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Antimicrobial susceptibility of anaerobic bacteria

Antimicrobial activity of stable hemiaminals against *Porphyromonas gingivalis*

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ABSTRACT

Porphyromonas gingivalis is a major etiologic agent and a key pathogen responsible for the development and progression of chronic periodontitis. Controlling the number of periodontal pathogens is one of the primary actions for maintaining oral health; therefore, active compounds with a capacity to exert antimicrobial activity have received considerable attention as they may represent potential new therapeutic agents for the treatment of chronic periodontitis. Heterocyclic compounds possessing 1,2,4- or 1,2,3-triazoles are known for several biological activities, including antibacterial properties. Among them are stable hemiaminals which can be obtained in reaction between nitrobenzaldehyde derivatives and 4-amino-1,2,4-triazole or 4-amino-3,5-dimethyl-1,2,4-triazole. In this study, we selected two relatively stable hemiaminals: (2,4-dinitrophenyl)(4*H*-1,2,4-triazole-4-ylamino)methanol (24DNTAM) and (2,4-dinitrophenyl)(4*H*-3,5-dimethyl-1,2,4-triazole-4-ylamino)methanol (24DNDMTAM). Both compounds showed promising anti-*P. gingivalis* activity, higher against ATCC 33277 strain as compared to A7436 strain. The lowest hemiaminal concentration inhibiting visible planktonic bacterial growth under high-iron/heme conditions was ~0.06 mg/ml, and the lowest hemiaminal concentration showing killing of bacteria was ~0.25 mg/ml. Antimicrobial activity was also observed against *P. gingivalis* grown on blood agar plates. Slightly higher antimicrobial activity of both compounds was observed when *P. gingivalis* was grown in co-cultures with epithelial HeLa cells under low-iron/heme conditions, which mimic those occurring *in vivo*. 24DNTAM was more effective against *P. gingivalis*, but exhibited higher cytotoxic activity against epithelial and red blood cells, as compared with 24DNDMTAM. We conclude that both hemiaminals might originate a novel group of biologically important molecules.

1. Introduction

Periodontal diseases are major public health problem due to their high prevalence and incidence in all regions of the world [1,2]. They are caused by a shift in the oral microbiota leading to a dysregulated host immune response, which results in irreversible destruction of tooth supporting tissues and tooth loss [3]. Cross-sectional studies in Europe demonstrated that moderate periodontitis occurs in 27–38% and severe periodontitis in 11–13% of the population [4,5]. In mild and moderate forms periodontitis affects 30–50% of the adults and in chronic form 5–15% of adults in the United States [6], and is responsible for up to 60% of tooth loss in the UK [7]. *Porphyromonas gingivalis*, a black-pigmented Gram-negative anaerobic bacterium, is a major etiologic agent and a key pathogen responsible for the development and progression of chronic periodontitis [8]. Moreover, the bacterium is considered a risk factor for cardiovascular and respiratory diseases, diabetes mellitus, osteoporosis, rheumatoid arthritis [9,10]. *P. gingivalis* can invade gingival epithelial and immune cells, remain viable and capable of spreading among host cells [11–13], and can spread systemically to other tissues [14,15].

Conventional treatment of periodontitis involves reduction of the total periodontal bacterial load by supragingival and subgingival mechanical debridement by scaling and root planing

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therapy [16]. However, bacterial deposits in the deep pockets and bacteria growing in the form of biofilm are difficult to remove and may be responsible for a poor treatment outcome, thus antibiotic treatment is often used as a systemic antimicrobial intervention. Among antibiotics, metronidazole and the combination of metronidazole and amoxicillin are usually used [16]. However, the antibiotic resistance of oral pathogens is increasing, especially in bacterial species isolated from patients with recurrent periodontitis [17–19]. In addition, these antibiotics are less effective because intracellular *P. gingivalis* are protected from antimicrobial activity. As a preventive or adjunct therapy, alternative therapeutic options are considered. Antibacterial mouthwashes include several compounds with chlorhexidine being commonly used because of its bactericidal and bacteriostatic properties [20]. Other plant-derived compounds comprise polyphenols [21] and resveratrol [22]. It has been shown that non-iron metalloporphyrins, which may be transported into the bacterial cell *via* hemophore/outer-membrane heme receptor systems, are potent inhibitors of functions of several proteins and are potential therapeutics for targeting bacterial infections [23,24]. The antimicrobial activity of non-iron metalloporphyrins was demonstrated also against *P. gingivalis* [25,26]. In addition, conjugates comprising antibiotic and porphyrins have been proposed to treat *P. gingivalis*-caused infections [27,28].

Heterocyclic compounds possessing 1,2,4- or 1,2,3-triazoles are known for several biological activities, including antibacterial properties [29–31]. Moreover, Schiff bases originating from triazole compounds also possess biological activities [32]. Hemiaminals are usually unstable intermediates in the nucleophilic addition reaction between carbonyl group of aldehydes or ketones and amines [33]. These compounds contain a tetrahedral carbon atom connected with a hydroxyl group and nitrogen atom. In the next step of the reaction, hemiaminal undergoes a process of dehydration which leads to the formation of stable imines or related compounds (Fig. 1A). The first stable hemiaminal was obtained in the reaction between 4-cyclohexyl-3-thiosemicarbazide and di-2-pirydyl ketone [34]. Its structure is stabilized by an intramolecular hydrogen bond. Recently, we have found that stable hemiaminals can be obtained in reaction between nitrobenzaldehyde derivatives and 4-amino-1,2,4-triazole or 4-amino-3,5-dimethyl-1,2,4-triazole [35,36] (Fig. 1B). The molecular stability of these intermediates results from the presence of both electron-withdrawing nitro groups as substituents on the phenyl ring (R_1) and 1,2,4-triazole or 3,5-dimethyl-1,2,4-triazole ring (R_2) (Fig. 1A), so no further stabilization by inter- nor intramolecular interaction is required. They are stable for a long time (in year scale) in the crystalline form as racemic crystals. On the other hand, hemiaminals dissolved in polar solvents undergo a slow decomposition to substrates, *e.g.* in DMSO solution, chemical half-life for hemiaminals obtained from nitrobenzaldehyde derivatives and 4-amino-1,2,4-triazole or 4-amino-3,5-dimethyl-1,2,4-triazole is between one to two weeks. Such stability of hemiaminals enables testing of their biological activity. In this study, we selected two relatively stable hemiaminals with chemical half-life about two weeks (2,4-dinitrophenyl)(4*H*-1,2,4-triazole-4-ylamino)methanol (24DNTAM) [35] and (2,4-dinitrophenyl)(4*H*-3,5-dimethyl-1,2,4-triazole-4-ylamino)methanol (24DNDMTAM) [36] and determined their potential antimicrobial activity against *P. gingivalis*.

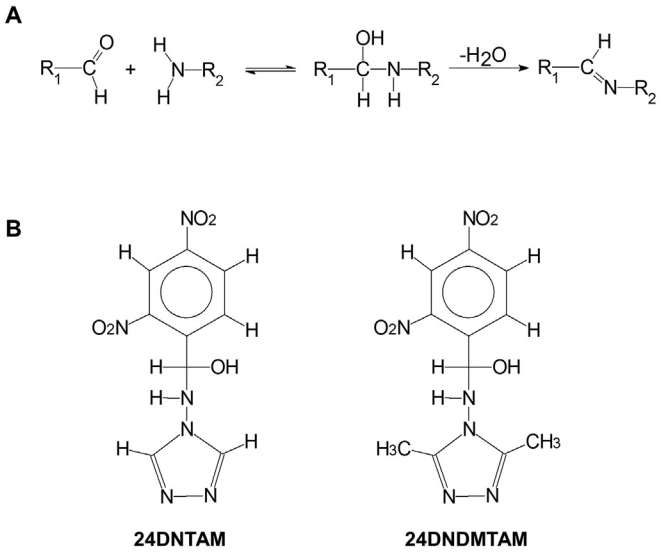

Fig. 1. Reaction scheme of the nucleophilic addition of a primary amine to a carbonyl group of aldehyde. Obtained in the first step hemiaminal (usually unstable intermediate) undergoes subsequent dehydration leading to formation of stable imines or related compounds (A). Chemical structures of examined stable hemiaminals: (2,4-dinitrophenyl)(4*H*-1,2,4-triazole-4-ylamino)methanol (24DNTAM); (2,4-dinitrophenyl)(4*H*-3,5-dimethyl-1,2,4-triazole-4-ylamino)methanol (24DNDMTAM) (B).

2. Material and methods

2.1. Bacterial cultures

P. gingivalis wild-type and mutants strains were maintained on blood agar plates (ABA, Schaedler anaerobe LAB-AGAR, supplemented with 5% sheep blood and vitamin K, Biocorp) at 37 °C under anaerobic conditions [37,38]. Mutant strains were grown in the presence of 1 μg/ml of erythromycin (ICN Biomedicals). After growing bacteria for 3–5 days on ABA plates, cultures were inoculated into liquid basal media (BM; tripticasein soy broth, 30 g/l and yeast extract, 5 g/l, Biocorp), supplemented with vitamin K (0.5 μg/ml) (Sigma), L-cysteine (0.5 mg/ml, Sigma), and hemin (5 μg/ml) (ICN Biomedicals).

P. gingivalis wild-type A7436 strain (encapsulated, non-fimbriated), extensively studied in our laboratory, was originally isolated from a refractory periodontitis patient [39] and its genome was recently sequenced [40]. This strain belongs to a group of more virulent *P. gingivalis* isolates [41], which are often found in patients with chronic periodontitis [42]. In this study, we also aimed to examine influence of hemiaminals against less virulent *P. gingivalis* wild-type ATCC 33277 strain (non-encapsulated, fimbriated) [43]. *P. gingivalis* TO4 mutant strain lacks functional *hmuY* gene (encoding hemophore-like HmuY protein) and TO6 mutant strain lacks functional *pgfur* gene (encoding PgFur transcriptional regulator of expression of several genes in *P. gingivalis*). The *hmuY* TO4 mutant strain exhibits similar growth rates under high-iron/heme conditions as compared to the wild-type strain [37,44], suggesting that the absence of HmuY protein production does not affect bacterial viability. The *pgfur* TO6 mutant strain is affected in expression of many genes, mostly important for *P. gingivalis* virulence under low-iron/heme conditions, which results in slightly retarded growth during the early growth phase, but production of higher bacterial biomass after prolonged growth [38].

2.2. Human cell cultures

Human cervical epithelial HeLa cells (ATCC CCL-2; The Polish Collection of Cell Lines of Polish Academy of Sciences, Wroclaw, Poland) were grown in Dulbecco's Modified Eagle's Medium (DMEM, Cytogen), supplemented with 10% heat-inactivated FBS (Cytogen), 100 μg/ml streptomycin, 100 U/ml penicillin (Cytogen), and 4 mM L-glutamine (Cytogen) at 37 °C in the presence of 5% CO_2.