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Inhibition of methionine gamma lyase deaminase and the growth of *Porphyromonas gingivalis*: A therapeutic target for halitosis/periodontitis

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ABSTRACT

Background and objectives: Pathogenic infections caused by *Porphyromonas gingivalis*, *Treponema denticola*, *and Tannerella forsythia* can result in the production of volatile sulfur compounds (VSC's) and other toxic compounds from methionine catabolism that can lead to halitosis and periodontitis. Our aim is to block the activity of methionine gammalyase-deaminase (Mgld) of methionine catabolism to prevent halitosis/periodontitis.
Designs: Cloned, expressed, Mgld protein was tested for purity by SDS-PAGE and western blotting. Mgld activity was tested by UV–vis spectroscopy and DTNB assay. Effects of Mgld inhibitor propargylglycine (PGLY) was tested on *P. gingivalis* growth by turbidity measurements. The effects of PGLY on oral epithelial and periodontal ligament cells in culture at different concentrations and time were tested for cell viability by MTT and Live-Dead assays. Amino acid comparisons of Mgld from different oral pathogens were done using standard bioinformatics program.
Results: Propargylglycine (PGLY) inhibited purified Mgld activity completely. *In vivo*, PGLY is a potent inhibitor on the growth of the *P. gingivalis* over 24 h, grown at 25 °C and 37 °C. Correspondingly *in vivo* Mgld activity was also affected by PGLY. Amino acid comparisons of oral pathogens showed 100% identity on the key residues of Mgld catalysis. Mammalian oral cell lines with PGLY, showed no difference in cell death over untreated controls assessed by MTT and Live-Dead assays.
Conclusions: PGLY arrest's VSC's production by *P. gingivalis*. Since initial Mgld activity is inhibited subsequent enzymatic and nonenzymatic products formed will be prevented. PGLY showed no toxicity towards cultured mammalian oral cells. Thus, PGLY can serve as a mouthwash ingredient to prevent halitosis/periodontitis.

1. Introduction

Halitosis, commonly called bad breath, is one of the dental care problems that people approach dentists for treatment (Bosy, 1997; Cortelli, Barbosa, & Westphal, 2008; Eli, Baht, Koriat, & Rosenberg, 2001; Fedorowicz, Aljufairi, Nasser, Outhouse, & Pedrazzi, 2016; Rosenberg, 1996, 2002; Tangerman, 2002; Tonzetich, 1977; Winkel, 2008; Yaegaki & Coil, 2000; Yaegaki, Coil, Kamemizu, & Miyazaki, 2002; Zalewska et al., 2012). Roughly, 20% of the people from general populations seem to suffer from this conditions in varying degrees. Of these, about 90% suffer from halitosis due to colonization of bacteria in sublingual regions, gingival margins, and the dorsum of the tongue (Rosenberg, 1996, 2002; Zalewska et al., 2012). Some of the pathogenic bacteria include *Porphyromonas gingivalis*, *Treponema denticola*, *and Tannerella forsythia* (Miyazaki et al., 1999). Halitosis can be easily controlled by minor adjustments in oral hygiene including brushing or gently scraping the dorsum of the tongue and improving the general oral health of the periodontium (Cortelli et al., 2008; Eli et al., 2001; Tangerman, 2002; Tonzetich, 1977; Yaegaki et al., 2002). Classification of genuine halitosis into different types involves: a) physiological or b) pathological (Miyazaki et al., 1999). Another classification of genuine halitosis is based only on a) intra-oral halitosis and b) extra-oral halitosis (Tangerman & Winkel, 2010). Different classification is based on blood-borne: (a) systemic diseases (b) metabolic diseases (c) food or (d) medication. Or Non-blood borne: (a) upper respiratory tract and (b) lower respiratory tract infections. Classification of halitosis according to the type of odor produced involves sulfurous odor caused by Volatile Sulfur Compounds (VSC's) most notably methylthiol/mercaptan,

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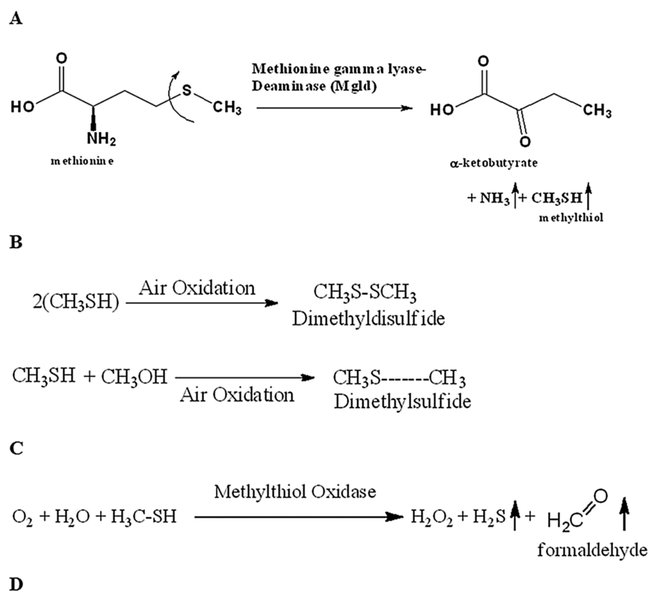

Fig. 1. A. Methionine gammalyasedeaminase (Mgld)-pyridoxal phosphate (PLP) catalyzed reaction, (Formation of methylthiol and ammonia). B. Non-enzymatic formation of Volatile Sulfur Compounds (VSC). C. Conversion of methylthiol into reactive compounds by methylthiol oxidase. D. If Mgld lyase activity is inhibited what enzymatic and non-enzymatic products of methionine degradation can be prevented?.

dimethyl sulfide, dimethyldisulfide and hydrogen sulfide. Or fruity odor (acetone breath) of diabetics and/or odor due to ammonia, dimethylamine and trimethylamine (fish odor syndrome) (Mountain, Brisbane, Hooper, Burnett, & Goldblatt, 2008). Chronic halitosis can be managed by a) cleaning the tongue surfaces gently b) rinsing c) maintaining proper oral hygiene, including daily tongue cleaning, brushing and flossing (Loesche & Kazor, 2002). Mouthwashes often contain antibacterial agents such as cetylpyridinium chloride, chlorhexidine, zinc gluconate, essential oils, and chlorine dioxide. Nevertheless, the existing mouthwashes are not successful in curing chronic halitosis and new improved inventions are warranted.

Methionine gamma lyase deaminase (Mgld) cleaves the sulfur amino acid methionine into methylthiol (CH_3SH) and forms the deaminated product 2-ketobutyrate (alpha-ketobutyrate) and ammonia (Fig. 1A). Two moles of methylthiol can air oxidize to form dimethyl disulfide (H_3C-S-S-CH_3) or non-enzymatically can react with fermentative product methanol to form dimethyl sulfide (H_3C-S-CH_3) (Fig. 1B). Methylthiol can also be converted into reactive compounds such as formaldehyde (H2C=O), hydrogen peroxide (H_2O_2) and hydrogen sulfide (H_2S) by uncharacterized methylthiol oxidase (Fig. 1C). Our aim is to inhibit the production of the starting product methylthiol and ammonia so that all downstream formations from methylthiol (Fig. 1D) can be arrested. In this paper, we describe the *in vitro* (purified Mgld) and *in vivo* inhibition of Mgld from *Porphyromonas gingivalis* by PGLY. In addition, we show severe growth inhibition of cultured *P. gingivalis* by PGLY for nearly 24 h. Thus, PGLY proves to be a very good candidate as a mouthwash ingredient to target halitosis and periodontitis.

2. Materials and methods

2.1. Mgld purification

E. coli BL21(DE3) *containing pET-Mgld* vector was grown in Terrific Broth (TB) with continuous shaking at 37 °C supplemented with kanamycin (40 μg/ml) until A600 of > 0.9 which indicates turbidity due to growth. Protein expression was then induced with 0.5 mM IPTG and the cultures were further incubated at 37 °C with shaking for 9 h. Cells were harvested at 5500 g for 30 min and the centrifuged cells were lysed with a mixture of: lysis buffer [50 mM KP buffer, 1 mM EDTA, 0.02 mM PLP, 2% beta-mercaptoethanol, 1U/1 ml Dnase I, protease inhibitor (1%) and lysozyme (1 μg/ml)]. Lysed cells were centrifuged at 5000*g* for 40 min at 4 °C. Crude supernatant was transferred into fresh tubes. 100 ml of crude extract was purified through a nickel column (bed volume of 11 ml) using a linear gradient of 20–200 mM of imidazole. Protein was measured using Bradford and UV–vis methods. Mgld-pyridoxal phosphate (PLP) has an absorption maxima at ~425 nm. The purity of Mgld was confirmed by 280:425 nm absorbance ratio. A 280:425 nm ratio of four or greater indicates higher purity. Pure protein fractions were then pooled and concentrated to ~(0.5–1 ml) using a 0.2 μm Amicon filter. The concentrated Mgld fractions were TEV protease cleaved (1:100 ratio) at 4 °C, in the dark, for 12 h. The resulting cleaved enzyme was subjected to DEAE column chromatography (15 ml bed volume). The column was then washed with PBS 10 mM + 100 mM NaCl and then a linear gradient of 100–600 mM of NaCl was applied. Protein fractions of interest were assayed independently through Bradford and DTNB assay, using the UV–vis (PLP) absorbance method. Protein fractions were then pooled, and passed through again on a 0.2 μm Amicon filter to remove all small molecular weight compounds. Exogenous PLP 0.1 mM 1:1 ratio with protein concentration was added and incubated in the dark at 4 °C, for 12 h. Free PLP was removed through an additional Amicon filtration. The purity of Mgld was tested using SDS-PAGE and western blot with PLP antibody. Mgld lyase activity was tested using a DTNB assay. Briefly, 10 μl of 2 mg/ml purified Mgld, 10 μL of 10 mM methionine, 20 μl of 5 mM DTNB and 60 μL of PBS were gently mixed on a microtiter plate and incubated with mineral oil overlay and incubated at 25 °C for 10 min. Immediately following incubation the product formation was detected by absorbance at 412 nM using a Synergy microtiter plate reader. Detailed procedures of Mgld purification, and assays were followed according to the procedures of Venkatachalam et al.

2.2. Inhibition study

Purified Mgld (2 mg/ml) was preincubated with 30 μM aqueous solution of PGLY at room temperature for 10 min. Control tubes contained water instead of PGLY. After 10 min of incubation, PGLY reacted and unreacted enzyme samples were assayed for lyase half reaction according to the mineral oil overlay DTNB assay devised by Venkatachalam (patent pending).

2.3. Cell culture

The oral (tongue) squamous epithelial cells (TR 146, Sigma-Aldrich) and periodontal ligament (PDL) (Sciencell) fibroblasts were used for this study.

TR 146 cells were cultured in HAMS F12 medium supplemented with Glutamine, 10% Fetal Bovine Serum (FBS) and 1% Antibiotics. PD fibroblasts were grown in Dulbecco's Modified Eagle Medium (Thermofisher, Waltham, MA) supplemented with 10% FBS and 1% Antibiotics. Cells in T75 flasks were incubated at 37 °C, 5% CO_2. The cell cultures were maintained by changing the medium twice a week.

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