

Taurolidine acts on bacterial virulence factors and does not induce resistance in periodontitis-associated bacteria

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Abstract

Background Taurolidine is thought to be an alternative antimicrobial in periodontal therapy. The purpose of this follow-up taurolidine study was to determine in more detail the mode of action of taurolidine against bacterial species being associated with periodontal disease. Further, a potential development of resistance against taurolidine in comparison with minocycline was to evaluate. Results Visualizing the mode of action of taurolidine to *Porphyromonas gingivalis* by scanning and transmission electron micrographs showed in part pores and release of constituents from the cell wall. The interaction of taurolidin with bacterial cell wall is also supported by the finding that taurolidine inhibited in a concentration-dependent manner the activities of LPS and of the *P. gingivalis* arginine-specific gingipains. However, an effect on *A. actinomycetemcomitans* leukotoxin was not found. When transferring 14 clinical isolates from subgingival biofilm samples (4 *P. gingivalis*, 2 *A. actinomycetemcomitans*, 2 *Tannerella forsythia*, 2 *Fusobacterium nucleatum*, 4 oral streptococci) on agar plates containing subinhibitory concentrations of taurolidine up to 50 passages, one *P. gingivalis* strain developed a resistance against taurolidine which was probably linked with efflux mechanisms. When antimicrobial pressure was removed, MIC reverted to baseline value. Testing development of resistance to minocycline in a similar way, an increase of MIC values occurred in five of the 14 included strains after exposure to subinhibitory concentrations of the antibiotic. Efflux might play a role in one *A. actinomycetemcomitans* strains, but obviously not in the other four strains. Removing antimicrobial pressure for a few passages did not revert the increased MIC values. Conclusion Taurolidine interacts with LPS and gingipains. Development of resistance seems to be a rare event when applying taurolidine. A potential development of resistance might be associated with efflux mechanisms.

Background

According to the latest reported data about 42% of the non-institutionalized U.S. population had periodontitis [1]. Periodontitis is a disease which destructs the tooth-supporting tissues. In pathogenesis of this disease host inflammatory response to subgingival bacteria leading to a pathogenic microbiota is central [2]. Here, *Porphyromonas gingivalis* is meanwhile postulated being a key stone pathogen in this transition [3], the major virulence factors are its cysteine proteases called gingipains [4]. Other bacteria involved in pathogenesis of periodontitis are *Tannerella forsythia* [5] and *Aggregatibacter actinomycetemcomitans* synthesizing a leukotoxin [6]. *A. actinomycetemcomitans* strains differ in their ability to produce leukotoxin, highly leukotoxin-producing strains (JP2-clone) have a deletion in the promotor region [7].

Antimicrobials are widely used in treatment of periodontal diseases. Adjunctive use of chlorhexidine digluconate results in better clinical outcomes [8]. The use of adjunctive systemic antibiotics appears to be beneficial in advanced and severe cases of periodontitis [9]. However, the long-term clinical benefit following the use of antibiotics is still unclear [10]. As a topical antibiotic minocycline (a tetracycline derivative) in microspheres has shown additional clinical benefits compared with nonsurgical periodontal therapy alone [11].

One potential alternative antimicrobial agent is taurolidine. Taurolidine is a derivative of the amino acid taurine, as an antimicrobial it has been proven to be safe and effective for prevention of central venous catheter infection [12]. In vitro-studies indicate an antimicrobial activity against oral microorganisms also when being organized in a biofilm. [13, 14]. An application in dentistry was discussed already several years ago. Rinsing with 2% of taurolidine solution depressed growth of dental biofilm by about 50% [15]. In several studies we evaluated the potential of taurolidine in vitro. The minimal inhibitory concentration (MIC)s of taurolidine were all below 1 mg/ml taurolidine with the exception of *Candida albicans* [16]. One percent of taurolidine inhibited clearly the formation of 12-species biofilms; however, the effect on an established biofilm was as limited as shown as for minocycline [17]. In an ex-vivo model using subgingival biofilm samples from periodontitis patients, the decrease of bacterial counts in biofilms was 0.87 log₁₀ cfu, corresponding 86.5% following application of 3% taurolidine gel after 60 min [18].

Development of resistance against antimicrobials is meanwhile a global problem, more than half a millions deaths annually are attributed to infections caused by antibiotic-resistant micro-organisms [19]. Development of resistance is clearly associated with antibiotic consumption and it is depending on the used antibiotic; the relationship is strong when quinolones are used and rather weak when beta-lactams were applied [20]. In addition, a considerable number of studies report the development of resistance to commonly used antiseptics, in part cross-resistance with antibiotics was found [21]. The increasing prevalence and spread of antimicrobial resistant bacteria is the natural consequence of genetic bacterial evolution. The more an antimicrobial agent is used, the higher is the probability of resistance formation [22].

Antimicrobial agents' resistance in bacteria can be of different origin, with a distinction between intrinsic and acquired resistance mechanisms. Intrinsic resistance mechanism is a natural property of microorganisms. Acquired resistance mechanism is based on a genetic modification of the bacterium [23]. It can occur as a result of a mutation or the ingestion of foreign resistance genes. Besides of the genetic potential of the microorganism a present selection pressure of an antimicrobial is of importance, e.g., mutations can be induced by acclimating bacteria to increasing concentrations of antimicrobial agents [23]. Gene transfer, such as the acquisition of extra-chromosomal gene elements by transposons or plasmids, can occur within a few hours [24]. Bacterial antimicrobial resistance mechanisms are based on target alteration, impermeability, enzymatic modification or destruction and efflux [25], in case of resistance to biocides (e.g. chlorhexidine) membrane modification or efflux is involved [26].

The purpose of this follow-up study was first to determine in more detail the mode of action of taurolidine against bacterial species being associated with periodontal disease and second to verify a potential development of resistance against taurolidine in comparison with minocycline.

Methods

Antimicrobials

In all experiments, taurolidine 2% solution (Geistlich Pharma AG, Wolhusen, Switzerland) was used and diluted until to the necessary dilution. As positive control minocycline (Sigma-Aldrich, Buchs, Switzerland) and as negative control dH₂O were used.

Microorganisms

Several oral bacterial strains (laboratory strains and clinical isolates; mainly *P. gingivalis* and *A. actinomycetemcomitans*) were included in the experiments (Table 1). The clinical isolates (4 *P. gingivalis*, 3 *A. actinomycetemcomitans*, 2 *T. forsythia*, 2 *Fusobacterium nucleatum*, 4 oral streptococci) were obtained from subgingival biofilm samples. Samples were only obtained from individuals who did not receive antibiotic treatment 2 months prior to the date of collecting. Culturing subgingival biofilm, and isolation of respective bacterial strains was approved by the Ethical Committee of the Canton Bern (KEK 096/15). Without removing of supragingival biofilm, sterile paper point (ISO 50) were inserted into the periodontal pocket for 30 s. Then the pooled paper points were placed into 1 ml of reduced transport fluid. After culturing, colonies typical for the respective species were isolated and subcultivated. Identity was confirmed by nucleic acid-based methods [27].

Bacterial strains were kept frozen at -80°C. About one week before experiments, they were subcultured and passaged 2 -3 times on tryptic-soy-agar plates with 5% of sheep blood (Oxoid, Basingstoke, GB).

Cells

Monocytic cells of human origin (MONO-MAC-6; DSMZ no. ACC 124) were maintained in RPMI 1640 medium containing 10% fetale bovine serum (FBS). For experiments cells were used in M199 media without FBS (Invitrogen; Carlsbad, CA, USA).

TEM and SEM photographs after exposure to 1% taurolidine

Three bacterial strains (*P. gingivalis* ATCC 33277, *T. forsythia* B13237 and *P. micra* ATCC 33270) were included. They were suspended to a density of about McFarland 4 in nutrient broth (Wilkins-Chalgren broth (Oxoid) supplemented with 5 mg/l β-NAD and 10 mg/ NAM (both Sigma-Aldrich, Buchs, Switzerland)) without and with 1% of taurolidine. After an incubation for 2 h in an anaerobic atmosphere at 37°C, these bacteria were prepared for transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

The samples were centrifuged and washed twice with 0.9% w/v NaCl and finally suspended to a 20-fold concentration of bacteria. For SEM, this suspension was transferred to slides and thereafter fixed with 2%

glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.4) for 15 min. Then samples were washed 3-fold with 0.1 M cacodylate buffer and dehydrated in ascending ethanol concentrations (30, 50, 70, 90 and 100%) for 10 minutes each. Subsequently, the samples were critical-point dried using liquid CO₂ and sputter coated with platinum (thickness approx. 1 nm) using a SCD005 sputter coater (BAL-TEC, Liechtenstein) to avoid surface charging. Finally, the specimens were investigated with a field emission (FE) scanning electron microscope LEO-1530 (Carl Zeiss NTS GmbH, Oberkochen, Germany).

For TEM, bacteria suspensions were fixed with 0.5% formaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 30 min. After washing 3-fold with 0.1 M cacodylate buffer and post-fixating with osmiumtetroxide for 1 h, dehydration in ascending ethanol series with post-staining in uranylacetate was performed. Afterwards the samples were embedded in epoxy resin (Araldite) and sectioned using a Leica Ultracut E (Leica, Wetzlar, Germany). Ultrathin sections (60 nm thickness) were mounted on filmed Cu grids, post-stained with lead citrate, and studied in a transmission electron microscope (EM 900, Zeiss, Oberkochen, Germany) at 80 kV and magnifications of 3,000x to 20,000x.

SEM and TEM photographs were taken at the Center for Electron Microscopy of the Jena University Hospital, Germany.

Interaction with bacterial virulence factors

To determine a potential effect of taurolidine on lipopolysaccharide (LPS), 10 µg and 100 µg LPS originated from *Escherichia coli* (Sigma-Aldrich) and 10 ng LPS from *P. gingivalis* (InvivoGen, Toulouse, France) were used. Taurolidine in the final concentrations of 0.01%, 0.25% and 1% and as control 16 µg/ml minocycline were added to the LPS for 2 h at 37°C. Thereafter, endotoxin activity was measured by Limulus ameobocyte lysate assay (LAL) QCL-1000™ test (Lonza, Basel, Switzerland) according to the manufacturer's recommendation. Also the two bacterial strains *P. gingivalis* ATCC 33277 and *A. actinomycetemcomitans* Y4 (about 10⁸/ml) were exposed to same concentrations of antimicrobials for 2 h before measuring endotoxin activity.

Leukotoxin was purified as described by Kachlany et al. [28], added by a final centrifugation by using 10 kDa centrifugal filter to remove proteins of lower weights. Leukotoxin in a concentration of 4 µg/ml and two *A. actinomycetemcomitans* strains (10⁸/ml in M199 media) were exposed to three concentrations of taurolidine (0.01%, 0.5%, 1.5%) and to 16 mg/ml minocycline for 2 h before monocytic cells were added. (*A. actinomycetemcomitans* leukotoxin is well known to affect viability of leukocytes, such as monocytes [6]). Vitality of MONO-MAC-6 cells was determined after 1 h and 20 h of incubation at 37°C with 5% of CO₂ by using MTT assay according to Mosmann [29].

Experiments on LPS and leukotoxin activity were made in independent triplicates. One-way ANOVA with post-hoc Bonferroni compared different antimicrobials with controls each. Further, t-test was used to find differences in viability of MONO-MAC-6 cells after exposing *A. actinomycetemcomitans* strains and

leukotoxin with non-stimulate cells (only controls). A difference with a p-value of <0.05 was considered as being statistically significant each.

A direct inhibitory effect on gingipain activity was assessed by adding three concentrations of taurolidine (0.01%, 0.25%, 1%) and of minocycline (0.5 µg/ml, 16 µg/ml, 128 µg/ml) for 2 h to 10 nM activated RgpB (being an arginine-specific gingipain; the RgpB was kindly provided by Jan Potempa, Jagiellonian University Krakow, Poland). Thereafter, *P. gingivalis* strains (10^8 /ml Wilkins-Chalgren broth) were exposed to the same concentrations of the antimicrobials for 2 h before measuring arginine specific amidolytic activity in the suspensions by adding the chromogenic N- α -benzoyl-DL-arginine-*p*-nitroanilide (BApNA) substrate (Sigma-Aldrich), USA) with a final concentration of 2 mM in the assay buffer (0.2M Tris-HCl, 0,1M NaCl, 5 mM CaCl₂, pH 7.6, supplemented with 10 nM cysteine hydrochloride solution in a ratio 1 : 1. The absorbance was read at 405 nm (37°C) at 30 s intervals for 1 h by using a spectrophotometer (BioTek EL808, BioTek, Luzern, Switzerland).

Potential development of resistance

Initially all clinical isolates were included in this experiment. However, the *A. actinomycetemcomitans* Be12206 strain did not survive the passages. So results from 14 strains were available. The method was adapted to the procedures made before [30, 31]. For that strains were transferred on Wilkins Chalgren agar plates (Oxoid) containing 1/4 - 1/8 of the MICs of taurolidine or minocycline up to 50 passages (about one passage each five days). Before and after each 10 passages MICs were determined again by agar dilution technique. If there was an increase or decrease of MICs after passaging, the concentration of the antimicrobial was adapted. Further strains with altered MICs were passaged 3 times on media free of antimicrobials to see the stability of the altered MIC. Strains showing changed MIC before and after 50 passages with subinhibitory concentrations of taurolidine were prepared for TEM and SEM as described above.

Strains with increasing MICs were screened for potential efflux resistance mechanisms. The efflux inhibitors 50 µg/ml NMP, 10 µg/ml CCCP, 100 µg/ml verapamil and 20 µg/ml reserpine (all inhibitors Sigma-Aldrich) were added to the nutrient media (Wilkins Chalgren broth) before adding antimicrobials and determining MIC again after 18 h of incubation.

Results

Visualization of taurolidine action

Differences of scanning electron microscopy (SEM) photographs of *P. gingivalis* ATCC 33277, *T. forsythia* Be13237 and *Parvimonas micra* ATCC 33270 without and with 2 h exposure to 1% taurolidine were small (Fig. 1A). After exposing to taurolidine, *P. gingivalis* ATCC 33277 showed in part pores and release of constituents from the cell wall, similar dense structures were attached to *P. micra* ATCC 33270, which

might be a sign of a loss of bacterial structure. Transmission electron microscopy (TEM) photographs (Fig. 1B) underline a dissolution of cell plasma constituents by taurolidine.

Interaction with most important virulence factors

Taurolidine inhibited concentration dependent the endotoxic activity of *Escherichia coli* LPS, *P. gingivalis* LPS and of the two gram-negative strains *P. gingivalis* ATCC 33277 and *A. actinomycetemcomitans* Y4. Statistically significant differences were always found when comparing 1% taurolidine with control. In contrast, 16 µg/ml of minocycline did not have any effect (Fig. 2).

Next, viability of monocytic MONO-MAC-6 cells measured by MTT assay underlines the toxicity of purified *A. actinomycetemcomitans* leukotoxin. Already after 1 h of incubation, viability of the cells tended to be lower, after 20 h the difference was statistically significant ($p=0.004$). Among the two used bacterial strains, the Be12206 strain belonging to the JP2 clone decreased the viability of the cells after 20 h of incubation ($p=0.006$). However, in this assay using the cell line of MONO-MAC-6 cells, an exposure to taurolidine clearly decreased the MTT activity, the effect is visible for 0.25% and 1% of taurolidine already after 1 h and for all three used concentrations after 20 h (each $p<0.01$). An inhibiting effect of the added antimicrobials (both taurolidine in three concentrations and 16 µg/ml minocycline) on toxicity of leukotoxin to MONO-MAC-6-cells was never measured (Fig. 3).

Taurolidine blocked the activity of the purified *P. gingivalis* arginine specific cysteine protease (RgpB). Using bacterial strains of *P. gingivalis*, the results were different. Applying 1% taurolidine to *P. gingivalis* ATCC 33277 and J374-1, the measured arginine-specific amidolytic activity clearly decreased, whereas 0.01% did not exert any effect on the enzyme activity. In contrast, when using the HG66 strain nearly no arginine-specific amidolytic activity was found already after bacterial culture was exposed to 0.01% taurolidine. Results for minocycline were similar when using the purified gingipain and the HG66 strain but there was no inhibition of gingipain activity when using the ATCC 33277 and the J374-1 strains (Fig. 4).

Potential development of resistance

Analyzing a potential development of resistance, an increase of MIC of taurolidine was found only against one *P. gingivalis* strains. But it is of interest to note that four strains, among them the two included *T. forsythia* strains became more sensitive to the antimicrobial after certain passages (Table 2). Resistance development against minocycline was found in five of the 14 included strains after exposure to subinhibitory minocycline (Table 3).

Next, strains exhibiting an altered sensitivity after certain passages were characterized further. The increase of the MIC of taurolidine against one strain (*P. gingivalis* J374-1) might be linked to efflux

mechanisms. It seems that this strain contains several efflux pumps. After removing antimicrobial pressure for three passages, MIC values were equal or close to baseline values (Table 4). Efflux mechanisms might play also a role in two *A. actinomycetemcomitans* strains showing an increase in resistance against minocycline. It is obviously not of importance in the other three strains with increased MIC values. Removing antimicrobial pressure for a few passages did not revert the increased MIC values (Table 5).

SEM photographs of *P. gingivalis* J374-1 show more vesicles at the surface after developing a higher resistance to taurolidine. After being exposed to taurolidine and becoming more sensitive, *T. forsythia* Be13237 has more rounded ends and *S. constellatus* BeTa7-1 lost obviously the ability to form chains. Highly dense particles are visible in the TEM photographs of *P. gingivalis* J374-1 after exposure to subinhibitory taurolidine. Those seems to be located in the cells and closely attached to the surface. TEM photographs of *T. forsythia* Be13237 show dense structures surrounded a halo attached to the outer membrane (Fig. 5).

Discussion

In the present study, the mode of action of taurolidine against bacterial species associated with periodontitis and the potential development of resistance were analysed.

TEM and SEM photographs and the inhibition of endotoxin activity underline a potential interaction of taurolidine with the bacterial cell wall. Taurolidine is active against gram-positive and gram-negative bacteria [32]. Taurolidine undergoes several hydrolysis reactions, first a cationic taurolidine is formed [33], which may attack the negatively charged bacterial cell wall. This is a mechanism known for other antimicrobials, *e.g.* charged chlorhexidine binds to the bacterial cell membranes [34]. For taurolidine, non isolable carbinolamine is the major bactericidal compound, the antimicrobial activity originates from the methylene iminium ion production [35]. An endotoxin-inactivation has been discussed for many years. Taurolidine inhibited concentration-dependent the expression of inflammatory cytokines induced by LPS in peripheral blood mononuclear cells [36]. In contrast, no clear reactivity of taurolidine and its derivatives with peptidoglycan was found [33].

In our study taurolidine neutralized the endotoxic activity of LPS. This included the LPS derived from *E. coli* and *P. gingivalis* LPS which showed less endotoxin activity. *P. gingivalis* synthesizes two types of LPS, the O-LPS with O-antigens and an anionic A-LPS with nonphosphorylated penta-acylated and nonphosphorylated tetra-acylated species, the latter one is less active in stimulating cytokine production in MONO-MAC-6 cells than total *P. gingivalis* LPS [37].

LPS plays a role in secretion of *A. actinomycetemcomitans* leukotoxin, an LPS with altered O-antigen polysaccharide leads to an increase of membrane-bound leukotoxin [38]. The MTT tetrazolium salt colorimetric assay described by Mosmann (1983) was used to measure viability of MONO-MAC-6 cells, it confirmed the toxicity of leukotoxin and of a highly-leukotoxic *A. actinomycetemcomitans* belonging to the JP2 clone. However, an inhibition of leukoxin activity by taurolidine was not found and thus, the result

might be related to decreased viability of the MONO-MAC-6 cells after being in contact with taurolidine. The observed toxicity is in contrast to a report using peripheral blood monocytes where no toxicity was found up to 10% taurolidine [36]. The difference might be explained by the origin of cells. Although MONO-MAC-6 cells are very similar in their expression profile to mature monocytes they originate from a leukemia patient [39]. Following they show properties of a tumor. Taurolidine promotes apoptosis of tumor cells, *e.g.* of liver cancer [40].

Except for the *A. actinomycetemcomitans* leukotoxin we focused on a potential interaction with *P. gingivalis* gingipains. Gingipains are membrane associated proteases functioning in proteolytic processing of nutrients, adhesion to host cells, evasion of immune response and more attributes [41, 42]. The arginine specific amidolytic activity of the purified cysteine protease RgpB and of different strains was clearly inhibited until blocked by taurolidine. Total blocking was found when RgpB and bacterial culture of the HG66 strain were exposed to 1% taurolidine. The HG66 strain is the only exception of all *P. gingivalis* strains where gingipains are not glycosylated and bound to outer membranes or outer membrane vesicles but released in a non-glycosylated soluble form into extracellular milieu [4]. This underlines that taurolidine inhibits directly gingipain activity. Also the as control used minocycline inhibited purified RgpB but there was obviously no effect on membrane-bound gingipains which suggests that the potential interference of taurolidine with the cell wall might be more of importance also in inhibition of gingipain activity.

In the present study, we compared the potential development of resistance of taurolidine versus minocycline on species being associated with periodontitis. Exposing microbial strains to subinhibitory concentrations of an antimicrobial for certain passages is a widely used method to evaluate the potential of an antimicrobial for development of resistance [31, 43]. The results have shown an increase of MIC of taurolidine against only one *P. gingivalis* strain (374-1), whereas a resistance development against minocycline was found in five of the 14 included strains after exposure to subinhibitory concentrations of the respective antimicrobial.

The increase of resistance of taurolidine against one strain (*P. gingivalis* J374-1) might be linked to efflux mechanisms. The efflux seems to be a first line of defense against an antimicrobial by pumping out the antimicrobial and decreasing its level within the bacterial cell [44]. Efflux pumps can be switched on when even low concentrations of an antimicrobial are in the cell leading to an adaptive resistance and expression is downregulated when the antimicrobial agent is absent [44]. The process of active efflux is one of the important mechanisms of resistance against antibiotics but also against antiseptics [45]. A *Chryseobacterium indologenes* strain which was isolated from oral biofilm up-regulated expression of an efflux-pump system in the presence of chlorhexidine digluconate [46]. Higher MIC values of chlorhexidine digluconate against *Pseudomonas aeruginosa* and *Staphylococcus aureus* strains were decreased by adding efflux pump inhibitors [47]. Efflux pumps belong to five families, regarding antimicrobial resistance the ABC pumps in gram-positive bacteria and the RND pumps in gram-negative bacteria are most important [48]. All used inhibitors on efflux pumps increased the sensitivity of the *P. gingivalis* J374-1 strain to taurolidine. Among the used inhibitors, 1-(1-naphthylmethyl)piperazine (NMP) and cyanide 3-

chlorophenylhydrazone (CCCP) are known to inhibit RND pumps, whereas reserpine and verapamil act on non-RND pumps [49, 50]. This leads to the assumption that both RND and non RND-pumps were involved in the adaptive resistance. After three passages of *P. gingivalis* J374-1 on media free of antimicrobials, the MIC of taurolidine the MICs became lower than at baseline which suggests a certain activity of the pumps already before antimicrobial's exposure.

Interestingly, a few strains became more sensitive to taurolidine when being exposed to subinhibitory concentrations of the antimicrobial. However, this phenomenon is difficult to explain. TEM photographs depicted areas of dense particles closed to cell bacterial cell wall leading to the assumption of an enrichment of the antimicrobial there. The *S. constellatus* BeTa7-1 strain after 50 passages on subinhibitory taurolidine lost the ability to form chains probably by a diminished expression of adhesins or receptors.

A resistance development against minocycline was found in five of the 14 included strains after exposure to subinhibitory minocycline. As doxycycline minocycline belongs to the 2nd generation of tetracyclines, an antibiotic class which target is the small subunit of bacterial ribosome where t-RNA binds to the respective nucleic acids of mRNA [51]. Acquired resistance is linked with efflux pumps, but also bacterial proteins removing tetracyclines from its binding site, enzymes degrading tetracyclines or mutations within 16S rRNA preventing binding of tetracyclines to ribosomes exist [51]. Resistance to tetracyclines among oral strains is reported differently. Recently about 30% of *Streptococcus intermedius* and *S. constellatus* strains and 47% of the *A. actinomycetemcomitans* were reported to be resistant against doxycycline [52, 53]. In contrast, less than 10% of *A. actinomycetemcomitans* strains isolated from aggressive periodontitis patients in Great Britain showed a resistance against tetracyclines [54] and MIC₉₀ values were generally low for *P. gingivalis* and for *F. nucleatum* [55]. A higher resistance of the oral streptococci than of other oral strains might be confirmed by our baseline MIC values. Those were 8 µg/ml minocycline or higher against three of the four included *Streptococcus* strains, but only against one strain (*A. actinomycetemcomitans*) of the 10 other strains. Five of the 14 strains developed increased MIC values against minocycline over 50 passages with subinhibitory concentrations. In the two *A. actinomycetemcomitans* strains an efflux mechanism might be involved, in one strain this might be related mainly to RND pumps, in the other strain only to non-RND pumps. In the other three strains (one *T. forsythia*, one *F. nucleatum* and one oral streptococcus) other mechanisms which are not investigated in detail may account for the increased MIC values. But removing antimicrobial pressure for a few passages did not revert the increased MIC values. In a study made several years ago, most oral bacteria survived transitory 2-8-fold MIC values of minocycline, however finally MIC values were not different from baseline (at the highest one step) [56].

Conclusions

Taurolidine interacts with LPS of periodontopathogens and inhibits activity of gingipains. Since development of resistance against taurolidine occurs very rarely when compared with that induced by

minocycline, further studies are warranted to evaluate its potential use in the treatment of periodontal and peri-implant infections.

Abbreviations

A. actinomycetemcomitans: *Aggregatibacter actinomycetemcomitans*

BApNA: N- α -benzoyl-DL-arginine-*p*-nitroanilide

CCCP: cyanide 3-chlorophenylhydrazone,

Cfu: colony forming units

E. coli: *Escherichia coli*

FBS: fetale bovine serum

F. nucleatum: *Fusobacterium nucleatum*

FE: field emission

LAL: Limulus amebocyte lysate assay

LPS: lipopolysaccharide

MIC: minimal inhibitory concentration

NMP: 1-(1-(naphthylmethyl)piperazine

P. gingivalis: *Porphyromonas gingivalis*

P. micra: *Parvimonas micra*

RgpB: arginine-specific gingipain B

S. constellatus: *Streptococcus constellatus*

SEM: scanning electron microscopy

T. forsythia: *Tannerella forsythia*

TEM: transmission electron microscopy

Declarations

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SE supervised and designed the study. SR, SS, NA conducted the experiments. SN carried out and analyzed the SEM and TEM analyses. SR, SS, NA processed and analyzed the data. SE, JZ and AS interpreted the results. All authors wrote, reviewed and approved the final manuscript.

Ethical approval and consent to participate

Culturing subgingival biofilm, and isolation of respective bacterial strains was approved by the Ethical Committee of the Canton Bern (KEK 096/15). All study participants signed an informed consent.

Consent for publication

Consent for publication was obtained from Geistlich Pharma AG.

Competing interests

Jürg Zumburrn is an employee of Geistlich Pharma AG. Anton Sculean is section editor and Sigrun Eick is associate editor of BMC Oral health. The other authors declare that they have no competing interests.

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Tables

Table 1 Strains included in the experiments

Species	Strain	Origin	Gram property
<i>Porphyromonas gingivalis</i>	ATCC 33277	Laboratory	Negative
<i>P. gingivalis</i>	HG66	Laboratory	Negative
<i>P. gingivalis</i>	J374-1	Clinical	Negative
<i>P. gingivalis</i>	BeOR6	Clinical	Negative
<i>P. gingivalis</i>	BeOR14	Clinical	Negative
<i>P. gingivalis</i>	BeOR15	Clinical	Negative
<i>Aggregatibacter actinomycetemcomitans</i>	Y4	Laboratory	Negative
<i>A. actinomycetemcomitans</i>	Be12206	Clinical	Negative
<i>A. actinomycetemcomitans</i>	OMZ 444	Clinical	Negative
<i>A. actinomycetemcomitans</i>	Be14207	Clinical	Negative
<i>Fusobacterium nucleatum</i>	BeOR1	Clinical	Negative
<i>F. nucleatum</i>	BeTa9-1	Clinical	Negative
<i>Tannerella forsythia</i>	Be13237	Clinical	Negative
<i>T. forsythia</i>	Be13216	Clinical	Negative
<i>Parvimonas micra</i>	ATCC 33270	Laboratory	Positive
<i>Streptococcus oralis</i>	BeJM933	Clinical	Positive
<i>S. constellatus</i>	BeTa7-1	Clinical	Positive
<i>S. mitis</i>	BeTa7-2	Clinical	Positive
<i>S. gordonii</i>	BeTa9-2	Clinical	Positive

Table 2 Minimal inhibitory concentration (MIC) of taurolidine (%) determined at baseline and after certain passages on agar plates containing subinhibitory concentrations of the compound

	Baseline	After 10 passages	After 20 passages	After 30 passages	After 40 passages	After 50 passages
<i>P. gingivalis</i> BeOR14	0.025	0.025	0.025	0.025	0.025	0.025
<i>P. gingivalis</i> BeOR6	0.025	0.05	0.05	0.05	0.05	0.05
<i>P. gingivalis</i> BeOR15	0.025	0.025	0.025	0.025	0.025	0.025
<i>P. gingivalis</i> J374- 1	0.025	0.025	0.1	0.1	0.1	0.1
<i>S. constellatus</i> BeTa7-1	0.1	0.1	0.1	0.1	0.1	0.025
<i>S. mitis</i> BeTa7-2	0.05	0.05	0.05	0.05	0.05	0.05
<i>S. gordonii</i> BeTa9- 2	0.1	0.1	0.1	0.1	0.1	0.05
<i>S. oralis</i> JM933	0.1	0.1	0.1	0.1	0.1	0.1
<i>A. actinom</i> OMZ 444	0.1	0.1	0.1	0.1	0.1	0.05
<i>A. actinom.</i> Be14207	0.05	0.05	0.05	0.05	0.05	0.05
<i>F. nucleatum</i> BeOR1	0.05	0.05	0.05	0.05	0.05	0.05
<i>F. nucleatum</i> BeTa9-1	0.05	0.1	0.1	0.1	0.1	0.1
<i>T. forsythia</i> Be13237	0.025	0.003	0.003	0.003	0.003	0.003
<i>T. forsythia</i> Be13216	0.025	0.003	0.003	0.003	0.003	0.003

Table 3 Minimal inhibitory concentration (MIC) of minocycline ($\mu\text{g/ml}$) at baseline and after certain passages on agar plates containing subinhibitory concentrations of the compound

	Baseline	After 10 passages	After 20 passages	After 30 passages	After 40 passages	After 50 passages
<i>P. gingivalis</i> BeOR14	1	1	1	1	1	0.5
<i>P. gingivalis</i> BeOR6	1	1	1	1	1	1
<i>P. gingivalis</i> BeOR15	1	1	1	1	1	1
<i>P. gingivalis</i> J374- 1	1	1	1	1	1	1
<i>S. constellatus</i> BeTa7-1	8	8	8	8	8	16
<i>S. mitis</i> BeTa7-2	8	8	16	16	16	16
<i>S. gordonii</i> BeTa9- 2	16	16	16	16	16	16
<i>S. oralis</i> JM933	1	1	4	4	4	4
<i>A. actinom</i> OMZ 444	1	2	16	32	32	32
<i>A. actinom.</i> Be14207	8	16	16	32	32	32
<i>F. nucleatum</i> BeOR1	1	1	1	1	1	1
<i>F. nucleatum</i> BeTa9-1	1	1	1	8	8	4
<i>T. forsythia</i> Be13237	0.5	0.25	0.25	0.25	0.25	0.25
<i>T. forsythia</i> Be13216	0.5	2	2	2	2	2

Table 4 MIC of taurolidine (%) after addition of efflux inhibitors against strains showing increase of MIC after exposing to subinhibitory concentrations of taurolidine as well as MIC of taurolidine after five passages on agar plates free of antimicrobials against strains showing deviated MIC after exposing to subinhibitory concentrations of taurolidine

	Base- line	After 50 passages	With 20 µg/ml reserpin	With 50 µg/ml NMP	With 10 µg/ml CCCP	With 100 µg/ml verapamil	After 3 passages w/o taurolidine
<i>P. gingivalis</i> J374-1	0.025	0.1	0.013	0.013	0.006	0.006	0.025
<i>S. constellatus</i> BeTa7-1	0.1	0.025	n.d.	n.d.	n.d.	n.d.	0.05
<i>T. forsythia</i> Be13237	0.025	≤0.003	n.d.	n.d.	n.d.	n.d.	0.025
<i>T. forsythia</i> Be13216	0.025	≤0.003	n.d.	n.d.	n.d.	n.d.	0.013

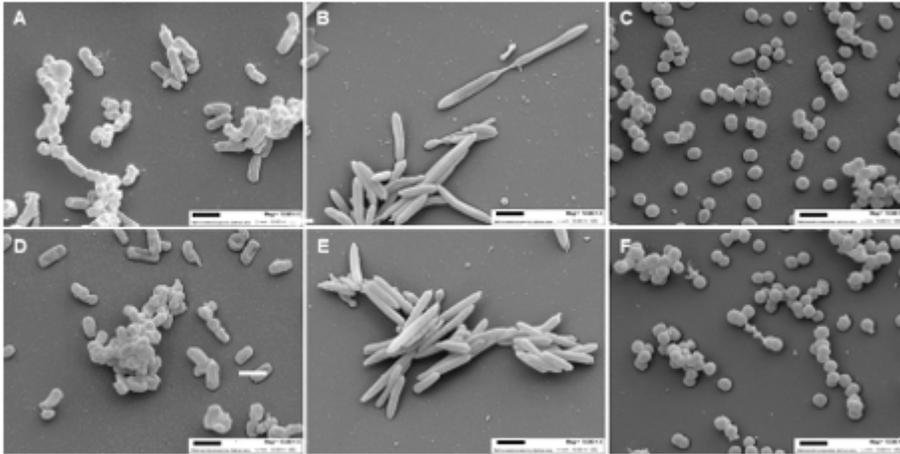
n.d. not done

Table 5 MIC of minocycline (µg/ml) after addition of efflux inhibitors against strains showing increase of MIC after exposing to subinhibitory concentrations of minocycline as well as MIC of minocycline after five passages on agar plates free of antimicrobials against strains showing increased MIC after exposing to subinhibitory concentrations of minocycline

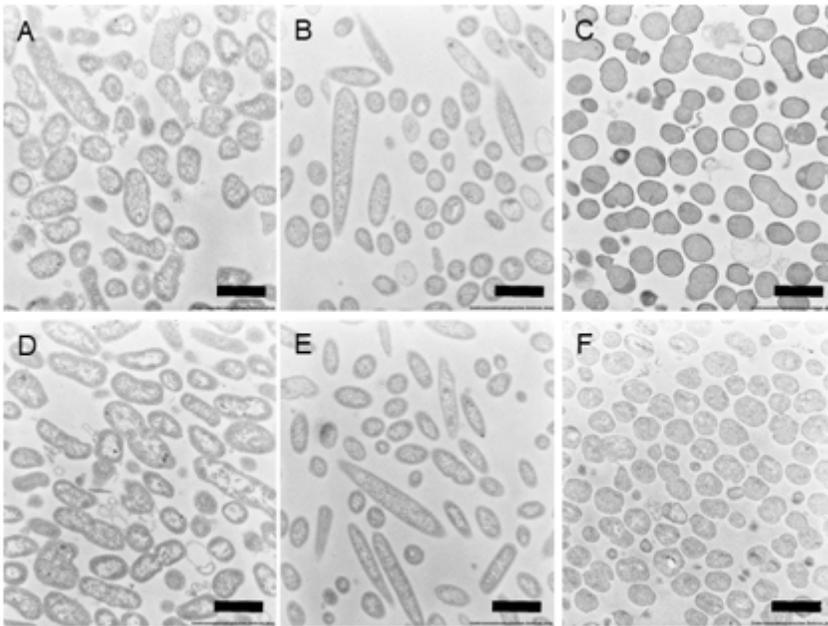
	Base- line	After 50 passages	With 20 µg/ml reserpine	With 50 µg/ml NMP	With 10 µg/ml CCCP	With 100 µg/ml verapamil	After 3 passages w/o minocycline
<i>S. oralis</i> JM933	1	4	4	4	4	4	4
<i>A. actinomycetemcomitans</i> OMZ 444	1	32	8	≤0.5	≤0.5	8	32
<i>A. actinomycetemcomitans</i> Be14207	8	32	8	16	32	4	32
<i>F. nucleatum</i> BeTa9-1	1	4	4	4	4	4	4
<i>T. forsythia</i> Be13216	0.5	2	2	2	2	2	2

Figures

SEM photographs



TEM photographs



length of the black bar 1 μm

Figure 1

SEM and TEM photographs of *Porphyromonas gingivalis* ATCC 33277 (A, D), *Tannerella forsythia* Be13237 (B, E) and *Parvimonas micra* ATCC 33270 (C, F) without (A, B, C) and with 2 h exposure to 1% tauroldine (D, E, F)

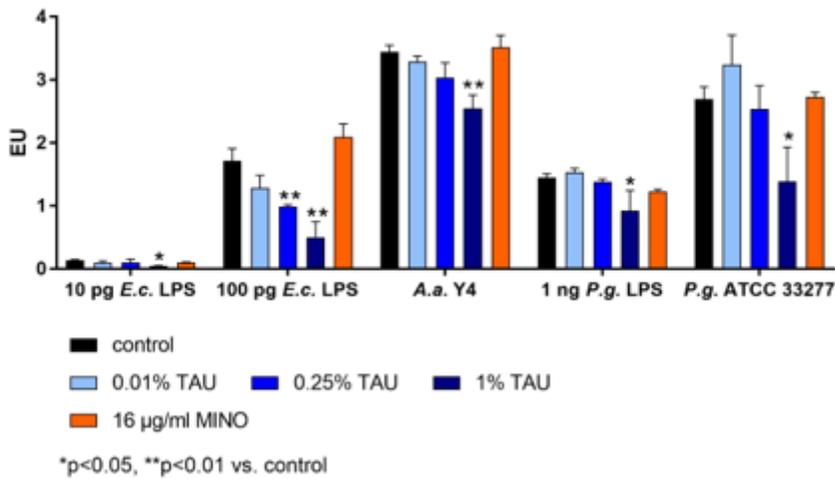


Figure 2

Endotoxin activity (endotoxin units (EU)) measured by Limulus amoebocyte lysate assay of *Escherichia coli* LPS, *Porphyromonas gingivalis* LPS as well as *P. gingivalis* ATCC 33277 and *Aggregatibacter actinomycetemcomitans* Y4 without and with 2 h pre-exposure to 0.01%, 0.25%, 1% taurolidine (TAU) and

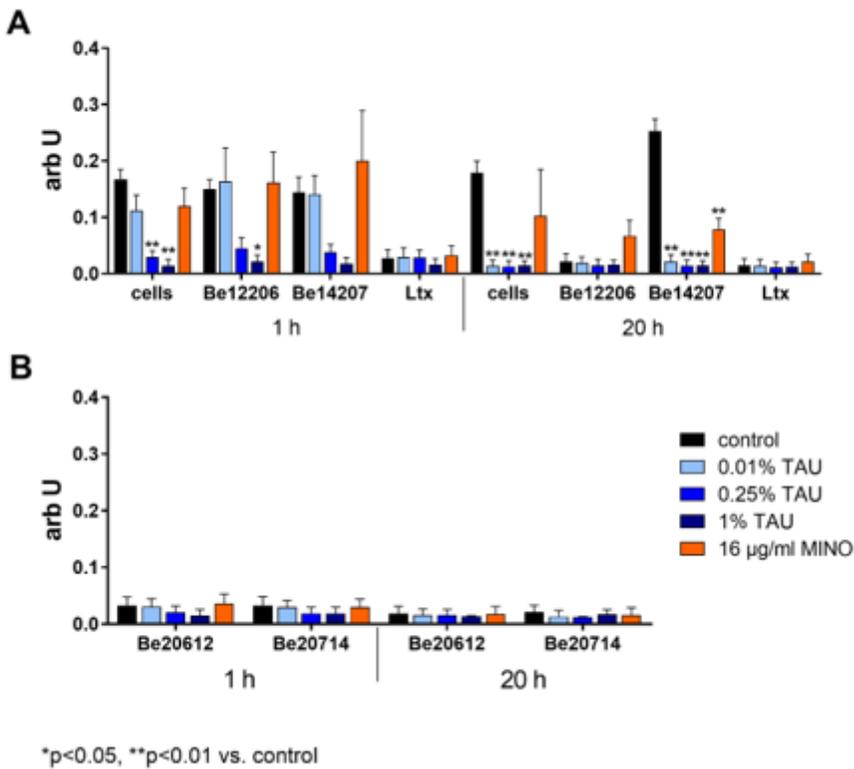


Figure 3

MTT activity of MONO-MAC-6 cells after infection with different *Aggregatibacter actinomycetemcomitans* strains (Be12206, Be14207), after addition of leukotoxin (Ltx, 4 µg/ml) and exposure to 0.01%, 0.25% and 1% taurolidine (TAU) as well as to 16 µg/ml minocycline (MINO)

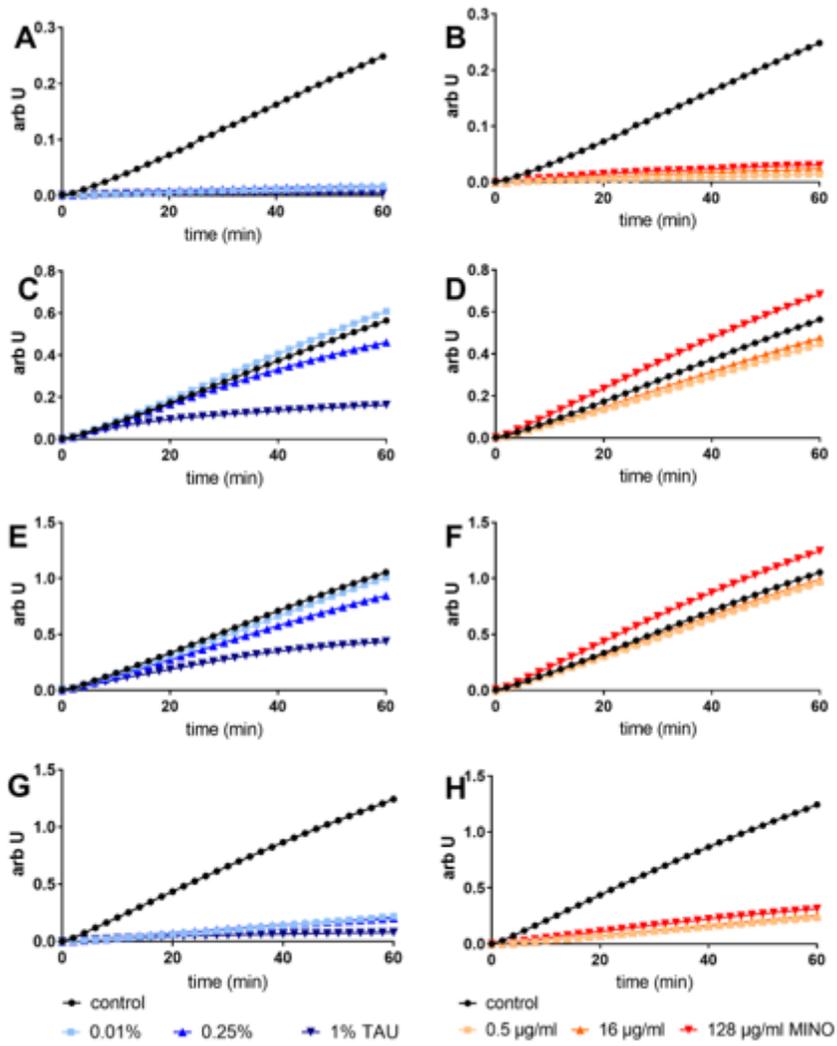
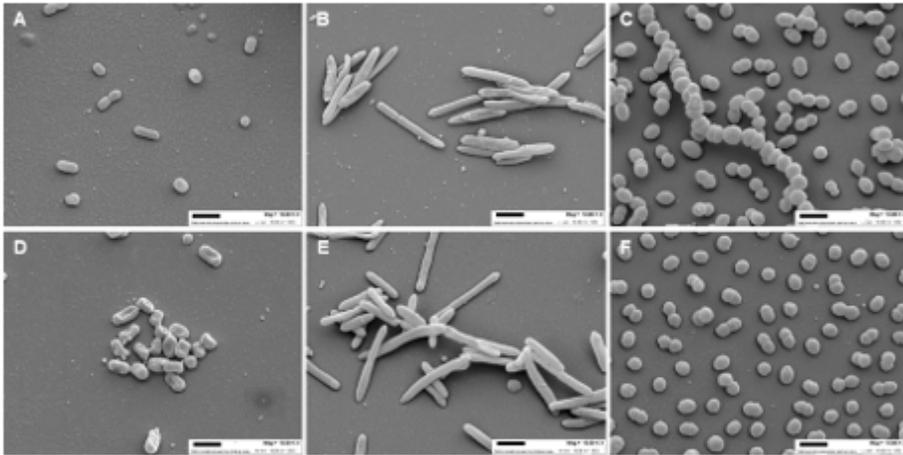


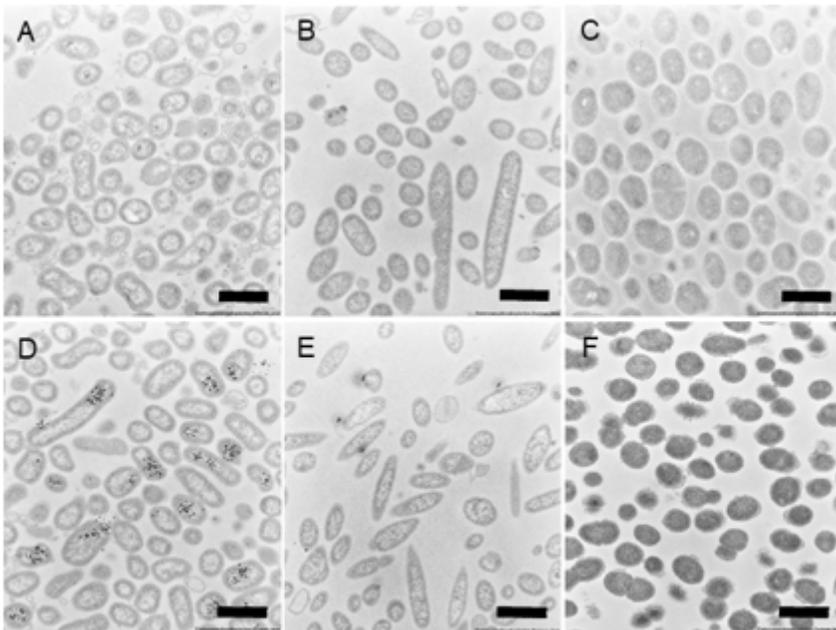
Figure 4

Arginine specific amidolytic activity after addition of taurolidine (TAU; A, C, E, G) and minocycline (MINO; B, D, F, H) in different concentrations to 10 nM RgpB (A, B), *Porphyromonas gingivalis* ATCC 33277 (C, D), *P. gingivalis* J374-1 (E, F) and *P. gingivalis* HG66 (G, H)

SEM photographs



TEM photographs



length of the black bar 1 μm

Figure 5

SEM and TEM photographs of *Porphyromonas gingivalis* J374-1 (A, D), *Tannerella forsythia* B13237 (B, E) and *Streptococcus constellatus* BeTa7-1 (C, F) before (A, B, C) and after 50 passages on agar plates containing subinhibitory concentrations of taurolidine (D, E, F)