an aqueous suspension of SiO₂

nanoparticles functionalized with silver. The silver ions are covalently bound to the silica dioxide surface through a bridging ligand. The antimicrobial action is further amplified by the presence of benzalkonium.

Microrganisms tested

We tested the activity of SIAB-GV3 on 14 different strains of microorganisms: methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA), *Enterococcus* spp., *Streptococcus pyogenes* 1 and *S. pyogenes* 2, *Streptococcus salivarius*, *Streptococcus mitis*, *Streptococcus mutans*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Candida albicans* 1 and *C. albicans* 2. These bacterial and fungal clinical isolates are considered the most commonly representative of the microbial flora responsible for inflammatory diseases of the oral cavity (Avila *et al.*, 2009). All these recent clinical isolates were identified by conventional laboratory procedures and speciation was performed by standard biochemical tests.

Determination of antibacterial activity

Determination of the minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of SIAB-GV3 was performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (Wayne, 2006). Briefly, aliquots of 5×10⁵ colony-forming units (CFU) were prepared in Mueller-Hinton broth (MH, Biomérieux, Italy) for each of the tested microorganisms, with the exceptions of *C. albicans*, which was cultured in Sabouraud broth (Biomérieux, Italy) and *P. gingivalis* and *A. actinomycetemcomitans*, which were cultured in Schaedler broth (Biomérieux, Italy). Eleven two-fold serial dilutions of SIAB-GV3 prepared in sterile water, were added, ranging a concentration from 1 mg/ml to 0.0007 mg/ml, to a final volume of 2 ml. The incubation was performed in aerobic condition, with the exceptions of *P. gingivalis* and *A. actinomycetemcomitans*, where it was performed in anaerobic condition. Positive (no SIAB-GV3) and negative (no inoculum) growth controls were included. The lowest SIAB-GV3 concentration inhibiting visible bacterial growth after 18 h of incubation at 37°C was considered the MIC.

Inocula not showing any visible bacterial growth in the MIC determination experiment described above were subcultured onto MH agar plates (Biomérieux, Italy) and incubated for 18 h at 37°C. For *P. gingivalis* and *A. actinimycetemcomitans* the test was carried out in Schaedler agar plates (Biomérieux, Italy) and the incubation was performed in anaerobic condition. The lowest SIAB-GV3 concentration showing 99.99% killing of bacterial cells was considered the MBC.

Evaluation of cytotoxicity of SIAB-GV3 against human cells

The cytotoxicity of SIAB-GV3 was evaluated against cultures of HELF cells (human embryo lung fibroblasts) in 24-well microplates.

Twenty one concentrations of SIAB-GV3, ranging from 50 mg/ml to 0.0008 mg/ml, were tested, starting from the following composition: 1 ml of SIAB-GV3 diluted in 4 ml of Earle broth 2% FBS (Fetal Bovine Serum) (Life Technologies, CA, USA) with 1% PSG (penicillin-streptomycin glutamine 100X solution (EuroClone, Pero, Milan Italy). The cells were incubated at 37°C in 5% CO₂. When confluence was reached, cells were inoculated with 1 ml of the different dilutions of the product SIAB-GV3.

The cytotoxicity effect, characterized by cell rounding and detachment, was evaluated under an inverted microscope 10x magnification at 1, 2, 3, 4, 24, 48, 72, 96, 120, 144 and 168 hours.

RESULTS

Antimicrobial activity of SIAB-GV3

The results of the antimicrobial activity of SIAB-GV3 against the strains tested in this study are shown in Table 1.

The SIAB-GV3 compound exhibits high antibacterial activity against ten of the eleven organisms. Among the clinical pathogens, *S. pyogenes* was the most susceptible followed by *P. gingivalis*, *S. mitis*, MRSA, *S. salivarius*, *S. mutans*, *A. actinimycetemcomitans*, MSSA, *Enterococcus* spp., *E. coli*, *C. albicans* 1, *C. albicans* 2 and *P. aeruginosa*. The MIC and MBC values, ranging from <0.0007 mg/ml to 0.25 mg/ml concentrations, show that the preparation has a strong inhibitory activity on bacte-

TABLE 1 - Antimicrobial activity of SIAB-GV3.

Microorganisms	MIC Concentration of SIAB (mg/ml)	MBC Concentration of SIAB (mg/ml)
<i>Staphylococcus aureus</i> MR	0.015	0.007
<i>Staphylococcus aureus</i> MS	0.03	0.015
<i>Enterococcus</i> spp.	0.03	0.015
<i>Streptococcus pyogenes</i> 1	≤0.0007	≤0.0007
<i>Streptococcus pyogenes</i> 2	≤0.0007	≤0.0007
<i>Streptococcus salivarius</i>	0.015	0.015
<i>Streptococcus mitis</i>	0.003	0.003
<i>Streptococcus mutans</i>	0.015	0.015
<i>Pseudomonas aeruginosa</i>	0.25	0.25
<i>Escherichia coli</i>	0.06	0.06
<i>Porphyromonas gingivalis</i>	0.0015	0.0015
<i>Aggregatibacter actinimycetemcomitans</i>	0.015	0.015
<i>Candida albicans</i> 1	0.06	0.06
<i>Candida albicans</i> 2	0.06	0.06

rial and fungal growth. The SIAB-GV3 showed highest MIC and MBC against *P. aeruginosa* (0.25 mg/ml), followed by *E. coli*, *C. albicans* 1, *C. albicans* 2 (0.06 mg/ml). The lowest values of MIC and MBC was recorded against *S. pyogenes* 1 and *S. pyogenes* 2 (<0.0007 mg/ml). The MIC values were 0.0015 mg/ml against *P. gingivalis*, 0.003 mg/ml against *S. mitis*, 0.015 mg/ml against MRSA, *S. salivarius*, *S. mutans* and *A. Actinimycetemcomitans*, and 0.03 mg/ml against MSSA and *Enterococcus* spp. The MBC have the same MIC value except for MRSA, MSSA and *Enterococcus* spp. that showed a twofold variation (one dilution), the MBC values being 0.007, 0.015 and 0.015 respectively.

Cytotoxic effect of SIAB-GV3

We evaluated the effect of SIAB-GV3 on the growth of human HELF line cells and considered the lowest concentration of SIAB-GV3

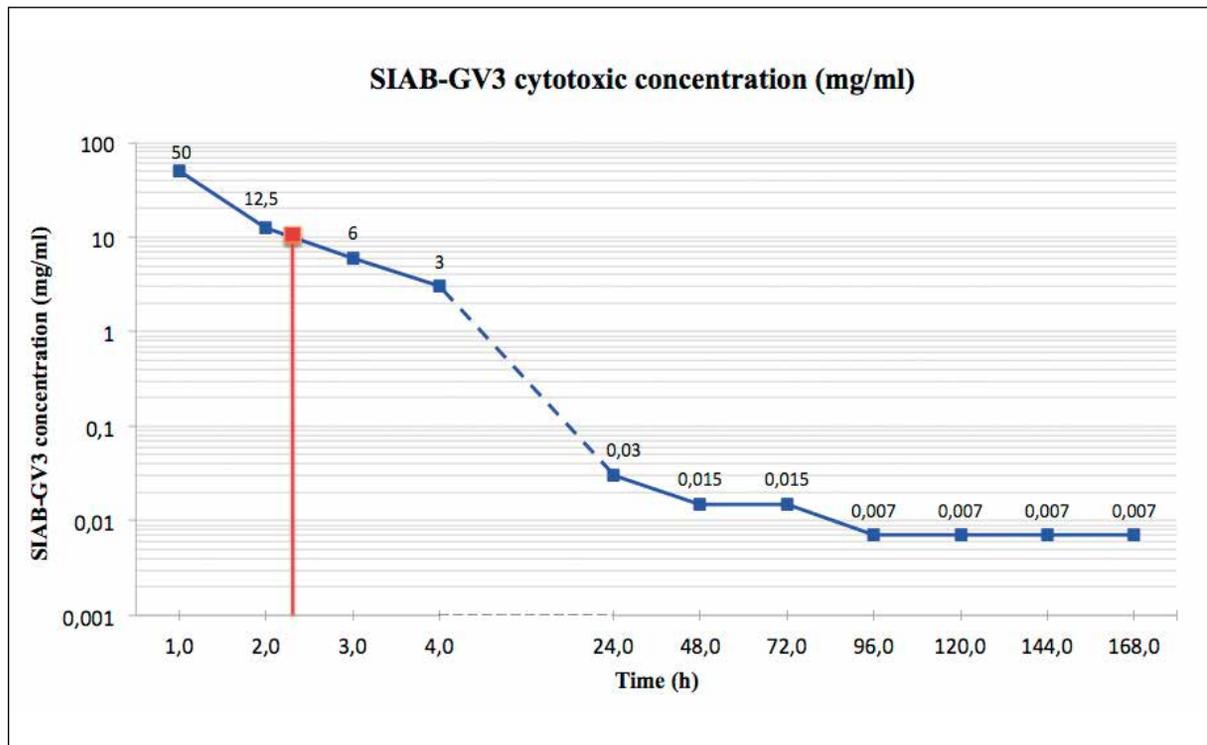


FIGURE 1 - Cytotoxic effect of SIAB-GV3. The graph shows the concentrations and times at which the product SIAB-GV3 proves lethal. The red square represents the concentration of SIAB-GV3 10 mg/ml, this is the concentration that is normally used as a dressing. The contact time at which the 10 mg/ml concentration becomes cytotoxic is about 2 and a quarter of hours, much longer than the exposure in clinical use.

with a cytotoxic activity that determined the complete destruction of the monolayer.

As shown in Figure 1, the cytotoxic activity of the product increases with the increase of its concentration and time of contact with the cells. The cytotoxic effect at the highest tested concentration (50 mg/ml) appears after an exposure time of one hour. At 2, 3 and 4 hours the lowest cytotoxic concentration is 12.5 mg/ml, 6 mg/ml and 3 mg/ml respectively. At 24 hours the cytotoxic effect is already apparent at a concentration of 0.3 mg/ml while at 48 and 72 hours it is 0.015 mg/ml. Starting from 96 hours up to 168 hours the lethal concentration settles at 0.007 mg/ml.

DISCUSSION

Infections of the oral cavity are important in human pathology as they may constitute foci for the remote transmission of severe infec-

tions such as endocarditis and glomerulonephritis. Periodontal infection constitutes a source of documented cardiovascular risk that must be prevented by effective means and control of foci, mainly with antibiotic treatments (Offenbacher *et al.*, 2005; Mäntylä *et al.*, 2013). In the presence of inflammation the microorganisms involved in periodontal diseases can invade host tissues and spread through the bloodstream with a pathogenic effect on various organs, also due to the production of toxins (Offenbacher and Beck, 2005). Haraszthy *et al.* (2000), using PCR, demonstrated periodontal pathogens in atherosclerotic plaques, and suggested an important role of the microorganisms in the subsequent atherosclerotic progression. As stated above, antibiotics are one of the main lines of defence against infections of the oral cavity, however antibacterial treatments can likely lead to the insurgence of resistance phenomena. Designing and testing alternative approaches, such as the use of

nanomolecular-based compounds, is thus of pivotal importance. Metallic nanoparticles, for example, are being studied extensively due to their proven antimicrobial activity.

The preparation we tested, named SIAB-GV3, constitutes a valid tool capable of inhibiting bacterial and fungal infections of the oral cavity. The compound has the properties to act at very low concentrations thanks to the combination of three agents each presenting antimicrobial activity. These agents are: silicon dioxide (SiO_2), silver ions (Ag^+) and benzalkonium. It was recently demonstrated that silver nanoparticles make pores on the bacterial cell walls. The cytoplasmic content is released in the medium, leading to cell death (Sondi and Salopek-Sondi, 2004) while having no negative effect on human cells. The activity of silver nanoparticles has already been assessed against the most common human pathogens. In particular, a strong action against Gram-positive bacteria, such as MRSA, MSSA and *S. pyogenes* has been reported, while only a moderate bactericidal effect was assessed against Gram-negative pathogens such as *Salmonella typhi* and *Klebsiella pneumoniae* (Nanda and Saravanan 2009; Lara *et al.*, 2010; Shahverdi *et al.*, 2007; Shrivastava *et al.*, 2007). It is important to note that there are no reports of induced resistance to nanoparticles, in contrast to antibiotics.

SIAB-GV3 exploits the synergistic effect of the combination of cationic species and silica dioxide surfaces, to improve the bactericidal effect of the product. The silver ions are covalently bound to the silica dioxide surface through a bridging ligand, providing an unprecedented photochemical and thermal stability to the complex. Silver ions are thus able to interact with functional groups of the microbial membrane, such as $-\text{COOH}$, $-\text{NH}_2$, $-\text{SH}$. The antimicrobial action is further amplified by benzalkonium which has been shown to decrease intermolecular interactions causing the dissociation of the cellular membrane lipid bilayers (McDonnel and Russell, 1999). The synergistic action of nanoparticles and benzalkonium cations has also been demonstrated to improve the antibacterial activity, even at low concentrations (Aymonier *et al.*, 2002).

The product SIAB-GV3 is formulated as an adjuvant for the treatment of inflammatory dis-

eases of the oral cavity such as stomatitis, gingivitis, periodontitis, mouth ulcers and small lesions from dental equipment. We tested the action of SIAB-GV3 against a panel of bacteria chosen among the most common species responsible for a high percentage of infections in the oral cavity and isolates part of the oral resident flora.

A previous study tested the activity, against a panel of bacteria, of silver cations alone, without the synergistic effect of benzalkonium and silica dioxide surfaces (Lara *et al.*, 2010). The obtained results showed higher bactericidal activity against a Gram-positive (*S. pyogenes*) than against Gram-negative bacteria (*P. aeruginosa* and *E. coli*). We included in our panel the microorganisms tested previously, but we also added bacteria normally present in the mouth, including the Gram-negative *P. gingivalis* and *A. Actinimycetemcomitans*. Our results show that the activity of the SIAB-GV3 is low against *P. aeruginosa* and *E. coli*, in accordance with previously published results on the activity of silver cations alone, but it is high against the Gram-negative oral pathogens *P. gingivalis* and *A. Actinimycetemcomitans* (Table 1). The synergistic activity of the SIAB-GV3 compound could also be partially responsible for this higher efficacy. When considering Gram-positive species, the effect of SIAB-GV3 is always strong: staphylococci and enterococci exhibit good sensitivity, and even higher sensitivity is seen in streptococci, in particular for *S. pyogenes*, the most sensitive of the tested organisms.

We also tested the sensitivity of *Candida albicans* to the SIAB-GV3 compound, obtaining a MIC value of 0,06 mg/ml of active ingredient (Table 1). The mechanism of action of nano-Ag on fungi is the targeting of the yeast cell membranes and the disruption of membrane potential. The formation of pores subsequently leads to cell death (Gajbhiye *et al.*, 2009). Although there are limited studies on the effect of silver nanoparticles on yeasts, the value we obtain, which indicates high efficacy of the compound, is in line with literature data (Kim *et al.*, 2008). It is well known that the size of the Ag nanoparticles plays a central role in antimicrobial activity (Morones *et al.*, 2005). Various studies evaluated the effect of Ag nanoparticles of dif-

ferent sizes, ranging from 25-50 nm (Panacek *et al.*, 2006) to 300 nm (Hyuk *et al.*, 2009; Ai *et al.*, 2011). The consensus result is that smaller particles exhibit higher antimicrobial activity, probably due to higher penetration in the bacterial cells.

The antibacterial properties are in fact related to the total surface area of the nanoparticles, smaller particles with larger surface to volume ratios have greater antibacterial activity (Baker *et al.*, 2005). We tested the cytotoxic activity of the SIAB-GV3 compound against HELF cells originated from the lung, as it is reported in the literature that nanoparticles can show cytotoxic effect in particular in lung and intestine cells (Ai *et al.*, 2011). Silver nanoparticles are particularly cytotoxic because they produce greater quantities of reactive oxygen species (ROS) than nanoparticles with different composition (Fubini, 1997). Furthermore smaller nanoparticles have clearly been shown to present higher cytotoxic activity (Ai *et al.*, 2011). Our results show that the compound presents cytotoxic effects only if used at high concentrations (50 mg/ml) or if the cells are exposed for extended periods of time (more than two hours). The SIAB-GV3 concentration used for dental treatment is 10 mg/ml. As seen in Figure 1 this concentration is cytotoxic after exposure of approximately 2.5 h, this time being much higher than that used in dental treatment.

To summarize, Ag nanoparticles have strong antimicrobial activity, coupled however with potential cytotoxic effects. Smaller particles have stronger antimicrobial activity but present stronger cytotoxic effects. In order to maximize the antimicrobial activity without high cytotoxicity, we coupled Ag nanoparticles with benzalkonium and silica dioxide membranes. Thanks to the synergistic effect of the three components of our compound, it is possible to obtain high antimicrobial activity with larger nanoparticles, thus reducing cellular penetration and the subsequent cytotoxic effects.

The results of this study demonstrate that very low concentrations of SIAB-GV3 have a strong antibacterial activity against the various representatives of the oral microbial flora, both Gram positive and negative microorganisms as well as fungi, placing the compound among

those of extreme interest in the treatment of inflammatory diseases of the oral cavity caused by bacteria and fungi.

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