Bactericidal effect of S-nitrosothiols against clinical isolates from keratitis

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Background: The purpose of this study was to evaluate the antimicrobial activity of two nitric oxide donors, ie, S-nitrosoglutathione (GSNO) and S-nitroso-N-acetylcysteine (SNAC), against clinical isolates from patients with infectious keratitis.

Methods: Reference broth microdilution assays were performed to determine the minimum inhibitory and bactericidal concentrations for GSNO and SNAC against four American Type Culture Collection strains and 52 clinical isolates from patients with infectious keratitis as follows: 14 (26.9%) Pseudomonas species; 13 (25.0%) coagulase-negative Staphylococci; 10 (19.2%) Staphylococcus aureus; nine (17.3%) Serratia marcescens; and six (11.5%) Enterobacter aerogenes. Sterility control and bacterial growth control were also performed.

Results: SNAC showed lower minimum inhibitory and bactericidal concentrations than GSNO for all clinical isolates from patients with infectious keratitis. For Gram-positive bacteria, mean minimum inhibitory and bactericidal concentrations were 2.1 ± 1.3 and 8.6 ± 3.8 mM for SNAC and 4.6 ± 3.2 and 21.5 ± 12.5 mM for GSNO (P, 0.01). For Gram-negative bacteria, mean minimum inhibitory and bactericidal concentrations were 3.3 ± 1.4 and 6.1 ± 3.4 mM for SNAC and 12.4 ± 5.4 and 26.5 ± 10.1 mM for GSNO (P, 0.01). The minimum bactericidal to inhibitory concentration ratio was ≥8 in 100% of all isolates tested for SNAC and in 94.2% tested for GSNO.

Conclusions: SNAC and GSNO had effective inhibitory and bactericidal effects against bacterial isolates from keratitis. SNAC showed greater antimicrobial activity than GSNO against all bacteria. Gram-positive bacteria were more susceptible to the inhibitory and bactericidal effects of the S-nitrosothiols.

Keywords: antimicrobial activity, S-nitroso-N-acetylcysteine, S-nitrosoglutathione, nitric oxide donors, infectious keratitis

Introduction

Bacterial infectious keratitis is a sight-threatening disease and one of the leading causes of blindness throughout the world.¹ Due to the large spectrum of possible microorganisms, its treatment requires a combination of topical fortified antibiotics.² Fluoroquinolones, mainly the 8-methoxyfluoroquinolones, gatifloxacin and moxifloxacin, have shown effectiveness as monotherapy.² However, the eminent risk of widespread resistance³,⁴ induced by inappropriate treatment has stimulated a growing interest in new antibacterial strategies.

Nitric oxide (NO) is an endogenous molecule that gained notoriety at the end of the 1980s after the discovery of its effects on smooth muscle relaxation, vasodilatation, and vascular pressure flow control.³ In the eye, NO has been shown to be a key...
regulator of vascular tone in the ophthalmic arteries involved with increasing blood flow to the retina, choroid, and the optic nerve head. Beyond control of vascular tone, NO was shown to be involved in several bioregulatory systems in the human body, including modulation of the immune response and toxicity of macrophages toward neoplastic cells and pathogens. Under physiologic conditions, NO reacts with thiol-containing molecules such as glutathione to form S-nitrosothiols (RSNOs), which are considered to be NO carriers and donors in humans.

The NO-mediated antimicrobial activity of RSNOs has been demonstrated against several pathogens, including protozoa and bacteria. S-nitrosoglutathione (GSNO) and S-nitroso-N-acetylcysteine (SNAC) have also been shown to have potent antimicrobial activity against trophozoites of *Acanthamoeba castellanii*, the etiological agent responsible for devastating sight-threatening keratitis. These data suggest that NO donors are important candidates for treating infectious eye diseases.

Although many studies have carried out microbiological tests with bacteria at neutral pH, the bactericidal activity of reactive nitrogen intermediates seems to be remarkable in lower pH environments. The aim of the present study was to evaluate the antimicrobial activity of two RSNOs, ie, GSNO and SNAC, against clinical bacterial strains isolated from patients with infectious keratitis.

**Materials and methods**

**Materials**
Glutathione (γ-Glu-Cys-Glu), N-acetyl-cysteine, sodium nitrite (NaNO₂), hydrochloric acid (HCl), sodium hydroxide (NaOH), phosphate buffer, and acetone were purchased from Sigma (St Louis, MO) and used as received. Cation-adjusted Mueller-Hinton broth (CAMHB) and tryptic soy agar were purchased from Oxoid (Basingstoke, England).

**Synthesis of GSNO and SNAC**
GSNO and SNAC were synthesized as previously described. GSNO was stored at freezer temperature (−20°C) and protected from light. freshly prepared stock acidic SNAC solution was diluted in phosphate-buffered saline (pH 7.4) in order to obtain 80.0 mM solutions and was used immediately. Solid GSNO was dissolved in phosphate-buffered solution to obtain 80.0 mM solutions. The final pH values of both solutions were adjusted to 5.0, 6.0, and 7.0 by titration with diluted HCl and NaOH solutions and measured using a pH meter (Hanna Instruments, Ann Arbor, MI).

**Stability of GSNO and SNAC solutions**
Decomposition levels for GSNO and SNAC incubated in CAMHB at 37°C and at pHs 5.0, 6.0, and 7.0 were characterized by monitoring the absorption band of GSNO and SNAC at 336 nm assigned to the −SNO moiety with a diode-array spectrophotometer (HP-8453, Hewlett-Packard, Palo Alto, CA) in a 1 mm optical path cuvette. GSNO and SNAC solutions, previously dissolved in phosphate-buffered saline at pH 7.4, were added to the culture medium to achieve a final concentration of 40.0 mM. The pH of the medium was further adjusted to the desired values by addition of 1 M NaOH or HCl solutions. Spectra of the solutions in the culture medium were taken at 2, 3, 5, 7, and 24 hours. The remaining concentrations of the GSNO and SNAC solutions were calculated on the basis of their molar absorption coefficients. This evaluation was performed in duplicate.

**Bacterial isolates**
The bacteria used in this study were randomly selected from 222 clinical isolates from patients with infectious keratitis seen at the Department of Ophthalmology of Federal University of São Paulo and enrolled from January to December 2009. Fifty-two (23.4%) clinical isolates, including 14 (26.9%) *Staphylococcus aureus* species, 13 (25.0%) coagulase-negative Staphylococci, 10 (19.2%) *Staphylococcus epidermidis*, nine (17.3%) *Serratia marcescens*, and six (11.5%) *Enterobacter aerogenes* were recovered twice on blood sheep agar (5%) from the Ocular Microbiology Laboratory Culture Collection (tryptic soy broth, 15% glycerol). American Type Culture Collection (ATCC) *S. aureus* 29213, *Staphylococcus epidermidis* 12228, *Pseudomonas aeruginosa* 27853, and *E. aerogenes* 13048 strains were also included. Harvested colonies (3–5) from fresh and pure cultures were inoculated in 5 mL of CAMHB and incubated at 37°C for 3–4 hours. The bacterial suspension was adjusted to a 0.5 McFarland standard (about 1.5 × 10⁶ cfu/mL) using a Baxter digital turbidimeter (Sacramento, CA).

**Preliminary determination of GSNO and SNAC antibacterial activity against ATCC strains**
In order to investigate the influence of pH on the antibacterial activity of GSNO and SNAC, four ATCC strains, *S. aureus* 29213, *S. epidermidis* 12228, *P. aeruginosa* 27853, and *E. aerogenes* 13048, were tested by broth microdilution using CAMHB with adjusted pH values of 5.0, 6.0, and 7.0. The minimum inhibitory concentrations (MIC)
of both compounds were determined by reference broth microdilution, according to the Clinical Laboratory Standard Institute. The final concentrations of GSNO and SNAC tested ranged from 40.0 mM to 0.31 mM. The plates were sealed and incubated at 37°C for 24 hours. Each experiment was carried out using properly sterile broth controls and controls for bacterial growth in CAMHB without drug. After incubation, the wells were examined and the MIC values (the lowest concentration without visible growth) were assessed.

**GSNO and SNAC susceptibility assay against clinical isolates**

Reference broth microdilution assays were performed to determine the MIC of GSNO and SNAC against 52 keratitis isolates as previously described, using CAMHB adjusted at a final pH of 5.0 (based on the results of the preliminary test against ATCC strains). The final concentrations of GSNO and SNAC tested ranged from 40.0 mM to 0.31 mM. Plates were sealed and incubated for 24 hours at 37°C in an aerobic atmosphere. Sterile broth controls and controls for bacterial growth in CAMHB without drug were also included. In addition, glutathione and N-acetyl-cysteine were also incubated with the ATCC strains in order to determine their possible antibacterial activity.

**Minimum bactericidal concentration**

Minimum bactericidal concentration (MBC) values were determined for both the ATCC strains (tested at three pH values) and clinical isolates. The contents of each well showing an inhibitory effect on the broth microdilution assay were assessed for viable bacteria by plating the contents of the wells onto tryptic soy agar plates. A colony count was performed after 24 hours of incubation. After overnight incubation, the bacterial colony was counted to determine the MBC (the lowest concentration showing ≥99.9% killing). Sterility control and bacterial growth control at pH 5.0 were also measured.

![Figure 1 Kinetic curves of GSNO and SNAC decomposition during incubation in cation-adjusted Mueller-Hinton broth (37°C) at pHs 5.0, 6.0, and 7.0 over 24 hours in a normalized arbitrary unity scale, based on their absorption bands assigned to the –N0 group. Notes: Initial concentration: 40 mM. Inset: Percentages of GSNO and SNAC remaining after 24 hours at pHs 5.0, 6.0, and 7.0 over 24 hours. Abbreviations: GSNO, S-nitrosoglutathione; SNAC, S-nitroso-N-acetylcysteine.](image-url)
Statistical analysis
The MIC and MBC values of each genus of bacterium were expressed as the mean ± standard deviation, MIC and MBC 50% and 90%, and MBC:MIC ratio. The Kruskal-Wallis test was used to identify differences in the percentage of molecular remnants after 24 hours at the three different pH levels. Analysis of variance was used to compare the means of MIC and MBC at the three different pH values, and the Tukey-Kramer statistical test was used in conjunction with analysis of variance for pairwise comparisons. Comparisons between MIC or MBC for GSNO and SNAC against the clinical isolates were analyzed using the paired-samples t-test. P values <0.05 were considered statistically significant.

Results
Stability of GSNO and SNAC
Figure 1 shows the kinetic curves for GSNO and SNAC decomposition in CAMHB at 37°C and at pHs 5.0, 6.0, and 7.0 over 24 hours in a normalized arbitrary unity scale, based on their absorption bands. After 24 hours, variation of pH in the range 5–7 did not lead to significant changes in the extent of decomposition of either GSNO (22.9% ± 2.3%, P = 0.964) or SNAC (17.2% ± 2.9%, P = 0.115). The graph bars of the inset show the quantification of the remaining amounts of GSNO and SNAC after their incubation in these conditions for 24 hours.

Influence of pH on antibacterial activity
GSNO and SNAC had significantly more antibacterial activity against ATCC strains when tested at pH 5.0 in comparison with pHs 6.0 and 7.0 (P < 0.05). However, there was no difference between the results obtained at pHs 6.0 and 7.0 (P > 0.05). Table 1 shows the MIC and MBC values for GSNO and SNAC against the ATCC strains at the three different pH levels.

Antibacterial activity of GSNO and SNAC against clinical isolates
SNAC showed lower MIC and MBC values than GSNO for all the different clinical isolates from patients with infectious keratitis. MIC values for SNAC ranged from <0.3 to 5.0 mM, with MIC_{50} and MIC_{90} values of 2.5 and 5.0 mM, respectively, while MIC values for GSNO ranged from <0.3 to 20.0 mM, with MIC_{50} and MIC_{90} values of 10.0 and 20.0 mM, respectively. MBC values for SNAC ranged from 1.25 to 20.0 mM, with MBC_{50} and MBC_{90} values of 5.0 and 10.0 mM, respectively, while MBC values for GSNO ranged from 0.6 to 40.0 mM, with MBC_{50} and MBC_{90} values of 40.0 and 40.0 mM, respectively.
Sterility control wells showed no contamination (all wells showing no visible growth and no colony was examined on the tryptic soy agar plates) and bacterial growth control wells showed adequate growth conditions (all wells showing visible turbidity and full colony formation were examined on the tryptic soy agar plates).

**Discussion**

Although numerous studies have documented the bactericidal action of RSNOs against several bacteria responsible for gastrointestinal, urinary, and respiratory diseases, each microorganism has a particular pathogenic mechanism and tissue relationship. The present study involving specific causative agents of keratitis confirmed the antimicrobial action of SNAC and GSNO against bacteria responsible for this pathologic condition, indicating that such NO donors might be useful for the topical treatment of ocular infections.

The strict mechanisms of NO-dependent antimicrobial activity are still unclear. Previous studies have demonstrated that NO, a transitory free radical, can directly inhibit bacterial respiratory chain proteins and disrupt iron-sulfur clusters in bacterial proteins, forming free iron radicals that catalyze toxic oxidative reactions. Iron depletion can also be induced by interaction of NO with metalloenzymes. Moreover, nitrosylation of free thiol groups may result in inactivation of metabolic enzymes.

Reactive nitrogen intermediates can also react with heme groups, phenol, or aromatic amino acid residues, thereby inactivating the actions of proteins. Additionally, reactive nitrogen intermediates can react with a tyrosyl radical in the ribonucleotide reductase enzyme or with metalloproteins, leading to inhibition of bacterial DNA replication. Deamination, strand breaks, and basic sites are other DNA alterations induced by NO-mediated oxidative damage.
Table 3 MIC and MBC 50% and 90% and MBC to MIC ratio of GSNO and SNAC against different clinical isolates from patients with infectious keratitis

<table>
<thead>
<tr>
<th>Organism</th>
<th>Clinical isolates n (%)</th>
<th>SNAC (mM)</th>
<th>GSNO (mM)</th>
<th>Percent of isolates with MBC to MIC ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>MBC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
</tr>
<tr>
<td>Gram-positive</td>
<td></td>
<td>1.25</td>
<td>10</td>
<td>2.5</td>
</tr>
<tr>
<td>CN Staphylococcus</td>
<td>23 (44.2)</td>
<td>1.25</td>
<td>10</td>
<td>2.5</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>10 (19.2%)</td>
<td>2.5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Gram-negative</td>
<td></td>
<td>2.5</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Serratia marcescens</td>
<td>9 (17.3)</td>
<td>1.25</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Pseudomonas species</td>
<td>14 (26.9%)</td>
<td>1.25</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>6 (11.5%)</td>
<td>1.25</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>52 (100%)</td>
<td>2.5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Note: % of isolates with MBC:MIC ratio ≥ 2.

Abbreviations: CN, coagulase-negative; mM, millimolar; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; GSNO, S-nitrosoglutathione; SNAC, S-nitroso-N-acetylcysteine; SD, standard deviation.

In spite of their potential therapeutic applications, the ocular toxicity of RSNOs has not been well characterized yet. Previous studies performed on rats have reported no adverse effects in the concentration range 1–2 mM. Our laboratory undertook a preliminary study to investigate the ocular toxicity of topical administration of SNAC and the non-toxic effect of other topical NO donors like sodium nitroprusside and S-nitroso-N-acetylpenicillamine. However, the effect of higher concentrations is not known. It is interesting to note that in the present study the inhibitory and bactericidal effect of NO donors in lower concentrations.

Considering that many targets of NO-mediated antimicrobial actions are inessential, it is possible that the antipathogenic effect of RSNOs works as a barrier, avoiding the penetration of bacteria through the lipopolysaccharide bilayer membrane of Gram-negative bacteria.
spontaneous thermal decomposition of GSNO and SNAC under the same incubation conditions as those used for the control strains at pHs 5.0, 6.0, and 7.0 over 24 hours (Figure 1) showed that both RSNOs undergo decomposition of 22.9% ± 2.7% (GSNO) and 17.2% ± 1.0% (SNAC) after 24 hours. Therefore, the remaining concentrations of GSNO and SNAC after 24 hours are still very high and in the millimolar range, which is well above the cytotoxic levels previously observed to kill Leishmania major and Leishmania amazonensis \(^{13}\) (IC\(_{50}\) in the range 54.6–161 \(\mu\)M) and trophozoites of Acanthamoeba castellanii \(^{16}\) (500–1000 \(\mu\)M) in vitro. Therefore, if the bactericidal actions are due to intact RSNO molecules, we can assume that the incubation media were exposed to high RSNO concentrations over the 24-hour incubation period. On the other hand, free NO release due to 17.2%–22.9% decomposition rate is also a possible factor contributing to the bactericidal action observed. In this sense, a combined action of intact GSNO and SNAC and free NO may operate to promote the observed antimicrobial actions.

In addition to therapeutic topical ocular instillation of GSNO and SNAC, the antimicrobial effect of these compounds could be helpful as a component of contact lens care systems and medical disinfection solutions. However, further studies are necessary to characterize the ocular toxicity, the in vivo effect in infectious keratitis, and the possible mechanisms involved in the bactericidal actions of these RSNOs.

**Conclusions**

GSNO and SNAC had bactericidal activity against clinical isolates from patients with infectious keratitis. SNAC showed greater antimicrobial activity than GSNO against all bacteria. Gram-positive bacteria were more susceptible to the inhibitory and bactericidal effects of the S-nitrosothiols.

**Disclosure**

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