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Effect of selected biofilm inhibitors, *N*-acetylcysteine and DNase, on some biological properties of taurine haloamines (TauCl and TauBr)

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Abstract

Antibiotic resistance is a common problem accompanying biofilm-associated chronic infections. New therapeutic strategies are based on a combined application of antiseptics with anti-biofilm agents. Taurine haloamines, taurine chloramine (TauCl) and taurine bromamine (TauBr), show antimicrobial and anti-inflammatory properties, which have been examined in a variety of local infections, including biofilm-associated infections. In contrast to beneficial antimicrobial effects of taurine haloamines against the planktonic form of bacteria, their efficacy against bacteria hidden in biofilm need to be enhanced. One possibility is to use them together with agents capable of destroying components of biofilm matrix. In this study we ask a question whether TauCl or TauBr are effective in killing Streptococcus mutans and Porphyromonas gingivalis, major oral bacteria responsible for the development of dental plaque and pathogenesis of periodontal diseases. Moreover, we have examined TauBr and TauCl stability in the presence of N-acetylcysteine (NAC) and DNase, agents with known anti-biofilm activity. We have found that TauBr was much stronger than TauCl microbicidal agent against both tested bacterial strains. However, TauBr was less stable than TauCl. NAC readily decomposed TauBr but not TauCl. In addition, TauBr inhibited DNase activity, when used in excess. This preliminary study confirms previous opinions that taurine haloamines have great potential in killing oral bacteria. However, further studies are necessary to find anti-biofilm agent(s) which together with TauCl/TauBr will give at least an additive therapeutic effect in the treatment of chronic infections, to support or replace ineffective antibiotic therapy.

Key words: *biofilm, N-acetylcysteine (NAC), DNase, taurine bromamine (TauBr), taurine chloramine (TauCl), oral bacteria, periodontal diseases.*

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Introduction

Taurine chloramine (TauCl) and taurine bromamine (TauBr), the major haloamines of the MPO-halide system of neutrophils, exert anti-microbial and anti-inflammatory properties, as documented in a number of *in vitro* studies [1-11]. Importantly, clinical studies have shown that both haloamines are also effective in the local treatment of inflammatory diseases, including biofilm-related infections, such as otitis externa, chronic rhinosinusitis [13, 14] or acne vulgaris [15]. Moreover, TauCl has been proposed as a novel therapeutic agent in the treatment and prevention of periodontal diseases, chronic infectious diseases related to the excessive development of the polymicrobial oral biofilm [16]. However, mature biofilms, consortia of bacteria attached to biological surfaces (e.g. dental plaque) and enclosed in self-generated matrix, are characterized by a high resistance to antibiotics and antiseptics. Therefore, new alternative therapeutic strategies should be tested. One possibility is to use antiseptics along with anti-biofilm agents to increase effectiveness of antimicrobial therapy [17, 18].

Only recently we have reported that TauBr is able to inhibit *in vitro* the formation of the *Pseudomonas aeruginosa* biofilm, but cannot eradicate the mature biofilm and

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cannot effectively kill bacteria hidden in the biofilm matrix [18]. Therefore, it is tempting to test the anti-biofilm effect of taurine haloamines in combination with agents which can destroy components of biofilm matrix (extracellular polysaccharides and/or DNA).

The aim of this study was to investigate the effect of *N*-acetylcysteine (NAC) and DNase, well-documented anti-biofilm agents, on stability and some biological properties of taurine haloamines. This laboratory study design is the first step in testing therapeutic potential of taurine haloamines combined with other anti-biofilm agents for a local treatment of biofilm-associated chronic infections, such as periodontal diseases.

Material and methods

Anti-biofilm agents

N-acetylcysteine [2-Acetamido-3-sulfanylpropanoic acid] (NAC), bovine pancreatic DNase I (DNase). Both reagents purchased from Sigma, Aldrich, Germany.

Taurine haloamines: taurine chloramine (TauCl) and taurine bromamine (TauBr)

TauCl (*N*-chlorotaurine sodium salt) a kind gift from Professor Waldemar Gottardi and Professor Marcus Nagl from the Division of Hygiene and Medical Microbiology, Innsbruck Medical University, Austria. TauCl as a crystalline sodium salt (molecular weight 181.57) was prepared as described previously [1]. Each preparation of TauCl was monitored by UV absorption spectra (λ = 200 to 400 nm) to assure the authenticity of TauCl (λ = 252 nm) and the absence of dichloramine ($TauCl_2$) (λ = 300 nm) and unreacted $HOCl/OCl^-$ (λ = 292 nm). The concentration of synthesized TauCl was determined using the molar extinction coefficient 429 $M^{-1}cm^{-1}$ at A_{252}.

TauBr was prepared in a two-step procedure. First, NaOBr was synthesized in reaction between equimolar amounts of NaOCl and NaBr (*POCH*, Poland) in the PBS solution. In such conditions virtually all the OCl^- present reacts with Br^- to form OBr^- and Cl^-. The presence and concentration of OBr^- was confirmed by UV spectra (λ = = 200 to 400 nm). In the second step, 20 mM NaOBr was added dropwise to an equal volume of 400 mM taurine. UV absorption spectrum was checked to exclude the formation of taurine dibromamine or chloramines and to estimate the concentration of TauBr (molar extinction coefficient – 430 $M^{-1}cm^{-1}$ at A_{288}). Stock solutions of TauCl and TauBr were kept at 4°C for a maximum period of 3 days before use.

Bacterial strains

All tests were performed on *Streptococcus mutans* ATCC 35 668 (*S. mutans*) and *Porphyromonas gingivalis* ATCC 33 277 (*P. gingivalis*).

Animals

The study was performed on CBA male/female mice, between 6 and 8 weeks of age, from the breeding unit, Department of Immunology UJ CM, Kraków. The mice were fed commercial, granulated food and water *ad libitum*. The study protocol was approved by the Local Ethics Committee in Kraków (No. 91/2011).

Peritoneal exudate cells

Peritoneal exudate cells (PECs) isolated from CBA mice were induced by intraperitoneal injection of 1.0 ml of thioglycolate. The cells were collected 18h later by washing out the peritoneal cavity with 5 ml of PBS (phosphate buffer solution) containing 5U heparin/ml. Then the cells were centrifuged and red blood cells were lysed by osmotic shock using distilled water; osmolarity was restored by addition of 2 × concentrated PBS.

Measurement of TauCl and TauBr stability

To determine the influence of anti-biofilm agents on stability of taurine haloamines, TauBr and TauCl were mixed with equimolar concentrations of NAC or DNase (3 mM). Reagents were kept in PBS for 60 minutes and then the stability of the tested agents was evaluated by analysis of their UV spectra, as described above.

Analysis of DNAse activity

Digestion of plasmid DNA with DNase I in the presence of TauCl or TauBr

One microgram of plasmid DNA pEGFP (Clontech) (6500 base pairs) was digested in 20 microliters of the reaction mixture, containing one microgram of DNase I (2.15 Kunitz units) and varied concentrations of TauCl or TauBr, in the presence of 40 mM Tris-HCl (pH 8.0), 10 mM $MgSO_4$ and 1 mM $CaCl_2$. Reactions were carried out for 40 minutes at 37°C and terminated by a thermal inactivation of DNase I (10 minutes at 70°C).

Electrophoresis

Digestion mixtures were separated by electrophoresis in 0.8% agarose gel, under constant voltage (4V per centimeter of distance between electrodes). Tris-borate buffer was used as a conductive medium (10.8 g/l Tris base, 5.5 g/l boric acid, 10 mM EDTA pH 8.0) and ethidium bromide was used for DNA visualization. Images were collected with UV transiluminator (Vilber Lourmat) and BioCapt 97.05s software.

Antimicrobial activity of the tested agents

Streptococcus mutans strain was grown in a Mueller-Hinton II Agar (Difco, USA) with 5% sheep blood addition at 35°C for 72 hours in aerobic conditions. *P. gin-*

givalis was grown in a *Schaedler Agar Base* (Difco, USA) at 35°C for 7 days in anaerobic conditions. Bacteria were centrifuged at 1800 × g, washed twice with 0.9% NaCl and diluted in saline to a concentration of 1×10^8 c.f.u./ml. Before use, bacteria were diluted in a phosphate buffer (PBS) (pH 7.4) to achieve a final concentration of 1×10^5 c.f.u./ml and then incubated with different concentrations of TauBr and TauCl. Immediately after the incubation (30 min), aliquots were removed and the viable cell count was determined by a pour-plate method, as described previously [2].

Measurement of reactive oxygen species generation: luminol-dependent chemiluminescence

The effect of TauCl, TauBr and NAC on the generation of reactive oxygen species (ROS) by neutrophils was evaluated *in vitro* using luminol-dependent chemiluminescence (LCL). LCL was counted at 37°C in temperature-stabilized luminometer Lucy 1 (Anthos, Austria). Briefly, 18-hour PECs induced by thioglycolate (5×10^5/well), were preincubated with tested agents in Hank's balanced salt solution (10 min at 37°C in an atmosphere of 5% CO_2) on a 96-well flat-bottom black plate (Nunc, Denmark). Then, the cells were mixed with luminol (0.8 mg/ml) at the 1 : 1 volume ratio (both Sigma-Aldrich, Germany) and incubated at 37°C for another 30 min. After incubation, the cells with TauCl, TauBr and/or NAC were immediately stimulated with opsonized zymosan 200 µg/ml (Sigma-Aldrich, Germany) or killed bacteria *(S. mutans or P. gingivalis*) at a ratio of 100 : 1 (bacteria to the cells). Photon emission over 75 min with 3-min intervals was measured. Results are expressed as relative unit light (RUL) where photons were counted every 5 seconds.

Table 1. Minimal inhibitory concentration of TauCl and TauBr [19]

Minimal inhibitory concentration MIC]	*S. mutans*	*P. gingivalis*
TauCl	3 mM	188 µM
TauBr	24 µM	48 µM

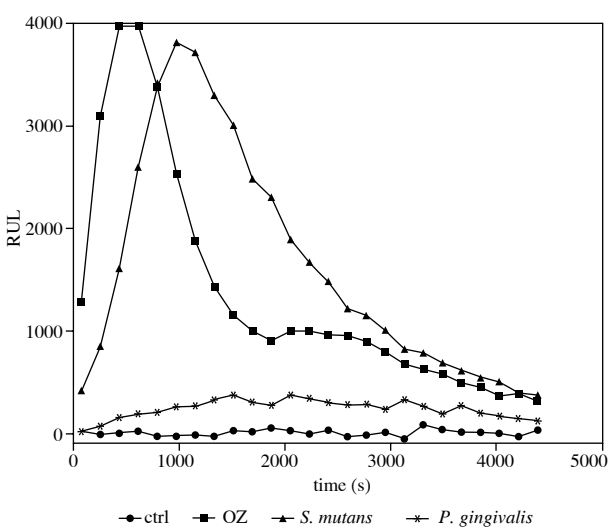


Fig. 1. Generation of ROS by PECs stimulated with opsonized zymosan (OZ) or killed bacteria (*S. mutans* or *P. gingivalis*). Ctrl – non-stimulated cells. LCL was performed and measured as described in methods. The figure shows one representative experiment

Results

Antimicrobial capacity of TauCl, TauBr against *S. mutans*, *P. gingivalis*

To confirm that taurine haloamines are good candidates for the treatment of periodontal diseases, their antimicrobial activity against the planktonic form of selected dental plaque bacteria was tested *in vitro*.

TauCl presents much weaker antimicrobial potential against tested bacterial strains than TauBr, as shown in Table 1. Minimal inhibitory concentration (MIC) of TauCl

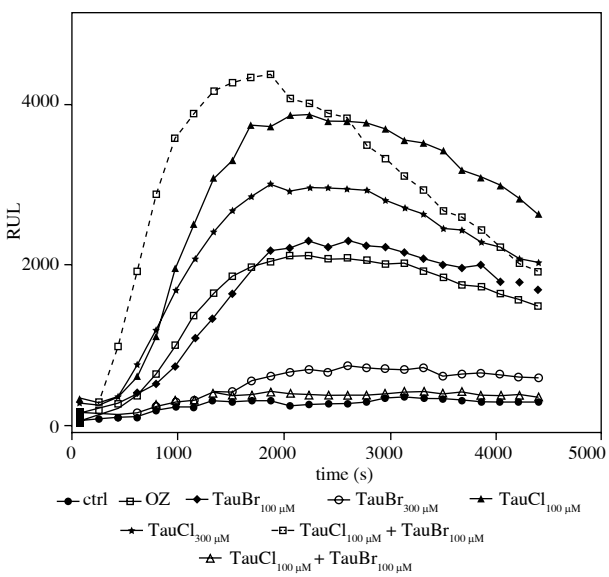


Fig. 2. Effect of TauCl and TauBr on ROS production by PECs stimulated with opsonized zymosan. PECs (5×10^5/well), were preincubated with the tested agents, TauCl, TauBr or TauCl+TauBr for 10 minutes. Then, LCL was performed and measured as described in methods. Ctrl – non-stimulated cells. OZ – stimulated cells, positive control LCL = 100%. The figure shows one representative experiment

for *P. gingivalis* was ~3.5 times higher than that of TauBr. Much bigger differences between TauBr and TauCl antimicrobial activities against *S. mutans* were observed. MIC of TauBr was 188 μM while TauCl effectively killed *S. mutans* at concentrations above 3 mM.

Generation of ROS by PECs in the presence of TauCl, TauBr and NAC

To determine whether tested agents affect ROS generation by inflammatory cells, peritoneal exudate cells were stimulated either with opsonized zymosan (OZ) or bacteria (*S. mutans, P. gingivalis*) in the presence of the agents. Both, opsonized zymosan and *S. mutans,* induced massive generation of ROS, as measured by LCL (Fig. 1). In contrast, *P. gingivalis,* an anaerobic bacterial strain, was a much weaker inducer of ROS generation (Fig. 1).

Both haloamines show a significant, dose-dependent reduction of LCL induced with all stimuli (OZ – Fig. 2; *S. mutans*, *P. gingivalis* – Fig. 4, Table 2), with the strong additive effect when added together (inhibition > 90%). At the used concentrations NAC was a much weaker antioxidant than both haloamines. Importantly, there was no additive antioxidant effect when NAC was incubated together with TauCl (Fig. 3 and Fig. 5B). DNase did not affect ROS generation (data not shown).

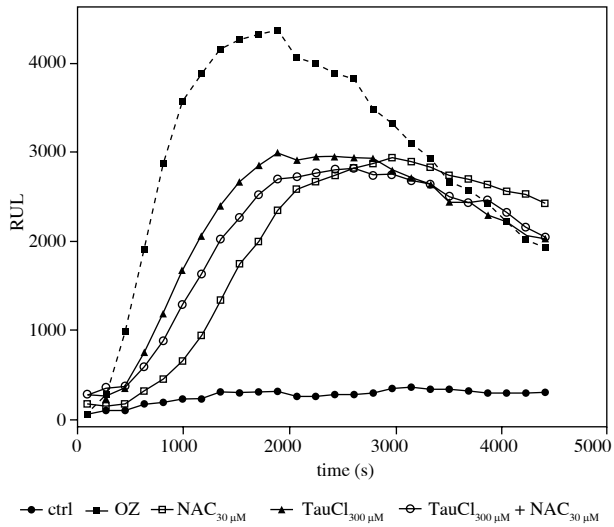


Fig. 3. Effect of TauCl and NAC on ROS production by PECs stimulated with opsonized zymosan. PECs (5×10^5/well) were preincubated with tested agents TauCl, NAC and TauCl + NAC for 10 minutes. Then, LCL was performed and measured as described in methods. Ctrl – control, non-stimulated cells. OZ – stimulated cells. The figure shows one representative experiment

Effect of NAC and DNase on the stability of TauCl and TauBr

To answer the question whether NAC and DNase affect stability of TauCl and TauBr and in consequence affect their expected biological properties, the UV spectra were analyzed. As shown in Fig. 6, at neutral pH the UV spectrum of TauBr disappeared after NAC addition, while there was no influence of NAC on the UV spectrum of TauCl when both reagents were used at the equimolar con-

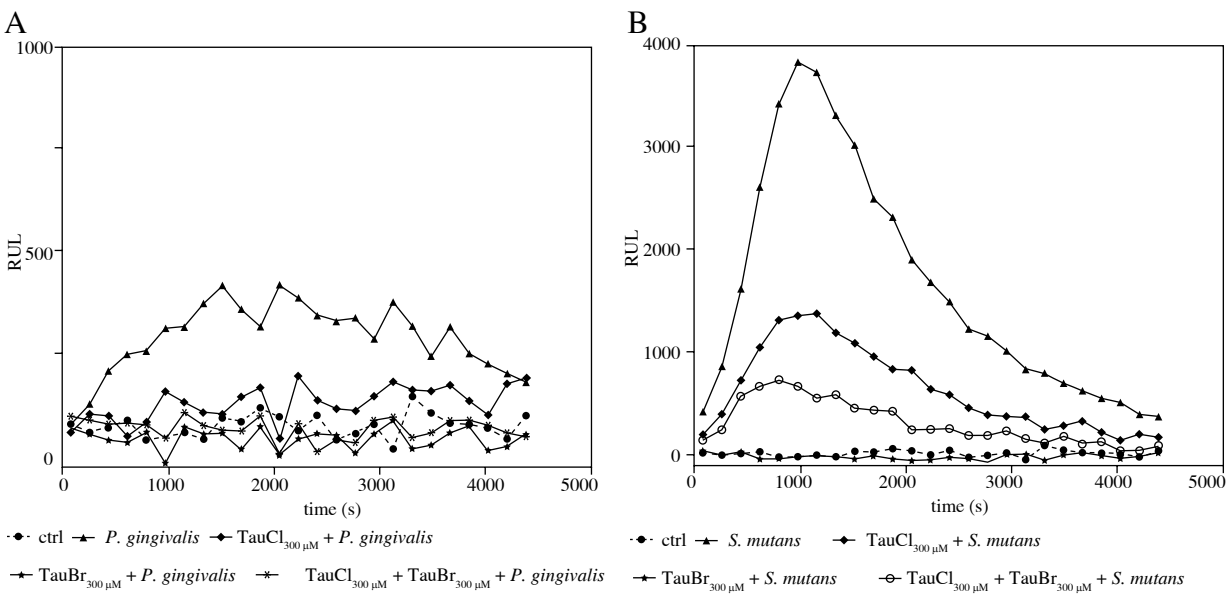


Fig. 4. Effect of TauCl and TauBr on ROS production by PECs stimulated with *P. gingivalis* (A) or *S. mutans* (B). PECs (5×10^5/well) were preincubated with TauCl and/or TauBr for 10 minutes. Then, LCL was performed and measured as described in methods. Ctrl – control, non-stimulated cells. The figure shows one representative experiment

Table 2. The inhibitory effect of TauCl, TauBr and NAC on the ROS production by PECs

	% inhibition of ROS production*							
	$TauCl_{100}$	$TauCl_{300}$	$TauBr_{100}$	$TauBr_{300}$	$TauCl_{100}$ + $TauBr_{100}$	$TauCl_{300}$ + $TauBr_{300}$	$TauCl_{300}$ + NAC_{30}	NAC_{30}
Induction of ROS generation with opsonized zymosan	8	27	47	83	48	90	35	31
Induction of ROS production with *S. mutans*	-	72	-	64	-	98	74	35

** % inhibition has been calculated from the results shown in Figs. 1-5*

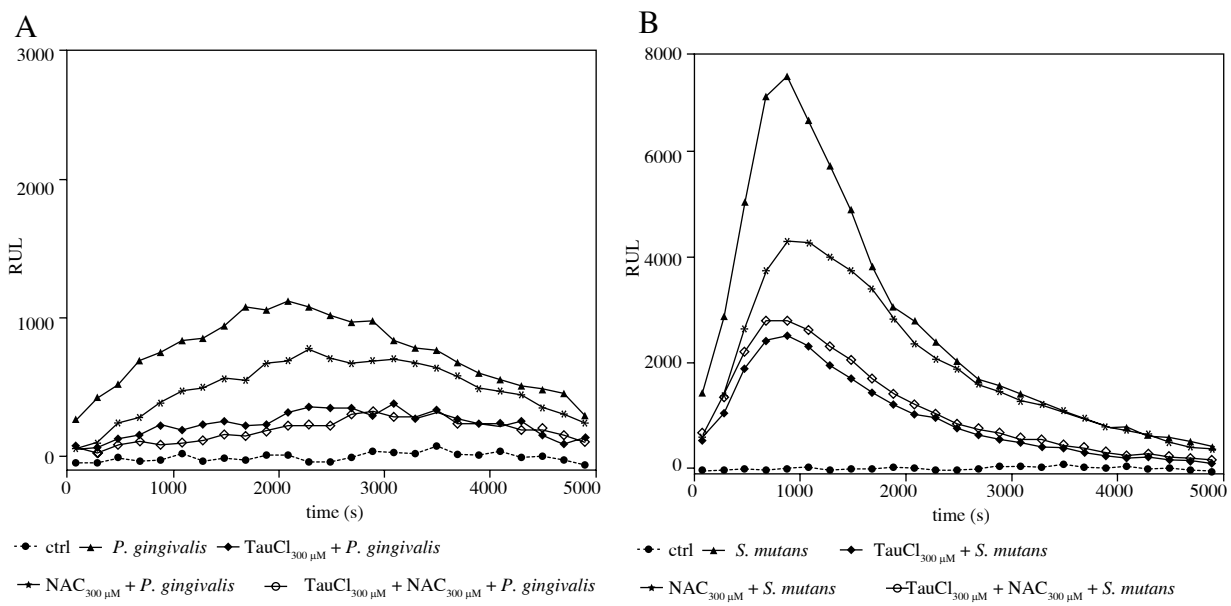


Fig. 5. Effect of TauCl and NAC on ROS production by PEC stimulated with *P. gingivalis* (A) or *S. mutans* (B). PECs (5×10^5/well) were preincubated with the tested agents TauCl and/or NAC for 10 minutes. Then, LCL was performed and measured as described in methods. Ctrl – control, non- stimulated cells. The figure shows one representative experiment

centrations. DNase had no effect on UV spectra of TauCl and TauBr (Fig. 7). These results clearly indicate that NAC but not DNase strongly affects stability of TauBr.

Effect of TauCl and TauBr on the activity of DNase

To examine the effect of TauCl and TauBr on DNase activity, plasmid DNA was digested with DNAse alone or with DNase in the presence of various concentrations of TauCl or TauBr. Digestion mixtures were separated by electrophoresis in 0.8% agarose gel as described in methods. DNAse used at a concentration of 50 μg/ml digested plasmid DNA (lanes 2-9). Either TauCl or TauBr alone did not affect the structure of plasmid DNA (data not shown). TauCl (lanes 8, 9) did not affect the activity of DNAse. Interestingly, TauBr in contrast to TauCl inhibited the activity of DNAse. The inactivation of DNAse was observed when TauBr was added in excess to DNAse (TauBr > 500 μM).

Discussion

Oral biofilm (dental plaque) is a polymicrobial biofilm growing on hard and soft dental surfaces and is considered the main etiological factor for gingivitis and periodontitis [17]. Among a variety of oral bacteria, *Streptococcus mutans* and *Porphyromonas gingivalis* play a crucial role in the formation of dental plaque and in the pathogenesis of periodontal diseases, respectively [17, 18, 20-22]. Modern concepts for the prevention and therapy of biofilm-associated infections are based on eradication of the biofilm by using antibiotics combined

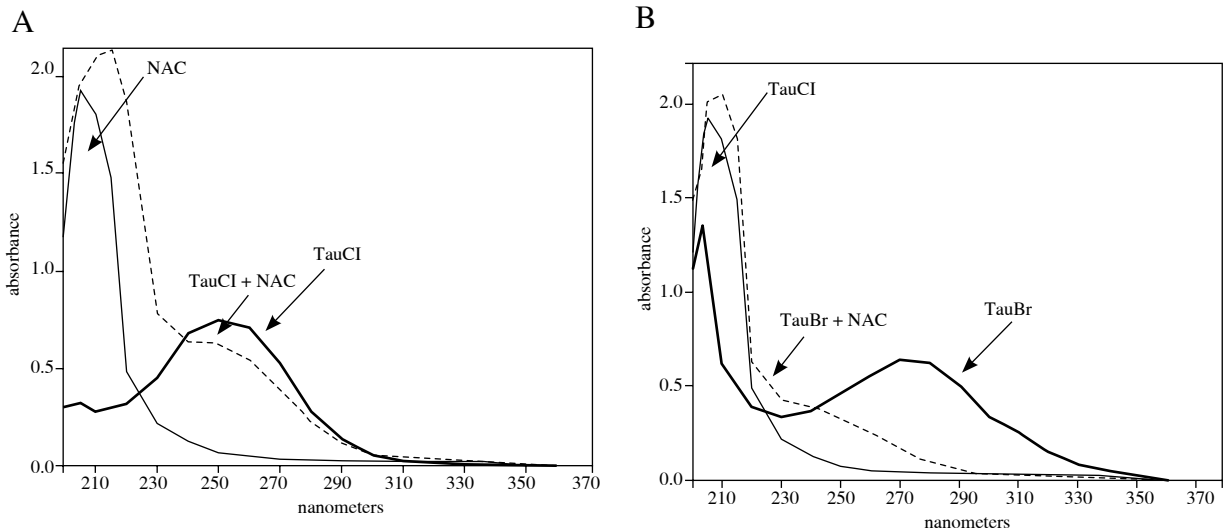


Fig. 6. Effect of NAC on the stability of TauCl (A) and TauBr (B) performed at pH 7,4. TauCl, TauBr and NAC solutions were mixed in equimolar concentrations (3 mM). The UV spectra were measured as described in methods

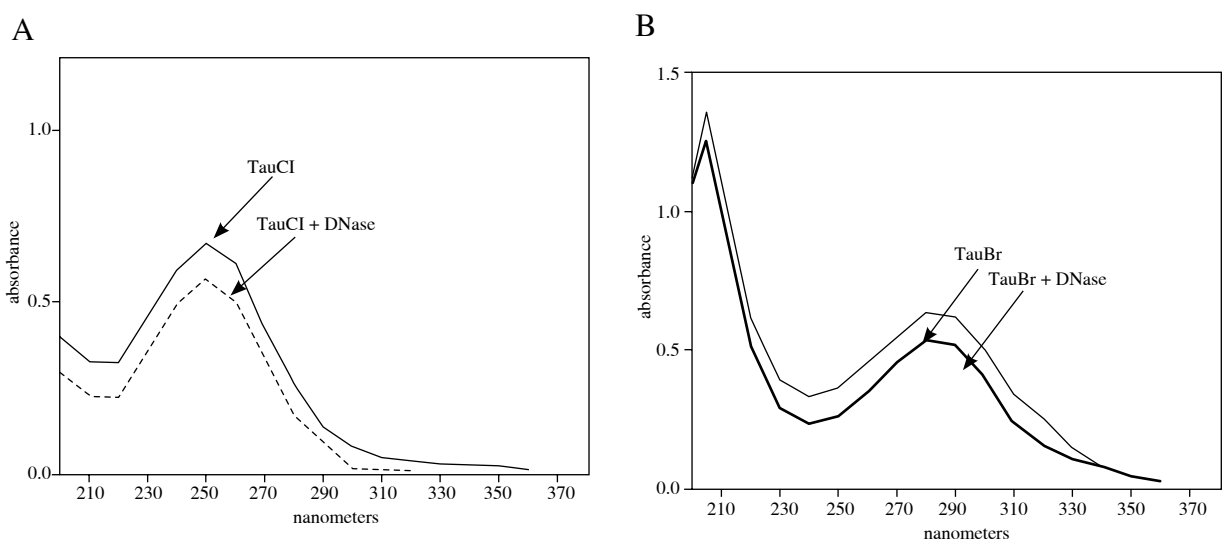
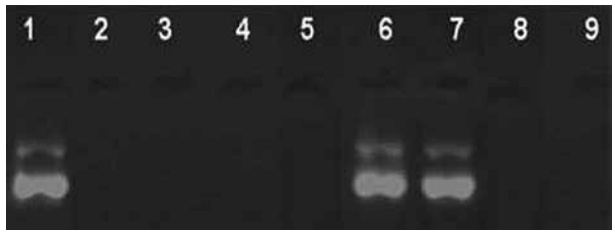


Fig. 7. Effect of DNase on the stability of TauCl (A) and TauBr (B) at pH 7.4. TauCl, TauBr and DNase solutions were mixed in equimolar concentrations (3 mM). The UV spectra were measured as described in methods

with adjuvant substances, capable of destroying biofilm matrix. Nevertheless, none of the currently available therapies are effective due to extreme resistance of biofilm bacteria to antimicrobial agents. It has been documented that bacteria within a biofilm can be even 1000 times more resistant to antibiotics than their planktonic counterparts [18]. To overcome this problem a variety of substances with a capacity to reduce biofilm growth has been tested, including NAC and DNase.

N-acetylcysteine is a thiol (sulfhydryl groups) – containing antioxidant with antibacterial properties and is a precursor in the formation of the antioxidant glutathione in the body. NAC, due to its mucus-dissolving properties, is used in medical treatment of chronic bronchitis. Recently, it has been found that NAC can increase therapeutic efficacy of antibiotics by degrading the extracellular polysaccharide matrix of biofilms [23-26]. However, the efficacy of NAC in reduction of dental plaque (oral biofilm bacteria) has not been tested yet. DNase, the second antibiofilm agent we have used in this study, also has the capacity to destroy components of the biofilm matrix, such as extracellular DNA. For example, the capacity of DNase to disrupt *P. aeruginosa* biofilm has been shown by Mohammed *et al*. [27, 28].

The main task of this study was to determine whether NAC or DNase can be used in combination with taurine



Plasmid DNA	+	+	+	+	+	+	+	+	+
DNase	–	+	+	+	+	+	+	+	+
$TauBr_{25\ \mu M}$	–	–	+	–	–	–	–	–	–
$TauBr_{50\ \mu M}$	–	–	–	+	–	–	–	–	–
$TauBr_{150\ \mu M}$	–	–	–	–	+	–	–	–	–
$TauBr_{500\ \mu M}$	–	–	–	–	–	+	–	–	–
$TauBr_{1500\ \mu M}$	–	–	–	–	–	–	+	–	–
$TauCl_{25\ \mu M}$	–	–	–	–	–	–	–	+	+
$TauCl_{1500\ \mu M}$	–	–	–	–	–	–	–	–	+

Fig. 8. Gel electrophoresis of plasmid DNA in the presence of DNAse at concentration of 50 mg/ml (lanes 2-9) and TauCl at concentrations 25, 1500 μM (lanes 8, 9) or TauBr at concentrations 25, 50, 150, 500, 1500 μM (lanes 3-7). Time of digestion of plasmid DNA was 40 minutes. Line 1 plasmid DNA without digestion

haloamines in the local treatment of biofilm-associated infections, especially in the treatment of periodontal diseases. We have chosen taurine haloamines (TauCl and TauBr) as previously our group and the others have shown their anti-inflammatory and antibacterial properties and promising potential in the local treatment of chronic (biofilm-associated) infectious diseases [13-16].

This study shows that at a neutral pH both monohaloamines, TauCl and TauBr, can effectively kill the planktonic form of dental plaque bacteria, namely, *S. mutans* and *P. gingivalis*. However, TauCl antimicrobial activity was observed at very high concentrations. It indicates that TauCl is a relatively weak antiseptic agent. On the other hand, in acidic milieu (pH 4-6), which is typical of an inflammatory environment, the ability of TauCl to kill pathogens increases significantly due to formation of a more potent $TauCl_2$ (taurine dichloramine). $TauCl_2$ has stronger bactericidal properties than TauCl, especially against Gram-negative bacteria probably due to a better penetration into bacteria. In addition, transfer of the active chlorine (transchlorination) from TauCl to amino groups of other molecules enhances its activity, mainly because of the formation of monochloramine (NH_2Cl) [29, 30].

TauBr, in contrast to TauCl, shows the microbicidal activity at very low concentrations < 10 μM, even as taurine monobromamine. Therefore, these results, together with our previous study [2, 20], suggest that TauBr is a more promising candidate than TauCl for killing bacteria hidden in the biofilm matrix, but, as TauCl is a more stable molecule than TauBr, we were tempted to examine the effect of anti-biofilm agents on activity of both taurine haloamines.

Previously, we have shown that TauBr but not TauCl is decomposed by H_2O_2 [7]. In this study, the low stability (high reactivity) of TauBr has been confirmed. Analysis of UV spectra clearly indicates that NAC completely decomposed TauBr without any effect on the stability of TauCl. The effect of NAC on TauBr may be explained by its antioxidant/scavenging properties. NAC is a powerful scavenger of some oxidants, such as hypochlorous acid and hydrogen peroxide [31]. In contrast to NAC, DNase, the second tested antibiofilm agent, did not affect the stability of either TauCl or TauBr. On the other hand, TauBr inhibited DNase enzymatic activity, when used at a concentration similar to that of DNase or in excess.

To prove whether NAC and DNAse are able to enhance anti-inflammatory properties of taurine haloamines, we also examined their ability to reduce ROS generation by activated inflammatory cells. Our previous [6, 7] and present results clearly indicate that both taurine haloamines can reduce generation of ROS by neutrophils (major cells of PECs) stimulated with opsonized zymosan (activation through FcR). The similar antioxidant effect of TauCl and TauBr was observed after stimulation of PECs with *P. gingivalis* and *S. mutans* (activation through TLRs) (Table 2). Therefore, we suggest that TauCl and TauBr may *in vivo* ameliorate inflammatory response induced by oral biofilm bacteria. Importantly, DNase did not show any effect on ROS production, while NAC confirmed its own antioxidant potential. However, the additive effect of NAC on antioxidant properties of TauCl *in vitro* was negligible, in our experimental set-up.

In conclusion, this preliminary study confirms previous suggestions that taurine haloamines, due to their anti-microbial and anti-inflammatory properties, are good candidates for a local treatment of periodontal diseases. It has become clear that they should be used in a combination with anti-biofilm agents to be more effective in

killing of biofilm-associated bacteria (e.g. *P. gingivalis* in the subgingival biofilm). However, expected positive therapeutic efficacy of such combination treatment requires good stability of both components and synergistic or at least additive antimicrobial effect. Therefore, NAC can be combined with TauCl but not with TauBr. On the other hand, the outstanding anti-microbial properties of TauBr indicate that TauBr with another adjuvant agent should be used in prevention and eradication of bacterial biofilms. Therefore, further studies should examine influence of matrix-degrading compounds on anti-inflammatory and anti-bacterial properties of taurine haloamines, especially on TauBr, the more promising local antiseptic.

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