Proteinase Activity in Human and Murine Saliva as a Biomarker for Proteinase Inhibitor Efficacy

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ABSTRACT

As molecularly targeted agents reach the clinic, there is a need for assays to detect their presence and effectiveness against target molecules in vivo. Proteinase inhibitors are one example of a class of therapeutic agent for which satisfactory methods of identifying successful target modulation in vivo are lacking. This is of particular importance while these drugs are in clinical trials because standard maximum-tolerated dose-finding studies often are not suitable due to lack of toxicity. Saliva represents a readily accessible bodily fluid that can be repeatedly sampled and used for assaying in vivo effects of systemic drugs. Here we show the development of a simple assay that can be used to measure proteinase activity in saliva and proteinase inhibition after systemic treatment with three different proteinase inhibitors. A variety of gelatinolytic activities present in human and murine saliva have been assayed with a fluorescent dye-labeled substrate and assigned to different proteinase categories by inclusion of specific classes of inhibitors. Treatment of mice with either matrix metalloproteinase inhibitors or a urokinase inhibitor for a period as short as 48 hours results in levels of the drugs that can be detected in saliva by mass spectrometry and concomitant decreases in salivary proteinase activity, thus demonstrating that these inhibitors successfully modulate their targets in vivo.

INTRODUCTION

Proteinase inhibitors have been developed as therapeutic drugs for many different diseases. Successful inhibitors in clinical use include HIV viral proteinase inhibitors and angiotensin-converting enzyme inhibitors. There are, however, many other disease targets for which proteinase inhibition has been proposed as a therapeutic approach but for which successful compounds are still lacking. For example, a number of inhibitors of the matrix metalloproteinases (MMPs) have been tested in large-scale clinical trials in cancer patients; however, none have been successful (1–3). The possible reasons for lack of efficacy of MMP inhibitors (MMPIs) are multiple but include the possibility that these drugs did not inhibit their anticipated targets in vivo.

The activity of secreted proteinases thought to be relevant pharmaceutical targets is regulated at multiple levels. In particular, the enzymes are produced as zymogens requiring subsequent cleavage for activation. There are also families of endogenous inhibitors for the different proteinase classes, e.g., tissue inhibitors of metalloproteinases (TIMPs) for MMPs or serpins for serine proteinases, as well as general inhibitors such as the serum component α2-macroglobulin (4, 5), all of which reduce the pool of active proteinases. Measurement of total proteinase expression levels does not discriminate between latent enzyme, inhibitor-complexed enzyme, and proteolytically active enzyme. Presumably, it is the pool of active proteinase that represents the real target of pharmacological proteinase inhibitors, and therefore methods of measuring activity rather than levels of expression are required to accurately assess the effects of inhibitory drugs.

For future clinical development of proteinase inhibitors, a biomarker that can indicate inhibition of proteinase activity after systemic administration in vivo will be extremely valuable. Molecules suitable for use as biomarkers should be present in easily sampled bodily fluids and preferably validated in preclinical models (6). Biomarkers can be used for pharmacodynamic studies to assess optimal biological doses of compounds that may not produce toxicity and therefore cannot be assessed with traditional maximum tolerated dose-driven dose-finding phase I clinical trials. Additionally, biomarkers can be used to assess patient compliance with the dosing regimen. This is of benefit because many molecularly targeted agents are designed to be self-administered on a chronic basis. Previous studies have used serum, plasma, or urine as sources of potential biomarkers that could reflect proteinase activity and proteinase inhibition (7–10); however, such studies have not yet resulted in the identification of a robust marker.

In our current study, we have investigated the use of proteinase activity itself in saliva as a suitable marker for testing the in vivo efficacy of proteinase inhibitors. We have validated the assay in human samples and shown that whereas there is variation of activity from individual to individual, the levels remain relatively stable in any single individual over time. To investigate the usefulness of salivary proteinase activity as an indicator of in vivo inhibitor efficacy, we have examined saliva from mice before and after treatment with three different pro-
teinase inhibitors. Two are MMPs [BB-94 (batimastat) and BMS-275291], both of which have broad-spectrum activity against the MMP family (11). As the third drug, we have used an inhibitor of a serine proteinase, the urokinase inhibitor B428 (12). Like the MMPs, B428 has shown some antitumor efficacy in preclinical models (13, 14). However, clear indications that any of these drugs do inhibit their target proteinases when administered systemically have not previously been presented. Here we demonstrate for the first time significant inhibition of salivary proteinase activity after in vivo administration of any of the three drugs.

MATERIALS AND METHODS

Human Saliva Collection. Saliva samples (~2 mL) were collected from healthy volunteers using a commercially available Salivette device (Sarstedt, Newton, NC). Samples were collected after subjects had fasted for at least 1 hour. Subjects were instructed to rinse their mouths with water before chewing the Salivette waf for about 1 minute so as to saturate it with oral secretions, principally saliva. The samples were either used fresh or stored at −20°C, a process demonstrated not to affect activity (data not shown).

Mice. Male and female mice of the FVB strain aged 6 to 10 weeks were maintained and treated according to Institutional Animal Care and Use Committee regulations. MMP-9–null mice on the FVB background were obtained from Dr. Lisa Coussens (University of California, San Francisco, CA).

Murine Saliva Collection. Mice were anesthetized with 90 mg/kg ketamine and 5 mg/kg xylazine, and saliva was induced with a subcutaneous injection of 100 µL of 0.04 mg/mL pilocarpine (Sigma, St. Louis, MO). Saliva was collected in glass capillary tubes held sublingually. An average of 250 µL of saliva was harvested from every animal at each collection.

Zymography. Twenty microcrystals of each saliva sample were mixed with 5 µL of a sample buffer containing 250 mmol/L Tris (pH 6.8), 25% glycerol, 10% (w/v) SDS, and 0.01% bromphenol blue and loaded onto 10% SDS-PAGE gels containing 0.25% porcin gelatin (Sigma). Molecular mass markers (Biowhittaker Molecular Applications, Rockland, ME) and a positive control sample of conditioned medium from the human fibrosarcoma cell line HT-1080 were also included on the gel. After electrophoresis, the gels were washed twice for 15 minutes each in 2.5% Triton X-100 and then incubated overnight at 37°C in a substrate buffer containing 50 mmol/L Tris-HCl (pH 7.6) and 10 mmol/L CaCl2, in the absence or presence of proteinase inhibitors EDTA (30 mmol/L), BB-94 (20 µmol/L), BMS-275291 (20 µmol/L), or B428 (20 µmol/L). After incubation, the gels were stained with a solution of 0.5% (w/v) Coomassie Blue R250 prepared in 50% methanol:10% acetic acid. Once stained, the gels were destained briefly in the same solution without the Coomassie Blue and then stored in distilled water. Areas of gelatinolytic activity appeared cleared against the blue background of the Coomassie Blue-stained undigested gelatin.

Western Blotting. Thirty microcrystals each of the saliva samples were mixed with a sample buffer as described above with the addition of 0.15 mol/L β-mercaptoethanol and boiled for 10 minutes before loading onto 10% SDS-PAGE gels. After electrophoresis, the separated proteins were transferred to nitrocellulose at 100 V for 2 hours. The nitrocellulose was blocked with 10% milk in Tris-buffered saline [0.15 mol/L NaCl and 0.01 mol/L Tris-HCl (pH 7.5)] for 1 hour at room temperature and then incubated overnight at 4°C with antihuman MMP-9 antibody (Ab-1; Calbiochem, San Diego, CA) diluted 1:1,000 in blocking solution. After multiple washes in Tris-buffered saline containing 0.05% Tween 20 (TBS-T), the blots were incubated for 30 minutes at room temperature with biotinylated antimouse antibody (Vector Laboratories, Burlingame, CA) diluted 1:15,000 in blocking solution. After a final series of washes in TBS-T, the blots were analyzed by chemiluminescence using Western Lightening reagents (Perkin-Elmer, Boston, MA), according to the manufacturer’s instructions and exposure to film (Fuji Super RX; Fuji, Stamford CT).

Matrix Metalloproteinase-9 Activity Assay. The commercially available Biotrak MMP-9 immunocapture assay (Amersham Pharmacia Biotech, Piscataway, NJ) was used to determine levels of active MMP-9 in saliva samples according to the manufacturer’s instructions. Samples were assayed without additional activation to assess the levels of endogenously active MMP-9, although it should be noted that the assay is reported by the manufacturer to have some cross-reactivity with pro–MMP-9.

Proteinase Inhibitor Treatment. BMS-275291 was administered twice daily by oral gavage at a dose of 30 mg/kg for 2 days. BB-94 was given once daily by intraperitoneal injection at a dose of 35 mg/kg for 2 days. B428 was given once daily as an intraperitoneal injection at a dose of 30 mg/kg for 3 days. This drug was also supplied in the drinking water at a concentration of 0.5 mg/mL during the 2-day treatment period.

Amylase Assay. To normalize pre- and posttreatment saliva samples, total amylase activity was measured using the Enz-Chek amylase assay kit (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions.

Proteinase Activity Assay. Total proteolytic activity in saliva samples was measured by fluorometry using the generic substrate dye-quenched (DQ) gelatin (Molecular Probes) and a protocol extensively modified from our recently reported in vitro assay for MMP-7 activity (15). An aliquot (usually 50 µL per 160 µL of total assay volume for human saliva and half these volumes for murine saliva) of each sample was mixed with substrate (10 µg/mL) in Tricine buffer [50 mmol/L Tricine (pH 7.4), 200 mmol/L NaCl, 10 mmol/L CaCl2, 0.05 mmol/L ZnSO4, and 0.005% Brij 35] and incubated at 37°C. An inhibitor mixture composed of inhibitors of serine, cysteine, and aspartyl proteinases (Complete-EDTA free; Roche Biochemicals, San Diego, CA) was included in the reactions for all of the human saliva samples, but not for the murine samples. Fluorometric measurements (excitation at 485 nm and emission at 538 nm) were recorded every 10 to 20 minutes for 2 hours. These values were used to calculate an initial rate of enzyme activity (Δfluorescence units/minute) that, for the murine samples, was then normalized for amylase concentration. To measure non-metalloproteinase activity, EDTA at a concentration of 30
mmol/L was included in the assay mixture. MP activity was then determined as total activity minus the EDTA-inhibited activity. Similarly, MMP activity, defined as the BB-94–inhibitable activity, was determined by subtracting the activity in the presence of BB-94 (25 μmol/L) from total proteinase activity. To measure non-serine proteinase activity, aprotinin (2 μg/mL) and leupeptin (100 μmol/L) were included in the assay.

Liquid Chromatography-Mass Spectrometry Analysis. Chromatographic separation of the saliva samples was performed using Surveyor high-performance liquid chromatography in tandem with a Thermo Finnigan (San Jose, CA) TSQ quantum triple quadrupole mass spectrometer equipped with a standard electrospray ionization source outfitted with a 100 micrometer internal diameter deactivated fused silica capillary. All chromatographic separations were performed on a Phenomenex (Torrance, CA) Prodigy high-performance liquid chromatography column [ODS (3) 100A 150 × 2 mm 5 μm]. The mobile phase component for the assays consisted of A = 5% acetonitrile:2% EtOH:93% MilliQ water:0.3% formic acid and B = 80% acetonitrile:10% EtOH:10% MilliQ water:0.3% formic acid. The gradient for all analyses began at 10% B, gradually changing the composition of the mobile phase to 95% to 100% B and returning to the starting composition. The flow rate was 200 μL/min. Nitrogen was used for both the sheath and auxiliary gas, which were set to 45 and 24 (arbitrary units), respectively. Mass spectrometer operation was in the positive ion mode, and the ion transfer tube was operated at 35 V and 280°C (for BMS-275291) or 320°C (for B428 and BB-94). The tube lens voltage was set to 112 V. Source collision induced dissociation was used at 15 V. The mass spectrometer was used in the selected reaction monitoring mode. Ions were collisionally activated with argon at an indicated pressure of 1.5 mTorr. The mass-spectral resolution was set to a peak width of 0.7 u for both precursor and product ions. Mass transitions at the specified collision energy m/z 609.3→195.1, 42 eV; m/z 302.7→159.1, 24 eV; m/z 605.4→461.2, 25 eV; and m/z 478.2→267.1, 20 eV were monitored for reserpine (used as an internal standard in all analyses), B428, BMS-275291, and BB-94, respectively. The scan width for product ions was 1.0 u, and the cycle time for each ion was 0.5 second. The electron multiplier gain was set to 2 × 10⁶. Data were acquired in profile mode. Xcalibur software, version 1.3 (Thermo Finnigan) was used on a Dell Optiplex GX240. The proteins contained in the saliva samples were precipitated out using a volume of acetonitrile equal to 5× the volume of the sample. The acetonitrile eluant was taken to dryness using a Savant Speed Vac and brought to 100 μL using mobile phase A. For analysis of BMS-275291, samples were not precipitated, and 50 μL of these samples and standards were used for a reduction/alkylation reaction.

This was initiated by the addition of 25 μL of 1.0 mol/L N-ethylmorpholine, 10 μL of Tris (2-carboxy-ethyl)-phosphin-HCl, and 50 μL of the internal standard reserpine. The mixture was heated to 60°C for 30 minutes using a standard heat block, after which 10 μL of 4-vinylpyridine were added. After a second 30-minute incubation at 60°C, the sample was ready for analysis. The calibration ranges were 1 to 1,132 ng/mL for B428, 28 to 360 ng/mL for BB-94, and 25 to 499 ng/mL for BMS-275291.

RESULTS

Assay Development. We initially focused on measuring proteinase activity in human saliva samples because we were aware of reports of MMPs in this fluid (16–19). Human saliva samples were collected using a Salivette device; the test subject chews a provided synthetic wad for 1 minute to fully saturate it with saliva, and then the saliva is extracted by a brief centrifugation. Proteinase activity was then assayed using DQ gelatin, a commercially available gelatin substrate heavily labeled with fluorescein such that it is minimally fluorescent due to self-quenching until it is cleaved by proteinase (hence DQ). The assay is relatively straightforward and involves the addition of 50 μL of human saliva to 0.8 μg of DQ gelatin at neutral pH in the presence of a mix of general inhibitors of serine, cysteine, and aspartyl proteinases and the monitoring of increases in fluorescence (excitation, 485 nm; emission, 538 nm) over time. The initial increase in fluorescence (from which proteinase activity, expressed in arbitrary fluorescence units, was calculated) was approximately linear with time usually for 30 minutes or longer (Fig. 1A), indicating that cleavage of the substrate approximates zero-order kinetics. For each saliva sample, proteinase activity was also measured in the presence of either EDTA, a general divalent cation chelator, or BB-94, a hydroxamic acid-based synthetic inhibitor. This allowed us to distinguish MP activity identified as EDTA-inhibitable and MMP activity defined as BB-94-inhibitable (Fig. 1B). In the human samples tested, the MP and MMP activities were generally equivalent (Figs. 1B and 2A), indicating that the MPs in saliva that cleave DQ gelatin are predominantly MMPs. The MP and MMP activities of saliva collected using the Salivette were comparable with those of saliva collected directly from the same subject and cleared by centrifugation (data not shown). BB-94 was originally developed as a broad-spectrum inhibitor of MMPs; however, it has also been shown to have activity against sheddases (20, 21), proteinases of the ADAM family that share homology with MMPs. A second synthetic inhibitor of MMPs, BMS-275291, designed to avoid sheddase inhibition (22), gave results comparable with those for BB-94 (data not shown), indicating that, in human saliva samples, the MP activity can be ascribed to the MMP subclass. To facilitate the planning of future saliva collections, we assessed the effect of storage at −20°C on the MP or MMP activity of saliva samples and found no significant difference between measured MP or MMP rates for fresh samples versus those stored at −20°C (data not shown). However, multiple rounds of freezing and thawing did lead to appreciable changes (data not shown), so this was avoided.

Assay Validation. Proteinases present in the oral cavity can originate from a variety of sources including bacterial infection, inflammatory cells present in response to infection, or secretions from glandular epithelium (19). It is therefore likely that activity levels will vary among individual donors. The levels of total gelatinase as well as MP and MMP activity among a group of 11 healthy donors (14 total samples), both male and female (age range, 25–50 years), exhibit significant subject-to-subject variability (61%), with 2 of the 11 donors having no appreciable MMP activity (Fig. 2A). This variation did not correlate with gender or age of the donors. We also
assayed five samples from each of three individuals collected over a 3-week period to assess intradonor variability (Fig. 2B). Under these conditions, the variability within any one individual was relatively small, with a maximum of 22%. Overall, the interassay precision was excellent, with a coefficient of variation of 6%, whereas the intra-assay coefficient of variation was 10%. Based on these data, we predict that a sample size of 15 would be needed to be confident of detecting an average reduction of 50% proteinase activity with a 5% level of significance and 80% power. Recruitment of this limited number of participants should be achievable in a clinical trial setting, including phase I trials with small numbers of subjects.

**Identification of Matrix Metalloproteinase-9 as the Major Gelatinase in Human Saliva.** Because our primary interest in developing this assay was to use it in clinical trials of synthetic inhibitors of MMPs, we tested the saliva samples for the presence of gelatin-degrading MMPs. We first subjected the samples to gelatin zymography to visualize the proteinase activity on a gelatin substrate and determined, based on molecular mass, that the major gelatin-degrading activity present in human saliva is MMP-9 (Fig. 3A). The majority of the gelatinase activity has an apparent size of ~120 kDa, which corresponds to the MMP-9/lipocalin complex reported previously in saliva (23, 24). The presence of MMP-9 was confirmed by Western blot analysis of the saliva samples using an antibody specific for human pro-MMP-9 (Fig. 3B). Finally, the saliva samples were assessed using a commercially available immunocapture activity assay specifically for human MMP-9. This assay has previously been used to demonstrate increased MMP-9 activity in saliva samples from patients with Sjogrens syndrome (25). Using this method, active MMP-9 was detected in all of the samples tested, and concentration ranged from 0.19 to 0.48 ng/mL. When the data from the fluorometric activity assay were compared with that from the MMP-9 specific activity assay, there was a reasonable correlation between the two measurements (Fig. 3C). However, the sample with the lowest (although clearly detectable) MMP-9 level by the immunocapture assay was one that showed minimal MP or MMP activity by the fluorometric assay, which may reflect some detection of the pro-form of MMP-9 in the immunocapture assay. Taken together, the levels of salivary proteinase activities vary among individuals but are relatively consistent in each individual over time. A. Human salivary samples were incubated with the substrate DQ gelatin, and fluorescence was measured over time in the presence of an inhibitor mixture (see Materials and Methods) either without additional inhibitor (total activity) or in the presence of either 30 mmol/L EDTA or 25 μmol/L BB-94. Data are displayed as mean ± SE (triplicate assays). The initial rate of fluorescence increase corresponds to gelatinolytic enzyme activity. B. MP and MMP activities in saliva determined from the difference in fluorescence in the absence versus presence of either 30 mmol/L EDTA or 25 μmol/L BB-94, respectively, from the data shown in A. The initial rates of increase in fluorescence for either total activity (A) or MP and MMP activities (B) were calculated by linear regression and, for this sample, are 0.58, 0.33, and 0.26 arbitrary unit/min, respectively.

**Fig. 1** Gelatinolytic activity in human saliva can be inhibited by EDTA and BB-94, indicating a MMP origin. A. Samples of human saliva (50 μL) were incubated with the substrate DQ gelatin, and fluorescence was measured over time in the presence of an inhibitor mixture (see Materials and Methods) either without additional inhibitor (total activity) or in the presence of either 30 mmol/L EDTA or 25 μmol/L BB-94. Data are displayed as mean ± SE (triplicate assays). The initial rate of fluorescence increase corresponds to gelatinolytic enzyme activity. B. MP and MMP activities in saliva determined from the difference in fluorescence in the absence versus presence of either 30 mmol/L EDTA or 25 μmol/L BB-94, respectively, from the data shown in A. The initial rates of increase in fluorescence for either total activity (A) or MP and MMP activities (B) were calculated by linear regression and, for this sample, are 0.58, 0.33, and 0.26 arbitrary unit/min, respectively.
together, these data point to MMP-9 as being the major MP source of gelatinolytic activity in the human saliva samples.

**Proteinases in Murine Saliva.** To validate our approach of using saliva to measure in vivo effects of proteinase inhibitors, we collected saliva from mice before and after inhibitor treatment. Basal salivary output from mice is extremely low, and thus salivation was stimulated by subcutaneous administration of the adrenergic agonist pilocarpine to animals sedated with ketamine/xylazine as described previously (26, 27). After approximately 5 minutes, we could begin to collect saliva in capillary tubes held sublingually, and we collected an average volume of 250 μL of saliva per animal. As with the human samples, murine saliva contains proteolytic activities that could be detected with the fluorescent gelatin substrate assay. Unlike the human samples, murine samples showed lower background, probably due to a lower level of oral infection and inflammation, and were thus generally assayed in the absence of any inhibitors. When the murine saliva was examined by zymography, the principal gelatin-degrading enzyme present had a molecular size consistent with MMP-9, and the activity could be inhibited by incubation in the presence of either of the MMPIs (BB-94 and BMS-275291), but not by incubation in the presence of the urokinase inhibitor B428 (Fig. 4A). Interestingly, saliva from animals in which MMP-9 was genetically ablated did not show decreased activity in the fluorometric assay despite a complete absence of MMP-9 as detected by zymography (data not shown).
In contrast to wild-type samples in which the gelatinolytic activity could be significantly decreased by the addition of exogenous MMPI to the DQ gelatin reaction, addition of BB-94 to MMP-9–null saliva samples did not decrease gelatinolytic activity. However, the samples from MMP-9–null mice showed greater inhibition with the addition of an inhibitor of serine proteases, aprotinin, than did samples from wild-type mice. These results suggest that alternative proteolytic activities, probably of the serine family, are present in increased amounts in the saliva of MMP-9–null mice.

**Measuring Proteinase Inhibition after Drug Treatment.** Saliva was collected from groups of mice both before treatment on day 1 to establish a baseline and after 2 days of treatment with either of the MMPIs (BB-94 or BMS-275291) or with the urokinase inhibitor B428. The final saliva collection was 2 hours after the last dose of drug on day 3. Because the inhibitors used in these studies target activity and not expression, it was not unexpected that levels of gelatinase expression were not altered between the pre- and posttreatment samples as determined by gelatin zymography (Fig. 4B). It should be noted that although enzymatic processing of substrate (gelatin) is visualized on a zymographic gel at molecular masses equivalent to latent enzyme, this activity is generated as a result of the sample processing for zymography and is not indicative of inherent proteolytic activity within the sample. Activity assays were then performed using the DQ gelatin substrate and 50 µL of saliva from animals before and after drug treatment in either the absence of any additional proteinase inhibitors or the presence of a general inhibitor of the proteinase family targeted by the drugs. In this way, we could monitor any effects of the administered drug and test whether they targeted the expected proteinase families. As can be seen in Figs. 5 and 6, we observed decreased gelatinolytic activity in the majority of animals after 2 days of treatment with the various drugs. To normalize the pre- and posttreatment samples from each individual mouse, amylase activity was measured, and the data are presented as the rate of DQ gelatin hydrolysis per unit of amylase activity. In general, the MMPI BMS-275291 (Fig. 5A and C) resulted in inhibition of salivary proteinase activity, with an average decrease of 49%. In almost all samples, the activities affected by this drug appeared to be both MMP and other classes of proteinases because comparisons of pre- and post-treated samples analyzed in the presence of an exogenous MMPI showed inhibitory effects of the administered MMPIs on residual salivary proteinase activity (Fig. 5B and C). We note that within the BMS-275291–treated group, two animals did not show the expected decrease in proteinase activity after drug exposure, although in both of these animals, the activity in the posttreatment saliva could be somewhat inhibited by the addition of exogenous MMPI. These results suggest that in these two animals, BMS-275291 was insufficient to inhibit salivary proteinase activity and likely reflect ineffective in vivo exposure to drug, an interpretation corroborated by measurement of levels of the drug in the saliva from these two animals (see below).

Although only a limited number of animals could be treated with the MMPI BB-94 due to a limited drug supply, we found this drug to be a moderately effective proteinase inhibitor in vivo (Fig. 6A), with an average percentage inhibition of 59%.

The serine proteinase inhibitor B428 produced a consistent inhibition of approximately 40%, which may be due to its direct effect on serine proteinases as well as possible downstream effects on the activity of proteinases of other classes. When the pre- and posttreatment saliva of animals treated with B428 was analyzed for gelatinolytic activity in the presence of the serine proteinase inhibitor aprotinin, there was still detectable proteo-
lytic activity that was impacted by the treatment with B428, indicative of a non-serine proteinase activity modulated by B428 (Fig. 6).

Presence of Administered Drugs in Saliva Samples. Reductions in the detectable proteinase activity present in saliva after systemic administration of protease inhibitors could occur by means of several mechanisms. The most straightforward is that the administered drug is also present in the saliva and actively inhibits enzyme activity. The detection in saliva of other therapeutic protease inhibitors (specifically, the HIV protease inhibitors squanivir, ritonavir, and idinavir) has been reported (28–31). To determine whether this occurred in the treated animals, a subset of the saliva samples from drug-treated mice was analyzed by liquid chromatography-mass spectrometry (LC-MS). Drug was detected by a mass spectrometer operating in the selected reaction monitoring mode using an electrospray ion source and reserpine as an internal standard. For each of the three drugs tested, the method was linear within the ranges 1 to 1,132 ng/mL for B428, 0 to 360 ng/mL for BB-94, and 0 to 499 ng/mL for BMS-275291 (data not shown). In this way, we confirmed the presence of each of the three drugs in saliva samples from correspondingly treated animals (Fig. 7A and B), although the BB-94 level was extremely low (1.5 ng/mL) and not readily evident in the chromatograms (data not shown). This is likely related to the poor solubility of BB-94. Interestingly, the two samples from BMS-275291–treated animals that failed to show reduced salivary proteinase activity after drug treatment had much lower concentrations of drug in the saliva compared with those in samples from the two responding animals (Table 1). Thus, the failure of these two animals to respond to drug with inhibition of proteinase activity in vivo may reflect inadequate dosing and suggests the utility of this assay for monitoring dose compliance.

Fig. 6 Effects of systemic treatment with BB-94 and B428 on murine salivary proteinase activities. Saliva was collected from FVB mice stimulated with pilocarpine (pretreatment), and the animals were treated for 2 days with either of the protease inhibitors [BB-94 (n = 2; A) or B428 (n = 4; B)] as described in Materials and Methods. Two hours after the final dose on day 3, saliva was again collected after pilocarpine stimulation (after 48 hours of treatment). Saliva samples, both pre- and posttreatment, were analyzed for amylase activity and then for proteinase activities using DQ gelatin substrate as described in Materials and Methods. Each saliva sample was assayed for total proteinase activity in the absence of additional inhibitor (A and B, black symbols and lines) and in the presence of exogenous inhibitor (A and B, gray symbols and lines; either BB-94 (A) or aprotinin/leupeptin (B)). Different symbols represent different animals.

Fig. 7 Proteinase inhibitors detected by LC-MS in the saliva from treated animals. Saliva was collected from animals after 48 hours of treatment with the proteinase inhibitors BMS-275291 or B428 and analyzed by LC-MS as described in Materials and Methods. Shown are the chromatograms obtained for (A) BMS-275291 (peak at 6.96 minutes) in a sample from a BMS-275291–treated animal and (B) B428 (peak at 6.31 minutes) in a sample from a B428–treated animal. The internal standard reserpine is the second peak in each chromatogram.

Table 1 Drug concentrations as determined by LC-MS and the corresponding changes in murine salivary gelatinolytic activity after 2 days of treatment with protease inhibitor

<table>
<thead>
<tr>
<th>Sample</th>
<th>Drug treatment</th>
<th>% Inhibition *</th>
<th>Drug concentration by LC-MS (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BMS-275291</td>
<td>34</td>
<td>1026</td>
</tr>
<tr>
<td>2</td>
<td>BB-94</td>
<td>80</td>
<td>386</td>
</tr>
<tr>
<td>3</td>
<td>B428</td>
<td>54</td>
<td>425</td>
</tr>
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* Negative values indicate activity increased in these samples.
DISCUSSION

We have developed and tested a simple and rapid assay for testing the \textit{in vivo} effectiveness of proteinase inhibitors. Using the generic substrate gelatin, we can assess proteolytic activities from at least two classes of enzyme in samples of an easily obtainable bodily fluid, saliva. In preclinical studies to measure drug efficacy \textit{in vivo} using animals, we have demonstrated inhibition of the salivary proteolytic activity after short-term administration of three different pharmacological proteinase inhibitors.

MMPs have been touted as representative of a new class of nontoxic agents that could be given to cancer patients chronically to prevent the progression of their disease. Clinical trials of a number of agents of this type, however, were unsuccessful for a number of reasons that have been discussed elsewhere (1–3). Nevertheless, the potential utility of this class of agents should not be ignored, especially given the wealth of preclinical data, all of which indicated efficacy of this therapeutic strategy. One of the issues that arose during the clinical trials was the lack of a means to determine optimal biological dose. Although a number of approaches have been tried using serum, plasma, urine, or saliva samples (7–10, 32), the majority of the reported methods detect levels of enzyme expression rather than enzyme activity or its inhibition. Such an assay is not likely to be useful in the case of many proteinase inhibitors that function by binding to the active site of the proteinase, thus blocking its enzymatic activity, but do not interfere with expression. An exception to this is the tetracycline-based group of inhibitors that can affect MMPs at multiple levels including expression, conversion of pro-enzyme to the active form, and direct inhibition of catalytic activity. For example, in a clinical trial of the chemically modified tetracycline Col-3 in Kaposi’s sarcoma, changes in levels of MMP-2 could be determined, and these were correlated with responses to the drug (33). As an alternative to measuring proteinase levels, there have been attempts to measure proteolytically generated substrate fragments in bodily fluids. Among the most successful markers of this type are fragments of collagen \textit{e.g.}, collagen type II C telopeptide fragments (CTX-II and ICTP) that can be generated by MMP and cathepsin activities (34–36). Levels of these fragments have been shown to be increased in the urine of patients with various pathologies associated with cartilage and bone destruction (34, 35, 37) and can be modulated after inhibitor administration in experimental animals (38). Other proteolytically generated soluble fragments of proteins such as E-cadherin or Fas ligand can also be detected in serum, for example (39–42). The solubilization of these proteins can be mediated by several different proteinases, thus making their detection of limited value when examining the effects of a class- or enzyme-specific proteinase inhibitor.

The concept of using oral fluids to measure impact of an antiproteolytic treatment on pathological proteinases is not new. Tetracycline and tetracycline derivatives can be used for treatment of gingivitis and periodontitis, diseases associated with increased levels and activity of MMPs, notably MMP-8 and MMP-9. Gingival crevicular fluid samples collected from patients before and after treatment with tetracycline for periodontitis have demonstrated decreased collagenolytic activity that correlated with therapeutic efficacy (43–46). In a different disease setting, that of rheumatoid arthritis, which is also associated with dysregulated proteolytic activity, salivary samples from 12 patients treated with doxycycline for 3 months also indicated reduced collagenolytic activities (32).

The assay described here uses gelatinolytic activity as a means of assessing various different proteinase activities and their pharmacological modulation. A number of reports in the literature describe the various proteinases that have been detected in oral fluids, including MMP-9, which we discerned to be the most prominent gelatinolytic enzyme present, and MMP-8 (17, 18, 23, 44, 47, 48). Additionally, saliva contains cathepsins (49, 50), kallikreins, and other serine proteinases (47, 51–54). The use of different kinds of general protease substrates may allow for detection and/or discrimination of some of the other activities present that are not detected by using gelatin. Nevertheless, we have shown here that gelatin is a useful indicator of the modulation of representative proteinases targeted by the drugs we have used.

Both the MMPI BMS-275291 and the urokinase inhibitor B428 appeared to directly impact their target proteinases but also had effects on other activities present in the saliva. Proteinase activity can be regulated by a complex cascade of activators and inhibitors in which proteolytic processing of pro-proteinases as well as proteolytic degradation of endogenous inhibitors can increase the pool of active proteinases. For example, the serpin α1 proteinase inhibitor has been reported as a substrate of multiple MMPs, including MMP-9 (55), whereas pro–MMP-9 can be activated by the serine proteinase plasmin and MMP-3 (56). Thus, interfering with any particular proteinase can have significant impact on activities downstream.

In the limited number of murine salivary samples that were analyzed by both LC-MS and fluorometric proteinase activity assay before and after treatment with proteinase inhibitors, there was not a positive correlation between drug concentration and degree of inhibition. However, the samples from the two BMS-275291–treated animals that contained particularly low concentrations of drug also showed ineffective inhibition of proteolytic activity. One interpretation of these results is that there is a threshold level of drug that must be reached before effective proteinase inhibition occurs. Our sample size is too small to confirm this idea, but when more proteinase inhibitors become available, it will be interesting to test whether a clear dose-response occurs or if a threshold effect is manifest.

The collection of the human samples with the Salivette device as used in our studies does not discriminate between true salivary gland secrections and gingival crevicular fluid. Because both fluids have been reported as sources of proteolytic activities, we would contend that such discrimination is irrelevant for assessing the \textit{in vivo} efficacy of the drugs used here because it is overall change in proteolytic activity in an individual subject that is important. Additionally, the ease with which the samples can be collected and handled would make this assay easily manageable in the setting of a large clinical trial.

As mentioned previously, elevated levels of certain MMPs, particularly MMP-8 and MMP-9, in saliva or gingival crevicular fluid have been reported to be associated with periodontal disease (18, 44, 45, 57). Hence, within a population of cancer patients or apparently healthy controls, there are likely to be
individuals with some degree of periodontal disease having intrinsically higher baseline levels of MMP activity, as was observed in our small cohort of apparently healthy control subjects. For this reason, it would be important that changes in proteolytic activity detected by the saliva assay during inhibitor treatment be determined on an individual basis rather than over a group of subjects. To ensure that levels are stable before enrollment in a drug trial, several baseline samples could be collected from each individual patient over a 2-week predrug period. An additional point that should be considered before applying the saliva assay to a clinical trial is the pharmacokinetic profile of the agent being tested. This would certainly impact the timing at which optimum inhibitory activity would be measurable after drug administration.

In addition to the potential use of the saliva assay for monitoring patients in drug trials so as to assess the in vivo efficacy of specific doses of protease inhibitors or other therapeutic agents, this assay or variations thereof could also be valuable for preclinical testing of novel inhibitors. Our results in mice demonstrate that in vivo efficacy of protease inhibitors can be determined in a relatively short time frame, with results in 3 days. Thus, the same type of assay could be used as a rapid screen for small amounts of new compounds to demonstrate their ability to modulate a target in vivo and as a means of monitoring dosing in clinical trials. Furthermore, salivary activity assays have the potential to be used for monitoring patient compliance.

In conclusion, we have developed and validated a simple assay for determining the in vivo effectiveness of protease inhibitors. We have tested three different drugs that inhibit two different classes of proteinases and demonstrated successful target modulation after systemic administration. Additionally, we have detected the presence of the administered drugs in the saliva of treated animals using LC-MS and compared levels of detection with degree of proteinase inhibition. Assay of salivary protease activity could be used to monitor dosing of patients in clinical trials, thus supplying one of the components missing from the original clinical testing of MMPs.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the kind gifts of BB-94 from British Biotech (Oxford, United Kingdom), BMS-275291 from Bristol-Myers Squibb (Raritan, NJ), and B428 from Dr. Galina Kuznetsov and colleagues at Eisai Research Institute (Andover, MA). We are also grateful to Dr. Lisa Coussens for the MMP-9 null mice. We thank Dr. Shiva Gautam for assistance with statistical analysis.

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Proteinase Activity in Human and Murine Saliva as a Biomarker for Proteinase Inhibitor Efficacy

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