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Antidiabetic "gliptins" affect biofilm formation by *Streptococcus mutans*

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ABSTRACT

Streptococcus mutans, a dental caries causing odontopathogen, produces X-prolyl dipeptidyl peptidase (Sm-XPDAP, encoded by *pepX*), a serine protease known to have a nutritional role. Considering the potential of proteases as therapeutic targets in pathogens, this study was primarily aimed at investigating the role of Sm-XPDAP in contributing to virulence-related traits. Dipeptidyl peptidase (DPP IV), an XPDAP analogous enzyme found in mammalian tissues,is a well known therapeutic target in Type II diabetes. Based on the hypothesis that gliptins, commonly used as anti-human-DPP IV drugs, may affect bacterial growth upon inhibition of Sm-XPDAP, we have determined their *ex vivo* antimicrobial and anti-biofilm activity towards *S. mutans*. All three DPP IV drugs tested reduced biofilm formation as determined by crystal violet staining. To link the observed biofilm inhibition to the human-DPP IV analogue present in *S. mutans* UA159, a *pepX* isogenic mutant was generated. In addition to reduced biofilm formation, CLSM studies of the biofilm formed by the *pepX* isogenic mutant showed these were comparable to those formed in the presence of saxagliptin, suggesting a probable role of this enzyme in biofilm formation by *S. mutans* UA159. The effects of both *pepX* deletion and DPP IV drugs on the proteome were studied using LC–MS/MS. Overall, this study highlights the potential of Sm-XPDAP as a novel anti-biofilm target and suggests a template molecule to synthesize lead compounds effective against this enzyme.

1. Introduction

Dental caries is the most prevalent, multifactorial, globally increasing oral health problem among children and adults (Bagramian et al., 2009; Selwitz et al., 2007). It is a manifestation of biofilm formation by certain members of the indigenous oral microbiota that are aciduric and acidogenic. Among them, *Streptococcus mutans* is one of the key etiological agents of dental caries. *S. mutans* is known to code for several peptidases and exoglycosidases that can facilitate utilization of human saliva as a source of nutrition (Ajdić et al., 2002). X-prolyl dipeptidyl aminopeptidase (XPDAP) is one such narrow substrate range cytoplasmic endopeptidase found in *S. mutans*, which help in utilization of proline-rich salivary polypeptides (Cowman and Baron, 1997, 1993). Collagenolytic and caseinolytic activities demonstrated by XPDAP further suggest its importance as a putative virulence factor in *S. mutans* (Cowman et al., 1975; Rosengren and Winblad, 1976). In *Streptococcus suis* and *Streptococcus gordonii* extracellularly present XPDAP play a role also in cellular invasion (Ge et al., 2009; Goldstein et al., 2001). Similarly, periodontal pathogen *Porphyromonas gingivalis* also had altered virulence in absence of XPDAP (Yagishita et al., 2001).

An analogous enzyme to XPDAP, DPP IV is also found in mammalian tissues and is a target for maintaining glucose homeostasis in Type II diabetic patients (Cowman and Baron, 1997; Green et al., 2006). Diabetes, an abnormal metabolic disorder, is an epidemic of significant healthcare concern among both developed and developing countries (King et al., 1998). Certain drugs, namely saxagliptin, vildagliptin and sitagliptin, are commonly used anti-human DPP IV (AHD) molecules used in the treatment of Type II diabetes (Green et al., 2006). DPP IV targets incretin hormones such as GLP-1, thereby decreasing their plasma levels. Inhibition of DPP IV leads to the opposite effect, which results in a restoration of glucose homeostasis in diabetic patients (Wang et al., 2012). As a novel approach, our recent investigation has shown that *S. mutans* XPDAP (Sm-XPDAP, encoded by *pepX*) is inhibited by saxagliptin *in vitro* (De et al., 2016). In an extension of this work and hypothesising a probable role of Sm-XPDAP in virulence, herein we have evaluated the *ex vivo* effect of these molecules on cell growth and

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biofilm formation by *S. mutans*. A *pepX* (SMU.35) isogenic mutant was also generated. Furthermore, whole cell proteome analysis of AHD treated cells and the isogenic mutant was performed to identify possible consequences of Sm-XPDAP inhibition or deletion.

2. Materials and methods

2.1. MIC and biofilm formation assay

The MIC was determined by microdilution assay according to the Clinical and Laboratory Standards Institute (CLSI, 2011), with the exception of the medium used, which was BHI (Ahn et al., 2012; da Silva et al., 2013). The highest concentration of 2048 μg/mL of each AHD was serially diluted down to 4 μg/mL and the final density of mid-exponential phase cells used was 10^6 CFU/mL. The drugs were dissolved in sterile water and erythromycin was used as a positive control.

Biofilm formation was assessed by a semi-quantitative crystal violet method in polystyrene 96-well cell culture plates (Costar 3595; Corning Inc., NY) as previously described (Ahn et al., 2008). An overnight culture of *S. mutans* UA159 (ATCC 700610) was transferred into pre-warmed BHI and grown till mid-exponential phase and then diluted 50 fold in semi-defined biofilm medium (SDM) supplemented with 20 mM glucose or sucrose. Aliquots (100 μL) of this culture were added to serially diluted drug in water (2048 μg/mL to 4 μg/mL), to make a final volume of 200 μL (with a 100-fold final dilution of cells) and incubated for 20 h. The adhered cells were stained with 1% crystal violet for 15 min and the extract was quantified at 495 nm. The viability of the cells in the culture medium (planktonic phase over the formed biofilm) at each concentration of drug was determined by CFU counting.

2.2. Construction of pepX deletion mutants

A *pepX* deletion mutant was generated by PCR ligation mutagenesis method (Lau et al., 2002). Briefly, erythromycin cassette, upstream and downstream flanking regions (about 600 bps) of *pepX* was amplified using specific primers (Table 2). The amplicons were digested using NcoI and SacI and ligated at 16 °C overnight. The resulting ligation mixes were used for PCR to obtain a mutagenic construct using primers pepX-Up-F and pepX-Dn-R. This fragment was naturally transformed into mid-log phase *S.mutans* UA159, grown in Todd-Hewitt broth containing 10% sucrose and the recombinants were selected on BHI agar containing 10 μg/mL erythromycin (Petersen and Scheie, 2000). *The pepX* mutant was confirmed by colony PCR and Sanger sequencing.

2.3. CLSM examination of biofilm

The structure of biofilm of the *pepX* mutant and wild-type *S. mutans*, grown on polystyrene discs in the presence of SDM and glucose and AHD drugs as described above, was evaluated using an LSM-510-META laser scanning microscope attached to an Axioplan-II microscope (Zeiss). The non-adherent cells were washed in saline, biofilms were stained with Live/Dead BacLight-(1X) (Molecular Probes Inc.) for 20 min and rinsed three times in saline to remove excess stain. Subsequently, the stained discs were examined with an alpha Pan-Fluor 100X objective under excitation at 488 nm (Argon-laser) and 543 nm (He-Ne-laser), and emission filter ranging 585–615 nm and 505–530 nm for Propidium iodide and SYTO 9, respectively. ImageJ v1.48 (NIH, USA) was used to process images. The proportion of viable cells (green) versus dead cells (red) was determined based on the intensity at each pixel using ImageJ (Nance et al., 2013). The proportion of green signal and the red signal was calculated by multiplying the total number of pixels with the given intensity (0 −255) at each channel and then dividing it by the sum of the intensity value for each signal measured at each image stack.

2.4. Proteome analysis of biofilm-grown cells

The proteome of biofilm-grown cells in absence or presence of an AHD drug and that of the Δ*pepX* mutant were analysed from 48 mL of culture. In brief, after 20 h incubation, the harvested biofilm cells were lysed. The lysate was used to acetone precipitate all proteins overnight at −20 °C. The precipitate was washed in 80% and 40% acetone successively, air dried and then partially pre-fractionated by 1D PAGE. The protein containing gel was stained, destained and sectioned into pieces for treatment in 100 mM NH_4HCO_3 and acetonitrile (ACN) for complete removal of Coomassie stain. The gel slices were dehydrated in ACN and digested in 40 μg/mL trypsin (Trypsin Gold, MS Grade, Promega) at 37 °C overnight. Digestion was then stopped by adding 50% ACN (v/v) and 5% formic acid (v/v) with shaking for 30 min. The peptides-containing digested extract was removed and the gel pieces were further extracted in ACN and formic acid before freeze drying. The lyophilized peptide digest was mixed in 5% ACN and 0.1% formic acid (v/v) and then run in a LC-NanoPump coupled to a tandem mass spectrometer (Thermo-Q-Exactive attached to HPLC Ultimate-3000-RSLCnano system), through an Easy Spray C18 column (PepMap-RSLC, 75 μm × 500 mm, Thermo Scientific) in a gradient solvent mixture of water and ACN containing 0.1% formic acid. The run was carried out for 215 min at a flow rate of 0.3 μL/min with a scan range of 350–1800 m/z. The mass spectrum data (MS and MS^2) was then processed in Progenesis LC–MS v4.1 and the peptides identified in MASCOT database (Matrix Science, UK).

2.5. Statistical analysis

Statistical analysis was performed using the software Statgraphics Centurion ver. XV (Statpoint Technologies, Inc., Virginia, USA). Fitting of data was done using Origin ver. 8.1 (Origin Lab Corporation, MA, USA). Inhibition of biofilm formation data were fitted using a dose/response function (Boltzmann), according to the following Eq. (1):

$$y = A2 + \frac{A1 - A2}{1 + e^{(x - EC50)/dx}} \quad (1)$$

where A2 and A1 are the maximum and minimum level of biofilm formed, respectively, and EC_{50} is the concentration of the drug affecting 50% biofilm formation. EC_{50} values from the four replicates of the same drug were derived and their mean calculated. Distribution of EC_{50} values of the same drug was checked for normality. Analysis of variance (ANOVA) test was run to compare means from different drugs and to check variance. Probability value threshold was set to 0.05. Multiple comparison analysis was performed between pairs of data sets (considered as independent), corresponding to biofilm formation level ($OD_{490\,nm}$) at each and every concentration of the given drug. F-test (ANOVA) was used to test for the rejection of the null hypothesis ($P < 0.05$).

3. Results

3.1. AHD drugs inhibited biofilm formation by S. mutans

AHD drugs did not show a visible growth inhibition effect in the concentration range studied (4–2048 μg/mL). In the presence of sucrose, *S. mutans* sessile growth was not affected by any of the drugs, whereas all three AHD drugs inhibited biofilm formation in the presence of glucose, albeit with different potency (Fig. 1A). At concentrations > 128 μg/mL of saxagliptin, there was 50% reduction of biofilm formation. Vildagliptin inhibited biofilm formation at concentrations ≥ 256 μg/mL, while sitagliptin demonstrated at least 50% inhibition at 256 μg/mL, albeit with a substantial increase in biofilm biomass at 2048 μg/mL. The EC_{50} values of all drugs fell within the range of 128–512 μg/mL. However, in the case of sitagliptin, the data point at 2048 μg/mL was not considered for fitting due to the