Research letter

Antimicrobial activity of branched oligo(hexamethyleneguanidine) hydrochloride on oral pathogens

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Abstract: The present work is devoted to study of the antimicrobial activity of a new promising synthetic biocidal compound, branched oligohexamethylene guanidine hydrochloride. The studies were carried out using optional anaerobic bacteria Staphylococcus aureus and Klebsiella pneumoniae, as well as obligate anaerobic bacteria Actinomyces pyogenes, Actinomyces odontolyticus, Peptostreptococcus micros, Finegoldia magna, Veillonella parvula, Prevotella disiens and Fusobacterium nucleatum. It was shown that branched oligohexamethylene guanidine hydrochloride inhibits the growth of these bacteria, and facultative anaerobic bacteria are more sensitive to it. The assumptions about the mechanism of this activity of branched oligohexamethylene guanidine hydrochloride are made. The results of the study show that the substance can be recommended as a broad-spectrum biocide.

Keywords: branched oligo(hexamethyleneguanidine) hydrochloride, antimicrobial activity, oral pathogens.


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Introduction

Dental diseases are among the most prevalent and costly diseases affecting industrialized societies, and yet are highly preventable. For example, in the UK, the National Health Service spends over £1.6 billion per annum on dental treatment, and this figure increases to £2.6 billion if the burgeoning private sector costs are included [1, 2].

Oral pathogens are microorganisms on the oral surface that play an important part in the development of caries and periodontal diseases [3, 4]. It is now clear that microorganisms play an essential role in the pathogenesis of dental caries and consequently provides a prime target for the prevention of this disease by antibiotics and vaccine [5, 6].

Most active pharmaceutical ingredients (API) have highly targeted specific mechanisms of action and interfere with one particular cellular function such as cell wall synthesis, protein or RNA synthesis, DNA replication or energy metabolism [7-10]. Drugs are widely used in humans and animals, resulting in the increasing emergence of resistant bacterial strains. Bacteria can be intrinsically resistant to certain substances or can overcome susceptibility by genetic adaptation. Hence the development of new generation antibiotics is a high unmet medical need [7], especially in dental care.

Modern antiseptics containing chlorhexidine that are commonly used in dentistry are known to stain teeth, composite restorations and dorsum of the tongue. They often cause taste sensitivity distortion. If used longer than 14 days, they might lead to disbiotic changes. Hence new active materials are needed. New promising candidates under development as alternatives for existing API are the synthetic guanidine-based polymers, with a new physicochemical mechanism of action and favorable toxicological properties.

One of the emerging classes of guanidine-based polymers belongs to the branched oligomers of hexamethylenediamine and guanidine which have been in use mainly as antiseptics, surface disinfectants and topical antimicrobials for over 80 years within clinical, industrial and domestic hygiene delivery [11]. Branched oligo(hexamethyleneguanidine) (OHMG) hydrochloride (Figure 1) can be used in medical practice for treating viral and infectious dental diseases because of their highly effective action and low toxicity [11-13].

The aims of the present investigation were to assess the antimicrobial activities of the branched OHMG hydrochloride on main oral pathogens.

Material and Methods

Microorganisms

Bacterial reference strains used in this research are Staphylococcus aureus ATCC 29213, Klebsiella pneumoniae ATCC 13883, Actinomyces pyogenes ATCC 49698, Actinomyces odontolyticus ATCC 17929, Actinomyces israelii ATCC 12102, Peptostreptococcus micros ATCC 33270, Veillonella parvula ATCC
The sensitivity of \textit{S. aureus}, \textit{K. pneumoniae} was determined by the standard broth macrodilution method. Strains were tested in the cation-balanced Mueller-Hinton broth (BD, USA) \cite{16, 17}.

During the examination of the MIC there was used the 0.5 \% (5000 \mu g/ml) solution of the OHMG as a basic solution out of which there was prepared a working solution – 500 \mu g/ml, with the following twofold dilutions in the Muller-Hinton broth.

The inoculum of test strains is prepared similarly to the preceding examination in the concentration of 1.0x10^9 CFU/ml.

In the test tubes with prepared dilutions of the OHMG (1 ml), there is added the suspension of the agar culture of the examined bacteria in the Muller-Hinton broth in the volume of 1 ml. After addition of the bacteria culture, the test tubes are incubated at the temperature 36°C for 24-48 hours.

The detection of the MIC was estimated on the absence of growth in the medium containing the least concentration of the branched OHMG hydrochloride.

The detection of the MIC of the obligate anaerobic bacteria was determined by the agar dilution method \cite{18, 19}.

During the examination of the MIC of the obligate aerobes, there was also used the branched OHMG hydrochloride 0.5\% (5000 \mu g/ml) solution in the capacity of the basic solution out of which the series of twofold diminishing concentrations are prepared. In the liquefied Brucella agar (17 ml) there is sequentially added 2 ml of each the branched OHMG hydrochloride dilution and 1 ml of laced blood.

The microbe’s suspension containing about 1-2x10^9 CFU/ml of examined microorganisms is prepared similar to the preceding examination and in the volume of 0.5 \mu l is placed on the surface of the agar with various concentrations of the branched OHMG hydrochloride. After the placing of the germ culture, the plates are incubated at the temperature of 36°C for 72 to 108 hours in anaerobic conditions.

The detection of the MIC is estimated according to the absence of the growth on the plate with the medium containing the least concentration of the branched OHMG hydrochloride.

The experiment was performed three times for each concentration of the branched OHMG hydrochloride in each type of microorganisms.

**Determination of the influence of branched OHMG hydrochloride on bacterial membranes**

To determine the effect of the OHMG on the permeability of bacterial membranes for external substances, the bacteria were cultured for 24 hours in culture medium CASO agar (Sigma-Aldrich, Germany) at +36°C for 20-24 hours (\textit{S. aureus} and \textit{K. pneumoniae}) or in CASO agar supplemented with 0.1\% cysteine in anaerobic conditions at +36°C for 3 days (\textit{A. odontolyticus} and \textit{F. nucleatum}). Bacteria were washed twice by precipitation by centrifugation (4000 g, 3 min) and resuspension in saline, and then resuspended in saline until the final density of the suspension was about 109 CFU/ml. The bacterial suspension was mixed with an equal volume of water containing fluorescent dyes LIVE/DEAD (10 \mu M SYTO 9 Green Fluorescent Nucleic Acid Stain (Invitrogen, Thermofisher Scientific, USA) and 60 \mu M propidium iodide (Invitrogen, Thermofisher Scientific, USA) and OHMG (control without OHMG), incubated for 15 min at room temperature, and then 3 \mu l of the suspension was applied to the slide, covered with a coverslip and visualized using an EVOS fluorescence microscope FluoD cell imaging station (Thermofisher Scientific, USA). The resulting images were processed using the Fiji program as follows. To obtain representative images, the brightness...
and contrast of the green and red channels were adjusted in such a way that when the channels were down, the intensity of the green and red color was comparable. For the quantitative processing of images, the brightness and contrast of the green and red channels were adjusted in such a way as to isolate the bacteria and their aggregates and to remove the background. After that, the images were binarized, the bacteria and their aggregates were detected using the analyze particles function, counting the number of bacteria and aggregates and their total area.

Results

Examination of the bactericidal activity of the 0.1%, 0.2% and 0.5% water solution of the OHMG hydrochloride

Table 1 presents the data on the bactericidal effects of a water solution of the branched OHMG hydrochloride on the tested microorganisms in three concentrations. As seen from Table 1, aqueous solutions of 0.05%-0.25% possess bactericidal activity against all the studied microorganisms. The investigated solutions caused 100% death of ten of the eleven species. In the study, K. pneumoniae – water solution of the OHMG hydrochloride 0.05% and 0.1%, causing the death of 99.90% and 99.96% of bacterial cells K. pneumoniae, during the second study found no growth increase in this type of microorganisms.

Determination of MIC of branched OHMG hydrochloride

The values of the MIC of branched OHMG hydrochloride shown in Table 2.

The results present in Table 2 indicate, that MIC of branched OHMG hydrochloride has a range 0.5-250.0 μg/ml. MIC-values of sample of facultative anaerobic microbiota (S. aureus ATCC 29213, K. pneumoniae ATCC 13883) was the lowest concentration which we have. Fusobacterium nucleatum ATCC 25586 and Prevotella disiens №1116/3 were most susceptible to branched OHMG hydrochloride, from obligate anaerobes.

Determination of the influence of branched OHMG-HC on bacterial membranes

To determine the effect of the OHMG on the permeability of bacterial membranes to external substances, the bacterial suspension was incubated for 15 minutes in the presence of 20 μg/ml of the OHMG and fluorescent dyes LIVE/DEAD. The mixture of LIVE/DEAD dyes consists of two components: the green fluorescent dye SYTO 9 and the red fluorescent dye propidium iodide (PI). Both dyes fluoresce only after binding to DNA, while SYTO 9 has the ability to penetrate into cells, while PI is unable to pass through intact cell membranes and only penetrates into cells whose membranes are damaged. Thus, living cells with intact membranes are colored green, while cells in which the integrity of the membranes are broken are colored red. This process is used to study the integrity of the bacterial cell wall.

To quantify this effect, the number of cells and aggregates stained with SYTO 9 (green) or PI (red), as well as their total area, and the ratio of these values for each image obtained, were counted. The data are given in Table 3.

Discussion

At present, a large number of synthetic biocidal compounds are known to fight the pathogenic microflora. These include antibiotics [20], quaternary ammonium bases [21-24], compounds of phenolic series [25-27] and some others. However, in modern works [8, 28-34] it is shown that in different microorganisms, with respect to these substances, resistance is developed sooner or later. This prevents good treatment. A new type of synthetic biocides to which resistance is not developed in microorganisms are guanidine compounds such as chlorhexidine salts [35-37], alexidine [38, 39] and polyguanidines [40].
Preliminary studies [41] show that one of the promising compounds exhibiting biocidal activity against aerobic and anaerobic microflora can be partially branched oligohexamethyleneguanidine hydrochloride. It is believed that the partially branched OHMG hydrochloride has a lower toxicity and corrosive activity than its linear counterparts [40]. At the heart of its action, apparently, is the interaction of positively charged guanidine fragments with negatively charged sites of the peptidoglycan layer of the bacterial cell [42]. As a result of this interaction, the integrity of the outer membrane of the cell is broken, and it collapses. Our studies (Figure 1) confirm this assumption, indicating the destruction of cell membranes under the action of the branched OHMG hydrochloride.

This study shows that branched OHMG hydrochloride has a pronounced antimicrobial effect against various bacterial strains. Aqueous solutions of branched OHMG hydrochloride 0.05-0.25% have bactericidal activity against all tested species of microorganisms (Table 1), which are the main types of pathogenic microflora in the oral cavity. Taking into account the low toxicity of branched OHMG hydrochloride, its aqueous solutions can be recommended as an active component of ready-made dosage forms for the treatment and prevention of inflammatory diseases of the oral cavity.

However, the effect of the branched OHMG hydrochloride on different types of microorganisms investigated is not the same. Based on the analysis of individual values of MIC (Table 2), it can be concluded that the antiseptic in question is different in relation to different types of microorganisms. Individual MIC values vary widely (from 0.5 μg to 250.0 μg/ml of antiseptic), with facultative anaerobic bacteria (Staphylococcus aureus and Klebsiella pneumoniae) more sensitive to the action of branched OHMG hydrochloride, compared to obligate anaerobic (Actinomyces pyogenes, Actinomyces odontolyticus, Actinomyces israellii, Peptostreptococcus anaerobius, Peptostreptococcus micros, Finegaldia magna, Veillonella parvula, Prevotella disiens and Fusobacterium nucleatum). The results of the experiment indicate that branched OHMG hydrochloride is more active against gram-negative bacilli among the mandatory anaerobes (Prevotella disiens and Fusobacterium nucleatum).

**Conclusion**

Data of the present study indicate that branched OHMG hydrochloride has potential as new candidate which can be used in medical practice for treating viral and infectious dental diseases because it has possessed a wide antimicrobial spectrum.

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**Conflict of interest**

The authors declare that they have no conflict of interest.

**Ethical approval**

For this type of study formal consent is not required. This article does not contain any studies with human participants or animals performed by any of the authors.

**References**


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