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The Inhibitory Effects of Quaternary Ammonium Methacrylates on Soluble and Matrix-bound MMPs

ABSTRACT

Matrix metalloproteinases (MMPs) bound to dentin contribute to the progressive degradation of collagen fibrils in hybrid layers created by dentin adhesives. This study evaluated the MMP-inhibiting potential of quaternary ammonium methacrylates (QAMs), with soluble rhMMP-9 and a matrix-bound endogenous MMP model. Six different QAMs were initially screened by a rhMMP-9 colorimetric assay. For the matrix-bound endogenous MMPs, we aged demineralized dentin beams for 30 days in calcium- and zinc-containing media (CM; control), chlorhexidine, or QAMs in CM to determine the changes in dry mass loss and solubilization of collagen peptides against baseline levels. The inhibitory effects of QAMs on soluble rhMMP-9 varied between 34 and 100%. Beams incubated in CM showed a 29% decrease in dry mass ($p < 0.05$), whereas beams incubated with QAMs showed only 0.2%-6% loss of dry mass. Significantly more solubilized collagen was detected from beams incubated in CM ($p < 0.05$). It is concluded that QAMs exhibited dentin MMP inhibition comparable with that of chlorhexidine, but required higher concentrations.

KEY WORDS: dentin, hydroxyproline, MDPB, matrix metalloproteinase, quaternary ammonium methacrylates.

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INTRODUCTION

Matrix metalloproteinases (MMPs) bound to dentin are thought to contribute to the progressive degradation of collagen fibrils in hybrid layers formed during dentin bonding. Dentin matrix contains MMP-2, 8, 9, and 20 (Sulkala *et al*., 2007). During the acid-etching phase of bonding, MMPs that are normally bound to mineralized collagen fibrils become exposed and activated (Mazzoni *et al*., 2006; Tay *et al*., 2006). Inhibition of host-derived MMPs may retard the degradation of resin-dentin bonds over time (Pashley *et al*., 2004; Hebling *et al*., 2005; Carrilho *et al*., 2007a,b). Chlorhexidine (CHX) has broad anti-MMP activity (Gendron *et al*., 1999) in addition to its antimicrobial activity. When demineralized dentin was incubated with 0.2 wt% CHX, collagen degradation was almost completely blocked (Pashley *et al*., 2004; Breschi *et al*., 2010). However, CHX is water-soluble and may leach out of hybrid layers and compromise its long-term anti-MMP effectiveness.

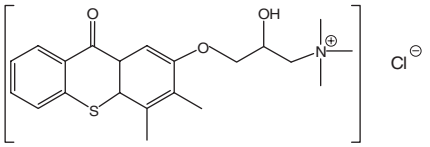
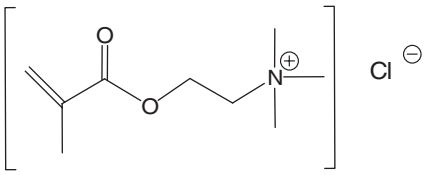
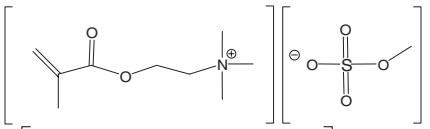
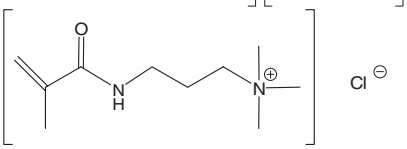
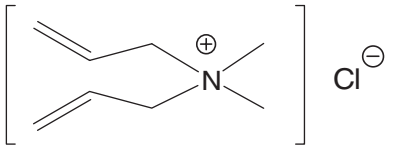
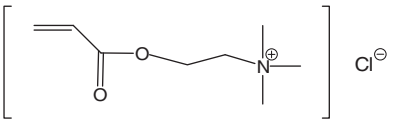
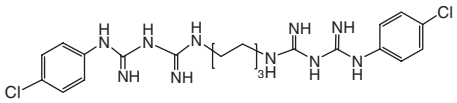
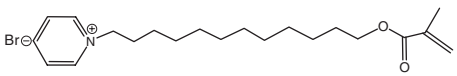
Quaternary ammonium compounds possess antimicrobial properties (Pernak *et al*., 2001) and have been incorporated into dental resins (Imazato *et al*., 1997; Xiao *et al*., 2008; Imazato, 2009; Namba *et al*., 2009). Similar to CHX, these compounds are water-soluble and may leach out of bonded interfaces. The use of polymerizable quaternary ammonium methacrylates (QAMs) such as 12-methacryloyloxydodecylpyridinium bromide (MDPB) (Imazato *et al*., 1997, 2007; Imazato, 2009; Li *et al*., 2009) is advantageous in that they can copolymerize with adhesive monomers. Since cationic CHX inhibits MMPs, we speculate that cationic quaternary ammonium methacrylates would also inhibit the endogenous dentin MMPs.

The purpose of this study was to screen the MMP-inhibiting potential of 6 quaternary QAMs using a soluble recombinant MMP-9 (rhMMP-9). The effects of QAMs on matrix-bound endogenous MMPs were also evaluated in demineralized dentin. The null hypothesis tested was that QAMs have no effect on MMP inhibition.

MATERIALS & METHODS

The chemical structures and abbreviations of the potential inhibitors are listed in the Table. All inhibitors except MDPB were purchased from Sigma-Aldrich

Table. The Chemical Structures of the Materials Used in This Study

OTX	3-(3,4-dimethyl-9-oxo-9H-thioxanthen-2-yloxy)-2-hydroxypropyl trimethylammonium chloride	
METMAC	[2-(Methacryloyloxy)ethyl] trimethylammonium chloride	
MCMS	Methacryloyl choline methyl sulfate	
MAPTAC	[3-(Methacryloylamino)propyl] trimethylammonium chloride	
DDAC	Diallyldimethylammonium chloride	
ATA	2-Acryloxyethyltrimethylammonium chloride	
CHX	Chlorhexidine digluconate	
MDPB	12-methacryloyloxydodecylpyridinium bromide	

(St. Louis, MO, USA) and used as received. The MDPB was used as a powder as received from the manufacturer (Kuraray Medical Inc., Tokyo, Japan). All QAMs except for MDPB were used as 30 wt% solutions for screening. The MDPB was used as 5 wt% based on the concentration utilized in commercial products containing this resin monomer (Imazato, 2009). Chlorhexidine digluconate and OTX, a hydrophilic tertiary amine photo-accelerator (Ye *et al*., 2008), were used at 0.2 wt%.

We used two screening levels to evaluate the inhibitory activity of QAMs on MMPs. The first level involved using soluble human rhMMP-9 in a colorimetric assay. The second level used matrix-bound endogenous MMPs present in completely demineralized dentin.

rhMMP-9 Assay

Purified human rhMMP-9 and a generic MMP Assay kit (Sensolyte, AnaSpec Inc., Fremont, CA, USA) were used. The procedures involved incubating a constant concentration of human MMP-9 with a proprietary chromogenic substrate from the assay kit. The latter is a thiopeptolide that can be cleaved by MMPs and collagenases to release a sulfhydryl group. The sulfhydryl group reacts with 5,5′-dithiobis(2-nitrobenzoic acid) to produce a colored reaction product (2-nitro-5-thiobenzoic acid) that can be detected spectrophotometrically at 412 nm.

The thiopeptolide substrate solution was diluted to 0.2 mM with the assay buffer in a 1:50 volume ratio. The rhMMP-9 was activated with trypsin (10 μg/mL) at 37°C for 2 hrs immediately before the experiment, after which the trypsin was inactivated with trypsin inhibitor. The assay was performed in quinta-replicates in a 96-well plate for each experimental QAM inhibitor and control. In each experimental well, 2 μL of rhMMP-9 (19.6 ng/well), and 10 μL of the potential MMP inhibitor were pre-incubated for 20 min to avoid the burst of MMP-9 activity that occurs when all reagents are mixed together simultaneously. After pre-incubation, additional assay buffer and 50 μL of the

thiopeptolide solution were added to reach a total of 100 μL for each well. We mixed the reagents completely by vibrating the plate gently for 30 sec, and readings were taken every 10 min for 60 min. Absorbance was measured at 412 nm by means of a plate reader (Synergy HT, BioTek, Winooski, VT, USA).

Background absorbance of the controls was determined from the mean corresponding absorbance readings and subtracted from the readings of the positive control. For the QAMs, the background of each QAM was determined separately and subtracted from the wells containing the MMP-9 and thiopeptolide substrate. The potencies of MMP-9 inhibition by the proprietary MMP kit inhibitor (GM6001) and QAMs groups were expressed as percentages of the adjusted absorbance of the "positive control", which was taken to be 100% inhibition. Neither OTX nor CHX could be used to inhibit MMP-9, because they created a yellow color with the substrate, even in the absence of MMP-9. We analyzed the data statistically to examine the effects of QAMs on MMP-9 inhibition. Since the normality and homoscedasticity assumptions of the data appeared to be valid, % inhibition in the six groups was analyzed by one-way ANOVA and Tukey multiple comparison tests at $\alpha = 0.05$.

Matrix-bound MMPs in Demineralized Dentin

Ninety extracted unerupted human third molars were obtained from 18- to 21-year-old patients (Martin-de-las Heras *et al*., 2000a,b) with their informed consent under a protocol approved by the Human Assurance Committee of the Medical College of Georgia. Ninety percent of the teeth had completely formed roots. None of the teeth was carious. The teeth were stored at 4°C in 0.9% NaCl supplemented with 0.02% NaN_3 to prevent bacterial growth and were used within 1 mo of extraction. The enamel and superficial dentin of each tooth were removed from the crown by means of an Isomet saw (Buehler Ltd., Lake Bluff, IL, USA) under water cooling. Dentin beams with dimensions 6 x 2 x 1 mm were sectioned from the middle of each dentin disk (*i.e*., 90 beams).

The beams were completely demineralized in 10 wt% H_3PO_4 (pH 1) for 18 hrs at 25°C. We used digital radiography to confirm the absence of residual minerals. They were randomly divided into 9 groups (N = 10). The groups included 6 QAMs (ATA, MCMS, MAPTAC, METMAC, DDAC, 30 wt% each and 5 wt% MDPB), as well as 0.2 wt% CHX or OTX prepared in a calcium- and zinc-containing complete storage medium (CM), or CM only. The CM contained 5 mM HEPES, 2.5 mM $CaCl_2.H_2O$, 0.05 mM $ZnCl_2$, and 0.3 mM NaN_3 (pH 7.4). Each beam was placed in 1.0 mL of respective incubation medium in individually labeled polypropylene tubes. The control groups were incubated in CM only. The tubes were incubated in a shaker-water bath (60 cycles/min) at 37°C for 30 days.

Matrix-bound MMP Activity Assessment

Loss of Dry Mass over Time

This attribute was used as an indirect measure of MMP-induced hydrolysis of endogenous matrix collagen (Carrilho *et al*., 2009; Tezvergil-Mutluay *et al*., 2010). After incubation, the beams were transferred to individually labeled, uncapped polypropylene tubes and placed in a sealed desiccator containing anhydrous calcium sulfate (Drierite, W.A. Hammond Company, Xenia, OH, USA). They were desiccated to a constant weight within 8 hrs. The initial dry mass was measured to the nearest 0.001 mg. After dry mass determination, the desiccated dentin beams were rehydrated with water for 1 hr (Agee *et al*., 2006) to their original dimensions before returning to the corresponding polypropylene tubes containing the original incubation medium. After 30 days of incubation, dry mass was re-measured under the same conditions. Since the normality and homoscedasticity assumptions of the data had been violated, loss of dry mass from the demineralized dentin beams was evaluated by Kruskal-Wallis one-way ANOVA and Dunn's multiple comparison tests at $\alpha = 0.05$.

Solubilized Collagen Peptides

The other index of matrix degradation was determined by measurement of the quantity of collagen peptide fragments that were solubilized over the 30-day incubation period. The collagen in demineralized dentin is insoluble type I collagen (Carrilho *et al*., 2009; Tezvergil-Mutluay *et al*., 2010). Demineralization of the mineralized dentin matrix with 10% phosphoric acid exposes and activates endogenous MMPs, even though they remain bound to the collagen (Martin-de-las Heras *et al*., 2000a). At the end of the incubation period, a 400-μL quantity of the medium was collected from each vial and placed in an individually labeled ampule, diluted with an equal volume of 12 N HCl to give a final concentration of 6 N HCl. The ampules were sealed (Ampulmatic, Biosciences Inc., Allentown, PA, USA), and the media were hydrolyzed at 120°C in an oil bath for 18 hrs. After hydrolysis, the ampules were opened and placed in glass desiccators containing anhydrous Drierite and NaOH pellets to trap the HCl vapor released from the hydrolysates as they were evaporated to dryness. The hydroxyproline content of each hydrolysate was analyzed spectrophotometrically at 558 nm by the method of Jamall *et al*. (1981). The hydroxyproline content was used to estimate the percent of degraded collagen, assuming that 90% of the dry mass of demineralized dentin consists of type I collagen, and that dentin collagen contains 9.6 mass% of hydroxyproline (Butler, 2000). For each specimen, the solubilized collagen was expressed as micrograms of hydroxyproline/mg of the dry mass of the demineralized dentin before incubation. Since the normality and homoscedasticity assumptions of the data had been violated, the data were evaluated by Kruskal-Wallis one-way ANOVA and Dunn's multiple comparison tests at $\alpha = 0.05$.

RESULTS

Inhibition of Soluble MMP-9

The inhibitory effects of QAMs on soluble rhMMP-9 varied between 34 and 100% (Fig. 1). The inhibitory effects of ATA, MCMS, and METMAC were from 97 to 100%, whereas MAPTAC and DDAC inhibited MMP-9 by only 34 and 55%, respectively. Inhibition by MDPB was approximately 89%, which was comparable with the kit inhibitor, GM 6001 (88%).

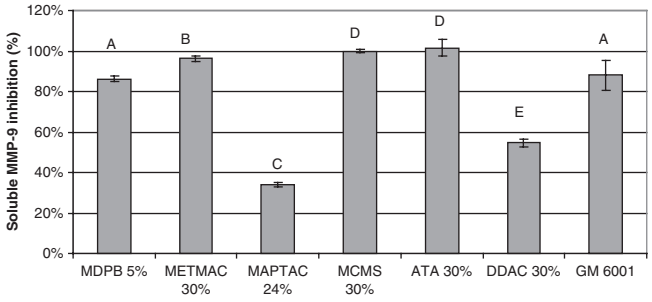


Figure 1. A bar chart comparing the percentage inhibition of rhMMP-9 by the control (GM 6001) and 6 potential MMP inhibitors. Values are means and standard deviations. Groups with the same letters on top of the bars are not statistically different ($p > 0.05$). See the Table for definition of abbreviations.

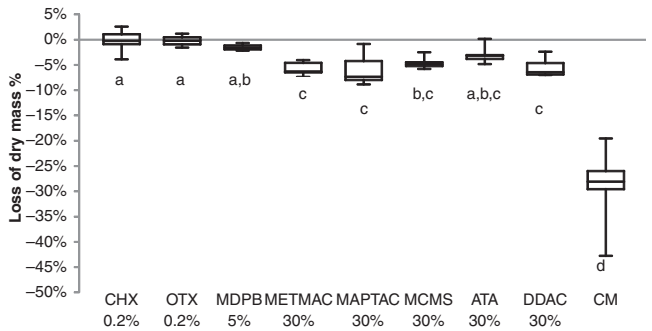


Figure 2. The loss of dry mass (%) from demineralized dentin beams incubated over a 30-day period in the control (CM) *vs.* QAM-, CHX-, or OTX-containing media. The loss of dry mass from each beam was calculated as a percentage of the dry mass of that beam at baseline. Groups with the same lower-case letter are not statistically significant ($p > 0.05$) by Kruskal-Wallis and Dunn's multiple comparisons. See the Table for definition of abbreviations.

Inhibition of Matrix-bound MMPs

Loss of dry mass (Fig. 2) and solubilization of collagen peptides (Fig. 3) over the 30-day incubation period showed significant differences between the CM control and the QAMs. Dentin beams incubated with QAM showed a 0.2-6% decrease in dry mass, compared with a 29% decrease in the CM control. If one assumes that the loss of dry mass is due to the collagenolytic action of endogenous MMPs, the QAMs inhibited those enzymes 79.3- 99.3% compared with the CM control.

A similar trend was observed with the dissolution of collagen peptides determined by hydroxyproline analysis. The beams stored in the CM medium liberated 35 μg hydroxyproline/mg dentin, whereas beams incubated in media containing inhibitors liberated 0.2-1.5 μg hydroxyproline/mg dentin. Thus, over the incubation period, endogenous MMPs released 39.9% of the total hydroxyproline available in the control beams, which is greater than one-third of the total insoluble collagen present in those beams (Appendix). Conversely, the inhibitors exhibited 98.6-99.4% inhibition of the insoluble, matrix-bound MMPs.

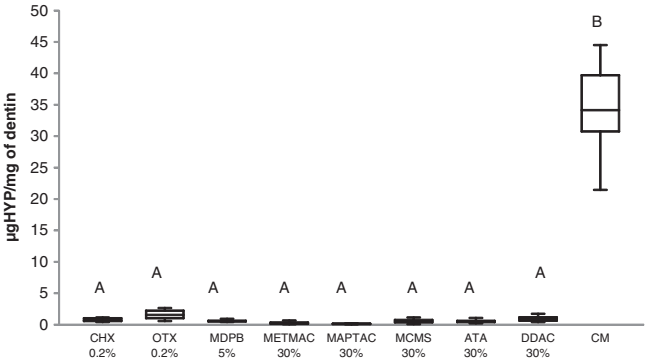


Figure 3. Hydroxyproline contents derived from the aging media (QAMs, CHX, and OTX) and the control (CM) after the 30-day incubation period. For each specimen, the dissolved collagen from the demineralized dentin beam was expressed as micrograms of hydroxyproline/mg of the dry mass of the baseline demineralized dentin. Groups with the same upper-case letter are not statistically significant ($p > 0.05$) by Kruskal-Wallis and Dunn's multiple comparisons. See the Table for definition of abbreviations.

DISCUSSION

In our previous papers, we have reported that acid-etching dentin powder with 37 wt% phosphoric acid (PA) has produced 65% (Pashley *et al*., 2004) or 98.1% inhibition (Mazzoni *et al*., 2006) in collagenolytic activity. We speculated, at that time, that those results indicated that although 37% PA uncovered the collagen matrix and exposed MMPs, it may have denatured the enzymes as soon as they were exposed. Later, work by Nishitani *et al*. (2006) using self-etching adhesives showed that they too exposed MMPs and activated them without denaturing the enzymes, because the pH was only 2-3 instead of -0.37 for 37% PA (Pashley *et al*., unpublished observations).

Preliminary unpublished experiments by Nishitani on the effects of 37% PA on the gelatinolytic activity of mineralized dentin powder revealed an initial enzyme activity of 126 ± 15 RFU/80 mg/24 hrs. When that dentin powder was acid-etched with 37% PA for 15 sec, the enzyme activity fell to 61 ± 28 RFU/80 mg/24 hrs. He continued to incubate the acid-etched dentin powder in pH 7.4 buffer at 37°C for 1, 2, and 4 wks. The enzyme activity slowly rose from 61 ± 28 to 369 ± 58 to 873 ± 126 and to 962 ± 115 RFU/80 mg/24 hrs over the ensuing 4 wks (n = 22 wells *per* time period). Clearly, acid-etching with PA initially lowered MMP activity but later increased it 16-fold. We now believe that acid-etching dentin with 37% PA raises the local calcium ion concentrations so high that it forms insoluble reaction products (*i.e*., $CaHPO_4$) that precipitate as fine crystals over exposed collagen and MMPs, thereby blocking access of fluorescent substrates to the enzyme. Within days, these relatively soluble reaction products can dissolve, thereby allowing substrates access to the enzymes. Thus, the notion that 37% PA denatures all MMPs may not be correct. Gelatinase A (MMP-2)

is known to resist extremes in temperature and pH (Sulkala *et al*., 2007).

This is why we can successfully completely demineralize mineralized dentin beams in 10 wt% PA without denaturing MMPs. If they were denatured, the beams would not lose dry mass over time. They would not slowly solubilize collagen peptides over time. However, beams incubated in simulation body fluids did lose dry mass and did solubilize collagen. Beams incubated in MMP inhibitors like chlorhexidine (Carrilho *et al*., 2009) or polyvinylphosphoric acid (Tezvergil-Mutluay *et al*., 2010) do prevent loss of dry mass and solubilization of collagen. Thus, this simple *in vitro* model is very useful in such studies. We may have altered the spectrum of MMPs by inadvertently denaturing some MMPs, but sufficient activity remains to screen a wide variety of potential MMP inhibitors.

Since the experimental QAMs inhibit both soluble rhMMP-9 and matrix-bound MMPs in the experimental model, the null hypothesis that QAMs have no effect on MMP inhibition has to be rejected. This is the first report on antibacterial QAMs being effective in inhibiting MMPs. The ability of QAMs to inhibit micro-organisms and MMPs at similar concentrations makes them very attractive from a therapeutic perspective.

Quaternary ammonium compounds (QACs) and the related biguanide CHX are cationic (*i.e*., they have positive charges). Most QACs have only one positive charge, while biguanides such as CHX have two fixed charges. Both mineralized and demineralized dentin substrates have net negative charges, due to the presence of trivalent phosphates in apatite and carboxylic groups in the collagen molecules, respectively. Thus, when cationic QACs are applied to dentin, they bind electrostatically to dentin and give it a net positive charge (Markowitz and Rosenblum, 2010). This changes the 3-D configuration of proteins, which rely, in part, on electrostatic attractions and repulsions to stabilize their ternary structure. We speculate that non-specific binding of QACs and biguanides alters the configuration of the active site of MMPs, making them unable to accept the complementary peptide sequence for collagen. That QAM-MMP complex remains inactive as long as the QAC is bound to the MMPs and the insoluble collagen. The substantivity of CHX has recently been shown to be greater than one might expect (Kim *et al*., 2010). In a recent study, binding of 0.2% CHX to acid-etched dentin resulted in the retention of 98% of the CHX for up to 8 wks (Carrilho *et al*., 2010). Presumably, QAMs mixed with adhesive monomers and polymerized *in situ* after their infiltration into demineralized dentin will be retained for years. That assumption requires further validation with long-term studies.

The catalytic site of MMPs contains cysteine-rich repeats necessary for substrate binding. The cysteine-rich repeat includes a glutamic acid residue that is adjacent to a histidine molecule, both of which are essential for its catalytic activity (Visse and Nagase, 2003). Since glutamic acid is a dicarboxylic acid, it contains a free carboxylate group at physiological pH and retains a negative charge even after formation of a peptide bond. We speculate that cationic QAMs electrostatically bind to such negative charges. The presence of cationic QAMs may sterically block the active site from adjacent collagen peptides, thereby inhibiting MMP activity.

The resin monomer MDPB has strong bactericidal activity as a monomer, and demonstrates cavity-disinfecting effects when incorporated into a dentin primer (Imazato *et al*., 1997; Imazato, 2009). Previous research on adhesives containing MDPB has shown more durable interfaces than conventional adhesives in the oral environment (Nakajima *et al*., 2003; Donmez *et al*., 2005). The increase in durability associated with MDPB-containing adhesives may be partially explained by the inhibitory effect of 5% MDPB on soluble and matrix-bound MMPs, as demonstrated in the present study.

The results on soluble MMP-9 for some of the QAMs were slightly different from those obtained with matrix-bound MMPs. Dentin is known to contain MMPs-2, -8, and-9, and cysteine cathepsins (Tersariol *et al*., 2010). The latter is a class of proteases that can also hydrolyze collagen. The inhibitory potential of QAMs may not be the same for all the endogenous enzymatic components of dentin. This may account for the discrepancy in results obtained for the soluble MMP-9 and the matrix-bound MMP model. Further research is needed to identify the effects of these inhibitors on cathepsins.

Within the limitations of this study, it may be concluded that experimental QAMs and the commercially available QAM (MDPB) demonstrate inhibitory effects on both soluble rhMMP-9 and matrix-bound MMPs. However, the experimental aliphatic QAMs require much higher concentrations to inhibit dentin MMPs than the pyridine ring-containing MDPB. The anti-MMP effects of QAMs on long-term durability of resin-dentin bonds require further substantiation.

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